Contributions of chemoautotrophic bacteria to the acid thermal waters of the geyser springs group in Yellowstone National Park
by James Alan Brierley

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Microbiology
Montana State University
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Abstract:
The Geyser Springs Group in Yellowstone National Park was selected for a study of the thermal water chemistry and the influence of the chemosynthetic autotrophic bacteria on the properties of these waters. A comparison of the analyses of certain thermal springs with a description published in 1935 showed no appreciable difference.

Two acid water drainages from thermal springs were studied to determine the effect of the chemosynthetic autotrophic bacteria on the water chemistry of these drainages.

Strains of sulfur-oxidizing Thiobacillus and iron-oxidizing Thiobacillus were found in the drainage designated GS-VI, VII. Only sulfur-oxidizing thiobacilli were found in the second drainage, designated GS-I. The highest temperature at which sulfur-oxidizing bacteria were found in these drainages was 69.5°C. A sulfur-oxidizing bacterium with a spherical cell shape was isolated. It resisted heating at 80°C for 90 minutes. Retractile bodies, which stained with a spore stain, were present in the cells of this unusual bacterium. This indicated that this organism may be able to form spores.

The chemosynthetic autotrophic bacteria influence the composition of the thermal drainage waters by their energy metabolism. The sulfuric acid, produced as a result of the oxidation of sulfur compounds, decreased the pH and increased the titratable acidity of the drainage waters. The aluminum concentration was increased in the water of the GS-VI, VII drainage probably as a result of the chemosynthetic autotrophic bacterial activity. The acid produced solubilized the aluminum from the kaolinite of the drainage channel.

The activity of the chemosynthetic autotrophic sulfur-oxidizing bacteria, as determined by gas uptake, was contained in the drainage channel material and not in the water flowing down the drainage. The two acid water drainages differed in their bacterial activity. Samples of the GS-VI, VII drainage taken from locations at which the temperature was between 43 and 53°C and between 20 and 26.5°C had the same activity. On the other hand a sample from the GS-I drainage at the location which the temperature was between 50 and 53°C had no measurable activity. But, there was a low rate of gas uptake in a sample from a location of temperature between 34 and 42.5°C. It was suggested that the difference in activity between the two drainages was because of the difference in water chemistry.

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in

Microbiology

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August, 1966

ACKNOWLEDGMENTS

The author would like to take this opportunity to express his gratitude to those who have been especially of help during the course of his graduate program.

Sincere thanks must be given to Dr. James E. Ogg of Colorado State University for without his confidence and encouragement this thesis would never have been accomplished.

The author expresses his gratitude to Dr. Kenneth L. Temple for his guidance in this study and help in the preparation of this thesis. Thanks are also due to Dr. John Wright for his support and suggestions throughout the duration of this project.

The cooperation extended by Mr. John McLaughlin, Superintendent, and Mr. John Good, Chief Park Naturalist, of Yellowstone National Park was greatly appreciated.

Sincere thanks are given to Dr. Bikash C. Raymahashay for his aid in the field and laboratory work and the information concerning the geochemical nature of the Geyser Springs Group.

Thanks to my wife, Corale, for her patience, excellent technical help, and aid in preparation of this thesis.

This project was supported by National Science Foundation Grant GB2062 and by Public Health Service Training Grant T1-WP-1, from the Division of Water Supply and Pollution Control.

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ABSTRACT

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Two acid water drainages from thermal springs were studied to determine the effect of the chemosynthetic autotrophic bacteria on the water chemistry of these drainages.

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INTRODUCTION

One of Nature's most unusual features is the discharge of thermal water in the form of hot springs and geysers. The beautiful colors of thermal water pools, the vivid sequence of coloration of drainages from hot springs and the eruptions of geysers are some of the striking features of thermal water phenomena. To the casual observer these spectacles provide impressions of a superficial nature. For those interested in the fundamental aspects of these phenomena, there is an area for study by a cross section of many scientific disciplines--physics, chemistry, geology, and biology. The study of thermal springs is especially interesting to the biologist, for here is an environment that provides bizarre conditions.

The major concentrations of thermal springs occur in five areas--Iceland, New Zealand, Japan, New Guinea, and Yellowstone National Park. The proximity of Yellowstone National Park to the laboratories at Montana State University, Bozeman, Montana, facilitates study of these thermal phenomena and almost makes it an obligation.

The most extensive chemical and physical description of Yellowstone National Park was published by Allen and Day (1935). They listed some characteristics of selected thermal springs of almost every thermal area within the Park. Their work will be quoted throughout this thesis for descriptive and comparative purposes. Recently the New Zealand thermal areas have been the subject of extensive chemical investigations by Ellis and Anderson (1961), Ellis and Sewell (1963), Golding and Speer (1961),

Mahon (1962, 1964, 1965), Ritchie (1961), and Surbutt (1964). These studies were concerned with the determination of the physical-chemical origin of the constituents of thermal waters. Uzumasa (1965) has reviewed the literature of the thermal water chemistry of Japanese thermal springs. His review presented analyses, classifications of thermal springs based on chemical constituents, and origins and mechanisms of flow of thermal springs.

Many forms of life are found in thermal springs and drainages from thermal springs. The early studies of thermal environments has been reviewed by Weed (1889 a, b).

The ecological studies of the blue-green algae of the thermal springs in Yellowstone National Park have received the most attention. Copeland (1936) and Nash (1938) made extensive descriptive surveys of the Myxophyceae.

The animal life of the hot springs of Yellowstone National Park and other hot springs in North America has been reviewed and described by Brues (1924, 1932). He listed the animal types found, but did little to relate these types to their environment. His description of the thermal springs was limited to water temperature, pH, and specific gravity.

Most studies of life in thermal waters are of a descriptive nature. However, some information has been obtained regarding upper temperature limits of life by the observations of biological activity in thermal waters. Setchell (1903) made observations and collected samples from the thermal springs of Yellowstone National Park and California to

determine this upper temperature limit. He found only blue-green algae and bacteria in water of temperatures above 45°C. He recorded 89°C as the highest temperature at which living organisms could exist, and he found these organisms to be filamentous bacteria. Setchell was able to find life only in alkaline waters and stated, "No organisms were found in springs reputed to have a decided acid reaction."

Kempner (1963), who studied upper temperature limits of life in Yellowstone National Park waters, found that 73°C was the upper limit for algal material scraped from the sides of pools and pool drainages. His evidence was based on the uptake of P^{32} labelled phosphate at 73°C. Although his evidence is considered reliable, his study was very limited. There is a wide divergence of thermal environments present in the Park. Some of the areas may yield organisms with an increased temperature tolerance. Temple (unpublished data) has found a filamentous bacterium apparently growing at 78.5°C in a small alkaline spring.

Brock and Brock (1966) determined that the optimum temperature for algal development in thermal springs of Yellowstone National Park is between 51° and 56°C. However, their investigation considered temperature as the only variable in thermal spring environments and they neglected the importance of water chemistry. It should also be pointed out that this temperature optimum is for algae of alkaline waters and not for algae of acid waters.

The study of the bacteria in thermal springs of Yellowstone National Park has not been extensive. Species of the genus Bacillus have been

found in springs of many thermal areas, (Walter 1952, Walter and Northam 1952, Marsh and Larsen 1953, Brierley and Walter 1963, and Brierly 1963). All of the bacteria were obtained from pools or pool and geyser drainages of alkaline pH except three, which were obtained from waters of acid reaction. All the studies with the exception of Walter (1952) were limited to the thermophilic species of Bacillus. The interaction of these bacteria with their environment has not been determined.

The bacteria and algae present in the thermal springs of Japan have probably received greater study than those of either New Zealand, Iceland or Yellowstone National Park. Most of the literature dealing with the life in the thermal springs of Japan is of a descriptive nature and gives little information about the activities of the organisms. Miyoshi's (1897) study of the Beggiatoa, Chromatium and sulfur bacteria was one of the earliest in this area. Molisch (1926) found Beggiatoa, Chromatium, Thiothrix, and iron bacteria, probably of the Leptothrix type. Algae, amoebae, infusoria, flagellates, rotifers and Anguillua of thermal springs were also described.

Emoto (1933a) made an extensive study to determine the presence or absence of thiobacilli in the thermal waters of Japan. He found four species--Thiobacillus thermitanus, T. crenatus, T. lobatus, and T. umbonatus. They were present only in water with detectable H_2S, however these organisms could tolerate a pH range of 1.4 to 7.5 and a temperature range of 5° to 80°C. T. thermitanus was the only species reported to exist in water of 80°C and pH 2.8.

The four species of thiobacilli described by Emoto have not been generally accepted, since their classification is based wholly on colony morphology. Vishniac and Santer (1957) believe that these four species are all T. thiooxidans. The Russian workers, Zavarzin and Zhilina (1964), studied the sulfur-oxidizing bacteria in the thermal areas of the Kunashir and Kurile Islands. They had hoped to find the Japanese species of Thiobacillus, but they were unable to isolate any bacteria which could be specifically identified. The bacteria which they isolated demonstrated a range of characteristics between T. thiooxidans and T. thioparus.

Emoto and Hirose (1942a) described 15 species of Cyanophyceae and two species of Chlorophyceae from the thermal springs of Narugo. They found Beggiatoa leptomitoformis in water having a pH of 6.6 to 6.7 and a temperature of 33° to 37°C. They also found the four Japanese species of Thiobacillus. T. thermitanus, T. crenatus, and T. lobatus were found at water temperatures between 33° and 76°C and T. umbonatus was found between the temperatures of 44° and 61°C. All of these species apparently live in waters having a pH between 3.0 to 7.1.

Emoto and Hirose (1942b) found Leptothrix ochraceae and three species of Thiobacillus in the thermal waters of the Onikobe springs. The Leptothrix was found only in a sample of water with a pH of 3.2 and at 43°C. T. thermitanus, T. crenatus and T. lobatus were only found in acid waters between pH 1.8 and 3.6 and at temperatures ranging from 31° to 69°C. In addition to the bacteria, there were 43 other forms of plant life which included members of the Cyanophyceae, Heterocontae, Chlorophyceae and

Conjugatae.

The most extensive survey of the acidic thermal waters of Japan was made by Negoro (1944), who gave chemical data of these thermal springs and presented information on the bacteria, algae and other plants of these waters. This paper presents the most complete descriptive survey of acidic thermal waters.

Schwabe (1936) made an extensive survey of the bacteria, algae, higher plants and insects of the thermal springs of Iceland. He supplemented his work by presenting physical descriptions, temperature conditions and chemical data for those thermal springs which were studied. Schwabe noted that bacteria were the only organisms in water at a temperature of 80°C and a pH of 9.4. This study dealt with alkaline waters which Schwabe says are the most important in Iceland's thermal areas.

There are many reports of a limited nature dealing with investigations of local thermal environments which occur throughout the world.

Czurda (1935) isolated two species of Thiobacterium (Thiobacillus) and a single species of Thiospirillum from the sediment of the Pystian hot spring in the Southern Moravian mountains of Czechoslovakia. In this spring drainage he found bacterial and blue-green algal growth at temperatures below 60°C.

Vouk (1960) reported that he had found an iron bacterium, Gallionella schmenzkii n. sp., in the thermal waters of Bad Gastein located in Austria.

A morphologically unusual bacterium was obtained from the hot springs of Tiberias (Israel) (Kahan, 1961). The cells were spherical, ovoid or

pearshaped and reproduction was by means of budding. These organisms grew in the laboratory between the temperatures of 37° and 50°C on low concentrations of yeast extract. The author believed that this organism was a member of the order Hyphomicrobiales.

A spore forming Thiobacillus, T. thermophilica n. sp., was isolated from the Bragunskie Hot Springs of Russia by Egorova and Derygina (1963). This chemoautotrophic bacterium has the characteristic of being the only Thiobacillus known to form spores. This bacterium grew at temperatures between 40° and 80°C, but only under alkaline conditions.

There are many references available regarding descriptive work on thermal waters. However, there is little information available regarding the interaction between the biological entities and their thermal environment. Weed (1889a) was probably the first worker to consider the role of life in thermal geochemistry. Weed speculated that plant life in the thermal springs of Mammoth Hot Springs in Yellowstone National Park causes the deposition of travertine. He also believed that vegetation of the alkaline thermal areas along the Firehole River in Yellowstone National Park were responsible for production of some deposits of siliceous sinter.

Kaplan (1956) surveyed the Rotorua-Taupo and White Island geothermal areas of New Zealand. His study determined the presence of sulfur-oxidizing bacteria, photosynthetic sulfur bacteria, sulphate-reducing bacteria, and algae of these thermal waters. He furthered his study by determining the conditions under which these populations exist. The

sulfur-oxidizing thiobacilli, which Kaplan studied, appeared to be the most geochemically active form of life present, and these bacteria may actually bring about the acidification of many pools and drainages. These sulfur-oxidizing thiobacilli may also be important in the formation of gypsum in the White Island area. It is known that gypsum is formed by the action of sulfuric acid on feldspar. Kaplan suggested that the gypsum, which was closely associated with sulfur, may be formed, at least in part, from sulfuric acid produced by the bacterial oxidation of sulfur.

Hariya and Kikuchi (1964) implicated the activity of bacteria in precipitating manganese as manganese hydroxide in hot and cold springs in Japan. The bacteria in their laboratory cultures were described as peritrichously flagellated rods, which were able to oxidize manganese as a source of energy. This organism is exceptional in that the group of organisms ordinarily associated with inorganic oxidations are of the order Pseudomanadales which all possess terminal flagella.

The bacteria of the Thiobacillus-Ferrobacillus group have been shown to catalyze a number of important geochemical transformations other than those which occur in thermal springs. The classic example (Razzell and Trussell, 1963) of degrading activity is the leaching of chalcopyrite ore by Thiobacillus ferrooxidans, which releases copper. Ehrlich (1964) demonstrated that a member of the Thiobacillus-Ferrobacillus group is able to catalyze the oxidation of arsenopyrite. During this oxidation there is a concurrent solubilization of arsenic as arsenite and arsenate. Ehrlich (1963) also demonstrated that orpiment can be oxidized by

Ferrobacillus ferrooxidans with a concurrent release of arsenite. T. ferrooxidans and T. thiooxidans have been shown to oxidize molybdenite with a simultaneous increase of molybdenum in solution. (Bhappu, et. al., 1965). Temple and Koehler (1954) demonstrated that T. thiooxidans and T. ferrooxidans can produce sulfuric acid by their oxidative attack on pyrite and marcasite in coal.

The purpose of this study is to describe some of the chemical and physical properties of a small thermal area within Yellowstone National Park, and to consider in detail its environment of acid thermal waters. The chemosynthetic autotrophic bacteria isolated from the acid thermal waters will be characterized. Any relationships, of a geochemical nature, between these bacterial populations and their environment will be explored.

MATERIALS AND METHODS

Water Chemistry

Water samples were collected from the sampling stations during all seasons from 1964 to 1966. The samples were filtered in the field through "Millipore" filters with a pore size of 0.45μ or "Flotronic" silver membrane filters with pore sizes of 0.2μ, 0.45μ, 0.8μ, and 1.2μ. After filtering, the samples were placed in 1 liter polypropylene plastic screw-cap bottles, which had been rinsed with a small amount of filtered sample. About 100 ml of each sample was also placed in clean glass stoppered pyrex bottles for iron analysis.

The samples were returned to the laboratory. When chemical analyses could not be done immediately after returning to the laboratory the samples were stored at 4°C.

The chemical analyses of all samples were conducted as follows: sodium, potassium, calcium and magnesium were determined with a Beckman Model DU Flame Spectrophotometer, following the procedures given in the Beckman Instruction Manual #334-A, and the magnesium of some samples was determined by atomic absorption spectroscopy using the Beckman Spectrophotometer; total alkalinity, aluminum, chloride, ferrous and total iron, fluoride, phosphate, silica, and sulfate determinations were made as described by the American Public Health Association (1960). The samples in which fluoride was determined, were distilled by procedure II of the American Public Health Association (1960). Ammonia was determined by the direct Nessler

method. The total or titratable acidity of acid water samples was determined by titration with 0.1 N NaOH to the phenolphthalein end point.

All laboratory pH values were taken with samples at 25°C using a Radiometer model 25 expanded scale pH meter which was standardized with two buffers to bracket the pH value of the sample. The laboratory Eh values were also determined using the Radiometer with the sample at 25°C.

The standard deviation (s) reported for the results were computed using the formula, $s=\sqrt{\frac{\Sigma x^2}{N-1}}$, in which $\Sigma x^2 = \frac{N\Sigma x^2 - (\Sigma x)^2}{N}$ (Crabtree, 1962).

Field Measurements

Field temperature measurements were taken using a "Tri-R" electronic temperature probe with a 20 foot lead.

Field pH and Eh determinations were made with Beckman or Corning high temperature glass electrodes, Beckman calomel and platinum electrodes. Readings were made with a "Keithley" VTVM and a standard reference EMF box, at the temperature of the water.

The rate of flow of water in one drainage was determined. A piece of pliable plastic was used to divert the water from the drainage into a 2 liter plastic beaker for a measured period of time determined with the sweep second hand of a wrist watch.

Bacteriological Studies

Samples for bacteriological analysis were collected in sterile, 100 ml, screw cap polypropylene bottles. Each sample consisted of the spring water and a portion of the drainage channel material at the site of collection. The samples were returned to the laboratory and stored at 4°C.

Microscopic observations were made with a Nikon phase-interference microscope.

Sulfur and iron-oxidizing bacteria were selected for inoculating enrichment media with 1 ml of sample. The enrichment media were incubated at temperatures which were within the range of temperature of the sample in the field. After three transfers in enrichment media, plates of sulfur and iron agar were streaked to obtain isolated colonies. Colony types were picked and restreaked at least three times to ensure purity of the culture.

Media

All media consisted of one of two basal salts solutions which were similar in composition to the water of the two acid thermal springs investigated.

GS-I basal medium:

Distilled water	1 L
$(NH_4)_2SO_4$	0.5 g
NaCl	0.3 g
KH_2PO_4	0.1 g
$Ca(NO_3)_2.4H_2O$	0.01 g
1 N H_2SO_4	2 ml
Trace element solution	0.5 ml
pH	2.7

GS-VI, VII basal medium

Distilled water	1 L
$(NH_4)_2SO_4$	0.3 g
NaCl	0.025g
KH_2PO_4	0.07 g
$Ca(NO_3)_2.4H_2O$	0.01 g
1N H_2SO_4	1.5 ml
Trace element solution	0.5 ml
pH	2.9

Appleby's trace element solution (Appleby, C. A. Division of Plant Industry, C. S. I. R. O., Canberra, Australia, personnel communication) was used in the above basal media.

Appleby's trace element solution

Distilled water	1 L
$FeCl_3.6H_2O$	3.60 g
H_3BO_3	0.57 g
$ZnSO_4.7H_2O$	0.44 g
$CoCl_2.6H_2O$	0.20 g
$CuSO_4.5H_2O$	0.02 g
$MnCl_2.4H_2O$	0.02 g
$Na_2MoO_4.2H_2O$	0.05 g

The enrichment media consisted of one of the basal salts solution and either sulfur or ferrous iron as an energy source.

The sulfur was sterilized by intermittent steaming for 4 consecutive days. Sulfur in 0.25 g quantities was steamed for 35 minutes and sulfur in 1 g quantities was steamed for 60 minutes. The basal salts solutions were dispensed in 50 ml quantities in 125 ml Erlenmeyer flasks and sterilized by autoclaving. The sterile 0.25 g quantity of sulfur was added to produce the sulfur enrichment medium. The 1 g quantities of sulfur were used for larger quantities of basal salts solution.

A solution of $FeSO_4.7H_2O$, 25 g in 100 ml of distilled water, was sterilized by autoclaving. Two ml of this solution was added to 50 ml of sterile basal salts solution in a 125 ml Erlenmeyer flask to provide the iron enrichment medium.

A medium with thiosulfate as the energy source, employing either of the basal media, was devised. It was necessary to modify each of the basal media to bring their pH near neutrality. Acid conditions change

thiosulfate to sulfite and sulfur. The GS-I basal medium was modified by using 0.1 g K_2HPO_4 in place of KH_2PO_4 and adding 3 ml of 0.01 N H_2SO_4 instead of the 1N H_2SO_4. The GS-VI, VII basal medium was modified by using 0.07 g K_2HPO_4 in place of KH_2PO_4 and 10 ml of 0.01 N H_2SO_4 in place of 1N H_2SO_4. The pH of the modified basal media was near 7. Two ml of a filter sterilized $Na_2S_2O_3.5H_2O$ solution, 12.5 g in 100 ml of distilled water, was added to 50 ml of the sterile modified basal media.

Solid media with either thiosulfate, iron or sulfur as energy sources and with respective basal salts solutions were also used.

A 0.9% concentration of Oxoid "Ionagar" No. 2 was added to the modified GS-I or GS-VI, VII basal media which was then autoclaved and cooled to 50°C. Ten ml of a filter sterilized solution of $Na_2S_2O_3.5H_2O$, 50 g in 100 ml distilled water, was added to each liter of medium. Plates were then poured.

It was necessary to separately sterilize the components of ferrous iron agar. The components for 1 L of basal medium were dissolved in 250 ml of distilled water. Five grams of $FeSO_4.7H_2O$ was mixed in a second 250 ml quantity of distilled water, and 9 g of Oxoid "Ionagar" No. 2 was placed in a third 500 ml quantity of distilled water. All of the components were sterilized by autoclaving for 15 minutes at 121°C, cooled to 50°C, combined and plates poured.

A new method was devised for the incorporation of colloidal sulfur in agar to provide a solid medium for the isolation of sulfur-oxidizing bacteria. The method described below is rapid and the medium, unlike the

thiosulfate agar medium, can be adjusted to any desired pH.

The colloidal sulfur suspension was prepared as follows: 1 g of flowers of sulfur in 50 ml of acetone was heated using a hot plate. The mixture was boiled for 3 minutes with constant swirling. After boiling, undissolved sulfur was allowed to settle and the hot solution of sulfur in acetone was decanted into 500 ml of distilled water at room temperature. This produced a milky colloidal suspension of sulfur. The amount of sulfur in suspension was increased by repeating the boiling procedure with two more portions of hot acetone. This suspension of sulfur in water was autoclaved at 121°C for 15 minutes to remove the acetone and sterilize the sulfur suspension. It was important to form the suspension in distilled water only, as the presence of salts caused the colloidal particles to aggregate.

The colloidal sulfur suspension was used to prepare solid media by mixing the sulfur suspension with an appropriately prepared concentration of agar and salt solution when all of the components had cooled to 50°C following autoclaving. The agar solution was prepared by adding 9 g of Oxoid "Ionagar" No. 2 to 250 ml of distilled water. Either the GS-I or GS-VI, VII basal salt solutions was used. The salts for 1 liter of medium were dissolved in 250 ml of distilled water. This provided 1 liter of an acidified agar medium with finely divided sulfur for the energy source.

When 1 or more liters of liquid medium were used in an experiment the vessels were sparged with air which had passed through sterile cotton

filters. A "Dyna-Vac" pump was used to deliver air to the flasks. The pump was not reliable enough to provide a constant regulated supply of air although it was continuous and vigorous.

The heat resistance of two strains of the sulfur-oxidizing bacterial isolates was determined.

One hundred milliliters of culture with sulfur as an energy source was incubated for 7 days at the same temperature used for the isolation of the strains. Each culture was then placed in a sterile dilution bottle with an Escher stopper and vigorously shaken for 1 minute. Five milliliters of the mixed suspension was placed in sterile screw cap tubes, which measured 115 mm x 16 mm. The tubes were placed in a 1 L beaker with water at 80°C. A tube containing 5 ml of distilled water and a thermometer was also placed in the bath. When the water in the tube reached 80°C the timing was begun. It was possible to maintain the temperature of the water in the tube at 80$\pm$ 1°C by adjusting the flame of the Bunsen burner used for heating and by constant stirring of the bath with a glass rod. Tubes were removed at 10 minute intervals. Immediately after the removal of a culture tube from the bath, 1 ml was transferred to a sterile sulfur enrichment medium and incubated at the temperature at which the culture was grown. The sulfur enrichment cultures were examined for growth after 7 days of incubation. Microscopic observation of each flask was made. The pH of the medium of each flask was determined and compared with an uninoculated control flask.

The importance of the chemoautotrophic bacterial populations in causing changes in water chemistry was assessed. For this experiment the bacteria were grown in the presence of drainage bed material to determine if any biologically initiated alteration of this material could bring about a change in water chemistry.

Two and one-half liter Fernbach flasks, filled with 2 L or either GS-I or GS-VI, VII basal medium, were used as reaction vessels. Fifteen grams of dried drainage bed material from either the GS-I or the GS-VI, VII drainages was added to respective flasks with the corresponding basal medium. The flasks with media and drainage bed material were sterilized by autoclaving at 18# for 30 minutes. The flasks were cooled and either 3 g of sterile sulfur or a sterile solution of $FeSO_4.7H_2O$, 5 g in 100 ml of distilled water, was added. These compounds served as bacterial energy sources.

Each flask was stoppered and connected to an air pressure manifold. Figure 1 shows the arrangement of each flask. Air was bubbled through distilled water in order to saturate the air with water and to prevent evaporation from the reaction vessel. The air was passed from the flask containing distilled water, through a sterile cotton filter, and into the reaction vessel.

The flasks were incubated (at temperatures corresponding to the temperature at which the inoculum was originally obtained) for a period of 3 days to allow for equilibration of basal medium with the drainage bed material. The flasks were then inoculated with 50 ml of culture.

Enrichment culture samples were used for inoculation rather than pure cultures. It was believed that these cultures would more closely duplicate the population in the field environment than a pure culture.

Each flask was aseptically sampled immediately after inoculation by closing the exhaust tube and removing the tube cover of the sampling tube. The air from the sparger increased the pressure and forced the sample through the sampling tube. The exhaust was reopened and the sterile tube cover replaced over the sampling tube.

The reaction vessels, inoculated with iron enrichment cultures, were sparged with air immediately after inoculation. The reaction vessels with the sulfur enrichment cultures were sparged with air only after a 3 day

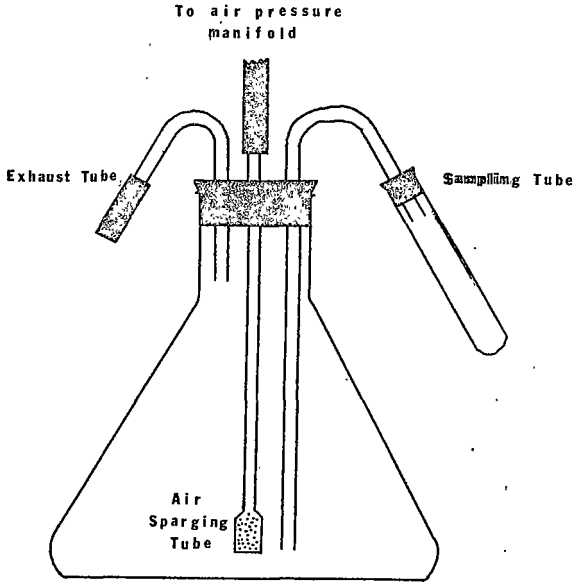

Figure 1. The reaction vessel used for determining geo-microbial activity.

incubation period. Cook (1964) showed that this period was necessary in order to establish growth. Immediate vigorous aeration or shaking prevents the contact of the bacterium with the sulfur and thus inhibits growth.

After 2 weeks of incubation, the final sample was removed for determination of the level of chemical constituents.

Both the initial and final samples were filtered through 0.45μ "Millipore" filters immediately after removal from the reaction vessels.

Manometric Measurements

Manometric experiments were performed with a Gilson Differential Respirometer. Single side arm reaction vessels were used.

Plug Sampler

It was necessary to compare samples from the thermal spring drainages to determine their relative biological activity. A sampling device was constructed so that equal sized samples of the drainage channel beds could be obtained. This device was used to obtain a "plug" of the channel bed material. The plug sampler (Figure 2) was assembled with a Bunsen burner, rubber stoppers, steel rod, and scrap metal. The core, which was 2.6 cm in diameter, was removed from a size 12 rubber stopper. The stopper was slipped over the bottom end of the Bunsen burner, so that 1 cm of the end of the Bunsen burner protruded. This stopper served as a collar to prevent sampling deeper than 1 cm. A size 6 rubber stopper was shaped to give a tight fit within the Bunsen burner. This rubber stopper was mounted on the end of a steel rod 3/8 inches in diameter and 9 inches long. The piston thus formed was placed in the Bunsen burner to

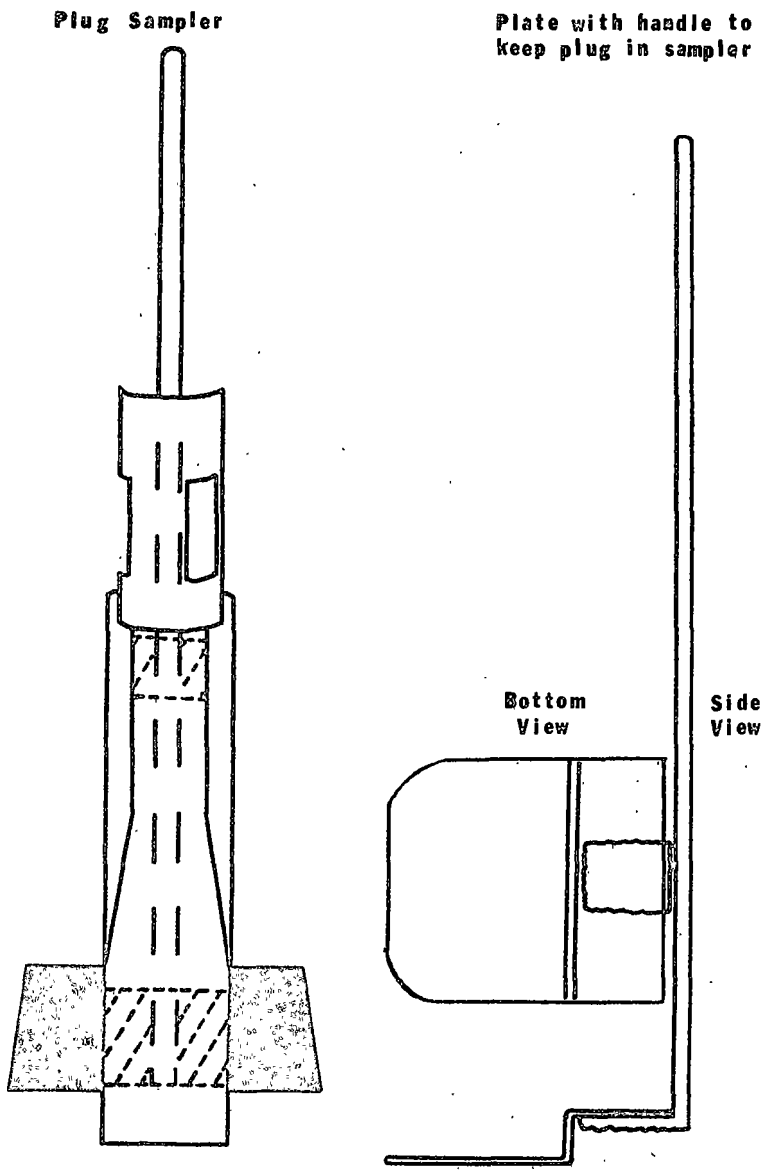

Figure 2. The plug sampler used for obtaining uniform samples from drainage beds.

provide a means for removing the sample plug. A small rubber stopper with the center removed was placed in the Bunsen burner opposite the sampling end. This stopper served as a guide for the piston. A metal plate with a handle (Figure 1) was used to close the sampling end to prevent the sample plug from dropping out when the sample was removed. The plugs were placed in sterile glass jars. This sampler removed a plug with a 5.3 cm^2 surface area and 5.3 cm^3 volume.

The following procedure was used to compare the activity of the sulfur-oxidizing chemoautotrophic bacteria in the two thermal water drainages investigated.

Each plug sample was mixed with 20 ml of sterile GS-I or GS-VI, VII basal medium, depending on the drainage from which the plug was obtained. The drainage material and basal medium were vigorously shaken for 1 minute. Ten milliliters of this suspension was sterilized by autoclaving at 18# for 15 minutes. Three milliliters of the basal medium with the plug sample and 3 ml of the sterilized suspension were placed in respective manometric flasks. The pH of the suspensions in each flask was stabilized at pH 7 by adding 1.5 ml of sterile 0.005 M Na_2CO_3. One-half milliliter of a sterile solution of $Na_2S_2O_3.5H_2O$, 0.125 g/ml, was added to each flask to provide an energy source. To serve as a control for endogenous respiration, one manometric flask contained 0.5 ml of sterile distilled water in place of the thiosulfate. The manometric flasks were then connected to the Gilson Differential Respirometer and the flasks were allowed to equilibrate with the water bath temperature for 15 minutes before the

start of each experiment. The change of gas pressure which was measured, was probably caused by the uptake of O_2 during the oxidation of thiosulfate. However, some of this change may have been due to the uptake of CO_2 since the chemoautotrophic sulfur-oxidizing bacteria are able to fix CO_2.