A study of the sulphur bacteria of the hot springs of Yellowstone National Park
by Raymond H Howard

A THESIS Submitted to the Graduate Committee in partial fulfillment of the requirements for the degree of Master of Science in Bacteriology
Montana State University

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A differential staining technic was developed for sulphur organisms utilizing a mordanted malachite green or methylene blue stain and counterstaining with sodium nitroprusside. With this technic, the cell outline retains the primary stain, and the sulphur granules assume a contrasting red color.

Representative species of gram positive and negative organisms were studied in order to show that this stain did not indicate sulphur complexes present in small concentrations in bacterial protoplasm.

Thiobacilus thiooxidans was stained with the above technic and polar red granules were observed. These red granules indicated that the sulphur complexes in Thiobacillus thiooxidans are similar in nature to the granules found in the true sulphur bacteria studied in the present work.

To test this technic, organisms collected from thermal waters of Yellowstone National Park and immediate vicinity were studied

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INTRODUCTION

Since so few studies have been made on the methods of differentiating and staining of sulphur bacteria, it is the purpose of the writers to determine a differential staining technic which will facilitate further observations on these organisms found in thermal waters in Yellowstone National Park and immediate vicinity.

REVIEW OF LITERATURE

The study of sulphur bacteria is a comparatively recent study of microorganisms. Although casual observations of composite sulphur bacteria in hot springs were made as early as 1860, studies as to their isolation and pure culture, morphology and physiology were not made until several years later. Cramer ('70) was the first to suggest that granules in Beggiatoa, a genus of the sulphur bacteria, consisted of sulphur. From investigations carried out in 1869-71 on the vegetation of the Yellowstone Hot Springs, J. W. Harshbarger ('97) reported the presence of bacteria able to deposit sulphur as granules within their cells. Cohn ('75) then postulated the theory that the Beggiatoa and the purple bacteria produce hydrgen sulfide by reduction of sulfates. W. H. Weed ('89) in his article entitled "The Vegetation of Hot Springs" shows that "travertine" is the result of sulphur deposition by the

Beggiatoa. This work was substantiated by B. M. Davis ('97). His observations were that "travertine" and "felt", a closely woven mass of filamentous bacteria in which crystals of calcium carbonate were imbeded, were responsible for many of the colored deposits in the park. Controversy as to the origin of organisms in sulphur springs was the result of investigations carried out by W. A. Setchell, ('03). His contention was that no organisms were found in strictly thermal waters nor in springs which were reputed to have a decided acid reaction.

Engelmann ('87) first postulated the theory that purple and green sulphur bacteria belonged to the photosynthetic group of organisms. This theory was strongly opposed by Winogradsky ('88) who proposed the theory of chemosynthetic metabolism. In these processes, the energy supply of the organism is not furnished by decomposition of organic matter, but by the oxidation of inorganic substances. In these processes, also, hydrogen sulfide is oxidized by the organism to sulphuric acid. Molisch ('07) published his monograph of the purple bacteria in which he concluded that purple bacteria assimilate organic compounds in the light. This was his attempt to defeat the theory of an autotrophic mode of life for these organisms, as outlined by previous investigators. Such a view was in direct support of the work of Nadson ('03) who stated, also, that hydrogen sulfide is not required for nutrition, and sulphur is not accumulated. Buder ('19) in discussing the value of the

various theories presented up until this time, was inclined to believe that the metabolism of the purple bacteria should be considered as a combination of photosynthetic and chemosynthetic modes of life, independent of each other, but providing the organisms with the faculty to live and thrive under divergent conditions. This idea is called "only a well founded assumption" but even at the present time, we have come no further in our knowledge of this function.

Warming ('75) and Lankester ('76) in their early investigations, drew the conclusion that all the various forms and shapes of colored organisms with droplets inside the cells represented different developmental stages or "phases of growth" of one species. This idea was attacked by Cohn ('75) who held to the monomorphistic viewpoint, as did Winogradsky ('87). Such a viewpoint stressed the fact that distinct variations were characteristic of different species. On this basis, Winogradsky established an elaborate system of classification of the sulphur bacteria based upon the shape and size of the cells, as well as upon their mode of colony formation. The excellence of this system is shown by the fact that it has been perpetuated--with only minor modifications--to the present day. Van Niel ('30), through extensive investigation, concluded that variations as to size, shape, and growth are often encountered and are the result of environmental effects such as hydrogen sulfide concentration, pH of the medium, age of

the culture, and presence of free oxygen.

Frobisher ('44) outlines the classification of sulphur bacteria in which he places them all under the Order Thiobacteriales. Criteria for further subdivision were the presence of photosynthetic pigments and the presence of free sulphur as granules within the cell walls. Those organisms which possessed photosynthetic pigments and store sulphur within their cell walls were classified under the Family Thiorhodaceae. Those organisms which possessed photosynthetic pigments and did not store sulphur within their cell walls were classified under the Family Athiorhodaceae. Those organisms which possessed no photosynthetic pigments but stored sulphur within their cell walls were divided into the filamentous organisms under the Family Beggiatoaceae and the non-filamentous organisms under the Family Acromatiaceae. Bergey ('46), however, has reclassified the sulphur bacteria. In his classification, he has placed the sulphur bacteria which resemble true bacteria in morphology under Order I, Eubacteriales, Suborder III, Rhodobacteriineae, and the sulphur bacteria which resemble algae in morphology under Order III Chlamydobacteriales.

Ellis ('32) in his investigations of the sulphur bacteria was the first to use a chemical compound to prove the existence of sulphur granules by color indication. When a smear of the sulphur containing organisms was treated with a concentrated

solution of sodium nitroprusside ($Na_2FeNO(CN)_5$), the rings of sulphur assumed a blood red color. This procedure proved the existance of sulphur granules; however, it was of no value in determining cell morphology since it failed to show the cell outline.

MATERIALS AND METHODS

Media employed:

Inorganic Medium according to Kiel ('12)

$CaH_2(CO_3)_2$	0.34%	$Ca_3(PO_4)_2$	0.02%
$MgH_2(CO_3)_2$	0.27%	KCl	0.01%
$CaSO_4$	0.31%	K_2S	0.01%
$MgSO_4$	0.51%	FeS	0.01%
Na_2SO_4	0.21%	CaS	0.01%

A small amount of ammonium sulphate was added; also, oxygen, hydrogen sulphide, and carbon dioxide were introduced.

Inorganic Medium according to van Niel ('30)

NH_4Cl	0.1%
K_2HPO_4	0.05%
$MgCl_2$	0.02%
$NaHCO_3$	0.1%
$Na_2S \cdot 9H_2O$	0.1%

This medium was adjusted to a pH of 8.0-8.5 by the addition of sterile Na_2CO_3 or H_3PO_4.

Samples investigated:

1. 24 samples were collected from thermal springs in southwestern portion of Montana.
2. 42 samples were collected from thermal sulphur springs in Yellowstone National Park.

Stains employed:

Ziehl's carbol fuchsin (Conn '40)

Crystal violet - 1:50,000 aqueous solution

Methylene blue - 1:50,000 aqueous solution

Malachite green - 1:50,000 aqueous solution

Huckers Gram Stain - (Hucker '23)

Sodium nitroprusside - 50% saturated aqueous solution

Bismark brown Y - concentrated solution

Fast green - concentrated solution

Congo red - concentrated aqueous solution

Cultures used for comparison tests:

Escherichia coli (Migula) Castellani and Chalmers

Proteus vulgaris Hauser

Bacillus subtilis Cohn emend. Praxmowski

Bacillus mycoides Flügge

Rhodospirillum rubrum (Esmarch) Molisch

Thiobacillus thiooxidans Waksman and Joffe

These cultures were obtained from stock cultures of the Botany and Bacteriology Department, Montana State College, Bozeman, Montana. The original cultures were obtained

from the American Type Culture Collection.

Review of Technics Employed

One of the greatest difficulties encountered by investigators in the field of sulphur bacteria was the development of media and technics suitable for the isolation and cultivation of pure cultures. Molisch ('07) employed a solid medium containing river water, peptone, dextrin, and agar. However, this medium contained an excess of organic material which would not permit exact studies of autotrophic forms.

Kiel ('12) employed a strictly inorganic medium containing the following constituents:

$CaH_2(CO_3)_2$	0.34%	$Ca_3(PO_4)_2$	0.02%
$MgH_2(CO_3)_2$	0.27%	KCl	0.01%
$CaSO_4$	0.31%	K_2S	0.01%
$MgSO_4$	0.51%	FeS	0.01%
Na_2SO_4	0.21%	CaS	0.01%

A small amount of ammonium sulphate was added; also oxygen, hydrogen sulfide and carbon dioxide were introduced.

Van Niel ('30) obtained very satisfactory results with a medium with the following composition:

NH_4Cl	0.1%
K_2HPO_4	0.1%
$MgCl_2$	0.1%
$NaHCO_3$	0.1%
$Na_2S \cdot 9H_2O$	0.1%

The quantities of K_2HPO_4 and $MgCl_2$ were changed to 0.05% and 0.02% respectively, because during sterilization, the original medium deposited $MgNH_4PO_4$. The medium was adjusted to a pH of 8.0-8.5 colorimetrically using a phenol sulfo red indicator, by the addition of sterile Na_2CO_3 or H_3PO_4.

In the culturing of sulphur bacteria by van Niel, comparative studies showed that cultures which developed under natural daylight conditions in the laboratory in three to four weeks showed the same growth in four to five days in case of continuous illumination by an ordinary electric bulb of 25-50 Watt, placed at a distance of 20-30 cm. The use of completely filled, glass stoppered bottles provided for fully anaerobic conditions which are necessary for the development of the purple and green sulphur bacteria.

In the culturing of sulphur bacteria, the authors made inoculations into a medium as described by Kiel ('12). Samples were collected from a hot spring situated at the Department of Interior Fish Hatchery, Bozeman, Montana; an outdoor pool at the residence of Mr. F. B. Cotner, Bozeman, Montana; and from a marsh located southwest of Bozeman on the road to Hyalite Canyon.

One mililiter amounts of these samples were inoculated into 30 ml. portions of the above medium. These tubes were then incubated at 32° C., in the dark, for eight days, and at this time a definite bacterial plate was noticed suspended

in the liquid above the precipitated compounds in those samples collected at the residence of Mr. F. B. Cotner. Stained smears from these bacterial plates showed the presence of gram positive short rods possessing no granules. These cultures were held under the above conditions for eight more days in the hope that a more definite bacterial plate would develop. However, the additional time proved too long and all of the cultures were lost.

Further samples were collected from the same localities and similar results were obtained. The medium as outlined by Kiel ('12) was employed, and the first signs of bacterial plate formation were noticed after six days incubation at 32° C. in the dark. These organisms were stained with Ziehl's carbol fuchsin, and the organisms found were typical Chromatium type sulphur bacteria as described by van Niel ('30). They occurred as slightly bent, ellipsoidal cells, containing granules characteristic of the above type. When stained by Hucker's modification of the Gram stain, the outline of the cell appeared faintly gram positive while the granular material showed a strong gram positive reaction. Further studies on these organisms, however, showed that these granules were of nature other than sulphur.

To further our studies, collections were made from hot springs located at four points in the southwestern portion of Montana. Five samples were collected from Norris Hot Springs,

Norris, Montana, in temperatures ranging from 24-44° C; one sample from Barkell Hot Spring, Silver Star, Montana at a temperature of 35° C; four samples from the spring outlet and overflow, Warm Springs, Montana at temperatures ranging from 35-74.5° C; and five samples from Gregson Hot Springs, Gregson, Montana at temperatures ranging from 33-62° C. All the samples were inoculated into medium as described by Kiel ('12) and incubated in a light cabinet as described by van Niel ('30). The temperature remained quite constant at 31-32° C. These samples were incubated for eleven days under aerobic and anaerobic conditions. Crystal violet stains and hanging drop preparations were made of these cultures and it was noticed upon examination of these slides that extreme quantities of vegetative life--algae, diatoms--were present, an indication that this type of media was not specific for bacterial growth.

The original samples were held in the light cabinet and after 7-10 days, much activity was noticed in these bottles. The five samples taken from Gregson Hot Springs exhibited large amounts of gas production. Also a heavy growth of algae was observed. After incubating for 11 days, from the time of collection, small amounts of each sample were inoculated into a medium as outlined by van Niel ('30). The tubes were then placed in a large vacuum jar, the lid sealed and the air withdrawn to provide strict anaerobic conditions. The vacuum jar

was then placed in the light cabinet and incubated at 32° C. for six days. Similar samples were incubated under aerobic conditions. After incubation, smears were made of the samples and stained with Zeihl's carbol fuchsin. It was noticed upon examination of the slides that the majority of forms were small rods and cocci, none of which appeared to contain sulphur granules within the cell wall. The specific activity of this medium may be indicated by the fact that fewer numbers of algae and diatoms were observed than in the medium used by Kiel. Repeated transfers were made into new media by inoculating one mililiter of the culture into 20 ml. of fresh media. Observation of the repeated transfers showed a marked decrease in the number of organisms present and continued incubation did not seem to increase the numbers. After four transfers, no organisms were found to be present, indicating that this type of medium was not suitable for growth and reproduction of the organisms under observation.

Since none of the samples collected up to this time showed the presence of sulphur granules, the authors decided to make collections in Yellowstone National Park from thermal springs having a distinctive odor of hydrogen sulfide. Two samples were collected from Upper Geyser Basin at temperatures of 47 and 76° C; one sample from Mirror Pool at Biscuit Basin at a temperature of 57° C; three samples from Lower Norris Geyser Basin at temperatures ranging from 59-81° C; two samples from

Mammoth Terraces at temperatures ranging from 47-80° C; and four samples from Frying Pan Springs at temperatures ranging from 32-80° C.

These samples were inoculated into van Niel's medium and incubated under aerobic and anaerobic conditions at an increased temperature of 42° C. An additional set of cultures was sealed with vaspar and incubated at 61° C. in a temperature oven under artificial illumination. All cultures were incubated for five days after which time smears were made and stained with Ziehl's carbol fuchsin. Upon observation of the stained smears, the sample collected from Mirror Pool, Biscuit Basin, showed numerous organisms which resembled those described under the suborder Rhodobacteriineae and a few of the filamentous type described under the Family Beggiatoaceae. Those cultures which were incubated at 61° C. showed similar results in types and numbers of organisms. Repeated transfers were carried out on all samples and observation of these transfers indicated that no organisms seemed to be present after the second transfer. However, the original samples which had been incubated under similar conditions showed no decrease in number.

The presence of sulphur containing organisms in the sample from Biscuit Basin indicated to the authors that further samples should be collected from this area, and similar areas as yet not investigated. Seven samples were collected from pools on the Formation Loop Road behind Mammoth Terraces at

temperatures ranging from 36.5-69° C; four samples from the region southeast of the museum at Norris Geyser Basin at temperatures ranging from 69-84° C; five samples from the mud volcanoes at temperatures ranging from 49-71° C; and eight samples from pools in Biscuit Basin at temperatures ranging from 22.5-65° C. Two liters of water were collected at the pool at Norris Geyser Basin and four liters from Mirror Pool at Biscuit Basin to be used as a medium for the cultivation of these organisms.

These samples were inoculated into van Niel's medium and incubated anaerobically at 42° C. In addition to these, all samples collected from Yellowstone National Park up to this time were inoculated into van Niel's medium, placed in an atmosphere of hydrogen sulfide and incubated at 42° C. in the light cabinet. The use of a hydrogen sulfide atmosphere was recommended by Ellis ('32) as a means of increasing the numbers of sulphur-containing organisms. After six days incubation, smears were prepared and stained with Ziehl's carbol fuchsin. Observation of these slides revealed the presence of many large rods and filamentous granular organisms in samples collected from the Formation Loop Road and at Biscuit Basin. Further studies showed that these granules were sulphur. Transferring was continued under conditions as outlined above and again a marked decrease in number of organisms was noticed after the second transfer. No organisms were found beyond

this second transfer.

Indications of continued growth in the original samples under incubation led to the use of natural water samples, collected from those particular areas, as a medium for cultivation. However, further work proved that this medium, under the above conditions, was insufficient for the continued growth and culture of sulphur bacteria.

While cultivation methods were in progress, a technic for a differential stain was being developed. Ellis ('32) had shown the existance of sulphur granules by the use of sodium nitroprusside in microscopic preparations. Johansen ('40) outlined a macroscopic test for the presence of sulphur by treating cultures with a 10% solution of potassium hydroxide and a 10% aqueous solution of sodium nitroprusside. A red color indicated the presence of sulphur in both tests. It is essential that these tests be carried out under optimum conditions as outlined above. Porter ('46) has shown that upon depletion of the H_2S supply, the sulphur granules undergo oxidation and diminish in size. When all the intracellular sulphur disappears, the organisms die.

In staining with sodium nitroprusside, various concentrations were used by the authors, at time intervals ranging from one minute to two hours. It was found that slides treated for five minutes with a 50% saturated solution (2 grams $Na_2FeNO(CN)_5$ in 10 ml. distilled water) gave the best results.

Heat-fixed smears of all samples were treated with a 50% aqueous solution of sodium nitroprusside, and red granules were observed in the samples obtained from Biscuit Basin and Formation Loop Road, Mammoth Terraces. However, this procedure failed to show cell outline, and it was difficult to tell whether the granules were contained within the cell wall. The next step was to develop a counterstain which would reveal cell outline.

Three well known dyes were first used as a counterstain: crystal violet, methylene blue and malachite green. Heat-fixed smears were stained for five minutes with a 50% solution of sodium nitroprusside. They were then blotted dry and counterstained with dilutions of the above mentioned stains ranging in concentration from 1:100 to 1:100,000 for intervals varying from one to three minutes. This procedure proved unsatisfactory since the red sulphur granules were either masked by the counterstain, or the aqueous solution of stain tended to wash out the sodium nitroprusside. When the smears were stained with the dyes first, then counterstained with sodium nitroprusside, the same negative results were observed. It was found that alcoholic solutions of the dyes could not be used due to crystallization of the sodium nitroprusside by the alcohol. Another technic employed was decolorizing with acetic acid. In this procedure, the smear was stained with high dilutions of crystal violet or methylene blue, decolorized

with different dilutions of acetic acid and counterstained with sodium nitroprusside. Treatment with acetic acid appeared to decolorize the cell completely and no outline could be seen.

Since counterstaining with the above dyes seemed to mask the sulphur granules, it was thought that a combination of sodium nitroprusside and negative stain might prove satisfactory. In the first attempt, the smears were stained with sodium nitroprusside in the regular manner and then treated with nigrosin. No results were obtained since the nigrosin covered the entire smear. A regular nigrosin smear was then prepared and after drying, was stained with sodium nitroprusside. This also proved unsuccessful since the nigrosin was washed off by the aqueous sodium nitroprusside solution. The next trial consisted of mixing the nigrosin and the sodium nitroprusside with a wet preparation of the culture and then allowing the smear to dry. On observation, it was noted that the sodium nitroprusside had crystallized leaving nothing but cracks and crystals.

Three dyes mentioned by Johansen ('40) as being satisfactory for cellulose cell wall stain were then used in the hope that their specificity would not interfere with the treatment of the granules with sodium nitroprusside. These dyes were Bismark brown Y, Fast green and Congo red. The same procedure was followed for these dyes as for the crystal violet

group already mentioned. Results were unsuccessful in that the granules were again masked by the counterstain.

In the studies with malachite green and methylene blue with sodium nitroprusside, it was found that the counterstain remained in each case. This indicated that the first-used stain was removed by the aqueous solution of the counterstain. Kendal ('37) stated that a mordant such as tannic acid is often used with basic dyes, i.e. malachite green and methylene blue, to precipitate the dye in an insoluble form. With this in mind, investigations were carried out in which tannic acid was used as a mordant for these organic dyes. Counterstaining was accomplished by treatment with sodium nitroprusside. Technics employed and results obtained are summarized in Tables 1 and 2.

It was found that the best results were obtained by staining a heat-fixed smear for three minutes with a 1:50,000 dilution of malachite green or methylene blue. The excess stain was then tipped off and the smear flooded with a 1:50 dilution of tannic acid. This was held for one minute, tipped off, flooded with fresh tannic acid solution and held for an additional minute. The smear was then blotted dry and counterstained with a 50% saturated solution of sodium nitroprusside for five minutes. The smear was blotted dry without washing and observed microscopically. Results obtained indicated that malachite green gave a greater color contrast than

TABLE I

Results of Staining with Malachite Green and Tannic Acid Mordant

Method of Treatment				Results
Trial	Malachite Green	Tannic Acid	$Na_2FeNO(CN)_5$	
1.	1:100,000* for 2 min	1:100** for 2 min.	50%*** sat.sol. for 5 min.	Faint outline of cell wall. Granules red in color.
2.	1:100,000 for 2 min	2:100 for 2 min.	50% sat. sol. for 5 min.	Cell outline slightly darker than trial #1.
3.	1:100,000 for 2 min	3:100 for 2 min.	50% sat.sol. for 5 min.	No appreciable difference from trial #2
4.	1:50,000 for 2 min.	2:100 for 2 min.	50% sat.sol. for 5 min.	Cell outline quite distinct. Granules red in color
5.	1:33,333 for 2 min.	2:100 for 2 min.	50% sat.sol. for 5 min.	Cell outline very distinct but granules slightly masked.
6.	1:50,000 for 3 min.	2:100 for 2 min.	50% sat.sol. for 5 min.	Cell outline very distinct and granules red in color.
7.	1:50,000 for 3 min.	3:100 for 2 min.	50% sat.sol. for 5 min.	No apparent change from that of trial #6.

* 1:100,000 - .01 gram malachite green powder in 1000 ml distilled water.

** 1:100 - 1 gram Acid, Tannic U.S.P. powder in 100 ml distilled water.

*** 50% saturated solution - 2 grams sodium nitroprusside in 10 ml distilled water.

TABLE 2

Results of Staining with Methylene Blue and Tannic Acid Mordant

Method of Treatment				Results
Trial	Methylene Blue	Tannic Acid	$Na_2FeNO(CN)_5$	
1.	1:100,000 for 2 min.	*1:100** FOR 2 min.	50% ***sat.sol. for 5 min.	Faint outline of cell wall. Granules red in color.
2.	1:100,000 for 2 min.	2:100 for 2 min.	50% sat.sol. for 5 min.	Cell outline slightly darker than trial #1.
3.	1:100,000 for 2 min.	3:100 for 2 min.	50% sat.sol. for 5 min.	No appreciable difference from trial #2.
4.	1:50,000 for 2 min.	2:100 for 2 min.	50% sat.sol. for 5 min.	Cell outline quite distinct. Granules red in color.
5.	1:33,000 for 2 min.	2:100 for 2 min.	50% sat.sol. for 5 min.	Cell outline very distinct but granules slightly masked.
6.	1:50,000 for 3 min.	2:100 for 2 min.	50% sat.sol. for 5 min.	Cell outline very distinct and granules red in color.
7.	1:2 5,000 for 3 min.	2:100 for 2 min.	50% sat.sol. for 5 min.	Granules take on a deep purple color.
8.	1:50,000 for 3 min.	3:100 for 2 min.	50% sat. sol. for 5 min.	No apparent change from that of trial #6.

* 1:100,000 - .01 gram methylene blue powder in 1000 ml distilled water.

** 1:100 - 1 gram Acid, Tannic U.S.P. powder in 100 ml distilled water.

*** 50% saturated solution - 2 grams sodium nitroprusside in 10 ml distilled water.

did methylene blue and malachite green is therefore recommended by the authors for use in this technic.

To prove that sodium nitroprusside does not indicate the presence of sulphur complexes such as glutathione and sulfhydryl groups in proteins, the developed technic was employed on non-sulphur gram negative and positive organisms. Cultures of Escherichia coli and Proteus vulgaris were used as typical gram negative organisms, and it was found that these organisms stained a solid green, with no visible effects from the sodium nitroprusside. Typical gram positive organisms used were Bacillus subtilis, Bacillus mycoides and Rhodospirillum rubrum, which gave similar results in all instances. Although not a true sulphur bacterium, Thiobacillus thiooxidans contains sulphur granules and was therefore used as a check for the developed technic. Upon staining of this organism, it was found that the cell outline stained green or blue depending upon the stain employed, and that red granules were located at the poles indicating the presence of sulphur.

DISCUSSION

In order to make observations of a physiological and morphological nature, it is desirable to control the conditions of growth and development; therefore means of cultivation have been studied. The latest works on cultivation methods for sulphur bacteria are those of van Niel ('30) and Ellis ('32). The authors attempted to duplicate these works but obtained negative results. This was due, perhaps, to the inability to provide the exact environmental conditions required by these organisms for growth and reproduction. The failure of these organisms for growth in artificial media was alleviated, however, by the fact that the original samples continued to survive under the temperatures and light conditions used, as outlined in the review of technics. Optimum conditions were necessary, since according to Porter ('46) an unfavorable environment causes oxidation of the sulphur and a decrease in the size of the granules. Complete oxidation or loss of the granules may occur and result in the death of the organism.

As previously indicated, Ellis ('32) and Johansen ('40) utilized sodium nitroprusside for detecting the presence of sulphur. Upon treatment with this compound, the sulphur granules assume a blood red color. The investigations carried out were based upon the work of these two men, and it was found that this color did appear upon treatment of the granules

with sodium nitroprusside. Scagliarini and Monforte ('34) attributed the existence of the red color to the action of sodium nitroprusside on the sulphide ion. Substantiation of this may be indicated by the fact that sodium nitroprusside does not give a red color in the presence of elemental sulphur, but does in the presence of sulphides, shown in experiments conducted by the authors. A possible mechanism for this reaction is outlined by Scagliarini and Pratesi ('28).

$$\left[Fe(CN)_5NO\right] + 2\ OH^- = \left[Fe(CN)_5N\begin{matrix}=O\\ \searrow O\end{matrix}\right]$$

$$\left[Fe(CN)_5N\begin{matrix}=O\\ \searrow O\end{matrix}\right] + S^{--} = \left[Fe(CN)_5N\begin{matrix}=O\\ \searrow S\end{matrix}\right]$$

The above formulation shows that upon hydrolysis of the sodium nitroprusside, an (NO_2) group is formed which in turn reacts with the sulphide ion to form an (NOS) group. In this instance, the (NOS) group is characterized by a red color. Von Deines ('33) has found after extensive microchemical tests that the material contained within the granules of sulphur bacteria was characteristic of a highly sulphured polysulphide and not of elemental sulphur. This seems to substantiate the above mechanism and may account for the red color upon treatment of sulphur bacteria with sodium nitroprusside. Many authors regard the sulphur granules as elemental sulphur. However, in view of the work of Scagliarinia and Monforte ('34) and of von Deines ('33) it seems likely that the sulphur exists in the granules in the form of highly sulphured polysulphides,

and it is this group with which the sodium nitroprusside is reactive.

Since aqueous sodium nitroprusside decolorized any dyes used to stain the cell outline, the use of a mordant for these dyes was indicated. Studies summarized in Tables 1 and 2 above showed that tannic acid would be a satisfactory mordant for malachite green and for methylene blue. Many theories have been proposed as to the specific action of mordants when employed in staining procedures. Although most of these theories have a sound basis, no one of them can satisfactorily explain all phenomena. Burke and Barnes ('44) in their work with the Gram reaction have presented the theory that restrictive cell membrane permeability may account for the inability to remove the mordant-dye complex which has formed within the cell. Gortner ('44) presented the theory of electrical charge. In this theory, the action of the mordant results in the alteration of the basic charge of the cell and allows attraction between positively and negatively charged colloids. This theory is further strengthened by the findings of Conn ('46) who stated that staining may be the result of adsorption (the property possessed by a solid body of attracting to itself minute particles of matter from a surrounding fluid), and that mordants contain ions that are known to have a decided influence upon the rate of adsorption. Hill and Kelley ('43) state that mordants such as tannic acid or tannic acid salts are used

to precipitate colored insoluble salts from basic dyes.

Any of the above mentioned theories could apply to the technic described in this paper for staining cell outline. According to the theory of Burke and Barnes, as outlined above, the tannic acid-malachite green complex cannot be removed from the cell due to restrictive permeability. Since adsorptive properties and ionic charge could not be measured, it is difficult to explain the theories of Gortner and Conn as related to the developed technic. Upon test tube observation of a malachite green and tannic acid mixture, a finely divided precipitate was noticed, which may be of some significance in the light of Hill and Kelley's statement.

Investigations were carried out which indicated the specific action of sodium nitroprusside on sulphur granules. According to Sponsler and Bath ('42), sulphur complexes such as glutathione and sulfhydryl groups are present in the protoplasm of bacterial cells. In order to prove that these compounds exist in amounts below the level of detectable reaction with sodium nitroprusside, typical gram positive and negative organisms were subjected to the staining procedure as previously outlined. Observation of these organisms showed that the entire cell retained the green color and in no case was there found a red color which might indicate activity with sodium nitroprusside. The specific action of sodium nitroprusside for sulphur granules was further shown by the results of stain-

ing the organism Thiobacillus thiooxidans. As mentioned by Porter ('46), sulphur granules are located at the poles of this organism, giving a characteristic bipolar stain. Upon observation of these organisms stained with malachite green and sodium nitroprusside, it was found that the outline of the organisms retained the green color and granules located at the poles were red. These red granules indicated that the sulphur complexes in Thiobacillus thiooxidans are similar in nature to the granules found in the true sulphur bacteria studied in the present work.