Membrane-associated Sulfur Oxidation by the Autotroph *Thiobacillus thiooxidans*

FRANK W. ADAIR

Department of Bacteriology, Rutgers, The State University, New Brunswick, New Jersey

Received for publication 21 April 1966

ABSTRACT

ADAIR, FRANK W. (Rutgers, The State University, New Brunswick, N. J.). Membrane-associated sulfur oxidation by the autotroph *Thiobacillus thiooxidans*. J. Bacteriol. 92:899-904. 1966.—Washed cell wall-membrane fragments derived from sulfur-grown cells of the strictly autotrophic bacterium, *Thiobacillus thiooxidans*, oxidized elemental sulfur to sulfate without the addition of cofactors. The oxidation was optimal at pH 7.0 and was increased by the presence of wetting agents. Oxygen uptake was inhibited by cyanide, azide, and thiol-binding agents. Sulfite was also oxidized, and both the sulfur- and sulfite-oxidizing systems were heat-labile. Neither thiosulfate nor tetrathionate was oxidized by soluble or membrane preparations. The fragments fixed $C^{14}O_2$ in the presence of ribose-5-phosphate, Mg^{++} , and adenosine triphosphate. Sulfur oxidation did not provide energy for $C^{14}O_2$ fixation in this system.

Previous studies (13, 16) revealed that sonically prepared cell-free extracts of the sulfur-requiring autotroph, *Thiobacillus thiooxidans*, oxidized elemental sulfur to thiosulfate only in the presence of reduced glutathione. A direct oxygenation of sulfur occurs in this system (14), and sulfur oxidation is blocked by the presence of KCN, resulting in the formation of thiocyanate in concentrations directly proportional to the amount of glutathione present. Vogler et al. (18) demonstrated sulfur oxidation in whole cells to be blocked by sodium cyanide, sodium azide, and carbon monoxide, which are well-known inhibitors of the cytochrome system. Recently, the presence of ubiquinone, cytochrome *c*, and cytochrome reductase have been reported in *T. thiooxidans* (2, 7). Other investigators have shown the oxidation of sulfide, thiosulfate, and tetrathionate by cell-free extracts of thiosulfate-grown cells of *T. thiooxidans* (8).

Cell-free extracts of the organism have also been found to fix CO_2 (3, 15); this fixation was dependent upon ribose-5-phosphate (R-5-P), Mg^{++} and adenosine triphosphate (ATP). Substrate oxidation has not been linked to the production of energy for CO_2 fixation in these extracts.

Experiments with whole cells (1, 10, 19) revealed that direct contact occurred between cells and sulfur in growing cultures of *T. thiooxidans*. It was further demonstrated that a sulfur-wetting substance was produced (5); the phospholipid nature of this compound has been described

(4, 11). Since oxidation of sulfur by whole cells is associated with a direct contact between substrate and organism, it seemed possible that sulfur oxidation might be linked in some manner to the cell wall-membrane complex. The purpose of this report is to describe the preparation of such a system, to describe its characteristics in relation to other systems, and to show that sulfur oxidation is a function associated with the cell membrane.

MATERIALS AND METHODS

Organism. The organism used in this study was *Thiobacillus thiooxidans* (Waksman strain), originally obtained from R. L. Starkey. A principal characteristic of the organism is its inability to grow on solid media, including *Thiobacillus* agar (Difco).

Culture technique. Cells were grown in 1-liter Erlenmeyer flasks containing 500 ml of a sterile basal salts medium as described by Vogler and Umbreit (19), with the addition of 1% (w/v) of sterile, sublimed elemental sulfur (Merck & Co., Inc., Rahway, N.J.). The cultures were allowed to stand for 3 days and then were placed on a rotary shaking machine at 23 C for 6 days (1).

Preparation of membrane fractions. The cells were harvested by means of a Sharples continuous flow centrifuge. The cell paste was then resuspended in distilled water and centrifuged at $1,000 \times g$ for 5 min to remove elemental sulfur. After centrifugation at $10,000 \times g$ for 10 min to obtain a cell pellet, 0.1 M tris(hydroxymethyl)aminomethane (Tris)-Cl buffer, pH 7.5, was added in a volume (in milliliters) equal to one-half the wet weight of the pellet in grams. This sus-

pension was passed twice through a cold French pressure cell (American Instruments Co., Inc., Silver Spring, Md.) at 16,000 psi. The viscosity of the broken cell paste was reduced by the addition of 2 mg of deoxyribonuclease (40,000 dornase units per mg) for 15 min at 4 C. Centrifugation at $30,000 \times g$ for 30 min at 0 C yielded a clear, rust-colored (soluble) fraction which was frozen in 1.0-ml portions. The pellet was washed twice and resuspended in cold 0.1 M Tris buffer (pH 7.0). Excess washing decreased enzymatic activity. This suspension was centrifuged at $1,000 \times g$ for 20 min to remove any residual whole cells. The particulate fraction was removed and frozen in 1.0-ml samples. Evidence that the particulate fraction is free from whole cells and represents cell-free material is the following: whole cells could not be seen microscopically, the pH optima for sulfur oxidation were markedly different (below pH 5, the particulate fraction does not oxidize sulfur), R-5-P and ATP are required for appreciable CO_2 fixation in the cell fragments, and sulfur oxidation enhances CO_2 fixation in whole cells, but not in membrane fragments.

Respiration. Oxygen uptake was measured in a standard Warburg apparatus at 28 C with shaking (140 oscillations per minute, amplitude at 4 cm). The Warburg flasks had a capacity of 15 ml; NaOH was not present in the center well. Unless indicated otherwise, all Warburg flasks contained 200 μ moles of 0.1 M Tris-Cl buffer (pH 7.0), 30 mg of elemental sulfur wetted with 2.0 mg of phosphatidylinositol, and distilled water to make a total volume of 1.0 ml.

CO_2 fixation. That amount of CO_2 fixed was determined by first adding a known quantity of $\text{NaH}^{14}\text{CO}_3$ to one arm of a double side arm Warburg flask. The second arm contained R-5-P, Mg^{++} , ATP, or a combination of these, depending on the experiment. One side arm was fitted with a glass plug, the other with a rubber serum plug. After equilibration for 10 min, the reactions were started by tipping the contents of the side arms into the cups of the flasks. To terminate the reactions, 0.3 ml of 5% HClO_4 was added to the serum-capped side arm by means of a needle and a 2.5-ml syringe; it was then tipped into the flask cups. NaOH (0.3 ml, 10%) was added to the side arm in the same manner to remove all unused C^{14}O_2 . The reaction mixtures were measured for radioactivity in a Tracerlab Multi/matic, automatic, gas-flow counter.

Sulfate measurements. Sulfate production was determined qualitatively by the addition of BaCl_2 and quantitatively by adding a known amount of labeled elemental sulfur (S^{35}) (Volk Radiochemical Co., New York, N.Y.) to the reaction mixture and then, after a given time, stopping the reaction with 0.3 ml of 5% HClO_4 . The reaction mixture was centrifuged at $5,000 \times g$ for 10 min; the pellet was then discarded. The clear supernatant fluid was then removed and treated with excess 1.0 M BaCl_2 . The labeled BaSO_4 precipitate was washed twice and resuspended in distilled water. A portion was placed in an aluminum planchet which was then heated to 600 C for 0.5 hr to remove any contaminating S^{35} . Radioactivity on the planchets was measured, and the values were corrected for self absorption.

Chemical determinations. Protein was determined by the Folin-Ciocalteu method (6) and thiocyanate by the method of Sörbo (12), without the addition of cyanide.

RESULTS

Growth. Approximately 0.20 g (wet weight) of cells per liter of medium was obtained after 9 days. During this time, the cultures would reach a pH value of 1.5. The organism did not use thiosulfate as an energy source for growth. Cell yields were increased and the incubation time was decreased when 0.5 ml of a 5% Tween-80 solution was added as a wetting agent (1).

Nature of the extract. Cells of *T. thiooxidans* which were broken in the French pressure cell and centrifuged at $30,000 \times g$ yielded a clear, deep rust-colored supernatant fluid, indicating the formation of relatively large cell fragments. These appeared microscopically as pieces of irregular shape. They are most probably cell wall-membrane complexes, and will be referred to as cell fragments.

When the cells were sonically treated for 10 min in a 10-kc Raytheon sonic oscillator: the $30,000 \times g$ supernatant fluid was opalescent in appearance. This indicates that, with sonic disintegration, the cells were broken into smaller fragments. The $1,000 \times g$ supernatant fluid obtained after resuspension of the $30,000 \times g$ pellet did not oxidize sulfur. The reason for this may be that the cell fragments have to retain a certain structural integrity for sulfur oxidation to occur.

Oxidation of elemental sulfur by cell fragments. When washed cell fragments were incubated with elemental sulfur, oxygen was consumed and sulfate was formed. The rate of oxygen uptake was linear for at least 1 hr (Fig. 1). It was also found that a slight endogenous oxygen uptake occurred in sulfur-free systems. The soluble fraction ($30,000 \times g$ supernatant fluid) consumed oxygen in the presence of sulfur only when reduced glutathione was present, and compares with the system described by Suzuki (13). Neither soluble preparations nor cell fragments oxidized thiosulfate or tetrathionate.

Oxygen utilization during sulfur oxidation was greatly increased by the addition of wetting agents (Table 1). Although all the compounds caused an increase in oxygen uptake, Tween-80 had the greatest effect. Visual inspection showed that the wetting agents allowed the elemental sulfur to become very finely divided. When no wetting agent was present, sulfur remained in a layer on the surface of the reaction mixtures in the Warburg flasks and was apparently only slightly usable in this form.

If cell fragments were incubated with sulfur at

FIG. 1. Oxidation of elemental sulfur by cell fragments of *Thiobacillus thiooxidans*. The reaction mixture contained 600 μ moles of Tris-Cl buffer (pH 7.0), 12.9 mg of S^{35} , and 37.5 mg of protein in a total volume of 3.0 ml.

TABLE 1. Effect of wetting agents on oxygen absorbance by cell fragments of *Thiobacillus thiooxidans*^a

Wetting agent	O ₂ uptake (μ liters/40 min)
None	28.0
Lecithin	139.0
Cephalin	151.0
Phosphatidylinositol	157.0
Tween-80	483.0

^a Cell fragments (20.0 mg of protein) were incubated as described in Materials and Methods, except that 2.0 mg each of the above compounds was supplied as wetting agents.

various hydrogen ion levels, it was found that oxygen was absorbed from pH 5.0 to 9.0, and was optimal at pH 7.0. Whole cells, in contrast, oxidized sulfur at pH levels from 1.0 to 9.0, with the greatest absorption occurring at pH 5.0 (Fig. 2). Cell fragments actively oxidizing sulfur at pH 7.5 irreversibly lost this ability when adjusted to pH 2.0.

Inhibition of sulfur oxidation. Incubation of the cell fragments with 10^{-3} M KCN or 10^{-3} M NaN₃ for 10 min prior to the addition of sulfur completely inhibited oxygen absorption and sulfate formation. Treatment of the reaction mixture in the same manner with the thiol-binding agents, *p*-chloromercuribenzoic acid (CMB; 2×10^{-3} M), or the sodium salt of iodoacetic acid (IAA; $2 \times$

10^{-3} M) also caused complete inhibition of oxygen uptake and sulfate formation. CMB added in a final concentration of 2×10^{-5} M caused approximately a 50% inhibition of oxygen uptake 20 min after sulfur was added. When the cell fragments were allowed to oxidize sulfur before the addition of CMB and IAA, inhibition of oxygen consumption still occurred (Fig. 3). Comparable results were obtained with whole cells. In contrast, it was reported (17) that whole cells of *T. neopolitani*, which oxidize thiosulfate, were protected against thiol-binding agents if incubated with thiosulfate before such agents were added.

Oxidation of other substrates. Table 2 shows the sulfur compounds oxidized by cell fragments of sulfur-grown cells of *T. thiooxidans* in comparison with results reported for a reduced glutathione-dependent sulfur-oxidizing system derived from sulfur-grown cells (13) and a system which does not require glutathione derived from thio-sulfate-grown cells (8).

In addition to sulfur, the fragments oxidized

FIG. 2. Effect of pH on elemental sulfur oxidation by cell fragments and whole cells. Each Warburg flask contained 30.0 mg of wetted sulfur and 2.0 ml of the following buffers: 0.2 M KCl-HCl (pH 1.0 and 2.0), 0.2 M glycine-HCl (pH 3.0), 0.2 M acetate (pH 4.0, 5.0), 0.2 M phosphate (pH 6.0, 7.0), and 0.2 M Tris-Cl (pH 8.0, 9.0). Whole cells (13.0 mg) and cell fragments (25.0 mg) were added to make a final volume of 3.0 ml.

FIG. 3. Effect of thiol-binding agents on sulfur oxidation. The reaction mixtures contained 120 μ moles of Tris-Cl buffer (pH 7.0), 30 mg of sulfur, and cell fragments (22.5 mg of protein) in a total volume of 1.2 ml. IAA was added from the flask side arm after 30 min (○). CMB was added after 60 min (■). Abbreviations: IAA = iodoacetic acid; CMB = *p*-chloromercuribenzoic acid.

TABLE 2. Comparison of substrate oxidations by extracts of *Thiobacillus thiooxidans*

Substrate	Fragments from sulfur-grown cells		Glutathione-requiring soluble extract from sulfur-grown cells ^a	Nonrequiring soluble extract from $S_2O_3^{2-}$ -grown cells
	Un-boiled	Boiled		
S^{2-}	+	+	+	+
S^0	+	—	+	? ^c
SO_3^{2-}	+	—	+	+
$S_2O_3^{2-}$	—	—	—	+
$S_4O_6^{2-}$	—	—	? ^c	+

^a Data obtained from Suzuki (13, 16).

^b Data obtained from London and Rittenberg (8).

^c Not reported.

sulfite. During this oxidation, approximately 0.23 μ mole of O_2 was taken up for every μ mole of SO_3^{2-} present. Sulfite oxidation was blocked completely by KCN and NaN_3 (10^{-3} M) when the inhibitors were added 10 min prior to the addition of the substrate. No thiocyanate was formed in the presence of KCN. Sulfide was also oxidized, and the system was completely heat-stable, whereas the sulfite- and sulfur-oxidizing systems were heat-labile. The oxidation of sulfide was carried out by extracts vigorously boiled for as long as 30 min. Similarly prepared extracts of *T. thioparus*, and the nonsulfur-oxidizing heterotroph *Pseudo-*

monas fluorescens also contained a heat-stable sulfide-oxidizing system.

Sulfide oxidation was inhibited by KCN, but not by NaN_3 , and thiocyanate was formed both in the presence and absence of cell fragments. Neither thiosulfate nor tetrathionate was oxidized by the membrane fraction.

Glutathione-requiring cell-free extracts of sulfur-grown cells of *T. thiooxidans* were reported to oxidize sulfur, sulfite, and sulfide to thiosulfate (13, 16). Partial purification of these extracts eliminated the oxidation of sulfide (13). The fate of thiosulfate is still undetermined, however, since an adenosine-5'-phosphosulfate reductase system capable of converting thiosulfate to sulfate, such as that found in *T. thioparus* (9), has not been found in extracts of sulfur-grown cells of *T. thiooxidans* (Cook, Ph.D. Thesis, Rutgers Univ., New Brunswick, N. J., 1963).

In contrast, noncofactor-requiring cell-free extracts of thiosulfate-grown cells of *T. thiooxidans* reportedly oxidized sulfide, thiosulfate, and tetrathionate to sulfate by way of polythionate intermediates (8). Elemental sulfur oxidation was not reported.

Carbon dioxide fixation. Since CO_2 fixation by *T. thiooxidans* is apparently similar to that of green plants (3), ATP is essential to the operation of this pathway. Substrate oxidation undoubtedly provides the high energy phosphate compounds for CO_2 fixation. For this reason, the ability to fix CO_2 was used to determine whether the oxidation of sulfur by cell fragments was producing any usable energy. The cell fragments fixed $C^{14}O_2$ and had a strict requirement for R-5-P and ATP (Fig. 4). However, sulfur did not stimulate $C^{14}O_2$ fixation in the presence of ATP (5×10^{-3} M). In systems where the amount of ATP used was as low as 0.1 μ mole/ml of reaction mixture, the active oxidation of sulfur still did not stimulate $C^{14}O_2$ fixation. Figure 4 shows that whole cells were relatively unaffected by R-5-P, Mg^{++} , or ATP in relation to $C^{14}O_2$ fixation, and sulfur oxidation did cause an increased fixation of $C^{14}O_2$ as expected.

The cell fragments fixed 38.2 μ moles of $C^{14}O_2$ per mg of protein, whereas whole cells fixed only 9.2 μ moles of $C^{14}O_2$ per mg of protein. The fragments, therefore, fixed about 4.15 times more CO_2 per mg of protein than whole cells indicating that the CO_2 -fixing enzymes were concentrated in the cell-fragment fraction.

DISCUSSION

The data indicate that the complete oxidation of elemental sulfur and sulfite to sulfate are functions

FIG. 4. $^{14}\text{CO}_2$ fixation by cell fragments and whole cells of *Thiobacillus thiooxidans*. The reaction mixtures contained either cell fragments (18.3 mg of protein) or whole cells (17.5 mg of protein), 121 μmoles of Tris-Cl buffer (pH 7.4), $\text{NaH}^{14}\text{CO}_3$ (5.6 μmoles at 23,410 counts per min per μmole), and combinations of R-5-P (10 μmoles), ATP (10 μmoles), Mg^{++} (30 μmoles), sulfur (S ; 30 mg), and water to make a total volume of 2.0 ml. The reactions proceeded for 2 hr.

closely associated with the cell wall-membrane complex.

The ability of NaN_3 and KCN to inhibit completely sulfur and sulfite oxidation by cell fragments indicates blockage of the cytochrome system. Further evidence for the location of the cytochrome system will be presented elsewhere. The glutathione-dependent oxidation system described by Suzuki (13, 14), which incorporates molecular oxygen directly into thiosulfate, was also inhibited by KCN, but thiocyanate was formed in direct proportion to the amount of glutathione present.

It seems, therefore, that at least two sulfur-oxidizing systems occur in sulfur-grown cells of *T. thiooxidans*. The first is dependent upon the presence of the cytochrome system in the cell wall-membrane complex. The other utilizes molecular oxygen directly with sulfur or an organosulfur substrate to form thiosulfate (14). However, the lack of a system to convert thiosulfate to sulfate implies that the complete oxidation of sulfur to sulfate is dependent on enzymes other than those found in the cytoplasm.

Sulfide is oxidized by a heat-stable system occurring in cell fragments, as well as in whole cells of *T. thiooxidans*, *T. thioparus*, and the nonsulfur-oxidizing heterotroph *P. fluorescens*. This suggests that the boiled whole cell and extract preparations are in some way stimulating the nonenzymatic oxidation of sulfide, and that sulfide oxidation is not the function of a conventional enzymatic reaction related to elemental sulfur oxidation in

T. thiooxidans. The inhibitory effect of KCN here can be explained by the formation of thiocyanate, which apparently cannot be oxidized. Since NaN_3 does not inhibit sulfide oxidation, it is not likely that cytochromes are involved.

The apparent discrepancy between extracts of *T. thiooxidans* in relation to the oxidation of thiosulfate and tetrathionate could stem from the fact that more than one strain of the organism exists. Schaeffer (Ph.D. Thesis, Rutgers Univ., New Brunswick, N.J., 1963), using the Waksman strain of *T. thiooxidans* and a strain obtained from J. London, Jr., demonstrated that only the latter strain grew when thiosulfate was used as an energy source. Since neither soluble extracts nor cell fragments derived from the Waksman strain oxidized tetrathionate or thiosulfate, it is reasonable to assume that the requisite enzymes are lacking. In contrast, extracts derived from cells capable of using thiosulfate for growth readily oxidized thiosulfate and tetrathionate (8).

The increased oxidation of sulfur by both whole cells and cell fragments in the presence of wetting agents is probably due in part to the ability of these compounds to increase the forces of adhesion between water and sulfur, thus causing the sulfur to become very finely dispersed and, therefore, more available for oxidation. However, it is possible that a specific biochemical reaction, as well as a purely physical reaction, is taking place. Further studies will be required to determine why Tween-80 has an effect three times greater than that of the other compounds used.

The data show that thiol groups located on the cell wall-membrane complex of *T. thiooxidans* are completely essential for elemental sulfur oxidation. Thiol-binding agents also inhibited the complete oxidation of thiosulfate by whole cells, but not by soluble extracts of *T. neopolitanus*, implying that the site of inhibition is at the cell-membrane level (17). Incubation with sulfur, before adding CMB and IAA, did not protect thiol groups in whole cells or membranes of *T. thiooxidans* as thiosulfate did for whole cells of *T. neopolitanus* (17). The reason for this lack of protection might be attributed to the particulate nature of elemental sulfur. Thiosulfate, on the other hand, is soluble, and would be more accessible to substrate oxidation sites.

Whole cells oxidized sulfur at pH levels ranging from 1.0 to 9.0, whereas cell fragments were active in a pH range from 5.0 to 9.0. This may be explained by the fact that the system responsible for oxygen uptake is located on the interior of the cell membrane and is exposed, in whole cells, to pH level near neutrality. In conjunction with this, the

exterior of the cell membrane has to be stable at very acid pH levels.

$C^{14}O_2$ fixation was used as an assay system to determine whether the oxidation of elemental sulfur by cell fragments was producing energy in the form of ATP. It was found, however, that sulfur oxidation did not stimulate $C^{14}O_2$ fixation in fragments, as it does in whole cells. The reason for this is not clear, and experiments are being undertaken to try to couple these two functions in cell-free preparations.

ACKNOWLEDGMENTS

I am greatly indebted to Wayne W. Umbreit for his suggestions and stimulating guidance throughout the course of this study. This investigation was supported by grant GB-1191 from the National Science Foundation.

LITERATURE CITED

1. COOK, T. M. 1964. Growth of *Thiobacillus thiooxidans* in shaken culture. *J. Bacteriol.* **88**:620-623.
2. COOK, T. M., AND W. W. UMBREIT. 1963. The occurrence of cytochrome and coenzyme Q in *Thiobacillus thiooxidans*. *Biochemistry* **2**:194-196.
3. ELSDEN, S. R. 1962. Photosynthesis and lithotrophic carbon dioxide fixation, p. 1-40. In I. C. Gunsalus and R. Y. Stanier [ed.], *The bacteria*, vol. 3. Academic Press, Inc., New York.
4. JONES, G. E., AND A. A. BENSON. 1964. Phosphatidyl glycerol in *Thiobacillus thiooxidans*. *J. Bacteriol.* **89**:260-261.
5. JONES, G. E., AND R. L. STARKEY. 1961. Surface-active substances produced by *Thiobacillus thiooxidans*. *J. Bacteriol.* **82**:788-789.
6. LAYNE, E. 1957. Spectrophotometric and turbidometric methods for measuring proteins, p. 448-450. In S. P. Colowick and N. O. Kaplan [ed.], *Methods in enzymology*, vol. 3. Academic Press, Inc., New York.
7. LONDON, J. 1963. Cytochrome in *Thiobacillus thiooxidans*. *Science* **140**:409-410.
8. LONDON, J., AND S. C. RITTENBERG. 1964. Path of sulfur in sulfide and thiosulfate oxidation by *Thiobacilli*. *Proc. Natl. Acad. Sci. U.S.* **52**:1183-1190.
9. PECK, H. D., JR., AND E. FISHER, JR. 1962. The oxidation of thiosulfate and phosphorylation in extracts of *Thiobacillus thioparus*. *J. Biol. Chem.* **237**:190-197.
10. SCHAEFFER, W. I., P. E. HOLBERT, AND W. W. UMBREIT. 1962. Attachment of *Thiobacillus thiooxidans* to sulfur crystals. *J. Bacteriol.* **85**:137-140.
11. SCHAEFFER, W. I., AND W. W. UMBREIT. 1962. Phosphatidylinositol as a wetting agent in sulfur oxidation by *Thiobacillus thiooxidans*. *J. Bacteriol.* **85**:492-493.
12. SÖRBO, B. 1957. A colorimetric method for the determination of thiosulfate. *Biochim. Biophys. Acta* **23**:412-416.
13. SUZUKI, I. 1965. Oxidation of elemental sulfur by an enzyme system of *Thiobacillus thiooxidans*. *Biochim. Biophys. Acta* **104**:359-371.
14. SUZUKI, I. 1965. Incorporation of atmospheric oxygen-18 into thiosulfate by the sulfur-oxidizing enzyme of *Thiobacillus thiooxidans*. *Biochim. Biophys. Acta* **110**:97-101.
15. SUZUKI, I., AND C. H. WERKMAN. 1958. Chemoautotrophic carbon dioxide fixation by extracts of *Thiobacillus thiooxidans*. II. Formation of phosphoglyceric acid. *Arch. Biochem. Biophys.* **77**:112-123.
16. SUZUKI, I., AND C. H. WERKMAN. 1959. Glutathione and sulfur oxidation by *Thiobacillus thiooxidans*. *Proc. Natl. Acad. Sci. U.S.* **45**:239-254.
17. TRUDINGER, P. A. 1965. Effect of thiol-binding reagents on the metabolism of thiosulfate and tetrathionate by *Thiobacillus neopolitanus*. *J. Bacteriol.* **89**:617-625.
18. VOGLER, K. G., G. A. LEPAGE, AND W. W. UMBREIT. 1962. Studies on the metabolism of autotrophic bacteria. *J. Gen. Physiol.* **26**:89-102.
19. VOGLER, K. G., AND W. W. UMBREIT. 1941. The necessity for direct contact in sulfur oxidation by *Thiobacillus thiooxidans*. *Soil Sci.* **51**:331-337.