Glycolate production and consumption
by M Bateson

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Microbiology
Montana State University
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Abstract:
Synechococcus lividus is the sole oxygenic phototroph inhabiting the surface of the cyanobacterial mat at 55°C in Mushroom Spring, Yellowstone National Park. Its photosynthesis causes superoxic and alkaline conditions, which should promote glycolate production and flux from S. lividus to heterotrophs. In NaH14CO3-labeled suspensions from the mat surface, 10-60% of the 14C fixed by S. lividus was detected as extracellular acid stable water soluble compounds. Decreasing CO2 by gas stripping, or increasing O2 by incubation under pure O2, increased the percent of photosynthate in the aqueous phase. Addition of CO2 decreased the percent of photosynthate in the aqueous phase. Two dimensional thin layer chromatograms showed that glycolate was an important extracellular product. At oxygen concentrations characteristic of the top 2 mm of mat during the day, glycolate comprised 25 to 58% of the extracellular carbon. At lower oxygen concentrations characteristic of the mat during early morning and late evening, glycolate comprises 8.5 to 46% of the extracellular carbon. Extracellular glycolate accounted for up to 12% of photosynthetically fixed carbon detectable in this experimental system.

The importance of photoexcretion and glycolate production may be underestimated because of glycolate flux to heterotrophs in the mat community where organisms are closely associated. Autoradiograms showed that filamentous bacteria incorporated glycolate, and that S. lividus cells did not. S. lividus cultures exhibited no uptake or respiration of 1-14C-glycolate. Inhibition of glycolate metabolism and addition of excess glycolate increased the percent of photosynthate in the aqueous phase, suggesting the flux of glycolate from producer to consumer. The results suggest that glycolate production and crossfeeding to heterotrophs is important in this community during daylight hours. In samples incubated in the dark, no glycolate was detected, although dark CO2 fixation leads to synthesis of one major unidentified compound which is excreted.
GLYCOLATE PRODUCTION AND CONSUMPTION
IN A HOT SPRING CYANOBACTERIAL MAT

by
Mary Margaret Bateson

A thesis submitted in partial fulfillment of the requirements for the degree of
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Microbiology

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November, 1985
APPROVAL

of a thesis submitted by

Mary Margaret Bateson

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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November 26, 1985
Children of steam and scalded rock, a story
you have to tell,
Writ in the glare of sunshine bright,
Sculptured and etched in marble white,
Illuminated in colors bold,
Richer than ever parchment old,
Children of steam and scalded rock, what is
the story you have to tell?

Our legends are old, of greater age than the
mountains round about.
We have kept our secrets epochs long,
They are not to be read by the passing throng.
It is nothing to us what men may say.
If they wish our story a price they must pay
In hard brain work, ere the tales are told.
We challenge mankind to draw them out.

Children of steam and scalded rock, your
challenge must rest for the present age.

I have scarcely broken the outer crust
That covers the greater truth, but I trust
Some man shall follow and therein find
Knowledge, that to the Present shall bind
The Past with cords wherein entwine
Threads of the perfect truth, divine.
Children of steam and scalded rock, some
man will come to accept thy gage.

Bradley Moore Davis (1897)
Science 6:145-157
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ABSTRACT

*Synechococcus lividus* is the sole oxygenic phototroph inhabiting the surface of the cyanobacterial mat at 55°C in Mushroom Spring, Yellowstone National Park. Its photosynthesis causes superoxic and alkaline conditions, which should promote glycolate production and flux from *S. lividus* to heterotrophs. In NaH\(^{14}\)CO\(_3\)-labeled suspensions from the mat surface, 10-60% of the \(^{14}\)C fixed by *S. lividus* was detected as extracellular acid stable water soluble compounds. Decreasing CO\(_2\) by gas stripping, or increasing O\(_2\) by incubation under pure O\(_2\), increased the percent of photosynthate in the aqueous phase. Addition of CO\(_2\) decreased the percent of photosynthate in the aqueous phase. Two dimensional thin layer chromatograms showed that glycolate was an important extracellular product. At oxygen concentrations characteristic of the top 2 mm of mat during the day, glycolate comprised 25 to 58% of the extracellular carbon. At lower oxygen concentrations characteristic of the mat during early morning and late evening, glycolate comprises 8.5 to 46% of the extracellular carbon. Extracellular glycolate accounted for up to 12% of photosynthetically fixed carbon detectable in this experimental system.

The importance of photoexcretion and glycolate production may be underestimated because of glycolate flux to heterotrophs in the mat community where organisms are closely associated. Autoradiograms showed that filamentous bacteria incorporated glycolate, and that *S. lividus* cells did not. *S. lividus* cultures exhibited no uptake or respiration of \(^{1-14}\)C-glycolate. Inhibition of glycolate metabolism and addition of excess glycolate increased the percent of photosynthate in the aqueous phase, suggesting the flux of glycolate from producer to consumer. The results suggest that glycolate production and crossfeeding to heterotrophs is important in this community during daylight hours. In samples incubated in the dark, no glycolate was detected, although dark CO\(_2\) fixation leads to synthesis of one major unidentified compound which is excreted.
INTRODUCTION

The research described in this thesis is part of a continuing effort to describe the ecology of microbial mat communities present in the effluent channels of alkaline siliceous hot springs found in Yellowstone National Park. Past research has improved our understanding of anaerobic decomposition and the accumulation and fate of fermentation products in these hot spring microbial mats. This investigation focuses on the importance of glycolate, a compound which may be produced by the photosynthetic community members and assimilated directly by heterotrophic microorganisms of the cyanobacterial mat community. Before stating specific research objectives I will first review literature related to hot spring cyanobacterial mats and algal excretion and crossfeeding.

Rationales for Study of Hot Spring Microbial Mats

Considerable interest in hot spring microbial mats has stemmed from their structural similarity to Precambrian stromatolites (Doemel and Brock, 1974; Walter, 1976, 1977; Walter, et al, 1972, 1976). Stromatolites are laminated sedimentary rocks often containing fossilized structures morphologically similar to modern microorganisms that form
microbial mats (Bridgewater, et al, 1981; Cloud, 1965; Cloud and Hagen, 1965; Golubic, 1976; Golubic and Barghoorn, 1977). Stromatolites are abundant from the Precambrian era, when only microorganisms were present and no evidence of more differentiated life forms appears in the fossil record (Awramik, 1984). The extremely high temperatures of hot spring microbial mats provide an environment which animals that might graze upon microorganisms cannot tolerate (Brock, 1978; Wickstrom and Castenholz, 1973). Thus, these completely microbial communities provide relevant models of Precambrian stromatolite-forming communities (Ward, et al, 1984), which would have also lacked animals. Studying hot spring microbial mats provides information which aids in interpreting the types of microorganisms and microbial activities which may have been present throughout early earth history, before the evolution of more complex organisms.

Interest in hot spring microbial communities has also resulted from the potential for discovering useful thermophilic microorganisms or understanding commercially useful microbial processes. Thermophilic conversion of organic wastes to methane (Sandbeck and Ward, 1981, 1982; Ward, 1978; Ward and Olson, 1980), and thermophilic fermentations (Anderson, 1984; Zeikus, 1980) are examples of such processes. Two thermophilic ethanol-producing
fermentative bacteria which may prove to be industrially useful (Wiegel, 1980) have recently been isolated from these hot spring mats (Ben-Bassat and Zeikus, 1981; Wiegel and Ljungdahl, 1981).

Hot spring cyanobacterial mats also provide an ideal system for ecological studies. In Yellowstone National Park, no metazoan organisms are found at temperatures above 40°C, and although one eucaryotic alga, *Cyanidium caldarium*, inhabits acidic springs up to 57°C, no eucaryotic algae are known to grow above 30-40°C in neutral or alkaline springs. It is likely that except in very acid waters where cyanobacteria are not found, the more well adapted procaryotes out-compete the eucaryotic algae (Brock, 1973). The extreme parameters of the system not only limit the community to procaryotes, but also restrict species diversity (Brock, 1985). For example, many springs have only one or a few photoautotrophs (Ward, et al, 1984). Limited diversity, combined with constancy of temperature and source water chemical composition, reduces the complexity of the ecosystem, so that ecological relationships among the community members may be more easily studied. Fundamental research in microbial ecology, especially the development of whole ecosystem models, can enhance our understanding of biochemical processes in other environments.
The research described here focuses on the major photoautotroph in the community. This cyanobacterium, Synechococcus lividus, inhabits an environment in which chemical conditions encourage photorespiration, a process which substantially decreases the overall rate of net photosynthesis in higher plants. Since photorespiration is not limited to cyanobacteria, but also occurs in algae and higher plants, further understanding of the process in this microbial ecosystem could improve our comprehension of photorespiration in general.

**Description of a Hot Spring Cyanobacterial Mat**

Cyanobacterial mat communities form in the effluent channels of alkaline hot springs at temperatures below approximately 70°C. The mats of Octopus Spring and Mushroom Spring have been extensively studied and are representative of one common type of mat community found in Yellowstone National Park. The formation of the microbial mats by photosynthetic organisms has been explored in many studies. Rates of primary production and decomposition of the mat are maximal between 50 and 61°C (Doemel and Brock, 1977; Revsbech and Ward, 1984; Sandbeck and Ward, 1982). The major primary producer found in the thin green surface layer of the mats is the cyanobacterium Synechococcus lividus, a nonmotile, unicellular organism classified in the order Chroococcales, family Chroococcaceae (Rippka,
1979, Stanier and Cohen-Bazire, 1977). These narrow, often slightly curved rods which exhibit autofluorescence characteristic of chlorophyll a are rarely found in layers deeper than the upper millimeter of the mats (Doemel and Brock, 1977). At least four stable temperature strains of S. lividus appear alike morphologically, but have temperature optima of 45, 50, 55, and 65°C (Castenholz, 1973, 1969; Peary and Castenholz, 1971). This organism is widely distributed in hot springs of Africa, Asia, New Zealand, and the Americas, but is absent from hot springs in Iceland and the Azores (Castenholz, 1978).

Another photosynthetic community member present at the mat surface and comprising much of the 1 to 3 mm thick orange layer below is the filamentous, gliding bacterium, Chloroflexus aurantiacus. The various carotenoid pigments of Chloroflexus produce the bright orange color of the mat (Pierson and Castenholz, 1974). In the light, this organism assimilates organic compounds during photoheterotrophic growth. It also exhibits heterotrophic activity in the dark, but only in the presence of oxygen. Some strains can grow photoautotrophically with H₂S as an electron donor, however H₂S is usually absent in the photic zone of mats in low sulfide springs due to oxygen production from oxygenic photosynthesis (Jørgensen, et al, 1979; Revsbech and Ward, 1984). Using immunofluorescence probes, Tayne (1983) showed that Chloroflexus is an
abundant filamentous organism in the mat. Two morphologically distinct, thinner filamentous organisms are also abundant in the Octopus Spring mat, but these failed to react with antiserum specific for Chloroflexus. The narrowest filaments were found almost entirely near the mat surface, while slightly wider filaments were distributed throughout the mat. These filamentous organisms remain to be identified.

Decomposition of the photosynthetically formed mats occurs through both aerobic and anaerobic processes. The aerobic decomposition process has received less attention (Ward, et al, 1984). An aerobic bacterium, Thermus aquaticus, has been isolated from various hot springs, including Mushroom and Octopus Springs in Yellowstone National Park, by incubating microbial mat samples in a dilute nutrient medium at 70 to 75°C (Brock and Freeze, 1969). In many cases, enrichments attempted at 55°C using the same inocula yielded motile, spore-forming bacilli which were not characterized further. Although the temperature optimum of T. aquaticus isolates is 70 to 72°C, they have been isolated from and grow at lower temperatures (50 to 60°C). T. aquaticus isolates are initially filamentous, with diameters similar to those described by Tayne (1983) for the unidentified filamentous organisms in the mat.
Isocystis pallida, a filamentous organism consisting of long chains of spherical cells, has been isolated from Oregon and Yellowstone hot springs (Giovannoni and Schabtach, 1983). Classification of this aerobic, chemoheterotrophic Gram negative bacterium was initially confused because cell division occurs by budding. Microscopic observation of morphologically similar organisms suggests that I. pallida is an inhabitant of mats in both Mushroom and Octopus springs in Yellowstone National Park.

Anaerobic decomposition appears important for the complete mineralization of the organic matter formed by photosynthesis. Fermentation products such as acetate, butyrate and propionate accumulate during dark, anaerobic conditions in the mat (Anderson, 1984). Three fermentative eubacteria have been isolated from Octopus Spring: Thermoanaerobium brockii (Zeikus, et al, 1979, 1980), Thermobacteriodes acetoethylicus (Ben-Bassat and Zeikus, 1981), and Thermoanaerobacter ethanolicus (Wiegel and Ljungdahl, 1981). The major fate of fermentation products is photoincorporation into Chloroflexus cells (Tayne, 1983). However, hydrogen, another fermentation product which is produced during anaerobic conditions in this mat, is consumed in the terminal steps of anaerobic decomposition. A sulfate-reducing bacterium, Thermodesulfotobacterium commune (Zeikus, et al, 1983), and
a methanogenic archaebacterium, *Methanobacterium thermoautotrophicum* (Zeikus, et al, 1980; Sandbeck and Ward, 1982) are isolates from the mats which may be carrying out the terminal steps of anaerobic decomposition.

Other as yet undetected organisms may be present in the microbial mat communities of alkaline silicious hot springs, in addition to the two filament types and motile spore-forming bacilli which have not been characterized. Since research has emphasized anaerobic degradation processes in the mat, relatively little effort has been made to isolate aerobic organisms which may be present in the mat. Although characterization of the community composition of microbial mats in alkaline hot springs is presently biased by limitations of pure culture techniques, further research utilizing biochemical markers such as lipids and nucleic acids could reveal more complete information concerning the organisms present and their relative abundance in microbial mats (Ward, et al, in press).

**Crossfeeding from Phototrophs to Heterotrophs in Microbial Communities**

Although carbon flow through decomposition of phototrophs in the mat ecosystem has been well studied in alkaline silicious hot spring microbial mats (Anderson, 1984; Doemel and Brock, 1977; Sandbeck and Ward, 1981,
1982; Tayne, 1983; Ward, 1978; Ward, et al, 1984; Ward and Olson, 1980), carbon flow from phototrophs to heterotrophs is less well understood. Oxygen and pH microelectrode studies (Revsbech and Ward, 1984) suggest that environmental conditions should favor processes leading to photoexcretion and consequential carbon flow to heterotrophs. These possibilities will be explored in this thesis.

Bauld and Brock (1974) suggested that heterotrophic microorganisms in hot spring microbial mats derive organic nutrients from products excreted by the cyanobacterium *S. lividus*. They detected between 3 and 12 percent of $^{14}$CO$_2$ fixed by cells in intact cores of the top green layer of mat as excreted $^{14}$C-organic material. However, photoexcretion products could be quickly taken up by heterotrophs, such as the photoheterotroph *Chloroflexus*, whose cells intertwine with *S. lividus* in the top layer of the mat. In acidic hot spring microbial mats dominated by the alga *Cyanidium caldarium*, 2 to 6% of $^{14}$CO$_2$ fixed by cells was detected as excreted material (Belly, et al, 1973). Similar results have been found in other aquatic environments; for example, natural populations of marine algae excreted 4 to 16% of photoassimilated carbon (Hellebust, 1965). These measurements of photoexcretion products were probably underestimates of the actual excretion of photosynthetically fixed carbon, due to the
close association of organisms in these mat communities, since products could have been taken up by heterotrophs before they were detected.

Many investigators have studied microbial assimilation of photoexcretion products in other ecosystems using size fraction filtration techniques to separate photosynthetic from presumably heterotrophic bacterial populations (Brock and Clyne, 1984; Cole, et al., 1982; Coveney, 1982; Haack and McFeters, 1982; and Paerl, 1984). The main conclusion from these studies is that significant carbon flow between phototrophs and heterotrophs occurs in very different aquatic environments. For example, in two eutrophic lakes in southern Sweden, estimated gross extracellular release of fixed carbon was low (1 to 7% of total $^{14}$C uptake), however, from 28 to 80% of the $^{14}$C released was recovered in the small particulate fraction rather than the aqueous fraction (Coveney, 1982). In a study of algal photoexcretion in Lake Mendota, Wis., estimations of the percent of photosynthate excreted were twice as high when bacterial uptake was taken into account (Brock and Clyne, 1984). In an epilithic algal-bacterial community in a pristine mountain stream, the percent of algal extracellular products incorporated by bacteria during short term incubations in the light ranged from 5.9% to 24.8% during a summer season (Haack and McFeters, 1982).
Many autotrophic organisms excrete organic compounds. The production of a variety of extracellular substances by algae, cyanobacteria, photosynthetic and chemolithotrophic bacteria is well established (Asami and Akazawa, 1974; Codd and Smith, 1974; Cohen, et al, 1979; Fogg, 1963; Fogg, et al, 1965; Hellebust, 1965; Tolbert and Zill, 1956). In one report (Hellebust, 1965), a few algal species excreted as much as 10 to 25% of their photoassimilated carbon during logarithmic growth, but most of the algae tested excreted 3 to 6%. Simple and complex polysaccharides are excreted by a large number of taxonomically diverse algae, and by several genera of cyanobacteria, including Anabaena, Nostoc, and Oscillatoria (Hellebust, 1974; Fogg, 1963). Tolbert and Zill (1956) showed that in acidic media, especially at pH values below 3.5, most of the labeled organic matter excreted by Chlorella pyrenoidosa during short term photosynthesis with $^{14}$C-bicarbonate was in the form of sucrose.

Algae commonly excrete amino acids and peptides, although these compounds generally represent a small fraction of the total excreted material (Fogg, 1963). In contrast, several cyanobacteria liberate very large portions of their assimilated nitrogenous substances into the medium in which they are cultured (Hellebust, 1974; Fogg, 1963). A large percentage of this material is in the form of polypeptides, with only trace amounts of free amino
acids found (Fogg, 1963; Whitton, 1965). The extracellular polypeptides of four different cyanobacterial species (Anacystis nidulans, Anabaena cylindrica, Nostoc sp., and Oscillatoria planctonica) had similar amino acid compositions, consisting of about fourteen amino acids with serine and glycine predominating, and no basic amino acids present (Whitton, 1965). Proteinaceous substances with unique properties, such as growth inhibitors or promoters, enzymes, and toxins have been detected as excretion products of various algae and cyanobacteria (Fogg, 1963; Hellebust, 1974).

Organic acids, including formic, acetic, lactic, oxalic, tartaric, and succinic acids, have been detected in filtrates from cultures of various cyanobacteria and algae growing autotrophically (Fogg, 1963; Hellebust, 1974). Glycolic acid, or glycolate, is the organic acid most commonly liberated by algae and cyanobacteria (Hellebust, 1974).

During the assimilation of $^{14}$CO$_2$ by photosynthesizing Chlorella pyrenoidosa (at pH 8), all of the $^{14}$C excreted into the medium was glycolate. The excreted material constituted 9.8% of the total $^{14}$C fixed. As the pH of the medium was lowered the percent of the excreted material that was glycolate decreased, and sucrose excretion increased (Tolbert and Zill, 1956). Hellebust (1965) found
glycolate in filtrates of all but one of 23 marine phytoplanktonic algal species studied. In four algal species, glycolate formed 9 to 38% of the carbon excreted, however, the average percent of excreted carbon as glycolate was only 5%.

Investigators have reported glycolate excretion by several cyanobacterial species: Coccochloris sp. (Hellebust, 1965), Anacystis nidulans (Dohler and Braun, 1971), Anabaena flos-aquae and Oscillatoria sp. (Cheńg, et al, 1972), and Anabaena cylindrica (Bergman, 1984). Glycolate excretion has also been detected in the purple photosynthetic bacterium Rhodospirillum rubrum (Codd and Smith, 1974), the purple sulfur bacterium Chromatium (Asami and Akazawa, 1974), and the chemolithotrophic bacterium Thiobacillus neopolitanus (Cohen, et al, 1979).

Glycolate Production during Photosynthesis

Organisms which utilize the Calvin-Benson cycle for autotrophic carbon reduction (see Figure 1) synthesize glycolate when oxygen competes with carbon dioxide for carbon fixation. During the Calvin cycle, CO$_2$ condenses with ribulose 1,5-diphosphate (RuDP) to form a transient six carbon compound which is rapidly hydrolyzed to two molecules of glycerate-3-phosphate. This carboxylation of RuDP is in competition with the oxygenation of RuDP to glycerate-3-phosphate and 2-phosphoglycolate. One enzyme,
Figure 1. The Calvin-Benson Cycle of autotrophic carbon reduction.

RuDP carboxylase-oxygenase, catalyzes both of these reactions, employing the same or adjacent sites on the enzyme (Chollet, 1977). The phosphoglycolate formed by the oxygenation of RuDP is hydrolyzed to glycolate by phosphoglycolate phosphatase.

In higher plants, and in many algae and cyanobacteria, glycolate is the substrate for photorespiration, a process in which O₂ is consumed and CO₂ evolved via the glycolate pathway. Photorespiration differs from mitochondrial respiration in that it does not occur in the dark, does not produce ATP or NADPH, and does not utilize the intermediates of the tricarboxylic acid cycle (Tolbert, 1974).
In plants and multicellular algae, glycolate is oxidized to glyoxylate by a flavin-linked glycolate oxidase found in peroxisomes or microbodies (Tolbert, 1971). As a result of the reaction, O$_2$ is consumed and the H$_2$O$_2$ produced must be destroyed by catalase, which is also localized in peroxisomes (Nelson and Tolbert, 1970). In cyanobacteria and most unicellular algae, glycolate is oxidized by an anaerobic glycolate dehydrogenase which does not produce H$_2$O$_2$ and would not require close proximity to catalase (Frederick, et al, 1973; Grodzinski and Colman, 1970). The total catalase activity of unicellular green algae is significantly less than found in plant leaves (Nelson and Tolbert, 1970). The total activity of glycolate dehydrogenase per unit of photosynthesis is far less (<10%) than that of glycolate oxidase in plants, and may account for the tendency for unicellular algae and cyanobacteria to excrete glycolate (Tolbert, 1980). Tolbert (1980) speculated that glycolate may be produced faster than it can be metabolized.

Five environmental factors are known to effect the extent of glycolate formation during photosynthesis: CO$_2$, O$_2$, pH, light intensity, and temperature. Low CO$_2$ partial pressure and high O$_2$ partial pressure enhance glycolate biosynthesis and excretion (Asami and Akazawa, 1974; Han and Eley, 1973; Storrø and McFadden, 1981; Tolbert, 1980; Beck, 1979). At a constant O$_2$ partial pressure, glycolate
production is inversely proportional to the CO₂ concentration up to levels which saturate CO₂ fixation. At saturating levels of CO₂, oxygen concentrations higher than that of air increase the proportion of fixed carbon which is incorporated into glycolate, whereas at lower O₂ levels the proportion is unchanged (Eickenbusch and Beck, 1973). At low CO₂ levels, glycolate formation depends on the oxygen concentration. These observations appear to result from competition between CO₂ and O₂ for the carboxylation or oxygenation of RuDP by the enzyme RuDP carboxylase-oxygenase.

Glycolate formation and excretion in algal cultures increase with the pH of the medium, reaching maximal rates at pH values between 8 and 9 (Orth, et al, 1966). This may be explained by the observation that in some plants the pH optimum of RuDP oxygenase activity is near 9.3, whereas the pH optimum of RuDP carboxylase activity is approximately 7.8 (Andrews, et al, 1973).

Light is required for photorespiration and glycolate production in plants and algae, due to their need for the regeneration of RuDP, which depends ultimately on light generated ATP and NADPH. Although the carboxylation reaction during photosynthesis is saturated at intermediate light intensities, the oxygenation reaction does not become saturated even in full sunlight (Tolbert, 1974).
High temperature stimulates glycolate synthesis and photorespiration in plant leaves, partially because increasing temperature reduces the affinity of RuDP carboxylase-oxygenase for CO$_2$ (Ogren, 1984). Responses to elevated temperature are also due to changes in relative solubilities of CO$_2$ and O$_2$ (Hall and Keys, 1983). As temperature increases, the solubilities of both atmospheric O$_2$ and CO$_2$ in pure water decrease, but the relative solubility of O$_2$ is greater (Ku and Edwards, 1977).

**Research Objectives**

The conditions within hot spring microbial mats should favor glycolate production by phototrophs and crossfeeding to heterotrophs. Temperature is high, and light intensity above the water often reaches 2000 microeinsteins m$^{-2}$ s$^{-1}$. Studies with O$_2$ and pH microelectrodes show that conditions in hot spring microbial mats such as Mushroom and Octopus Springs in Yellowstone National Park are near the optimum for glycolate synthesis and excretion in the photic zone of the mats (Revsbech and Ward, 1984). Superoxic conditions prevail in the top 3 mm of the mat during many of the daylight hours. The peak oxygen concentration in the Octopus Spring mat at 55°C occurs shortly after solar noon and reaches approximately six times that of the overlying water. For the majority of the daylight hours, the pH of the photic zone is above 8.4, and pH remains greater than
9.4 for several hours. At the pH levels found in the mat, most of the inorganic carbon present would be in the form of $\text{HCO}_3^-$, rather than $\text{CO}_2$, the chemical species used by RuDP carboxylase-oxygenase. The environmental conditions of extremely high $\text{O}_2$ concentrations, high pH (thus low $\text{CO}_2$), high light intensity and high temperature suggest that production and photoexcretion of glycolate by *S. lividus* could be an important process in this mat. The major objective of my research was to investigate the importance of glycolate production and consumption in an alkaline silicious hot spring microbial mat and in pure cultures of the major mat phototroph, *S. lividus*. 
MATERIALS AND METHODS

Study Area

Experiments were carried out at Mushroom Spring in the Lower Geyser Basin of Yellowstone National Park. This alkaline hot spring (pH 8.3) is located about 0.2 km northeast of Great Fountain Geyser along the Howard Eaton Trail. Experiments were conducted on samples collected from the cyanobacterial mat in a 55-58°C region of the effluent channel which fanned out approximately 10 m from the source pool. The site was chosen because Revsbech and Ward (1984) and Doemel and Brock (1977) showed that primary productivity was maximal in this temperature range. Results of pilot experiments also indicated that photosynthetic uptake of $^{14}$CO$_2$ was higher at Mushroom Spring than at Octopus Spring, where previous studies have been performed by researchers from this laboratory.

Pure Culture Methods

An axenic culture of *S. lividus* was provided by Dr. Richard Castenholz. This strain, Y-7c7b-S, was isolated from Clearwater Spring in Yellowstone National Park. Stock cultures were maintained in 125 ml Erlenmeyer flasks containing 80 ml of liquid medium D (Castenholz, 1969).
inoculation of cultures was performed so that the initial concentration of cells was $10^5$ to $10^6$ cells/ml. Flasks were incubated at 45°C with constant illumination by two 20 watt cool white fluorescent lights placed 15 cm above the liquid in the flasks.

For radiolabeling experiments, cultures were grown in Roux bottles containing 800 ml liquid medium DG (Castenholz, 1969), and incubated in a 30 liter aquarium water bath maintained at 50°C with a circulator immersion heater. The aquarium was illuminated with three 20 watt cool white fluorescent lights on one side and two 60 watt incandescent light bulbs on the opposite side. Aeration was provided by an aquarium air compressor with variable flow control. The flow rate was approximately 150 ml/min. The air was passed through three sterile filters of glass wool, and then bubbled through sterile distilled water to provide humidity. Air was bubbled into the culture medium through an air stone, the air outflow was connected to a sterile test tube. Samples could be removed aseptically through a glass tube which was immersed in the culture by stopping the air outflow, so that the pressure created in the vessel forced the culture sample out through the tubing into a test tube or flask.

Inoculation of *S. lividus* was performed so that the initial concentration of cells in the Roux bottle was approximately $1 \times 10^6$ cells/ml. Cell densities were
determined by counting cells in a Petroff-Hauser cell counter. During logarithmic growth, the doubling time of the culture was approximately 6 hours. Portions of the culture were harvested for radiolabeling experiments during the middle or late logarithmic phase of growth. Cells removed from the Roux bottle were kept at 50°C in a flask immersed in the water bath while samples were prepared as described below.

**Sampling**

At the hot spring study area, suspensions of cells from the mat surface were prepared by scraping off portions of the top 0-2 mm of the mat surface with a spatula, and gently homogenizing in spring water using a tissue grinder (Wheaton Scientific, No. 357546, using pestle "B" with clearance of 0.140-0.655mm). Some samples were diluted further with additional spring water. Pure cultures of *S. lividus* grew as a homogeneous suspension of cells, so that only gentle mixing was necessary to prepare cells. Some suspensions were diluted in supernatant liquid of the culture medium. The supernatant liquid was prepared immediately before the radiolabeling experiments (described below) by removal of cells by centrifugation.

In experiments on both the pure culture and mat samples, two or five ml aliquots of cell suspension were added to 1 or 2-dram (ca. 3.7 or 7.4 ml) vials. Recessed
butyl rubber stoppers (A. H. Thomas; size 00) were used to seal vials. Stoppers were secured by taping over the top and around the vial neck with electrician's black tape. For dark incubations, vials were completely wrapped with black tape.

Mat samples contained in vials were incubated in natural sunlight, immersed in water at least 1.5 cm deep over the mat from which the cells had been collected. Experiments were performed between two hours before and three hours after solar noon and light intensities above the water surface ranged from 800 to 2000 microeinsteins m\(^{-2}\) s\(^{-1}\) as measured by a LICOR quantum sensor (Lambda Instruments), which senses only in the 400 to 700 nm region. Pure culture samples were incubated on racks immersed 2 cm deep in the 50°C aquarium water bath. Light for the pure culture samples was provided by three 20 watt cool white fluorescent lights set 10 cm above the incubation rack. Light intensity at the surface of the water was approximately 10% of full sunlight.

A variety of incubation conditions were employed for the different experiments described in the Results section. In some vials, oxygen concentration was increased by running a stream of pure oxygen into the vial headspace for 15 seconds while the stopper was being secured. In other vials, 0.2 ml 100% CO\(_2\) was injected into the air headspace of the vials, resulting in approximately 20% CO\(_2\) in the
headspace. When testing the effect of CO$_2$ addition it was necessary to add glycylglycine to 0.1 M to buffer pH at 8.2. In this case controls also contained glycylglycine. In other samples CO$_2$ concentration was decreased by removal of carbon dioxide species from the spring water with which the cell homogenate was prepared. Spring water was acidified to pH 3, sparged with nitrogen for 30 minutes, and returned to the original pH by addition of 1 N NaOH. The CO$_2$-free water was used to make an extremely thick homogenate of the Mushroom Spring mat, as described above. Control samples were prepared by diluting the thick homogenate in natural spring water thus restoring CO$_2$ concentrations to near the natural level. The samples with decreased CO$_2$ were prepared by diluting the homogenate in an equal volume of CO$_2$-free spring water. This produced similar cell densities in the control and CO$_2$-depleted samples.

In some experiments 2-pyridylhydroxymethanesulfonic acid, or HPMS (Sigma Chemical Co.) was added to inhibit glycolate oxidase. Vials were injected with 0.2 ml of ten-fold concentrated aqueous stock solutions adjusted to the pH of Mushroom Spring water. The range of final concentrations tested was from $10^{-1}$ M to $10^{-6}$ M HPMS.

Some pure culture samples of $S. \text{ lividus}$ were preincubated with unlabeled glycolate (Sigma Chemical Co.) for four hours before incubation with radiolabeled
glycolate (see Radiolabeling Experiments). Vials were injected with 0.2 ml of a $10^{-3}$ M aqueous stock solution adjusted to the pH of the culture medium, so that the final concentration was $10^{-4}$ M glycolate.

In one experiment on Mushroom Spring samples, unlabeled glycolate (Sigma) or sodium acetate (Baker Chemicals) was added before incubation with radiolabeled NaH$^{14}$CO$_3$ (see below). Vials were injected with 0.2 ml of ten-fold concentrated aqueous stock solutions adjusted to pH 7.5. Final concentrations were $10^{-2}$ or $10^{-3}$ M.

Radiolabeling Experiments

Photosynthetic uptake of CO$_2$ and excretion of organic carbon was measured by addition of 0.2 ml (2 uCi) of a 10 uCi/ml stock solution of NaH$^{14}$CO$_3$ (50 mCi/mmol, New England Nuclear) diluted in sterile distilled water (pH 8.0). Fates of 1$^{14}$C-labeled glycolate (50 mCi/mmol, ICN Pharmaceuticals, Inc.), 2$^{14}$C-acetate (54 mCi/mmol, New England Nuclear) or U$^{14}$C-protein hydrolysate (57 mCi/mAtom, Amersham Corp.) were measured by addition of 0.2 ml (2 uCi) from 10 uCi/ml stock solutions. The homogenized samples, described above, were preincubated on their sides for 15-30 minutes before injection of radiolabeled substances, then incubated for 2 hours.
Three different methods were used for terminating biological activity after 2 hour incubations with NaH$^{14}$CO$_3$. In most experiments performed in the field and lab, samples were poisoned by injection of 0.1 ml of formaldehyde solution (37.1%, Baker Chemicals). Samples to be analysed for glycolate excretion were usually frozen on dry ice. These two methods were compared to immediate separation of cells and supernatant by filtering and centrifuging as described below. Biological activity in samples labeled with $^{14}$C labeled glycolate, acetate or protein hydrolysate was always terminated by injection of 0.1 ml formalin. In abiological controls, the same amount of formalin was added before adding radiolabel.

Analyses of gas headspace subsamples for $^{14}$CO$_2$ were made as described in the Analytical Methods section. Radioactivity incorporated into cells was determined by filtering a homogeneous aliquot (0.1-0.5 ml) of each sample through a 0.45 um membrane filter (Millipore Corp.). To remove background radioactive material, the filters were rinsed with at least 3.0 ml of either Medium D, or prefiltered Mushroom Spring water. The filters were dried, and exposed to concentrated HCl fumes overnight to remove radioactive carbon dioxide species. The filters were placed in 10 ml of Aquasol (New England Nuclear), and
radioactivity was determined with a Packard Tri Carb 460 CD Liquid Scintillation System using the sample channels ratio method to determine disintegrations per minute (dpm).

Radioactively labeled organic carbon present in the aqueous phase was also determined. To obtain the cell-free aqueous phase the homogenate was centrifuged at 3000 g for 30 minutes in a Sorvall GLC-1 centrifuge. In one field experiment to compare killing methods, centrifugation was for 2 minutes at 15,600 g in a Brinkmann Eppendorf Centrifuge 5414. After centrifugation, 1.0 ml of the supernatant liquid was acidified with 0.1 ml 6N HCl and bubbled with air for 30 minutes, a period sufficient to remove NaH\textsuperscript{14}CO\textsubscript{3} from test vials. Aquasol (2.0 ml) was then added and radioactivity was determined as described above, using the internal standard method to determine dpm. After calculating radioactivity in cells and aqueous phase per milliliter of original homogenate, the percent of total photosynthate in the aqueous phase was determined by dividing the radioactivity in the aqueous phase alone by that in cells and the aqueous phase.

**Analytical Methods**

\textsuperscript{14}CO\textsubscript{2} Analysis

Total amounts of radioactively labeled carbon dioxide species were determined by analysis of subsamples of the gas headspace of vials following the method of Ward and
Olson (1980). 0.2 ml gas samples were removed using an air-flushed Glasspak syringe (Becton-Dickinson) attached to a Mininert valve (Supelco) to eliminate loss of sample due to pressure differences. Samples were injected into a Carle model 8500 thermal conductivity gas chromatograph connected by a teflon line to a Packard model 894 gas proportional counter (GPC). The gas chromatograph contained a stainless steel column (2.3 meters by 3.18 mm O. D.) packed with Poropak N, 80-100 mesh, through which helium flowed at a rate of 21 ml/minute. Helium make-up gas was added to increase the flow rate to 70 ml/minute. Propane was added at 10% of the total flow rate as a quench gas. Radioactivity of labeled gasses was quantified as area units on a Spectra-Physics Minigrator. The conversion factor of area units to dpm was determined by comparison of standard samples analysed by the GPC or counted by liquid scintillation counting. Total amounts of $^{14}\text{CO}_2$ per vial were determined by correction for the difference between subsample and total volume, and for gas solubility and dissociation equilibria by the method of Stainton (1973).

**Autoradiography**

Autoradiograms of cell material labeled with $1^{-14}\text{C}-\text{glycolate}$ were prepared according to the method of Brock and Brock (1968). Homogeneous samples were fixed to slides, rinsed to remove residual $1^{-14}\text{C}-\text{glycolate}$, coated
with liquid photographic emulsion (Kodak NTB-2), and exposed in the dark for 3 weeks at room temperature. The slides were developed in Kodak D-19 developer for 2 minutes and fixed with Kodak acid fixer for 5 minutes at 22°C. Slides were observed using interference contrast microscopy at 500X total magnification with a Leitz Ortholux II microscope. Photomicrographs were taken as described by Sandbeck and Ward (1981), with a Nikon Microflex model EFM semi-automatic photomicrograph attachment using Kodak Plus-X or Panatomic-X film. Enlargements were made on high contrast silver bromide print paper (Koda Bromide F-4).

$^{14}$C-Glycolate Determination

The amount of radioactively labeled glycolate present in the aqueous phase of homogenized samples from Mushroom Spring mat and pure cultures of $S.\ lividus$ after incubation with NaH$^{14}$CO$_3$ was determined. Material analysed for $^{14}$C-glycolate was the supernatant liquid of samples that had been killed by freezing or by immediately filtering as described in the section on radiolabeling experiments. Samples were clarified by centrifugation, acidified and bubbled to remove inorganic carbon, then analysed by ion exchange and thin layer chromatography as outlined in Figure 2.
Figure 2. Analysis of radioactivity in various fractions of homogenized samples. Radioactivity of those fractions enclosed in boxes was counted and used to quantify glycolate production.
Ion Exchange Chromatography

The aqueous phase samples (4.0 ml) were partially purified by passage through a strongly basic anion exchange column (Dowex 2X8-400, Sigma Chemical Co.) preconditioned with formate. The resin bed, prepared in a disposable Pasteur pipet with 6 mm inside diameter, was 5 cm in length. Samples were made alkaline by addition of one drop of 6 N NaOH before they were applied to the column. After application of the sample, the column was washed with 4.0 ml of distilled water, and then eluted with 8.0 ml 1 M formate. Fractions of 1.0 ml were collected in 1.5 ml microcentrifuge tubes (Arthur H. Thomas, Co.). Radioactivity present in 50 ul aliquots of each fraction was counted as described above.

Standards of 1-14C-glycolate diluted to 0.3 uCi/ml were run through the column to determine which fractions to collect from experimental samples for further analysis by thin layer chromatography. Fractions 10 through 14 contained 97.7% of the radiolabeled glycolate eluted from the column. This was 94.6% of the glycolate that had been applied to the column.

For each sample, the glycolate-containing fractions were combined in a 15 ml plastic centrifuge tube, frozen at -70°C, and lyophilized. Samples were resuspended in 0.5-1.0 ml methanol, transferred to 1.5 ml microcentrifuge tubes, and centrifuged for 2 minutes in a Brinkmann
Eppendorf Centrifuge (Model 5414) to remove insoluble material. The methanol solution was transferred to another 1.5 ml microcentrifuge tube and evaporated under a stream of nitrogen gas. The sample was resuspended in 50 or 100 ul methanol. The average percent recovery of these procedures was 81.9% for glycolate standards.

**Thin Layer Chromatography**

Using the thin layer chromatography (TLC) method of Bleiweis, et al (1967), radioactively labeled glycolate was separated from other compounds in the samples purified by ion exchange chromatography. Gelman ITLC, type SG chromatography medium (Gelman Instrument Co., Ann Arbor, Michigan) was used. The medium consists of glass microfiber support sheets which are impregnated with silica gel as the adsorbent. The sheets (20 X 20 cm) were spotted with 5.0 ul of the methanol solutions described above. Replicate 5.0 ul samples of the solutions were counted by liquid scintillation counting as described above.

The following solvent mixtures were used for two-dimensional development: (1) petroleum ether (b.p. 30-60°C), anhydrous diethyl ether, and formic acid (28/12/1), followed by (2) chloroform, methanol, and formic acid (80/1/1) (reagent grade solvents from J. T. Baker Chemical Co.). The development chamber was rectangular with the following dimensions: 7 X 27 X 24 cm. The first solvent
mixture was allowed to ascend 15 minutes, sheets were air
dried in a hood for 5 minutes, and the second solvent
mixture was allowed to ascend for 20 minutes in the
direction perpendicular to solvent 1.

Radioactively labeled spots were visualized by
autoradiography. Sheets were treated with surface
autoradiograph enhancer (En³HANCE spray, New England
Nuclear, Inc.). In complete darkness, the sheets were
placed in cardboard film holders with 8 X 10 in Kodak X-
OMAT AR film and clamped firmly together. The film was
exposed for 7 days at -70°C, then developed for 5 minutes
at 21°C in Kodak GBX Developer-Replenisher and fixed in
Kodak liquid fixer.

Dark spots visible on the autoradiogram were traced on
the glass microfiber chromatography sheet. The spots were
cut out of the sheet and placed in 10 ml Aquasol liquid
scintillation cocktail for determination of radioactivity
as described above. ¹⁴C-labeled-glycolate was identified
by comigration with 1-¹⁴C-glycolate and unlabeled glycolate
visualised by spraying with 0.04% bromphenol blue
(ethanolic).

Recovery of radioactivity from all spots on a TLC
plate averaged 81.8% of that spotted on the plate; recovery
of glycolate standards was not significantly different,
77.6%. Since the loss of glycolate was equivalent to the
loss of all compounds, total radioactivity recovered from
all spots on a TLC plate was divided into the amount of radioactivity in the glycolate spot of the plate to calculate the proportion of glycolate in the sample that had been spotted. The sample spotted on the TLC plate was the acid fraction of the total aqueous photosynthate that had been separated by ion exchange chromatography. Thus, to determine the percentage of glycolate in the entire aqueous photosynthate, the proportion of radioactivity in glycolate on the TLC plate was multiplied by the percentage of radioactive material applied to the ion exchange column which eluted in the acid fraction. The importance of glycolate can also be expressed as a percentage of total photosynthetically fixed carbon (\(^{14}\text{C}\) in cells and aqueous phase). This was determined by multiplying the percent of glycolate in the aqueous phase by the percent of total photosynthate present in the aqueous phase.

Dissolved Oxygen Assay

Dissolved oxygen in cell homogenates was assayed iodometrically using the azide modification (Am. Pub. Hlth. Assoc., 1985). The procedure was modified to accommodate small sample sizes. The manganous sulfate solution and the alkali-iodide-azide reagent were diluted to one tenth strength. All reagents were stored under a N\(_2\) headspace. After incubation, 2.0 ml of the liquid sample was removed with an N\(_2\)-flushed syringe and injected into an N\(_2\)-flushed,
stopped 2-dram vial containing 0.2 ml manganous sulfate solution. Alkali-iodide-azide reagent (0.2 ml) was then injected. After the precipitate settled to approximately half the vial volume, 0.2 ml 6N H₂SO₄ was injected. Samples were returned to the laboratory and titrated within eight hours of the end of the experiment using sodium thiosulfate diluted to one twentieth strength. A standard curve obtained by measuring O₂ in solutions equilibrated with N₂, air or O₂ was used to calculate the conversion factor from ml titrant to mM O₂. Control samples of Mushroom Spring or distilled water, which had been bubbled with N₂ to remove O₂, showed that no O₂ contamination occurred using this technique.

**Statistical Methods**

In all experiments, two sample t-test was used to compare means of replicate samples. Statistical programs were from MSUSTAT, version 2.20 (Lund, 1983). All experiments were repeated at least once, unless otherwise noted.
RESULTS

Inorganic Carbon Fixation in the Light and Dark

Homogenized samples of Mushroom Spring mat and pure cultures of *S. lividus* exhibited 40 to 50 times higher incorporation of NaH\(^{14}\)CO\(_3\) into cells when incubated in the light than when incubated in the dark (Table I). In all samples, radioactively labeled organic carbon was detected in the extracellular or aqueous phase of the homogenate. In Mushroom Spring mat samples, the amount of radioactive material detected per ml was over three times greater in the light samples than in samples incubated in the dark, indicating the production of extracellular photosynthetically derived carbon. The percent of total radioactive material fixed that was detected in the aqueous phase was much higher in the dark samples, 75.7% compared to 21.7% (Table I), because less \(^{14}\)C was fixed into cells.

In pure cultures of *S. lividus*, the amount of radioactive material detected in the aqueous phase was 35 times greater in the light than in the dark. A high percentage of photosynthate was detected in the aqueous phase of samples incubated in light and dark.
Table 1. Inorganic carbon fixation in the light and dark. Samples were from the Mushroom Spring mat and a pure culture of *S. lividus*.a

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cells dpm/ml</th>
<th>Aqueous dpm/ml</th>
<th>% $^{14}$C in the Aqueous Phase b</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mushroom Spring</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Light</td>
<td>1,250,000</td>
<td>340,500</td>
<td>21.7</td>
</tr>
<tr>
<td>Dark</td>
<td>32,000 *</td>
<td>100,000 *</td>
<td>75.7 *</td>
</tr>
<tr>
<td><strong>S. lividus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Light</td>
<td>164,700</td>
<td>185,800</td>
<td>53.0</td>
</tr>
<tr>
<td>Dark</td>
<td>3,200 *</td>
<td>5,300 *</td>
<td>64.6</td>
</tr>
</tbody>
</table>

a Biological activity terminated with formalin. In samples injected with formalin before addition of NaH$^{14}$CO$_3$, radioactivity detected in cells and aqueous fractions was less than 1.0% of that in light samples.

b The percent $^{14}$C in the aqueous phase aqueous equals:

$$\frac{\text{aqueous phase dpm/ml}}{\text{cells dpm/ml + aqueous phase dpm/ml}}$$

* indicates significant differences (p<0.05) of dark samples compared to light samples.
Environmental Effects on Production of Extracellular Photosynthate

Effects of Oxygen

In both Mushroom Spring mat and S. lividus samples, oxygen addition resulted in a statistically significant increase in the percent of total photosynthate detected in the extracellular fraction (Table 2). In the oxygen enriched samples from Mushroom Spring, the sum of radioactivity detected in cell and aqueous fractions is less than that of the control (30% of the control). Thus, oxygen enrichment not only increased the percent in the extracellular fraction, but it decreased photosynthetic activity in general. In the pure culture of S. lividus, the decrease in photosynthetic activity (cells plus aqueous fraction) of the oxygen enriched samples was not as dramatic (76% of the control). In both Mushroom Spring and S. lividus samples, $^{14}$C in both cells and aqueous phase decreased upon oxygen enrichment, but there was a greater proportionate decrease in the cellular fraction.

To evaluate the effectiveness of oxygen addition to the headspace on the dissolved oxygen concentration within samples, dissolved oxygen was measured directly. Oxygen concentration varied with time during incubation of homogenized mat samples in closed vials (Figure 3). The
Table 2. The effect of oxygen addition on the percent of total photosynthate in the aqueous phase. Mushroom Spring mat samples and pure cultures of *S. lividus* were incubated in the light.a

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cells dpm/ml</th>
<th>Aqueous dpm/ml</th>
<th>% $^{14}C$ in the Aqueous Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mushroom Spring</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>89,100</td>
<td>42,000</td>
<td>35.3</td>
</tr>
<tr>
<td>+ O$_2$</td>
<td>17,800 *</td>
<td>22,200 *</td>
<td>56.1 *</td>
</tr>
<tr>
<td><strong>S. lividus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>23,300</td>
<td>28,000</td>
<td>54.1</td>
</tr>
<tr>
<td>+ O$_2$</td>
<td>14,900</td>
<td>24,400</td>
<td>62.4 *</td>
</tr>
</tbody>
</table>

*a* Biological activity was terminated with formalin.

* indicates significant differences (p<0.05) between oxygenated samples compared to the controls; n=6.
Figure 3. Dissolved oxygen concentration during incubation of homogenized samples of Mushroom Spring mat with O₂ or air headspace. Bars indicate standard error (n=3).
oxygen concentration in samples with oxygen added to the headspace of the vials was on average 2.6 times higher than the concentration in samples incubated with air in the headspace. Mushroom Spring water overlying the mat had an oxygen concentration of 150 uM. The peak oxygen concentration in samples with added oxygen was 800 uM, or 5.3 times that of the overlying spring water.

Effects of Carbon Dioxide

Removal of CO₂ by gas stripping of Mushroom Spring water increased the percent of total photosynthate detected in the aqueous phase of homogenized mat samples (Table 3). The greater uptake of radioactivity in these samples compared to controls was expected due to increased specific activity of the ¹⁴CO₂, and suggests that the CO₂ concentration was effectively lowered.

In the pure culture of S. lividus, the percent of total photosynthate in the aqueous phase decreased when CO₂ concentration was increased (Table 3). In the CO₂-enriched samples, less radioactivity was detected in both cells and aqueous phase, due to the decreased specific activity of NaH¹⁴CO₃ which occurred upon addition of CO₂.

In another experiment performed with Mushroom Spring mat samples (data not shown), 1.0 ml CO₂ was added to a 1.0 ml headspace to give about 50% CO₂ in the gas phase. As with the pure culture, the percent of photosynthate in the
aqueous phase decreased in the CO₂-enriched samples, but the difference was not statistically significant. Radioactivity incorporated into cells in both the control and the CO₂-enriched samples was low in this experiment. In other experiments (also not shown), addition of CO₂ to achieve 2% CO₂ in the headspace did not affect the percentage of photosynthate in the aqueous phase.

Table 3. The effects of carbon dioxide removal or addition on percent of total photosynthate in the aqueous phase. Mushroom Spring mat samples and pure cultures of S. lividus were incubated in the light.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cells dpm/ml</th>
<th>Aqueous dpm/ml</th>
<th>% ¹⁴C in the Aqueous Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mushroom Spring</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>37,700</td>
<td>15,200</td>
<td>29.8</td>
</tr>
<tr>
<td>CO₂-free</td>
<td>154,500 *</td>
<td>84,800 *</td>
<td>37.4 *</td>
</tr>
<tr>
<td>S. lividus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>23,500</td>
<td>28,100</td>
<td>54.1</td>
</tr>
<tr>
<td>+ CO₂</td>
<td>8,700 *</td>
<td>8,700 *</td>
<td>48.3 *</td>
</tr>
</tbody>
</table>

a Biological activity was terminated with formalin.

* indicates significant differences (p<0.05) between treated samples compared to the control; n=6.
The Importance of Glycolate as an Excretion Product

Using ion exchange and thin layer chromatography (TLC), \(^{14}\text{C}\)-glycolate was separated from other compounds in the photosynthate of the aqueous fraction following a \(\text{NaH}^{14}\text{CO}_3\) labeling experiment. Figure 4 shows two TLC plates on which compounds from the organic acid fraction of the aqueous phase were separated. Only the identity of glycolate was determined. Other radioactive compounds, including those which remained at the origin, and compounds which eluted from the ion exchange column in the neutral fraction were not identified.

Extracellular Products in the Light and Dark

Glycolate was the major product detected in the aqueous phase of samples incubated in the light (Figure 4 and Table 4). In the example shown in Figure 4a glycolate comprised 85% of the radiolabel detected on the TLC plate. Compounds other than glycolate were also detected in the organic acid fraction. Figure 4a illustrates the locations of these acidic radioactively labeled compounds on TLC plates. The relative importance of each of the extracellular compounds for this sample was calculated and is listed in Table 4 as a percent of the total carbon in the aqueous phase. Glycolate represented 55.4% of the radioactivity in the aqueous phase for this sample. Note that the material spotted on the TLC plates was only 65% of
Figure 4. Thin layer chromatograms of radioactively labeled products of CO$_2$ reduction found in the aqueous phase of samples from the Mushroom Spring mat incubated in the (a) light (with added oxygen) or (b) dark. The position of glycolate, as determined by analysis of standards, is indicated by the dashed line. The solvent systems employed were: (1) petroleum ether (b.p. 30-60$^\circ$), anhydrous ether, and formic acid (28/12/1) and (2) chloroform, methanol, and formic acid (80/1/1).
Table 4. Importance of glycolate and other compounds as extracellular products of CO₂ reduction in the Mushroom Spring mat. Samples were incubated in the light (with added oxygen), or dark, and results are expressed as a percent of the total aqueous organic carbon.a

<table>
<thead>
<tr>
<th>Compound(s)</th>
<th>% of Aqueous Fixed Carbon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Origin</td>
<td>5.2</td>
</tr>
<tr>
<td>Glycolate</td>
<td>55.4</td>
</tr>
<tr>
<td>1</td>
<td>2.3</td>
</tr>
<tr>
<td>Light</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.4</td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
</tr>
<tr>
<td>4</td>
<td>1.2</td>
</tr>
<tr>
<td>Total</td>
<td>65.0</td>
</tr>
<tr>
<td>Origin</td>
<td>3.0</td>
</tr>
<tr>
<td>Dark</td>
<td></td>
</tr>
<tr>
<td>D1</td>
<td>65.2</td>
</tr>
<tr>
<td>D2</td>
<td>26.8</td>
</tr>
<tr>
<td>Total</td>
<td>95.0</td>
</tr>
</tbody>
</table>

aData derived from the thin layer chromatograms illustrated in Figure 4. Biological activity was terminated by filtration and centrifugation.

The total material excreted in the light (Table 4). The neutral portion of the extracellular material may contain sugars, amino acids, or other uncharged compounds which did not bind to the ion exchange column.

In samples incubated in the dark, glycolate was not detected (Figure 4b). Practically all (95%) of the excreted material eluted with the acidic fraction from the ion exchange column (Table 4), and most (65%) of the radioactivity was found in one spot (D1) on the TLC plate.
Effects of Oxygen on Glycolate Production

Table 5 shows that oxygen enrichment increased glycolate production in Mushroom Spring mat samples incubated in the light. In this experiment, the percent of total photosynthate in the aqueous phase increased with oxygen addition, by 1.67 times that of the control. The importance of glycolate expressed as a percentage of extracellular photosynthate increased three-fold with oxygen addition. In these same oxygen-rich samples, the importance of glycolate expressed as a percentage of total photosynthetically fixed carbon increased nearly five-fold compared to controls.

Table 5. Effects of oxygen addition on the importance of glycolate as an extracellular photosynthetic product in Mushroom Spring mat samples.\(^a\)

<table>
<thead>
<tr>
<th>Condition</th>
<th>( ^{14})C in the aqueous phase</th>
<th>% of aqueous photosynthate as glycolate</th>
<th>% of total photosynthate as glycolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18.3</td>
<td>8.5</td>
<td>1.5</td>
</tr>
<tr>
<td>+ O(_2)</td>
<td>30.5</td>
<td>25.2 *</td>
<td>7.4 *</td>
</tr>
</tbody>
</table>

\(^a\) Samples were frozen to stop biological activity.

* indicates significant differences (p<0.05) of oxygenated samples compared to the control; n=3.
Factors Influencing Determination of Glycolate Production

In experiments conducted at Mushroom Spring, biological activity was terminated either by freezing or by formalin addition. It seemed likely that these killing methods might cause cells to leak intracellular fixed carbon, so that organic carbon analysed in the aqueous phase would not represent excreted photosynthate. Using the pure culture of *S. lividus*, the two methods used to terminate biological activity in field experiments were compared to immediate separation of cells and aqueous phase by filtration and centrifugation (Table 6). Freezing and formalin killing increased the percentage of photosynthate.

Table 6. Effects of killing methods on the determination of percent of total photosynthate in the aqueous phase of *S. lividus* cultures.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cells dpm/ml</th>
<th>Aqueous dpm/ml</th>
<th>% (^{14}\text{C}) in the Aqueous Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filtered</td>
<td>560,400</td>
<td>99,400</td>
<td>18.2</td>
</tr>
<tr>
<td>Formalin</td>
<td>285,600 *</td>
<td>163,200</td>
<td>43.9 *</td>
</tr>
<tr>
<td>Frozen</td>
<td>542,000</td>
<td>448,700 *</td>
<td>47.9 *</td>
</tr>
</tbody>
</table>

* indicates significant differences (p<0.05) of formalin-killed or frozen samples compared to filtered samples; n=6.
recovered in the aqueous phase approximately 2.5 times compared to those determined after immediate separation of cells and aqueous phase by filtration and centrifugation (Table 6).

In another experiment with *S. lividus*, the effect of freezing on the importance of glycolate in the aqueous fraction was investigated. As in the previous experiment, the immediately filtered samples exhibited a significantly lower percent of total photosynthate in the aqueous phase, 7.9% compared to 35.5% in the frozen sample (Table 7). However, the importance of glycolate expressed as a percentage of excreted photosynthate was significantly greater in the immediately filtered samples, 7.1% compared to 0.6% in the frozen samples (Table 7), suggesting that $^{14}$C-glycolate was diluted by other radioactive material

<table>
<thead>
<tr>
<th>Condition</th>
<th>$^{14}$C in the aqueous phase</th>
<th>% of aqueous photosynthate as glycolate</th>
<th>% of total photosynthate as glycolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frozen</td>
<td>35.5</td>
<td>0.62</td>
<td>0.22</td>
</tr>
<tr>
<td>Filtered</td>
<td>7.9 *</td>
<td>7.1 *</td>
<td>0.56 *</td>
</tr>
</tbody>
</table>

* indicates significant differences (p<0.05) of filtered samples compared to frozen samples.
extracted from the cells by freezing. The low percentage of glycolate expressed as a percent of the total fixed carbon (0.2%-0.6%) suggested that laboratory incubation conditions did not favor glycolate production by *S. lividus*.

A similar experiment was run to test the effects of freezing on the importance of glycolate in the aqueous phase of Mushroom Spring mat samples under the various incubation conditions used in field experiments. Samples were incubated in the light, with or without oxygen addition (Table 8). As in previous experiments (see Tables 6 & 7), freezing increased the amount of total photosynthate detected in the aqueous fraction. In both control and oxygenated samples, the percent of aqueous photosynthate as glycolate was higher when samples were filtered and centrifuged immediately, but the increases were not statistically significant. Thus, dilution of glycolate by other radioactive material extracted from the cells by freezing was not as dramatic as demonstrated in the pure culture of *S. lividus* (see Table 7). Glycolate comprised 46% of the excreted photosynthate in filtered controls, and 58% of excreted photosynthate in the presence of added O₂. Glycolate was apparently extracted from the cells by freezing as well as excreted from the cells, since the percent of total photosynthate as glycolate was higher in the frozen samples (Table 8).
Table 8. Effects of freezing on the importance of glycolate as an extracellular photosynthetic product in Mushroom Spring mat samples. Samples were incubated with and without oxygen addition.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Condition</th>
<th>% \textsuperscript{14}C in the aqueous phase</th>
<th>% of aqueous photosynthate as glycolate</th>
<th>% of total photosynthate as glycolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frozen</td>
<td>20.8</td>
<td>37.9</td>
<td>6.8</td>
</tr>
<tr>
<td>Filtered</td>
<td>9.4 *</td>
<td>45.8</td>
<td>4.3 *</td>
</tr>
<tr>
<td>+ O\textsubscript{2}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frozen</td>
<td>25.4</td>
<td>50.1</td>
<td>12.8 #</td>
</tr>
<tr>
<td>Filtered\textsuperscript{b}</td>
<td>11.8 * #</td>
<td>57.9 #</td>
<td>6.8 * #</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Dissolved oxygen concentration was measured in samples of the same homogenate used in this experiment; data presented in Figure 3.

\textsuperscript{b} Includes example shown in Figure 4a and Table 4.
Interactions Within the Mat Community

A series of experiments was designed to assess the flux of glycolate between the organisms which produce it and those which consume it in the natural mat community. These experiments concerned the fate of glycolate and the effect of interrupting flow between producer and consumer by various techniques.

Fate of $1^{-14}$C-glycolate

In homogenized, aerobic samples of Mushroom Spring mat incubated in the light, the major fate of $1^{-14}$C-glycolate was incorporation into cells as opposed to oxidation to $^{14}$CO$_2$ (Table 9). In pure cultures of *S. lividus*, incorporation of $1^{-14}$C-glycolate into cells or oxidation to $^{14}$CO$_2$ was negligible. Extending the incubation time to the doubling time of the culture (6 hours), did not increase the uptake or metabolism of glycolate. Preincubation with unlabeled glycolate did not induce the metabolism of glycolate by *S. lividus*.

Autoradiograms prepared from light incubated aerobic samples of Mushroom Spring mat homogenates revealed that filamentous cells incorporate $1^{-14}$C-glycolate. Cells shaped like *S. lividus* did not exhibit incorporation of the compound. Figure 5 shows representative photomicrographs of autoradiograms.
Table 9. Fate of $^{14}$C-glycolate in Mushroom Spring mat samples and pure cultures of *S. lividus*.

<table>
<thead>
<tr>
<th>Condition</th>
<th>CO₂</th>
<th>Cells</th>
<th>Aqueous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mushroom Spring</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Light, 2 hr</td>
<td>16.77 ± 1.28</td>
<td>50.30 ± 4.76</td>
<td>32.90 ± 5.59</td>
</tr>
<tr>
<td>Formalin$^b$</td>
<td>2.37 ± 0.18</td>
<td>0.67 ± 0.15</td>
<td>97.00 ± 0.26</td>
</tr>
<tr>
<td><em>S. lividus</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Light, 2 hr</td>
<td>0.18 ± 0.06</td>
<td>0.68 ± 0.31</td>
<td>99.13 ± 0.32</td>
</tr>
<tr>
<td>Dark, 2 hr</td>
<td>0.20 ± 0.03</td>
<td>0.08 ± 0.01</td>
<td>99.72 ± 0.01</td>
</tr>
<tr>
<td>Light, 6 hr</td>
<td>0.20 ± 0.04</td>
<td>0.11 ± 0.01</td>
<td>99.70 ± 0.03</td>
</tr>
<tr>
<td>Dark, 6 hr</td>
<td>0.27 ± 0.02</td>
<td>0.06 ± 0.01</td>
<td>99.67 ± 0.02</td>
</tr>
<tr>
<td>Light, induced$^c$</td>
<td>0.19 ± 0.02</td>
<td>0.04 ± 0.01</td>
<td>99.77 ± 0.03</td>
</tr>
<tr>
<td>Dark, induced$^c$</td>
<td>0.23 ± 0.04</td>
<td>0.03 ± 0.01</td>
<td>99.74 ± 0.05</td>
</tr>
</tbody>
</table>

$^a$ 96.5% of the added $^{14}$C was recovered. Values expressed are mean ± standard error; n=3.

$^b$ Samples were injected with formalin before incubation with $^{1-14}$C-glycolate. Biological activity in all other samples was terminated with formalin following incubation.

$^c$ Samples were preincubated with 0.1 μM glycolate for four hours before two hour incubation with $^{1-14}$C-glycolate.
Figure 5. Autoradiograms prepared from homogenized cells from the 55°C mat of Mushroom Spring labeled with 1-14C glycolate. Typical *S. lividus* cells are indicated by arrows. Magnification 2900x.
Effects of Decreasing Population Density

As homogenized samples from the Mushroom Spring mat were further diluted in spring water, the percent of total photosynthate recovered in the aqueous phase increased (Figure 6). The percent extracellular photosynthate also increased when *S. lividus* was diluted in the supernatant liquid of the culture medium in which the cells had been growing. As cells from both the pure culture of *S. lividus* and the Mushroom Spring samples were diluted, the total photosynthate detected in cells and the aqueous fraction decreased by a factor greater than the dilution factor (data not shown).

Effects of 2-pyridylhydroxymethanesulfonic Acid

In cell homogenates from Mushroom Spring, 2-pyridylhydroxymethanesulfonic acid (HPMS), an inhibitor of glycolate oxidation, prevented the incorporation of 1-14C-glycolate into cells, and the oxidation of the glycolate to 14CO2 (data not shown). At a concentration of 10^-4 M, HPMS did not inhibit oxygen production measured using oxygen microelectrodes in the Mushroom Spring mat (Ward, personal communication), but did inhibit glycolate uptake and oxidation more than acetate or protein hydrolysate uptake and metabolism. This concentration was used to interrupt glycolate consumption during NaH14CO3 labeling experiments. Experiments were performed on both dense and dilute mat
Figure 6. Effect of dilution on the percent of total photosynthate recovered in the aqueous phase of Mushroom Spring mat samples and pure cultures of S. lividus. Biological activity was terminated with formalin in each experiment.
homogenates reasoning that glycolate flow from cell to cell may be influenced by the degree of separation between cells. Total photosynthate (\(^{14}\)C in cells and aqueous fraction) detected in Mushroom Spring samples treated with \(10^{-4}\) M HPMS was 40% of the total photosynthate detected in control samples (Table 10). HPMS did not directly inhibit photosynthesis by \textit{S. lividus}; in fact, HPMS often increased total photosynthesis in the pure culture. In dense suspensions of the top layer of mat, the percent of total photosynthate in the aqueous phase increased significantly upon HPMS addition (Table 10). In the more dilute suspensions, the percent extracellular photosynthate increased, but the increase was not statistically significant.

When dense or dilute cultures of \textit{S. lividus} were treated with \(10^{-4}\) M HPMS, the percent of total photosynthate in the aqueous phase did not increase (Table 10), supporting the interpretation that in the mat glycolate flows between producer and consumer.

**Effects of Glycolate and Acetate Additions**

When homogenized samples from the Mushroom Spring mat were incubated in the presence of 10 mM glycolate or acetate the percent of total photosynthate in the aqueous phase increased significantly (Table 11). The amount of total carbon fixed decreased to approximately one half of
Table 10. The effects of HPMS on total carbon fixation and percent of total photosynthate in the aqueous phase. Mushroom Spring mat samples and pure cultures of *S. lividus* were incubated in the light.a

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Cells + Aqueous dpm/ml</th>
<th>% $^{14}$C in the Aqueous Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mushroom Spring</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.6x10⁹ cells/ml</td>
<td>control 776,800</td>
<td>9.2</td>
</tr>
<tr>
<td></td>
<td>+ HPMS 293,400 *</td>
<td>22.0 *</td>
</tr>
<tr>
<td>7.3x10⁸ cells/ml</td>
<td>control 322,400</td>
<td>19.9</td>
</tr>
<tr>
<td></td>
<td>+ HPMS 122,900 *</td>
<td>29.8</td>
</tr>
<tr>
<td><strong>S. lividus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.8x10⁷ cells/ml</td>
<td>control 672,870</td>
<td>47.4</td>
</tr>
<tr>
<td></td>
<td>+ HPMS 579,800</td>
<td>43.1</td>
</tr>
<tr>
<td>2.6x10⁷ cells/ml</td>
<td>control 157,100</td>
<td>52.3</td>
</tr>
<tr>
<td></td>
<td>+ HPMS 341,800</td>
<td>47.9</td>
</tr>
</tbody>
</table>

* indicates significant differences (p<0.05) of treated samples compared to control; n=6.

a Biological activity was terminated with formalin.
the control level, possibly due to a lower specific activity of $^{14}$CO$_2$ caused by nonradioactive CO$_2$ produced through respiration of added glycolate or acetate. This experiment was performed only once.

Table 11. The effects of glycolate or acetate addition on the percent of total photosynthate in the aqueous phase of Mushroom Spring mat samples.$^a$

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Cells + Aqueous dpm/ml</th>
<th>% $^{14}$C in the Aqueous Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>264,400</td>
<td>21.6</td>
</tr>
<tr>
<td>1 mM glycolate</td>
<td>248,700</td>
<td>24.8</td>
</tr>
<tr>
<td>10 mM glycolate</td>
<td>135,100</td>
<td>30.0 *</td>
</tr>
<tr>
<td>10 mM acetate</td>
<td>106,100</td>
<td>29.9 *</td>
</tr>
</tbody>
</table>

* indicates significant differences (p<0.05) of treated samples compared to the control; n=6.

$^a$ Biological activity was terminated with formalin.
DISCUSSION

The major objective of my research was to investigate the importance of glycolate production and consumption in the Mushroom Spring microbial mat. The glycolate detected was photosynthetically derived, as the precursor was $^{14}$CO$_2$ and production was light dependent (Figure 4, Table 4). S. lividus is the major CO$_2$-fixing phototroph in the Mushroom Spring mat. While the other known mat phototroph, Chloroflexus, is capable of H$_2$S-dependent, anoxygenic photosynthetic CO$_2$ fixation, this process is probably not significant in the mat during the majority of the daylight hours because high O$_2$ concentrations exclude H$_2$S from the photic zone (Revsbech and Ward, 1984).

Photoassimilated carbon can be excreted as organic compounds by phototrophs, including cyanobacteria such as S. lividus. Sharp (1977) has drawn attention to various sources of error which may invalidate determinations of excreted products of photosynthesis using radiolabeling methods. I also found that certain methods overestimated excreted photosyntheate. The percent of total photosyntheate in the aqueous phase of samples treated with formalin or frozen to stop biological activity was much higher than in samples whose cells and aqueous phase were separated by
filtration and centrifugation (Table 6). Freezing and formalin addition evidently caused the cells to leak. Even in cells that have not been treated with formalin or frozen, damage of cells during filtration may release soluble \(^{14}\text{C}\)-labeled compounds into the filtrate (Sharp, 1977). While performing filtration, I minimized damage to cells by taking precautions such as rinsing with spring water or medium in which the cells had been growing, keeping cells constantly wet, and using a mild vacuum. Also, radioactivity in the aqueous phase was determined from samples of large volume derived from centrifuged homogenates, to avoid magnifying errors resulting from analysis of small filtrate samples.

Experiments in which filtration and centrifugation were used to separate cells from the aqueous phase produced results which reflect the carbon excreted by cells. In order to be precise, the phrase "photosynthate detected in the aqueous phase" has been used rather than the phrase "excreted photosynthate" when formalin or freezing methods were used to terminate biological activity. Although the freezing and formalin treatments increased the percent of total photosynthate detected in the aqueous fraction, experiments conducted using these methods produced valid comparisons of various treatments on the production of photosynthate which either was or could be excreted.
The addition of oxygen to Mushroom Spring mat samples increased the percent of photosynthate detected in the aqueous phase in samples that had been formalin treated (Table 2), and in samples which were filtered and centrifuged (Table 8). Pure cultures of \textit{S. lividus} also responded to oxygen addition with an increase in the percent of photosynthate detected in the aqueous, although the increase was not as dramatic. Addition of carbon dioxide had the opposite effect on the \textit{S. lividus} culture; the percent of photosynthate detected in the aqueous fraction decreased. This was also observed in the Mushroom Spring homogenates, though the results were not statistically significant. The removal of carbon dioxide increased the percent of photosynthate in the aqueous fraction in Mushroom Spring samples.

Other investigators have demonstrated that high oxygen and low carbon dioxide partial pressure enhance glycolate biosynthesis and excretion of photoassimilated carbon by a variety of phototrophic organisms (Asami and Akazawa, 1974; Beck, 1979; Eickenbusch and Beck, 1973; Han and Eley, 1973; Storrø and McFadden, 1981; Tolbert, 1980). The results of experiments on the Mushroom Spring microbial mat community are consistent with the hypothesis that some of the extracellular material detected is a product of the oxygenation of the Calvin-Benson cycle intermediate, RuDP.
Analysis of the photosynthate in the aqueous phase by ion exchange and thin layer chromatography revealed that glycolate was a major product in the Mushroom Spring mat homogenates incubated in the light. Other compounds were also detected, including four compounds in the acidic fraction which were separated on the TLC plates. A portion of the compounds in the aqueous phase of samples incubated in the light did not bind to the ion exchange column; these presumably include neutral sugars or amino acids.

The percent of aqueous photosynthate as glycolate in mat samples increased with $O_2$ addition, and the increase in glycolate detected in the aqueous phase was greater than the increase in excretion in general (Table 5). In the mat samples, the percent of aqueous photosynthate as glycolate was higher when samples were filtered and centrifuged than when samples were frozen, but the difference was not statistically significant. Although glycolate may have been diluted by other organic material extracted from cells in the freezing procedure, glycolate was also extracted, because glycolate as a percent of total photosynthate increased significantly with freezing (Table 8).

In four experiments on homogenized samples of Mushroom Spring mat in which the samples were frozen before filtration, the percent of aqueous photosynthate as glycolate ranged from 8.5 to 31.4% in control samples. In samples with added oxygen, the values ranged from 25 to 50%
as glycolate. In the final experiment with samples filtered and centrifuged at the spring site, the percent of aqueous photosynthate as glycolate was slightly higher in both the control (46%) and the oxygenated samples (58%). Thus, glycolate is a major compound produced and excreted by photoautotrophs in this mat environment.

Glycolate is also important when considered as a percentage of the total photosynthate. In control samples, the percent of total photosynthate as glycolate ranged from 1.5 to 6.1%. In samples with added oxygen, the values ranged from 7.4 to 12.8%. In samples not frozen, the percent of total photosynthate as glycolate was lower (control, 4.3%; oxygen, 6.8%) due to the fact that less glycolate was extracted from the cells when samples were not frozen.

Unlike the Mushroom Spring mat, cultures of *S. lividus* did not produce much glycolate (Table 7). Reasoning that low CO$_2$ partial pressures should enhance glycolate production, *S. lividus* cultures used for all of the experiments were grown without CO$_2$ enrichment. However, it has been demonstrated that air-grown algal and cyanobacterial cultures contain high levels of carbonic anhydrase, an enzyme which concentrates bicarbonate in cells (Berry, et al, 1976). Cultures grown at high CO$_2$ levels have low carbonic anhydrase activity and have low carbon-fixation rates when carbon is mainly available as
bicarbonate and not dissolved CO$_2$ (Berry, et al., 1976). Jahnke (1981) showed that air-grown Chlorella with high carbonic anhydrase activities released only small quantities of glycolate even when incubated with 100% O$_2$ and saturating light intensities. Results of an experiment on the pure culture did show that estimations of glycolate as a percent of photosynthate in the aqueous phase were effected by the methods of terminating biological activity in the samples (Table 7). In pure cultures of *S. lividus*, freezing increased the percent of photosynthate detected in the aqueous fraction compared to samples that were immediately filtered and centrifuged (Table 6). The organic material extracted from cells by freezing diluted out glycolate as a component of the aqueous photosynthate, since the percent of excreted photosynthate as glycolate was ten-fold higher in the filtered than in the frozen samples (Table 7). In the mat samples, the dilution of glycolate was not as dramatic as in the pure culture. This presumably reflects the greater relative importance of glycolate production compared to the production of other intracellular intermediates in the *S. lividus* cells in the natural mat community.

The validity of estimations of the importance of glycolate in carbon flow within the Mushroom Spring mat depends on many factors. One consideration is how closely the experimental system mimics the in situ conditions. In
the top 1 mm of the mat, the peak oxygen concentration is greater than 900 uM for several hours of a cloudless summer day, but oxygen concentration varies both with time of day and spatially within the layers of the mat (Revsbech and Ward, 1984). In the homogenized samples with added oxygen, the concentration of dissolved oxygen ranged from 625 to 975 uM after one hour of incubation; similar to the upper mat layers in full sunlight. During further incubation the oxygen concentration decreased. Considering the vertical variation of oxygen in the mat and the temporal variation during incubation of homogenized samples, the oxygen concentrations in the samples with added oxygen seem to be comparable to those of the top 2 mm of mat during midday, while the oxygen concentrations in the homogenized control samples were closer to those of the top 2 mm of mat in early morning or late evening. Thus estimates of the importance of glycolate obtained from filtered samples suggest that glycolate may comprise up to 46% of the excreted photosynthate in low light periods, and as much as 58% of the photosynthate excreted in high light (ca. 1000-1800 hr in summer).

Interactions within the mat community will also affect the estimations of excretion of glycolate and other extracellular products. Excreted compounds, such as glycolate, can be taken up by heterotrophs in the mat, reducing the amount of product detected in the aqueous
phase, and increasing the radioactivity detected in cells. In ecosystems lacking large populations of filamentous heterotrophs, it has been possible to separate the phototrophic populations from the majority of the heterotrophs using serial filtration techniques (Brock and Clyne, 1984; Cole, et al, 1982; Coveney, 1982; Haack and McFeters, 1982; and Paerl, 1984). These investigators have concluded that failure to account for heterotrophic utilization of excreted carbon can lead to serious underestimates of total excretion in measurements of photosynthetic production. Estimations of the percent excretion by algae in Lake Mendota were twice as high when bacterial uptake was taken into account (Brock and Clyne, 1984). Coveney (1982) detected up to 80% of the $^{14}C$ excreted in the fraction containing heterotrophic bacteria. The best estimate of excreted photosynthate in Mushroom Spring mat samples is that observed in experiments in which filtration and centrifugation were used to separate cells and aqueous phase. Depending on oxygen concentration, 9.4 to 11.8% of the CO$_2$ fixed was excreted. These percentages are probably lower than the actual percent excretion in the mat because of rapid transfer of carbon from phototroph to heterotroph. A similar effect would be expected for glycolate production expressed as a percentage of total fixed CO$_2$. 
In the Mushroom Spring mat, glycolate was incorporated by filamentous organisms and not by *S. lividus* (Figure 5). *S. lividus* in axenic culture also did not incorporate or metabolize glycolate (Table 10). Rippka (cited by Stanier and Cohen-Bazire, 1977) demonstrated that exogenous glycolate in the presence of DCMU in the light did not support growth of cyanobacteria (over one hundred strains were examined).

Tayne (1983) described filaments of three different widths that were present in the mat, two of which failed to react with antiserum specific for *Chloroflexus*. The autoradiographic procedure used here to determine which organism takes up glycolate does not distinguish between these three possible filament types. Glycolate incorporation may be carried out by *Chloroflexus*, and/or by the unidentified filamentous organisms. Light stimulated incorporation of 1-\(^{14}\text{C}\)-glycolate in Octopus Spring mat samples (Tayne, 1983), indicating photoheterotrophic activity. In samples incubated in the dark, glycolate was also partially metabolized to \(\text{CO}_2\). *Chloroflexus* is capable of both photoheterotrophic uptake of compounds and aerobic growth in the dark.

Though uptake of photosynthetically produced and excreted compounds, such as glycolate, by the filamentous heterotrophs would decrease estimates of excretion and glycolate production, it may not alter the composition of
the excreted products detected in the aqueous phase. Bell (1983) concluded that prolonged exposure to a single alga may favor the development of bacterial populations whose enzyme-mediated transport systems are adapted to the compounds excreted by the alga. Products other than glycolate which are excreted in the mat are probably also assimilated and metabolized by heterotrophic community members. Thus, the composition of excreted photosynthate would not be selectively altered. If this is true, the importance of glycolate expressed as a percentage of the aqueous fraction may be a close estimate of the importance of glycolate in the excreted photosynthate.

Several different approaches were taken to attempt to interrupt carbon flow between producer and consumer in the Mushroom Spring mat in order to better understand and estimate excretion. One approach involved spatially separating the organisms by diluting the homogenized cell suspensions. Bauld and Brock (1974) hypothesized that the decrease in photoexcretion products detected in samples with a higher density of organisms can be explained by the presence of heterotrophs which take up the products more readily when cells are in closer proximity. Although this may be occurring, evidence presented here indicates that the increase in excretion upon dilution must be mediated by
factors effecting the photoexcreting organism itself, since an axenic culture of *S. lividus* exhibited the same phenomenon (Figure 6).

Other investigators have also shown that cell density in pure cultures of algae can influence the percentage of excretion (Huntsman, 1972; Ignatiades and Fogg, 1973). It has been suggested that algae "condition" their media through photoexcretion. Monahan and Trainor (1970) showed that the filtrates of the algae *Hormotila blennista*, containing carbohydrate, ninhydrin positive compounds and glycolate, were autostimulatory. Nalewajko, et al (1963) obtained evidence that glycolate decreased the lag period for growth of *Chlorella* cultures started from small inocula. Fogg (1977) has argued that if low molecular weight compounds freely diffuse between the interior of cells and the medium, then laboratory cultures with high population densities will inevitably show that little photosynthate appears extracellularly, whereas relatively high proportions will be released by less dense populations.

Another effect of dilution observed in both axenic cultures and mat samples was that the total carbon fixed on a per cell basis decreased with dilution (data not shown). Sharp (1977) interpreted these effects of dilution (lowered carbon fixation and high excretion) as the result of cultural shock to the organisms on transfer to a medium of
slightly different nutrient composition or salinity. However, changes in medium composition in the experiments on axenic cultures of *S. lividus* were minimized by diluting the organisms in the medium in which the organisms had been growing just 30 minutes before the labeling experiment.

Although dilution experiments did not necessarily link production and consumption of excreted photosynthate, the addition of HPMS to mat homogenates did provide evidence that glycolate flows from producer to consumer. The percent of photosynthate in the aqueous phase increased with HPMS addition, especially in dense suspensions (Table 10). HPMS is known to inhibit the enzymes which oxidize glycolate, either glycolate oxidase or glycolate dehydrogenase (Jahnke, 1981; Nelson and Tolbert, 1970; Zelitch, 1971). Inhibition of glycolate oxidation in the mat could result in increased excretion of glycolate by *S. lividus*, however, excretion was not increased when axenic cultures of *S. lividus* were treated with HPMS (Table 10). HPMS probably decreased metabolism of glycolate by filamentous heterotrophic organisms that normally incorporate and oxidize glycolate, interrupting the flow of glycolate and thus increasing the percent excretion detected. Thus, the estimate of glycolate production in this mat community may be low due to the consumption of glycolate by heterotrophs.
Another effect of HPMS was to decrease total photosynthetic activity in the mat. In the pure cultures of *S. lividus*, a decrease in production of total photosynthate was not evident. These results suggest that in the mat, glycolate consumption by heterotrophs may stimulate photosynthesis. In the mixed community of the microbial mat, the heterotrophs could be "milking" the phototrophs of diffusible, low molecular weight compounds such as glycolate by assimilating such compounds as quickly as they appear extracellularly. Respiration of glycolate to CO₂ by heterotrophs could then increase the inorganic carbon available for photosynthesis. Another benefit may be that glycolate consumption may stimulate energy conservation through development of proton motive force in *S. lividus*. This would be analogous to the generation of proton motive force by excretion of fermentation products in some fermentative bacteria (Otto, et al, 1980). A concentration gradient of the compound to be excreted exists between the inside and outside of the cell; consumption of the excreted product increases such a gradient.

Another method of interrupting uptake of excreted material by heterotrophs is to flood the system with presumed excretion products, such as glycolate. Glycolate addition to mat homogenates increased the percent of photosynthate in the aqueous phase. The radioactive
glycolate produced and excreted during $^{14}\text{CO}_2$ labeling was presumably taken up less efficiently by heterotrophs and accumulated in the aqueous phase. Nonradioactive acetate addition also increased percent of photosynthate in the aqueous phase. This may indicate that acetate is one of the other products excreted by *S. lividus* or other organisms fixing carbon dioxide in the mat. Another possibility is that acetate may interfere with glycolate uptake. However, the kinetics of glycolate and acetate uptake in mat homogenates were different, implying that separate proteins are responsible for uptake of each compound (Tayne, 1983).

Although the most significant route for CO$_2$ fixation in the Mushroom Spring mat was light-dependent CO$_2$ fixation (Table 1), CO$_2$ fixation and the production of extracellular organic compounds in the dark was appreciable. Glycolate production is a light-dependent process (Figure 4). However, there was extremely high production of an unknown organic acid (D1) in the dark samples from the Mushroom Spring mat (Table 4). In the samples incubated in the dark, the percentage of total fixed carbon in the aqueous phase was higher for samples that were frozen, however, the percent of aqueous fixed carbon as the unknown compound (D1) was the same in frozen and filtered samples (data not shown). This suggests that the compound D1 is not merely a compound extracted from cells, but is excreted. Although
extracellular photosynthate from the *S. lividus* culture incubated in the dark was not analysed, the compound did not appear to be produced to the same extent (if at all) by the pure culture. In the *S. lividus* cultures, the percent of total fixed carbon in the aqueous phase in the dark was not significantly different than in the light, whereas in the mat samples, the percent in the dark was much higher than in the light (Table 1).

In the dark, mechanisms other than photoautotrophic CO$_2$ fixation must be in effect in this microbial community. Chemolithotrophic autotrophs, such as iron-, sulfur-, and hydrogen-oxidizing and nitrifying bacteria fix carbon dioxide via the Calvin cycle enzymes (Bowien and Schlegel, 1981). CO$_2$ is also utilized by heterotrophic organisms in anaplerotic carboxylation of pyruvate to form oxaloacetate. CO$_2$ assimilation occurs via a reductive tricarboxylic acid cycle in *Hydrogenobacter thermophilus* (Shiba, et al, 1985), the only obligate autotroph among all aerobic hydrogen-oxidizing bacteria so far reported (Bowien and Schlegel, 1981). This extremely thermophilic bacterium was isolated from a hot spring in Japan (Kawasumi, et al, 1984). Excretion of fixed carbon by this thermophilic organism has not been reported.

CO$_2$ reduction also occurs in catabolic and anabolic pathways of some anaerobes which might inhabit this mat. Methanogens, such as the hot spring isolate *Methano-*
bacterium thermoautotrophicum, which reduce CO₂ to methane and also reduce CO₂ for biosynthesis (Fuchs and Stupperich, 1982), would be unlikely to excrete such a high percentage of soluble fixed carbon. Clostridium thermoautotrophicum, like other homoacetogenic bacteria (Thauer, et al, 1977; Zeikus, 1983), produces acetate from molecular hydrogen and carbon dioxide, and has been isolated from a water sample from an alkaline silicious hot spring (possibly Octopus Spring) in Yellowstone National Park (Wiegel, et al, 1981). Because most of the carbon fixed in the dark was excreted, it seems likely that catabolic CO₂ reduction by such an organism might be occurring in the mat. Identification of the compound or compounds produced in the dark should reveal the microbial process responsible for production of the material, and possibly reveal the nature of the organisms involved.

The results of this research have increased the understanding of carbon flow within the microbial mat ecosystem. In the light, a significant proportion of the carbon dioxide fixed by S. lividus is excreted as organic carbon. Estimations of the actual percent of carbon excreted are complicated by uptake of the excretion products by heterotrophs in the mat, and the effects of cell density on the detection of extracellular carbon. Photosynthetic oxygen production and presumably CO₂ consumption by S. lividus creates an environment which
stimulates the production and excretion of glycolate. This compound constitutes a major portion (25 to 58%) of the excreted material when oxygen concentration is close to that of the top layers of the microbial mat throughout most of the day. The importance of glycolate as a percent of excreted material would be likely to be somewhat lower in the early morning or late evening, when light intensity and oxygen production are lower. In the dark, degradation of polymeric organic matter by anaerobic bacteria leads to production of fermentation products (Anderson, 1984). Based on data from experiments on uptake kinetics, Tayne (1983) suggested that these fermentation products may be more important sources of organic carbon to mat heterotrophs than is glycolate. Another fermentation product, hydrogen, drives \( CO_2 \) reduction to methane. Although methane is a major product (Sandbeck and Ward, 1981), another product of dark \( CO_2 \) reduction (unknown acid D1) may also be an important intermediate which flows between autotrophs and heterotrophs in the microbial mat community.
Conclusions

1. Photoexcretion occurs in alkaline silicious hot spring mat communities and is regulated by oxygen and carbon dioxide levels in the mat.

2. Glycolate, while not the only photosynthetic product excreted, is a major product, comprising up to 58% of the extracellular carbon, depending on the oxygen concentration.

3. At high oxygen concentrations, characteristic of the top 2 mm of mat between late morning and evening, glycolate comprises 25 to 58% of the extracellular carbon.

4. At oxygen concentrations characteristic of the mat during early morning and late evening, glycolate comprises 8.5 to 46% of the extracellular carbon.

5. Glycolate excretion is considerable; extracellular glycolate accounts for up to 12% of photosynthetically fixed carbon detectable in the mat.

6. Filamentous heterotrophic bacteria consume glycolate in the mat.

7. The link between phototroph and heterotroph may lead to underestimates of glycolate production and percent excretion, and may stimulate total CO₂ flux in the mat.

8. Dark CO₂ fixation leads to synthesis of one major organic compound which is excreted.
LITERATURE CITED


Bateson, Mary Margaret
Glycolate production and consumption in a...