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

Master of Science

in

Microbiology

MONTANA STATE UNIVERSITY
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Date 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 $1\text{-}^{14}C$-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 H_2S as an electron donor, however H_2S 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 (Anacystic 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. (Cheng, 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 Akazawi, 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,

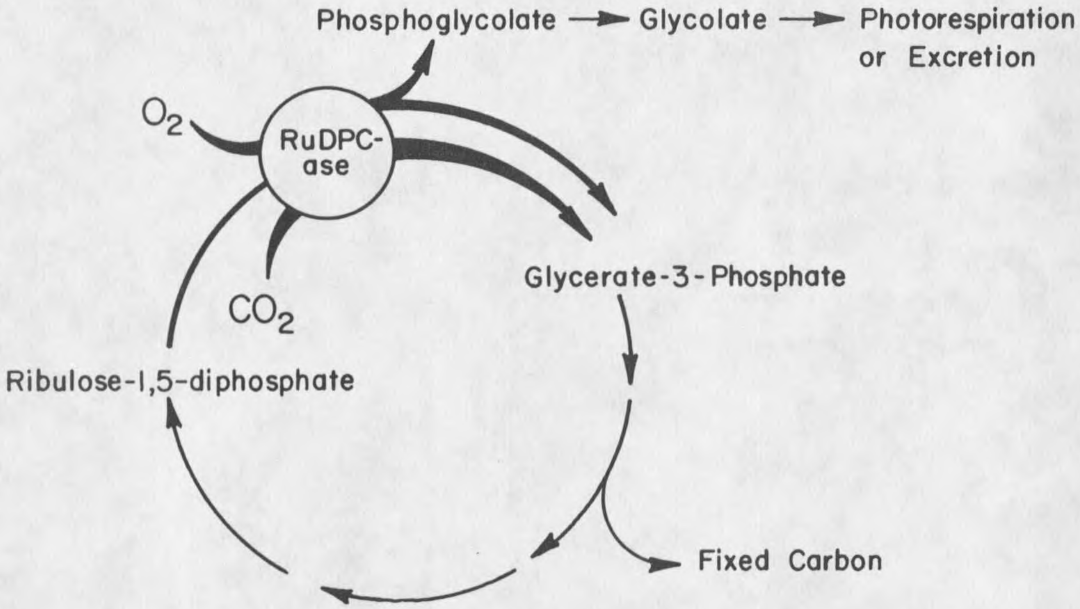
Phosphoglycolate → Glycolate → Photorespiration or Excretion
O_2
RuDPC-ase
CO_2
Glycerate-3-Phosphate
Ribulose-1,5-diphosphate
Fixed Carbon

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 O_2 is consumed and CO_2 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_2O_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_2O_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 CO_2 concentration up to levels which saturate CO_2 fixation. At saturating levels of CO_2, oxygen concentrations higher than that of air increase the proportion of fixed carbon which is incorporated into glycolate, whereas at lower O_2 levels the proportion is unchanged (Eickenbusch and Beck, 1973). At low CO_2 levels, glycolate formation depends on the oxygen concentration. These observations appear to result from competition between CO_2 and O_2 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 reachs 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 HCO_3^-, rather than CO_2, the chemical species used by RuDP carboxylase-oxygenase. The environmental conditions of extremely high O_2 concentrations, high pH (thus low 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 x 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 homegenate 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. 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 $NaH^{14}CO_3$ (50mCi/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 10uCi/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 disintigrations 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 $NaH^{14}CO_3$ 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

$^{14}CO_2$ 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 $^{14}CO_2$ 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}C-glycolate were prepared according to the method of Brock and Brock (1968). Homogeneous samples were fixed to slides, rinsed to remove residual 1-^{14}C-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.

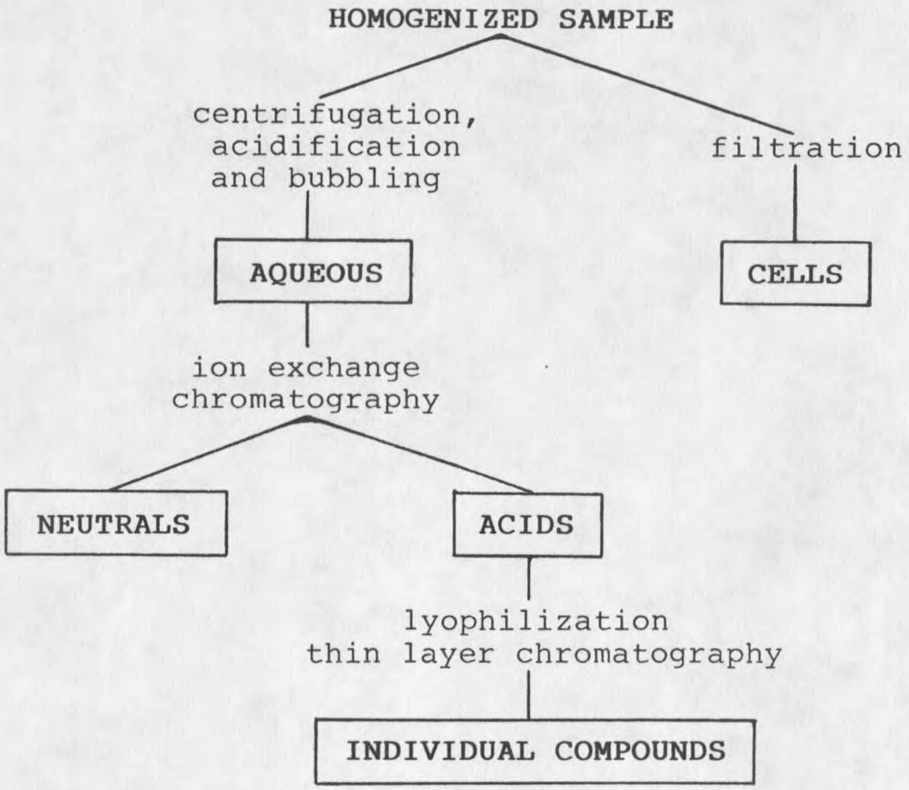
HOMOGENIZED SAMPLE
centrifugation, acidification and bubbling
filtration
AQUEOUS
CELLS
ion exchange chromatography
NEUTRALS
ACIDS
lyophilization
thin layer chromatography
INDIVIDUAL COMPOUNDS

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-^{14}C-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, transfered 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^{o}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