A CYTOLOGICAL AND MICROCHEMICAL STUDY OF THIOBACILLUS THIOOXIDANS

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Thiobacillus thiooxidans is characterized by certain physiological peculiarities which have aroused wide interest since it was first isolated and studied by Waksman and Joffe (1922). This organism can oxidize elementary sulfur and thiosulfates; it is able to grow at pH 1, and is inhibited by a pH $\geqslant$ 6. Until recently, however, the morphology of the organism was restricted to such routine studies as measurement of cell dimensions, motility, and the gram reaction. Recent interest in its cell structure was stimulated by the need of a morphological basis to explain the mechanism by which the organism is able to attack elementary sulfur (Umbreit, Vogel and Vogler, 1942). According to those investigators, there is, at each end of the cell of *Thiobacillus thiooxidans*, a droplet of highly unsaturated fat which is responsible for the "dipolar" staining of the cell by certain staining procedures. "This fat globule is placed in contact with the sulfur particle, in such a manner that sulfur dissolves in it and is taken into the cell for oxidation." Later studies with the electron microscope (Umbreit and Anderson, 1942), however, did not bring out the "dipolar appearance" of the cell. The electron micrographs showed principally a mixture of cell forms varying from short, ellipsoidal cells ($1 \times 0.5\mu$) to elongated, cylindrical cells ($2\text{–}3 \times 0.5\mu$). The cells showed also considerable variation in transparency to the electron beam (presumably at an accelerating potential of 60 kv.), the majority being uniformly opaque to the electrons. Of particular interest are three cylindrical cells which showed an internal structure in the form of helicoidal bands, for similar bands were observed in a few of the large bacteria by Swellengrebel (1909) and by Dobell (1911), both of whom considered them as cell nuclei.

PRESENT INVESTIGATION

Culture and medium

The culture of *Thiobacillus thiooxidans* used in this investigation was received through the courtesy of Doctor Umbreit, of the University of Wisconsin, to whom we are also thankful for placing at our disposal his original electron micrographs, and the necessary information for culturing and handling the organism. The medium we used most frequently was that of Vogler and Umbreit (1941) in which $(NH_4)_2SO_4$ was substituted for NH_4Cl in a concentration containing about the same amount of nitrogen. The mineral solution was distributed in test tubes, in 10 ml. amounts, and sulfur, in the form of sterile flowers of sulfur, was added to each tube with a sterile loop. Cultures were initiated by inocula-

tion of each tube with 1 ml. of a 1–2 day old culture of the organism in a similar medium, and incubation at the temperature of the laboratory which varied between 25° and 30°C. We also used cultures grown at laboratory temperature on the thiosulfate agar medium of Waksman (1922).

Methods

The methods used in the present investigation are similar to those previously used by the author in studying the cell structure of other bacteria (Knaysi, 1941, 1942). The buffered dye solutions were prepared mostly by adding 1 ml. amounts of 1 per cent solutions of the dye in water to 4 ml. portions of buffer solutions prepared by mixing, in various proportions, 1 per cent solutions of monopotassium and dipotassium phosphate, or 0.1 *n* acid potassium phthalate and 0.1 *n* HCl. The final pH was determined with the glass electrode. Smears were prepared from liquid cultures, usually filtered through No. 4 Whatman paper to remove the sulfur particles, and observations were made mostly on wet preparations, unfixed, fixed by heat or by 95 per cent alcohol. Whenever necessary, the fixed smear was immersed for a few moments in a beaker of water to remove the acids transported from the medium. The microscopic and photomicrographic combinations and films are the same as those used previously (Knaysi, 1942).

Form, size and grouping of the cells

The *form* and *size* of the cell were studied in smears fixed by heat and stained by the method described (Knaysi, 1941) for the demonstration of the cell-wall. In the liquid, *sulfur medium*, young, actively growing cultures (fig. 1) consist of cells the majority of which are fairly uniform and have the shape of a short ellipsoid of revolution with the following characteristics (see Knaysi, 1941):

$$E = 1 - \frac{b^2}{a^2} = +0.44 \text{ to } +0.7$$

$a = 0.6 \text{ to } 0.7\mu$
$b = 0.4 \text{ to } 0.5\mu$

where E is the excentricity, a half of the major axis, and b half of the minor axis of the principal section. The volume ($V = 4/3\ \pi ab^2$) of the cell varies, accordingly, from $0.34\mu^3$ to $0.6\mu^3$. Those cells probably correspond to "Type I" of Umbreit and Anderson (1942). As the cultures grow older, one observes a mixture of cellular forms including cylindrical or curved cells of different thickness, large spherical or ellipsoidal cells having the earmarks of chlamydospores (microcysts), irregular cells, ghost cells, and cell debris. It may therefore be concluded that the cultures of *Thiobacillus thiooxidans* in the liquid, sulfur medium present similar sequence and mixture of cell forms to those observed in cultures of other bacteria. On the *thiosulfate agar medium*, the cells generally tend to be more elongated (fig. 2), with a varying from 0.8 to 1.2μ, b from 0.5 to 0.6μ, and V from 0.84 to $1.26\mu^3$.

The cells are mostly single or in pairs, often held in close proximity by the masses of slime in which they are imbedded. Smears made from thiosulfate agar slants often show a tendency, on the part of the cells, to lie parallel to one another.

Structure of the cell

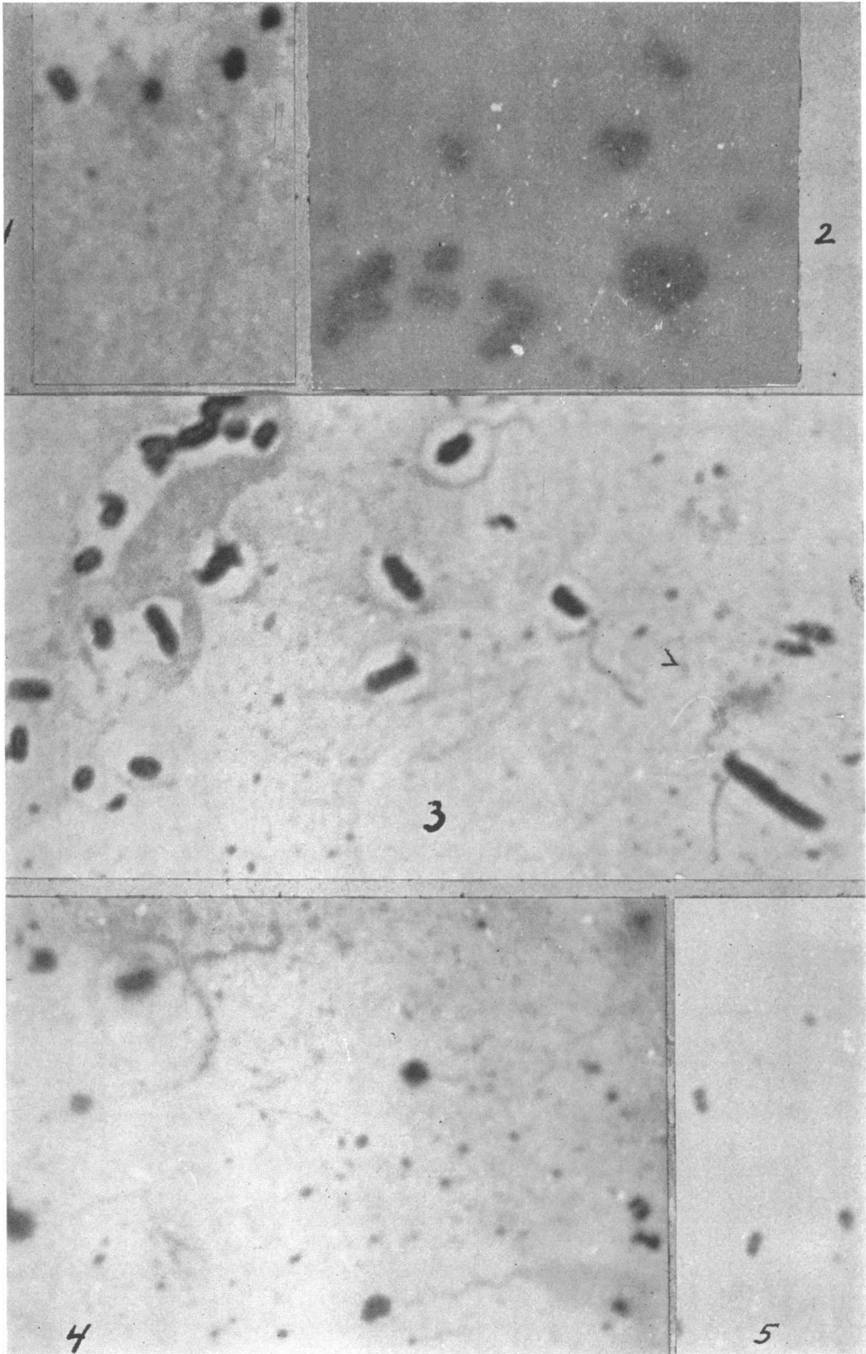
The cell of *Thiobacillus thiooxidans* consists of protoplasm surrounded by a thin cell-wall. It is heavily capsulated and provided with a flagellum. Depending on the form and size of the cell, the protoplasm may contain one, two, or several intraprotoplasmic structures, the nature and function of which will be discussed below.

a. The protoplasm. The protoplasm proper of *Thiobacillus thiooxidans* appears, both in bright and in dark-field, as a homogeneous system. Its volume is often greatly reduced due to the relatively large structures it includes. Its stainability with acid or basic dyes is similar to that of the protoplasms of other bacteria. It stains deeply with China blue (an acid dye) dissolved in *n*HCl, and with methylene blue dissolved in a phosphate buffer solution of pH 7; below pH 4, it stains with methylene blue only faintly. In this respect, the protoplasm of *Thiobacillus thiooxidans* behaves like those of the gram-negative bacteria (Stearn and Stearn, 1928).

b. The cell-wall and slime. Both the cell-wall and the slime were demonstrated by the method previously described (Knaysi, 1941). The cell-wall appears blue and the slime red. The cells are imbedded in the slime and held together *en masse* (fig. 2). The slime is probably secreted by the cell and is usually denser around the cells and appears, in smears, in the form of typical capsules. Typical capsulation can also be observed around single cells or pairs of cells which have broken away from the mass (figs. 1, 3 and 4).

In view of the extent of slime formation by *Thiobacillus thiooxidans* and of the ease with which that slime can be demonstrated, it appears surprising that no mention of it has been made by other workers. It is our belief that the "exceedingly faint halo", observed around the cells by Umbreit and Anderson (1942), and considered by them as an artifact, really represents the cell capsule. It is true that capsules are not usually demonstrated with the electron microscope when the cell suspension is prepared in the usual way. However, the work of Knaysi and Mudd (1942) on the pneumococcus shows that, when the cell suspension is prepared directly on the collodion film, the capsule of that organism can be readily observed. This procedure avoids the contact of the cells with relatively large volumes of water and probably preserves electron-scattering material occluded in the capsule. As Umbreit and Anderson are not specific about their technique, it is probable that they used liquid cultures followed by instantaneous washing which, in view of the nature of the capsule of *Thiobacillus thiooxidans*, was not sufficient to remove all material occluded in the capsule.

c. Motility and flagella. Waksman and Joffe (1922) described the organism as non-motile. However, Vogler and Umbreit (1941) explained the slight turbidity developed by the organism in liquid, sulfur-containing media, as due to



FIGS. 1, 3 AND 4. CELLS FROM A 4-DAY OLD CULTURE IN THE LIQUID, SULFUR MEDIUM, STAINED BY THE AUTHOR'S METHOD FOR THE DEMONSTRATION OF THE CELL-WALL

FIG. 2. CELLS FROM A 5-DAY OLD CULTURE ON THE THIOSULFATE AGAR MEDIUM, STAINED BY THE AUTHOR'S METHOD FOR DEMONSTRATING THE CELL-WALL

Note the poor contrast and definition and the cell masses due to excessive production of slime.

FIGS. 5 AND 6. CELLS FROM A 3-DAY OLD CULTURE, IN THE LIQUID, SULFUR MEDIUM, STAINED WITH METHYLENE BLUE AT pH 3.6–3.8

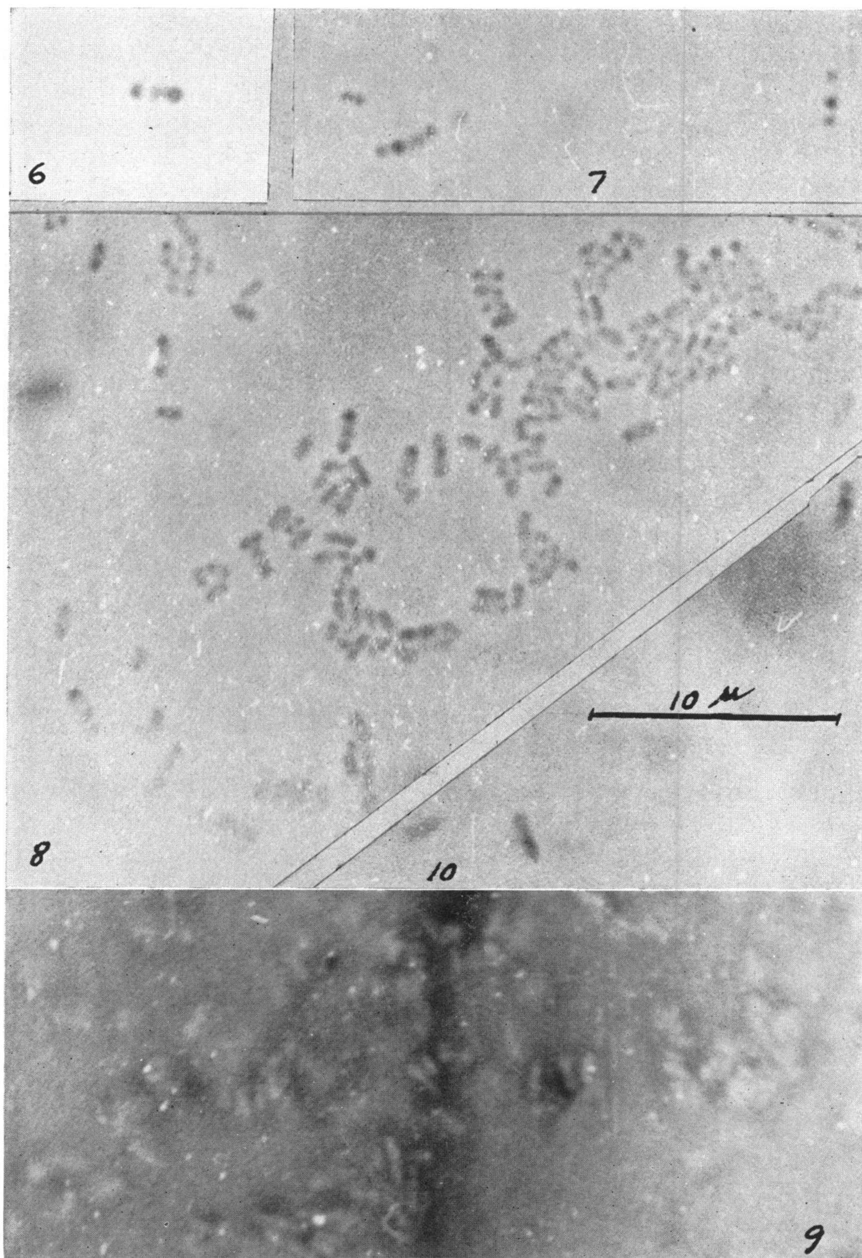


FIG. 7. CELLS FROM THE SAME CULTURE OF FIGURES 5 AND 6, STAINED WITH METHYLENE BLUE AT pH 1.6–1.8

FIG. 8. CELLS FROM A 5-DAY OLD CULTURE IN THE LIQUID, SULFUR MEDIUM, FIXED FOR 1 MIN. IN 95 PER CENT ALCOHOL, TREATED FOR 2 HRS. IN 0.02 PER CENT SODIUM BICARBONATE, AND STAINED WITH LUGOL'S SOLUTION

FIG. 9. CELLS FROM A 14-DAY OLD CULTURE ON THE THIOSULFATE AGAR MEDIUM, STAINED WITH LUGOL'S SOLUTION

Note the absence of granules stainable with iodine

FIG. 10. CELLS FROM A 15-DAY OLD CULTURE ON THIOSULFATE AGAR, STAINED BY A MODIFIED BURKE'S GRAM METHOD (OMISSION OF BICARBONATE; DECOLORIZATION WITH 95 PER CENT ALCOHOL FOR 10 SEC.)

Note the gram-positive vacuole in a gram-negative protoplasm

movement through the medium in search of sulfur. That implies their belief in the motility of the organism. Later, Umbreit and Anderson (1942) were able to observe an occasional flagellum with the electron microscope. However, the majority of the cells were non-flagellated.

In the present work, we have repeatedly observed the organism in dark-field, or in bright field stained by the method previously described (Knaysi, 1941) for the demonstration of the cell-wall. In dark-field, the majority of the cells are either non-motile or endowed with an extremely slow motion. Occasionally, however, one observes a cell with an extremely rapid motion. It is interesting to note that such cells are continuously shifting the direction of their motion, as if they were searching for something. This may be a justification for the hypothesis of Vogler and Umbreit outlined above. In stained preparations, one observes numerous free flagella and unflagellated cells (figs. 1, 3 and 4). There are, however, many cells provided with a single terminal, rarely lateral, flagellum. In capsulated cells, one often observes the flagellum throughout the capsule to its origin in the cell (figs. 1, 3 and 4). The lack of motility of many cells, and the extreme sluggishness with which many others move, is undoubtedly due to the fact that the majority of the cells are embedded in slime, for it has been shown (Knaysi, 1933) that a certain variant of *Bacillus megatherium* carried flagella but was non-motile because the cell chains were surrounded by a heavy capsule and grew in fascicles.

In the liquid, sulfur-containing medium, the flagellum is relatively thick, much thicker than on the solid medium. This confirms the early observations of Reichert (1909) on other bacteria. In the liquid culture, the thickness of the stained flagellum is about 0.15μ, about 0.15–0.19 of the cell width. With the exception of a few cases in which the flagellum is straight or bent into a loop, it is usually helicoidal in form and consists mostly of 5 turns. The width d of the helix is about 0.7μ and the altitude h of a turn is 1.3 to 1.8μ. This gives for the true length of a coil:

$$\lambda = \sqrt{h^2 + \pi^2 d^2} = \sqrt{6.53} \text{ to } \sqrt{8.08}\ \mu$$
$$= 2.6 \text{ to } 2.8\mu$$

a flagellum of five coils would thus measure 13 to 14μ or, often, about 10 times the length of the cell.

d. Intra-protoplasmic structures. The electron micrographs published by Umbreit and Anderson (1942) show principally two types of intracellular structures: 1) an opaque mass occupying a large portion of the cell volume, 2) a helicoidal body present in some of the cylindrical cells and extending from one end of the cell to the other. A cell containing the helicoidal body may also contain one or more round bodies. The nature of those structures was not determined, but it was thought likely that they would consist of reserve material.

In the course of the present investigation, we made repeated attempts to redemonstrate these structures and determine, if possible, their nature and their function. We were particularly interested in those having a helicoidal form, because such structures were observed in some of the large bacteria (Swellen-

grebel, 1906; Dobell, 1911), and were considered as nuclei. Unfortunately, we have been so far unable to observe clearly any intracellular structure which could unmistakenly be considered helicoidal; and we suspect that such forms are distinct in nature and in function from the ellipsoidal or spherical bodies regularly formed by the cell. Our study had, therefore, to be confined to the latter group.

Spherical or *ellipsoidal* intracellular *bodies* are formed by the majority of the cells; we believe that they may be potentially formed by all normal cells regardless of their form. Among the short, ellipsoidal cells (fig. 5), we noticed two groups; the first consists probably of resting cells containing each a single structure eccentrically located; the second consists of growing cells containing two such structures each, thus showing the "bipolar" appearance referred to by Umbreit and his co-workers. Among the elongated, cylindrical cells (figs. 6 and 7), we also distinguish two groups; the first includes relatively thick cells containing two (rarely one or three) terminal structures and probably consisting of resting cells; the second consists of more slender cells containing three or more structures each; when three structures are present, one is usually located in the center of the cell where partition is to be expected, and the other two are terminal. In young cultures growing on the thiosulfate agar medium, the majority of the cells are of this latter type.

In studying the *nature* of these intracellular structures, one is led to distinguish between those formed in the liquid, sulfur-containing medium and those formed on thiosulfate agar. In untreated cells, grown in the *liquid, sulfur medium*, the structures stain with methylene blue at pH 1.7, whether the cells were not fixed or whether they were fixed with heat, or with 95 per cent alcohol from 6 to 48 hours. They become unstainable with methylene blue at pH 1.7–4.0 after exposure of the cells to water at 80°C. for 10 minutes, to 0.02 per cent sodium bicarbonate for 2–3 hours, or when the cells were allowed to starve. Starvation was brought about by letting the culture stand from 16 to 24 hours after removal of the sulfur by filtration through paper. According to our present understanding, the above reactions indicate that the structures contain, or consist of, a reserve material having several of the properties of volutin (see Knaysi, 1942). This conclusion is strengthened by chemical analysis of the bicarbonate extract. According to Umbreit (1943), a solution of 0.20 per cent bicarbonate extracts, in 3 hours, 11.2 per cent of the organic phosphorus and 6.4 of the nitrogen of the cells. The extracted material also contains pentose in the molar ratios of 1/2.2/27 to phosphorus and nitrogen respectively. On the other hand, when the untreated cells are placed in contact with Lugol's solution (I_2-KI), the structures assume a deep brown color characteristic of fat; they also stain to a variable extent with Sudan III. In cells fixed in 95 per cent alcohol for six hours, staining with iodine and with Sudan III was still apparently unimpaired in the majority of the cells. However, treatment of the cells with 0.02 per cent sodium bicarbonate reduced but did not destroy, stainability with iodine and with Sudan III. In the majority of the cells, the structures usually appeared somewhat disorganized and stained less definitely, often presenting only a stained contour; in certain cells and cultures, however, they can be clearly dem-

onstrated (fig. 8). Stainability with iodine is generally lost upon starvation of the cells. In cultures 6 to 15 days old grown on *thiosulfate agar*, the intracellular structures stain yellow-brown with iodine (fig. 9) and only faintly with Sudan III; they take up methylene blue at pH 1.7–4 in untreated cells, but not in cells exposed to 0.02 per cent sodium bicarbonate for 2–3 hours. Consequently, in cells grown on thiosulfate agar, the structures seem to contain, or consist of, a single substance having the properties of volutin.

The above reactions lead to the *conclusion* that the intraprotoplasmic structures observed in the cell of *Thiobacillus thiooxidans* and sometimes responsible for the "dipolar" staining of the cells are large vacuoles containing reserve material. The existence of a vacuolar membrane, probably lipoid-containing, can also be demonstrated. When the cells are grown in a liquid medium containing elementary sulfur, the content of the vacuole consists either of a new substance having several of the properties of volutin, and other properties different from those of volutin, or of an intimate mixture of volutin with another substance. If it is a mixture, the two substances must be so dispersed that the individual particles are not resolved with the microscope. The nature of the second substance, whether it be free or combined with volutin, needs discussion. Its behavior toward iodine and Sudan III tends to indicate a fatty nature. However, it is our experience that reserve fat is usually removed when the cells are fixed in 95 per cent alcohol for 48 hours. Taking into consideration the fact that volutin alone is formed when the cells are grown on the thiosulfate medium, one would suspect that, in the presence of elementary sulfur, the vacuole contains either elementary sulfur, or a sulfur compound of the indicated properties, associated with volutin. This suspicion is strengthened by the fact that, when minute particles of flowers of sulfur are placed in the well of a hanging drop slide and allowed to melt by passing the slide over the flame, those particles give, after resolidification, deep brown coloration with iodine, stain with Sudan III, and are not dissolved in 95 per cent alcohol. We are therefore inclined to conclude that the reserve material normally formed by *Thiobacillus thiooxidans* is volutin, and that, in the presence of elementary sulfur, some form of sulfur may also be present in the vacuole as a reserve material. This would explain Vogler's finding (1941) that, in the presence of sulfur, "the organism synthesizes a reserve storage product which enables it to live in the absence of oxidizable sulfur."

The gram reaction

The literature contains contradictory statements regarding the gram reaction of *Thiobacillus thiooxidans*. Waksman and Joffe (1922) stated that the organism is gram-positive. On the other hand, Starkey (1935) found it gram-negative. Umbreit, Vogel and Vogler (1942) explain the discrepancy by a pH difference in the staining solutions and its influence on the chemical reactivity of fat.

In the present investigation we first used Burke's method (1922) which usually gives more clear-cut results than most other techniques. For reasons which will soon become obvious, we finally modified that method by omitting the addi-

tion of sodium bicarbonate, by increasing the time of contact with iodine to 2 minutes and by decolorizing with 95 per cent ethyl alcohol for 10 seconds. Acetone could also be used for only a few seconds.

The results of our study with both the liquid and the solid cultures have shown that the protoplasm of *Thiobacillus thiooxidans* is definitely gram-negative, whereas the vacuolar content is gram-positive (fig. 10). Young cells which have not had time to accumulate reserve material, and cells which do not contain reserve material, either because it was used up or removed with mild alkalies, are gram-negative throughout. In young cells one can often discern a spot, corresponding to the vacuole, showing a border-line color because of the presence of a small amount of reserve material. In view of the relatively large size of the vacuole and of the fact that the cultures generally used are at a stage when the cells are rich in reserve material, we can very well see how it is possible to overlook, in ordinary microscopic observations, the often thin protoplasmic layer and to call the organism gram-positive. On the other hand, most dye solutions used in gram-staining contain an alkali to intensify the staining (mordant action of OH^-), and the alkalinity of those solutions may be sufficiently high to remove the volutin from the vacuole. This is particularly true in Burke's method. By this method, the cell appears gram-negative, with merely a gray or border-line color at the locus of the vacuole. Certain cells may even show an empty vacuole and a vacuolar membrane of doubtful reaction.

DISCUSSION

We hope that the observations reported in this paper will be instrumental in bringing about a solution to the question: How is *Thiobacillus thiooxidans* able to attack elementary sulfur? The answer given by Umbreit, Vogel and Vogler (1942) is that the organism "oxidises insoluble sulfur by dissolving it in a fat globule, located at the end of the cell". This fat droplet is considered to be the same structure which may, "under given staining procedures give the organism a 'dipolar' appearance". The later work of Umbreit and Anderson (1942) with the electron microscope, however, did not reveal the existence of such a globule. The present investigation shows that the structure responsible for the dipolar staining of the organism is intra-protoplasmic; it is a vacuole, containing reserve material. Regardless of the nature of that reserve material it is difficult to see how such an intra-protoplasmic structure can be placed in contact with the sulfur particles of the medium, especially when the cell is embedded in slime.

Another question which may be asked regarding *Thiobacillus thiooxidans* is: By what mechanism is the organism able to survive, and even to grow, at the low pH developed in its medium (pH $\leqslant$ 1). The present investigation shows that the protoplasm of the organism is neither strongly acidic, nor do its constituents exist below their iso-electric point. It seems possible to us that the strongly acidic, relatively huge vacuole usually present in the cell, plays a role in the pH balance between the protoplasm and the exterior environment.

Finally, the results of the present investigation have a direct bearing on the theory of the gram reaction. They bring out the relation between the acidic

strength of a cell constituent and its gram reaction (Stearn and Stearn, 1928), and they show that a cellular structure may be gram-positive without being fatty in nature. Moreover, our observations on the young cells indicate that the results of the gram reaction may depend on the quantity of gram-positive material present in the cell; the larger is the quantity, the greater is the amount of dye bound and the greater is the mass of insoluble compound formed with the mordant; in the young cells the gram-positive material may be present, but its concentration is less than in older cells, and the final color is not so obvious under the microscope. Finally, the coexistence of both gram-positive and gram-negative structures within the same cell, is an indication that a difference in the gram reaction may exist without a corresponding difference in the permeability of the cell "membrane", and it is doubtful whether it can be attributed to the vacuolar membrane. In view of the brilliant work of Burke and Barnes (1929) it is not the author's intention to deny that a difference in permeability may exist between gram-positive and gram-negative cells. It can be pointed out, however, that, if such a difference exists, it would tend to intensify other qualitative differences already existent between the cells, for the gram reaction is probably not the result of a single factor, but an expression of the resultant of several inherent differences.

SUMMARY

The cell structure of *Thiobacillus thiooxidans* is fundamentally similar to that of other bacteria. It consists of a gram-negative protoplasm (pH of isoelectric point > 4) containing one or more large vacuoles. When the medium contains elementary sulfur, the vacuolar content gives the reactions of both volutin and sulfur; on Waksman's thiosulfate medium, only volutin is formed. The protoplasm is surrounded by a cell-wall, and the cells are imbedded in slime. The cells are actively motile and provided, each, with a single, thick terminal flagellum. The bearing of the present study on the mechanism of some of the physiological processes of the organism and on that of the gram reaction are discussed.

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