Free lipids of hot spring microbial mats of possible evolutionary significance
by Jentaie Shiea

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

Abstract:
The free lipid compositions of several hot spring microbial mats and bacteria were examined. These included a eucaryotic algal mat, two cyanobacterial mats, and three photosynthetic bacterial mats and the photosynthetic bacteria which build them. Generally speaking, the major free lipids of the mats reflect well the inputs of predominant mat-building photosynthetic microorganisms. Comparative study of these samples permitted evaluation of several unique lipids thought to serve as "chemical fossils" of certain microbial groups representing important steps in microbial evolution during the Precambrian period. Mid-chain branched monomethylalkanes were found only in cyanobacterial mats. The presence of whole suites of structural isomers of these compounds in these modern mats suggests that cyanobacteria may have been direct contributors of the monomethylalkane suites found in Precambrian sediments. Dimethylalkanes found in one of the cyanobacterial mats also resemble similar compounds previously extracted from Precambrian samples. Hopanoid compounds were found in anoxic mats and bacteria as well as in oxic mats, raising doubt that such compounds are produced solely by aerobic and facultative bacteria.

Sterols were abundant and varied only in the eucaryotic algal mat and appear to reflect the eucaryotic nature of this mat. Low levels of common sterols found in cyanobacterial mats are likely to be contaminants, as similar sterols were found in anoxic mats and bacteria which should be unable to synthesize them. Two classes of compounds appeared to be unique to the green bacteria studied. Long chain alkenes found in the green nonsulfur bacterium Chloroflexus aurantiacus appear to mark its presence in both hot spring and marine lagoonal mats. Wax esters were detected in both C. aurantiacus and the green sulfur bacterium Chlorobium sp., and mats which contain them. In most cases, free alcohols and fatty acids were less abundant than hydrocarbons and wax esters. Alcohol distributions seem to reflect the distributions of alcohols esterified in major chlorophylls and wax esters. Fatty acid distributions appear to reflect the complex lipid fatty acids of predominating photosynthetic mat-building microorganisms.

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ABSTRACT

The free lipid compositions of several hot spring microbial mats and bacteria were examined. These included a eucaryotic algal mat, two cyanobacterial mats, and three photosynthetic bacterial mats and the photosynthetic bacteria which build them. Generally speaking, the major free lipids of the mats reflect well the inputs of predominant mat-building photosynthetic microorganisms. Comparative study of these samples permitted evaluation of several unique lipids thought to serve as "chemical fossils" of certain microbial groups representing important steps in microbial evolution during the Precambrian period. Mid-chain branched monomethylalkanes were found only in cyanobacterial mats. The presence of whole suites of structural isomers of these compounds in these modern mats suggests that cyanobacteria may have been direct contributors of the monomethylalkane suites found in Precambrian sediments. Dimethylalkanes found in one of the cyanobacterial mats also resemble similar compounds previously extracted from Precambrian samples. Hopanoid compounds were found in anoxic mats and bacteria as well as in oxic mats, raising doubt that such compounds are produced solely by aerobic and facultative bacteria. Sterols were abundant and varied only in the eucaryotic algal mat and appear to reflect the eucaryotic nature of this mat. Low levels of common sterols found in cyanobacterial mats are likely to be contaminants, as similar sterols were found in anoxic mats and bacteria which should be unable to synthesize them. Two classes of compounds appeared to be unique to the green bacteria studied. Long chain alkenes found in the green nonsulfur bacterium Chloroflexus aurantiacus appear to mark its presence in both hot spring and marine lagoonal mats. Wax esters were detected in both C. aurantiacus and the green sulfur bacterium Chlorobium sp., and mats which contain them. In most cases, free alcohols and fatty acids were less abundant than hydrocarbons and wax esters. Alcohol distributions seem to reflect the distributions of alcohols esterified in major chlorophylls and wax esters. Fatty acid distributions appear to reflect the complex lipid fatty acids of predominating photosynthetic mat-building microorganisms.

INTRODUCTION

The question of the origin of life is one of the most important problems of natural history. Scientists have studied this question for many centuries. Of course, it is possible that life did not arise on the Earth but no one can prove or disprove this possibility. Much evidence has been found which strongly suggests that life might have originated on the Earth and the scheme by which life evolved can be speculated upon (Woese, 1987).

There is a rich fossil record of plants and animals which occurred in the last 600 million years (Phanaerozoic period 600 million years ago to the present). A less well preserved record of microbial evolution extends throughout the Precambrian period (prior to 600 million years ago) to at least 3.5 billion years (3.5 Ga) ago, only 1 billion years after the Earth was thought to have formed (Schopf and Walter, 1983). In 1983, Schopf and coworkers submitted a possible scheme for the evolutionary events in the Precambrian (Schopf, et al, 1983) as outlined in Figure 1. They suggested that the earliest living cells were presumably simple, aquatic, and small spheroidal anaerobic microorganisms which could have evolved very early (before 3.5 Ga). Only a trace of oxygen from decomposition of

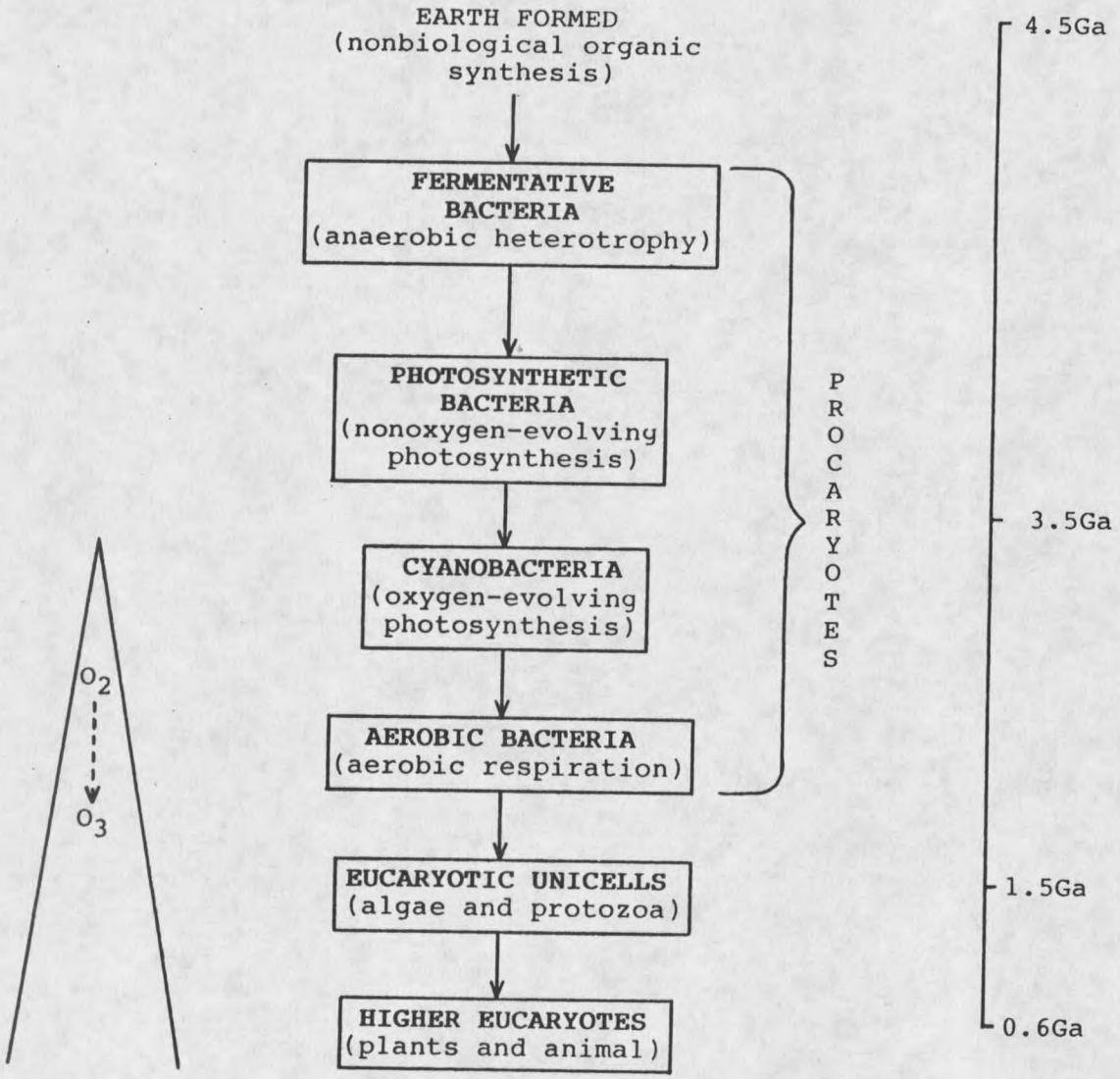

Figure 1. A suggested scheme for evolution during the Precambrian (modified from Schopf and Walter, 1983).

water by ultraviolet (UV) light probably was present in the atmosphere at that time. This earliest microbial life (heterotrophs) could have survived by fermenting organic molecules formed nonbiologically in the anoxic environment. The first photosynthetic organisms may have evolved when nonbiologically derived nutrients were diminished by primitive heterotrophs. Early phototrophs could have derived energy from photosynthesis by using a single photosystem which was entirely anaerobic and anoxygenic. Later, oxygenic photosynthesis could have followed, carried out by the ancestors of modern cyanobacteria. These microorganisms would have possessed photosystem II and thus generated oxygen. As the concentration of free oxygen in the atmosphere began to rise toward the modern level, anaerobic microorganisms were forced to retreat to anoxic habitats leaving the best space for photosynthesis to the cyanobacteria. The ozone (O_3) layer probably formed after oxygenation of the atmosphere. This protecting layer could filter out most ultraviolet (UV) radiation and eventually made it possible for organisms to live in shallow water or on the land with less depedence on the UV-absorbing protection of water. The first eucaryotic cells might have emerged around one and a half billion years ago (Schopf and Oehler, 1976). Eucaryotic cells contain more complex metabolic systems and organelles. When multicellularity and advanced sexual reproduction were evolved later, rapid

diversification of eucaryotic life forms (e.g. plants and animals) could have happened.

Although much evidence has been found to support parts of this suggestion, the major clues to connect all of the events are still missing. Indeed, more evidence is needed to completely understand life history in the Precambrian. This thesis deals with improving our interpretation of some of the evidence which suggests major events in microbial evolution during the Precambrian.

Approaches to Studying Microbial Evolution

Three approaches have been used in exploring the evolution of life: 1) study of the possible genetic and energetic relations among living organisms, 2) observation of ancient microfossils to understand morphological evolution, and 3) study of the inorganic and organic "chemical fossils" in ancient sediments in comparison to the chemistry of relevent modern environments and organisms. In order to understand the whole sequence of the evolution of the earliest life forms all three approaches must be taken.

The Phylogentic Record

Darwin, the father of evolution, pointed out that the various life forms can be produced through the interaction of a changing environment and a changeable organism, but

some clues from the antecedents will be retained and can be found in the descendents (Clark, 1976). From studying the fossil record, such changes have been observed to have occurred over long periods of geological time (Clark, 1976). However, even though no living organism is biochemically identical with its Precambrian antecedents, vestiges of earlier biochemistries have been retained. In the last fifty years, many living organisms have been examined for their genetic and biochemical characteristics, and the mechanism of change is partly understood.

The traditional approach of comparative biochemistry reveals a range of complexity in microbial biochemistry. It is logical to speculate that living organisms evolved from a simple metabolic system to more complex systems. Primitive life was presumed to possess the simplest fermentation system and the necessary organic materials for this metabolism were mainly produced nonbiologically (Schopf, 1978). Only little energy can be conserved in this process. When photosynthetic bacteria first evolved, light could be used to drive reactions for assimilation of exogenous nutrients. It was suggested that ancestral photosynthetic organisms contained only a cyclic electron transport system, the simplest known photosynthetic system in modern living organisms, for the efficient assimilation of external nutrients (Olson, 1970). Other photosynthetic systems such as those in cyanobacteria are more complex

(with a second photosystem) and should have evolved later. Recently, some cyanobacteria have been found in sulfide-rich anaerobic environments in which the cyanobacteria performed anoxygenic photosynthesis (Cohen, et al, 1975; Garlick, et al, 1977; Oren and Padan, 1978; Oren and Shilo, 1979; Castenholz and Utkilen, 1984; Cohen, et al, 1986). The ability to carry out both oxygenic and anoxygenic photosynthesis suggests that cyanobacteria may have played a transitional role between anoxygenic and oxygenic organisms. Eucaryotes with their greater cellular complexity are presumed to have evolved even later.

While comparative biochemistry may lead to speculation about the sequence of evolutionary change, more modern phylogentic approaches provide a record based on actual mutational change in a biochemical mechanism which has been conserved throughout evolution--protein synthesis. Risobomal RNA nucleotide sequences for living organisms are unique, and the extent of sequence difference depends on the phylogenic relatedness of the organisms being compared (Woese, 1987). A phylogenetic tree which reveals the evolutionary relationships among living things is shown in Figure 2. Three major kingdoms--eubacteia, archae-bacteria, and eucaryotes--have been suggested. Each of the kingdoms is quite distinct on the molecular level. Some details are of interest to the speculated scheme described in Figure 1. Fermentative eubacteria (e.g. Thermotoga) and

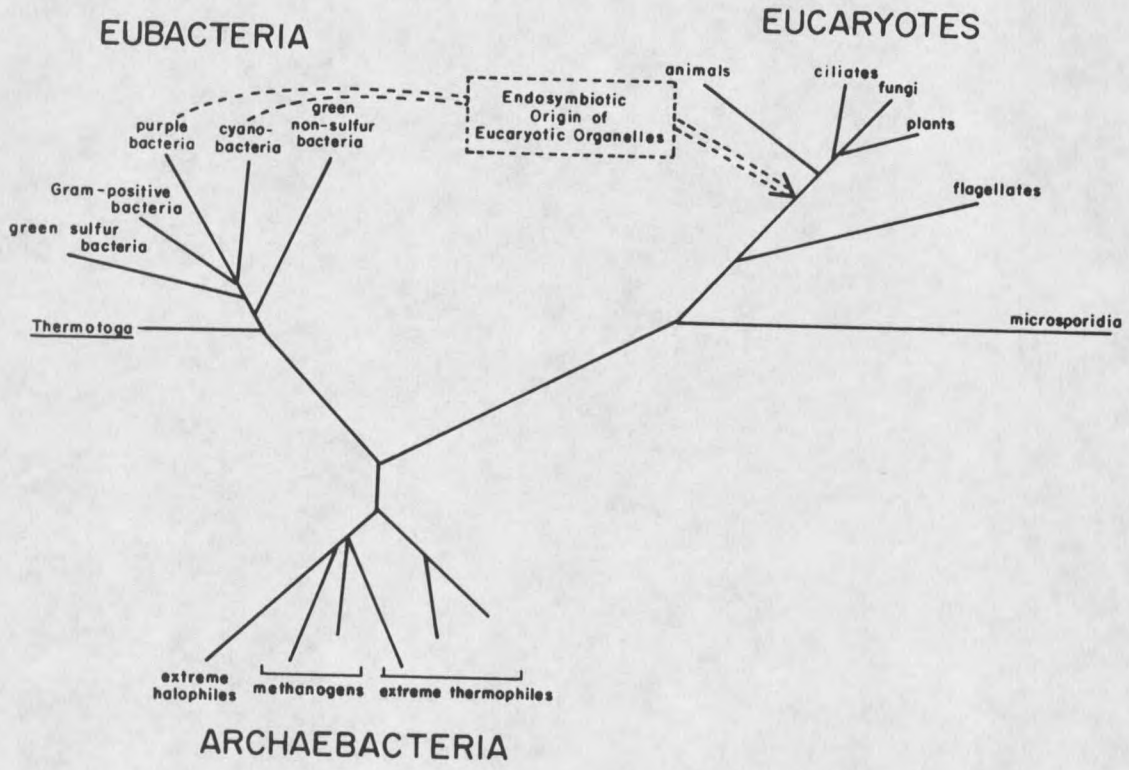

Figure 2. Phylogenetic tree determined from 16S rRNA sequence comparisons (modified from Woese, 1987).

archaebacteria (e.g. extreme thermophiles) may have predated the earliest photosynthetic microorganisms. Compared to some photosynthetic bacteria (e.g. green nonsulfur and green sulfur bacteria), cyanobacteria were relatively late to evolve. Unicellular eucaryotes (e.g. some flagellate and microsporidian protozoa) preceeded multicellular eucaryotes (e.g. animals and plants). Finally higher eucaryotes do seem to have derived their organelles (e.g. mitochondria and chloroplasts) from procaryotes (e.g. "purple bacteria" and cyanobacteria) via intracellular symbiosis.

The Fossil Record

Although the study of modern organisms may show the sequence of evolution, this approach cannot tell us the timing of events. To relate evolutionary events to time, the records (e.g. fossils) which were preserved in the Precambrian need to be examined. Fossils are commonly preserved as body fossils and trace fossils (e.g. tracks). There is a rich fossil record of Phanerozoic life, however large body and trace fossils are rarely found in only the latest part of the Precambrian record. The two best records of Precambrian organisms are microfossils and stromatolites. The oldest known and simplest life forms (procaryote-like) are preserved as microfossils in stromatolites about 3.5 billion years old.

The term "stromatolite", first used by Kalkowsky in 1908, is from the Greek "stroma", meaning bed or coverlet, and "lithos", meaning stone (Schopf, 1978). At first, stromatolites were thought to have had a nonbiological origin, but after a number of fossil microscopic organisms were found in them, stromatolites were thought to be one of the best remains by which to interpret the early evolutionary history of life. According to Walter (1977), a stromatolite is "an organosedimentary structure produced by the sediment trapping, binding and/or precipitation activity of microorganisms, primarily cyanobacteria". Krumbein (1983) restricted the term "stromatolite" to refer only to laminated geological structures, and defined unconsolidated laminated systems which are clearly related to the activity of modern microbial communities as "potential stromatolites". Although the definition of stromatolite is still in debate, this type of geological structure undoubtedly provides valuable clues for exploring early life. The oldest known stromatolites were found in the Warrawoona Group, Western Australia, of the 3.5-Ga-old Towers Formation (Schopf and Walter, 1983). Many other stromatolites have been found in Precambrian rocks (Walter, 1983). Stromatolites increased in abundance throughout the Precambrian, then decreased abruptly at the Precambrian-Phanerozoic boundary (Awramik, 1984).

Living microbial mat communities, with a laminated structure similar to stromatolites, have been found in hot spring and marine lagoon environments (Awramik, 1984). Because of the structural similarity between modern mats and ancient stromatolites, these modern microbial communities are considered as the best models by which to intepret the nature of stromatolite communities (Krumbein, et al, 1977). Most microbial mats are built by cyanobacteria living together with other bacteria (Castenholz, 1984a). However, many mats which are built by photosynthetic bacteria and which lack cyanobacteria have recently been found in hot springs (Ward, et al, in press b). The existence of photosynthetic bacterial mats implies that stromatolites could have been constructed by either anoxygenic or oxygenic photosynthetic microorganisms.

By using transmitted light microscopy or electron microscopy, microfossils can be observed in thin sections of silicified stromatolites. Some microbes are three-dimensionally preserved in the rock, hence it is possible to determine the shape and the size of microbes preserved in the stromatolites. Over one hundred types of stromatolitic microbes have been identified in such micropaleontology studies (Schopf and Walter, 1983). From these studies, the morphologic evolution of stromatolitic microbes has also been observed. It seems that the microbes found in the stromatolites exhibit increasing size

and structural complexity through the Precambrian (Hofmann and Schopf, 1983). This suggests that life might have originated from a simple and small style (procaryotes) and evolved to a more complex and large style (eucaryotes).

Although many stromatolitic microbes have been recognized from microscopic observation, the interpretation of the type of microorganism preserved is simply based on the shape of the microfossils. For example, many microfossils, including the oldest known, have been regarded by some micropaleontologists to represent the cells of cyanobacteria (Schopf, 1978; Schopf and Walter, 1983; Schopf and Packer, 1987). Microbiologists are well aware of the limited value of morphology in identification. Even if shape were meaningful the high pressure and temperature conditions of rock formation might distort the original structures and characteristic components of cells. So far, scientists still can't make a definitive conclusion about the types of these microbes based solely on shape. Other lines of evidence to better understand the sequence and timing of evolutionary events are needed.

The Geochemical Record

The value of geochemistry to the study of evolution is obvious. For example, the decay of radioisotopes is the basis for age dating of rocks (White and Wood, 1986). Many other inorganic geochemical transitions are thought to

record changes in environmental conditions during the Precambrian which were possibly caused by biological evolutionary events. For example, the depositional histories of three minerals-uraninite (UO_2), red beds, and banded iron formations are thought to record a transition of Earth's atmosphere from anoxic to oxic. In the presence of certain amounts of oxygen, grains of UO_2 will be oxidized to U_3O_8 and are thereby dissolved in water. In this case streambed deposits of uraninite can't accumulate any more. It is estimated that this will occur when the concentration of atmospheric oxygen is greater than about one percent (Grandstaff, 1976). Uraninite-bearing deposits have been found only in the sediments older than about 2 Ga, but not in younger sediments. This suggested that an increase in concentration of oxygen probably occurred at that time. Red beds (mostly the mineral hematite, Fe_2O_3) represent a kind of mineral deposit which shows just the opposite pattern as uraninite, having been found only in sediments younger than 2 Ga. This is also consistent with an increase in the oxygen concentration at that time. The oxidation required for hematite deposition would have demanded a large supply of oxidants, presumably O_2. Banded iron formations (BIF) are comprised of mostly ferric ion and can be accumulated under high oxygen concentration. The major BIF deposits were found to be around two billion years old (Awramik, 1984) also indicating the increased

availability of oxidants (e.g. O_2) at that time. Based on the record of these inorganic deposits, scientists suggested that huge amounts of oxygen must have been generated around two billion years ago and before that time the concentration of oxygen was probably very low. Though nonbiological sources of the oxygen can't be ruled out, a biological source--oxygenic photosynthesis performed by cyanobacteria--seems more plausible.

Organic geochemical transitions may also record information pertinent to biological evolutionary events. It is well known that biological preference for the lighter isotopes of the key elements of life (e.g. carbon, hydrogen, sulfur, and nitrogen) will alter stable isotope abundances. For example, when carbon has passed through a chain of metabolic process of bacteria, the ratio of ^{12}C to ^{13}C increases. Thus, the value of $\delta^{13}C$ defined as

$$\left[\frac{(^{13}C/^{12}C)\text{sample}}{(^{13}C/^{12}C)\text{standard}} - 1\right] \times 10^3 \text{ (o/oo, PDB)}$$

(where PDB is the Peedee belemnite reference standard which is used to report the isotopic composition of carbon in samples) (Hayes, et al, 1983; Schidlowski, et al, 1983), becomes more negative. For sediments comprised of organic matter from bacteria or plants the $\delta^{13}C$ value is around -30 to -10 (o/oo,PDB). Samples from marine carbonates and atmospheric carbon dioxide have $\delta^{13}C$ value around 0 to -10

(o/oo,PDB). The preferential selection of ^{12}C is mainly due to autotrophic CO_2 fixation (Schidlowski, et al, 1983). Through isotopic geochemistry studies, scientists suggest that autotrophic biological activity was present at least 3.5 billion years ago ($\delta^{13}C$ is close to -30 (o/oo,PDB) (Hayes, et al, 1983). Other isotopic ratios such as $\delta^{15}N$, δ D, and $\delta^{34}S$ can also be used as an index of biological activity, but very few data are available for Precambrian samples. To better understand ancient biochemistries, more stable isotope measurements are needed.

The deficiency of stable isotope geochemistry is that the specific types of autotrophs involve in the ancient biosphere can't be figured out. To overcome this problem, the "biochemical marker" approach, another aspect of organic geochemistry, has been developed. Through analysis of specific organic chemical remains of Precambrian life by modern analytical techniques, it is hoped that more specific information about the types of organisms present in the ancient fossils can be learned. In this type of organic geochemistry "biomarkers", compounds which are produced by distinctive groups of organisms, are sought. In a sense these could be considered "chemical fossils". At least two approaches must be taken. The first is to examine ancient and recent sediments in the hope that some of the characteristic organic material produced by the ancient organisms is still there in recognizable form. The

second is to examine living organisms and mats to understand which organic compounds are diagnostic of which organisms. Combining the results from both approaches, one can attempt to infer when different types of living things were present.

Some organic compounds are unique in certain organisms and can be used as very good biomarkers to reflect the presence of these organisms in sediments (Smith, et al, 1983). The survival of some organic materials such as nucleic acids, proteins, and carbohydrates may be very poor, because of rapid microbial degradation and diagenesis which destroy them (Brown, et al, 1972). Lipids, however, seem to have longer survival potential. The surface of both eucaryotic and procaryotic cells is covered with a mixture of lipids (Goldfine, 1984), including linear hydrocarbons and their oxygenated derivatives such as fatty acids, long chain alcohols, wax esters, sterols, triterpenoids, and other isoprenoids. Some of these compounds are weakly bonded and easy to extract and analyze in organic solvents, and thus are called "free lipids". In many cases, these substances combine with more polar compounds to form complex lipids such as phospholipids, sulfolipids and steroidal glycosides, which are harder to extract and analyze.

Most organic matter in sediments is in the form of kerogen, a macromolecular material with no regular

structure, and which can't be extracted by organic solvents (Farrington, et al, 1977). Analytical methods for analyzing kerogen are still under development (Nagy, 1982). However, many fossil free lipids have been found in ancient (including Precambrian) and recent sediments (Nes and Nes, 1980; ten Haven, et al, 1985). A major problem in such work is the possible contamination of ancient sediments from lipids of more recent origin which have migrated into the rock, or which are introduced during analysis. Careful analysis of well-chosen samples, in which lipids are likely to be derived from organic matter deposited when the sediment formed, still reveals evidence of a surviving biomarker record for at least part of the Precambrian period. Most of these free lipids are hydrocarbons, some formed by defunctionalization of cellular components (e.g. steranes from sterols, hopanes from hopanols and hopenes) which occurs through microbial alteration or during preservation over geological time (Barghoorn,et al, 1965). In comparison to other chemical bonds, the C-C bond in hydrocarbons is quite strong (the bond energy for C-C is 347 KJ/mole) so the carbon skeleton is often preserved when hydrocarbons pass through different organisms or become incorporated into sediments. The approximate lifetime of alkanes subjected to 150^{o}C in the absence of catalysts can be calculated to be about 10^{9} years (Nes and Nes, 1980). Hence, biological information represented by a particular

hydrocarbon skeleton should be preserved over very long periods of time. Many steranes, hopanes, monomethyl alkanes, acyclic isoprenoids and other hydrocarbons have been found in Precambrian samples (see, for example, Eglinton, et al, 1964; Barghoorn, et al, 1965; Hoering, 1978; Arefev, et al, 1980; Nes and Nes, 1980; Jackson, et al, 1986; Klomp, 1986; Fowler and Douglas, 1987; Summons, 1987). The major objective for organic geochemists is to correctly assign the origins of sediment free lipids to the organisms which contributed them.

Many living organisms have been surveyed to attempt to understand their free lipid composition. As a result, a number of organic compounds (or their hydrocarbon derivatives) have been suggested as good biomarkers for certain types of organisms. Some hydrocarbon biomarkers which are known to survive in Precambrian rocks, and their suggested biological sources are shown in Table 1. A few of these may be particularly useful in establishing the timing of major evolutionary events, such as the emergence of cyanobacteria and eucaryotes. For example mid-chain branched alkanes, such as 7- and 8-methylheptadecanes may be unique to cyanobacteria, hopanoid hydrocarbons may be unique to aerobic bacteria, and steranes may be unique to eucaryotes (and possibly cyanobacteria). The validity of the suggested assignments is only as good as the choice of organisms studied in such surveys, however. Thus, organic

Table 1. Hydrocarbons with potential as Precambrian biological markers (modified from McKirdy and Hahn, 1982).

Hydrocarbon type	Characteristic feature	Possible sources
normal alkanes	C_{12} to C_{21} with odd carbon-number preference, C_{15} or C_{17} dominant	algae, bacteria, cyanobacteria
branched alkanes	7- and 8-methylheptadecane	cyanobacteria
	C_{16} to C_{30} iso and anteiso alkanes	bacteria
	C_{13} to C_{20} regular isoprenoids	photosynthetic algae, certain bacteria
	C_{21} to C_{40} regular and irregular isoprenoids (incl. squalene)	archaebacteria
cyclic alkanes	C_{27} to C_{35} pentacyclic triterpanes of hopane series	aerobic prokaryotes[a]
	steranes	algae, cyanobacteria

[a] restriction to aerobes suggested by Taylor (1934) and Ourisson (1987).

geochemical studies have also been performed on modern mats, as communities of organisms analogous to stromatolite communities. The complexity of marine lagoonal mat communities, which contain cyanobacteria, anoxygenic photosynthetic bacteria, and eucaryotic microorganisms, obscures the assignment of the sources of lipids found there. In comparison to lagoonal mats, hot spring microbial mats are built by much simpler collections of microorganisms, making the assignment of sources of lipids easier. In addition, hot spring microbial mats are built by different types of photosynthetic microorganisms (e.g. photosynthetic bacteria, cyanobacteria, and eucaryotic algae). They are thus especially valuable in verifying biomarker assignmemt for these evolutionary important microbial groups. Comparing the free lipid distributions of different hot spring microbial mats has been the main point of my research.

Hot Spring Microbial Mats and Microorganisms Studied

For this project, six mats were examined (see Table 2). All of the mats were collected from hot springs located in Yellowstone National Park and New Zealand. In hot spring mats, the environmental conditions are usually too extreme for eucaryotic organisms. In general, eucaryotes can't tolerate temperatures above 40-50^{o}C. An exception occurs in mats of acidic hot springs such as