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# Fine Structure of *Thiobacillus thiooxidans*

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ABSTRACT

MAHONEY, ROBERT P. (Skidmore College, Saratoga Springs, N.Y.), AND MERCEDES R. EDWARDS. Fine structure of *Thiobacillus thiooxidans*. J. Bacteriol. **92**: 487–495. 1966.—Thin section analysis of the chemosynthetic autotroph *Thiobacillus thiooxidans* revealed structures comparable to gram-negative heterotrophic bacteria. Although this species is unique in that it oxidizes elemental sulfur for energy, uses carbon dioxide as its sole source of carbon, and can withstand a *p*H of less than 1, thin sections revealed a profile of the cell envelope (cell wall and plasmalemma) similar to other gram-negative species which have more common physiological traits. The cell wall is composed of five layers with an overall width of approximately 200 A, and the plasmalemma appears as a conventional "unit membrane" with a width of about 85 A. Volutin granules and less-dense bodies of similar shape and size were frequently observed in close association with the nucleoplasm. The nature and function of these bodies are unknown at this time.

One of the early publications on electron microscopy of bacteria dealt with the sulfur oxidizing bacterium *Thiobacillus thiooxidans* (28). Subsequent electron microscopy of chemosynthetic autotrophs has been concerned with *Hydrogenomonas* (24), *Ferrobacillus* (R.P. Mahoney and D. G. Lundgren, Bacteriol. Proc., p. 32, 1965), and *Nitrocystis*, *Nitrosomonas*, and *Nitrobacter* (17). The early micrographs of *T. thiooxidans* (28) were taken at low magnification prior to the development of methods for thin sectioning, and they reveal little detail that might explain this microorganism's unique way of life. In a recent study, electron micrographs of sulfur crystals before and after exposure to *T. thiooxidans* revealed erosion of the crystal where the cells had attached (22).

This species can live, grow, and reproduce under conditions which are intolerable, if not lethal, for most other biological systems. The organism oxidizes elemental sulfur for energy, utilizes carbon dioxide as its sole source of carbon, and can survive a *p*H of less than 1 (27).

Although data are extant as regards culture characteristics and biochemical aspects (31), many fundamental questions remain. The mechanism for sulfur utilization is not fully understood. A sulfur-oxidizing enzyme has been reported by Suzuki (25, 26) but the nature and significance of the actual process will require further investigation. The ability of the organism to withstand the low *p*H continues to be intriguing. It would be interesting to explore the internal anatomy of this species to see whether the fine structure might afford a reasonable explanation of the organism's unique characteristics.

## MATERIALS AND METHODS

The strain of *T. thiooxidans* used for this study was kindly supplied by R. M. Borichewski, Rutgers University. Cells were grown in Waksman's medium (32) at 28 C, on a reciprocal shake machine, and were harvested after 4 to 7 days by centrifugation after the culture fluid had been decanted away from the sulfur. The condition of the culture was checked with the aid of a light microscope, and all cultures used had a high percentage of actively motile cells.

In general, cells were fixed in 1% $OsO_4$ in Veronal acetate buffer (*p*H 6.1) with the addition of calcium and tryptone (11). In a few cases, magnesium ions were added along with calcium ions, and in other cases the tryptone was deleted, but no benefit resulted from these modifications. Also, some fixation was accomplished by use of 1% $OsO_4$ in a sulfuric acid-dipotassium phosphate mixture at a final *p*H of 5.8. Mixtures of the latter reagents at *p*H 3.5 and 4.5 were also used but did not yield good preservation of the cells. Osmium tetroxide was also employed with Waksman's salts, at a *p*H of 4.5. In a few cases, formaldehyde or glutaraldehyde in phosphate buffer, over a range of *p*H, was employed, followed by osmium fixation, but no improvement in the preservation was noted. The best results are those represented in the illustrations, whose legends indicate the methods used.

Dehydration was through graded strengths of ethyl alcohol and embedding was in methacrylate or in Epon. Some samples were treated with uranyl acetate

(0.5% solution in Veronal-acetate buffer, *p*H 5.0) for 30 to 40 min prior to dehydration. However, no notable benefit accrued from this treatment.

Thin sections were cut with a diamond knife and LKB or Porter-Blum ultramicrotomes.

Sections were poststained with lead citrate (20) or a combination of lead citrate-uranyl acetate (30). Chromyl chloride (3) was occasionally used with methacrylate sections.

Preparations were examined in a Siemens Elmiskop I electron microscope at an acceleration voltage of 80 kv and were photographed at magnifications of 20,000 to 40,000 times, with the use of Eastman Kodak high-contrast lantern-slide plates.

## RESULTS

*Cell envelope.* The peripheral structure of *T. thiooxidans* appeared as a loose-fitting, wavy or rippled envelope (Fig. 1–5). At low magnification, as shown in Fig. 12, the layers of the cells appeared so close to each other that it was difficult to determine the true profile. However, when cells were partially plasmolyzed (Fig. 4–5), distinct layers of cell wall and plasma membrane were revealed.

The cell wall appears as a five-layered structure (Fig. 4–5) similar to that reported for other gram-negative species (6, 16, 19). The outer portion of this five-layered structure appears to be a unit membrane; that is, in cross section it shows the typical dense-light-dense layering, approximately 85 A in width. Underlying this unit membrane is another less-dense layer of varying width (minimal thickness, 60 A), limited by yet a third dense layer about 30 A in width (Fig. 4–5, 8). In spite of variations in the thickness of the layers of the cell wall (especially the M layer, Fig. 4–5), values obtained from numerous micrographs yielded an average of 200 A.

The structure delimiting the cytoplasm, the plasmalemma or cytoplasmic membrane, is a typical unit membrane, 80 to 85 A in width (Fig. 4–5), as found in other gram-negative bacteria (2, 19).

Invagination of the plasmalemma into the cytoplasm has on occasion been observed (Fig. 10). However, tubular or vesicular organelles were not found in serial sections.

*Cytoplasm and nucleoplasm.* The granular nature of the cytoplasm (Fig. 1, 5, 9, 13) is due to electron-dense particles—ribosomes with an average width of 160 A. In some instances, the ribosomes are seen adhering to the plasmalemma (Fig. 11). The nuclear material is distributed diffusely throughout the cytoplasm (Fig. 1–3, 11, 13), as has been shown for other gram-negative bacteria (4, 6). This nuclear material appears as fine electron-dense fibrils with a diameter of 25 to 40 A, and is frequently seen to be associated with ribosomes (Fig. 4–5).

Also observed were large very electron-dense bodies ranging in diameter from 700 to 1,000 A (Fig. 8, 12), similar to volutin granules described in other bacteria (5, 9). Granules of unknown origin of similar size but lesser density were also observed (Fig. 2, 6–8), and could be seen in cells which also contained a volutin granule (Fig. 8, 12). In cells where the density of the cytoplasm has been reduced, these bodies are seen to be intimately associated with the nuclear fibrils (Fig. 6, 7, 12, 13). Discrete lipid or sulfur inclusions were not detected.

*Cytokinesis.* The constrictive mode of cell division of *T. thiooxidans* (Fig. 11–13) is typical of that initially described for gram-negative species in Robinow's (21) classic studies with a light microscope and subsequently well documented via thin-section analysis (2, 4, 5, 8). The cell wall and plasmalemma invaginate as a unit, and no plate or septum is observed to precede the division furrow (Fig. 11–12). Figure 13 exemplifies the mode of division, wherein a deep furrow is evident, and the process is nearing completion. In this figure, each daughter cell appears to possess its own continuous plasmalemma (white arrows), whereas the cell wall is yet common to both cells.

## DISCUSSION

No extramural structure (capsule or slime layer) was detected which might be cited as protective to *T. thiooxidans*. The cell wall is multilayered, similar to the five-layered structure observed in other gram-negative bacteria (6, 16, 19). Indeed, the profile observed in *T. thiooxidans* appears to be the same as that of other chemosynthetic autotrophs (17).

The fact that in this species the cell wall is not a unique structure tends to diminish the importance of the wall as a physical barrier between the cytoplasm and the acid environs of the cell. However, this should not rule out the cell wall-plasmalemma function based on chemical composition. Indeed, chemical analysis of the cell wall and plasmalemma appears to be a logical course, especially in light of the high lipid content reported for this species (29), and the detection of phospholipids both in the cell (10) and in the culture fluid (23).

Large electron-dense bodies (Fig. 12) characteristic of volutin granules (5, 9), and thought to be composed of polymetaphosphate (33), were frequently observed. Such bodies may well be the source of the hydrolyzable polyphosphate previously detected by Barker and Kornberg (1) in this species. These granules are found mostly in the nucleoplasm, in association with the fine dense

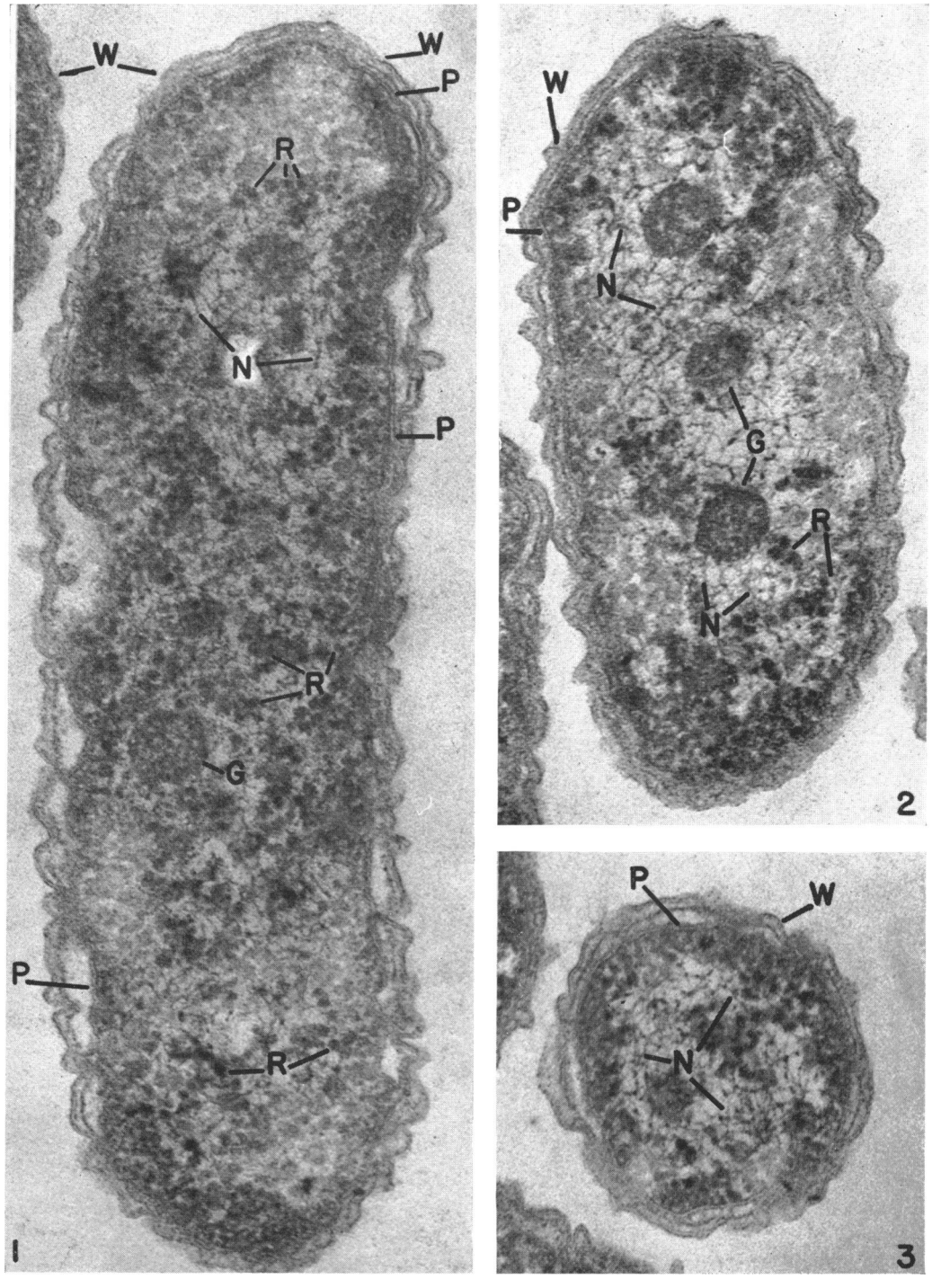



FIG. 1–3. *Longitudinal, oblique, and cross sections, respectively, showing cell wall (W), plasmalemma (P), nucleoplasm (N), ribosomes (R), and granules (G). Cells were fixed in osmium tetroxide (Kellenberger's method), embedded in Epon, and stained by lead citrate-uranyl acetate.* × *140,000.*

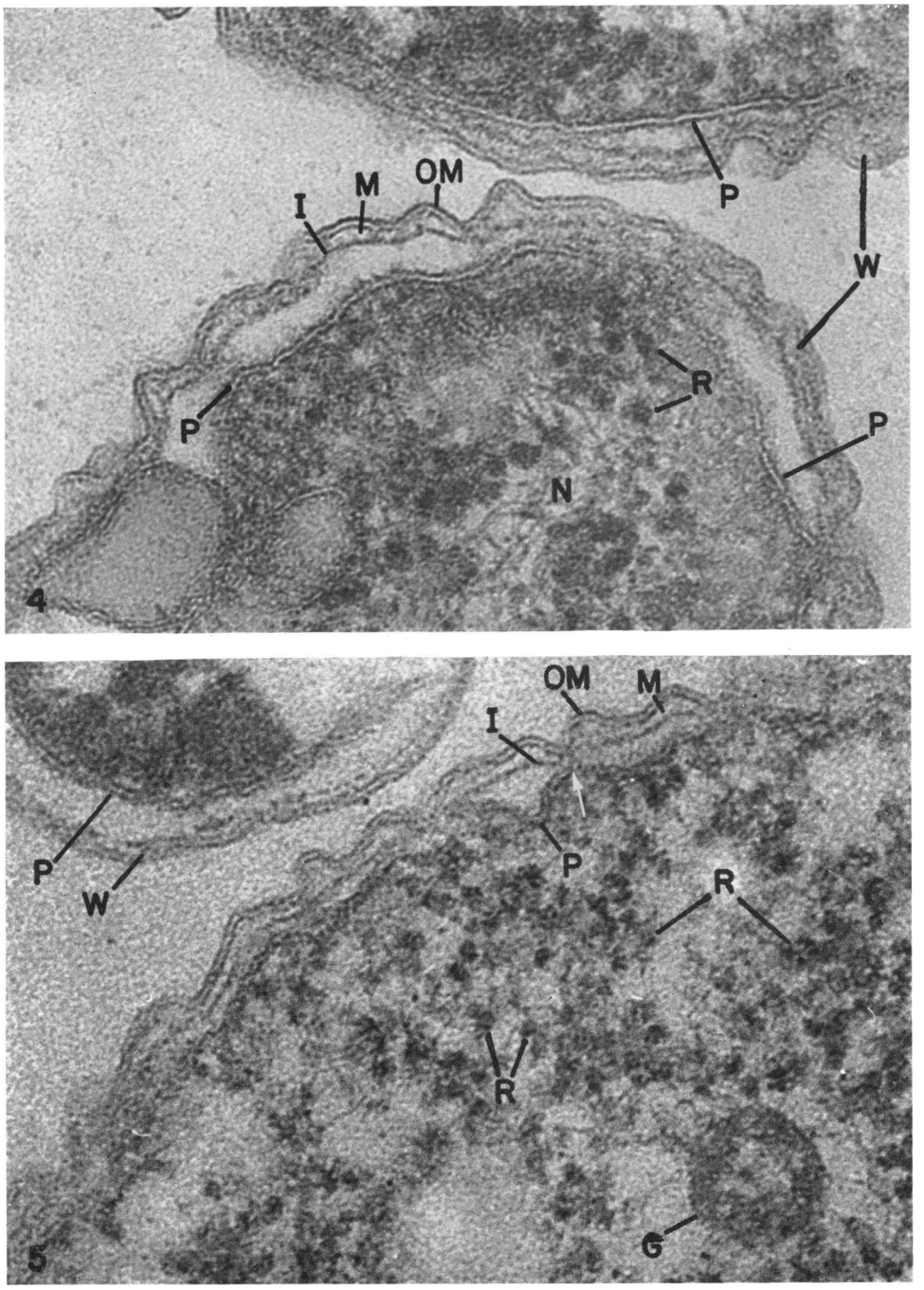



FIG. 4–5. *Portions of cells showing their multilayered envelopes, i.e., the cell wall (W) with an outer unit membrane (OM), a middle layer (M), an innermost layer (I), and the plasmalemma (P)—a typical unit membrane bordering the cytoplasm. Nucleoplasm with thin, dense filaments (N), ribosomes (R), and a granule (G) are also indicated. Cells in Fig. 4 were fixed in osmium tetroxide (Kellenberger's method), embedded in Epon, and stained by lead citrate-uranyl acetate. × 200,000. Cells in Fig. 5 were fixed in osmium tetroxide (in Veronal-acetate buffer, pH 6.1, with addition of Ca and Mg ions), embedded in Epon, and stained by uranyl acetate-lead citrate. × 200,000. White arrow indicates point of presumed adhesion between cell wall and plasmalemma.*

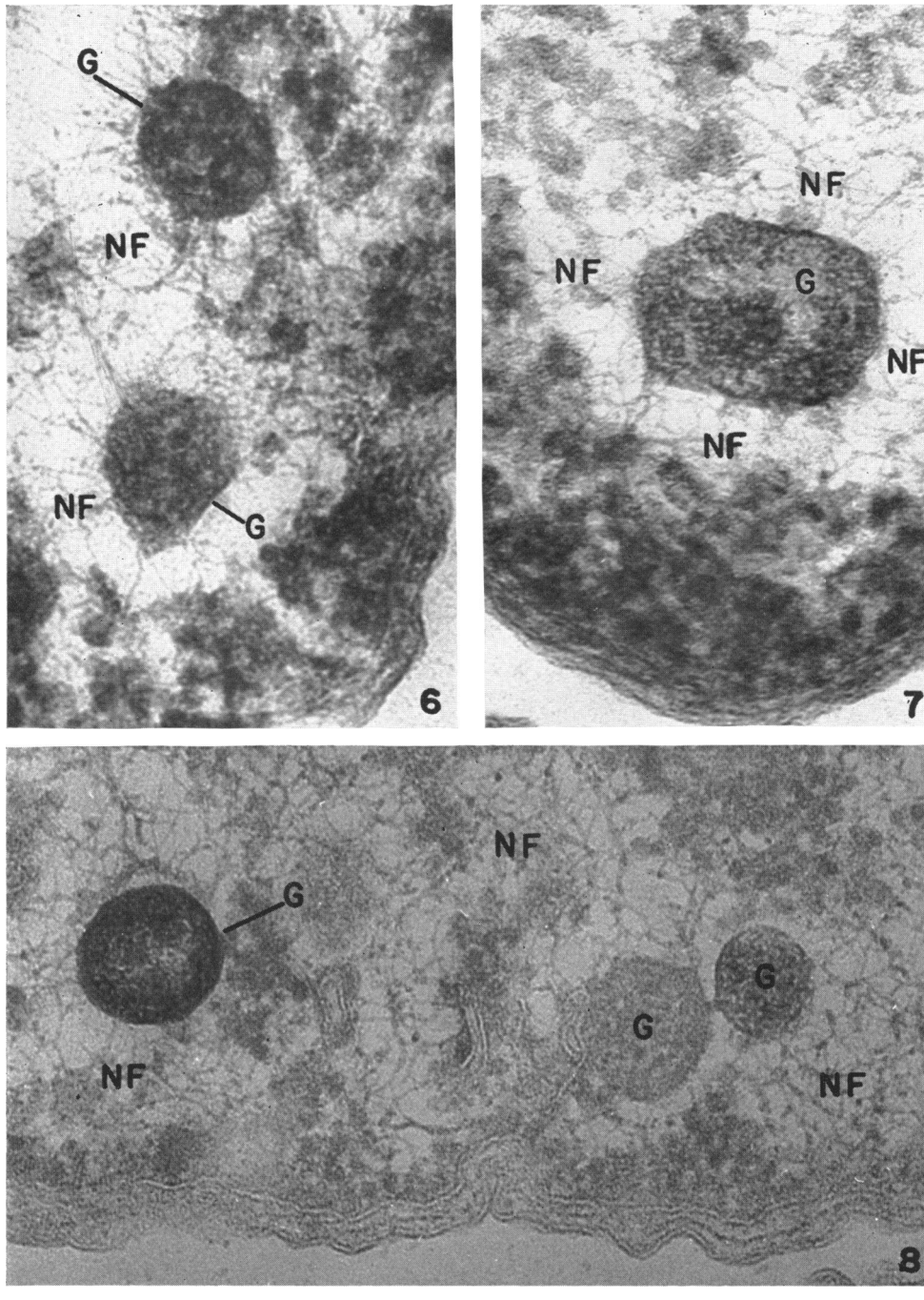
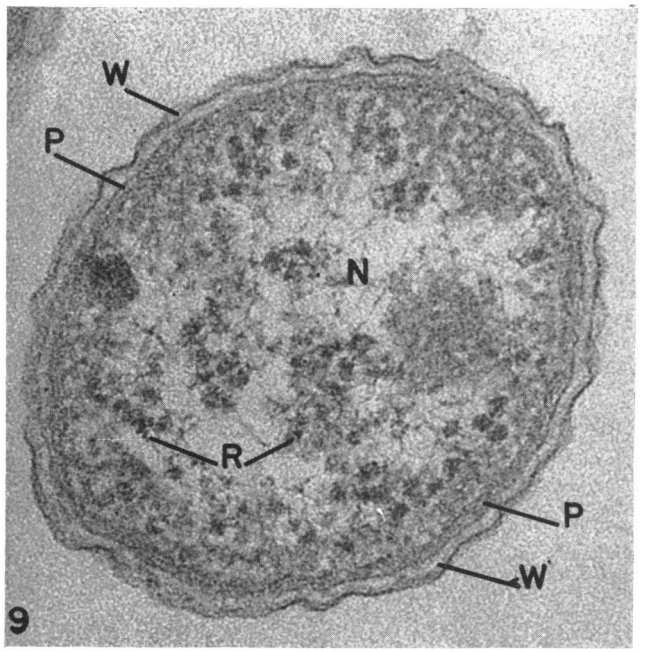
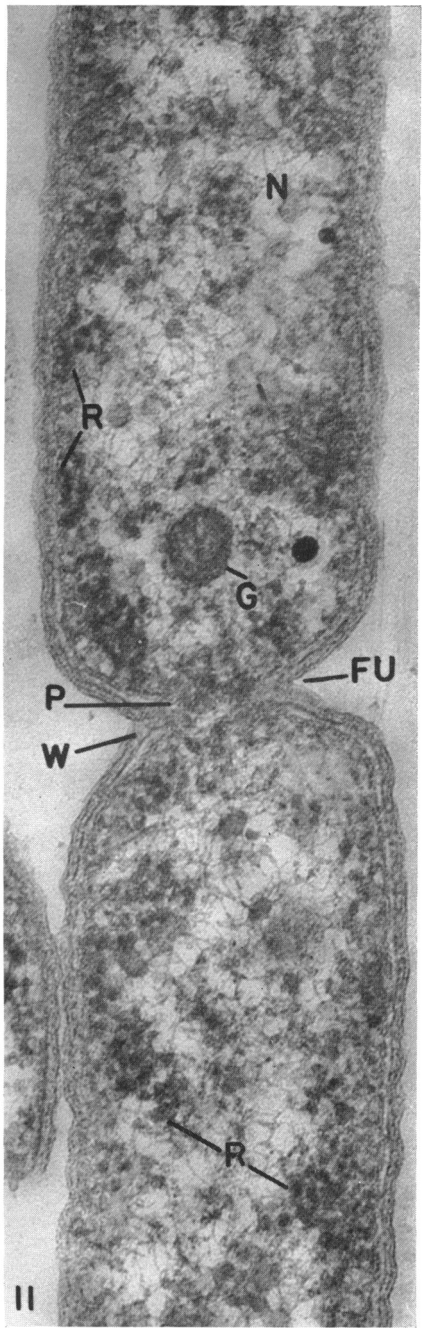



FIG. 6–7. *Portions of cells exhibiting large dense granules (G) associated with nuclear filaments (NF). The granule of Fig. 7 displays internal structure of a subgranular type. Cells fixed in osmium tetroxide (Kellenberger's method), embedded in methacrylate, and stained with lead citrate.* × *200,000.*

FIG. 8. *Portion of a cell showing granules (G) of variable density in association with nucleoplasm filaments (NF). Cells fixed in osmium tetroxide (Kellenberger's method), embedded in Epon, and stained with uranyl acetate-lead citrate.* × *160,000.*



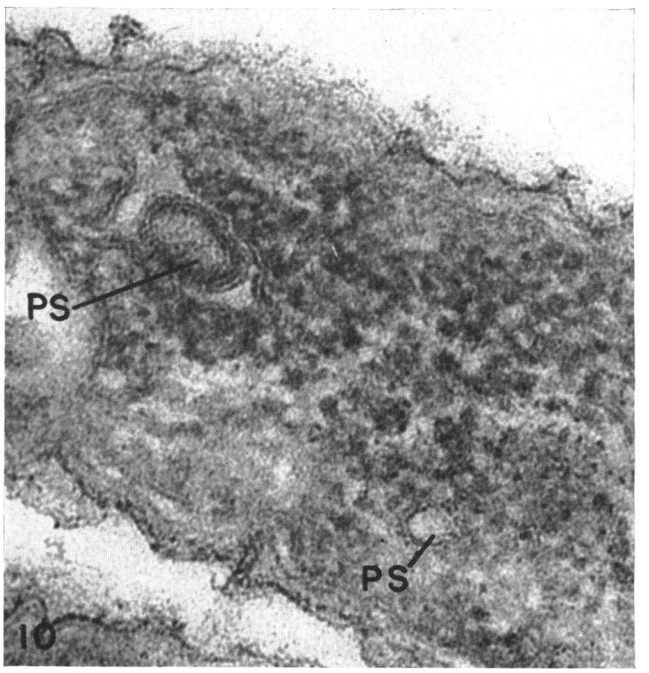




FIG. 9. *Cross section showing nucleoplasm (N) and ribosomes (R) scattered throughout the cell. The cell wall (W) and the plasmalemma (P) are seen in close apposition to each other. Cells were fixed with osmium tetroxide in a mixture of sulfuric acid and dipotassium phosphate, at pH 5.8, embedded in Epon, and stained with uranyl acetate-lead citrate. Note good preservation by this method.* × *160,000.*

FIG. 10. *Membranous structures similar to mesosomes or plasmalemmasomes (PS) are found but interpreted as superficial invaginations of the plasma membrane as cut in a longitudinal tangential section to the surface of the cell. Cells fixed in osmium tetroxide (Kellenberger's method), embedded in Epon, and stained with uranyl acetate-lead citrate.* × *200,000.*

FIG. 11. *Longitudinal, median section of a dividing cell. The furrow (FU) is formed by the invagination of plasmalemma (P) and cell wall (W). Note thin, dense filaments of nucleoplasm (N) diffuse throughout the cell, and ribosomes (R) frequently seen in apposition to the plasmalemma (R in upper half of the figure). A granule (G) is indicated. Cells fixed in osmium tetroxide (Kellenberger's method), embedded in methacrylate, and stained with lead citrate.* × *120,000.*

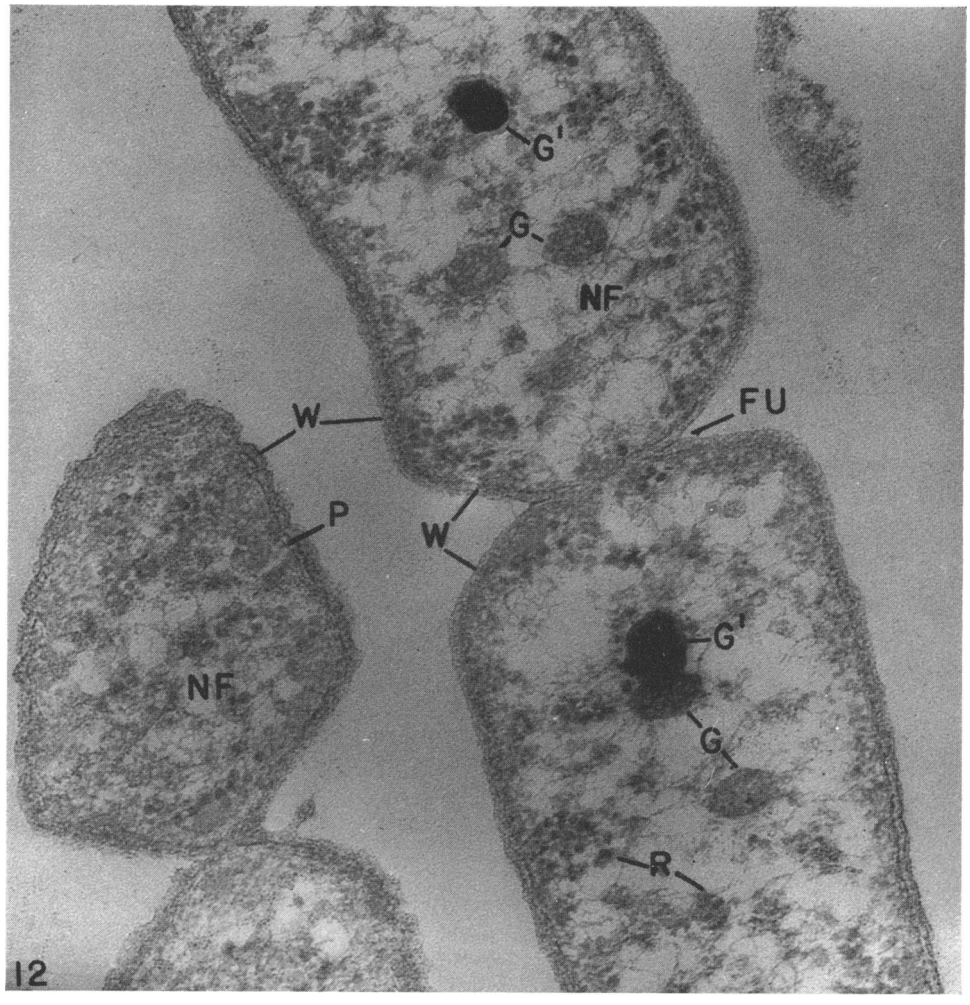



FIG. 12. *Cells at progressive stages of fission. Cell wall (W), plasmalemma (P), ribosomes (R), nuclear filaments (NF), and granules (G) are indicated; two opaque, very dense granules (G') display the characteristic appearance of volutin. Cells fixed in osmium tetroxide (Kellenberger's method), embedded in methacrylate, and stained with chromyl chloride.* × *100,000.*

fibrils (Fig. 6–7) interpreted as being the deoxyribonucleic acid material.

The less-dense bodies (Fig. 2, 8, 12, 13) in which some internal structure is sometimes detected (Fig. 7) may be similar to some granules reported in *Ferrobacillus ferrooxidans* (7, 14). The significance of these bodies and their chemical nature will require further investigation. They have been tentatively interpreted as stages of volutin (polymetaphosphate) deposition.

The lack of complex cytomembrane systems in *T. thiooxidans* is somewhat an anomaly when compared with the recent study of *Nitrocystis*, *Nitrosomonas*, and *Nitrobacter*, by Murray and Watson (17). Based on Murray's (15) suggested relationship between complexity of membranes and energetic processes, one would expect to find a wealth of membranous elements in this sulfur-oxidizing species. Instead, one finds a dearth of cytomembranes, and, for that matter, the same is true of an iron-oxidizing species, *F. ferrooxidans* (7, 14).

Our studies have revealed that *T. thiooxidans* is structurally similar to the many well-documented gram-negative heterotrophic bacteria. This similarity of internal anatomy integrates nicely with biochemical aspects reported by O'Kane (18) and LePage and Umbreit (12, 13), all of which points

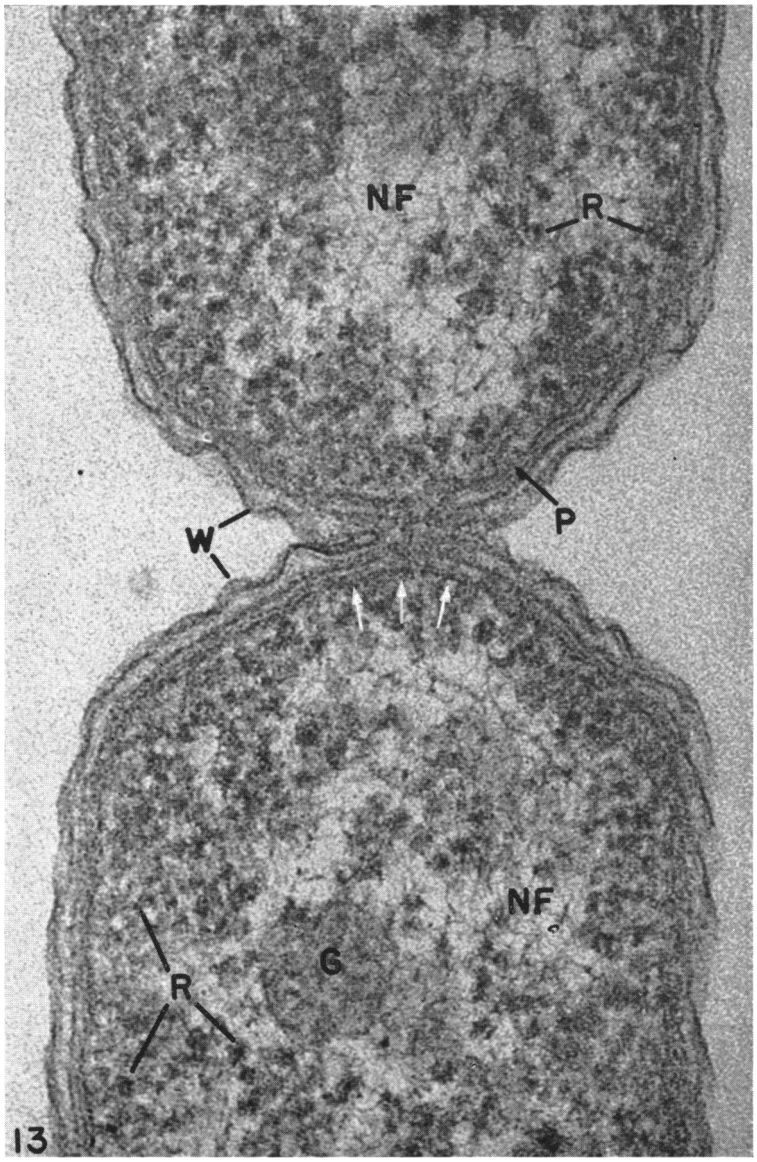



FIG. 13. *Final step of cell division seen in longitudinal, median section. Cell wall (W) and plasmalemma (P) are indicated at the furrow zone. Note that the plasmalemma already appears as a continuous limit (arrows), separating the two cytoplasms, but the cell wall is not yet completed at the splitting point. Filaments of the nucleoplasm (NF) are seen among numerous dense particles (ribosomes, R), and a granule (G) of middle electron density is present. Cells fixed in osmium tetroxide in a mixture of sulfuric acid and dipotassium phosphate, at pH 5.8, embedded in Epon, and stained with lead citrate-uranyl acetate.* × *200,000.*

to an internal metabolism that is heterotrophic. Thus, there is no structural mystique, and one might conclude that the answers to questions regarding sulfur oxidation and the organism's ability to withstand the acid environment will be found after further chemical analysis.

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