A STUDY OF ENZYMES INVOLVED IN THE SULFUR OXIDATION OF *Thiobacillus thiooxidans*

By

HECTOR M. LIZAMA

A Thesis

Submitted to

the Faculty of Graduate Studies and Research

The University of Manitoba

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

ISBN 0-315-34030-4

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ABSTRACT

Sulfur oxidation was studied using intact cells and cell-free systems of *Thiobacillus thiooxidans*. The cell-free extracts from which all preparations were made, were from cells broken by passage through a French pressure cell. Particular attention was given to the isolation and partial purification of sulfite oxidase and the sulfur-oxidizing enzyme. Sulfite oxidase was associated with cell envelopes but could be solubilized in active form by mild detergent treatment. The sulfur-oxidizing enzyme was released in soluble form but remained tightly associated with nucleic acid. Calcium and sucrose appeared to play a substantial role in the stabilization of the enzyme. In the absence of nucleic acid or calcium, the sulfur-oxidizing enzyme was very unstable and seemed to bind other proteins. Sulfite oxidase was able to reduce ferricyanide but not mammalian or *Saccharomyces cerevisiae* cytochrome *c*. The isolation of a native cytochrome *c* for the reconstitution of a sulfite-oxidizing system is needed.

ACKNOWLEDGEMENTS

The author wishes to thank Dr. I. Suzuki, Professor for his guidance, generosity, and patience during the course of this work.

The author also wishes to thank Mr. S.C. Kwok for his companionship and technical assistance. Many thanks to Dr. J.K. Oh are offered for his valuable advice also. Acknowledgements also go to the department of Microbiology where the author has found nothing but help and friendship.

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ABBREVIATIONS

AMP	- adenosine-5'-monophosphate
CFE	- cell-free extract
DEAE	- diethylaminoethyl
DNP	- dinitrophenyl
DTE	- dithioerythritol
DTT	- dithiothreitol
EDTA	- ethylenediaminetetraacetic acid
GSH	- reduced glutathione
h. heart	- horse heart
HOQNO	- hydroxyquinoline
NADH	- nicotine adenine dinucleotide
SDS	- sodium dodecyl sulfate
Tris	- Tris (hydroxymethyl) aminomethane

INTRODUCTION

INTRODUCTION

Thiobacillus thiooxidans is a Gram negative, rod-shaped bacteria found naturally in acidic soil environments. The organism obtains its energy requirements by oxidizing reduced or partially reduced sulfur compounds including sulfide, elemental sulfur, thiosulfate, polythionates, and sulfite to sulfuric acid. Its requirements for carbon are met solely by assimilating carbon dioxide from the atmosphere. *T. thiooxidans* belongs to the thiobacilli, a genera known for their ability to oxidize inorganic sulfur compounds. However, *T. thiooxidans* is set apart from other thiobacilli by being able to withstand an extracellular pH of less than 1.0. As a result, *T. thiooxidans* can be described as a true chemolithotrophic acidophile.

Such simplistic nutrient requirements necessarily warrant great biochemical complexity in the organism. Detailed studies on the mechanism of sulfur oxidation in *T. thiooxidans* have only been able to yield very basic outlines. The complexity of the organism, together with the complex chemistry of sulfur compounds have proven to be formidable barriers to biochemical studies of sulfur oxidations. Initial divergent reports on the sulfur-oxidizing system have only gradually given way to more established postulations.

The present study has been an attempt to characterize sulfur oxidation in *T. thiooxidans*. This characterization involved some studies with intact cells, but mostly concentrated on French pressure

cell-derived cellular components. Particular attention was given to the isolation and partial purification of sulfite oxidase and the sulfur-oxidizing enzyme. These two key enzymes are probably the clues to resolving the mechanisms involved in the metabolism of inorganic sulfur compounds.

HISTORICAL

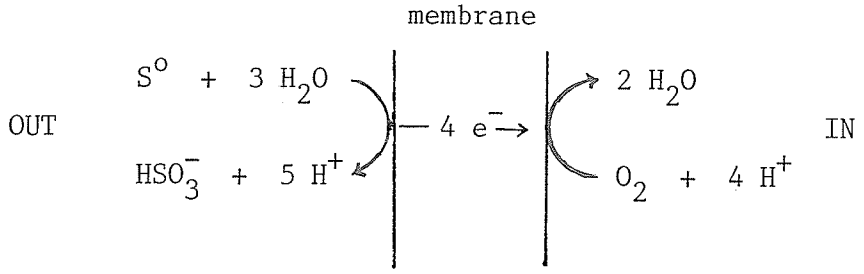
HISTORICAL

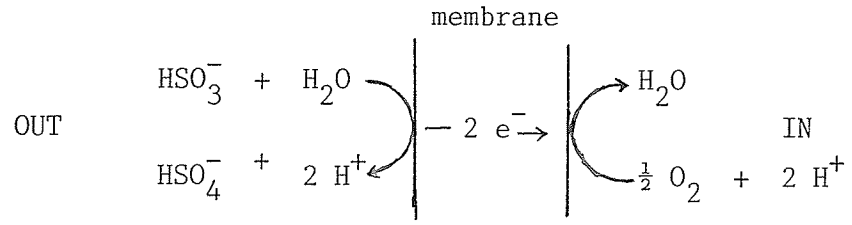
Thiobacillus thiooxidans was discovered in 1921 by Waksman and Joffe (58). It was further studied and described by Starkey, who developed a growth medium still used today (46). Of all the thiobacilli studied to date, *T. thiooxidans* has the lowest recorded G + C ratio. On the basis of this, as well as its membrane lipid composition, it has been classified as a class III thiobacillus. It is the only organism to occupy that class (56). Although *T. thiooxidans* has been the subject of many studies and reviews (1, 7, 12, 16, 17, 23, 40), most recent studies on the oxidation of sulfur compounds have bipased this organism in favour of other thiobacilli (15, 18, 23, 37, 47, 59). In spite of this, there is as of yet no clear understanding on the mechanism of sulfur oxidation.

One of the major problems involved in the oxidation of elemental sulfur is the nature of the substrate itself. Elemental sulfur is insoluble in water; as a result, the cell must come in contact with it in order for oxidation to occurr (15). *T. thiooxidans* is motile and has been observed "attacking" sulfur particles (12). Studies have shown that sulfur oxidation is facilitated by the use of wetting agents or surfactants; this is not only true for intact cells, but for cell fragments as well (1). The wetting agents probably increase the force of adhesion between water and sulfur, facilitating contact between sulfur and the enzymes involved in its oxidation. There has

been a search for a possible wetting agent used by *T. thiooxidans*, the evidence so far pointing to the lipid phosphotidylinositol, which is produced by growing cultures of the organism (43). In shaking culture experiments, attachment of active cells to sulfur particles is quite fast; attachment is complete within five minutes. This attachment seems to be associated with energy generation, as it is inhibited by anaerobiosis and metabolic inhibitors such as 2,4-DNP and HOQNO (51). The method of attachment is not yet clear but thiol groups which are bound to the cell surface appear to be essential for the initial adhesion process.

Once sulfur is attacked by the cell and attachment takes place, the problem of oxidation still has to be resolved. Because of its insolubility, sulfur has to be either oxidized right at the cell surface, dissolved in the cell membrane, or taken up as elemental sulfur by the cell and oxidized internally (15). No definite theory has been proposed, but methods of oxidation have been postulated for each of the three possibilities. An extracellular sulfur oxidation mechanism has been proposed in which the electrons derived from sulfur oxidation enter the cell through the inner membrane, leaving H^+ and HSO_3^- outside (7). A similar reaction would also take place in the oxidation of sulfite:





If this were true, then the protons produced by sulfur oxidation would be released outside the cell while cytochrome oxidase would use the electrons to consume H^+ within the cell, maintaining intracellular pH neutrality when protons enter the cell through the ATP synthetase (7). This proposed mechanism is somewhat new, but has received widespread support by many researchers (4, 10, 15). Other biochemical studies using cell components have so far yielded three sulfur-oxidizing systems: a cell wall membrane complex; a system made up of a soluble and membrane fractions; and a soluble sulfur-oxidizing enzyme (35). Interestingly, each of these three systems has been derived from studies using different methods of cell breakage; the first used breakage by French pressure cell, the second one by sonication under a nitrogen atmosphere, and the third by sonication after an ion-exchange resin treatment. It is quite likely that the same enzyme is involved in all three.

The sulfur-oxidizing enzyme now denoted as sulfur: oxygen oxidoreductase EC 1.13.11.18, was first partially purified during studies on the oxidations of elemental sulfur and of thiosulfate (48, 49). The cells were broken by sonication after an ion-exchange resin treatment and gave a cell-free extract capable of oxidizing colloidal sulfur to thiosulfate in the presence of glutathione (GSH). The actual substrate of this enzyme was postulated to be a sulfur-glutathione

complex. Colloidal sulfur is made up mostly of the octahedral molecule S_8. Glutathione opens the sulfur ring by nucleophilic attack on one of the sulfur atoms, forming a linear polysulfide which can be attacked enzymatically (48). The partially purified enzyme was found to be devoid of glutathione reductase activity, indicating the glutathione-polysulfide complex was present only as an intermediate. Later studies using preparations from *T. thiooxidans* and *Thiobacillus thioparus* however, demonstrated that the initial product of sulfur oxidation was not thiosulfate but sulfite (49). The formation of sulfite was proven by trapping it with formaldehyde; otherwise, a non-enzymatic condensation of sulfite with elemental sulfur would produce the thiosulfate as previously observed:

$$SO_3^{2-} + S \longrightarrow SSO_3^{2-}$$

These observations then, indicated the following mechanism for sulfur oxidation in the presence of catalytic amounts of glutathione:

1) $$S_8 + GSH \longrightarrow GS_8\text{-}SH$$

2) $$GS_8SH + O_2 + H_2O \xrightarrow{\text{enzyme}} GS_7\text{-}SH + SO_3^{2-} + 2\,H^+$$

overall: $$S + O_2 + H_2O \xrightarrow[GSH]{\text{enzyme}} SO_3^{2-} + 2\,H^+$$

The enzyme involved was considered as an oxygenase which contained non-heme iron and possibly labile sulfide as cofactors (49). Removal of bound iron with 2,2'-dipyridyl destroyed enzyme activity. This particular form of the enzyme has been found in a number of thiobacilli besides *T. thiooxidans* and *T. thioparus*. The enzyme has been found in in *Thiobacillus novellus* and forms an integral part of that organism's

thiosulfate oxidizing system (37). The iron/sulfur oxidizer *Thiobacillus ferrooxidans* has also been found to contain a sulfur-oxidizing enzyme similar to that of *T. thiooxidans* in that its optimum pH is 7.8 and requires catalytic amounts of glutathione for activity (45). As with the *T. thiooxidans* enzyme, both non-heme iron and labile sulfide were present and sulfite was the product of sulfur oxidation.

Apart from the soluble enzyme system, a sulfur-oxidizing system has been prepared which does not require the use of cofactors or sulfhydryl compounds such as glutathione (1). This system involves a cell wall membrane complex derived from cells broken by French pressure cell where sulfur is oxidized completely to sulfate. Thiol-binding agents were found to inhibit activity, indicating that protein-bound active sulfhydryl groups may be substituting for glutathione. Sulfur oxidation in this system seems dependent on a cytochrome system in the membrane complex, since it can be inhibited by such agents as NaN_3 and KCN. These however, are probably affecting the cytochrome-mediated oxidation of sulfite, rather than that of sulfur (1). Crude extracts of *T. thiooxidans* derived by French pressure cell breakage in our laboratory do oxidize colloidal sulfur in the absence of glutathione. However, activity increases substantially upon its addition, indicating that its catalytic efficiency is much greater than that of the protein-bound sulfhydryl groups.

A sulfur-oxidizing system composed of a soluble and membrane fractions has been prepared by sonication under a nitrogen atmosphere (21). It appears that the soluble fraction is involved with the oxidation of sulfur to sulfite while the membrane fraction (which can

oxidize sulfite, NADH, and NADPH) completes the oxidation to sulfate (20). The soluble fraction responsible for sulfur oxidation has been resolved to two components: a nonheme iron protein with a molecular weight of 23,000 (50). Nonheme iron is essential for sulfur oxidation since its removal by KCN or diethyldithiocarbamate decreases activity. To date however, all components including soluble and membrane preparations are required for activity; sulfur-oxidizing activity by the two soluble components alone has not been determined (50).

Sulfite is also oxidized by crude extracts of *T. thiooxidans* but does not appear to be a cellular substrate; rather, it is the key intermediate in the oxidation of sulfur to sulfate (35). To date there are two known mechanisms of sulfite oxidation: one is the APS reductase pathway which involves substrate-level phosphorylation; the other is the sulfite oxidase pathway which involves oxidative phosphorylation (35). Thus, it is the sulfite oxidation step which is associated with energy generation. Sulfur oxidation in *T. thiooxidans* then, can be perceived simply as a way for the cell to obtain sulfite for its energy requirements. The oxidation of sulfite in *T. thiooxidans* appears to proceed by the sulfite oxidase pathway which basically consists of channeling electrons through cytochrome *c* to cytochrome oxidase:

$$SO_3^{2-} + 2 \text{ cyt } c(ox) + H_2O \rightarrow SO_4^{2-} + 2 \text{ cyt } c(red) + 2 H^+$$

$$2 \text{ cyt } c(red) + \tfrac{1}{2} O_2 + 2 H^+ \rightarrow 2 \text{ cyt } c(ox) + H_2O$$

Both sulfite oxidase and cytochrome oxidase enzymes have been found in *T. thiooxidans* (28). Sulfite oxidation takes place in the membrane fractions where sulfite can be oxidized with either molecular oxygen

or a bacterial cytochrome *c*-552 from *Pseudomonas stutzeri* as electron acceptors (22). Active membrane fractions have been partially purified and found to contain various cytochromes of the a, b, and c types, indicating participation of the entire cytochrome system (22). The mechanism of sulfite oxidation in *T. thiooxidans* however, is still not clear since not as much research has been done as in other thiobacilli.

Thiosulfate oxidation in *T. thiooxidans* remains ambiguous to date. Many other thiobacilli are known to oxidize this compound and the mechanisms are well documented for *T. thioparus* (30, 31, 32), *T. novellus* (36, 37), and *Thiobacillus versutus* (59-65). These thiobacilli however, all grow at a pH close to neutrality, unlike *T. thiooxidans*. Thiosulfate is fairly stable at pH 7.0 (12), so there is little ambiguity about its use as a cellular substrate by the neutrophilic thiobacilli. Below pH 4 to 5 however, thiosulfate rapidly decomposes in a complex manner, the products depending on the acidity of the solution (41). Decomposition of thiosulfate involves nucleophilic displacement of sulfite ions from hydrogen thiosulfate by the thiosulfate ion. This results in the formation of sulfur plus bisulfite (41). Since *T. thiooxidans* grows at an acidic pH, it is impossible to determine whether the cells are oxidizing thiosulfate or one of its decomposition products: elemental sulfur(12). The problem is further complicated in that, as *T. thiooxidans* grows, the pH of the growth medium drops below one. Under such extremely acidic conditions, sulfane monosulfonate intermediates from the decomposition of thiosulfate interact with one another to give sulfur dioxide, sulfur, and poly-

thionates (41). During early studies, it was proposed that polythionates were intermediates in the pathways of thiosulfate and sulfur oxidation (26, 41, 52, 53, 54, 57). This created much confusion until the role of polythionates was finally ruled out.

Recent advances in the understanding of oxidation of sulfur compounds has shifted to organisms and substrates that are easier to study. Fundamental studies on *T. thiooxidans* and the oxidation of elemental sulfur is lagging behind such organisms as *T. versutus* and *T. ferrooxidans*, and such substrates as thiosulfate. But elemental sulfur is the building block of all the other sulfur compounds; before its metabolism can be clearly understood, progress with other sulfur compounds will be slow and limiting.

MATERIALS AND METHODS

MATERIALS AND METHODS

Materials

All chemicals and reagents used were of reagent grade and obtained commercially. Elemental sulfur was obtained from British Drug Houses. Ltd., Toronto. Sodium thiosulfate and sodium sulfite were obtained from Fisher Scientific Co., Fairlawn, N.J., U.S.A. GSH, AMP, NADH, bovine serum albumin (crystalline), DTE, cytochrome *c* (horse-heart, type III and *Saccharomyces cerevisiae* cytochrome *c*), trypsin (bovine pancreatic, crystalline), trypsin inhibitor (from soybean), catalase (bovine liver, crystallized), β-glucuronidase (as a component of snail gut juice or crystallized), ribonuclease (crystalline), Tris (Trizma base and Trizma hydrochloride), and EDTA (disodium salt) were obtained from Sigma Chemical Co., St. Louis, Missouri, U.S.A. Deoxyribonuclease (DNAse I from bovine pancreas, lyophilized) was obtained from Calbiochem, La Jolla, California, U.S.A. Ammonium sulfate (enzyme grade, ultra pure) was obtained from Schwartz/Mann, Cambridge, Massachussets, U.S.A. Triton X-100 (alkyaryl polyether alcohol) was obtained from Baker Chemical Co. Phillipsburg, N.J. DEAE-celulose was from Schleicher & Schnell Inc., Keene, New Hampshire, U.S.A. DEAE-Sephadex, Sephadex G-100 and G-150, and blue dextran were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Electrophoresis reagents were obtained from Bio-Rad Laboratories, Richmond, California, U.S.A.

All reagents, buffers included, were prepared using glass-distilled water.

Organism and growth conditions

Thiobacillus thiooxidans (ATCC 8085) cultures were grown under autotrophic conditions using Starkey's medium (46). The medium consisted of 0.3 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0. 018 g $FeSO_4 \cdot 7H_2O$, 3.5 g KH_2PO_4, and 0.25 g $CaCl_2$ in one liter of distilled water. The organism was grown stationary in 2.8 L Fernbach flasks containing one L of the medium at 28°C for four to five days. A 2.5% inoculum was used and 50 g of powdered sulfur were sprinkled on the liquid surface of each flask and distributed evenly. The flask was covered by a piece of tissue paper, allowing proper air exchange.

After incubation, the cells were passed through a Whatman No. 1 filter paper under vacuum in order to remove the powdered sulfur. The turbid cell suspension was then passed through a Sharples super centrifuge fitted with a water cooling system (7°) at 40,000 to 60,000 r.p.m. The resulting cell paste was washed once with distilled water and suspended at a concentration of 20 mg (wet weight) per mL in distilled water adjusted to pH 2.0 with dilute sulfuric acid. The cells were stored at 4°C and used for experiments within 20 days.

Methods

Preparation of sulfur suspension

A sulfur suspension was used as substrate for sulfur oxidation experiments. It was prepared by suspending 32 g of powdered sulfur in

100 mL of distilled water containing 0.05% Tween 80. The suspension was stirred with a magnetic stirring bar until the preparation was homogeneous, then stored at a 4°C until needed. Whenever the preparation was used, it was kept constantly stirring so as to maintain homogeneity.

Fine colloidal sulfur was prepared by sonicating the sulfur suspension three times for twenty seconds in a 10 kc/sec Raytheon sonic disintegrator. The milky white colloidal sulfur was decanted off from the sonicated sulfur and kept at 4°C until needed.

Preparation of DMSO-sulfur

Elemental sulfur dissolved in DMSO was used as a substrate in stoichiometric experiments. It was prepared by dissolving a few grains of sulfur in dimethylsulfoxide (DMSO). Whenever stoichiometry was tested, a few µL of DMSO-sulfur were added to a suspension of intact cells and the amount of oxygen uptake measured. The same amount was then added to the preparation being tested and the oxygen uptake compared to that of intact cells, which consumed 1.5 mols O_2 per mole S (I. Suzuki, personal communication). The DMSO-sulfur solutions were stored at room temperature.

Preparation of cell-free extracts

The cell suspension was centrifuged once and resuspended in 0.1 M potassium phosphate buffer at pH 7.5 so as to achieve a cell concentration of 200 mg (wet weight) per mL. The thick cell suspension was then passed three times through a French pressure cell at 20,000 p.s.i. The broken cell suspension was then centrifuged at 2,000 × *g* so as to precipitate the cell debris. The cell-free extract was carefully remo-

ved with a pasteur pipette. A batch of 23 liters of cells usually produced about 40 mL of extract. These crude extracts were normally stored at -20°C until needed.

Fractionation of extracts by ultracentrifugation

Crude extracts were treated with a few grains of deoxyribonuclease (111,000 units per mg) before being centrifuged at 120,000 × *g* for two hours at 4°C. The supernatant was collected and the resulting pellets were resuspended with 0.1 M phosphate buffer (pH 7.5) using a manual ground-glass homogenizer. The homogenized preparation was again centrifuged at 120,000 × *g* for two hours at 4°C. The resulting supernatant was collected and pooled with the first. The combined supernatants were denoted as the "Spinco supernatant". The final pellets were resuspended with a manual ground-glass homogenizer in the membrane storage buffer consisting of 10 mM potassium phosphate buffer + 1 mM $MgCl_2$ + 10% (v/v) glycerol (39). This preparation was labeled "cell envelopes".

Trypsin treatment

Partial digestion of intact cells with trypsin prior to breakage consisted of adding 1.5 mg trypsin (16,260 units per mg) per mg wet weight cells and stirring gently for 15 min at 25°C. The reaction was stopped by adding trypsin inhibitor.

Ammonium sulfate fractionation

Selective protein precipitation using ammonium sulfate was carried out as described by Schleif and Wensink (44). After precipitation, the fractions were resuspended in either 50 mM Tris-Cl, pH 7.5 buffer or S-buffer (10% w/w sucrose in 50 mM Tris-Cl, pH 7.5 buffer). The suspen-

ded samples were then dialyzed against their respective buffers in order to remove the ammonium sulfate. Dialysis was conducted for one hour against one liter of buffer, and repeated a second time against fresh buffer or against CS-buffer (0.1 M $CaCl_2$ in S-buffer) when the procedure involved the use of calcium.

Ion-exchange chromatography

Ion-exchange chromatography experiments were carried out using either DEAE-Sephadex A-50 equilibrated with 50 mM Tris-Cl, pH 7.5 buffer or DEAE-cellulose equilibrated with S- or CS- buffers. The columns used ranged in size from 4.0 × 1.0 cm to 10.0 × 3.5 cm, depending on the size of the applied sample. Elutions were always carried out stepwise by increasing the ionic strength with NaCl, KCl, or $CaCl_2$ in equilibrating buffer, as indicated.

Gel filtration chromatography

Gel filtration chromatography experiments were carried out using Sephadex G-150, unless indicated otherwise. The equilibrating buffers used were 50 mM Tris-Cl, S-, CS-, or AS-(0.1 M AMP in S-buffer) buffers. The sample volume never exceeded 7% of the total bed volume in columns measuring 2.5 × 45.0 cm. The experiments were always run at $4^{o}C$, using a flow rate of 4.2 $mL/cm^2/h$. Fractions were collected every 3.0 mL using a LKB 7000 Ultrorac fraction collector. The column was kept covered with aluminum foil during the experiment in order to protect any light-sensitive components. The void volume was determined using Blue Dextran 2000.

Digestion with β-glucuronidase

Crude extracts and cell envelope preparations were digested with

β-glucuronidase in crystalline form or as a component of snail gut juice using a modification of the method of Noguchi et al. (34). Samples of one to ten mL of crude extract or cell envelopes were incubated at 30°C for two hours in the presence of 4730 units or 15175 units of enzyme per mL of extract or cell envelopes, respectively.

Fractionation of digested membranes

The β-glucuronidase-digested cell envelopes were fractionated by sucrose density gradient centrifugation using a modification of the method of Osborn et al. (39). A 38 mL Beckman quick-seal ultracentrifuge tube was layered with six mL portions of 25, 30, 35, 40, 45, and 50% (w/w) sucrose in 10 mM Tris-Cl, pH 7.5 buffer. The 1.5 to 2.0 mL sample was carefully layered on top of the gradient. After sealing, the tubes were cooled for 15 minutes at 4°C prior to centrifugation. Centrifugation consisted of spinning the tubes at 120,000 × *g* for two hours at 4°C. At the end of the run, the individual bands were either withdrawn from the tube with a pasteur pipette or collected in fractions using a Beckman fraction recovery system. The membrane bands were usually diluted with distilled water and pelleted at 120,000 × *g* for two hours and resuspended in the membrane storage buffer.

Fractionation of undigested membranes

The undigested cell envelopes were fractionated also by sucrose density gradient centrifugation, this time using a modification of the method by Bodo and Lundgren (3). This modified method was exactly the same as that for the digested membranes except for the gradient, which consisted of 40, 45, 50, 55, 60, and 65% (w/w) sucrose in 10 mM Tris-Cl, pH 7.5 buffer.

Membrane solubilization

Selective solubilization using mild detergent treatment was carried out based on information given by the work of Helenius and Simons (14). The cell envelopes were diluted to a concentration of 25 mg dry weight per mL. The surfactant of choice, Triton X-100 was added to a 5% (w/w) concentration, also giving a weight by weight ratio of 2:1 detergent to cell envelope. The resulting suspension was stirred at $4^{o}C$ for one hour in order to ensure proper detergent-membrane interaction. The preparation was then centrifuged at 120,000 × *g* for two hours at $4^{o}C$ in order to pellet that portion of cell envelope which had not been solubilized. The supernatant was collected while the precipitate was resuspended in the membrane storage buffer using a ground-glass manual homogenizer.

Assay of sulfur oxidation

Sulfur oxidation was assayed in a Gilson Oxygraph. The reaction vessel consisted of a thermostated glass vessel equipped with a teflon-coated Clark oxygen electrode, which measured oxygen uptake at a constant temperature of $25^{o}C$. The reaction vessel contained in a total volume of 1.2 mL:

1) assaying cells-	0.35 mL	sulfur suspension
	0.1 mL	intact cells
	0.75 mL	assay buffer
2) assaying enzyme-	0.35 mL	sulfur suspension
	0.3 mL	sample
	0.55 mL	assay buffer
	0.01 mL	GSH (0.1 M)

Assay buffer in the case of intact cells consisted of either distilled water adjusted to pH 2.0 with dilute sulfuric acid or 0.1 M potassium phosphate buffer at pH 2.5 or 7.5. Assay buffer in the case of extracts or enzyme preparations consisted of either 0.1 M potassium phosphate buffer or 50 mM Tris-Cl buffer, both at pH 7.5. When DMSO-sulfur was used as a substrate, 10 µL of the solution were added, the rest of the reaction mixture being made up with assay buffer. One unit of activity was defined as one nmol O_2 consumed per minute. One unit of specific activity was defined as one nmol O_2 consumed per minute per mg protein.

When longer term sulfur oxidation was measured, the rates were measured manometrically using a Warburg apparatus as described by Suzuki and Silver (49). The reaction mixture contained in a total volume of 3.2 mL:

0.82 mL	0.1 M phosphate buffer, pH 7.5
1.0 mL	colloidal sulfur
1.0 mL	sample
0.3 mL	catalase (10 mg/mL)
0.02 mL	dipyridyl (20 mM)
0.05 mL	GSH (0.1 M)

Reaction was carried out at 25°C.

Assay of thiosulfate and sulfite oxidation

Thiosulfate and sulfite oxidation were assayed by measuring oxygen consumption in a Gilson oxygraph in the same manner as described for sulfur oxidation. In the case of thiosulfate, the reaction mixture contained in a total volume of 1.2 mL:

0.1 mL	intact cells
1.1 mL	distilled water at pH 2.0
0.01 mL	sodium thiosulfate (63 mM)

In the case of sulfite oxidation, the reaction mixture contained in a total volume of 1.2 mL:

0.3 mL	sample
0.9 mL	assay buffer
0.01 mL	0.1 M sodium sulfite in 50 mM EDTA

When intact cells were assayed, only 0.1 mL of cells was used, the rest of the reaction volume being made up with assay buffer which consisted of 0.1 M potassium phosphate buffer at pH 5.5. In the case of extracts and enzyme preparations, either 0.1 M phosphate buffer or 50 mM Tris-Cl buffer, both at pH 7.5, were used as assay buffers. In all cases, reactions were started with addition of substrate.

Sulfite oxidase assay using ferricyanide

Assay of sulfite: ferricyanide oxidoreductase activity was carried out using a modification of the method by Charles and Suzuki (5). The reaction mixture in a 3 mL, one centimeter cuvette to a total volume of 2 mL:

1.6 mL	50 mM Tris-Cl, pH 7.5 buffer
0.2 mL	5 mM $K_3Fe(CN)_6$
0.1 mL	sample
0.1 mL	Na_2SO_3 in 50 mM EDTA

The reaction was started with the addition of sulfite. Decrease in absorbance at 420 nm due to the reduction of ferricyanide was moni-

tored against a control cuvette containing all the reaction components except for substrate.

Sulfite oxidase assay using cytochrome *c*

Assay of sulfite:cytochrome *c* oxidoreductase activity using oxidized cytochrome *c* was carried out using a modification of the method described by Charles and Suzuki (5). The reaction mixture in a 3 mL, one centimeter cuvette had a total volume of 2 mL:

0.2	mL	sample
0.1	mL	Na_2SO_3 (0.1 M) in 50 mM EDTA
1.6	mL	50 mM Tris-Cl, pH 7.5 buffer
0.1	mL	type III h. heart cytochrome *c* or *Saccharomyces cerevisiae* cytochrome *c*

The reaction was started with the addition of sulfite. Increase in absorbance at 550 nm due to reduction of cytochrome *c* was monitored against a control cuvette not containing sulfite.

Cytochrome oxidase assay

The activity of cytochrome oxidase was assayed using a modification of the method described by Oh and Suzuki (36). The reaction mixture contained in a 3 mL, one centimeter cuvette:

1.7	mL	50 mM Tris-Cl, pH 7.5 buffer
0.2	mL	sample
0.1	mL	0.8 mM red. h. heart cytochrome *c*

The reaction was initiated with the addition of reduced cytochrome *c*. Decrease in absorbance at 550 nm due to the oxidation of reduced cytochrome *c* was monitored against a control cuvette without sample.

NADH oxidase assay

Assay of NADH oxidase activity was carried out using a modification of the method of Mackler (33). The reaction mixture in a 3 mL, one centimeter cuvette had a total volume of 1.5 mL:

0.75 mL	0.1 M Tris-Cl, pH 7.5 buffer
0.45 or 0.35 mL	distilled water
0.1 or 0.2 mL	sample
0.1 mL	NADH, 1mM

The reaction was started with the addition of substrate. Decrease in absorbance at 340 nm was monitored against a blank cuvette containing all the reaction components except substrate.

Protein determination

Protein concentration was determined by a modified Lowry method of Schacterle and Pollack (42) using a phenol reagent. Bovine serum albumin was used as reference.

KDO assay

Assay of 2-keto-3-deoxyoctonic acid for the detection of lipopolysaccharide was carried out using the method of Osborn (38).

Centrifugations

Low speed centrifugation was carried out using a Sorvall superspeed RC-2B autorefrigerated centrifuge at 4°C. High speed centrifugation was carried out using a Beckman Model L, type B ultracentrifuge using a Ti 60 fixed angle rotor.

Spectrophotometry

Absorption spectra as well as time-absorbance studies were carried out using a Beckman DU-8 spectrophotometer. For routine absor-

bance measurements, a Gilford 2400 spectrophotometer was used.

Tentative molecular weight determinations

Tentative molecular weights from gel filtration chromatography experiments were estimated using selectivity product data of Sephadex G-150 from a Pharmacia Fine Chemicals Co. product guide booklet (9).

Non-denaturing polyacrylamide gel electrophoresis

Purity of the various preparations was monitored using polyacrylamide gel electrophoresis under non-denaturing conditions. A 7.5% acrylamide gel was prepared following a standard method described by Keresen (19). The gel size used was 16 by 16 cm. Gels were run on a BRL vertical gel electrophoresis system for four hours at a constant voltage of 100 volts.

SDS polyacrylamide gel electrophoresis

Fractionation of protein samples under denaturing conditions were carried out using a 12% acrylamide gel prepared according to the Laemmli buffer system (24). The gel size was also 16 by 16 cm. Gels were run on a BRL vertical gel electrophoresis system for two hours at a constant voltage of 200 volts. Subunit molecular weight standards were from a Sigma SDS Molecular weight markers Kit no. MW-SDS-70L, covering molecular weights from 14,000 to 70,000 daltons.

RESULTS

RESULTS

Sulfur oxidation by intact cells

T. thiooxidans intact cells readily oxidized sulfur at either neutral (7.5) or acidic (2.0) pH. They were also able to oxidize sulfur dissolved in dimethyl sulfoxide (DMSO-sulfur). Sulfur oxidation experiments using DMSO-sulfur showed stoichiometry where $1\frac{1}{2}$ mol of O_2 were consumed per mol of sulfur.

The oxidation of sulfur was monitored spectrophotometrically in a Beckman DU-8 spectrophotometer. The experiment consisted of placing a suspension of intact cells in the spectrophotometer, then adding a small amount of colloidal sulfur and taking a difference spectrum every few minutes until the cells became anaerobic due to respiration. The onset of sulfur oxidation was marked by the apearence of a broad peak in the 360 nm region. As the sulfur oxidation continued, and the suspension became anaerobic, flavin and cytochromes were reduced (see Figure 1). There was also a general decrease in absorption probably due to decrease in turbidity caused by the settling of the colloidal sulfur in the cuvette.

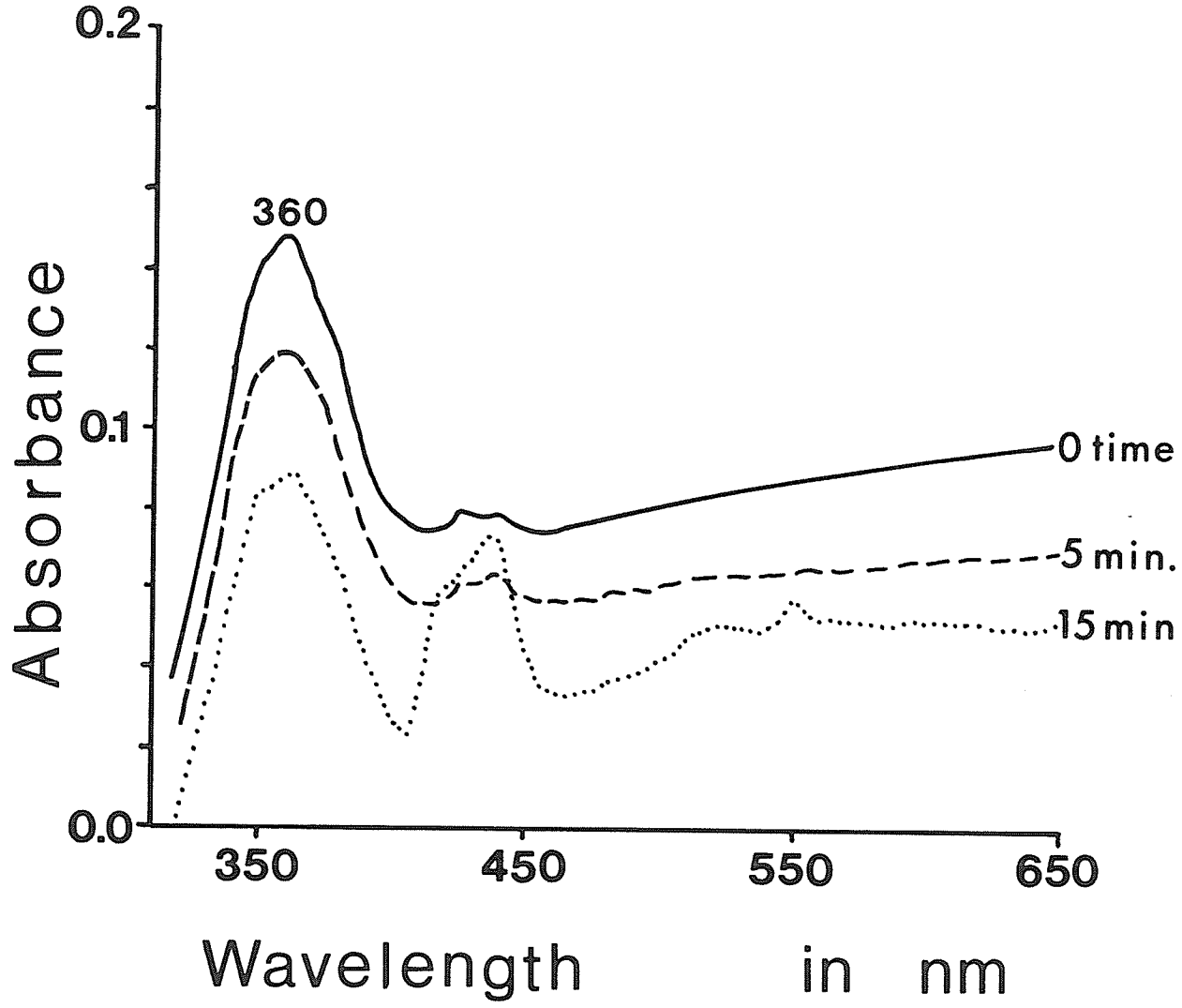
Sulfite inhibition of sulfur oxidation

Sulfite inhibited sulfur oxidation of intact cells at pH 2.0 but not at pH 7.5. Healthy cells were completely inhibited at pH 2.0 in the presence of 0.83 mM (one µmol per assay volume) sulfite, regardless of whether or not sulfur oxidation was already taking place. Addition of

FIGURE 1. Difference spectra of intact cells oxidizing colloidal sulfur in distilled water at pH 2.0.

The reaction mixture consisted of 1.2 mL of intact cells (20mg/mL) plus 15 µL of colloidal sulfur.

The time required for anaerobiosis to be achieved was determined to be 13 min (by measurement in oxygraph).


0.2
0.1
0.0
Absorbance
360
0 time
5 min.
15 min
350
450
550
650
Wavelength in nm

sulfite to cells oxidizing sulfur resulted in a rapid uptake of a small amount of oxygen and some inhibition of subsequent sulfur oxidation. The uptake of oxygen was not due to the oxidation of sulfite, since sulfite is not oxidized at this pH (28). As little as 0.08 mM (100 nmol per assay) sulfite was able to cause a small oxygen uptake of about 20 nmols oxygen (2 nmols oxygen per 10 nmols sulfite). Subsequent addition of sulfite did not induce any more short bursts of oxygen uptake, but increasingly inhibited sulfur oxidation. The presence of sulfite before the onset of sulfur oxidation did not cause the rapid oxygen uptake; sulfur oxidation had to be taking place in order for this to happen. This rapid oxygen uptake was a cell-sulfite interaction, not a sulfur-sulfite one. If cells were incubated with sulfite at pH 2.0 and then transfered to pH 7.5, the sulfur-oxidizing activity was restored.

Spectrophotometric studies revealed that addition of sulfite to intact cells at pH 2.0 resulted in the apearence of a broad peak at 360 nm, similar to the one seen at the onset of sulfur oxidation (see igure 2). In contrast to sulfur oxidation, however, there was no further spectral change with time. If sulfur or thiosulfate was added to the sample, the spectrum remained unchanged.

Thiosulfate oxidation by intact cells

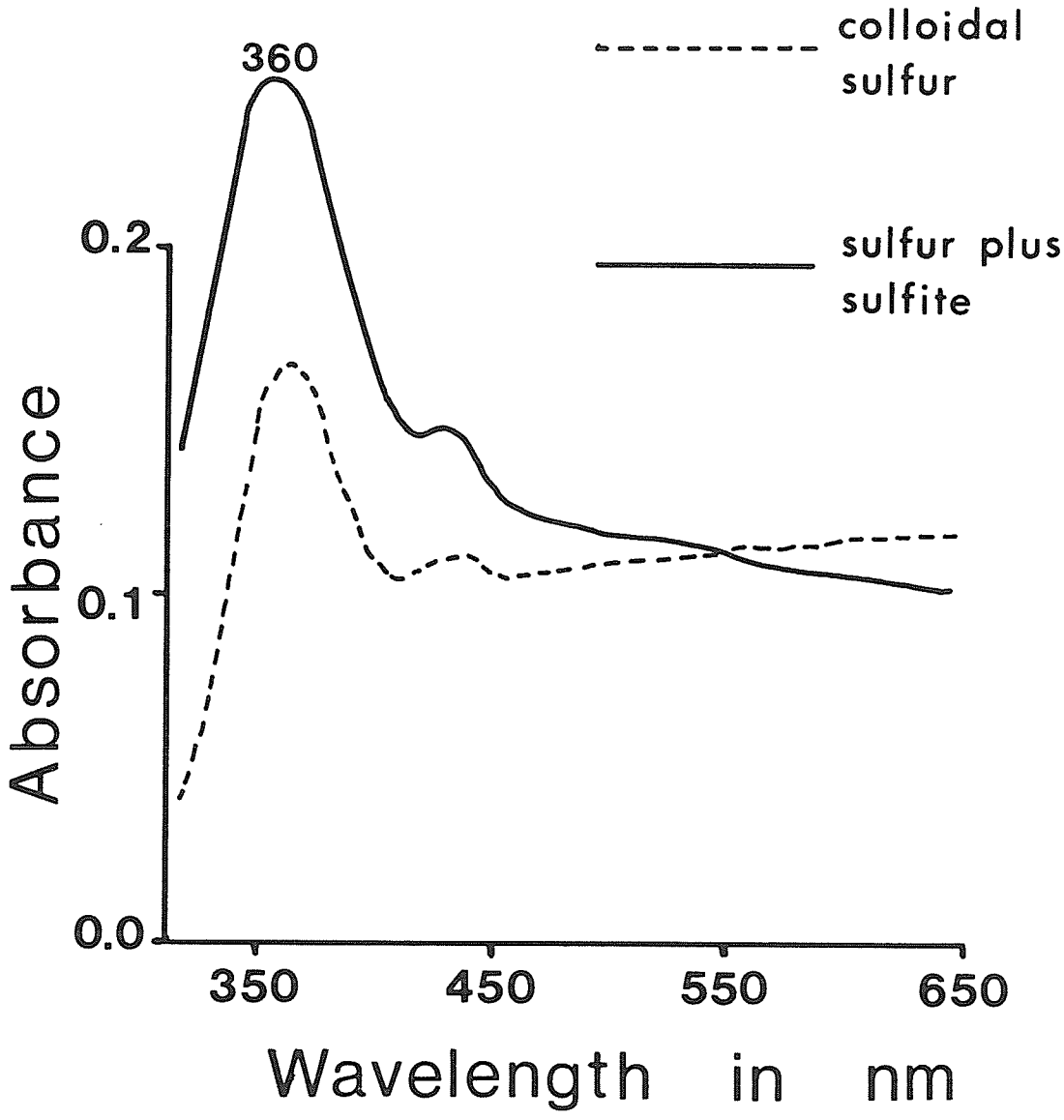
Intact cells were able to oxidize thiosulfate readily at acidic pH (2.0) but not at neutral pH (7.5). Good stoichiometry was observed in that 2 mols of molecular oxygen were consumed per mol of thiosulfate. The oxidation reaction was biphasic, with a rapid initial oxidation rate to 0.5 mols oxygen per mol thiosulfate, followed by a slower,

FIGURE 2. Difference spectra showing the effect of sulfite on colloidal sulfur oxidation by intact cells in distilled water at pH 2.0.

The reaction mixture consisted of 1.2 mL of intact cells (20 mg/mL) plus 15 µL of colloidal sulfur.

The spectrum was taken immediately upon addition of sulfur.

Sulfite was introduced by adding 1 µmol of sodium sulfite.


colloidal sulfur
sulfur plus sulfite
360
0.2
0.1
0.0
Absorbance
350
450
550
650
Wavelength in nm

semi-linear rate to completion (see Figure 3). The uptake of one oxygen atom corresponds to the oxidation of a sulfite ion. Sulfite was able to inhibit only the second phase of thiosulfate oxidation, not the first. Even if sulfite was present in the reaction mixture before thiosulfite, the first stage proceeded to completion. The spectrum of sulfite-inhibited, thiosulfate-oxidizing cells was identical to that of sulfur-oxidizing cells inhibited by sulfite. This strongly suggests that sulfite is inhibiting the sulfur-oxidation part of thiosulfate oxidation. In other words, thiosulfate oxidation probably occurs in the following manner:

$$S_2O_3^{2-} \xrightarrow{\text{rhodanese}} S^o + SO_3^{2-} \quad \text{phase 1} \downarrow$$

$$SO_3^{2-} + \tfrac{1}{2}\,O_2 \xrightarrow[\text{oxidation}]{\text{sulfite}} SO_4^{2-}$$

$$S^o + O_2 + H_2O \xrightarrow[\text{oxidation}]{\text{sulfur}} SO_3^{2-} \quad \text{inhibited by sulfite} \quad \text{phase 2} \downarrow$$

$$SO_3^{2-} + \tfrac{1}{2}\,O_2 \xrightarrow[\text{oxidation}]{\text{sulfite}} SO_4^{2-}$$

Spectrophotometric studies showed that during the thiosulfate oxidation there was no appearence of the 360 nm absorption peak, although a small extent of reduction was observed for flavins and cytochromes.

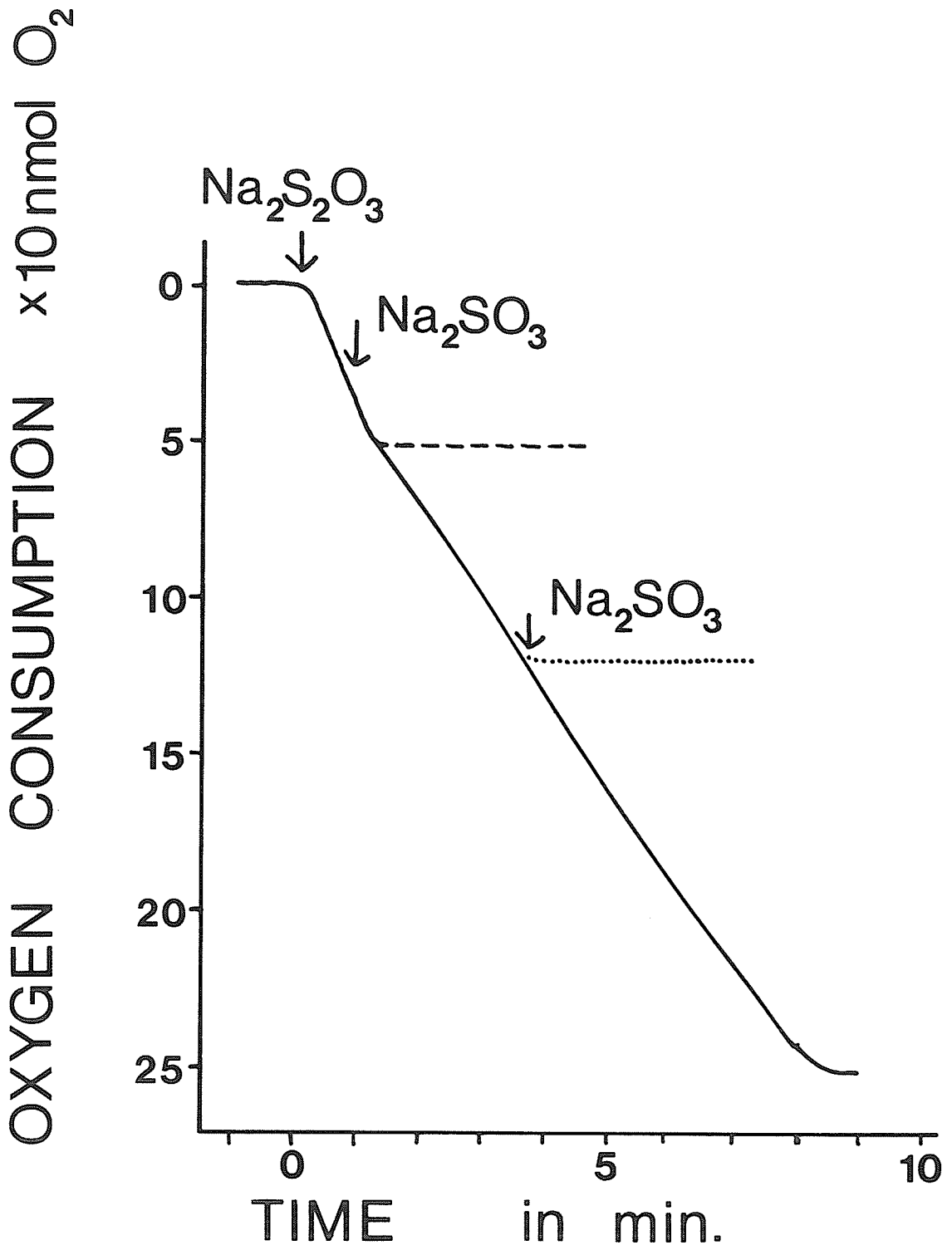
Intact cells did not oxidize thiosulfate at neutral (7.5) pH. This requirement of acidity for oxidation was on the part of the cells, not on the substrate. A thiosulfate solution was acidified by diluting with mild acid. With time, the formation of turbidity due to acid-

FIGURE 3. Biphasic oxidation of thiosulfate and the inhibition of the second stage by sulfite.

The reaction mixture consisted of 0.1 mL of cells (20mg/mL) plus 1.1 mL of distilled water at pH 2.0.

Addition of 12.5 nmols of thiosulfate resulted in the consumption of 25 nmols of oxygen (2 mols oxygen per mol thiosulfate).

Addition of 1 μ mol of sulfite inhibits only the second stage of thiosulfate oxidation, with only 0.5 nmols oxygen being consumed (0.5 nmols oxygen per mol thiosulfate).


OXYGEN CONSUMPTION x10 nmol O_2
$Na_2S_2O_3$
Na_2SO_3
Na_2SO_3
0
5
10
15
20
25
0
5
10
TIME in min.

mediated thiosulfate breakdown could be observed. If this acidified solution of thiosulfate was added to cells at neutral pH, both before and after thiosulfate breakdown could occur, no oxygen uptake could be observed.

Effect of freeze-thawing on intact cells

Freezing of intact cells destroyed ability to oxidize sulfur at acid or neutral pH. Addition of GSH did not restore the activity. Spectra of freeze-thawed cells at pH 2.0 showed that on contact with sulfur, a peak at 360 nm did appear, as with healthy cells. However, no other spectral features appeared since no anaerobiosis ensued. If the cells were frozen at pH 7.5, some lysis occurred. These partially lysed cells also showed no sulfur-oxidizing activity even in the presence of GSH. The 360 nm peak did appear upon contact with sulfur but no other features were present, as with cells frozen at pH 2.0. If the freeze-thawed cells (at acid or neutral pH) were broken in a French pressure cell, the resulting extracts showed no sulfur-oxidizing activity with or without GSH. The 360 nm peak did not appear when the extracts came in contact with sulfur.

Properties of Cell-Free Extracts

Intact cells broken by passage through French pressure cell gave extracts that were active in sulfur oxidation. The extracts were able to oxidize sulfur without GSH. However, this GSH-independent activity was very small (only 12% of the activity seen in the presence of GSH) and unstable, decreasing by 50% after only ten minutes under assay conditions. It was also very susceptible to storage, decreasing by 3/4 after only one freeze-thaw. Addition of catalytic amounts of GSH grea-

tly increased the sulfur-oxidizing activity. This GSH-stimulated activity in the crude extracts was about 20% of sulfur-oxidizing activity seen in the intact cells before breakage.

The extracts also showed a good sulfite-oxidizing activity; about 70% of sulfite-oxidizing activity seen in the intact cells before breakage was recovered in the crude extracts. Interestingly, the extracts had no ability whatsoever to oxidize thiosulfate. Even when the assay was done under acidic conditions, thiosulfate was not oxidized.

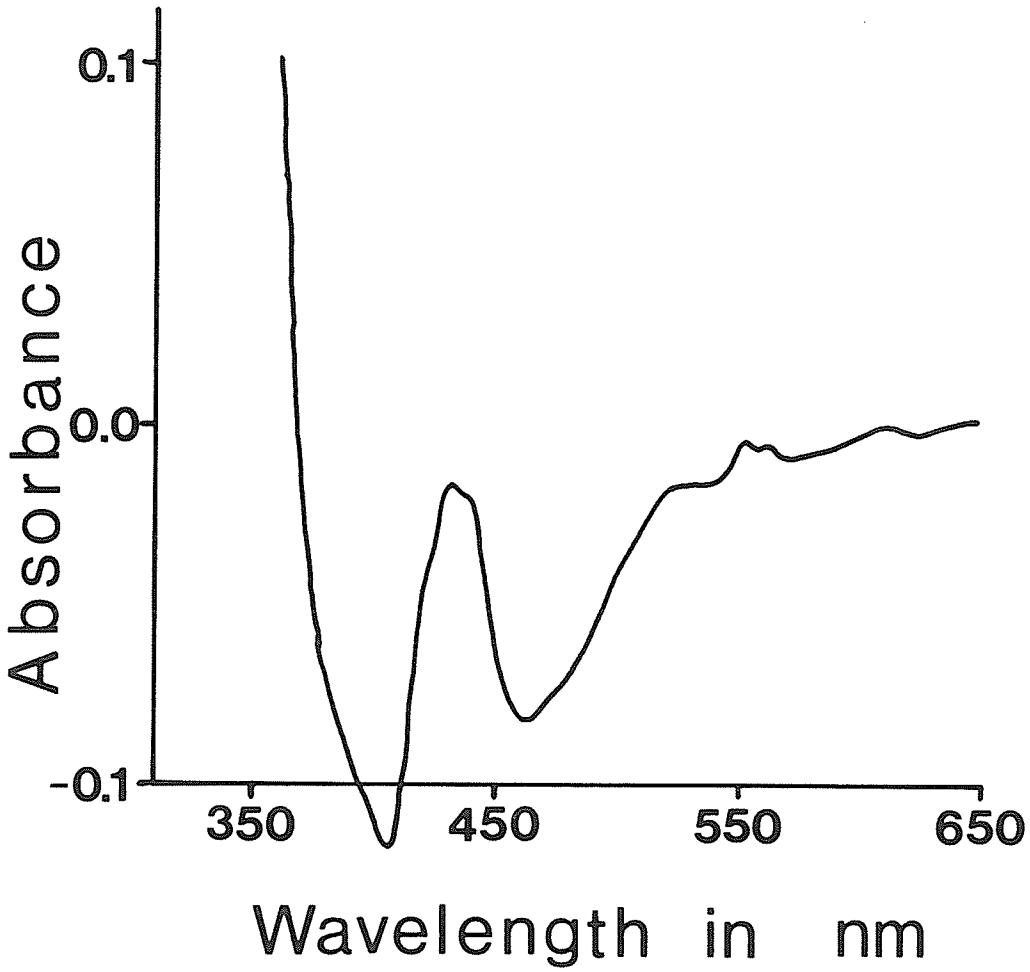
Visible spectrum of cell-free extracts were similar to those of intact cells; cytochromes *b* and *c*, as well as flavin could be seen (see Figure 4). Spectral changes due to sulfur oxidation could not be monitored due to the high turbidity caused by adding colloidal sulfur to the much less turbid extracts.

Clark Oxygen Electrode as method of assay

In the past, most published studies on sulfur oxidation have been carried out using manometric measurements with a Warburg apparatus. In these studies, the Clark oxygen electrode in an Oxygraph has been used almost exclusively, to measure the oxygen uptake. The cell-free extracts behaved identically in both. To illustrate this, the sulfur-oxidizing activity of extracts were compared by running duplicate experiments, using both methods. Assay reaction mixtures were standardized so that conditions in both would be identical. The two activities were different by only 3%, even though the typical Oxygraph assay lasted less than ten minutes while the Warburg experiment lasted about two hours.

FIGURE 4. Difference spectrum of cell-free extract in 0.1 M potassium phosphate buffer (pH 7.5).

The sample was reduced with a few grains of sodium dithionite


0.1
0.0
-0.1
Absorbance
350
450
550
650
Wavelength in nm

Effect of buffers

Extracts were normally prepared by breaking the cells in buffer at a pH of 7.5, be that Tris-Cl or potassium phosphate. In order to see which buffer was better, two batches of extract were prepared: one in 0.1 M Tris-Cl and one in 0.1 M potassium phosphate, both at pH 7.5. Both initially gave good sulfur-oxidizing activity, the one prepared in Tris buffer being slightly more active. In order to compare their stability, they were both tested for GSH-stimulated sulfur-oxidizing activity in their respective buffers in a Warburg apparatus. The results are illustrated in Figure 5. The extract prepared in phosphate buffer proved to be more stable than the one prepared in Tris buffer. The extract prepared in Tris buffer began to level off after about 30 minutes while the one in phosphate buffer remained almost linear for two hours. The phosphate buffer was selected for all subsequent experiments.

Ultracentrifugation of crude extracts

The brown-colored cell-free extract could be fractionated into a soluble (cytoplasmic) and a particulate (membrane) component by ultracentrifugation. Centrifugation at 120,000 × *g* for two hours gave a clear yellow Spinco supernatant, leaving behind a dark brown cell envelope pellet. The major part of sulfur-oxidizing activity was localized in the Spinco supernatant, while all of the sulfite-oxidizing activity was localized in the cell envelope (see Table 1). Apparently, the total sulfur-oxidizing activity, whether it be GSH dependent or or independent, increased on fractionation; the activities of the Spinco supernatant and the cell envelopes added together exceed the

FIGURE 5. Oxidation of colloidal sulfur by crude extracts prepared in 0.1 M potassium phosphate buffer (pH 7.5) or 0.1 M Tris-Cl buffer (pH 7.5).

Oxygen uptake was measured manometrically in a Warburg apparatus as described in Materials and Methods.

The reaction mixtures contained 29 mg of protein in the form of crude extract.

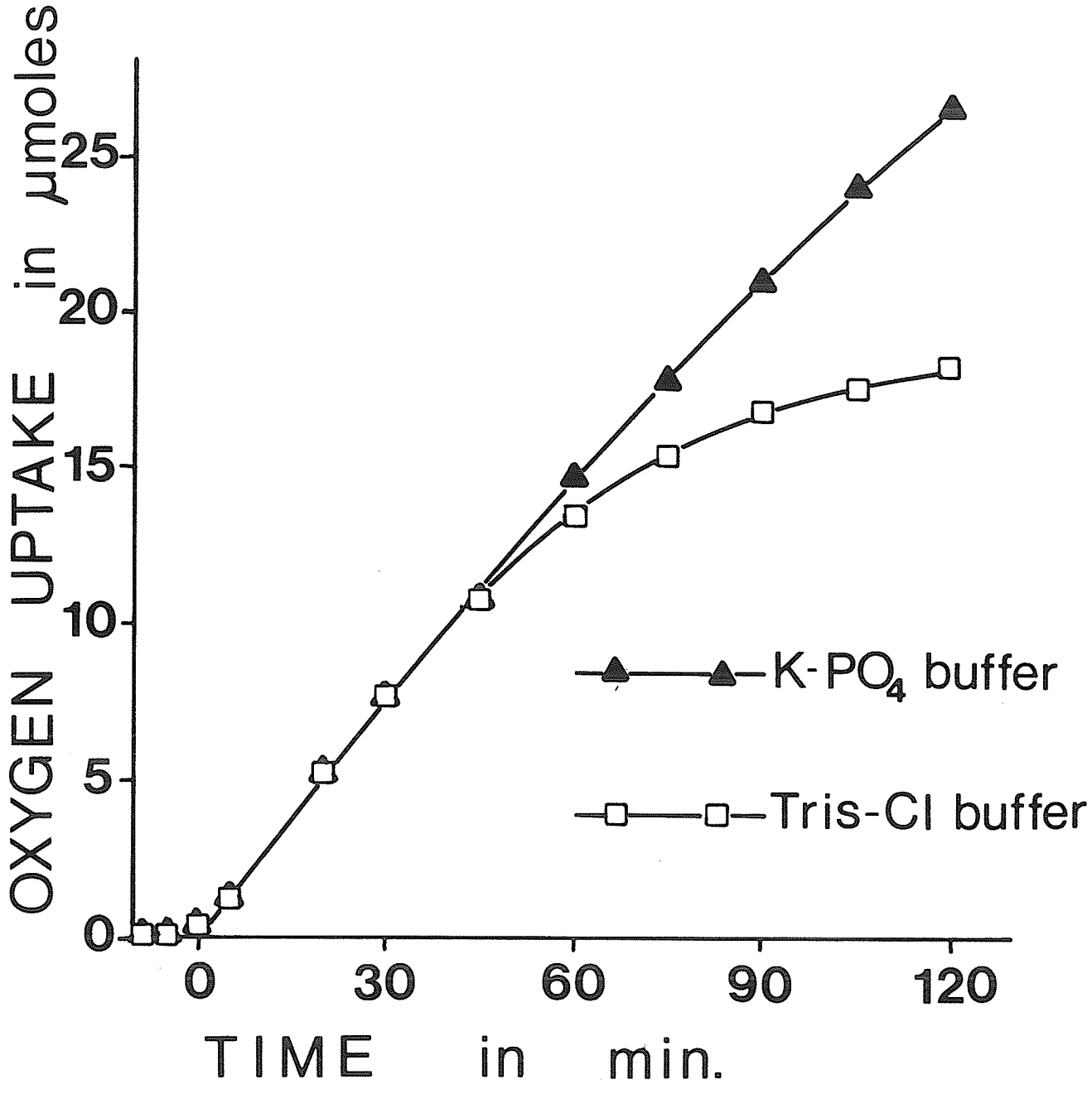

OXYGEN UPTAKE in µmoles
0
5
10
15
20
25
0
30
60
90
120
TIME in min.
K-PO4 buffer
Tris-Cl buffer

TABLE 1. Fractionation of crude extract by ultracentrifugation

Sample	Total volume (mL)	Total protein (mg)	Total sulfur oxidizing activity (nmol O_2 per min)		Total sulfite oxidizing activity (nmol O_2 per min)	Activity Distribution (%)	
			GSH	no GSH		Sulfur	Sulfite
CFE	12.5	264.	4,250	400.	10,700	100.	100.
Spinco supernatant	10.7	96.	3,470	240.	360.	82.	3.
Cell envelope	1.8	151.	1,060	200.	7,780	25.	73.

Activity was determined under the standard conditions described in the Materials and Methods. Glutathione was present at 1 µmol per 1.2 mL reaction mixture. Potassium phosphate was used as the assay buffer.

activity found in the crude extract. The sulfur-oxidizing activity of the cell envelopes appeared to be stimulated by GSH to a lesser extent than that of the Spinco supernatant. In the Spinco supernatant, GSH increased activity by 14 times compared to only five times in the cell envelopes.

Most of the protein found in the cell-free extract was precipitated with the cell envelopes. This ultracentrifugation step appeared to be very satisfactory for separating the sulfur from sulfite oxidizing activities. If the cell envelopes were homogenized and centrifuged for a second time, the resulting supernatant was also active for sulfur, but not sulfite, oxidation. If the two supernatants were pooled, the recovery of sulfur-oxidizing activities was greatly improved, as shown on Table 3.

Ammonium sulfate fractionation

The Spinco supernatant, which contained the major part of sulfur-oxidizing activity was fractionated using ammonium sulfate. A preliminary experiment showed that most of the protein precipitated at an ammonium sulfate concentration of 50% saturation, leaving most of the sulfur-oxidizing activity in the supernatant. The activity could be precipitated by increasing the ammonium sulfate concentration to 75% saturation. In order to deduce the optimum fractionation procedure, a fractionation profile of the Spinco supernatant was carried out. A serial ammonium sulfate precipitation from 25% to 85% saturation, increasing in steps of 10%, was done. Each precipitate was resuspended in 50 mM Tris-Cl to the original volume and assayed for sulfur-oxidizing activity using the same buffer. Visible spectra of each fraction

was also taken. The results are summarized in Table 2. Most of the activity was found in fractions precipitated between 45% and 75% ammonium sulfate saturation. It is interesting to note that the flavin content of the Spinco supernatant was precipitated early in the fractionation. At the same time, cytochrome *c* was present in varying amounts throughout the fractionation profile.

By doing a serial fractionation of 25%, 50%, 75%, and 100% ammonium sulfate saturation, it was possible to derive four distinct fractions: a 25% fraction with little activity or color; a 50% fraction containing the flavin and some sulfur-oxidizing activity; a 75% fraction containing most of the sulfur-oxidizing activity and some cytochrome *c*; and a 100% fraction with little activity, containing cytochrome *c*. The difference spectra of these four fractions can be seen on Figure 6. Isolation profile of the two fractions with activity is shown on Table 3. Similar profiles were obtained in six separate runs.

Effect of trypsin treatment

In the past, cell-free extracts of *T. thiooxidans* have been prepared by treating intact cells with trypsin prior to breakage by sonication (2). The effect of a trypsin treatment prior to breakage by French pressure cell was examined. A suspension of intact cells was partially digested with trypsin for 15 minutes prior to cell breakage by the usual procedure. Sulfur-, sulfite-, and thiosulfate-oxidizing activities were monitored for the cells, cells after trypsin treatment, and the resulting crude extract. The results are summarized in Table 4. Upon trypsin treatment, the cells immediately lost 71% of sulfur, 43% of sulfite, and 68% of thiosulfate-oxidizing activities. The treated

TABLE 2. Ammonium sulfate fractionation profile of the pinco supernatant.

Sample	Total activity (nmol O_2 per min)	Activity distribution (%)	[a]Presence of flavin	[a]Presence of cyt. c
CFE	3,670	100	+	+
Spinco supernatant	1,990	54	+	+
25% precipitate	115	3	-	+
35% precipitate	117	3	+	+
45% precipitate	103	3	+	+
55% precipitate	205	6	+	+
65% precipitate	527	14	-	+
75% precipitate	319	9	-	+
85% precipitate	60	2	-	+

[a]Presence of flavin or cytochrome *c* determined by taking the difference spectrum of each preparation.

FIGURE 6. Difference spectra of the ammonium sulfate fractions in 50 mM Tris-Cl buffer (pH 7.5).

The samples were reduced with a few grains of sodium dithionite.

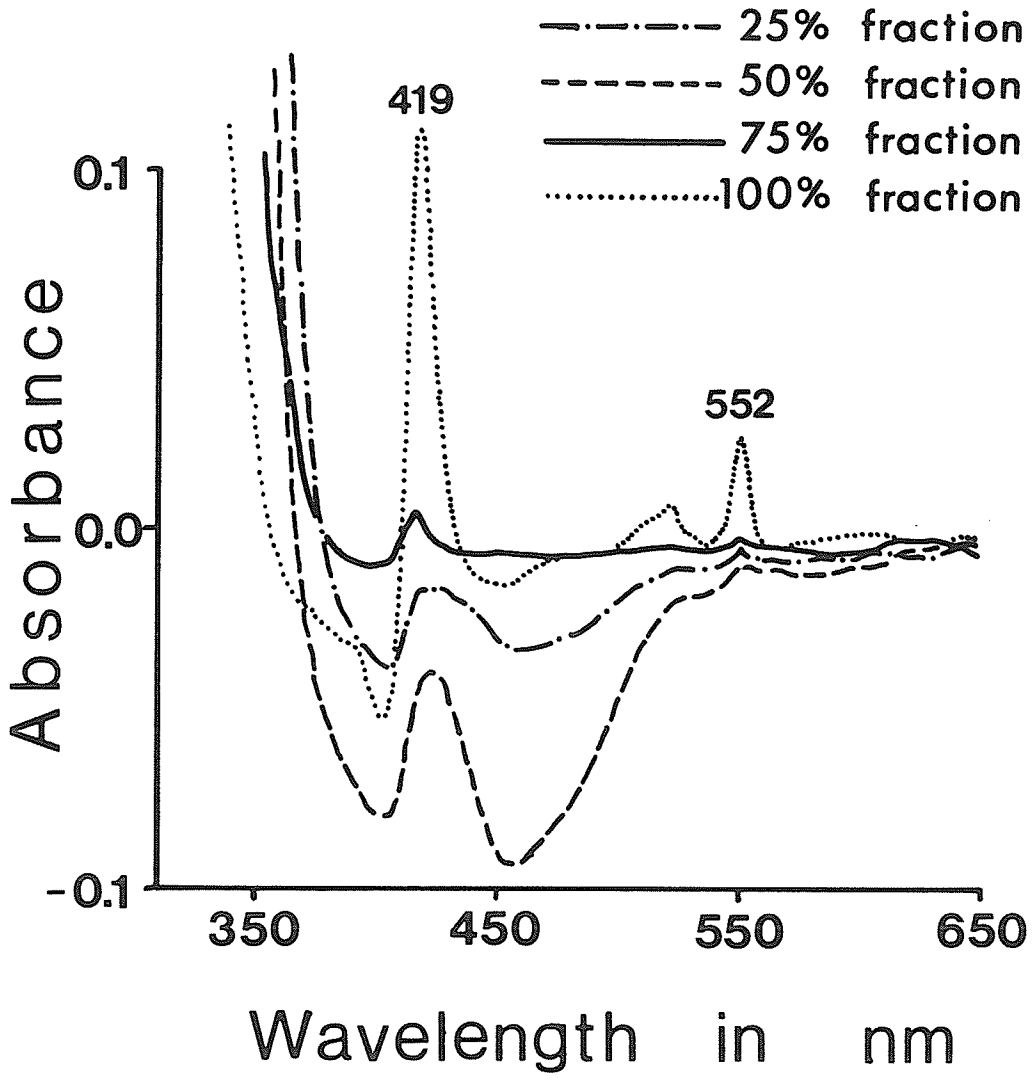

25% fraction
50% fraction
75% fraction
100% fraction
419
552
0.1
0.0
-0.1
Absorbance
350
450
550
650
Wavelength in nm

TABLE 3. Isolation profile of the sulfur-oxidizing enzyme

Sample	Total volume (mL)	Total activity (nmol O_2 per min)	Total protein (mg)	Specific activity (nmol O_2 per min per mg protein)	Activity distribution (%)	Purif. fold
CFE	71.0	38,100	2,520	15.1	100.	1.0
Spinco supernatant	99.0	44,200	874	50.6	116.	3.4
50% fraction	8.3	8,570	-	-	23.	-
75% fraction	7.5	15,200	211.	72.	40.	4.8

cells themselves became less turbid and took on the apearence of having undergone lysis. The extracts derived from these treated cells were bright yellow, as opposed to light brown for normal extracts. These extracts retained almost 40% of the initial untreated intact cell sulfur-oxidizing activity, almost double that of extracts derived from untreated cells. Similarly, almost all of the sulfite-oxidizing activity was recovered in the extracts, as compared to about 70% in extracts prepared in the usual manner. Most strikingly, when assayed at acidic pH, some thiosulfate-oxidizing activity was recovered (about one quarter that of intact cells). Usually, no thiosulfate-oxidizing activity was seen in crude extracts.

The resulting extracts were used for a crude isolation of the sulfur-oxidizing enzyme, including single step ultracentrifugation and ammonium sulfate fractionation. The results are summarized in Table 5. Upon centrifugation, more than 90% of the activity was distributed to the Spinco supernatant. This value was only slightly above the 80% mark for the extracts derived from untreated cells that underwent a single step ultracentrifugation. Upon ammonium sulfate fractionation, however, most of the sulfur-oxidizing activity was localized in the 50%, flavin containing fraction. About one third of the original crude extract activity was localized in this fraction, while only 12% of the crude extract activity was found in the 75% fraction. The amount of activity contained in the trypsin-treated 50% fraction then, was similar and not higher than that of the 75% fraction of the usual extracts.

TABLE 4. Effect of Trypsin treatment prior to cell breakage.

Sample	[a]Sulfur-oxidizing activity	[a]Thiosulfate-oxidizing activity	[a]Sulfite-oxidizing activity	Activity distribution (%)		
				Sulfur	Thio.	Sulfite
Intact cells	146.	132.	184.	100.	100.	100.
Trypsin-treated cells	42.	42.	104.	29.	32.	57.
Cell-free extract	54.	32.	180.	37.	24.	98.

[a]Activity expressed as nmol O_2 consumed per minute per mL of sample.

TABLE 5. Isolation of sulfur-oxidizing enzyme from trypsin-treated CFE.

Sample	Total sulfur-oxidizing activity (nmol O_2 per min)	Activity distribution (%)
Trypsin-treated CFE	5,040	100
Spinco supernatant	4,660	92
50% fraction	1,510	30
75% fraction	600	12

Effect of Dithioeryrhritol on enzyme isolation

There is a strong likelihood that the sulfur-oxidizing enzyme may involve sulfhydryl groups which during the isolation and purification procedure may be oxidized to form disulfides. An experiment was carried out in order to see if the sulfur-oxidizing enzyme could be stabilized by being kept in the reduced state with a thiol-protecting compound throughout the isolation procedure. Two batches of cell-free extracts were prepared: one was used as control, while to the other one was added 1 mg dithioerythritol (DTE) per mL of extract (6). They were then both ultracentrifuged once to obtain Spinco supernatants for ammonium sulfate fractionation. The various preparations were assayed for sulfur-oxidizing activity and protein content. From the results summarized in Table 6, there does not appear to be any improvement on the recovery of activity, nor on the degree of purification, upon isolation of the enzyme. Enzyme purification actually appears to be better in the control samples. The DTE-containing 75% fraction had a vivid pink color, much more so than its non-DTE containing counterpart, indicating the higher degree of reducing conditions in the preparation.

Ion exchange chromatography

Ion exchange chromatography was carried out for both the 50% and the 75% fractions. Prior to the experiment, both fractions were dialyzed for two hours against 50 mM Tris-Cl buffer in order to remove the residual ammonium sulfate and provide the proper counter-ion for an anionic exchange column. In both fractions, the enzyme was found to stick well to DEAE-Sephadex beads equilibrated with 50 mM Tris-Cl, pH 7.5 buffer. In the case of the 75% fraction, a brownish band remained

TABLE 6. Isolation profile of enzyme from crude extract with and without dithioerythritol (DTE).

Sample	Total sulfur-oxidizing activity ($nmol\ O_2$ per min)	Total protein (mg)	Specific activity ($nmol\ O_2$ per min per mg protein)	Activity distribution (%)	Purific. fold
with DTE					
Crude extract	5,310	656.	8.1	100.	1.0
Spinco supernatant	3,930	223.	17.6	74.	2.2
50% fraction	1,130	53.	21.3	21.	2.6
75% fraction	1,420	29.	48.8	27.	6.0
without DTE					
Crude extract	5,100	525.	9.7	100.	1.0
Spinco supernatant	4,010	187.	21.4	79.	2.2
50% fraction	1,010	52.	19.4	20.	2.0
75% fraction	1,390	19.	73.3	27.	7.6

stuck to the top of the bed while a pink-yellow band eluted with the wash. This wash contained a *c*-type cytochrome. Stepwise elution with increasing ionic strength revealed that some activity started to elute at 0.2 M NaCl, with most of the activity coming down as late as 0.35 M. As a result, following purification procedures involved thoroughly washing with the equilibrating buffer before pushing activity out with 0.35 M NaCl in the equilibrating buffer.

In the case of the 50% fraction, no visible protein eluted with the buffer wash; only a brownish-yellow band stuck tightly to the top of the bed. Stepwise elution with increasing ionic strength gave similar results as with the 75% fraction, therefore the activity was also eluted with 0.35 M NaCl after a thorough wash with equilibrating buffer.

The results from the ion-exchange chromatography experiments of the two fractions are outlined on Table 7. Slightly less than one third of the activity was lost in this purification step. However, the purification achieved was very slight. The enzyme did not behave as a single entity during chromatography; instead of eluting in a narrow range, it was dissociated from the ion-exchange medium over a wide ionic strength. In the case of the 50% fraction, loss of activity coupled to low removal of unwanted protein actually decreased the degree of purification. The active eluates themselves were very different from each other. The eluate derived from the 75% fraction contained *c*-type cytochrome, while the one derived from the 50% fraction contained flavin but no cytochrome. These are likely to be impurities, given the fact that they are mutually exclusive in the two prepara-

TABLE 7. Purification profile of enzyme on the ion-exchange chromatography of ammonium sulfate fractions.

Sample	Total activity (nmol O_2 per min)	Total protein (mg)	Specific activity (nmol O_2 per min per mg protein)	Activity distribution (%)	Purific. fold
a) 75% fraction					
75% fraction	1,760	46.	38.2	100.	1.0
DEAE prep.	1,210	28.	43.4	69.	1.1
b) 50% fraction					
50% fraction	1,320	67.	19.6	100.	1.0
DEAE prep.	954.	56.	17.0	72.	0.9

tions. The spectra of these are illustrated in igure 7. The results were quite reproducible.

Gel filtration chromatography

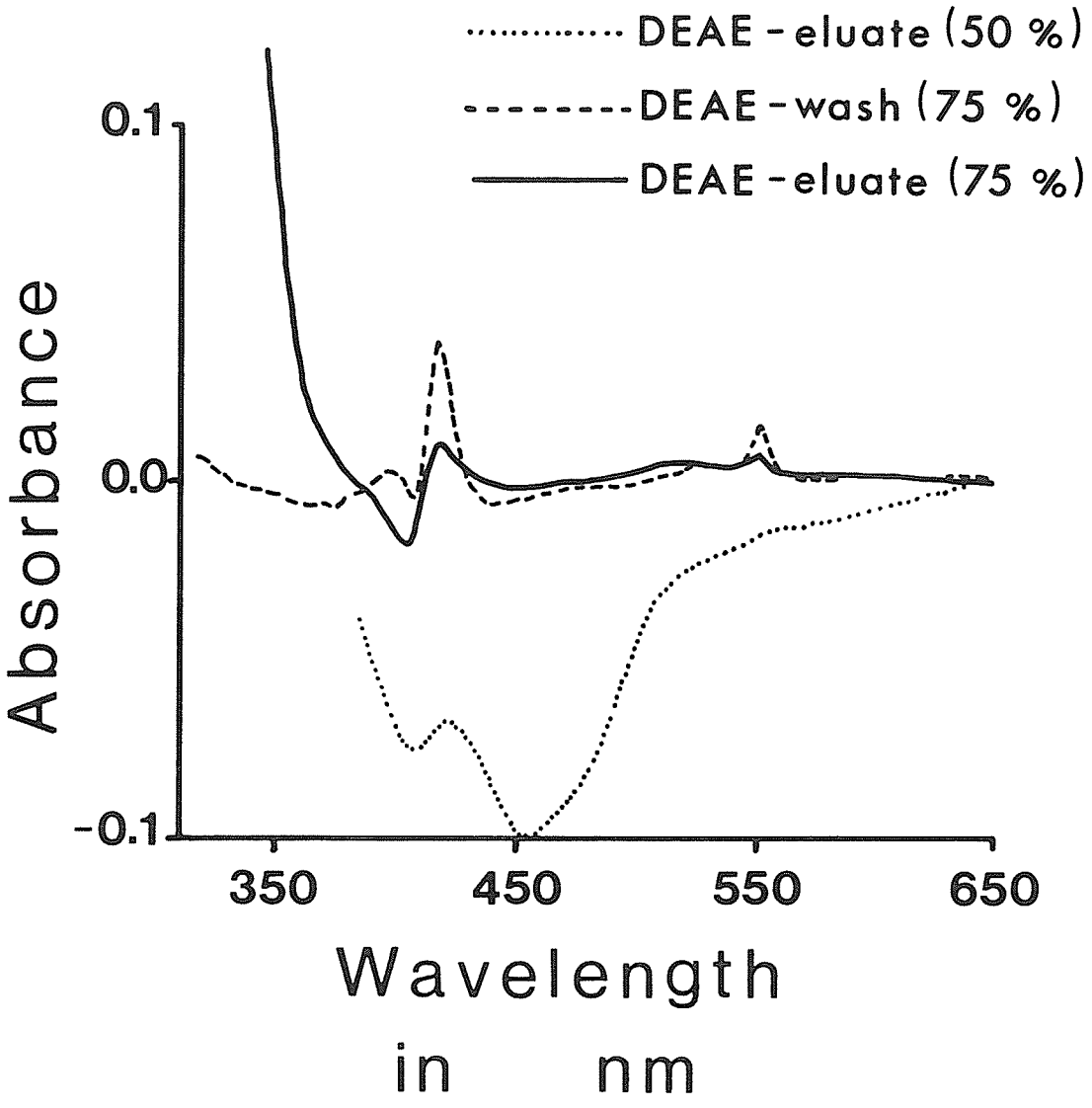
Further purification of the active preparations was attempted on the basis of molecular weight separation using gel filtration chromatography. The enzyme behaved as having a very large molecular weight. When the ion-exchange preparations were passed through a Sephadex G-100 column, activity started eluting within the void volume and was spread out over more than a quarter of the bed volume. Following experiments were carried out using Sephadex G-150 beads. With these columns, activity no longer eluted in the void volume. However, activity did not elute as a single protein peak; rather, it spread out over almost the entire bed. After several trials, using columns ranging in size from 35 to 200 mL bed volume, it became clear that separation on the basis of molecular weight would not be possible.

Membrane fractionation by sucrose density gradient

An attempt was made to separate the inner and outer membranes by using an isopycnic sucrose density gradient centrifugation step. For this procedure, the crude cell envelopes (one mL of cell envelope was derived from approximatly two mL of CFE) derived from the ultracentrifugation of crude extracts were used. Because these envelopes were ultimately derived from intact cells broken by French pressure cell, a gentle form of breakage, there was a good possibility that inner and outer membrane fragments were still bound together by peptidoglycan. As a result, the peptidoglycan had to be digested away in order to ensure proper separation of the membranes.

FIGURE 7. Difference spectra of DEAE preparations from the ammonium sulfate fractions that contained sulfur-oxidizing activity in 50 mM Tris-Cl buffer (pH 7.5).

The samples were reduced with a few grains of sodium dithionite.


DEAE - eluate (50 %)
DEAE - wash (75 %)
DEAE - eluate (75 %)
0.1
0.0
-0.1
Absorbance
350
450
550
650
Wavelength
in nm

In the past, *T. thiooxidans* cells have been reported to be insensitive to digestion by lysozyme (34). The enzyme β-glucuronidase from snail gut juice has been used on intact cells, giving spheroplasts which are acid stable and retain sulfur-oxidizing activity (34). Samples of crude extracts and cell envelopes were tested for any effects β-glucuronidase may have on them before using this step in the fractionation procedure. Duplicate samples of crude extract and cell envelopes were incubated for two hours at 30°C: one of each of the samples in the presence of β-glucuronidase. After incubation, each of the test and control samples were tested for sulfur and sulfite-oxidizing activities (see Table 8). Comparing the results, it was found that there was only a very slight difference between the test and control samples. Digestion with β-glucuronidase then, had only a minimal effect on enzyme activity and could be used in the fractionation procedure without any problem.

A density gradient centrifugation experiment was set up using both digested and undigested cell envelopes. For the digested sample, a density gradient of 25, 30, 35, 40, 45, and 50% (w/w) sucrose content was used. It was beleived that the undigested sample would not separate into two membrane bands with different densities, but migrate through the gradient as one large homogeneous band consisting of cell envelope vesicles as seen on studies with *T. ferrooxidans* cell envelopes (3). As a result, a denser linear density gradient of 40, 45, 50, 55, 60, and 65% (w/w) sucrose content was used for the undigested cell envelopes. The envelopes were carefully layered on top of the gradients and centrifuged for two hours at 120,000 × *g*. At the end of the run,

TABLE 8. Effect of β-glucuronidase treatment on sulfur and sulfite oxidizing activities.

Sample	Sulfur-oxidizing activity (nmol O_2 per min)	Sulfite-oxidizing activity (nmol O_2 per min)
Undigested crude extract	229	920
[a]Digested crude extract	195	880
% difference	15	4
Undigested crude membrane	35	[c]instantaneous
[b]Digested crude membrane	41	[c]instantaneous
% difference	15	-

[a]Digestion of crude extract involved the addition of 50 µL of snail gut juice (β-glucuronidase crude solution, 94,600 units/mL, Sigma Chemical Co.) to one mL of crude extract and incubation at 28°C for 2 h. (CFE = 200 mg wet wt. cells / mL)

[b]Digestion of crude membrane involved the addition of 0.25 g of β-glucuronidase (607000 units/g, Sigma Chemical Co.) to a 10 mL sample of crude membrane (0.16 g wet weight membrane/ mL , 28 mg protein/ mL) and incubation at 30°C for 2 h.

[c]Instantaneous activity: oxygen uptake was too fast to be accurately recorded by the apparatus being used.

two distinct bands could be seen on the tubes containing the digested cell envelopes. A heavy band could be seen at the 45-50% interface, while a lighter band could be seen just below the 40-45% interface. The tubes containing the undigested cell envelopes appeared to have a single band. On close examination, however, there turned out to be really two bands with a turbid zone in between. The heavy band was just below the 45-50% interface while the light band was just below the 40-45% interface (see Figure 8).

Each sucrose density gradient tube contained 38 mL of gradient. One tube each from the digested and undigested envelope gradients were put through a Beckman Fraction Recovery System, fractionating the gradient into one mL fractions. Each fraction was then assayed for protein content and sulfite-oxidizing activity. As a result, it was then possible to come up with a gradient profile for both digested and undigested cell envelopes (see Figures 9 and 10). There was a remarkable similarity between the digested and undigested cell envelope gradients. In both cases, two major protein peaks appeared, corresponding to the heavy and light bands seen on the gradient. The sulfite-oxidizing activity also peaked at each of the two protein peaks. Near the top of the gradients, some protein and sulfite-oxidizing activity was seen. This corresponded to some low density material which did not enter the gradient and appeared as a small yellowish band at the top of the tube. No sulfur-oxidizing activity could be detected in any of the fractions being tested.

Using the information from these preliminary experiments, it was possible to obtain the heavy and light bands in greater quantities

FIGURE 8. Location of membrane bands on sucrose density gradient tubes after fractionation.

The digested sample consisted of cell envelope preparation digested with β-glucuronidase as described in the Materials and Methods.

The undigested sample consisted of cell envelope preparation as a control.

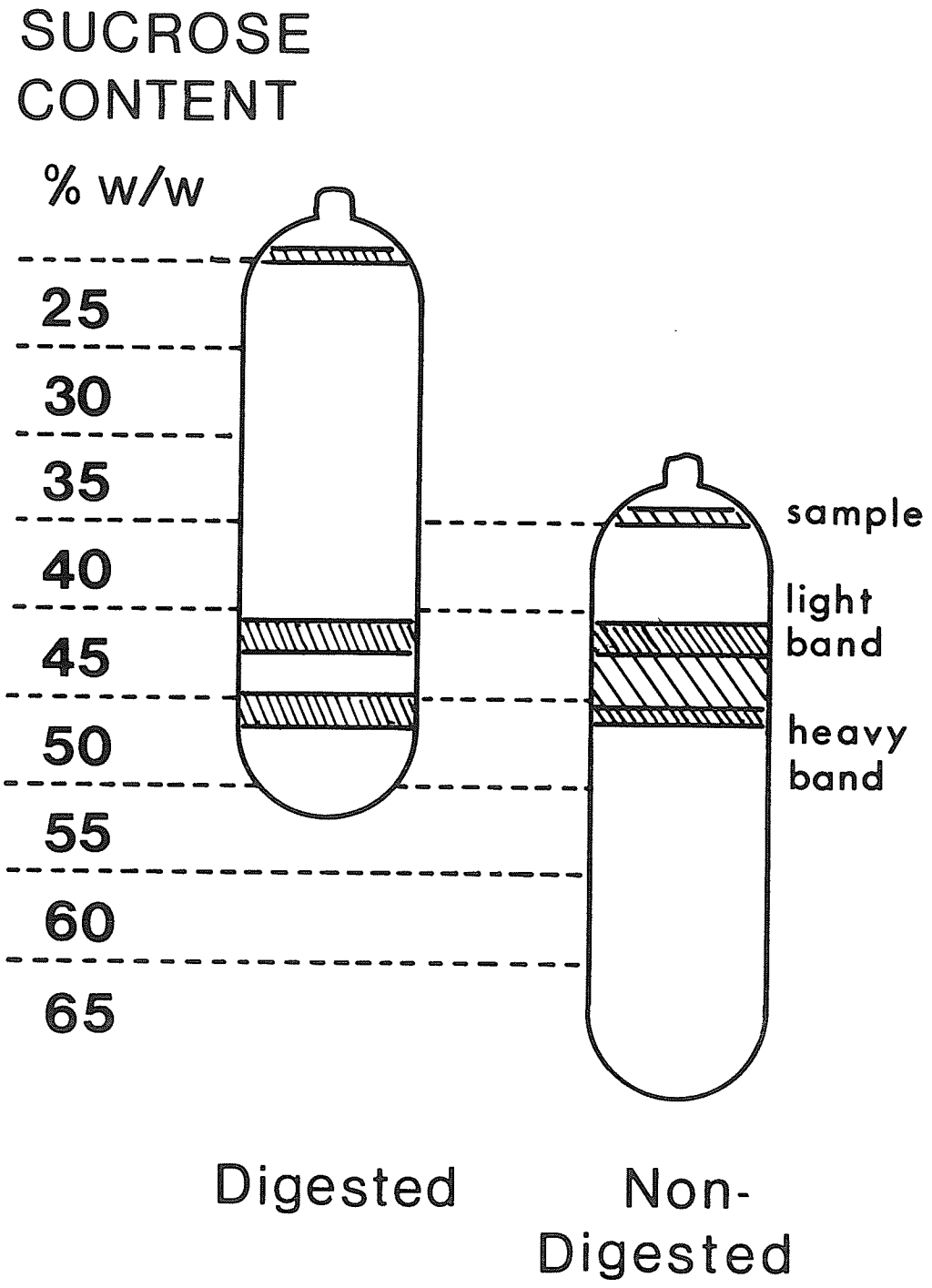

SUCROSE CONTENT
% w/w
25
30
35
40
45
50
55
60
65
sample
light band
heavy band
Digested
Non-Digested

FIGURE 9. Sucrose density gradient profile of untreated cell envelope sample.

The sucrose density gradient ultracentrifugation tube was fractionated into 1 mL fractions as described in the Materials and Methods.

The individual fractions were assayed for sulfite-oxidizing activity and protein content.

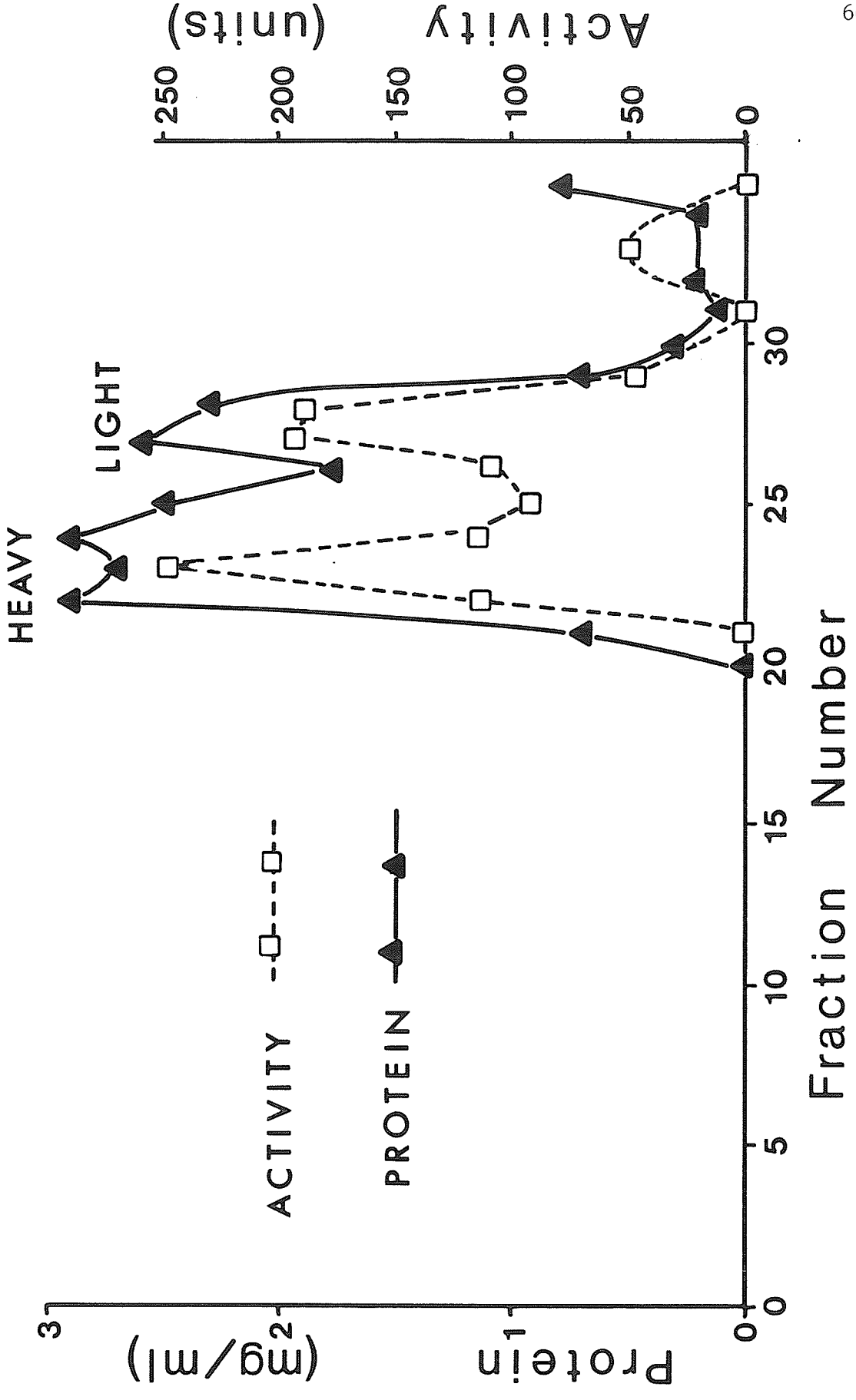
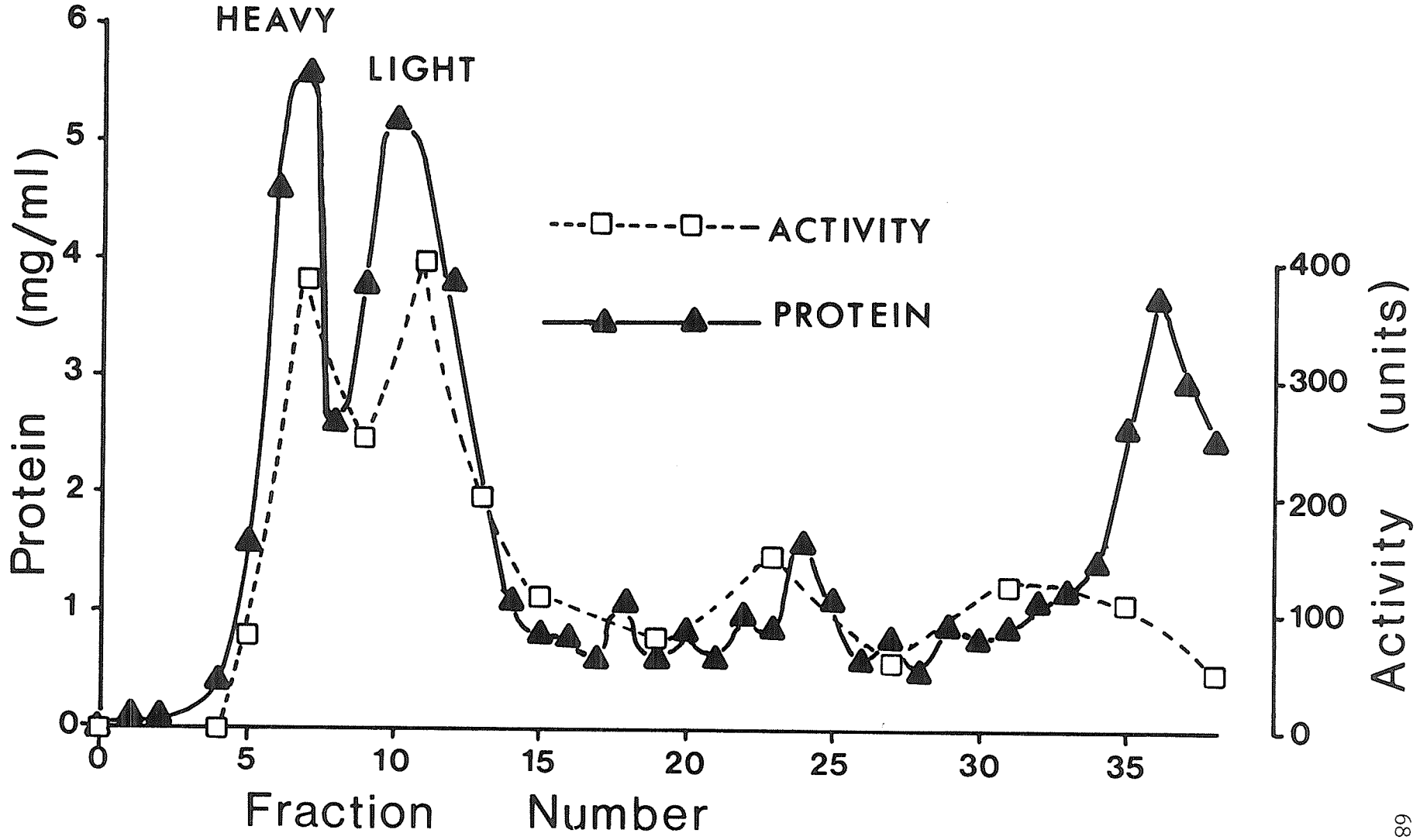

Activity (units)
250
200
150
100
50
0
HEAVY
LIGHT
ACTIVITY
PROTEIN
Protein (mg/ml)
3
2
1
0
0
5
10
15
20
25
30
Fraction Number

FIGURE 10. Sucrose density gradient profile of β-glucuronidase-treated cell envelope sample.

The sucrose density gradient centrifugation tube was fractionated into 1 mL fractions as described in the Materials and Methods.

The individual fractions were assayed for sulfite-oxidizing activity and protein content.


HEAVY
LIGHT
ACTIVITY
PROTEIN
Protein (mg/ml)
Activity (units)
Fraction Number
0
1
2
3
4
5
6
100
200
300
400
5
10
15
20
25
30
35

while at the same time simplifying the procedure. Duplicate gradients for digested and undigested cell envelopes were set up. This time the gradients were made up of 40, 45, and 50% (w/w) sucrose. As before, the envelopes were carefully layered on top of the gradients and then centrifuged at 120,000 × *g* for two hours, however, the envelopes had not migrated past the the 40% sucrose region. The tubes had to be centrifuged a further two hours, at which time the two distinct bands on all samples could be seen. The heavy and light bands for the respective samples were collected using a pasteur pipette. The collected samples were then diluted with distilled water in order to decrease sample density, and pelleted at 120,000 × *g* for 16 hours. The resulting pellets were resuspended with membrane storage buffer and used for further study. In both digested and undigested samples, there was very little heavy band pellet compared to light band pellet.

A number of assays were carried out on the different bands in order to determine whether the fractionation experiment had been successful in separating the inner and outer membranes. The bands were assayed for protein content as well as sulfur-, and sulfite-oxidizing activities. In order to identify the inner membrane, a NADH oxidase assay was used, whereas to identify the outer membrane, a KDO assay was used. A summary of the findings are illustrated in Table 9. In both digested and non-digested samples, over 90% of the protein was distributed to the light band. It was also in the light band where more than 90% of the sulfite-oxidizing activity was found. No sulfur-oxidizing activity could be found either in the heavy or light band of either sample. The NADH oxidase activity indicated that the light bands

TABLE 9. Trial purification of sulfite oxidizing system by membrane fractionation.

Sample	Total activity ($nmol\ O_2$ per min)	Total protein (mg)	Specific activity ($nmol\ O_2$ per min per mg protein)	Activity distribution (%)	Purification fold
Crude extract	35,300	331.	107.	100.	1.0
Spinco supernatant	488.	127.	4.	1.	-
Cell envelope	24,000	102.	235.	68.	2.2
Digested membrane	30,700	103.	298.	87.	2.8
Light band	10,400	92.	113.	30.	1.1

from both the digested and undigested cell envelopes at least contained the inner membrane. The heavy band could not be positively identified as the outer membrane because the KDO assay was unsuccessful in all the trials.

In order to determine if there really had been any separation of the inner and outer membranes in the density gradient centrifugation, all four membrane samples were run under denaturing conditions on SDS-polyacrylamide gel electrophoresis. On examination of the polypeptide band patterns (see Figure 11), there was very little difference between the light and heavy bands of both the digested and undigested samples. In both cases, a few polypeptides were missing from the heavy band; but there were no unique polypeptides in the heavy membrane band which could distinguish them as representing the outer membrane.

Based on the findings from the membrane fractionation experiments, a partial purification of the sulfite-oxidizing system was attempted. An eight mL sample of cell envelopes digested with β-glucuronidase was fractionated by sucrose density gradient centrifugation. The light bands from the various tubes were collected, pelleted, and resuspended in membrane storage buffer before protein and activity assays were carried out. The overall results for this purification procedure are outlined in Table 10. Although almost one third of total original sulfite-oxidizing activity does end up in the light membrane fraction, purification is not improved by this membrane fractionation procedure.

Membrane fractionation by mild-detergent solubilization

The use of surfactants for the selective solubilization of lipid bilayers and cell membranes has been widespread recently (14). Thus,

FIGURE 11. SDS-polyacrylamide gel electrophoresis of sucrose density gradient fractions.

Lane 1 : Heavy band from untreated sample (12.6 µg protein)
2 : Light band from untreated sample (11.6 µg protein)
3 : Crude membrane/cell envelope (16.8 µg protein)
4 : Heavy band from digested sample (12.6 µg protein)
5 : Light band from digested sample (14.8 µg protein)

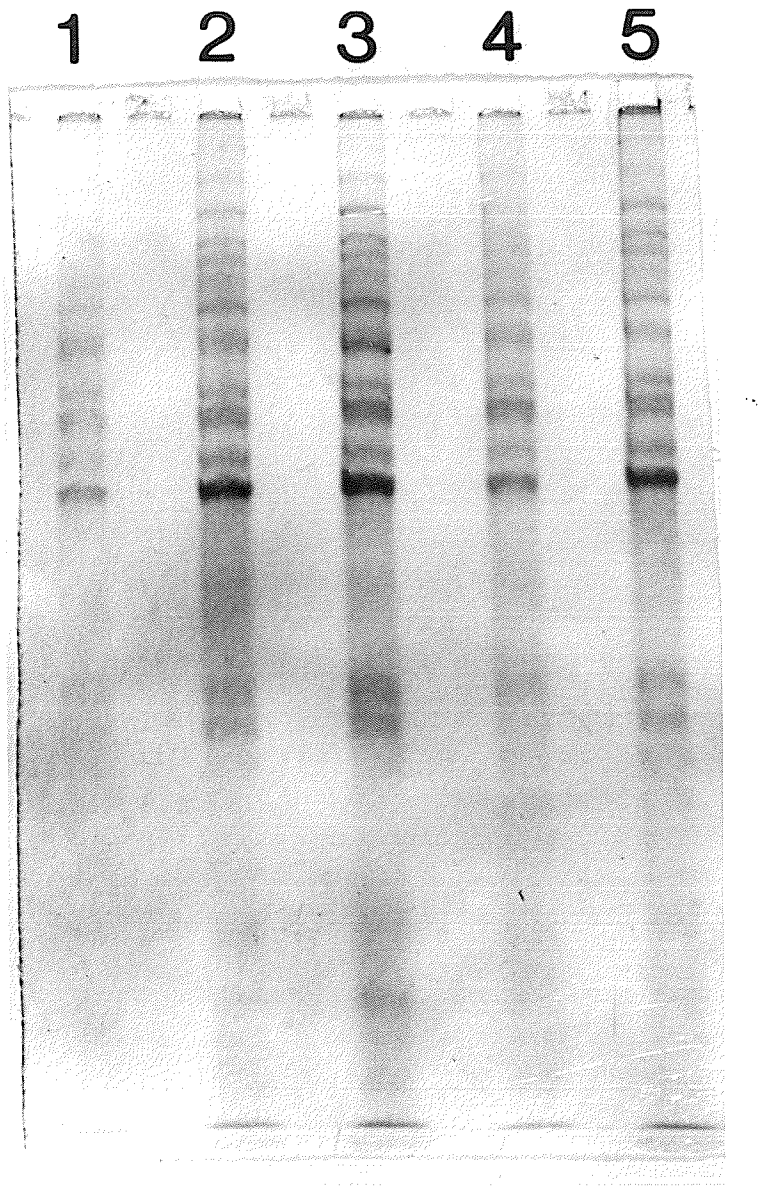

1
2
3
4
5

TABLE 10. Characterization of light and heavy bands from sucrose density gradient centrifugation.

	Cell envelope digested with β-glucuronidase		Undigested cell envelopes	
	Light band	Heavy band	Light band	Heavy band
Location in gradient	40-45%	45-50%	40-45%	45-50%
Density (g/cm^3)	1.21	1.24	1.21	1.24
Total protein (mg)	78.4	6.3	61.6	6.3
Sulfite oxidation total activity	8,680	285	6,440	177
Sulfite oxidation specific activity	110.7	45.2	104.5	28.1
NADH oxidase specific activity	2.73	0	2.73	0
KDO content	-	-	-	-
Protein distribution (%)	93		91	9
Sulfite oxidation activity distribution (%)	97	3	97	3

Sulfite oxidation specific activity expressed as nmol O_2 consumed per minute per mg protein.

NADH oxidase specific activity expressed as nmol NADH oxidized per minute per mg protein.

it has become possible to selectively solubilize the inner membrane of Gram-negative bacteria (14, 27) with the use of non-ionic detergents. The approach used here was to selectively solubilize the inner membrane of *T. thiooxidans* using Triton X-100 (14). A weight by weight ratio of 1-3 detergent to 1 of lipid is usually required for solubilization. In this case, a 2:1 (w/w) detergent to crude membrane (dry weight) in a 5% weight per volume detergent content was used.

The dry weight content of a crude membrane preparation was determined by drying 2 mL of a cell envelope preparation in a porcelain crucible. Once the dry weight content was established, the sample was diluted to 25 mg/mL and detergent added to a concentration of 50 mg/mL. The mixture was stirred for one hour at $4^{o}C$ to ensure interaction between detergent and membrane. This treated membrane sample was then fractionated by ultracentrifugation for two hours at 120,000 $\times$ *g* so as to separate the solubilized from the still particulate matter. A clear yellow-green supernatant was obtained and decanted. The light brown pellet was resuspended in the membrane storage buffer. The different preparations were compared by assaying for protein content and sulfite-oxidizing activity (see Table 11).

Upon treatment with Triton X-100, all sulfite-oxidizing activity was lost. A little more than 8.5% of the original, pre-treatment activity could be recovered if the assay mixture was supplemented with cytochrome *c* (oxidized, h. heart) to a concentration of 0.83 μM. When this treated membrane was fractionated, almost one half of the activity was recovered in the soluble fraction; no activity was found in the particulate fraction.

TABLE 11. Characterization of soluble and particulate fractions from surfactant-mediated membrane fractionation.

Sample	Total protein content (mg)	[a]Total sulfite-oxidizing activity (nmol O_2 per min)	Protein distribution (%)	Activity distribution (%)
Triton-treated membrane	704.	968.	100.	100.
Supernatant fraction	238.	425.	32.	44.
Precipitate fraction	507.	0.	68.	0.

[a]Sulfite oxidation required the addition of horse heart cytochrome *c* to a concentration of 0.83 μM.

Since the overall oxidation of sulfite involves the two enzymes sulfite oxidase and cytochrome oxidase, loss of oxygen uptake activity does not necessarily mean that both enzymes are absent. The various fractions were tested for these activities. Sulfite oxidase was assayed using either ferricyanide or oxidized cytochrome *c* as electron acceptor. Cytochrome oxidase was assayed using reduced mammalian cytochrome *c*. The results are summarized in Table 12. Activity for sulfite oxidase could be easily detected using the ferricyanide assay. No activity could be detected, however, if cytochrome *c* was used as the electron acceptor; neither mammalian nor *Saccharomyces cerevisiae* cytochrome *c* was reduced with sulfite. Addition of AMP did not stimulate the activity, either with ferricyanide or cytochrome *c* as electron acceptor. No activity whatsoever could be detected for cytochrome oxidase in any of the fractions, not even in crude extract.

Activity values for sulfite oxidase were obtained from cruder preparations so as to compare with the detergent-treated ones. The results are summarized in Table 13. Activity increased dramatically (about five fold) upon treatment with Triton X-100, most of the activity being solubilized. The soluble fraction was by far the purest preparation.

In order to determine the extent to which cell envelopes of *T. thiooxidans* had been fractionated using this mild detergent treatment, the three samples (treated membrane, soluble and particulate fractions) were run under denaturing conditions on SDS-polyacrylamide gel electrophoresis. On examination of the polypeptide band patterns (see Figure 12), it appeared that some fractionation had taken place. The

TABLE 12. Fractionation of enzyme activities involved in sulfite oxidation.

	Treated membrane	Soluble fraction	Particulate fraction
Total activity of sulfite oxidase with:			
a) ferricyanide	50.3	35.7	4.6
b) mammalian cytochrome *c*	0.0	0.0	0.0
c) *S. cerevisiae* cytochrome *c*	0.0	0.0	0.0
Total activity of cytochrome oxidase	0.0	0.0	0.0
Specific activity of sulfite oxidase (using ferricyanide)	0.07	0.15	0.01
Activity distribution of sulfite oxidase (using ferricyanide)	100.	71.	9.

Activities are expressed as absorbance change per minute under the conditions described in the Materials and Methods.

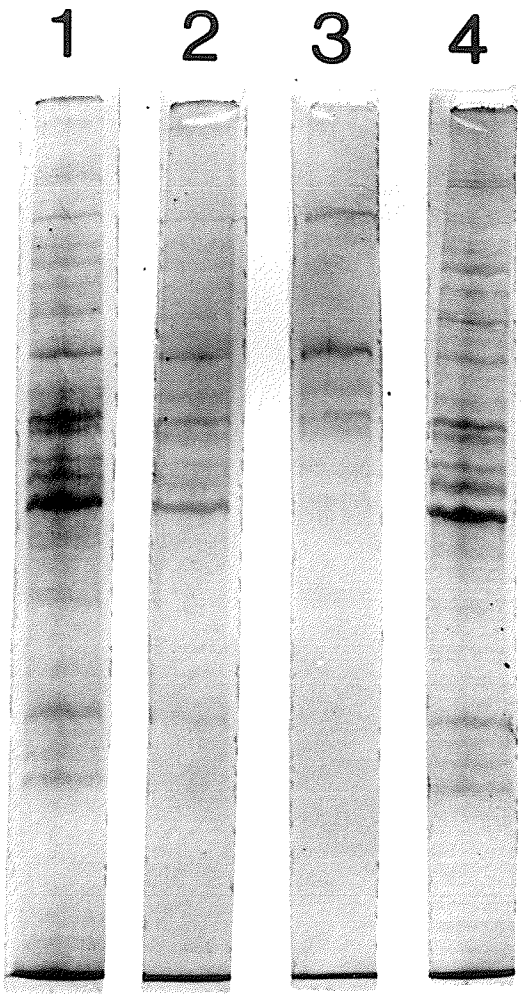
TABLE 13. Partial purification of sulfite oxidase.

Sample	Total volume (mL)	Total activity	Total protein (mg)	Specific activity (nmol O_2 per min per mg protein)	Activity distribution (%)	Purif. fold
Crude extract	33.	20.5	999.	0.021	100.	1.0
Spinco supernatant	46.	2.4	384.	0.006	12.	0.3
Cell envelope	16.	10.4	685.	0.015	51.	0.7
Treated membrane	88.	50.3	704.	0.071	245.	3.5
Soluble fraction	84.	35.7	238.	0.150	174.	7.3
Particulate fraction	10.	4.6	507.	0.009	23.	0.4

Activity expressed as decrease in absorbance units (A) per minute at 420 nm using the ferricyanide assay.

FIGURE 12. SDS-polyacrylamide gel electrophoresis of membrane solubilization experiment preparations.

Lane 1 : Crude membrane/cell envelope (38.5 µg protein)
2 : Detergent-treated membrane (24.0 µg protein)
3 : Soluble fraction (23.0 µg protein)
4 : Particulate fraction (39.0 µg protein)


1
2
3
4

majority of the polypeptide bands of the membrane remained in the particulate fraction upon ultracentrifugation, but some polypeptide bands were more concentrated in the soluble fraction. This Triton X-100 treatment, therefore, was able to selectively solubilize the enzyme sulfite oxidase as well as a few other proteins from the crude cell envelope preparation.

Effect of sucrose on sulfur-oxidizing enzyme

Sulfur-oxidizing activity was lost upon membrane fractionation by sucrose density centrifugation. It is possible that the sucrose had an effect on the enzyme. When a small sample of Spinco supernatant was made to 40% (w/w) sucrose and assayed for sulfur oxidation, activity had decreased by about 20%. It is therefore unlikely that the enzyme was inactivated by the presence of sucrose in the density gradient . On the other hand, it is possible that the enzyme was easily soluble in sucrose and was distributed throughout the gradient instead of migrating with the membranes. A small sample of the Spinco supernatant was made to 10% (w/w) sucrose before being precipitated with 75% ammonium sulfate saturation. Normally, all of the sulfur oxidizing activity is precipitated at this ammonium sulfate concentration. However, in this case more than 50% of the residual activity remained in the supernatant, with only slightly more than 40% going to the precipitate.

The effect of sucrose solubilization was further tested on the usual ammonium sulfate fractionation procedure. A sample of the Spinco supernatant was fractionated in the usual manner to yield the two active precipitates (50% and 75% ammonium sulfate fractions) which were resuspended in 50 mM Tris buffer and allowed to interact for 15

minutes before being precipitated again with their respective saturations of ammonium sulfate. The supernatants and precipitates were then assayed for protein content and sulfur-oxidizing activity. The results are summarized in Table 14.

In both cases, over 90% of the protein came down with addition of ammonium sulfate, indicating that solubilization by sucrose was very selective. The 50% fraction lost more than 80% of the activity through this sucrose treatment, although the ammonium sulfate could have contributed to this, since activity normally decreased with high ammonium sulfate content. Of that residual activity, most of it was solubilized. The 75% fraction lost 60% of the activity through this treatment. However, all of the residual activity was solubilized by sucrose raising the specific activity over 10 fold.

Ion exchange chromatography using sucrose

Ion exchange chromatography was carried out on the two active ammonium sulfate fractions (50% and 75%), this time in the presence of sucrose to see if the procedure could be improved. The two fractions were resuspended in S-buffer (see Materials and Methods) and dialyzed against the same. The two fractions were loaded on DEAE-Sephadex columns equilibrated with S-buffer. This time, no activity could be detected at an ionic strength of 0.2 M NaCl; as a result, the columns were washed extensively with 0.2 M NaCl in S-buffer before being eluted with 0.35 M. The activities recovered were 13% and 10% for the 50% fraction and 75% fraction, respectively. These values made the purification step extremely wasteful in terms of activity, and was not again used in the purification procedure.

TABLE 14. Solubilization of sulfur-oxidizing enzyme by sucrose

Sample	Total activity (nmol O_2 per min)	Total protein (mg)	Specific activity (nmol O_2 per min per mg protein)	Activity distribution (%)	Protein distibution (%)
a) 50% fraction					
50% fraction	464.	35.4	13.1	100.0	100.0
Sucrose supernatant	44.	1.6	27.5	9.5	4.5
Sucrose precipitate	22.	33.8	0.7	4.7	96.5
b) 75% fraction					
75% fraction	277.	7.8	35.5	100.0	100.0
Sucrose supernatant	112.	0.3	373.3	40.4	3.9
Sucrose precipitate	0.	7.5	-	0.	96.1

Fractions were made to 10% sucrose, then made to their respective ammonium sulfate saturations to precipitate protein. The supernatants and precipitates were then assayed for activity.

1st Sephadex G-150 column

A purification procedure based on molecular weight separation was carried out on the 75% fraction. The 75% ammonium sulfate precipitate was resuspended with a minimal ammount of S-buffer (see Materials and Methods) and dialyzed against the same. The very concentrated preparation was then passed through a Sephadex G-150 column equilibrated with either S- or CS- buffer. Activity was eluted as a broad peak at roughly one-half the bed volume. The Kav value for the activity peak was about 0.34 with S-buffer and about 0.46 with CS-buffer, corresponding to molecular weights of roughly six and four $\times 10^4$ daltons, respectively (see Table 15). The active fractions were pooled and assayed for sulfur oxidizing activity and protein content. The resulting preparation retained 83% of original activity and about 46% of original protein loaded; as a result, the purification achieved was only about a factor of two. These figures are illustrated in Table 16. Visible spectrum of the active eluate revealed the presence of *c*-type cytochrome plus possibly some non-heme iron.

Removal of nucleic acid from the 1st G-150 preparation

Ultraviolet spectra of the 1st G-150 preparation revealed that the nucleic acid content was extremely high: more than 20% by the A_{280nm}/A_{260nm} ratio (25). This nucleic acid had to be removed before further purification could take place. Short digestion of the preparation with ribonuclease or deoxyribonuclease with subsequent passage through DEAE-cellulose proved unsuccessful. When the nuclease digestion was aided by the presence of trypsin, the nucleic acid content still did not decrease.

TABLE 15. Sulfur-oxidizing elution characteristics from Sephadex G-150 with sucrose.

Column	Void volume Vo (mL)	Eluting volume Ve (mL)	Total bed volume Vt (mL)	Kav value $\frac{Ve - Vo}{Vt - Vo}$	Tentative molecular weight (daltons)
1st G-150	51	99	196	0.33	6×10^4
1st G-150	51	102	196	0.35	6×10^4
2nd G-150	60	99	191	0.30	7×10^4
1st G-150 (Ca)	60	117	191	0.44	4×10^4
1st G-150 (Ca)	63	132	184	0.57	3×10^4
1st G-150 (Ca)	51	117	187	0.48	4×10^4
2nd G-150 (AMP)	48	93	187	0.32	7×10^4
2nd G-150 (AMP)	51	93	187	0.31	7×10^4

Tentative molecular weights according to Pharmacia Fine Chemicals product guide on the Gel Filtration theory and practice booklet (9).

TABLE 16. Column chromatography of the 75% fraction for sulfur-oxidizing enzyme.

Sample	Total volume (mL)	Total activity (nmol O_2 per min)	Total protein (mg)	Specific activity (nmol O_2 per min per mg protein)	Activity distribution (%)	Purif. fold
75% fraction	90.0	7760	150	51.7	100	1.1
1st 0.1 M eluate	8.5	6470	69	93.8	83	1.8

The column used was a Pharmacia column (45.0 × 2.5 cm) packed with Sephadex G-150 and equilibrated with S-buffer. Similar results were obtained with CS-buffer.

Calcium is widely used in transformation experiments and is thought to interact with DNA (8, 11, 55). If the preparation in S-buffer was applied to a DEAE-cellulose column and eluted with increasing concentrations of calcium chloride, activity could be eluted before the nucleic acid. In another experiment, the G-150 preparation in S-buffer was made to 0.1 M calcium chloride and incubated at $4^{o}C$ for one hour. Subsequent passage of this preparation through a DEAE- equilibrated with CS-buffer resulted in the activity washing through while the nucleic acid remained bound to DEAE.

If calcium chloride at a concentration of 0.1 M was present from the very beginning during the Sephadex G-150 step, the nucleic acid removal step could be simplified to passing the active G-150 eluate through DEAE equilibrated with CS-buffer immediately after elution. As a result, CS-buffer was introduced to the procedure; the 75% fraction was dialyzed against CS-buffer prior to passage through the 1st G-150 column, also equilibrated with CS-buffer. The effect of calcium treatment is illustrated in Figure 13.

The DEAE-cellulose itself had a poor binding capacity for nucleic acid; in order to be able to decrease the nucleic acid content from more than 20% to about 3%, it was necessary to use one mL bed volume per mg protein of the 1st G-150 preparation.

Some observed calcium effects

When calcium was introduced to the 75% fraction during dialysis, some precipitate was formed. This precipitate was quite likely to include calcium and possibly some nucleic acid. If the precipitate was removed by centrifugation, activity remained in the supernatant. The

precipitate formed slowly with time; however, it could be accelerated by freeze-thawing of the sample.

In gel filtration chromatography using S-buffer, the sulfur-oxidizing enzyme did not truly behave as a single entity. However, the observed activity peak did correspond to a molecular weight in the neighborhood of 60,000. Yet if calcium was present in the form of CS-buffer, the activity peak observed behaved as if the molecular weight was only 40,000 (see Table 15).

2nd Sephadex G-150 column

With nucleic acid no longer bound to the enzyme, there was a likelihood that the molecular weight would be different. As a result, another separation on the basis of molecular weight was carried out. At this point, the enzyme was very dilute, requiring concentration before passing through a second Sephadex G-150 column. The nucleic acid-free enzyme was dialyzed against S-buffer in order to remove calcium. The dialyzed preparation was then loaded on a DEAE-cellulose column equilibrated with S-buffer. A tight yellow band could be seen sticking to the top of the bed while a colored fraction containing cytochrome passed through with the wash. After washing with more S-buffer, the yellow band was eluted with CS-buffer. This concentrated eluate was then passed through a second Sephadex G-150.

Activity was eluted as a broad peak, again at about one-half the bed volume. However, the activity peak corresponded to a molecular weight value of about 70,000 (see Table 15). The enzyme probably became heavier due to association with other proteins resulting from nucleic acid removal.

2nd Sephadex G-150 column using AMP

In the absence of nucleic acid, the enzyme appeared to bind other proteins, giving a heterogeneous mixture which was difficult to purify. As a result, the enzyme was allowed to bind to something resembling nucleic acid, but simple enough so that purification of the complex could still take place. Adenosine-5'-monophosphate (AMP) was the agent of choice and was introduced to the procedure in the form of AS-buffer (see Materials and Methods).

The concentrated DEAE eluate was dialyzed against AS-buffer in order to remove calcium and introduce AMP before passage through the second G-150 column, also equilibrated with AS-buffer. The results are illustrated on Tables 15 and 17.

From the values given on Table 17, the recovery of activity was quite poor. In the concentration step alone, more than 60% of activity was lost. Most of the protein, however, was also removed; the 1st 0.1 M eluate retained less than one fifth of protein eluted from the first G-150 column. The 2nd G-150(AMP) column step itself was characterized by a 50% loss in activity. The observed activity peak elutes at a position corresponding to a molecular weight of about 70,000 (see Table 15).

The active eluate was extremely dilute and had to be concentrated in order to be able to assay for protein content. As a result, the entire preparation was concentrated by an ion-exchange chromatography step, consisting of loading the sample on a DEAE-cellulose mini-column and eluting with CS-buffer. This concentration step however, resulted in a further 40% loss of activity. Even though this 2nd 0.1 M eluate

FIGURE 13. Ultra-violet spectra of sulfur-oxidizing enzyme before and after nucleic-acid removal by calcium.

The "before" sample consists of the 1st G-150 preparation in CS-buffer diluted ten times.

The "after" sample consists of the 1st 0.1 M eluate in CS-buffer diluted ten times.

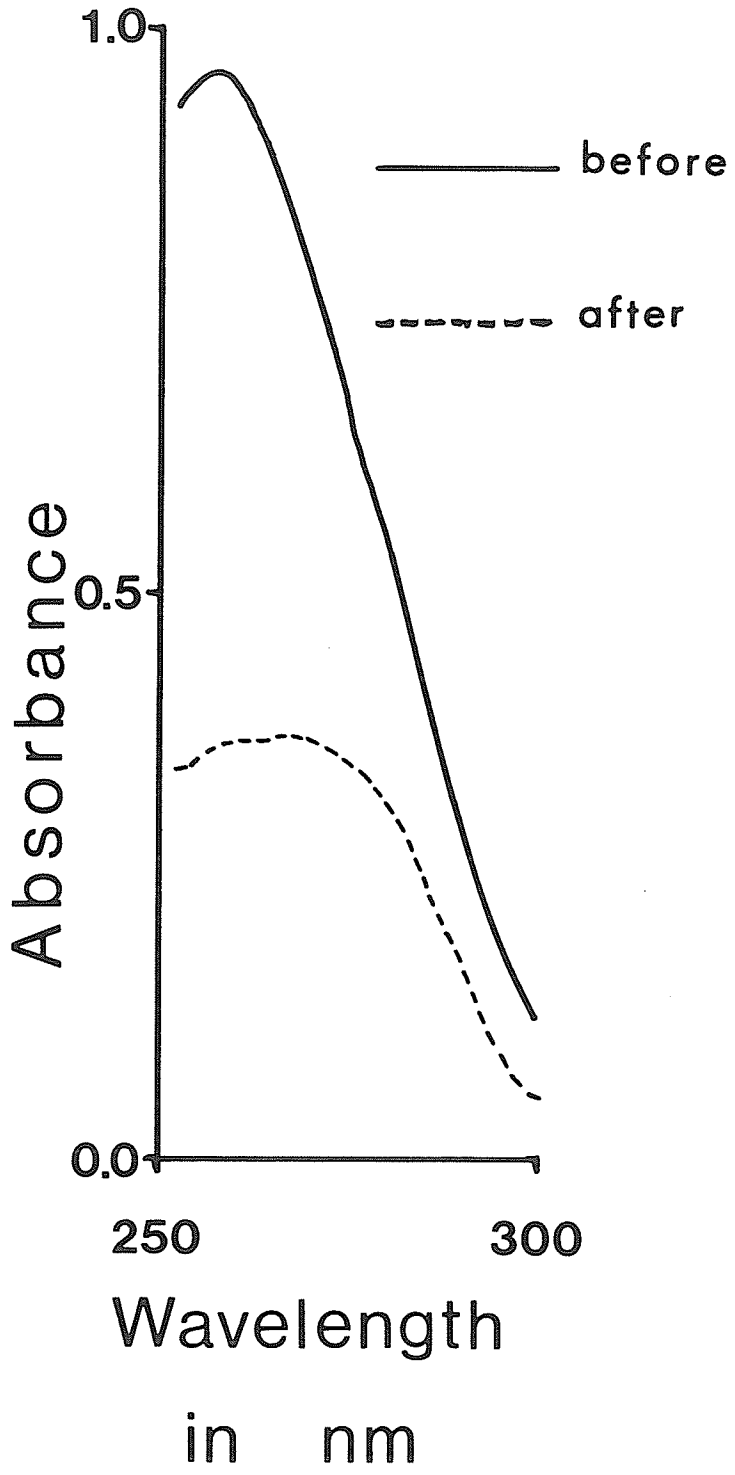

1.0
0.5
0.0
Absorbance
before
after
250
300
Wavelength
in nm

TABLE 17. Further purification profile of sulfur-oxidizing enzyme.

Sample	Total volume (mL)	Total activity (nmol O_2 per min)	Total protein (mg)	Specific activity (nmol O_2 per min per mg protein)	Activity distribution (%)	Purific. fold
1st G-150 preparation	85.0	8,810	81.3	108.4	100.0	1.1
1st 0.1 M eluate	7.5	3,000	16.1	186.5	34.1	1.7
2nd G-150 preparation	45.0	1,520	-	-	17.3	-
2nd 0.1 M	6.5	911	5.9	154.4	10.3	1.4

The 0.1 M eluate refers to a concentration step where a sample was loaded on a DEAE-cellulose column equilibrated with S-buffer and eluted with CS-buffer.

preparation only contained slightly more than 7% of the original active protein that eluted from the first Sephadex G-150 column, the accumulated loss of activity was so great that the degree of purification was less than that of the 1st 0.1 M eluate.

Characterization of the 2nd 0.1 M eluate

The 2nd 0.1 M eluate retained only 0.3% of the protein found in the crude extract, but only slightly more than 3% of the activity. The preparation was run on both denaturing and non-denaturing polyacrylamide gel electrophoresis in order to determine its purity. The results are illustrated in Figures 14, 15, 16. The presence of a number of bands on both the denaturing and non-denaturing gels indicate that the preparation was not pure. The two most prominent bands on the denatured gels correspond to molecular weights of 41,000 and 36,000. However it is impossible to determine whether one or any of these corresponded to the sulfur-oxidizing enzyme.

Further purification attempts of the 2nd 0.1 M eluate using ion-exchange chromatography on a small scale were unsuccessful; it was not possible to reduce the number of bands seen on the non-denaturing gel. The preparation itself was yellow in color, its visible spectrum revealing the presence of what may be non-heme iron (see Figure 17). Due to the presence of impurities however, it is impossible to determine if the features seen corresponded to the enzyme or not.

FIGURE 14. SDS polyacrylamide gel electrophoresis purification profile of sulfur-oxidizing enzyme.

Lane 1 : Crude extract (136 µg protein)
2 : Spinco supernatant (42 µg protein)
3 : 75% fraction (90 µg protein)
4 : 2nd 0.1 M eluate (7 µg protein)

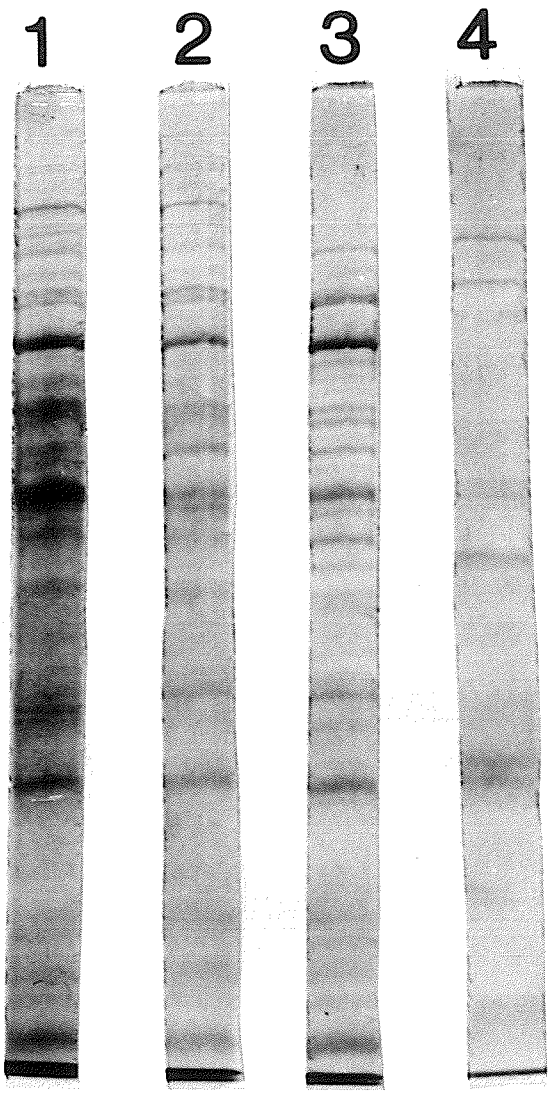

1
2
3
4

FIGURE 15. A different SDS-polyacrylamide gel electrophoresis purification profile of sulfur-oxidizing enzyme.

Lane 1 : Spinco supernatant (28 µg protein)
2 : 75% fraction (70 µg protein)
3 : 1st G-150 preparation (20 µg protein)
4 : 1st 0.1 M eluate (30 µg protein)
5 : 2nd 0.1 M eluate (10 µg protein)

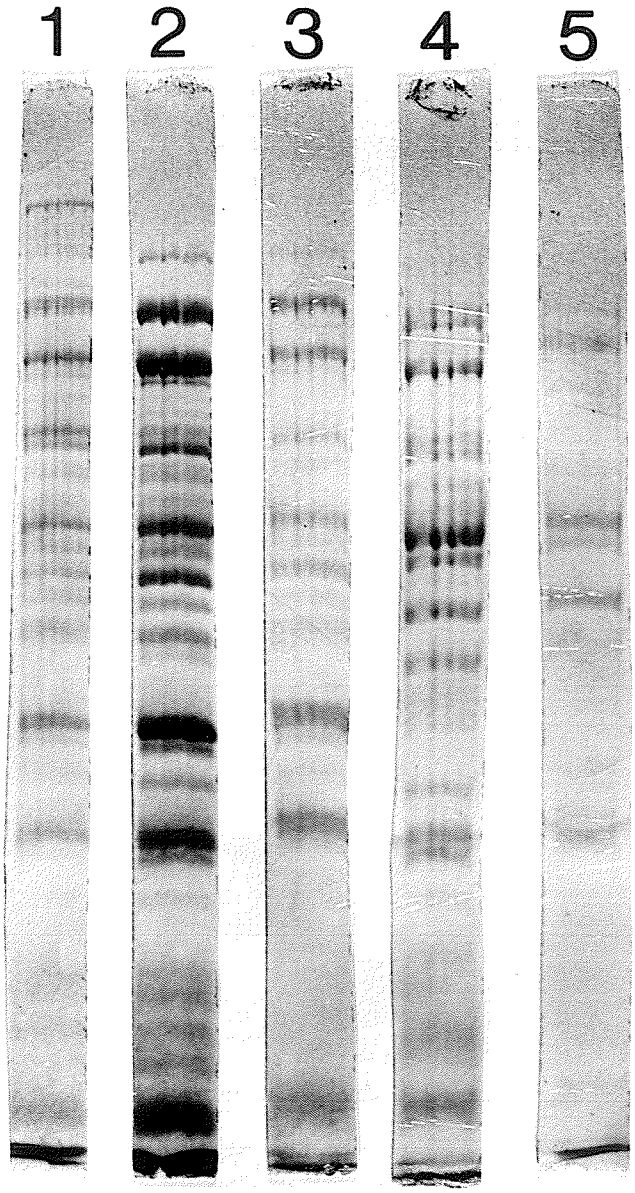

1
2
3
4
5

FIGURE 16. Non-denaturing polyacrylamide gel electrophoresis purification profile of sulfur-oxidizing enzyme.

Lane 1 : Spinco supernatant (35 µg protein)
2 : 75% fraction (87 µg protein)
3 : 1st G-150 preparation (50 µg protein)
4 : 1st 0.1 M eluate (75 µg protein)
5 : 2nd 0.1 M eluate (25 µg protein)
6 : a different 2nd 0.1 M eluate (25 µg protein)

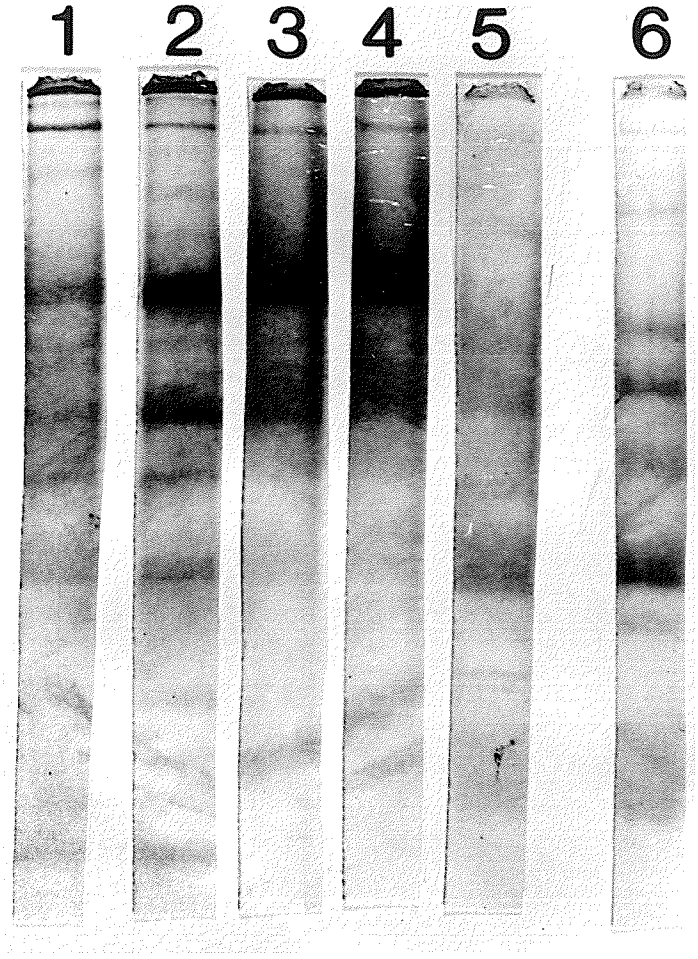
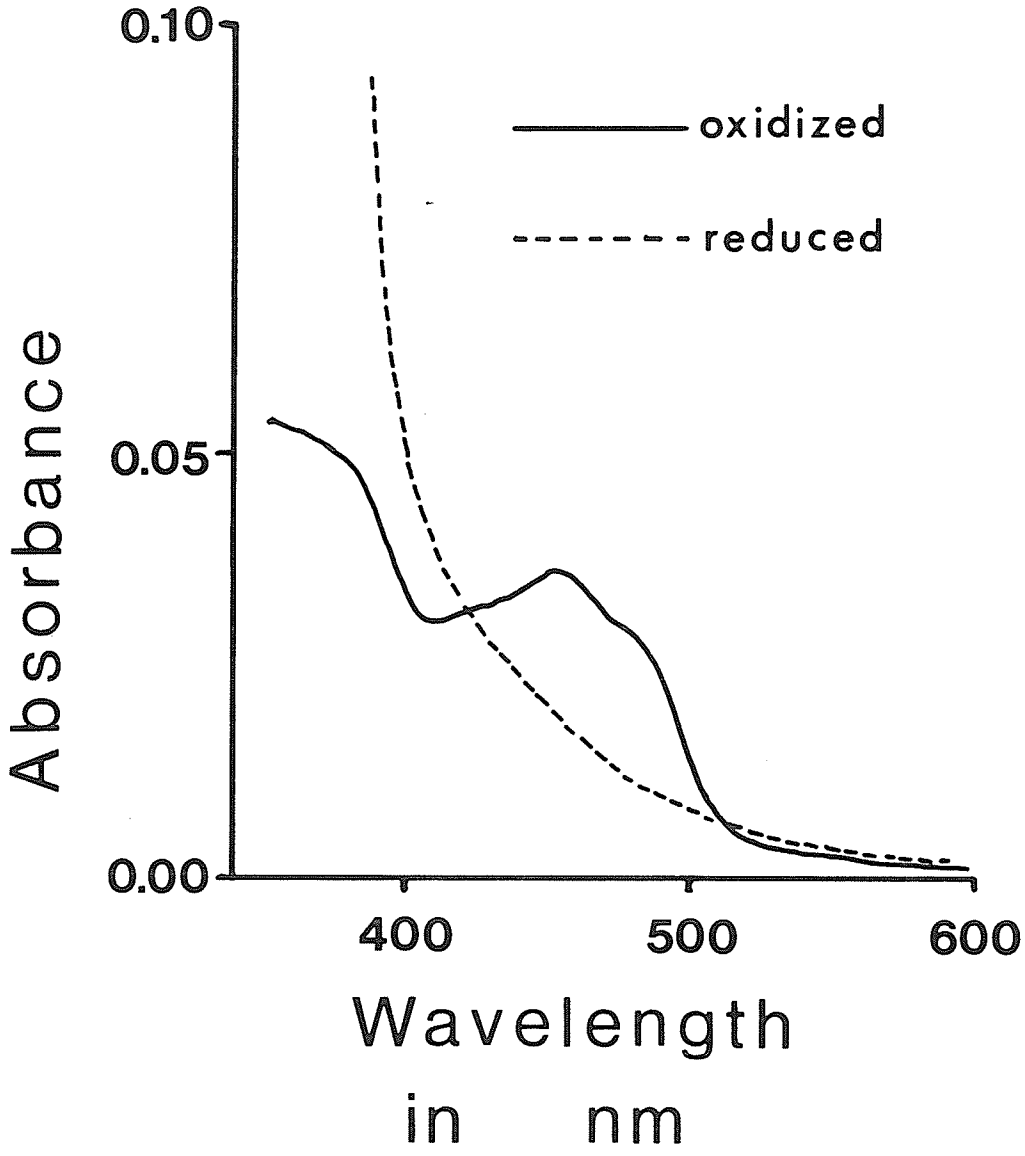

1
2
3
4
5
6

FIGURE 17. Oxidized and reduced spectra of the 2nd 0.1 M eluate in CS-buffer, undiluted.

The sample contained a protein content of 0.4 mg/mL


0.10
0.05
0.00
Absorbance
oxidized
reduced
400
500
600
Wavelength
in nm

DISCUSSION

DISCUSSION

Sulfur metabolism, including intact cells and cell-free extracts of *T. thiooxidans* was investigated, paying particular attention to the enzymes involved in the oxidation of sulfite and elemental sulfur. In the past, most studies carried out with cell-free systems have used sonic disruption as the method of breakage (2, 28, 48, 49). The work presented here differentiates itself along with that of Adair (1) in that the cells were broken by French pressure cell. This much gentler method of breakage was bound to have an effect in the isolation, characterization, and resolution of the enzymatic systems involved in sulfur metabolism. As it turned out, substantial differences were encountered in the subsequent behaviour of cell components, as compared to previous studies, this being especially true for the sulfur-oxidizing enzyme (2).

Intact cells were capable of oxidizing elemental sulfur to sulfate both at pH 2.0 and 7.5. Oxidation observed at neutral pH is not constant, however; if the cells are left at neutral pH for two to three hours, they lose activity (48). Sulfite is oxidized optimally at pH 5.5 (28). It can also be oxidized at pH 7.5, but not at 2.0. Sulfite also happened to inhibit sulfur oxidation at pH 2.0 but not at 7.5. By using the acid dissociation constants of the various forms of sulfite, it is possible to determine those which are most abundant at each pH. The abundance of these different forms of sulfite can be

visualized on Table 18. From that table, it can be seen that at optimum sulfite-oxidizing pH, by far the most abundant form of sulfite is the bisulfite anion (HSO_3^-). It stands to reason then, that the true substrate in sulfite oxidation is possibly bisulfite. At neutral pH, where oxidation still takes place, bisulfite is still the predominant species by a factor of three. At pH 2.0, bisulfite still predominates by $1\frac{1}{2}$ times over sulfurous acid (H_2SO_3), yet no oxidation takes place. This suggests that sulfurous acid is not oxidized by cells and is inhibiting sulfur oxidation and bisulfite oxidation. At pH 5.5 and 7.5, where sulfur oxidation is not inhibited by sulfite, sulfurous acid content is almost nonexistant.

Intact cells were able to oxidize thiosulfate completely to sulfuric acid at pH 2.0 but not at 7.5. Interestingly, this acidic pH was required on the part of the cells; cells at neutral pH were not able to oxidize acidified thiosulfate. This indicates that a known chemical decomposition of thiosulfate to elemental sulfur and sulfite in acid is not the only factor contributing to the observed activity. A further study of this observation would also clarify the existing ambiguity as to whether it is really thiosulfate or the products of its decomposition which are oxidized by *T. thiooxidans* under natural (acidic) conditions (12). Oh and Suzuki (36) observed that the thiosulfate cleaving complex in *T. novellus* was localized in the cell membrane. It may follow then, that thiosulfate binds to cells and is broken at the cell surface, where an acidic environment could play a substantial role. This arrangement is supported by the oxidation of thiosulfate being biphasic. The first stage in thiosulfate oxidation

TABLE 18. Abundance of sulfite species at different pH's

pH	H_2SO_3	HSO_3^-	SO_3^{2-}	Comments
2.0	0.6	1	10^{-5}	not oxidized, inhibits sulfur-oxidation
5.5	2×10^{-4}	1	3×10^{-2}	optimum oxidation, not inhibitory
7.5	2×10^{-6}	1	0.3	oxidized, not inhibitory

Values given according to dissociation constants for sulfurous acid according to values given by Lyons and Nickless (29).

appears to involve the cleavage of thiosulfate into its sulfur and sulfite components. If the sulfur oxidation part is inhibited by extracellular sulfite, thiosulfate oxidation terminates at the first stage and only the first sulfite is oxidized.

Spectrophotometric studies revealed that the oxidation of sulfur by intact cells was characterized by the appearence of a peak at 360 nm. It was not clear whether this peak was due to sulfur oxidation or sulfur binding; this peak however, was observed upon cells coming in contact with sulfur. Addition of sulfite to cells at pH 2.0 (inhibitory conditions) also gave the appearence of a peak at 360 nm which was much stronger than that peak given by sulfur. This is probably due to the fact that during sulfur oxidation, the sulfur-oxidizing system is at dynamic equilibrium, unlike during sulfite inhibition where the system has been stopped. There is a possibility that the 360 nm peak represents an intermediate sulfur complex, only seen during sulfur oxidation. Spectra of various sulfur compounds such as dithionite, tetrathionate, thiosulfate, sulfur, sulfite, and combinations of these failed to give a spectrum with the 360 nm peak (unpublished data). If this possibility is true, however, it is also quite possible that under inhibitory conditions, sulfite is able to form an irreversible version of this complex. It would be fairly simple to postulate that sulfurous acid, due to its electroneutral nature, is able to bind and inhibit the sulfur-oxidizing system. The fact that sulfite inhibition can be reversed by raising the extracellular pH suggests that inhibition is probably localized at a site that is close to, or at the cell membrane.

The freeze-thawed cells formed the 360 nm peak with sulfur, but did not oxidize it. It is possible that freeze-thawing results in the alteration of some component so that sulfur oxidation is inhibited in a manner similar to that of sulfite inhibition.

Cell breakage by passage through French pressure cell gave extracts which retained 20% of sulfur-oxidizing activity and 70% of the sulfite-oxidizing activity of intact cells. The extracts proved to be fairly stable, showing a constant rate of sulfur oxidation during periods extending over two hours. This stable activity, however, was not seen in the presence of Tris-buffer; as a result, potassium phosphate was established as the buffer of choice. As with earlier findings (1), these extracts exhibited some sulfur-oxidizing activity which was independent of GSH. However, this activity was very small and very susceptible to storage. It is quite possible that protein-associated thiol groups (1) are responsible for breaking the sulfur ring during GSH-independent oxidation. It appears though, that whatever is responsible for this is partially destroyed upon breakage. It is quite possible that this GSH-independent sulfur oxidation depends on a structural, rather than an enzymatic intactness of a cellular component.

The crude extracts could be fractionated by ultracentrifugation into a soluble and a particulate fraction, containing mostly membranous material. This fractionation was able to separate sulfur- and sulfite- oxidizing activities in a very efficient manner. All of sulfite-oxidizing activity was localized in the cell envelope, correlating with previous studies which localized the sulfite oxidation as

taking place in the cell membrane. Although some sulfur-oxidizing activity was also found in the cell envelope, most of the activity remained in the Spinco supernatant. Interestingly, sulfur-oxidizing activity increased upon fractionation. This could be due to the removal of some inhibitory component in the cell envelope. Also interesting is the fact that sulfur oxidation in the cell envelopes was less dependant on GSH. This indicates that most of those native sulfhydryl groups are localized in the cell envelope, probably membrane-bound.

The Spinco supernatant can be separated into four fractions using ammonium sulfate fractionation: a 25% fraction that had little sulfur-oxidizing activity; a 50% fraction that contained flavin and some activity; a 75% fraction that contained some cytochrome *c* plus most of the sulfur-oxidizing activity; and a 100% fraction with little activity that contained cytochrome *c*. Previous work on the sulfur-oxidizing enzyme using sonication found it to be strongly associated with flavin (2); in that study, it was suggested that flavin formed part of the active site, flavin being involved in other oxygenases (13). With the French pressure cell derived preparations described here, flavin is successfully separated from most of the activity at a fairly early stage in the purification.

Isolation of the sulfur-oxidizing enzyme was not improved by the use of a thiol-protecting compound, namely DTE.

If the intact cells were partially digested with trypsin prior to breakage, the resulting extracts at first displayed similar properties to those of untreated extracts. The main difference between the

two was that the trypsin-treated extracts were able to oxidize thiosulfate, as opposed to non-treated extracts which did not display thiosulfate-oxidizing activity. It is possible that a partial digestion with trypsin liberates a thiosulfate-oxidizing component, possibly rhodanese, from other cellular components, enabling it to survive cell breakage and possible entrapping within membranes. The possibility, however, will have to be proven by other experiments. Sulfur-oxidizing activity recovered upon cell breakage is almost twice that of extracts from untreated cells. Most of this activity, however, ends up in the 50% fraction, along with the flavin, similar to the results obtained with extracts derived by sonication (2). Since flavin can induce some non-enzymatic activity (I. Suzuki,personal communication), making the results harder to interpret, the trypsin treatment was not regularly used in this study.

The results obtained from ion-exchange and gel filtration chromatography experiments on the 50% and 75% ammonium sulfate fractions indicated that the sulfur-oxidizing enzyme did not behave as a single entity. During ion-exchange chromatography, the activity from both fractions did not elute at a critical ionic strength, but over a wide range from 0.2 to 0.35 M NaCl in equilibrating buffer. The resulting active eluates from each ammonium sulfate fraction did not resemble one another. The eluate derived from the 50% fraction still contained flavin whereas the eluate derived from the 75% fraction still contained cytochrome. The fact that flavin and cytochrome were mutually exclusive from each preparation suggests that they are not involved in activity. When these two eluates were passed through a Sephadex

G-150 column, no protein or activity peak could be seen. Instead, activity was spread out over almost the entire bed volume.

The use of sucrose during ion-exchange chromatography of the 50% and 75% fractions did improve chromatography in the sense that activity was eluted over a narrower range of salt concentration. However, the amount of activity lost was still very high, making the step too wasteful for use as a standard method. On the other hand, sucrose was remarkable at improving the behaviour of enzyme during gel filtration chromatography of the 75% fraction. The majority of activity initially loaded on the column could be recovered in the eluate, which contained about one-half of the original protein. Activity still eluted as a broad peak; but even then this was much more desirable than the results obtained without sucrose.

The active eluate from the first Sephadex G-150 column (containing sucrose) was found to have a very high content of nucleic acid. This high nucleic acid content was most likely also present in earlier preparations of the sulfur-oxidizing enzyme. This nucleic acid was very difficult to separate from activity, success being finally achieved by the use of calcium in the purification procedure. This strong association with a mostly heterogeneous population of nucleic acid was probably responsible for the enzyme not behaving as a single entity in previous experiments.

It appears that the sulfur-oxidizing enzyme tightly bound itself to nucleic acid upon cell breakage by French pressure cell. It is possible that the enzyme exists in close association with a cellular component, possibly membrane. Upon cell beakage, this association is

disrupted and the enzyme binds nucleic acid instead. The likelihood exists that the previously observed association of the enzyme with flavin (2) may be similar to its association with nucleic acid observed here. This could mean that upon cell breakage, the enzyme will bind either nucleic acid or flavin, depending on the method of breakage: French pressure cell in the former, sonication in the latter.

It remains unknown how sucrose can influence the behaviour of the sulfur-oxidizing enzyme so as to make it resemble a single protein species. Sucrose by itself is able to solubilize the enzyme as seen in sucrose density gradients and ammonium sulfate precipitation in the presence of sucrose (see Results). It is possible that a high sucrose content may have partially simulated an environment the enzyme normally experiences in the intact cell.

Calcium also seems to have a significant effect on the behaviour of the sulfur-oxidizing enzyme. First of all, as seen in the nucleic acid removal step, it was able to neutralize the enzyme's affinity for nucleic acid. Second, the presence of calcium in gel filtration chromatography experiments resulted in the enzyme behaving as having a molecular weight of 4×10^4; in the absence of calcium, the enzyme behaved as having a molecular weight of 6×10^4 daltons. Calcium then, seemed to mediate the dissociation of nucleic acid from the enzyme. Whether calcium interacted with nucleic acid or the enzyme itself, is not clear. It is also not clear whether this effect is a property of calcium alone, or whether the same would be observed with another divalent cation such as magnesium. In any case, the enzyme did behave

as having a smaller molecular weight when calcium was present. This observation suggests that the enzyme does have a molecular weight in the neighborhood of 4×10^4 daltons. This figure agrees with previous studies (2). In the absence of calcium, when the enzyme was free to associate with nucleic acid, its molecular weight necessarily increased.

Purification of the sulfur-oxidizing enzyme beyond the first Sephadex G-150 column proved unsuccessful. The enzyme could be concentrated by binding it to DEAE-celulose and eluting it with 0.1 M calcium. In terms of activity, however, this proved to be very expensive. Passage through a second Sephadex G-150 column again resulted in a high loss of activity. By the time the active eluate from the second Sephadex G-150 column was concentrated by another DEAE-cellulose column step, activity had decreased to roughly 3% of original crude extract activity. At this stage, the enzyme was still not pure, as confirmed by denaturing and non-denaturing polyacrylamide gel electrophoresis.

Failure to purify the sulfur-oxidizing enzyme probably lies on the enzyme's need to associate with other components so as to simulate its natural environment in the intact cell. Once nucleic acid was removed, the enzyme appeared to be stable so long as calcium was present. When calcium was removed and the enzyme was passed through a second Sephadex G-150 column, its apparent molecular weight increased from 4×10^4 to 7×10^4 daltons. This could be explained by a possible formation of a dimer, but a more accurate determination of molecular weights is required before any explanation can be given. It is also

possible that in the absence of calcium, the enzyme bound other proteins, increasing the apparent molecular weight. The introduction of AMP into the purification procedure did not help this situation. It is not clear, however, whether AMP could not be made to replace nucleic acid; it is possible that the amount of AMP used was not enough.

For the isolation and partial purification of the sulfite-oxidizing enzymes, two approaches were taken. Evidence pointed to the cell membrane as the site where sulfite oxidation took place. Since in typical Gram-negative organisms most membrane-bound enzymes are found in the inner membrane, a membrane fractionation experiment was carried out using sucrose density gradient centrifugation in order to separate the inner and outer membranes. At the same time, previous work done with surface-active agents indicated that whole, or portions of, inner membranes could be selectively solubilized by increased detergent content (14). Selective solubilization of the cell envelopes was carried out with Triton X-100.

In order to optimize membrane fractionation during sucrose density gradient, the remaining murein cell wall in the cell envelope was digested with β-glucuronidase (34), which did not affect sulfite-oxidizing activity. Cell envelopes not treated with β-glucuronidase were run as control. Upon fractionation, two distinct bands could be seen in both the control and digested samples. A light band with a density of 1.21 g/cm^3, containing more than 90% of the original protein and almost all of the sulfite-oxidizing activity, was positively identified as the inner membrane by the presence of NADH oxidase activity. A heavier band with a density of 1.24 g/cm^3, containing less

than 10% of the original protein and very little activity probably corresponded to the outer membrane.

There seemed to be virtually no difference between the digested and undigested samples. In the gradient tubes, the only difference to be seen was a turbid zone between the two bands in the undigested sample. SDS-polyacrylamide gel electrophoresis confirmed this by showing identical polypeptide band patterns for the respective digested and undigested membrane samples. In both cases, all of the polypeptides seen in the heavy band could also be seen in the light band, but some polypeptides seen in the light band were absent in the heavy band. Thus the light band contained heavy band components.

The membrane fractionation by sucrose density gradient centrifugation was therefore incomplete. Digestion with β-glucuronidase has been used mainly in the preparation of spheroplasts, taking the place of lysozyme (34). These procedures involve the permeabilization of the outer membrane with EDTA so that lysozyme or β-glucuronidase can digest the cell wall. In this case, the cells were already broken. The cell envelopes may have taken the form of vesicles, making it difficult for β-glucuronidase to act. Even if EDTA had been used, the mixed orientation of the vesicles might have had an adverse effect on the digestion by the enzyme. Nevertheless, the fact that two distinct bands were seen on the gradient supports the theory that *T. thiooxidans* has an inner and an outer membrane.

As far as sulfite-oxidizing activity is concerned, the fractionation does not look promising. Although most of the activity does end up in the light band, the degree of purification does not improve

after fractionation.

Selective solubilization using Triton X-100 involved treating the cell envelopes with the surfactant, followed by separation of the solubilized and non-solubilized components by ultracentrifugation. Treatment of the cell envelopes with Triton X-100 resulted in the loss of all sulfite-oxidizing activity. Addition of oxidized mammalian cytochrome *c*, however, allowed the recovery of almost 10% of the activity. Upon fractionation, almost one-half of this cytochrome *c* dependent activity could be recovered in the soluble fraction. Since the activity measured was so small, it is difficult to state that it was truly due to the enzymatic activity and not due to chemical interaction of components present.

Upon assaying for the individual enzymes involved in sulfite oxidation, it was found that sulfite oxidase activity increased by five fold and became soluble upon treatment with detergent; more activity could be found in the treated membrane than in the crude extract. It is apparent that this mild detergent treatment releases the enzyme from a complex, enabling it to interact more readily under artificial assay conditions. On the overall, this fractionation procedure was very efficient in the purification of this enzyme. This was confirmed by SDS-polyacrylamide gel electrophoresis, where it can be seen that some fractionation did take place.

No activity whatsoever could be detected for the enzyme cytochrome oxidase. It is possible that the reduced mammalian cytochrome *c* in the assay was not suitable for reaction with *T. thiooxidans* cytochrome oxidase. Similarly, neither mammalian, nor *S. cerevisiae* oxidized cyto-

chrome c was able to interact with sulfite oxidase. Only ferricyanide was reduced by sulfite. Both cytochrome oxidase and sulfite oxidase of *T. thiooxidans* may require a native cytochrome c for activity. Ferricyanide can apparently substitute for the cytochrome in the sulfite oxidase reaction. Purification of a native cytochrome and its demonstration in the cytochrome oxidase and sulfite oxidase reaction as electron donor and electron acceptor, respectively, will be essential to establish the roles of these enzymes in sulfite oxidation by *T. thiooxidans*.

CONCLUSION

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The enzymes involved in sulfur and sulfite oxidation: sulfur-oxidizing enzyme, sulfite oxidase, and cytochrome oxidase, all seemed to be membrane associated to some degree. Sulfite oxidase and cytochrome oxidase are probably membrane-bound, since they remain associated with the cell envelopes upon cell rupture. The sulfur-oxidizing enzyme was liberated as a soluble component upon cell breakage. However, because of its strong association to nucleic acid and sometimes to flavin, there is a strong possibility that the sulfur-oxidizing enzyme resides in the cell membrane . This and other evidence from sulfur, sulfite, and thiosulfate oxidation in intact cells, suggests that oxidation of inorganic sulfur compounds by *T. thiooxidans* occurs at the cell surface.

REFERENCES

REFERENCES

1. Adair, F.W. 1966. Membrane-associated sulfur oxidation by the autotroph *Thiobacillus thiooxidans*. J. Bacteriol. 92: 899-904.

2. Bhella, R.S. 1981. Purification and characterization of sulfur-oxidizing enzyme of *Thiobacillus thiooxidans*. M. Sc. Thesis, The University of Manitoba, Winnipeg, Manitoba.

3. Bodo, C. and D.G. Lundgen. 1974. Iron oxidation by cell envelopes of *Thiobacillus ferrooxidans*. Can. J. Microbiol. 20: 1647-1652

4. Booth, I.R. 1985. Regulation of cytoplasmic pH in bacteria. Microbiol. Rev. 49: 359 378

5. Charles, A.M. and I. Suzuki. 1966. Purification and properties of sulfite: cytochrome *c* oxidoreductase from *Thiobacillus novellus*. Biochim. Biophys. Acta. 128: 522-534

6. Cleland, W.W. 1964. Dithiothreitol, a new protective reagent for SH groups. Biochem. 3: 480-482

7. Cobley, J.G. and J.C. Cox. 1983. Energy conservation in acidophilic bacteria. Microbiol. Rev. 47: 579-595

8. Deich, R.A. and L.C. Hoyer. 1982. Generation and release of DNA-binding vesicles by *Haemophilus Influenzae* during induction and loss of competence. J. Bacteriol. 152: 855-864

9. Gel filtration theory and practice, a booklet by Pharmacia Fine Chemicals, Uppsala, Sweden. Rahms i Lund, Sweden, 1981

10. Goulbourne, E., et al. 1986. Mechanism of ΔpH maintenance in active and inactive cells of an obligatly acidophilic bacteria. J. Bacteriol. 166: 59-65

11. Grinius, L.. 1980. Nucleic acid transport driven by ion gradient across cell membrane. FEBS Let. 113: 1-10

12. Harrison, A.P. 1984. The acidophilic thiobacilli and other acidophilic bacteria that share their habitat. Ann. Rev. Microbiol. 38: 265-292

13. Hayashi, O. 1974. Molecular mechanisms of oxygen activation. Academic Press, New York. pp 8, 431-435, 441

14. Helenius, A. and K. Simons. 1975. Solubilization of membranes by detergents. Biochim. Biophys. Acta. 415: 29-79

15. Hooper, A.B. and A.A. DiSpirito. 1985. In bacteria which grow on simple reductants, generation of a proton gradient involves extracytoplasmic oxidation of substrate. Microbiol. Rev. 49: 140-157

16. Karavaiko, G.I., and T.A. Pivarova. 1974. Function of surface-membrane structure in *Thiobacillus thiooxidans*. Microbiol. 44: 269-271

17. Karavaiko, G.I. and T.A. Pivovarova. 1973. Mechanism of oxidation of reduced sulfur compounds by thiobacilli. Microbiol. 42: 389-393

18. Kelly, P.D. 1982. Biochemistry of the chemolithotrophic oxidations of inorganic sulfur. Phil. Trans. Royal Soc. London Ser. B298: 499-528

19. Keresen, I. 1984. Methods of Protein Analysis (translated by R.A. Chalmers). Ellis Howard / John Wiley and sons, Chichester, England pp 97-114

20. Kodama, A. 1969. Studies on the metabolism of a sulfur-oxidizing bacteria. VI. Fractionation and reconstitution of the elementary sulfur-oxidizing system of *Thiobacillus thiooxidans*. Plant Cell. Physiol. 10: 645-655

21. Kodama, A. and T. Mori. 1968. Studies on the metabolism of a sulfur-oxidizing bacteria. V. Comparative studies on sulfur and sulfite oxidizing systems of *Thiobacillus thiooxidans*. Plant Cell. Physiol. 9: 725-734

22. Kodama, A., et al. 1970. Studies on the metabolism of a sulfur-oxidizing bacteria. VII. Oxidation of sulfite by a cell-free extract of *Thiobacillus thiooxidans*. Plant Cell. Physiol. 11: 701-711

23. Kuenen, J.G. and O.H. Touvinen. 1981. The genera *Thiobacillus* and *Thiomicrospora*. In The Prokaryotes. Eds. M.P. Starr, et al. Springer Verlag, New York pp 1023-1036

24. Laemli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. Nature. 227: 680-685

25. Layne, E. 1957. Spectrophotometric and turbidimetric methods for measuring proteins. In Methods in Enzymolocy, vol III. Eds. S.P. Collowick and N.O. Kaplan. Academic Press, New York pp 451-454

26. London, J. and S.C. Rittenberg. 1964. Path of sulfur in sulfide and thiosulfate oxidation by thiobacilli. Proc. Nat. Acad. Sci. 52: 1183-1190

27. Lugtenberg, B. and L.V. Alphen. 1983. Molecular architecture and functioning of the outer membrane of *E. coli* and other gram-negative bacteria. Biochim. Biophys. Acta. 737: 51-115

28. Lukow, O.M. 1977. Studies on the sulfur metabolism of *Thiobacillus thiooxidans*. M. Sc. Thesis, The University of Manitoba, Winnipeg, Manitoba.

29. Lyons, D. and T.G. Nickless. 1968. The lower oxy-acids of sulfur. In Inorganic Sulfur Chemistry. Ed. G. Nickless. Elsevier Publishing Co., Amsterdam p 516

30. Lyric, R.M. and I. Suzuki. 1970. Enzymes involved in the metabolism of thiosulfate by *Thiobacillus thioparus*. I. Survey of enzymes and properties of sulfite: cytochrome *c* oxidoreductase. Can. J. Biochem. 48: 334-343

31. Lyric, R.M. and I. Suzuki. 1970. Enzymes involved in the metabolism of thiosulfate by *Thiobacillus thioparus*. II. Properties of adenosine-5'-phosphosulfate reductase. Can. J. Biochem. 48: 344-354

32. Lyric, R.M. and I. Suzuki. 1970. Enzymes involved in the metabolism of thiosulfate by *Thiobacillus thioparus*. III. Properties of thiosulfate-oxidizing enzyme and proposed pathway of thiosulfate oxidation. Can. J. Biochem. 48: 355-363

33. Mackler,B. 1967. DPNH oxidase of heart muscle. In Methods in Enzymology, vol X. Eds. R.W. Estabrook and M.E. Pullman. Academic Press, New York pp 261-262

34. Noguchi, A., et al. 1977. Acidostability of spheroplasts prepared from *Thiobacillus thiooxidans*. Arch. Microbiol. 112: 1647-1652

35. Oh, J.K. and I. Suzuki. 1980. Respiration in chemoautotrophs oxidizing sulfur compounds. In Diversity of Bacterial Respiratory Systems, vol III. Ed. L.J. Knowles. CRC Press. Inc. pp 113-138

36. Oh, J.K. and I. Suzuki. 1977. Isolation and characterization of a membrane-associated thiosulfate-oxidizing system of *Thiobacillus novellus*. J. Gen. Microbiol. 99: 397-412

37. Oh, J.K. and I. Suzuki. 1977. Resolution of a membrane-associated thiosulfate-oxidizing complex of *Thiobacillus novellus*. J. Gen. Microbiol. 99: 413-423

38. Osborn, M.J. 1963. Studies on the gram-negative cell wall. I. Evidence for the role of 2-keto-3-deoxyoctonate in the lipopolysaccharide of *Salmonella typhimurium*. Proc. Nat. Acad. Sci. 50: 499-506

39. Osborn, M.J., et al. 1972. Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. J. Biol. Chem. 247: 3962-3972

40. Peck, H.P. 1968. Energy coupling mechanism in chemolithotrophic bacteria. Ann. Rev. Microbiol. 22: 489-518

41. Roy, A.B. and P.A. Trudinger. 1970. The Biochemistry of Inorganic Compounds of Sulfur. Cambrige University Press, London pp 18-20

42. Schacterle, G.R. and R.L. Pollack. 1973. A simplified method for the quantitative assay of small amount of protein in biologic material. Annal. Biochem. 51: 654-655

43. Schaeffer, W.I. and W.W. Umbrett. 1963. Phosphotidylinositol as a wetting agent in sulfur oxidation by *Thiobacillus thiooxidans*. J. Bacteriol. 85: 492-493

44. Schleif, R.F. and P.W. Wensink. 1981. Practical Methods in Molecular Biology. Springer Verlag, New York p 62

45. Silver, M. and D.G. Lundgren. 1968. Sulfur-oxidizing enzyme of *Ferrobacillus ferrooxidans (Thiobacillus ferrooxidans)*. Can. J. Biochem. 46: 457-461

46. Starkey, R.L. 1925. Concerning the physiology of *Thiobacillus thiooxidans*, an autotrophic bacteria oxidizing sulfur under acid conditions. J. Bacteriol. 10: 135-163

47. Sugio, T., et al. 1985. Role of a specific iron-reducing system in sulfur oxidation of *Thiobacillus ferrooxidans*. Appl. Env. Microbiol. 49: 1401-1406

48. Suzuki, I. 1965. Oxidation of elemental sulfur by an enzyme system of *Thiobacillus thiooxidans*. Biochim. Biophys. Acta. 104: 359-371

49. Suzuki, I. and M. Silver. 1966. The initial product and properties of the sulfur-oxidizing enzyme of thiobacilli. Biochim. Biophys. Acta. 122: 22-33

50. Takanuwa, S. 1975. Studies on the metabolism of a sulfur-oxidizing bacterium. VII. Purification and characterization of soluble components indispensable for sulfur oxidation by *Thiobacillus thiooxidans*. Plant Cell Physiol. 16. 1027 1035

51. Takanuwa, S., et al. 1979. Some properties of cell-sulfur adhesion in *Thiobacillus thiooxidans*. J. Gen. Appl. Microbiol. 25: 21-29

52. Trudinger, P.A. 1969. Assimilatory and dissimilatory metabolism of inorganic sulfur compounds by microorganisms. In Advances in Microbial Physiology, vol 3. Eds. A.H. Rose and J.F. Wilkinson. Academic Press, New York pp 111-152

53. Trudinger, P.A. 1967. The metabolism of inorganic sulfur compounds by thiobacilli. Rev. Pure Appl. Chem. 17: 1-24

54. Trudinger, P.A. 1964. Evidence of a form-sulfur intermediate in thiosulfate oxidation by *Thiobacillus X*. Aust. J. Biol. Sci. 17: 577-579

55. van Die, I., et al. 1983. The influence of phase transition of membrane lipids on uptake of plasmid DNA in *E. coli* transformation. FEMS Microbiol. Let. 18: 127-130

56. Vishniac, W.V. 1974. In Bergey's Manual of Determinative Bacteriology, 8th ed. Eds. R.E. Buchanan and N.E. Gibbons. Williams and Wilkins Co., Baltimore p. 459

57. Vishniac, W. and M. Santer. 1957. The thiobacilli. Bacteriol. Rev. 21: 195-213

58. Waksman, S.A. and J.S. Joffe. 1921. The oxidation of sulfur by microorganisms. Proc. Soc. Exper. Biol. Med. 18: 1-3

59. Wei-Ping Lu. 1986. A periplasmic location for the thiosulfate-oxidizing multi-enzyme system for *Thiobacillus versutus*. FEMS Microbiol. Let. 34: 313-317

60. Wei-Ping Lu and D.P. Kelly. 1984. Purification and characterization of two essential cytochromes of the thiosulfate-oxidizing multi-enzyme system from *Thiobacillus* A_2 *(Thiobacillus versutus)*. Biochim. Biophys. Acta. 765: 106-117

61. Wei-Ping Lu and D.P. Kelly. 1984. Properties and role of sulfite: cytochrome *c* oxidoreductase purified from *Thiobacillus versutus* (A_2). Biochim. Biophys. Acta. 130: 1683-1692

62. Wei-Ping Lu and D.P. Kelly. 1983. Rhodanese: an enzyme not necessary for thiosulfate oxidation by *Thiobacillus* A_2. FEMS Microbiol. Let. 18: 289-292

63. Wei-Ping Lu and D.P. Kelly. 1983. Purification and some properties of two principal enzymes in the thiosulfate-oxidizing multi-enzyme system from *Thiobacillus* A_2. J. Gen. Microbiol. 129: 3549-3564

64. Wei-Ping Lu and D.P. Kelly. 1983. Partial purification and reconstitution of a thiosulfate-oxidizing system from *Thiobacillus* A_2. J. Gen. Microbiol. 129: 1673-1681

65. Wei-Ping Lu and D.P. Kelly. 1983. Thiosulfate oxidation, electron transport and phosphorylation in cell-free systems from *Thiobacillus* A_2. J. Gen. Microbiol. 129: 1661-1671