The fate of fermentation products and glycollate in hot spring microbial mats with emphasis on the role played by Chloroflexus aurantiacus

by Timothy Alden Tayne

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Microbiology
Montana State University

Abstract:
The fate of various fermentation products was investigated in a low sulfate hot spring microbial mat (Octopus Spring) that has methanogenesis as the terminal step in anaerobic catabolism, and in a high sulfate hot spring microbial mat (Painted Pool), which has sulfate reduction as the terminal anaerobic catabolic step. These mats were tested under light and dark aerobic and anaerobic conditions and also under preincubated conditions, which should favor acetogenic bacteria, important in catabolism of fermentation products in other systems. Filamentous bacteria, resembling Chloroflexus aurantiacus, incorporated the fermentation products in the light. For most compounds catabolism to CO2 was not significant in the dark, especially under anaerobic conditions. Glycollate, a product of cyanobacterial photorespiration, was tested and showed results similar to the fermentation products. Autoradiography did not reveal which cells incorporated glycollate. Antiserum was prepared against a strain of Chloroflexus isolated from Octopus Spring which reacted specifically with other Chloroflexus isolates and with the most abundant filamentous bacteria in the mats studied. It was used with combined immunofluorescence-autoradiography to show that Chloroflexus was responsible for photoincorporation of the fermentation products. Dark anaerobic catabolism was suggested by the conversion of butyrate to acetate and propionate to CO2. The sensitivity of these reactions to accumulation and enrichment of butyrate degrading acetogenic bacteria gave evidence of a potential for aceto-genesis. The heterotrophic potential technique was used to test the population potential for the uptake of these compounds in the light.

The greatest potential activity was found in the section 1-3 mm below the mat surface and this section showed a much higher potential for uptake of acetate and propionate than for other substrates tested including butyrate, ethanol, lactate and glycollate. Although there was a potential for photoincorporation of the fermentation products by Chloroflexus, or for catabolism of those products by acetogenic bacteria, it appeared only Chloroflexus was highly adapted for the product uptake and metabolism.

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24 May 1983
Date

David M. Ward
Chairperson, Graduate Committee

Approved for the Major Department

25 May 1983
Date

Norman D. Reed
Head, Major Department

Approved for the College of Graduate Study

27 May 1983
Date

Michael Malone
Graduate Dean

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ABSTRACT

The fate of various fermentation products was investigated in a low sulfate hot spring microbial mat (Octopus Spring) that has methanogenesis as the terminal step in anaerobic catabolism, and in a high sulfate hot spring microbial mat (Painted Pool), which has sulfate reduction as the terminal anaerobic catabolic step. These mats were tested under light and dark aerobic and anaerobic conditions and also under preincubated conditions, which should favor acetogenic bacteria, important in catabolism of fermentation products in other systems. Filamentous bacteria, resembling Chloroflexus aurantiacus, incorporated the fermentation products in the light. For most compounds catabolism to CO_2 was not significant in the dark, especially under anaerobic conditions. Glycollate, a product of cyanobacterial photorespiration, was tested and showed results similar to the fermentation products. Autoradiography did not reveal which cells incorporated glycollate. Antiserum was prepared against a strain of Chloroflexus isolated from Octopus Spring which reacted specifically with other Chloroflexus isolates and with the most abundant filamentous bacteria in the mats studied. It was used with combined immunofluorescence-autoradiography to show that Chloroflexus was responsible for photoincorporation of the fermentation products. Dark anaerobic catabolism was suggested by the conversion of butyrate to acetate and propionate to CO_2. The sensitivity of these reactions to H_2 accumulation and enrichment of butyrate degrading acetogenic bacteria gave evidence of a potential for acetogenesis. The heterotrophic potential technique was used to test the population potential for the uptake of these compounds in the light. The greatest potential activity was found in the section 1-3 mm below the mat surface and this section showed a much higher potential for uptake of acetate and propionate than for other substrates tested including butyrate, ethanol, lactate and glycollate. Although there was a potential for photoincorporation of the fermentation products by Chloroflexus, or for catabolism of those products by acetogenic bacteria, it appeared only Chloroflexus was highly adapted for the product uptake and metabolism.

INTRODUCTION

This study was a part of ongoing research on following decomposition in hot spring microbial mats. The major part of the work was done in Octopus Spring in the White Creek area of the Lower Geyser Basin of Yellowstone Park. This spring has a source temperature of approximately 91°C and luxuriant growth of mats at a temperature range from approximately 70°C to below 40°C. Octopus Spring is a very popular study site for several reasons. First, the system serves well for the study of high temperature decomposition of cells and polymers by thermophilic microorganisms. Second, it is free of metazoan grazers, such as flies, because the water temperatures are above those tolerated by animals. Third, the mat offers a low diversity of organisms, which simplifies the study of microbial interactions. Finally, the mat exists under steady state conditions (Brock, 1967b) because the effluent water is stable in terms of chemical and physical, features (Brock, 1967a).

The Octopus Spring mat is built by the combined effort of two photosynthetic organisms. The first, a unicellular cyanobacterium, Synechococcus lividus, makes up a green layer at the top of the mat which is approximately 1 mm thick. The other organism, Chloroflexus aurantiacus, is primarily a photoheterotrophic bacterium which is thought to form the thick orange mat that is found down to the siliceous

sinter, which makes up the bed of the effluent streams. The close proximity of these two organisms has led to studies concerning cross feeding between them (Bauld and Brock, 1974). Many cyanobacteria have been shown to excrete glycollate during a process called photorespiration. This could serve as a source of carbon and energy for Chloroflexus.

Other studies that deal with the activity of the phototrophs show that the actively growing layer of the mat is near the surface. Bauld and Brock (1973) showed chlorophyll a to be in highest concentration within the top 0.5 mm and to be absent below 1 mm. This corresponds to the vertical profile of oxygenic photosynthesis which occurs in the top 1 mm in Nymph Creek (Revsbech and Ward, 1983) and Octopus Spring (Revsbech and Ward, unpublished). In sunlight, oxygen levels of Octopus Spring exceed saturation in the top several mm, while in darkness the oxygen penetrates only the top 0.5 mm due to diffusion from the overlying water (Ward, et al., 1983). The highest concentration of bacteriochlorophylls a and c, found in Chloroflexus, lies between 0.5 and 3 mm below the mat surface.

Studies have been done to determine the rate of growth and decomposition of the Octopus mat (Doemel and Brock, 1977) by using silicon carbide powder. By placing a thin layer of carbide on the mat, they had a marker visible in subsequent sampling. Repeated layering of carbide at timed intervals and analysis of accumulation above the layer and degradation between the layers led them to the conclusions that (1) the mat builds to a certain height until a steady state is reached when the photosynthetic Synechococcus and Chloroflexus self-shade themselves,

(2) the upward growth of the mat results because Chloroflexus migrates up through the carbide and lays out a network probably to hold the Synechococcus against the flowing stream water, and (3) the steady state is achieved by complete decomposition of the lowest levels of the mat below the photic zone.

Previous studies on degradation of hot spring microbial mats have been primarily limited to the terminal processes of methanogenesis and sulfate reduction (Ward, 1978; Ward and Olson, 1980; and Sandbeck and Ward, 1981, 1982) so that the fate of fermentation products from polymers is open to speculation. A possible fate of the products from fermentation in this microbial ecosystem could be uptake by Chloroflexus in the light by photoincorporation. Studies by Pierson and Castenholz (1971), Sirevag and Castenholz (1979), and Madigan, et al. (1974) have shown photoheterotrophic activity in cultures of Chloroflexus using a variety of substrates.

The general objective of this study was to investigate the role of Chloroflexus in the fate of the products of fermentation and also glycollate which may be excreted by Synechococcus.

Anaerobic Decomposition and the Acetogenic Fate of Fermentation Products

The anaerobic degradation of organic matter has been studied in several types of systems by many people. As in the digestion of sewage, a cooperating interaction of anaerobic microbes exists. There are three basic groups of bacteria involved as described by Bryant (1976). The

first group ferments the primary substrates such as proteins, polysaccharides, and lipids to acetic and other fatty acids, alcohols, H_2, CO_2, NH_3, and H_2S. The second group, the obligate proton-reducing (H_2-forming) bacteria called acetogens (McInerney, et al., 1979, 1981) oxidize long chain fatty acids and alcohols to acetate, H_2 and CO_2. The reactions of the acetogenic bacteria under standard conditions (activities of substrates and products at 1 M, except H^+ at 10^{-7} M and gases at 0, 1 atmosphere, 25°C, at pH 7) are endergonic. This implies that energy is required for the reactions to proceed to completion. These reactions are made exergonic (energy producing) in anaerobic systems by the removal of the acetogenic products H_2 and acetate. This is carried out by the methanogenic or sulfate-reducing bacteria in a process called interspecies hydrogen transfer (McInerney and Bryant, 1981). Listed below are the acetogenic reactions (taken from Thauer, et al. 1977) of common fermentation products that were considered in this study as substrates for photoheterotrophic utilization by Chloroflexus or for acetogenic bacteria.

	$\Delta G°'$ (K cal/reaction)
$Propionate^- + 3H_2O \longrightarrow Acetate^- + HCO_3^- + H^+ + 3H_2$	+18.2
$Butyrate^- + 2H_2O \longrightarrow 2\ Acetate^- + H^+ + 2H_2$	+11.5
$Ethanol + H_2O \longrightarrow Acetate^- + H^+ + 2H_2$	+ 2.3
$Lactate^- + 2H_2O \longrightarrow Acetate^- + HCO_3^- + H^+ + 2H_2$	- 1.0

As shown, the only energy producing reaction is lactate oxidation. The other reactions become exergonic by the H_2-removing reactions previously discussed, allowing the acetogenic bacteria to obtain energy by acetate production from a fermentation product.

The third group found in the anaerobic degradation process, the methane-producing bacteria (methanogens), catalyze the conversion of acetate, H_2, and CO_2 to methane. The methanogens serve a very valuable function at the terminal step by removing hydrogen, as discussed above. H_2 consumption also allows the fermenters to produce more H_2 and lower levels of reduced electron sink products, such as lactate, ethanol, butyrate, and propionate (Wolin, 1974, 1976). This results in production of more oxidized compounds like acetate and CO_2 because of electron transfer to the methanogens instead of electron sink products (Gottschalk, 1979). This scheme of flow in the breakdown of organic compounds has been proposed for various anaerobic sytems such as aquatic sediments, muds, marshes, and anaerobic digesters (Mah, et al., 1977; and Bryant, 1976). In high sulfate sediments, such as marine sediments, sulfate-reducing bacteria replace methanogens in the terminal stages of decomposition. Another anaerobic system occurs in the rumen of the cow, and other ruminant animals. Here carbohydrates serve as the main substrates. The breakdown of carbohydrate polymers again results in production of volatile fatty acids (VFA), but they are removed by absorption and are utilized by the ruminant as an essential part of nutrition. In the rumen, methane production is almost entirely from CO_2 reduction by H_2 (Hungate, 1975) because acetate has been removed with the other VFA's.

Another goal of this study was to investigate any possible acetogenic activity that may exist in the Octopus Spring microbial mat.

There has never been any acetogenic activity shown before in this mat, but it could also be as important in determining the fate of fermentation products.

Algal Excretion Products

Cyanobacteria are generally limited to a strictly photoautotrophic metabolism, i.e., they can be grown only if CO_2 is the sole carbon source. However, some species can photoassimilate some organic compounds. Acetate is an example of an organic compound which can be utilized as a substrate for biosynthetic pathways (Hoare, et al., 1967). The uptake of acetate was shown to be dependent on the parallel uptake of CO_2. Many cyanobacteria also carry out photorespiration, the liberation of soluble organic substances or CO_2 (Fogg, et al., 1973; Fogg and Horne, 1970). Many studies have been done on marine phytoplankton (Helebust, 1965; Anderson and Zeutschel, 1970) and freshwater algae (Fogg, et al., 1965, 1973) with mixed interpretations as to whether or not photoexcretion was important.

It is well documented that CO_2 is evolved during photorespiration from the leaves of many plants (Hew, et al., 1969). There is also evidence that some algae excrete carbon in the form of glycollate during this photorespiratory activity. Glycollate, a two-carbon acid, is a photorespiration product when O_2 replaces CO_2 in the ribulose diphosphate carboxylase reaction, resulting in the oxidation of ribulose diphosphate, instead of its carboxylation (Chollet, 1977). A high pO_2 results in

stimulation of glycollate production while a high pCO_2 inhibits its synthesis. With the excretion of glycollate comes reduced photosynthetic efficiency. In many plants and algae the glycollate produced is further oxidized to CO_2 instead of being excreted (Cheng, et al., 1972; Döhler and Braun, 1971). Grodzinski and Colman (1970) found similar pathways present in both plants and algae. Codd and Stewart (1973) were able to show that a culture of Anabaena would excrete $^{14}CO_2$ in the light and to a lesser degree in the dark after incubation with ^{14}C-glycollate. The cyanobacterial genera Anabaena and Oscillatoria both have K_m's, or uptake affinities for glycollate, orders of magnitude higher than those reported for heterotrophic bacteria (Miller, et al., 1971). Thus, if both algae and bacteria were present in the same environment in which a low continuous supply of excreted glycollate were available, the bacteria would theoretically control its fate.

Glycollate has also been detected in the supernatant of photoheterotrophically grown cells of members of the families Rhodospirillaceae (the purple nonsulfur bacteria), Chromatiaceae (purple sulfur bacteria) and Chlorobiaceae (green sulfur bacteria) (Storrø and McFadden, 1981; Lorimer, et al., 1978). It is unknown if the glycollate production is from the condensation of two CO_2 molecules and thereby bypassing the Calvin Cycle, or from oxidation of the Calvin Cycle intermediates (Chollet, 1977).

Field studies were carried out by Bauld and Brock (1974) to examine the ability of the natural population of S. lividus in Octopus Spring to

excrete ^{14}C-labeled organic compounds after photosynthetic uptake and fixation of $^{14}CO_2$. They were able to show that excretion of ^{14}C-labeled compounds occurred both in the light and, to a lesser extent, in the dark, but with much higher percentage of photosynthate excreted in the dark. They also showed that the lower the cyanobacterial population density, the higher the percentage of excreted products (up to 50% compared to total ^{14}C fixed in the light). Thus, in the densely populated mat itself an underestimation of photoexcretion may result because of rapid uptake by closely packed bacteria (Bauld and Brock, 1974). In the dark, however, no difference due to population density was seen. This would seem to suggest that the Synechococcus cells that are self-shaded or are less densely populated in lower levels might excrete a higher percentage of the photosynthetically fixed CO_2. Microelectrode studies by Revsbech and Ward (unpublished) have shown high pO_2 and presumably low pCO_2 (as evidenced by a high pH in layers of active photosynthesis), which would suggest that the cyanobacteria in this environment might excrete a higher level of glycollate, if they do indeed excrete it (Chollet, 1977). This could support the theories of crossfeeding of other bacteria including Chloroflexus suggested by Bauld and Brock (1973).

The possiblity that glycollate is a source of carbon and energy for Chloroflexus was also studied in the present investigation of substrate sources and fates.

Chloroflexus aurantiacus

Chloroflexus aurantiacus is a filamentous bacterium with quite varied physiological capabilities. It can grow aerobically or anaerobically in the light as a photoheterotroph or aerobically in the dark as a heterotroph; some strains also are capable of a photoautotrophic growth using CO_2 with H_2S as an electron donor. Woese (1981) showed in 16s rRNA homology studies that this organism is quite unique and unrelated to the other eubacteria. Its classification in a separate family (Chloroflexaceae) from other photosynthetic bacteria is now justified by the 16s rRNA studies.

One of the earliest studies related to Chloroflexus was in 1966 before its discovery. Brock and Brock (1966) were determining the chlorophyll, protein and RNA content of Octopus Spring. They observed a large peak in the chlorophyll absorption spectrum scans at about 450 nm, presumably caused by the bright orange color of the mat. It is now known that the orange color is due to the carotenoid pigments of Chloroflexus. Halfen, et al. (1972) and Pierson and Castenholz (1974b) found that at least ten different carotenoids can be produced and that some are produced only at high light intensities. Thus, carotenoids may serve as protection against high light intensity. The major carotenoids produced are beta and gamma carotene and glycosides of gamma carotene.

In addition to the carotenoids, Chloroflexus contains bacteriochlorophyll c_s (Gloe and Risch, 1978) which is unlike the bacterio-

chlorophyll c found in members of the Chlorobiaceae. The bacteriochlorophyll c_s is found in reaction centers called chlorosomes, or 'chlorobium vesicles', which are small cylindrical bodies found in close association with the membrane (Cohen-Bazire, et al., 1964; Pierson and Castenholz, 1974b; Madigan and Brock, 1977c; Staehelin, et al., 1978). Bacteriochlorophyll a is also present but is found only in the reaction centers in the cytoplasmic membrane. Bacteriochlorophylls a and c_s have been shown to be independently produced (Pierson and Castenholz, 1974b). The production of both is reduced by high light intensity or high oxygen levels, but bacteriochlorophyll c_s decreases more in response to these parameters. The fact that the Octopus Spring undermats are bright orange shows that bacteriochlorophylls have been inhibited relative to carotenoids presumably by high oxygen concentration not by light, since the orange undermat is at low light (approximately 10% full sunlight under the first millimeter of mat (Doemel and Brock, 1977)), but is super oxic.

Chloroflexus, as previously stated, is capable of dark aerobic respiration. When high levels of bacteriochlorophylls are present, respiratory oxygen consumption is markedly decreased by light (Pierson and Castenholz, 1974a). This is probably because the bacteriochlorophyll mediated electron flow is in direct competition with oxidative electron flow caused by the sharing of several electron carriers, as is also the case in purple nonsulfur bacteria (Marrs and Gest, 1973). Chloroflexus is also able to use H_2S to support photoautotrophy or photomixotrophy

(assimilation of CO_2 and preformed carbon compounds at the same time) (Madigan and Brock, 1977a). Chloroflexus was also shown to carry out photoincorporation of $^{14}CO_2$ without an external reductant, such as sulfide, by utilizing an internal electron donor.

Since the original isolation of Chloroflexus from hot a spring, there have been several discoveries of related organisms which have been included in the Chloroflexaceae (Castenholz and Pierson, 1981). A mesophilic strain, Chloroflexus aurantiacus var. mesophilus (nom prof.) was isolated in Russia by Gorlenko (1975). Pivovarova and Gorlenko (1977) were able to show that strains BR-1 and KN-4 of the mesophilic Chloroflexus had many characteristics in common with the thermophilic strains. There is inhibition of bacteriochlorophyll a and c production at high light intensity or high partial pressure of oxygen, and both strains grow aerobically or anaerobically in the light and aerobically in the dark.

A strain known as "green" Chloroflexus was isolated from the top mat at 55-64°C in high sulfide hot springs of the Mammoth area of Yellowstone Park (Castenholz and Giovannoni, 1981). This mat has a green color because of high levels of bacteriochlorophyll c and lack of carotenoids (carotenoids are not produced in the absence of oxygen). Strain GC79 grows rapidly photoheterotrophically under anaerobic conditions, but fails to grow under 2% oxygen. The organism can withstand high levels of oxygen for long periods but will not resume growth until oxygen is removed (Castenholz and Giovannoni, 1981).

A filamentous organism resembling Chloroflexus, strain designation F-I, has been found in Oregon and Yellowstone hot springs. It forms bright orange mats with cyanobacteria usually underneath. As with all Chloroflexus strains, crosswalls can be seen about every 10 μm. The filament diameter has been found to be 1.5 μm. Granules are seen in strain F-I which may be poly-β-hydroxybutyrate. The gliding rate of filaments is about 0.1-0.4 μm/second compared to about 0.01-0.04 μm/second for the known strains of Chloroflexus (Pierson and Castenholz, 1971). F-I appears to possess bacteriochlorophyll c, d, or e but has only small amounts of bacteriochlorophyll a and lacks chlorosomes (Pierson, 1973). The carotenoids are essentially identical to those of aerobically grown Chloroflexus. F-I is thought by Castenholz and Pierson (1981) to be a member of the family Chloroflexaceae that has lost the chlorosomes.

Field Studies

Several investigators have attempted to study bacterial photosynthesis with the use of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), a herbicide which stops oxygenic photosynthesis. However, DCMU may increase the level of H_2S, usually absent in the photic zone due to O_2 produced by oxygenic photosynthesis (Jørgensen, et al., 1979; Revsbech and Ward, unpublished). Thus DCMU may artificially increase the level of bacterial photosynthesis measured. Using this approach uptake of $^{14}CO_2$ in hot spring microbial mats was linear for several hours in the light (Pierson and Castenholz, 1971; Bauld and Brock, 1973; Doemel and

Brock, 1977). Bauld and Brock (1973) showed that H_2S (0.01% w/v) increased bacterial photosynthesis. Dark fixation of $^{14}CO_2$ which was about 20% of that in light samples, and both light and dark CO_2 fixation decreased rapidly within the top 4 mm.

Bauld and Brock (1973) showed that $^{14}CO_2$ photoassimilation in a hot spring microbial mat was maximum at about 400 foot-candles but dropped about 5 fold at full sunlight (5000-8000 foot-candles). Using neutral density filters, Madigan and Brock (1977b) found that Chloroflexus and the cyanobacterium Synechococcus could adapt to low light intensity. Under full sunlight, cyanobacterial photosynthesis was reported to be slightly inhibited while bacterial photosynthesis was not. During $^{14}CO_2$ and ^{14}C-glutamate uptake studies on cells adapted to a 73% reduction of light, both organisms were inhibited by full sunlight and showed a maximum uptake at 14% light. Under a 98% reduction filter, only Chloroflexus remained because Synechococcus was inactive and washed out in the effluent flow. The maximum uptake in Chloroflexus was at about 5% full sunlight with a severe inhibition in bright light. This ability of both Chloroflexus and Synechococcus to adapt to light may well serve for survival in the hot spring mats because light intensities vary greatly diurnally and seasonally.

Studies have shown that Chloroflexus, or a filamentous bacterium resembling it, is capable of assimilating organic compounds or CO_2 in the light. Bauld and Brock (1973) showed photoassimilation of ^{14}C-glutamate, and ^{14}C-aspartate. Pierson and Castenholz (1971) showed a light induced uptake of ^{14}C-acetate and $^{14}CO_2$. Sandbeck and Ward

(1981) found similar results with ^{14}C-acetate. Autoradiograms by Pierson and Castenholz (1971), Sandbeck and Ward (1981), and Ward, et al., (1983) showed that only filamentous bacteria were radiolabeled by ^{14}C-compounds after the incubation.

Evolutionary Significance

Interest in Chloroflexus has also arisen because of the presence of microfossils similar to its morphology in laminated sedimentary rock structures called stromatolites (Walter, et al., 1972). For years the accepted theory was that stromatolites appeared with the first appearance of cyanobacteria. However, Bauld and Brock (1973) hypothesized that some stromatolites may be of bacterial origin. By 3,500 million years ago, microbial life was apparently advanced enough for stromatolite formation (Awramik, 1981; Cloud, 1978). The discovery of Chloroflexus, and its ability to form mats free of cyanobacteria (Castenholz, 1977; Ward and Revsbech, 1983), have warranted a more cautious interpretation as to whether or not such early stromatolites were formed by cyanobacteria (Walter, et al., 1980). During the Archean period, which ended 2,500 million years ago, oxygen-generating phototrophic life probably evolved. This could have caused an oxygen crisis for the obligate anaerobes and some facultative anaerobes when the reduced atmosphere became oxidized. Peschek (1978) put forth the theory that the early cyanobacteria lived on either photosystem I using sulfide or photosystems I and II splitting compounds such as reduced nitrogen compounds instead of H_2O. This would allow for the evolution of both

photosystems before the evolution of H_2O splitting and subsequent oxygen evolution.

Stromatolites are still being formed. They belong to three classes: columnar, columnar-layered, and conical (Awramik, 1982). These stromatolites are generally found in shallow water in many environments including tidal areas, thermal pools, and hot springs. Yellowstone Park has many of these formations once thought to be extinct. There are columnar and conical types formed primarily of silica with Chloroflexus filaments and Synechococcus cells permeating them. Phormidium, another cyanobacterium which is filamentous, is found in lower temperature stromatolites. The laminar nature of these structures could be due to the migration of microbes due to light and dark cycles (Doemel and Brock, 1974; Walter, et al., 1972).

Objectives and Approaches

The main objective of this research was to examine the fate of various fermentation products and the algal excretion product glycollate in hot spring microbial mats. I also wanted to study the role Chloroflexus played in the metabolism of these compounds, since previous studies have shown photoincorporation of a few compounds by an organism resembling Chloroflexus. This was accomplished by the simultaneous use of fluorescent antibody against Chloroflexus and autoradiography after incorporation of ^{14}C-labeled compounds. The combined fluorescent antibody-autoradiography technique was used previously by Fliermans and Schmidt (1975) to identify Nitrobacter in soil samples and to study their activity in the use of ^{14}C-$NaHCO_3$.

The heterotrophic potential technique of Parsons and Strickland (1962) was used to study the vertical distribution of photoheterotrophic activity and adaptation to the various organic compounds photoassimilated. In a radiolabeling experiment, the velocity (v) of an uptake reaction could be calculated if the fraction of label metabolized (F) during time (T) could be corrected for the concentration of added substrate (A) plus the naturally occuring pool level of that compound (S_N):

$$v = \frac{F(S_N + A)}{T}$$

Since S_N cannot be accurately measured, only the maximum potential for uptake (V_{max}) can be determined instead, assuming the compound is taken up by Michaelis-Menten saturation kinetics (Wright and Burnison, 1979; Sorokin and Kadota, 1972). The Michaelis-Menten expression can be rewritten in terms of F and A:

$$T/F = A/V_{max} + (K + S_N)/V_{max}$$

where V_{max} is the maximum rate of uptake of a compound when the enzyme is completely saturated with substrate, and K is the half saturation constant (Wright and Burnison, 1979). Thus a plot of T/F versus A gives a straight line of slope $1/V_{max}$, and the V_{max} value can be experimentally determined if F is measured at different values of A. Tempest and Neijessel (1978) describe V_{max} as the enzymatic capacity of a population of cells for the uptake and metabolism of a substrate.

MATERIALS AND METHODS

Study Areas

The primary study area was Octopus Spring found in the Lower Geyser Basin of Yellowstone Park. The specific location can be found in Brock (1978). Octopus Spring was chosen as a study site for several reasons. There are easily accessible sites of different temperatures all fed from the same source, but at any site, temperature varies only about 5°C. The algal-bacterial mat used in this study is well developed and has been studied in detail by others (see Brock, 1978 and Ward, et al., 1983). One sampling site, referred to as the shoulder, was above the southmost channel and was protected from the source by a siliceous sinter barrier. This site had a mat area of a few square meters and thickness of from 1 to 1.5 cm. The temperature at this mat varied less than at other sites (50°C $\pm$ 2° where samples were collected) and the mat was much more homogeneous than at other sites. Sampling was also done in the southmost effluent channel at average temperatures of 50°, 60°, and 70°C.

Another site, Painted Pool (see Castenholz, 1977) on the Mammoth Terrace Loop Road of Mammoth Hot Springs, was chosen because it offered a chance to study a microbial system that differs from Octopus Spring in the terminal stage of anaerobic metabolism (Ward and Olson, 1980). Octopus Spring, a low sulfate spring, has methanogenesis as the final

step in anaerobic decomposition. In Painted Pool, on the other hand, due to a higher sulfate concentration, sulfate reduction dominates over methanogenesis because of competition for the electron donor hydrogen. The temperature of the mat used for study was approximately 42°C. Cyanobacterial mats were also sampled at 51° and 54°C at New Spring (see Castenholz, 1977), also on the Mammoth Terrace Loop Road. Mats presumably formed by "green" Chloroflexus were sampled from high sulfide sources at 64°C in Painted Pool and 60°C in New Spring. White streamers of presumably Thermothrix, a filamentous H_2S oxidizing, chemolithotroph, were collected near the sources of both springs.

Fate of ^{14}C-Labeled Compounds

The first experiments carried out were designed to follow the fate of ^{14}C-labeled compounds that might be fermentation products or cyanobacterial excretion products (glycollate). These possible carbon and energy sources were incubated with mat samples under either natural or extreme conditions designed to advantage certain metabolic groups.

All fate of label experiments were done using the same procedures described here and in Analytical Methods. Cores were kept at a constant 1 cm x 50.3 mm^2 by using a No. 4 cork borer and by cutting each core off 1 cm below the mat surface. After cutting, the cores were inverted into one dram vials. Vials were flushed for approximately 30 seconds with helium then stoppered with butyl rubber stoppers for anaerobic conditions or were stoppered under air for aerobic conditions. Spring water (2.0 ml) from the collection site was added to aerobic samples. Anoxic

water, prepared by bubbling helium through water from the near boiling source (or Painted Pool water) for 15 minutes, was added (also 2.0 ml) to anaerobic samples. Dark incubation tubes were prepared by double wrapping the vial with black plastic tape. All vials were incubated inverted in racks in the hot spring to allow full sunlight to illuminate the mat surface and to keep the temperature at that of the collection site. All samples were preincubated for 30 minutes except for some vials which were preincubated 24 hours.

The radiolabeled substrates were added to the preincubated vials using helium flushed gastight syringes to obtain a final concentration of approximately 1 μCi/vial. After a two hour incubation period, 0.1 ml formalin was added to stop biological activity. Formalin controls were run initially to ensure that nonbiological background counts were insignificant. The analysis of samples, as explained in Analytical Methods, included measurement of ^{14}C in the gas headspace, cell fraction and filtrate. Sometimes further analysis of the filtrates was done to determine the ^{14}C-products from the metabolism of the ^{14}C-substrate (see below).

Experiments were set up to determine the fate of some labeled compounds after a longer dark preincubation before the addition of radiolabel. Again 1 cm x 50.3 mm^2 cores were put into dark wrapped vials and tubed under nitrogen. After the addition of 1 ml of anoxic Octopus Spring water, the samples were put into a thermos of 52° water. The thermos was then immersed into an insulated cooler containing 58°C water and transported back to the laboratory. This allowed the samples

to be transported without a decrease in incubation temperature over 5°C. Incubation continued in the laboratory at 50°C. At various times headspace samples were removed with a gastight syringe and analyzed for hydrogen and methane levels using a gas chromatograph (as described in Analytical Methods). Analysis continued until it was determined that methane was being produced. At this time 2-bromoethane sulfonic acid (BES) was added at a final concentration of 0.05 M to one-half the sample vials to inhibit methanogenesis (Gunsalus, et al., 1976). The inhibitory concentration was determined in preliminary experiments in which BES was added at various concentrations to vials for long term incubation immediately upon return to the laboratory (see Results). Methane and hydrogen levels were monitored for approximately 30 more hours until hydrogen levels in the vials with BES were approximately 16 times those in the control samples. At this time the ^{14}C-labeled compounds previously used were added to a final concentration of 1 μCi/vial. Twelve hours after the start of the incubation with the labeled substrates, gas headspace analysis revealed that $^{14}CO_2$ had been produced in all tubes; 0.1 ml formalin was added to stop biological activity. Gases, cellular fractions and filtrates were analyzed as described below.

Culture Methods

Acetogenic Bacteria Enrichment

Cultures were set up to try to enrich acetogenic bacteria from Octopus Spring. Anaerobic techniques for preparation and inoculation of

media were those of Hungate (1969) as modified by Bryant (1972). The media preparations were designed after McInerney, et al. (1979) with medium D (Castenholz and Pierson, 1981) used instead of Pfennig's medium. The basal medium contained (final concentration in grams/liter): NaCl,0.04; $NiCl_2 \cdot 6H_2O$,0.02; KH_2PO_4,0.5; $MgCl \cdot 6H_2O$,0.33; NH_4Cl, 0.4; $CaCl_2 \cdot 2H_2O$, 0.05; ferric chloride, 0.29; resazurin, 0.001; and Wolin's B-vitamin solution, 2 ml (McInerney, et al., 1979). One of the following substrates was added to each type of enrichment medium: sodium propionate, 18mM; n-butyrate, 18mM; or ethanol, 20mM. Controls with no added carbon and energy source were always inoculated in parallel.

Inoculation of media was by anoxically adding a 1 cm x 50.3 mm^2 core from the 50° mat at Octopus Spring into 10 ml medium. At this time $Na_2S \cdot 9H_2O$ was added to a final concentration of 0.05% giving the final pH 8.2. Cultures were transported as described above and kept in the dark at 50°C. Cultures were checked every few weeks by visual inspection for turbidity and gas headspace analysis for methane production. Once each month 1 ml of each positive enrichment culture was transferred from each culture to a fresh tube with or without the same substrate.

Chloroflexus

A strain of Chloroflexus was isolated from a microbial mat sample from the 50°C mat at Octopus Spring. Transport to the laboratory was as previously described and isolation procedures were initiated in the laboratory. Isolation was made on medium D with 0.5% yeast extract in 1.5% agar under cool white fluorescent lamps after Castenholz and Pierson

(1981), by streaking a core fraction in a straight line across the plate. Every five days the leading edge of growth was transferred with an inoculating loop until all visible contaminants were lost. After repeated transfers a pure culture was obtained as evidenced by (1) direct microscopic observation of only filamentous bacteria which did not autofluoresce, (2) lack of growth on standard plate count medium (Difco) at 24, 35, 50, or 60°C, and (3) absorption spectra and growth characteristic of Chloroflexus aurantiacus (Pierson and Castenholz, 1974b).

Growth was greatly enhanced in medium D with 0.5% yeast extract and 0.5% sodium acetate under 60 watt tungsten lamps as suggested by M. T. Madigan (personal communication). Media were contained in completely filled 500 ml prescription bottles containing 0.05% $Na_2S \cdot 9\ H_2O$ and adjusted to pH 8.2. Optimum growth resulted in bottles incubated within six inches of the tungsten lamps.

Preparation and Testing of Antiserum

Cultivation and Preparation of Culture

The strain of Chloroflexus isolated from Octopus Spring was grown to prepare antiserum for ecological studies. Twelve liters of medium D with 0.1% yeast extract, 0.1% sodium acetate, 1% $NaHCO_3$ and 0.05% cysteine hydrochloride (to replace the $Na_2S \cdot 9\ H_2O$) was sterilized in a 20 liter glass fermenter vessel which was put on a New Brunswick micro-ferm model MF-214. To help maintain purity and anaerobic conditions

over the long period of time needed to obtain a suitable yield of cells, a continuous flow of nitrogen was bubbled through the vessel. Temperature was maintained at 50°C and four 60 watt tungsten bulbs served as the light source. After eight days of continuously stirring, the cells were harvested by continuous centrifugation using a Sorvall model RC2-B centrifuge at 480 g (2,000 rpm). The cells were washed once in 0.85% NaCl then three times in distilled water to remove salts and medium residues before lyophilization of the cells. Lyophilization was done overnight in a Freezemobile 6 (Vitris Company) and the lyophilized cells were then kept in a desiccator.

Preparation of Anti-*Chloroflexus* Antiserum

Four rabbits were obtained for production of antiserum to the *Chloroflexus* strain isolated from Octopus Spring. After predose blood samples were drawn, two rabbits were given four 1 ml subcutaneous injections of complete Freund adjuvant each containing 1 mg lyophilized *Chloroflexus* cells. Both rabbits received another series of complete Freund-*Chloroflexus* injections three days later. One and two weeks later both received a series of incomplete Freund adjuvant with *Chloroflexus* cells. Forty-nine days after the initial injections, slide agglutinin titers were done on both rabbits by serially diluting blood serum and reacting it with 1 mg of the lyophilized *Chloroflexus* cells. Titers were 8 and 16 so injections were discontinued at this time. Slide titers were chosen over tube titers due to the tendency of the cells to autoagglutinate. Triton-X100 and Tween 80 were both tested to stop the autoagglutination reaction without effect.