Inorganic Sulfur Metabolism In The Heterotroph *Pseudomonas aeruginosa*

by

Lawrence B. Schook

A DISSERTATION

Submitted to the Office for Graduate Studies
Graduate Division of Wayne State University
Detroit, Michigan
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

1978

MAJOR: IMMUNOLOGY AND MICROBIOLOGY

Approved by:

R. S. Berk, Ph.D., Advisor — Date 2-9-78

L. Carrick, Jr., Ph.D. — Date

C. D. Jeffries, Ph.D. — Date

M. A. Leon, Ph.D. — Date

B. Zak, Ph.D. — Date

NAME:

MAJOR:

DEGREE:

DATE OF GRADUATION:

APPROVED BY: Richard L Berk

DATE OF APPROVAL: 2-9-78

Abstract

Pseudomonas aeruginosa was grown on a succinate-basal salts medium supplemented with various inorganic sulfur compounds as their sole source of sulfur. The organism was able to grow on the sodium salts of either sulfide, thiosulfate, tetrathionate, dithionite, metabisulfite, sulfite or sulfate, but not on dithionate. Analyses of the culture media after 24 h growth showed the accumulation of sulfate from each of the inorganic sulfur sources other than sulfate. Manometric studies with resting cells obtained by growth on each of the individual sulfur sources yielded net oxygen uptake for each substrate except for sulfite and dithionate. Similar results were obtained with cell-free extracts from these cells using spectrophotometric techniques. Thiosulfate oxidase activity appeared to be induced by growth on sulfide, thiosulfate and tetrathionate, with little or no activity observed when cells were grown on inorganic sulfur sources of higher oxidative states. Metabisulfite oxidase appeared to be associated with growth on all inorganic sulfur compounds. Rhodanese activity appeared to be constitutively present and its activity appeared to be independent of the growth medium employed and was only observed in a soluble fraction. Thiosulfate and tetrathionate oxidase activities were studied in greater detail than the other sulfur oxidases and both were found to be distributed between particulate and soluble fractions.

The soluble form of thiosulfate oxidase was purified 85-fold through ultracentrifugation, ultrafiltration, ion exchange chromatography and gel filtration. Attempts to solubilize the particulate activity by deoxycholate or Triton X-100 treatment were unsuccessful. The partially purified enzyme had a molecular weight of approximately 100,000 with a pH optimum of 6.0-6.5 and an optimum temperature of 37°C when utilizing ferricyanide as electron acceptor. Cytochrome c was utilized as an electron acceptor whereas NAD, NADP, FAD, methylene blue, and 2,6-dichlorophenol-indophenol, failed to replace ferricyanide. The Km values for thiosulfate and ferricyanide were 6.7×10^{-4}M and 1.1×10^{-3}M, respectively. Enzyme activity was inhibited 100% by 1 mM mercuric chloride and 53% by 1 mM pCMB. Sulfite also inhibited the oxidation of thiosulfate.

The uptake of ^{35}S-thiosulfate by resting cells showed typical saturation kinetics and a binding Km of 0.5 mM was determined. The uptake of ^{35}S-thiosulfate per mg dry weight was similar for both thiosulfate and sulfate grown cells. Uptake was stimulated by energy sources and was decreased by Group VI anions, sulfate, and inhibitors of oxidative phosphorylation.

Table of Contents

Acknowledgements

I would like to express my gratitude to each member of the department for helping me complete this dissertation. Particularly, I would like to offer my thanks to the lab for bearing with me through the years.

Most of all, I thank my advisor, Dr. Richard S. Berk, for patience and guidance both personal and professional through my development. I also express gratitude to my committee, Drs. M. A. Leon, C. D. Jeffries, L. Carrick, Jr., and B. Zak for their advice and criticism during the preparation of this dissertation.

A special acknowledgement is extended to my friends - Rick, Debbie, Lee, Cathy, and Karen - who have given much and taken nothing.

This dissertation is dedicated to my family whose constant love and support was always there. And to Louise who gave me a new life.

List of Tables

List of Figures

Introduction

The utilization of inorganic sulfur compounds by plants, microorganisms, and higher life forms has been studied extensively (55, 62, 73). The metabolism of these compounds is characterized by the oxidative and reductive nature of utilization. Peck (54) has recently evaluated the evolutionary significance of inorganic sulfur metabolism, particularly the two cycle system. He suggests that inorganic sulfur metabolism may be a primitive physiological pathway of organisms since reduced inorganic compounds must have been abundant in the primitive anaerobic atmosphere. Hence, an ecological system (Figure 1) evolved between those organisms able to oxidize reduced sulfur compounds and those able to gain energy by reduction of oxidized sulfur compounds. Although much literature (55, 62, 73) exists concerning the biology of organisms that oxidize and reduce inorganic sulfur compounds (Table I), little is known concerning the mechanisms involved in such reactions. Particularly puzzling are the regulatory mechanisms and significance of such systems under present ecological conditions.

The autotrophic utilization of reduced inorganic sulfur compounds allows the bacterium to use carbon dioxide as a sole source of carbon for growth. Heterotrophic organisms which require carbon sources more reduced than carbon dioxide also utilize these sulfur compounds. The thiobacilli, containing both autotroph and facultative autotrophs, are the best studied microorganisms using reduced inorganic sulfur compounds. These organisms are small gram-negative rod-shaped cells

Figure 1. The relationship between various organisms able to reduce or oxidize inorganic sulfur compounds.

SULFUR CYCLE

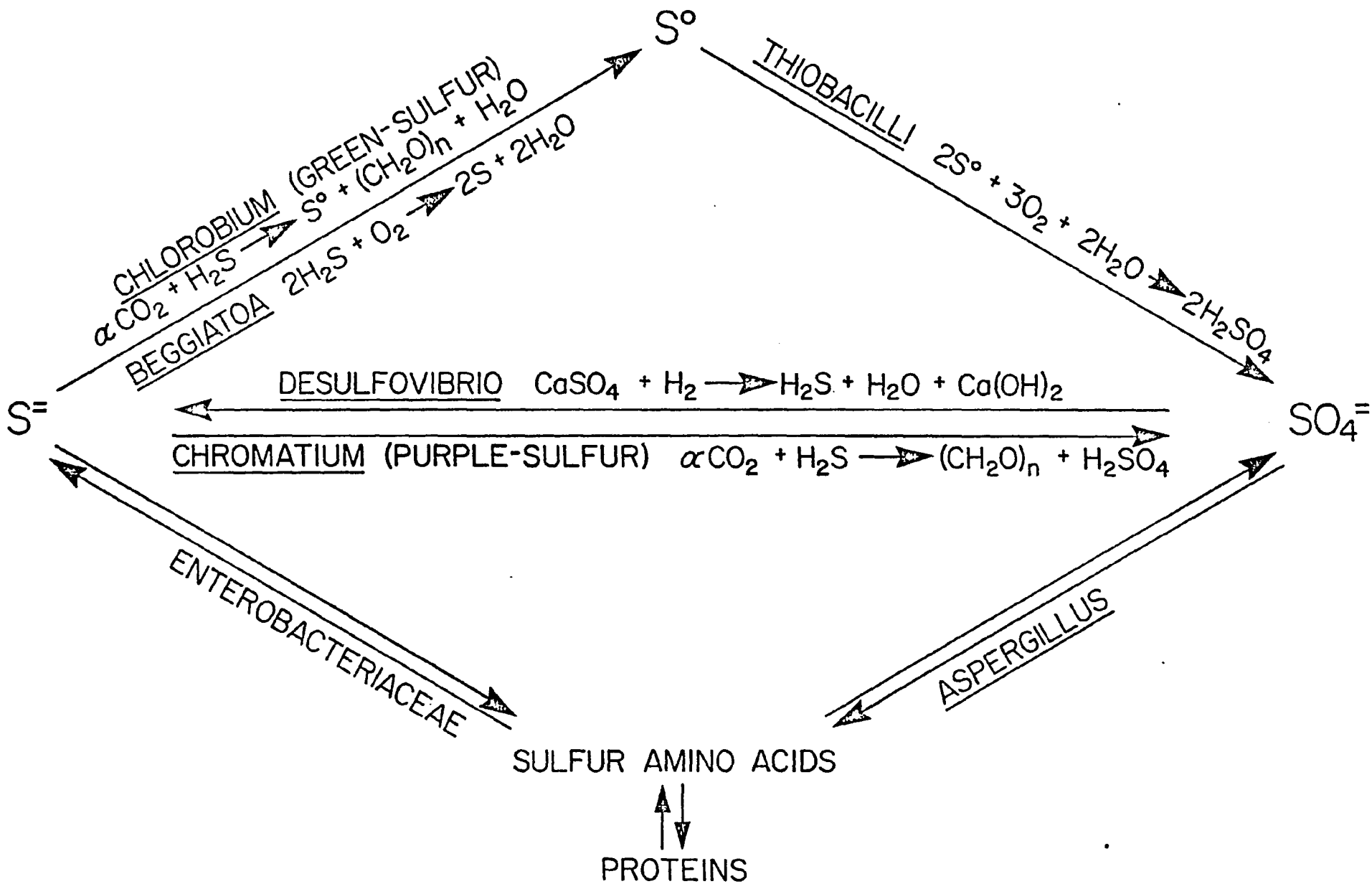


Table I
Nomenclature of inorganic sulfur compounds

$S^{=}$	Sulfide
S^{o}	Elemental sulfur
$S_2O_3^{=}$	Thiosulfate
$S_4O_6^{=}$	Tetrathionate
$S_3O_6^{=}$	Trithionate
$S_5O_6^{=}$	Pentathionate
$S_6O_6^{=}$	Hexathionate
$S_2O_4^{=}$	Dithionite (hydrosulfite)
$S_2O_5^{=}$	Metabisulfite
$S_2O_6^{=}$	Dithionate
$S_2O_7^{=}$	Pyrosulfate (disulfate)
$S_2O_8^{=}$	Persulfate
$SO_3^{=}$	Sulfite
$SO_4^{=}$	Sulfate

which are motile by means of a single polar flagellum. They derive energy from the oxidation of sulfides, elemental sulfur, thiosulfate, polythionates, and sulfite. The final oxidation product is sulfate, but sulfur and polythionates accumulate, sometimes temporarily, under certain conditions. Except for *Thiobacillus denitrificans*, which grows anaerobically with nitrate as an electron acceptor, they are strict aerobes. Thiobacilli are found in sea water, marine mud, soil, fresh water, sewage, sulfur springs and in or near sulfur deposits (83).

Although it has been long recognized that certain heterotrophic organisms such as *Pseudomonas fluorescens*, *P. aeruginosa*, *Bacillus* spp., *Achromobacter stutzeri*, and actinomycetes (62) can oxidize reduced sulfur compounds, only a few enzyme systems have been investigated.

In 1943, Pollock and Knox (57), isolated an enzyme from *P. aeruginosa* involved in the reduction of tetrathionate to thiosulfate. Trudinger (74) also found tetrathionate reductase activity associated with an aerobic gram-negative pleomorphic rod isolated from soil. In addition, this enzyme oxidized thiosulfate under appropriate conditions with tetrathionate as the major product. Hall and Berk (25) also isolated an organism, *Alcaligenes* sp., from soil which oxidized thiosulfate. Like Trudinger's, their purified enzyme had a pH optimum between 6.2 and 6.8, with tetrathionate as the major product. However, they did not investigate whether the enzyme, under physiological conditions, also reduced the product.

Another enzyme involved with heterotrophic sulfur metabolism that has been investigated is rhodanese. Rhodanese (thiosulfate:cyanide sulfur transferase, E.C.2.8.1.1) is involved in the intracellular

turnover of reduced sulfur (62). This enzyme has been detected in many bacteria including the autotrophic thiobacilli (88) and heterotrophic organisms such as *P. aeruginosa*, *Alcaligenes* sp. (27), and *Acinetobacter* sp. (Vandenbergh and Berk, unpublished). The enzyme is believed to be involved in the detoxification of cyanide or the terminal stages of cysteine biosynthesis (62). In addition to its cyanolytic properties, rhodanese has been demonstrated to possess thiosulfate reductase activity (82).

The oxidation of sulfide by thiobacilli has been known for years, but until recently little was known of the mechanisms involved. Suzuki and Werkman (71) showed that sulfide oxidation by *T. thiooxidans* resulted in the accumulation of elemental sulfur. In contrast, others (35, 85) have shown that thiosulfate and polythionates accumulated from both washed cell preparations and extracts of *T. thioparus* and *T. thiooxidans*. Adair demonstrated (4) that extracts of *T. thioparus* and *P. flourescens* boiled for thirty minutes still catalyzed oxidation of sulfide cast doubt as to the biological significance of these oxidations to the physiological activity of the thiobacilli. Moriarty (as cited by 73) has shown that with *T. concretivorus* sulfide oxidation is at least partly enzymatic. Peck's (55) pathway for thiosulfate oxidation also included the role of a sulfide oxidase for oxidation of sulfide to elemental sulfur.

Electron transfer during the aerobic oxidation of sulfide in *T. concretivorus* (42), *T. neapolitanus* C (20), and *T. denitrificans* (7) is coupled to cytochrome reduction. Drozd (20) has shown, using inhibitors, that this oxidation is associated with two sites of oxidative phosphorylation unlike the oxidation of sulfite and thiosulfite

which involves only one site. Spectral analysis by Aminuddin and Nicholas (7) of extracts prepared from *T. denitrificans* support this finding since both cytochrome c and d were reduced during sulfide oxidation.

Evidence now exists for the direct oxidation of sulfide to sulfite resulting in no sulfur, thiosulfate, or polythionate production. Schedel *et al.* (64) have found that *T. denitrificans* contains sulfite reductase. This enzyme was associated with both assimilatory and dissimilatory reductive pathways for sulfate utilization (62). Murphy *et al.* (44) have shown that this enzyme contains a new prosthetic group, siroheme, which catalyzes the six electron reduction from sulfite to sulfide. Schedel *et al.* (64) reported that their purified enzyme with a molecular weight of 160,000 daltons has an absorption spectrum similar to siroheme, and functioned during the oxidation of reduced sulfur compounds.

Although, elemental sulfur, like sulfide, can be oxidized by thiobacilli, little is known concerning this reaction. Suzuki and Werkman (71) showed that a soluble enzyme of *T. thiooxidans* produced polythionates during the oxidation of sulfur. However, this oxidation only occurred if substrate amounts of glutathione were added to the reaction mixture. Subsequently, Suzuki (70) partially purified the soluble enzyme of *T. thiooxidans* such that only catalytic amounts of glutathione were required to oxidize sulfur to thiosulfate. This oxidation strictly required oxygen and attempts to substitute other artificial electron acceptors failed. Furthermore, the glutathione requirement was not replaced by other thiols such as mercaptoethanol, cysteine, or ascorbic acid. Silver and Lundgren (68) also isolated

an enzyme from *T. ferrooxidans* which oxidized elemental sulfur in the presence of catalytic amounts of glutathione. In addition to the soluble-sulfur oxidizing system for *T. thiooxidans*, described by Suzuki (70), Adair (4) showed that this organism also has a cell membrane-associated enzyme which oxidizes sulfur directly to sulfite and sulfate without the addition of glutathione, and without production of thiosulfate or polythionates.

As previously mentioned, all strains of thiobacilli oxidize thiosulfate to sulfate. Because of thiosulfate's relative stability under physiological conditions, it has been the preferred growth substrate for research directed towards the elucidation of inorganic sulfur metabolism in the thiobacilli. The literature shows apparent conflicts for the mechanism(s) in the oxidation of thiosulfate exists. Currently, three major pathways have been proposed (Figure 2) to explain at least part of the experimental observations. The apparent contradictions may be related to species differences or differences in experimental design. Roy and Trudinger (62) have shown that many factors contribute to the production of various intermediates and products in the oxidation of thiosulfate regardless of the organism used. Their work has indicated that the complete oxidation of thiosulfate to sulfate does not always occur, since the amount of sulfate produced is affected by pH, protein concentration, phosphate concentration, and possibly feedback inhibition by certain intermediates (sulfite).

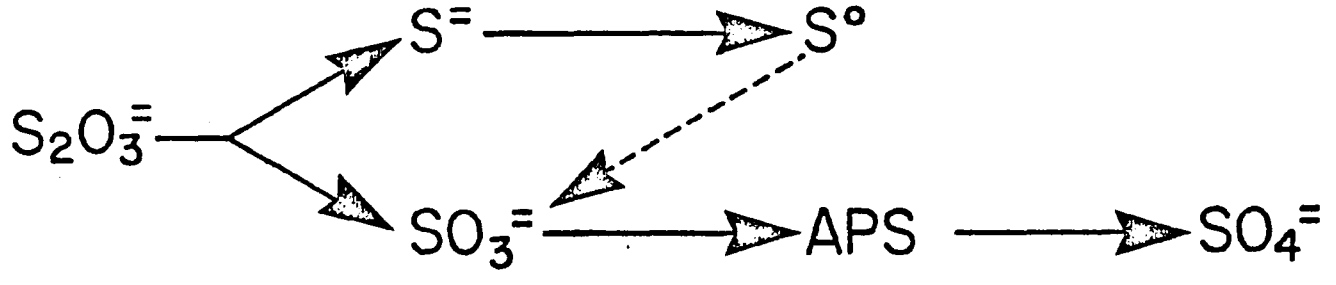
The proposed pathway of London and Rittenberg (35) (Figure 2a) is based primarily on the fact that most thiobacilli produce polythionates during thiosulfate oxidation. Working with cell-free extracts (CFE) of *T. thioparus* and *T. thiooxidans*, they showed that extracts

Figure 2. Proposed pathways for thiosulfate oxidation in *Thiobacilli*.

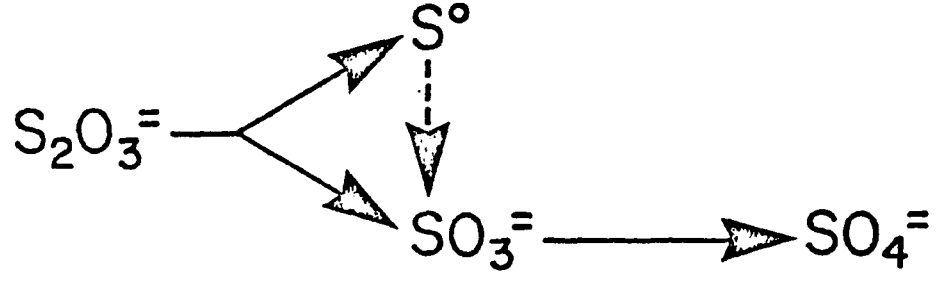
PROPOSED PATHWAYS FOR THIOSULFATE OXIDATION IN *THIOBACILLI*

A. $S_2O_3^= \longrightarrow S_4O_6^= \longrightarrow S_3O_6^= \longrightarrow SO_3^= \longrightarrow SO_4^=$

B.



C.



catalyzed the complete oxidation of sulfide, thiosulfate, tetrathionate, and trithionate to sulfate. They found the rates of oxidation were uniform and that the addition of cofactors, primers, or reducing agents were not required for oxidation. Dithionate, however, could not be oxidized by these extracts. The ability of thiobacilli to accumulate and oxidize polythionates during sulfide and thiosulfate oxidation supported London and Rittenberg's (35) postulated pathway in which thiosulfate is oxidized via the lower polythionates to sulfate.

Sinha and Warren (67) also detected polythionates during the oxidation of thiosulfate by *T. ferrooxidans*. By using ^{35}S thiosulfate labelled in the sulfane atom (outer position), they found that tetrathionate was labelled at the inner or sulfonate sulfur atom, as was trithionate, with ^{35}S-sulfate being formed. This finding, however, must be viewed cautiously since Roy and Trudinger (62) showed evidence that sulfate is preferentially formed from the sulfonate group of thiosulfate rather than the sulfane atom. Again, evident contradictions exist since Trudinger (73) himself found that during initial thiosulfate oxidation by intact *T. neapolitanus*, only two molecules of sulfate were formed from the sulfonate atom and at least one molecule from the sulfane atom. He suggested that two molecules of thiosulfate interact during the oxidation of thiosulfate but that tetrathionate was not the product of this interaction. Observations by Kelly and Syrett (32) also showed that ^{35}S-sulfate was more rapidly formed from S-^{35}S-thiosulfate than ^{35}S-S-thiosulfate by *Thiobacillus* strain C.

Tetrathionate is the most common polythionate to accumulate during the oxidation of thiosulfate, and according to London and Rittenberg,

(35), Trudinger (75), and Hall and Berk (25), is stoichiometrically produced by this direct oxidation. Trithionate and pentathionate, are also formed during the oxidation of thiosulfate but accumulate in varying amounts. Roy and Trudinger (62) state the extent of polythionate production is related to species or strain of organism tested, is stimulated by low phosphate levels and suppressed by high levels, and is a function of the concentrations of bacteria, substrate, and oxygen in the medium. These variables were shown by DeLey and VanPoucke (17) to determine the extent of thiosulfate oxidation to sulfate. The ultimate amount of thiosulfate oxidized dependent on the ratio of bacteria to the concentration of thiosulfate. A high ratio resulted in a rapid oxidation to sulfate, whereas, a low ratio resulted in thiosulfate remaining and sulfite accumulating. They found 10^{-5} to 10^{-3} M sulfite depressed activity in cell suspensions oxidizing thiosulfate. Other (38) experiments have shown this inhibition to be irreversible.

Vishniac (86) postulated that both trithionate and pentathionate are produced by the chemical disproportionation of tetrathionate which was enhanced by the presence of thiosulfate, allowing two nucleophilic displacement reactions to occur. The first between thiosulfate and tetrathionate producing pentathionate and sulfite; the second between tetrathionate and sulfite forming trithionate and thiosulfate. Kelly and Syrett (32), however, presented an alternative hypothesis for trithionate formation based on enzymatic reactions. They showed that trithionate was formed by an oxidative reaction: (1) between sulfite and thiosulfate and (2) between thiosulfate and tetrathionate. They were able to account for the distribution of ^{35}S within trithionate

using ^{35}S-thiosulfate. Their results are consistent with a mechanism of thiosulfate oxidation based on an initial reductive cleavage of the molecule and with a mechanism in which thiosulfate undergoes an initial oxidative reaction.

In 1966, Charles and Suzuki (12) presented evidence against the polythionate pathway of London and Rittenberg (Figure 2c). They found that extracts of *T. thioparus* oxidized thiosulfate to sulfate but failed to oxidize tetrathionate. They suggested that the organism used rhodanese, the enzyme involved in cyanolysis, to reductively cleave thiosulfate to "sulfur" and sulfite with the subsequent oxidation of these products by "sulfur-" and sulfite-oxidizing enzymes, respectively. Trudinger (75) has suggested that two different enzymes may be present. Rhodanese is greatly inhibited by thiol-binding reagents such as mercuric chloride, mercurochrome, and iodoacetic acid; whereas his soluble thiosulfate-oxidizing enzyme was not affected by these compounds.

A unique pathway involving *T. thiocyanoxidans* was described by Youatt (89) and like that of Charles and Suzuki (12) does not include tetrathionate yet involves polythionate intermediates. Youatt showed that during thiocyanate degradation by this organism sulfide is formed; the oxidation of sulfide to sulfate then resulted in the production of thiosulfate, metabisulfite and pyrosulfite.

Finally, Peck (55) has proposed (Figure 2b) that thiosulfate, in the presence of glutathione, AMP, and extracts of *T. thioparus* is reduced to sulfide and sulfite. These products are then oxidized to elemental sulfur and sulfate by sulfide-oxidase and adenine 5'-sulfatophosphate (APS)-reductase, respectively. Peck based this pathway on his observation that unsupplemented extracts of *T. thioparus* only

oxidized thiosulfate to polythionates and not to sulfate as an end product. However, the supplementation of extracts prepared by Charles and Suzuki (12) from *T. novellus* with glutathione inhibited the oxidation of thiosulfate. Peck's hypothesis also conflicts with observations made by London and Rittenberg (35), Kelly and Syrett (32), and Trudinger (74), because these investigators were unable to detect any elemental sulfur during the oxidation of thiosulfate.

Moreover, less information is known regarding the oxidation of polythionates as compared to that of thiosulfate. Although many thiobacilli are known to oxidize tetrathionate and trithionate to sulfate (37, 75), the pathways involved are still unknown. Although penta- and hexa-thionates are oxidized by *T. thiooxidans*, dithionate is not utilized (35). Cook (15) has stated, however, that dithionate oxidation by *T. denitrificans* occurred if thiosulfate was present. The effect of thiol-binding agents on tetrathionate and trithionate metabolism was evaluated by Trudinger (75). He found thiol groups are necessary for polythionate oxidations as opposed to thiosulfate oxidation. This finding explains how, as earlier stated, the production of polythionates is related to oxygen concentrations in the medium. The maintenance of reduced thiol groups is essential for polythionate oxidation to sulfate whereas thiosulfate oxidation to tetrathionate may occur.

At present, there are two pathways involved in the oxidation of sulfite by the thiobacilli. The first mechanism was reported to involve the formation of APS from AMP and sulfite by the enzyme APS-reductase (55, 56). The APS is then converted to sulfate and ATP by ADP-sulfurylase with the regeneration of AMP by adenylate kinase.

The specific properties of purified APS reductases from *T. denitrificans* (10) and *T. thioparus* (1, 38), showed it to be soluble to use both oxygen and ferricyanide as electron acceptors and apparently not to be inhibited by sulfhydryl-binding compounds. The formation of APS rather than sulfate directly is referred to as the AMP-dependent sulfite oxidase pathway. This pathway also is supported by the fact that energy is generated by substrate level phosphorylation through sulfite oxidation by thiobacilli.

However, other workers (13, 14, 35, 37) have shown that an enzymatic conversion of sulfite to sulfate was not dependent on AMP and thus involved a separate mechanism. The purifications of soluble sulfite oxidases from *T. novellus* (13) and *T. thioparus* (37) has shown both to require ferricyanide or cytochrome c as electron acceptors. Some investigators have also found AMP-independent sulfite oxidase activity associated with the particulate or cell-membrane fragments from *T. thiooxidans* (4) and *T. concretivorus* (42). More recently, Aminuddin and Nicholas (6) have evaluated the oxidation of sulfite in *T. denitrificans*. They have shown that sulfite was oxidized either to APS in the soluble fraction or to sulfate in the membrane fraction of *T. denitrificans*. The particulate enzyme was further purified 50-fold through solubilization with deoxycholate. The membrane-bound sulfite oxidase was not stimulated by AMP and used nitrate, oxygen, or ferricyanide as an electron acceptor. Studies using inhibitors of oxygen uptake (potassium cyanide, azide, iodoacetamide, and arsenite) indicated that an electron transport chain is involved during AMP-independent sulfite oxidation. The purified enzyme used only ferricyanide as an electron acceptor and neither nitrate nor

oxygen were reduced. Thiol-binding reagents, cyanide, and azide were potent inhibitors, which suggested a requirement for sulfhydryl groups and for the presence of a terminal oxidase. The inhibited enzyme was reactivated by dithiothreitol treatment. This AMP-independent sulfite oxidase had an optimum pH of 8.3 with a Km of 0.5 mM for sulfite.

Peck's (56) original evaluation of APS reduction and sulfite oxidation estimated that approximately 0.5-0.6 μmoles of substrate were metabolized per hr per mg protein. Much concern was expressed since these values were too low to account for sulfur metabolism in the intact bacterium; particularly since Charles' and Suzuki's (13) AMP-independent sulfite oxidase of *T. novellus* produced 60 μmoles per hr per mg. Peck's hypothesis, however, was further supported by Bowen *et al.* (10), who found the specific activity for APS reductase to be 498 μmoles ferricyanide reduced per hr per mg compared to 183 μmoles reduced by AMP-independent sulfite oxidase (6). Aminuddin and Nicholas (7) have shown that both the soluble and particulate fractions involved in "sulfite oxidation" are linked to electron transfer, suggesting that at least in *T. denitrificans*, oxidation of sulfite to sulfate can proceed via both the AMP-dependent and independent pathways.

The assimilation of inorganic sulfur compounds into organic cellular components is a phenomenon long recognized (62). The binding and transport of sulfate in *Salmonella typhimurium* has been studied extensively by Pardee *et al.* (50, 51, 52, 53). His results suggested that bacteria possess highly specific binding sites on the cell surface. The binding of sulfate was saturable at 4 μM and structurally similar anions specifically inhibited binding and transport. Pardee (52) showed, using mutants unable to grow on sulfate or thiosulfate,

that these anions cannot enter the cell. Pardee (53) showed that osmotic shock caused loss of the ability to bind sulfate and he purified the sulfate-binding protein. This protein had an unusual amino acid composition in that it contained no sulfur amino acids. Sulfate binding was dependent upon the ionic strength, and required calcium, tempting Pardee to speculate that calcium provides the positive charge necessary for binding at the active sites.

The active transport of sulfate (50) was also depressed by growth on cysteine, and could be inhibited by preventing energy production. Pardee (50) calculated that there were approximately 10^4 sulfate-binding proteins per *S. typhimurium*. Dreyfuss (19) also showed evidence for a sulfate transport system in *Salmonella* sp. which catalyzed thiosulfate transport. Deyrup (18) also showed using mammalian kidney that uptake of sulfate ions was inhibited by thiosulfate, suggesting that both types of ion bind to a common site. The uptake of sulfate in autotrophic bacteria has been demonstrated in *Desulphovibrio desulphricans* (24) and *T. ferrooxidans* (79). Kelley *et al.* (31) has described an active transport system in thiosulfate grown *T. ferrooxidans* where the outer ^{35}S from differentially labelled thiosulfate was incorporated into cell protein. Sulfate was also rapidly bound by these cells but unlike thiosulfate, ^{35}S-sulfate was completely removed from the thiosulfate-grown cells by washing. In addition, the sulfate was not incorporated into thiosulfate grown cells. The uptake of thiosulfate could be inhibited by structurally similar anions such as molybdate, tungstate, and selenated, but was enhanced by cysteine and glucose.

Perhaps the phenomenon most difficult to explain is the uptake

of elemental sulfur. Roy and Trudinger (62) presented two hypotheses for the uptake of insoluble sulfur by thiobacilli: (1) that sulfur may react with chemicals or enzymes released by the organism with the formation of soluble sulfur compounds or (2) that sulfur and a cellular component may react at the cell surface. The first hypothesis was supported by the fact that surface active agents such as phosphatidyl inositol are released by the bacteria which "solubilize" the sulfur. The second, by the demonstration that contact between the substrate and bacteria was essential for utilization.

Induction and repression of inorganic sulfur oxidation has been somewhat limited because of the chemoautotrophic nature of thiobacilli. Santer *et al.* (63) studied the facultative autotroph, *T. novellus*, which grown in heterotrophic media or in simple salts media containing carbon dioxide and inorganic sulfur-containing compounds, in an attempt to study induction of activity. They found that a lag period in growth occurred when the organism was sub-cultured from a glutamate-citrate medium to a thiosulfate medium, or vice-versa. They felt that this induction period was required for enzyme synthesis since manometric studies showed that autotrophically grown cells could oxidize both thiosulfate and tetrathionate whereas heterotrophically grown cells oxidized neither. Gutierrez and Ruiz-Herrera (as cited by 62) have found that the ability of *T. ferrooxidans* to oxidize elemental sulfur was induced by growth on sulfur.

Aleem (5) studied thiosulfate oxidation and electron transport in a soluble CFE preparation of *T. novellus*. He showed that both thiosulfate oxidase and thiosulfate:cytochrome c reductase were induced by growth on mineral media containing thiosulfate, and were

markedly reduced in heterotrophically grown cells. Vishniac and Trudinger (84) more specifically, demonstrated that the enzyme responsible for oxidation of thiosulfate to tetrathionate by *T. novellus* was induced by thiosulfate.

Catabolite repression of thiosulfate oxidation in *T. novellus* was studied by LeJohn *et al.* (34). They found that fermentable carbon sources caused repression of all enzymes implicated in thiosulfate oxidation in this facultative chemoautotroph. Glucose caused a strong repression while glycerol, lactate, lactose, ribose, and pyruvate exhibited less severe repression. In all cases, the repression was relieved by returning the organism to fresh medium with no fermentable substrates. Aerobically metabolized substrates, amino acids and organic acids, caused only temporary or no repression of the thiosulfate oxidative system. All enzymes believed to participate in thiosulfate oxidation, except tetrathionase, were found simultaneously induced or repressed, suggesting that tetrathionate is not a necessary intermediate in the thiosulfate-oxidation pathway of *T. novellus*.

Enzymatic comparisons between autotrophic and heterotrophic inorganic sulfur metabolism with *T. ferrooxidans* showed repression of thiosulfate oxidase activity. Tuovinen *et al.* (78) found no thiosulfate-oxidizing enzymes when the organism was grown autotrophically on ferrous iron and that high levels of thiosulfate-oxidizing enzyme were found with heterotrophic growth (glucose) suggesting that the enzyme was not induced by thiosulfate. Equal amounts of thiosulfate oxidizing enzyme were found in both soluble and particulate fractions when grown autotrophically with thiosulfate; however, approximately ten times more soluble enzyme than particulate was observed if the

cells were grown heterotrophically. The authors suggested that thiosulfate oxidizing enzyme is not essential for thiosulfate oxidation since another route for the oxidation via its cleavage to sulfide and sulfite (rhodanese) exists. This hypothesis was further supported by the high levels of AMP-independent sulfite oxidase levels which were seen when *T. ferrooxidans* was grown autotrophically with thiosulfate.

The metabolic regulation of inorganic sulfur oxidation in heterotrophic organisms has received even less attention than the thiobacilli. Trudinger (74) studied the oxidation of thiosulfate to tetrathionate by two heterotrophic organisms isolated from the soil. In one organism, the oxidizing activity was constitutive whereas in the other, activity was induced by thiosulfate or tetrathionate. Hall and Berk (25) detected thiosulfate oxidase, rhodanese, sulfite oxidase, and APS-reductase in crude CFE from an *Alcaligenes* sp. grown on mercaptosuccinate (MS). However, only thiosulfate oxidase which resulted in tetrathionate formation appeared to be induced by growth of the organism on MS as sole source of carbon and sulfur.

Palumbo (48, 49) has shown that sulfite affected the growth, pigment production, and slime formation of *P. aeruginosa*. He showed that sulfite regulates these secondary metabolites, rather than sulfur containing amino acids, thiosulfate, sulfate, or sulfide. The mechanism by which sulfite stimulated the fluorescent pigment and slime was interesting since neither contained any detectable amount of sulfur. The inhibition of *P. aeruginosa* growth by sulfite appeared to be a function of the medium pH. At pH 7.0-7.5, no growth (or subsequent pigment production) was observed; at pH 7.5 to 9.0, growth and pro-

duction of pigment and slime were observed. Palumbo (48, 49) believed that the inhibition of growth between pH 7.0-7.5 was due to the formation of bisulfite (HSO_3^-) from sulfite which is extremely inhibitory to most bacteria.

Because of the paucity of reports on "autotrophic sulfur metabolism" by heterotrophic organisms, the present investigation was initiated to further characterize the nutritional and metabolic properties of these organisms. Our study deals with the heterotroph *Pseudomonas aeruginosa* which has been shown previously to be capable of oxidizing reduced inorganic sulfur compounds (25, 74). Like the thiobacilli, *P. aeruginosa* is a gram-negative rod-shaped organism, motile by means of a single polar flagellum, and shares similar environmental habitats. Umbreit (81) stated that strict autotrophs, e.g., thiobacilli, are adapted to live in what is essentially a toxic environment and that it has, therefore, so changed its permeability properties that all but a few essential materials are excluded. Why then can heterotrophic organisms such as pseudomonas also survive in these environments? The answer to this question is perhaps the exceptional metabolic diversity and ability of *Pseudomonas* spp. to adapt to a wide range of environmental conditions (60). Biochemically, *P. aeruginosa* has been shown to utilize many inhibitory compounds such as benzalkonium chloride (3), centrimide (36), chlorohexidine (36, 61), and hexachlorophene (8) allowing this bacterium to contaminate hospital environments. The ubiquitous nature of this organism and its ability to utilize many compounds, both inorganic and organic, suggests that it can adapt to many toxic conditions. This adaptability appears particularly significant since some investigators feel that the soil heterotrophs

may play the dominant role in the oxidation of reduced soil sulfur (85).

The work of Jackson *et al.* (29) suggest using G+C% composition analysis of DNA, that a possible relationship exists between *T. trautweinii* (66%), *T. novellus* (66%), *T. denitrificans* (64%), *T. thioparus* (66%) and certain pseudomonads, e.g., *P. aeruginosa* (64-66%) and *P. flourescens* (60-65%) which oxidize sulfur compounds. They concluded it would be interesting to study pseudomonads and their relationship to the thiobacilli. We also feel that a further investigation into the ability of the heterotroph, *P. aeruginosa*, to oxidize inorganic sulfur compounds is warranted. Richmond and Clarke (60) have been interested in the evolutionary potential of this organism. Pseudomonas, they state is very flexible genetically and can carry more than one plasmid. The significance of plasmids in the organisms' evolutionary development is exciting, for *P. aeruginosa* (16, 47) has been shown to exchange plasmids with such soil organisms as *Rhodospirillium* sp., *Rhodopseudomonas* sp., *Rhizobium* sp., *Azotobacter* sp., and *Acinetobacter* sp.

The purpose of this study then is to determine the ability of *P. aeruginosa* to oxidize various inorganic sulfur compounds and to investigate the nutritional requirements necessary for these reactions. Concomitantly, the purification and characterization of a key enzyme such as thiosulfate oxidase will also be initiated. This characterization of the physiology of inorganic sulfur oxidation by *P. aeruginosa* should aid in furthering our understanding of the relationship between pseudomonads and other soil organisms and the significance of such metabolic diversity in the organisms' adaptability to "toxic" environments.

Materials and Methods

A. Organism: The organism used throughout these studies was *Pseudomonas aeruginosa* ATCC 17934. The original culture was stored in 2X Skim Milk (Difco, Detroit, MI) at -70°C in a Revco freezer. Working cultures were grown on Tryptose Agar (Difco, Detroit, MI) slants and kept at room temperature.

B. Media: The organism was grown in a 1% (W/V) sodium succinate sulfur free-basal salts medium which was singly supplemented with various inorganic sulfur sources. The basal salts medium contained (per 1): K_2HPO_4, 0.75 gm; KH_2PO_4, 0.25 gm; NH_4Cl (J. T. Baker Chemical Co.) 5 gm; $MgCl_2 \cdot 6H_2O$, 0.5 gm; NaCl, 0.5 gm; $FeCl_3 \cdot 6H_2O$, 10 µg; $CaCl_2 \cdot 2H_2O$, 2 µg. Fisher Scientific Co. was the source of all chemicals unless otherwise noted. The pH of the medium was adjusted aseptically after autoclaving with either 0.1 N HCl or NaOH to pH 7.0. The sodium succinate or mercaptosuccinate (MS), and sulfur sources were filter sterilized using a 0.22 µm Millipore filter and added aseptically to the autoclaved basal salts solution.

C. Growth Conditions: The organism was inoculated into the succinate-basal salts solution and incubated for 18 h at 35°C on a New Brunswick aerating shaker. The resulting cultures were either used to study initial growth factor requirements or used to reinoculate

media under similar conditions.

D. Resting Cells: For experiments requiring resting cells, the culture was centrifuged at 10,000 X G for 15 min at 4°C. The resulting cell pellet was suspended in the basal salts solution without a carbon and sulfur source and re-centrifuged. The resultant cell pellet was again suspended in the basal salts solution prior to its use in assay procedures.

E. Chemicals: The following inorganic sulfur sources were used to supplement the succinate-basal salts medium: $Na_2S_4O_6$, sodium tetrathionate (K & K Laboratories, Plainview, NY); $Na_2S \cdot 9H_2O$, sodium sulfide (Mallinckrodt Chemical, St. Louis, MO); $Na_2S_2O_5$, sodium bisulfite (General Chemical, New York, NY): Na_2SO_3, sodium sulfite; $Na_2S_2O_6 \cdot 2H_2O$, sodium dithionate; $Na_2S_2O_4$, sodium hydrosulfite (dithionite); $Na_2S_2O_3 \cdot 5H_2O$, sodium thiosulfate; Na_2SO_4, sodium sulfate; and S, elemental sulfur (sublimed) (Fisher Scientific Co., Fairlawn, NJ). The mercaptosuccinate (MS) was obtained from Nutritional Biochemical Co., Cleveland, Ohio. The highest available grade of purity for each compound was used.

F. Preparation of Cell-Free Extracts: Cell-free extracts (CFE) were prepared by passing cell suspensions, in 0.1 M phosphate buffer (pH 7.5), through a French pressure cell at 16,000 psi. All cell suspensions were treated twice and whole cells and debris were removed by centrifugation at 10,000 X G for 10 min prior to assay.

G. Manometric Techniques: Oxygen uptake was measured in a Warburg apparatus (Gilson Medical Electronics, Middleton, WS), using standard methods described by Umbreit (80). The manometry flasks contained 0.2 ml of 20% KOH, 0.2 ml of substrate, 1.0 ml of cells of CFE, and 1.8 ml of 0.1 M, phosphate buffer (pH 7.5). Substrate (auto-oxidation) and endogenous controls were routinely employed with each experiment.

H. Protein Determination: The protein concentration of CFE was determined using the micro-biuret method of Koch and Putnam (33). Armour bovine serum albumin was used as the standard.

I. Sulfate Assay: The amount of soluble sulfate accumulated during growth of the organism with the various inorganic sulfur compounds, with resting cells, or CFE was determined by the method of Berglund and Sörbo (9). Standard curves were used to determine the extent ot interference of these compounds on the determination of free sulfate.

J. Sulfide Assay: Quantitative determinations of free sulfide were made using the method described by Siegel (66). The extent of interference by media constituents on the measurement of sulfide was also determined.

K. Preparation of Cell Fractions: The crude CFE (see above) was fractionated at 4°C by differential centrifugation as outlined by Tuovinen *et al.* (78). A Sorvall RC-2 was used for the 10,000 X G

centrifugation and a Beckman model L5-50 ultracentrifuge (rotor type SW 50.1) for the 108,000 and 150,000 X G fractions.

L. Solubilization: The particulate fractions (P_{150}) obtained after ultracentrifugation were treated to solubilize thiosulfate or tetrathionate-oxidizing activity from the cell membranes. Treatment was either with 2% V/V Triton X-100 (41) or 1 mg/mg protein sodium deoxycholate (58).

M. Assay of Thiosulfate-Oxidizing Enzyme: Enzyme activity was assayed in micro-Thunberg tubes by following the reduction of ferricyanide as described by Trudinger (74). The standard assay mixture contained in 3.0 ml was: 3.0 µmole $K_3Fe(CN)_6$ Fisher Scientific Co.), 20 µmoles $Na_2S_2O_3$, 300 µmoles potassium phosphate buffer (pH 6.2) and and 0.2 ml of CFE. The assay system was mixed and the reaction initiated by tipping in the substrate from the upper reservoir of the Thunberg tube. Initial attempts to use this assay system in an aerobic environment were difficult to interpret because of non-enzymatic oxidation of thiosulfate. Therefore, before initiating the reaction, the sealed tubes were gassed with nitrogen (10 alternate cycles of evacuation and gassing using a three-port manifold and an aspirator pump). Ferricyanide reduction was followed at 420 nm in a Beckman DB spectrophotometer (10 mm light path) and the activity was recorded as absorbency change over a 5 min period. Controls consisted of a complete assay system minus either the CFE, potassium ferricyanide, or thiosulfate. One unit of enzyme was defined as the amount of enzyme required to reduce 1 µmole of ferricyanide per min under standard

assay conditions.

N. Assay of AMP-Independent Sulfite Oxidase: Sulfite Oxidase (sulfite:oxygen oxidoreductase, E.C.1.8.3.1) was detected by a modification of the method of Charles and Suzuki (14). The enzyme activity was determined by following the rate of reduction of ferricyanide at 420 nm using a Beckman DB spectrophotometer. Standard assay mixtures contained, in a 3 ml volume, 3 μmoles $K_3Fe(CN)_6$, 20 μmoles of Na_2SO_3, 150 μmoles tris (hydroxymethyl) aminomethane (Tris-HCl) buffer (pH 7.6) and 0.2 ml of the CFE preparation. Assays were performed in micro-Thunberg tubes under an atmosphere of nitrogen. One unit of enzyme was defined as the reduction of 1 μmole of ferricyanide per min under the standard assay conditions.

Sulfite oxidase activity was also measured by the method of Johnson and Rajagopalan (30). The enzymatic activity was determined by following the rate of reduction of cytochrome c (horse heart, type VI, Sigma Chemical Co., St. Louis, MO) at 550 nm using a Beckman DB spectrophotometer. Micro-Thunberg tubes contained 0.25 ml of CFE preparation, 0.04 mM ferricytochrome c, 0.4 mM sodium sulfite, and 0.1 M Tris-HCl buffer (pH 8.5) to a final volume of 2.5 ml. One unit of sulfite oxidase activity was defined as the amount of enzyme necessary to produce an absorbance change of 0.13/min which corresponds to 1 μmole of cytochrome c reduced per min.

The procedure of Aminuddin and Nicholas (6) was also used for assaying the presence of sulfite oxidase. The reaction mixture contained 10 μmole Na_2SO_3, 5 μmole $K_3Fe(CN)_6$, and enzyme in a final volume 3.0 ml made up with 50 mM Tris-HCl (pH 8.3) containing 0.2 mM NaEDTA.

The assays were performed as previously described (see above).

O. APS Reductase Assay: Adenosine 5'-phosphosulfate (APS) reductase (EC 1.8.99.2) activity was determined with ferricyanide or cytochrome c as the electron acceptor as described by Adachi and Suzuki (1). These assays were performed in Thunberg tubes under a nitrogen atmosphere to reduce non-specific oxidation.

P. Assay for Sulfide Oxidase, Tetrathionate Oxidase, Dithionite Oxidase, Metabisulfite Oxidase, and Sulfate Oxidase Activity: Enzyme activity was measured as previously described for the thiosulfate-oxidizing enzyme by following the reduction of ferricyanide at 420 nm under a nitrogen atmosphere with the respective sulfur source.

Q. Rhodanese Assay: Rhodanese (thiosulfate:cyanide sulfur-transferase, E.C.2.8.1.1.) assays were performed on the CFE from cells grown with the various inorganic and organic sulfur sources according to the method of Sörbo (69). One rhodanese unit (R.U.) is defined as that amount of enzyme which forms 10 microequivalents of thiocyanate (one microequivalent of thiocyanate equals 0.104 O.D. at 460 nm) after 5 min.

R. Sulfatase Assay: Sulfatase (E.C.3.1.6.1.) activity was measured by the procedure outlined by Rammler *et al.* (59). Both cells induced for growth on each of the inorganic sulfur compounds and those sulfate-starved were assayed.

S. Radioactive Studies:

1. Binding and Uptake of Thiosulfate.

Cells from induced cultures were washed and suspended in the inorganic sulfur-free basal salts medium. The washed cell suspensions were standardized by dry weight determinations (105°C for 6 h). The properties of the uptake system were studied by pre-incubating the cell suspensions for 5 min before adding the labelled ^{35}S-thiosulfate using a reciprocating water bath at 37°C. After a further incubation (1-120 min), 100 µl samples were filtered (Millipore GSWP, GS 0.22 µm, 25 mm) using a Millipore 3025 Sampling Manifold (No. XX2702530) and washed with 5 ml of cold (4°C) basal salts solution. For the washing experiments, ^{35}S-thiosulfate labelled cells were collected by centrifugation at 19,000 rpm for 2 min and the radioactivity in the supernatant fraction, after successive washings, was determined.

2. Effect of Thiosulfate Concentration on Uptake.

The incubation mixtures were as described above except that the ^{35}S-thiosulfate (outer) and cold thiosulfate were increased from 0.125-4.0 mM with respect to the standardized washed cells. After incubation, a 100µl sample was removed, filtered, and washed with 5 ml of cold basal salts. Dilutions of the isotope stock were also made to determine activity (CPM/µmole thiosulfate). The specific activity was expressed as nmoles of thiosulfate incorporated per mg of dry weight.

3. Effect of Various Compounds on Thiosulfate Uptake.

Various compounds were added to the washed cell suspensions and incubated for 5 min in a reciprocating water bath at 37°C prior to the addition of 0.5 mM ^{35}S-thiosulfate (0.5 µCi/µmol). After an additional

30 min incubation, 100µl samples were removed, filtered, and washed with 5 ml of cold basal salts.

4. Radioassay of Labelled Compounds.

The filtered radioactive samples were dried at 25°C and placed in 20 ml scintillation vials. The filters were immersed in 5 ml of scintillation fluor (Spectrofluor, Amersham/Searle, Arlington Heights, IL) containing 4 g POP and 50 mg POPOP per 1 of toluene (scintillation grade, Research Products, International Corp., Grove Village, IL). The vials were counted in a Packard Tricarb 3255 liquid scintillation spectrometer. Channel ratios allowed an 80% efficiency of counting.

5. Radiochemicals.

Sodium ^{35}S-thiosulfate labelled at the inner sulfur atom (S.A. 12.5 mCI/µmol) and the outer sulfur atom (S.A. 10.3 mCi/µmol) were obtained from Amersham/Searle. The purity of these isotopes was 98% and 95%, respectively.

T. Purification of Thiosulfate Oxidase: All fractionation steps were performed at 4°C. Reduction in volume of the various enzyme fractions was accomplished using Aquacide II (Cal Biochem). Following each fractionation procedure, an aliquot was removed and assayed for oxidase activity and protein concentration.

Step 1. Ultracentrifugation: The crude CFE (see Preparation of CFE) were centrifuged in a Beckman ultracentrifuge at 35,000 rpm (approximately 145,000 X G) for one h. The fractions were designated as Supernatant 150 (S_{150}) or Pellet$_{150}$ (P_{150}).

Step 2. DNase and RNase Treatment: The S_{150} fraction was enzymatically treated for the removal of nucleic acids. Deoxyribonuclease I (DNase I, B grade, bovine pancreas) and ribonuclease (RNase, A grade, bovine pancreas) both obtained from Calbiochem (La Jolla, CA) were added at 5 µg/ml fo the, S_{150} fraction. The mixtures were incubated for 60 min at 4°C.

Step 3. Ultrafiltration: The S_{150} fraction, after DNase I and RNase treatment, was added to a 50 ml Diaflo Ultrafiltration cell (Amicon, Lexington, MA) fitted with either an XM100A or XM300 ultrafiltration membrane. The S_{150} fraction was then washed with 5 volumes of 50 mM phosphate buffer (pH 7.0) under 10-15 psi of nitrogen.

Step 4. Ion Exchange Chromatography: The concentrated enzyme preparation was adsorbed to a column of DEAE-Sephadex A-25 equilibrated with 100 mM Tris-HCl buffer (pH 7.6). After the enzyme sample had been allowed to equilibrate for 30 min, the column was washed with the Tris-HCl buffer until no more unadsorbed protein could be eluted. Subsequent elutions were performed using a stepwise gradient of sodium chloride from 0.1 M to 0.8 M in 100 mM Tris-HCl buffer (pH 7.6). The chromatography was performed using a refrigerated fraction collector (Gilson Medical Electronics Corp.) equipped with a drop counter and an ultraviolet monitor (Instrument Specialties Co.). The chromatographic fractions (5 ml) containing the desired enzymatic activity were pooled, concentrated to approximately 10 ml, and dialyzed overnight against 100 mM phosphate buffer (pH 6.2).

Step 5. Molecular Sieving Chromatography: The concentrated post-DEAE fraction was then rechromatographed on a column of Sephacryl S-200 superfine (Pharmacia) equilibrated with 100 mM phosphate buffer (pH 6.2). The column was used under the same conditions as described above with a descending flow rate of 60 ml/hr. The resulting active fractions were pooled, concentrated, and dialyzed against the 100 mM phosphate buffer (pH 6.2).

U. Characterization.

Molecular Weight Determinations: The molecular weight of the partially purified thiosulfate oxidase was determined by gel filtration on a 2.5 cm x 90 cm column of Sephacryl S-200. A Schwarz-Mann calibration kit containing apo-ferritin, gamma globulin, bovine serum albumin, ovalbumin, chymotrypsinogen myoglobin, and cytochrome c was used. A 2.5 ml sample containing 2.5 mg of each protein per ml was applied to the column and eluted with 100 mM phosphate buffer (pH 6.2).

Disc Polyacrylamide Gel Electrophoresis: Disc electrophoresis of crude and purified preparations was performed utilizing the method of Brewer (11) with 50 mM Tris-Glycine buffer (8.2). Samples were applied to the gel tubes and subjected to a current of 2 mA per tube.

SDS-Electrophoresis: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of crude and purified preparations was performed utilizing the method of Weber and Osborn (87). In addition to visualizing purity of the samples, molecular weight markers were also run to establish a relationship between mobility and molecular weight.

Effect of pH on Thiosulfate Oxidase: The pH optimum for this enzyme was determined by using final concentrations of 50 mM phosphate-citrate, sodium phosphate, and Tris-HCl in their respective buffering ranges.

Effect of Temperature on Thiosulfate Oxidase: The temperature stability of purified thiosulfate oxidase was determined using 100 mM phosphate buffer (pH 6.2). Enzyme samples were incubated for 30 min at 10 degree intervals between 0° and 80°C in temperature-blocks. After incubation aliquots were removed and assayed at ambient temperature for thiosulfate oxidase activity. Similar studies were conducted to determine the optimal temperature for thiosulfate oxidase activity. In this case, each micro-Thunberg tube was pre-incubated for 5 min prior to addition of substrate and then further incubated during the 5 min reaction period.

Effect of Cations, Inhibitors, and Substrate Concentrations on Thiosulfate Oxidase: The affect various concentrations of cations and inhibitors have on thiosulfate oxidase was determined under standard assay conditions. Also determined was the effect that various substrate concentrations have on the reaction velocity of thiosulfate oxidase. Finally, substrate specificity was determined by using the different inorganic sulfur compounds as substrates.

Results

I. Mercaptosuccinate Studies

A. Growth Studies.

Preliminary studies were done to determine whether *P. aeruginosa* ATCC 17934 could utilize mercaptosuccinate (MS) as a sole source of carbon, sulfur, and energy as had been previously shown with *Alcaligenes* (25). Cultures of *P. aeruginosa* were inoculated onto plates containing varying amounts of MS. Since no growth was observed after 96 h, experiments were done supplementing the plates of agar medium with different amounts of citrate or succinate and either $FeCl_3$ or $FeCl_2$. Growth occurred on all plates supplemented with citrate or succinate and increased growth was observed with increasing amounts of either supplement. No differences were seen between plates containing $FeCl_2$ or $FeCl_3$. Also, no growth appeared on plates containing only citrate or succinate, as expected, since no source of sulfur was present.

The ability to induce MS utilization was studied by comparing *P. aeruginosa* grown on 5% peptone + 0.25% trypticase soy or 1% MS + 0.1% succinate. The cells were harvested, washed, and then sub-cultured into media containing 1% MS with or without $MgSO_4$ (0.05% w/v). Surprisingly, no difference in growth was observed whether supplemented with $MgSO_4$ or not. Also, no difference between cultures inoculated from either initial growth medium was noticed.

Because of the inability of *P. aeruginosa* to grow on medium containing only MS, further experiments were performed using a salts

medium containing MS supplemented with succinate. Nephelometer flasks containing 1% MS + 0.1% succinate were inoculated with cells grown for 18 h in 1% MS + 0.1% succinate and monitored for growth (absorbance at 440 nm). Aliquots were removed to determine the pH and the presence of sulfate ($SO_4^=$) (Figure 3). Both absorbance and pH increased together for the first 24 h; whereas, after this time, absorbance decreased while pH continued to increase. This continued increase in pH could represent the release of basic ribosomes from lysed cells. Sulfate accumulation was not detectable at any time during the course of the experiment. No change was observed in pH in uninoculated control flasks.

Because the increase in pH might be limiting growth, an attempt was made to increase the buffering capacity of the basal salts to maintain neutrality. Washed cells grown on 1% MS + 0.1% succinate were subcultured into flasks containing (a) 1% MS + 0.05 M phosphate buffer, (b) 0.1% succinate + 0.05 M phosphate buffer, and (c) 1% MS + 0.1% succinate + 0.05 M phosphate buffer. No growth was observed after 48 h in any of the flasks which contained the increased phosphates. However, since high levels of phosphates have been shown to inhibit growth of *P. aeruginosa*, similar experiments were done using basal salts (a) buffered with 50 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (pH 7.5) and (b) adjusting the pH every 6-12 h with sterile 0.1 N HCl to neutrality. Both of these attempts also failed to increase cell growth (yield) and lead us to believe that perhaps pH was not the limiting growth factor. No difference in growth was observed for inoculated flasks of 1% MS + 0.1% succinate whether inoculated under aerated or stationary growth conditions.

The following study (Figure 4) was done to determine what concen-

Figure 3. Response of *Pseudomonas aeruginosa* grown on 1% Mercapto-succinate + 0.1% succinate.

ABSORBANCE 440 nm
0.1
0.2
0.3
0.4
0.5
HOURS
24
48
72
96
6.0
6.5
7.0
7.5
8.0
8.5
pH
0
0.5
1.0
1.5
2.0
2.5
μ moles SO4= / ml

tration of MS would offer maximum growth since 1% MS might be toxic. However, the data suggests a direct relationship between concentration of MS and growth over the range tested. Surprising is the observation that 1% MS + 0.05% $MgSO_4$ actually sustained the lowest growth of the media used. The results indicate that *P. aeruginosa* grew best on 1% MS + 0.1% succinate. A study was done to determine the effect the source of inoculum had for *P. aeruginosa* growth on MS. The results indicated that in every case, cells grown in 1% MS + 0.1% succinate, washed, and used to inoculate test media, yielded higher results.

This study (Figure 5) was done to determine whether the organism was actually utilizing MS as a carbon source or was growing on succinate and using MS as a source of sulfur. The following (Figure 5B) shows that increased growth was observed when cells were grown on 1% MS + 0.1% succinate rather than 1% MS of 0.1% succinate. It appears that growth in 1% MS + 0.1% succinate is an additive effect of the two components. Also, it appears that the basic conditions observed in the media are caused by metabolism stimulated by succinate rather than MS, since no increase in pH was observed in cells grown only in 1% MS. Other experiments (Figure 5A) were done to determine whether *P. aeruginosa* grown on succinate as a carbon source could use MS as a sulfur source. The following suggests that, when MS is used as a carbon source, (1%) growth is faster than when it is used as a sulfur source (0.1%) with 1% succinate. It would also suggest that MS is inhibitory for growth and that inorganic sulfate is a better source of sulfur.

B. Manometric Studies

Since *P. aeruginosa* appears to grow best on supplemented MS media,

Figure 4. Effect of Mercaptosuccinate concentration on *P. aeruginosa* growth.

(o) 1% Mercaptosuccinate + 0.01% succinate

(△) 0.1% Mercaptosuccinate + 0.1 succinate

(□) 0.01% Mercaptosuccinate + 0.01% succinate

(■) 0.001% Mercaptosuccinate + 0.1% succinate

(●) 1% Mercaptosuccinate + 0.05% $MgSO_4$

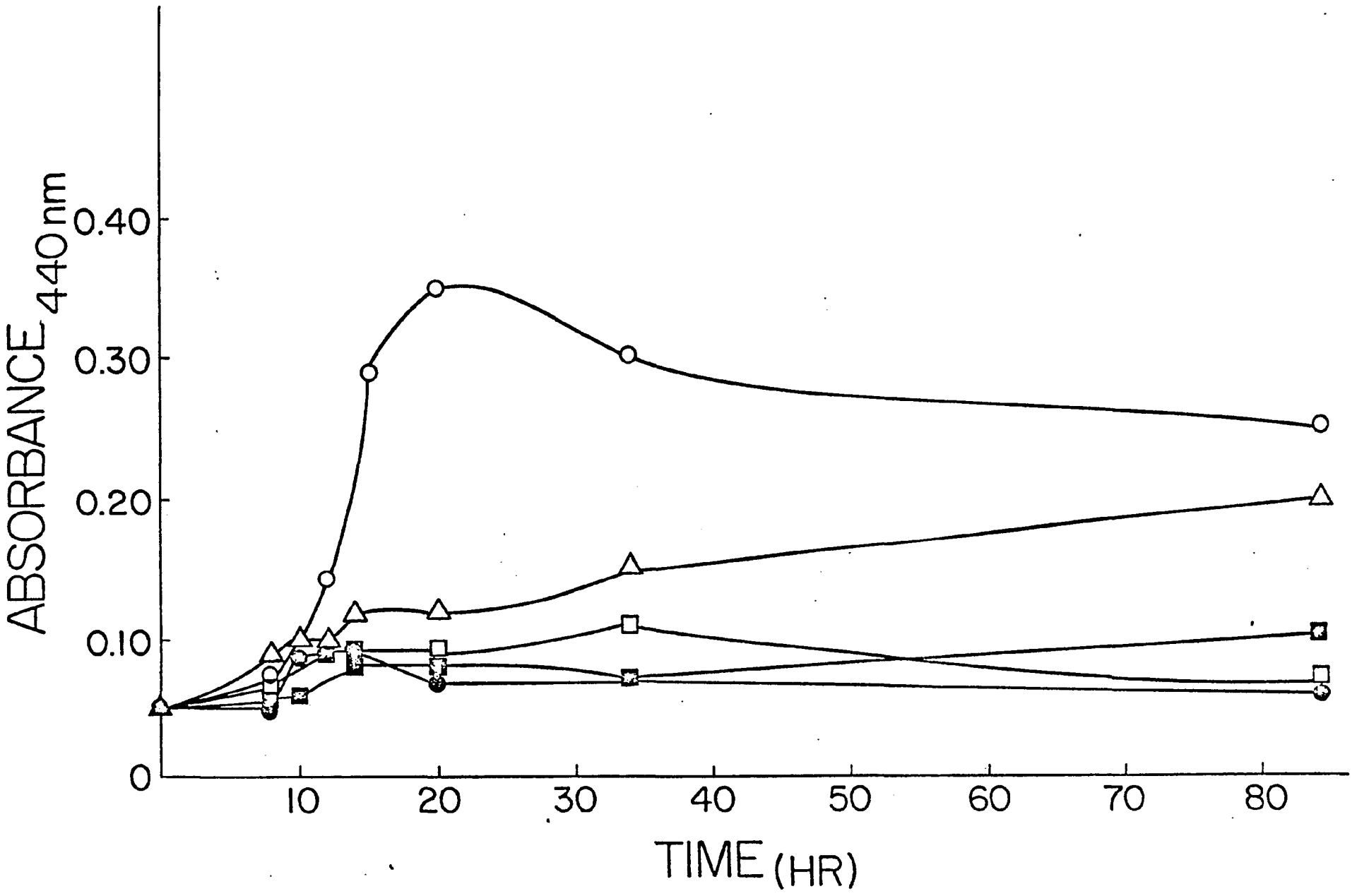
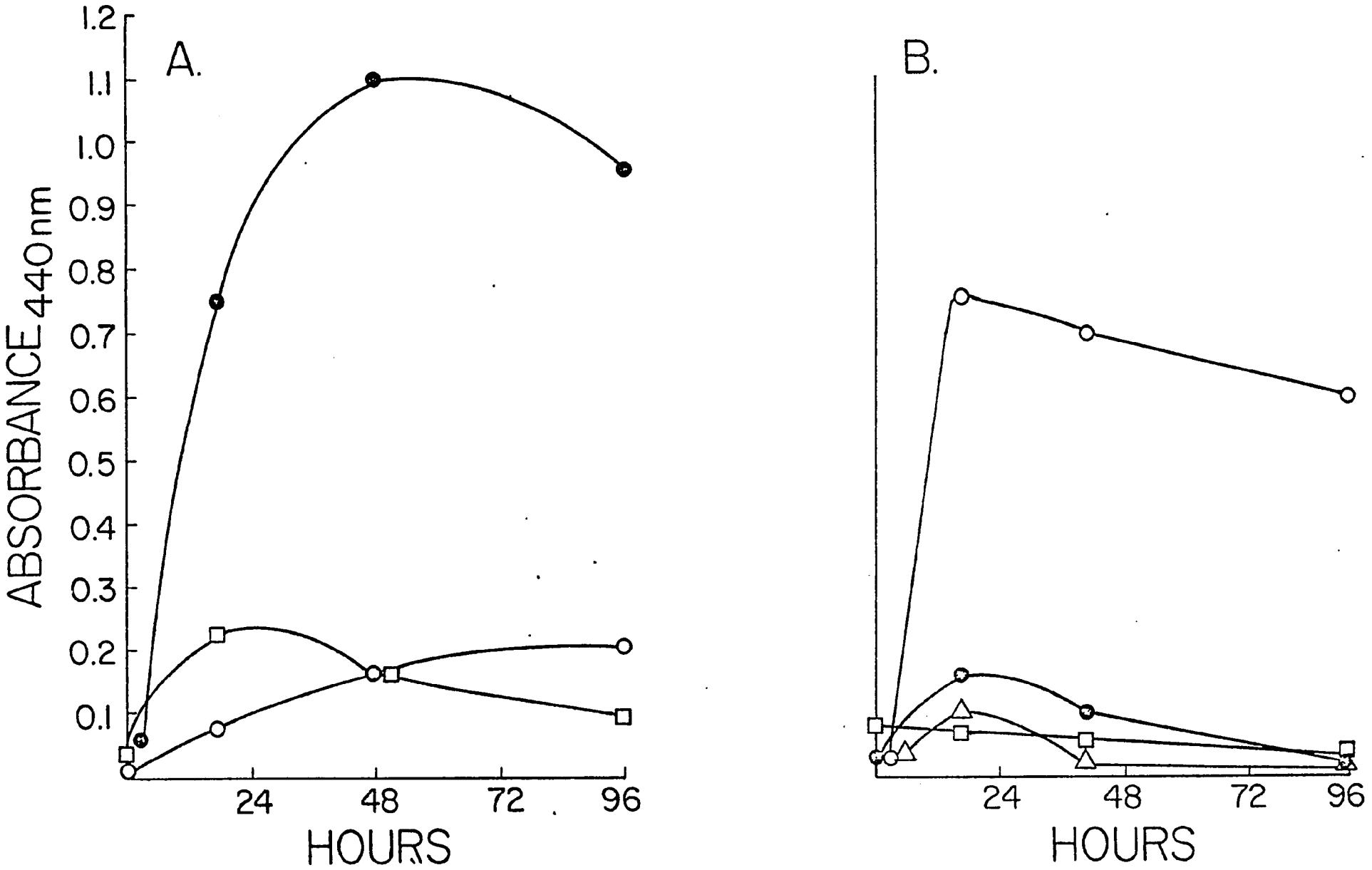

ABSORBANCE 440 nm
0.40
0.30
0.20
0.10
0
10
20
30
40
50
60
70
80
TIME (HR)

Figure 5. Effect of carbon and sulfur sources on growth of *Pseudomonas aeruginosa*.

A. Response of 1% mercaptosuccinate (MS) + 0.1% succinate (Suc) grown cells of *P. aeruginosa* sub-cultured on (●) 1% Suc + 0.05% $MgSO_4$, (○) 1% Suc + 0.1% MS, and (□) 1% MS + 0.1% Suc.

B. Response of 1% Succinate (Suc) + 0.05% Na_2SO_4 grown cells of *P. aeruginosa* sub-cultured on (●) 1% MS + 0.1% Suc, (○) 1% Suc + 0.05% Na_2SO_4, (□) 1% MS, and (△) 0.1% Suc.


A.
B.
ABSORBANCE 440nm
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
24
48
72
96
HOURS
24
48
72
96
HOURS

different pH's were used to determine the optimal pH for MS oxidation manometrically. *P. aeruginosa* sub-cultured on 1% MS + 0.1% succinate was harvested after 18 h at 35°C, and washed twice in the appropriate buffer.

The greatest net oxygen uptake during MS oxidation occurred in 100 mM phosphate buffer (pH 6.0-8.0) with maximum uptake at pH 7.0. Although endogenous respiration was at the lowest level. With the 100 mM phosphate buffered system, the endogenous respiration increased with pH increase, but the net uptake for MS decreased indicating a low optimum pH for maximum activity.

Manometric studies were done to compare cells grown on 1% succinate + 0.1% MS with cells grown on 1% MS + 0.1% succinate with regards to their ability to oxidize MS and succinate. The results showed that cells grown on 1% succinate + 0.1% MS had higher levels of net oxygen consumption for both substrates (MS and succinate) than cells grown on 1% MS + 0.1% succinate.

Cell free extracts (CFE) were prepared to determine whether a transport deficiency for MS may account for the organism's difficulty in metabolizing this substrate. CFE were added to flasks buffered at different pH's (100 mM phosphate buffer) as with resting cell studies. No additional activity, however, was observed, suggesting that transport of the substrate is not a limiting factor.

Cells grown on 1% MS + 0.1% succinate were suspended in 100 mM, phosphate (pH 7.0) and flasks were set up according to Umbreit (80) to determine if carbon dioxide (CO_2) was liberated from MS. Both the direct and indirect methods showed no CO_2 evolution, suggesting that decarboxylation is probably not a mechanism for MS utilization.

II. Inorganic Sulfur Studies

A. Growth Studies

Since it was apparent that *P. aeruginosa*, unlike the *Alcaligenes* sp. described by Hall and Berk (25), was unable to fully utilize MS, further experiments were designed to evaluate the extent of sulfur utilization by this organism. Because attempts to increase the growth on MS by (a) increasing the buffer capacity, (b) supplementing with different sulfur, iron, and carbon sources, (c) aeration, and (d) adjusting pH did not have any affect, the inhibitory effect of the high sulfur content of MS was evaluated.

Initially, studies were done to determine which inorganic sulfur compounds could be utilized as a source of sulfur by *P. aeruginosa*. Various inorganic sulfur sources were used to supplement a sulfur-free basal salts solution containing sodium succinate as the sole source of carbon. The sulfur compounds tested were sodium salts of sulfide, thiosulfate, tetrathionate, dithionite, metabisulfite, dithionate, sulfite, and sulfate. Primary cultures first grown using 0.5% sodium sulfate as the sulfur source were harvested, washed twice, and then used to inoculate nephelometer flasks containing the succinate-basal salts supplemented with different concentrations of the inorganic sulfur compounds. As shown in Figures 6 and 7, essentially similar results were achieved with each of the aforementioned compounds. Most of these compounds demonstrated an 18 h lag period before significant growth occurred at all of the concentrations tested (0.001%-1.0%). Two exceptions to this observation were: (a) no lag in growth was detected when either tetrathionate or metabisulfite was used as the sulfur source. Metabisulfite was inhibitory at higher concentrations,

Figure 6. Response of sulfate-grown cells of *P. aeruginosa* sub-cultured on different concentrations of sulfide (Na_2S), thiosulfate ($Na_2S_2O_3$), tetrathionate ($Na_2S_4O_6$), or dithionite ($Na_2S_2O_4$).

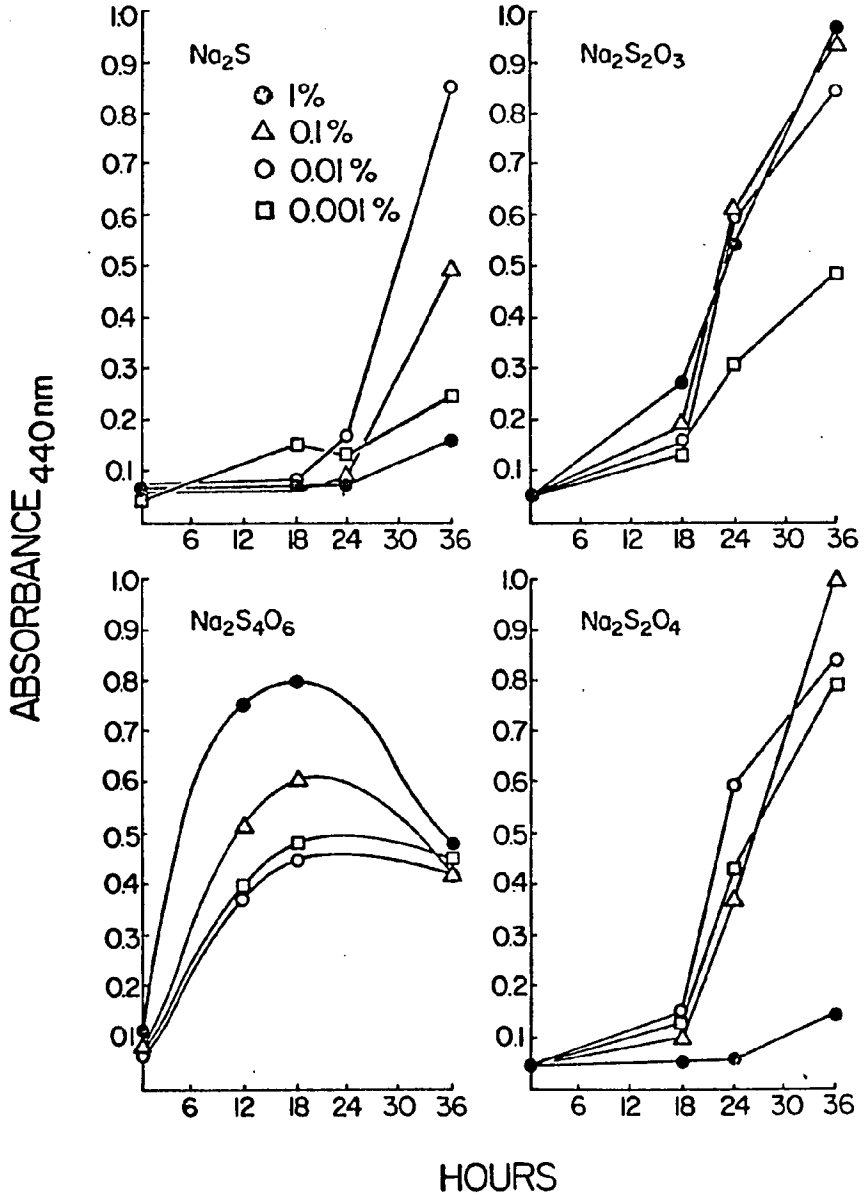
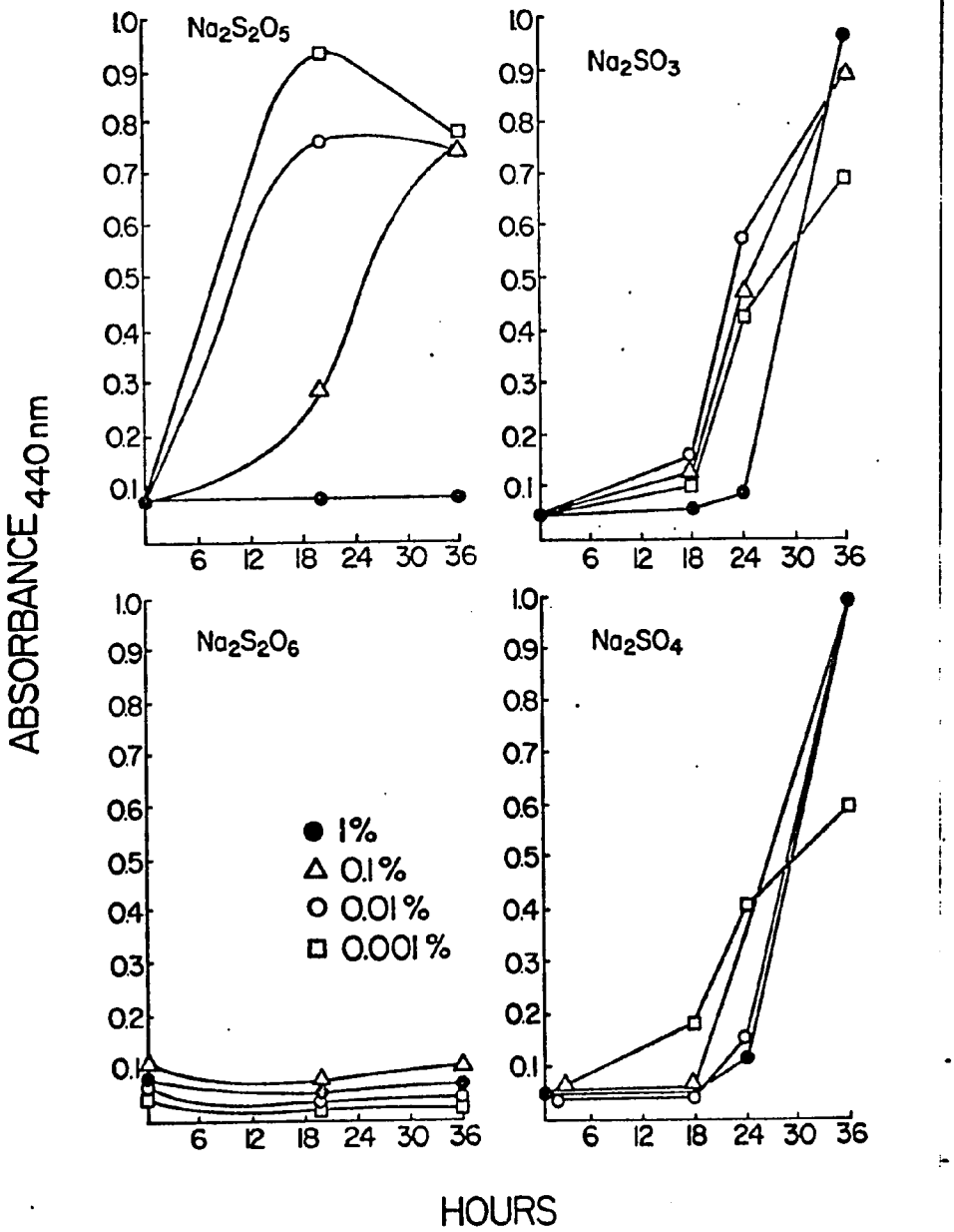

Na_2S
1%
0.1%
0.01%
0.001%
$Na_2S_2O_3$
$Na_2S_4O_6$
$Na_2S_2O_4$
ABSORBANCE 440nm
HOURS
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
6
12
18
24
30
36

Figure 7. Response of sulfate-grown cells of *P. aeruginosa* sub-cultured on different concentrations of metabisulfite ($Na_2S_2O_5$), sulfite (Na_2SO_3), or dithionate ($Na_2S_2O_6$). The response of tryptone-grown cells sub-cultured on different concentrations of sulfate (Na_2SO_4) is also shown.


$Na_2S_2O_5$
Na_2SO_3
$Na_2S_2O_6$
Na_2SO_4
1%
0.1%
0.01%
0.001%
ABSORBANCE 440 nm
HOURS
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
6
12
18
24
30
36

whereas with tetrathionate, growth was proportional to the amount present, (b) dithionate was not able to support growth at any of the tested concentrations.

A study was done to determine if *P. aeruginosa* could be stimulated by vitamins to utilize sodium dithionate as a source of sulfur. As shown in Table II, attempts to increase growth, even after 96 h, with any of the vitamins tested were not successful. These results are similar to those of London and Rittenberg (35) who were unable to show dithionate utilization with *T. thioparus* and *T. thiooxidans*. Although growth could not be stimulated for the organism to utilize dithionate at the concentrations tested, the inhibitory effect of these concentrations was tested. Supplements of dithionate (Figure 8) added to a culture growing on thiosulfate did not affect growth at any concentration tested. Flasks containing only dithionate again did not support growth. Thus, it appears that dithionate is not inhibitory or toxic but is a non-metabolizable sulfur substrate. In addition, the inability of resting cells and CFE to metabolize dithionate suggests the absence of a specific oxidase for this substrate in *P. aeruginosa*.

Experiments were then carried out on organisms, induced to grow on the succinate basal medium, using each compound as a sole sulfur source rather than sulfate alone. Because maximal growth was most frequently observed between 0.01% and 0.1% of the individual sulfur sources, the organism was grown for 18 h with each of the individual sulfur sources (0.05%), harvested, washed twice, and used to inoculate a medium containing the same respective sulfur source at 0.05%. In these induced cultures, as shown in Figure 9, no lag period was seen. Maximal growth was usually seen by 10-12 h except for sulfide grown cells where maxi-

Table II
Effect of vitamin stimulation on dithionate utilization
by *Pseudomonas aeruginosa*

Vitamin	Final Concentration µg/ml	O.D. 440 nm			
		0 h	24 h	48 h	96 h
Niacinamide	2 µg	0.03	0.03	0.03	0.03
Thiamine HCl	2 µg	0.03	0.03	0.03	0.03
Riboflavin	0.5 µg	0.03	0.03	0.03	0.02
Choline Cl	2 µg	0.03	0.03	0.02	0.02
Ascorbic acid	2 µg	0.03	0.02	0.02	0.02
Inositol	2 µg	0.03	0.03	0.03	0.02
Biotin	0.5 µg	0.03	0.03	0.03	0.03
Ca-pathothenate	2 µg	0.03	0.03	0.03	0.03
PABA	2 µg	0.03	0.03	0.03	0.03
B_{12} (cyanocobalamin)	1 µg	0.03	0.03	0.03	0.03
D_2 (calciferol)	2 µg	0.03	0.03	0.03	0.03
Vitamin A-acetate	1 µg	0.03	0.03	0.03	0.03
Pyridoxine	2 µg	0.03	0.03	0.03	0.03
Folic acid	2 µg	0.03	0.03	0.03	0.03
Yeast extract	0.1%	0.05	0.60	0.35	0.30
Yeast extract (no $S_2O_6^=$)	0.1%	0.05	0.60	0.30	0.25
No vitamin	----	0.03	0.03	0.03	0.03

Figure 8. Effect of dithionate ($S_2O_6^{=}$) on *Pseudomonas aeruginosa* growing on thiosulfate.

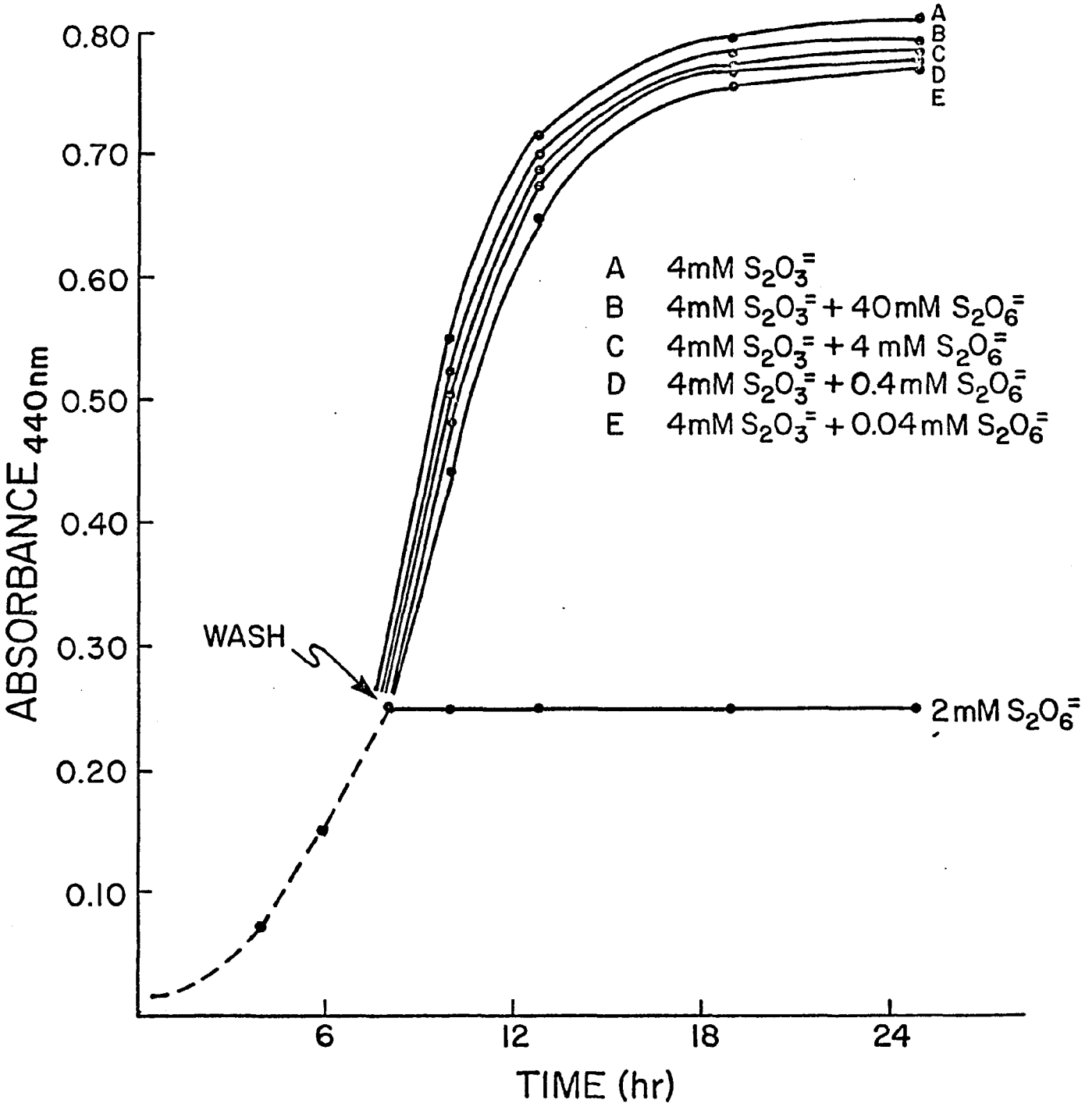
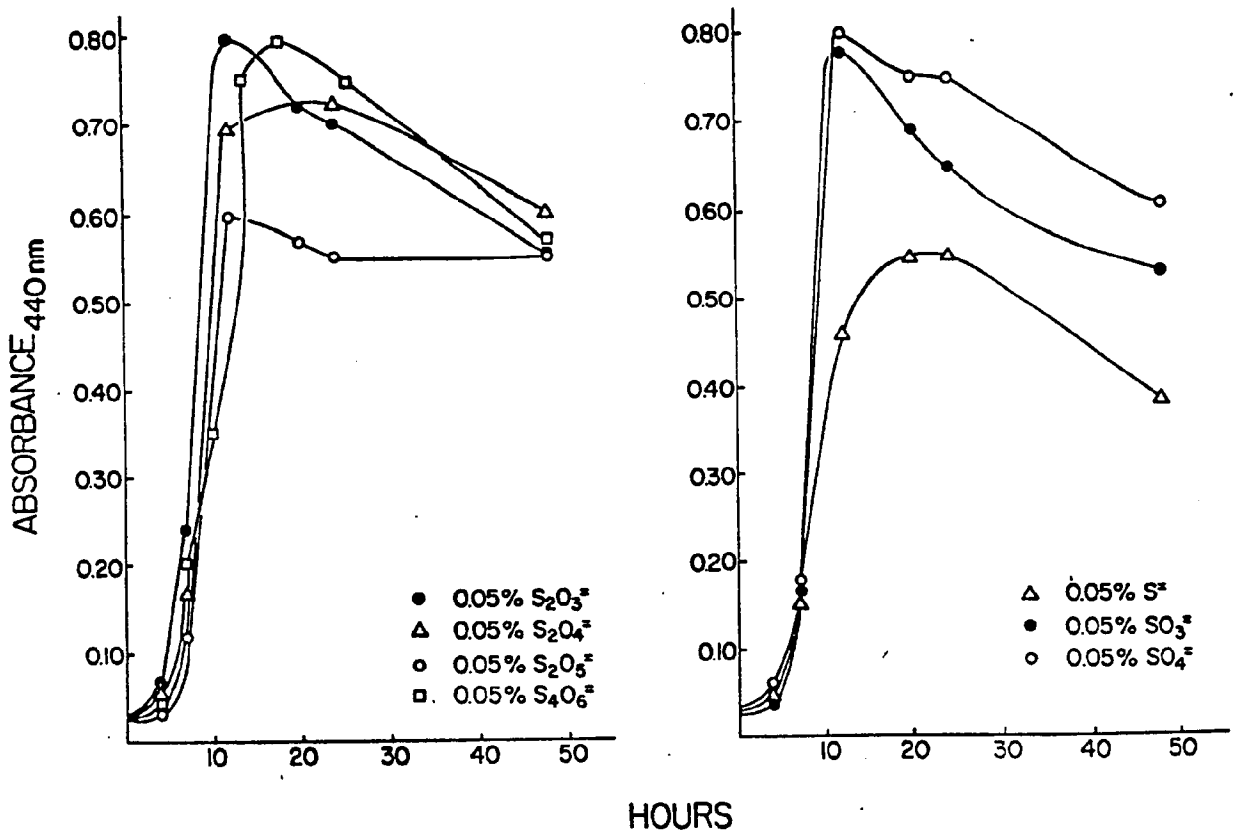

0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
ABSORBANCE 440nm
6
12
18
24
TIME (hr)
A
B
C
D
E
A 4mM $S_2O_3^=$
B 4mM $S_2O_3^=$ + 40mM $S_2O_6^=$
C 4mM $S_2O_3^=$ + 4 mM $S_2O_6^=$
D 4mM $S_2O_3^=$ + 0.4mM $S_2O_6^=$
E 4mM $S_2O_3^=$ + 0.04mM $S_2O_6^=$
WASH
2mM $S_2O_6^=$

Figure 9. Growth of *P. aeruginosa* induced for utilization of various inorganic sulfur sources. In this experiment the cells were grown on each individual sulfur source and then subcultured on the same sulfur source, respectively.


ABSORBANCE 440nm
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
10
20
30
40
50
HOURS
0.05% $S_2O_3^=$
0.05% $S_2O_4^=$
0.05% $S_2O_5^=$
0.05% $S_4O_6^=$
0.05% $S^=$
0.05% $SO_3^=$
0.05% $SO_4^=$

mal growth did not occur until 20 h.

Analysis of the supernatant fractions from these induced cultures (Table III) revealed that the pH of the medium increased from neutrality to pH 9.0 as a function of growth for all compounds tested. Again, as in the MS studies, the pH of the medium continued to increase as the growth began to decrease. Perhaps this drastic increase in pH is responsible for the rather short stationary phase of growth with rapid lysis of the cells occurring after maximum growth is achieved.

These supernatant fractions were also assayed for the accumulation of extracellular sulfate (Table IV). Where growth occurred all the inorganic sulfur compounds were oxidized by *P. aeruginosa* to sulfate to various extents. Unlike the *Alcaligenes* sp. previously described (27), this organism was not capable of oxidizing mercaptosuccinate with the formation of sulfate. Also, the rate of appearance of extracellular sulfate was not directly related to growth rates, but varied with the oxidative levels of the substrate. With sulfate grown cells, the medium sulfate decreased with cell growth but subsequently rose with declining cell growth, possibly indicating autolysis of the organism and liberation of intracellular sulfate. Also, there was never any detectable accumulation of sulfide in any growth media, nor were any morphological differences or in the organism seen regardless of the sulfur source. However, similar studies (Table V) using resting cells from induced cultures grown on each inorganic sulfur source revealed no accumulation of extracellular sulfate during incubation with the individual sulfur sources over an 18 h incubation period. Whether these resting cell cultures were unable to oxidize the respective sulfur compound because of a lack of an energy source for transport

Table III
Change of medium pH during growth
of *Pseudomonas aeruginosa*

Source of Sulfur for Growth[a] (4 mM)	pH of Culture Medium[b] Time (h)					
	0	8	10	12	36	84
$S^=$	7.4	7.4	7.5	7.5	7.9	9.0
$S_2O_3^=$	6.7	7.5	7.7	8.0	8.5	9.0
$S_4O_6^=$	6.7	7.5	7.8	7.9	8.6	9.0
$S_2O_4^=$	6.7	7.4	7.7	8.0	8.8	9.0
$S_2O_5^=$	6.2	6.2	6.5	7.5	8.5	9.1
$SO_3^=$	6.7	7.1	7.4	7.7	8.5	9.0
$SO_4^=$	6.7	7.5	8.0	8.2	8.8	9.0

[a] The organism was grown for 18 h with each individual sulfur source, harvested, washed twice, and used to inoculate the respective sulfur source.

[b] Values given have been corrected for spontaneous changes in uninoculated flasks.

Table IV
Accumulation of extracellular sulfate during growth of *Pseudomonas aeruginosa* on various sulfur compounds

Source of Sulfur for Growth[a] (4 mM)	Accumulation of Extracellular Sulfate[b] µmoles $SO_4^=$/ml of Growth Media					
	Time (h)					
	0	8	10	12	36	84
$S^=$	0	0	0	0	0	1.25
$S_2O_3^=$	0	0	0	0	1.0	1.0
$S_4O_6^=$	0	0	0	0.5	1.0	1.0
$S_2O_4^=$	0	0.5	0.5	3.25	4.0	4.0
$S_2O_5^=$	0	1.75	1.75	1.75	1.75	1.75
$SO_3^=$	0	0.75	1.25	1.25	2.0	2.25
$SO_4^=$	3.75	2.75	2.5	3.0	4.0	4.0

[a] The organism was grown for 18 h with each of the individual sulfur source, harvested, washed twice, and used to inoculate the respective sulfur source.

[b] Values given represent amount of $SO_4^=$/ml of growth media from induced cultures and have been corrected for spontaneous auto-oxidation.

Table V
Extracellular sulfate accumulation with resting cells

Source of Sulfur for Growth (4 mM)	Accumulation of Extracellular Sulfate (4 mM)[a]							
	$S^=$	S_2O_3	S_4O_6	S_2O_4	S_2O_5	S_2O_6	SO_3	SO_4
$S^=$	N.D.[b]	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
$S_2O_3^=$	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
$S_4O_6^=$	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
$S_2O_4^=$	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
$S_2O_5^=$	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
$SO_3^=$	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
$SO_4^=$	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

[a] Sulfate assays were performed at 0, 1, 2, 6, and 18 h from each resting cell suspension.

[b] N.D. means "none detected".

or because of the toxic inhibitory effects of accumulated free sulfite is not known. Control experiments with resting cells have shown, however, that the lack of extracellular sulfate was not due to binding of the anion to the bacterial cell surface.

Cell-free extracts (CFE) were prepared from individual cultures which had been induced to grow on the respective inorganic sulfur sources. These CFE behaved, however, like the growing cells and oxidized the various sulfur compounds to sulfate (Table VI). Also, note that none of the CFE were able to oxidize dithionate ($S_2O_6^=$) to sulfate. These results suggest that the inability of the resting cells to oxidize similar sulfur compounds is probably a transport problem rather than inhibition of oxidation by free sulfite.

B. Oxidase Activities

Manometric studies were done using resting cells which had been induced to grow on each of the individual sulfur sources. These resting cells showed (Table VII) net oxygen uptake for each substrate except sulfite and dithionate. In most cases, incomplete oxidation occurred for each substrate used based on the theoretical net oxygen uptake for sulfate production. These findings are consistent with the inability of resting cells to produce extracellular sulfate.

Because of the inability of resting cells to completely oxidize the sulfur compounds to sulfate, CFE were prepared as described using the French press. The ability of the organism to oxidize the different inorganic sulfur sources when grown on the individual sulfur compounds was then determined by following spectrophotometrically the reduction of ferricyanide. As shown in Table VIII, the CFE of *P. aeruginosa*

Table VI
Accumulation of sulfate from CFE prepared by growth of *Pseudomonas aeruginosa* on different inorganic sulfur sources

Source of Sulfur for Growth[a] (4 mM)	Time (hr)	Substrate (4 mM)[b]						
		$S^=$	$S_2O_3^=$	$S_4O_6^=$	$S_2O_4^=$	$S_2O_5^=$	$SO_3^=$	$S_2O_6^=$
$S^=$	1	2.0	2.0	1.5	1.0	2.5	2.5	0
	2	2.75	2.5	1.5	1.5	1.25	2.0	0
$S_2O_3^=$	1	1.0	3.75	1.0	1.5	3.0	3.25	0
	2	1.5	2.75	1.0	1.75	1.25	2.75	0
$S_4O_6^=$	1	3.0	2.75	2.0	2.5	2.5	2.0	0
	2	3.75	3.25	3.0	2.75	2.75	3.0	0
$S_2O_4^=$	1	1.5	1.75	1.25	1.0	2.5	2.5	0
	2	2.75	2.75	1.75	1.0	1.25	2.0	0
$S_2O_5^=$	1	1.0	0.25	0	0	0.5	0.5	0
	2	1.0	1.5	1.0	0	0.5	1.25	0
$SO_3^=$	1	1.0	2.25	1.5	0	2.0	2.0	0
	2	1.75	2.0	1.75	0	1.25	2.0	0
$SO_4^=$	1	1.5	2.25	0	0	2.0	2.0	0
	2	2.5	2.75	0.75	0	1.25	1.75	0

[a] CFE were prepared from individual cultures which had been induced to grow on the respective sulfur sources. (See Methods and Materials).

[b] The reaction mixture contained 4 mM of substrate, basal salts, and the respective CFE (2 mg/ml). Values given represent μmoles of $SO_4^=$/ml of reaction mixture and have been corrected for spontaneous auto-oxidation.

Table VII
Manometric studies with resting cells of *Pseudomonas aeruginosa* and inorganic sulfur compounds

Source of Sulfur for Growth (4 mM)	NET[a] µmoles O_2 Uptake with Resting Cells — Substrate (10 µmoles)							
	$S^=$	$S_2O_3^=$	$S_4O_6^=$	$S_2O_4^=$	$S_2O_5^=$	$SO_3^=$	$S_2O_6^=$	$SO_4^=$
$S^=$	3.5	0	0	6.0	2.83	0	0	0
$S_2O_3^=$	0	8.0	7.0	0	3.6	0	0	2.0
$S_4O_6^=$	0	7.0	10.0	7.65	16.2	0	0	0
$S_2O_4^=$	0	0	7.65	5.4	0	0	0	0
$S_2O_5^=$	0	0	16.2	0	0	0	0	2.0
$SO_3^=$	0	0	1.7	0	0	0	0	0
$SO_4^=$	0	1.2	0.3	1.7	0	0	0	2.6

[a] Corrected for endogenous and auto-oxidation; average of two separate experiments.

Table VIII
Oxidase activity of CFE for different inorganic sulfur compounds

Source of Sulfur for Growth (4 mM)	Specific Activity (Units/mg Protein)								
	$S^=$	$S_2O_3^=$	$S_4O_6^=$	$S_2O_4^=$	$S_2O_5^=$	$SO_3^=$	$S_2O_6^=$	$SO_4^=$	Rhodanese
$S^=$	-	12.8	4.6	59.2	46.4	9.6	0	7.2	0.10
$S_2O_3^=$	-	19.6	11.7	34.6	36.4	0	0	0	0.09
$S_4O_6^=$	-	11.7	7.0	11.3	21.0	5.8	0	4.9	0.11
$S_2O_4^=$	-	0	11.3	16.9	18.3	0	0	0	0.06
$S_2O_5^=$	-	2.3	21.0	21.2	23.8	3.2	0	0	0.09
$SO_3^=$	-	0	5.8	0	1.5	0	0	0	0.08
$SO_4^=$	-	0	4.9	15.6	16.9	0	0	0	0.12

One unit of enzyme was defined as the amount reducing 1 µmole of ferricyanide per min at 420 nm. Enzyme activity was assyed in micro-Thunberg tubes under a nitrogen atmosphere for 5 min. One rhodanese unit (R.U.) was defined as that amount which forms 10 microequivalents of thiocyanate after 5 min.

was able to oxidize each substrate except dithionate ($S_2O_6^=$). Sulfide oxidizing enzyme could not be assayed by this method, because the substrate sulfide completely reduced the ferricyanide used in the assay. It appeared, from this study, that thiosulfate oxidase activity was induced by the growth of the organism on sulfur sources of the lower oxidative states (i.e., sulfide, thiosulfate, and tetrathionate), while little or no thiosulfate oxidase activity was observed with *P. aeruginosa* grown on compounds of higher oxidative states (i.e., dithionite, metabisulfite, sulfite, and sulfate). The induction of thiosulfate oxidase by thiosulfate and tetrathionate was also shown by Trudinger (74) in his heterotrophic soil isolate for thiosulfate utilization. His study, however, did not study sulfide as a substrate for growth so no further comparisons can be made. Also, metabisulfite ($S_2O_5^=$) oxidase activity was associated with the oxidation of all the inorganic sulfur compounds tested in this study. Sulfite grown cells appeared to have only small amounts of this enzyme and no sulfite oxidase activity was found when thiosulfate was used as the sulfur source. In fact, very little sulfite oxidase activity was detected using this assay except in cells grown with sulfide, tetrathionate, and metabisulfite as the sulfur source. Rhodanese which may be involved in the intracellular turnover of reduced sulfur (62) appeared to be constitutively present, since equal amounts of activity regardless of the source of sulfur for growth was observed (Table VIII). Other studies (see following section) show that rhodanese activity is also constitutive in this organism in spite of the carbon source used for growth.

We examined the oxidase activities of different cell fractions by differential ultracentrifugation of CFE prepared from cells grown either

with thiosulfate or tetrathionate as a sulfur source. These cell fractions were then analyzed for thiosulfate and tetrathionate oxidase activities (Table IX). Thiosulfate oxidase activity was distributed in equal amounts in both the soluble (S_{108}) and particulate fractions (P_{108}) whether induced by growth on thiosulfate or tetrathionate. However, tetrathionate oxidase activity was higher (specific activity) in the particulate fraction (P_{108}) whether induced by growth on thiosulfate or tetrathionate with the highest activity associated with the particulate fraction (P_{108}) from tetrathionate grown cells. Tuovinen *et al.* (78) also found equal thiosulfate oxidizing enzyme activity in the supernatant (S_{105}) and particulate (P_{105}) fractions from *T. ferrooxidans* grown autotrophically on thiosulfate. Heterotrophic growth of this organism on glucose increased the activity of the S_{105} fraction three-fold and decreased the particulate fraction activity. Detection of rhodanese activity only in the supernatant (S_{108}) fraction was consistent with previous findings (72). Tuovinen *et al.* (78) however, did detect rhodanese activity in *T. ferrooxidans* particulate fractions (P_{108}).

C. Rhodanese

Since rhodanese has been implicated in thiosulfate utilization, we further investigated the enzyme's role in inorganic sulfur metabolism by *P. aeruginosa*. As shown in Table X, rhodanese appeared to be constitutively present regardless of the sulfur or carbon source used for growth. Tuovinen *et al.* (78) found that rhodanese was present in *T. ferrooxidans* whether growth was autotrophic on thiosulfate or ferrous iron, or heterotrophically grown on glucose. Hall and Berk (25) also

Table IX
Enzyme activities in various cell fractions
of *Pseudomonas aeruginosa*

Enzyme		Specific Activity (Units/mg Protein)	
		Grown on $S_2O_3^=$	Grown on $S_4O_6^=$
Thiosulfate Oxidase	BC	48.0	N.D.
	S_{10}	5.3	10.3
	P_{10}	22.2	N.D.
	S_{108}	85.3	113.5
	P_{108}	96.0	120.0
Tetrathionate Oxidase	BC	10.66	6.0
	S_{10}	42.7	44.6
	P_{10}	18.5	4.4
	S_{108}	37.3	43.6
	P_{108}	86.4	211.2
Rhodanese	BC	0.01	0.01
	S_{10}	0.04	0.03
	P_{10}	0.0	0.0
	S_{108}	0.02	0.05
	P_{108}	0.0	0.0

N.D. No activity detected

B.C. Broken cells

S_{10} Supernatant 10,000 x g, 10 min.

P_{10} Pellet 10,000 x g, 10 min.

S_{108} Supernatant 108,000 x g, 90 min. (prepared from S_{10})

P_{108} Pellet 108,000 x g, 90 min.

Table X
Effect of carbon and sulfur source
on activity of rhodanese

Media for Growth	Rhodanese Units[a]	Protein[b] (mg/ml)	Specific Activity (R.U./mg Protein)
1% Sodium succinate and 0.05% Na_2S	0.09	1.5	0.06
0.05% $Na_2S_2O_3$	0.11	1.6	0.07
0.05% $Na_2S_4O_6$	0.11	1.0	0.11
0.05% $Na_2S_2O_4$	0.06	1.6	0.04
0.05% $Na_2S_2O_5$	0.08	1.0	0.08
0.05% Na_2SO_3	0.06	1.6	0.04
0.05% Na_2SO_4	0.12	1.5	0.08
0.05% $MgSO_4$	0.18	2.3	0.08
0.05% L-cysteine	0.14	2.3	0.06
5% peptone and 0.25% trypticase soy	0.18	2.3	0.08
1% sodium citrate and 0.05% $MgSO_4$	0.11	1.0	0.11
1% mercaptosuccinate and 0.1% sodium succinate	0.12	1.0	0.12

[a] One Rhodanese Unit (R.U.) is defined as that amount of enzyme which forms 10 microequivalents of thiocyanate (one microequivalent of thiocyanate equals 0.104 O.D. at 420 nm).

[b] Protein determinations were made on CFE (23,000 X g supernatant).

showed that rhodanese was constitutive in *Alcaligenes* sp. and was unaffected by the addition of thiosulfate to the growth medium. Another explanation for the constitutive presence of rhodanese may be that none of the carbon or sulfur compounds tested repress rhodanese synthesis. Whatever the explanation, rhodanese was present in all cells, whereas, as previously mentioned, thiosulfate oxidase was induced only by growth on sulfide, thiosulfate, or tetrathionate.

Rhodanese utilizes both thiosulfate and β-mercaptopyruvate as a substrate (62, 65). This observation suggested that inorganic sulfur compounds other than thiosulfate may be utilized by this enzyme. The substrate specificity of rhodanese from *P. aeruginosa* was evaluated with CFE prepared from sulfide or sulfate grown cells (Table XI). As shown, neither CFE could utilize any substrate other than thiosulfate. These results suggest that rhodanese is not directly involved in the utilization of other inorganic sulfur compounds.

The effects of pH (Table XII) and temperature (Table XIII) were determined so comparisons to thiosulfate oxidase could be made. As has been previously shown (88), rhodanese has a very broad pH optimum, which is unlike previously reported optimal pH values of 6.2-6.4 for thiosulfate oxidase (25, 74). The enzymatic activity did appear to be affected by the reaction temperature. Rhodanese activity was highest at 37°C and still active at 4°C and 50°C.

D. Sulfatases

The sulfatases (62) are a large group of hydrolytic enzymes which catalyze the hydrolysis of sulfate esters ($R.O.SO_3^-$) forming alcohols (ROH) and sulfate ($SO_4^=$). Rammler *et al.* (59), Murooka *et al.* (43)

Table XI
Ability of different inorganic sulfur compounds to serve as electron donors for rhodanese

Electron Donors	Specific Activity (R.U./mg Protein)		
	Source of Sulfur for Growth		
	0.05% Na_2S	0.05% NaS_2O_3	0.05% Na_2SO_4
0.125M Na_2S	0	0	0
0.125M $Na_2S_2O_3$	0.07	0.10	0.12
0.125M $Na_2S_4O_6$	0	0	0
0.125M $Na_2S_2O_4$	0	0	0
0.125M $Na_2S_2O_5$	0	0	0
0.125M Na_2SO_3	0	0	0
0.125M $Na_2S_2O_6$	0	0	0
0.125M Na_2SO_4	0	0	0

Table XII
Effect of pH on activity of rhodanese
from *Pseudomonas aeruginosa*

Buffer pH	Rhodanese Units	Protein (mg/ml)	Specific Activity
0.1M cacodylate			
5.0	0.21	4.2	0.05
6.0	0.25	4.2	0.06
0.1M phosphate			
6.0	0.25	4.2	0.06
6.5	0.25	4.2	0.06
7.0	0.25	4.2	0.06
7.5	0.17	4.2	0.04
8.0	0.17	4.2	0.04
0.1M Tris-HCl			
8.0	0.17	4.2	0.04
9.0	0.08	4.2	0.02

CFE's (29,000 X g supernatant) were prepared from cells grown on 5% peptone + 0.25% trypticase soy broth at 35°C for 18 h.

Table XIII
Effect of temperature on activity of
rhodanese from *Pseudomonas aeruginosa*

Temperature °C	Rhodanese Units	Protein (mg/ml)	Specific Activity
4	0.52	4.0	0.13
25	1.16	4.0	0.29
37	1.80	4.0	0.45
50	1.04	4.0	0.26

CFE (29,000 X g supernatant) was prepared from cells grown on 5% peptone + 0.25% trypticase soy broth at 35°C for 18 h.

Rhodanese activity was determined in 0.1M phosphate buffer, pH 8.0.

and Adachi (2) have shown that these enzymes are synthesized during sulfate limiting growth conditions and are repressed by sulfate, thiosulfate, sulfite, and cysteine in normal growth concentrations. *Pseudomonas* sp. have been isolated (21, 22, 28, 43) which synthesize sulfatases. In order to exclude the possibility that *P. aeruginosa* was simply utilizing endogenous or exogenous sulfate esters rather than the various inorganic sulfur compounds for growth, sulfatase activity was measured (Table XIV). The organism was grown under sulfate-limiting conditions to see whether sulfatase activity was then derepressed. At no time was any sulfatase activity observed in this strain (ATCC 17934) of *P. aeruginosa*, suggesting that the organism was not able to utilize compounds with sulfate esters as sources of sulfur. Also, no activity, as would be expected, was detected in cells grown with reduced inorganic sulfur compounds.

E. Sulfite Oxidase

A more extensive evaluation of the role of sulfite oxidase seemed warranted because of rather conflicting results. *P. aeruginosa* was shown to grow on sulfite (Figure 7) yet showed low amounts of sulfite oxidase activity (Table VIII). Also, no sulfite oxidase activity was present in CFE from cells grown on thiosulfate although sulfate was accumulated by both growing cells (Table IV) and CFE (Table VI); neither was any net oxygen uptake demonstrated manometrically with cells grown on any of the inorganic compounds (Table VII). Previous work in our laboratory (25) had shown that with an *Alcaligenes* sp. both sulfite oxidase (AMP-independent) and APS reductase (AMP-dependent) were constitutively present.

Table XIV
Sulfatase activity in *Pseudomonas aeruginosa*

Source of Sulfur for Growth (mM)	O.D. 650 nm Bacterial Suspension	O.D. 420 nm Change After 30 min (p-nitrophenol)	Specific Activity (K')[a]
Na_2S (4)	0.08	0	N.A.
$Na_2S_2O_3$ (4)	0.40	0	N.A.
$Na_2S_4O_6$ (4)	0.55	0	N.A.
$Na_2S_2O_4$ (4)	0.28	0	N.A.
$Na_2S_2O_5$ (4)	0.42	0	N.A.
Na_2SO_3 (4)	0.58	0	N.A.
Na_2SO_4 (4)	0.55	0	N.A.
Na_2SO_4 (1)	0.58	0	N.A.
Na_2SO_4 (0.1)	0.53	0	N.A.
Na_2SO_4 (0.01)	0.20	0	N.A.
Na_2SO_4 (0.001)	0.15	0	N.A.
Control (*Aerobacter aerogenes*)		0.35	1.0

[a] S.A. (K') is defined as µmoles p-nitrophenol liberated per unit of O.D. 650 per minute.

Table XV lists the various assay systems used to detect both AMP-independent and -dependent sulfite oxidases. However, these procedures did not detect any sulfite oxidase in CFE of *P. aeruginosa* grown on thiosulfate. Trudinger (74) has also found that both cells and CFE from his heterotrophic soil isolate which had an inducible thiosulfate oxidase did not oxidize sulfite. Since it has been shown (6, 55) that these sulfite oxidases may be present in low levels, we felt that these assay conditions might not be sensitive enough to detect small quantities of enzyme. Therefore, CFE's from thiosulfate grown *P. aeruginosa* were fractionated using ammonium sulfate in an attempt to concentrate any enzyme activity (Table XVI). No APS-reductase activity was detected in any of the fractions whether cytochrome c or ferricyanide was used as an electron acceptor.

Investigations by Rajagopalan *et al.* (30, 65) have shown that sulfite oxidase is a molybdoenzyme requiring molybdenum for activation. They have shown, at least in humans, that deficiencies in sulfite oxidase may be corrected by increased dietary amounts of molybdate facilitating cofactor synthesis. Our attempts to stimulate sulfite oxidase activity with different concentrations of molybdate were unsuccessful (Table XVII).

F. Radioactive Studies

Studies were done to determine thiosulfate uptake by *P. aeruginosa*. Initial experiments showed (Figure 10) that uptake increased with time up to 30 min with either outer or inner labelled thiosulfate. Comparatively more inner than outer labelled thiosulfate was bound to the resting cells. However, this difference was greater with an increase

Table XV
Oxidation of sulfite by CFE of *Pseudomonas aeruginosa* induced for growth on thiosulfate

Enzyme	Assay System	Specific Activity
A. AMP-Dependent[a]		
APS-reductase (adenosine-5'phosphosulfate reductase)	1. $K_3Fe(CN)_6$, AMP, Phosphate (7.5)	None detected (N.D.)
	2. Cytochrome c, AMP, Tris-HCl (8.7)	N.D.
B. AMP-Independent		
Sulfite oxidase[b]	$K_3Fe(CN)_6$, Tris-HCl (8.3)	N.D.
Sulfite oxidase[c]	Cytochrome c, Tris-HCl (8.5)	N.D.
Sulfite oxidase[d]	$K_3Fe(CN)_6$, Tris-HCl (7.6)	N.D.
C. AMP-Dependent and Independent[c]	Monitoring of extract for absorption maximum of cytochrome b at 413 nm	N.D.
D. Control (sulfite grown)	$K_3Fe(CN)_6$, Tris-HCl (8.3)	3.2

[a] Adachi and Suzuki, Can. J. Biochem. (1977) *55*:91-98.

[b] Aminuddin and Nicholas, J. Gen. Microbiol. (1974) *82*:103-113.

[c] Johnson and Rajagopalan, J. Clin. Invest. (1976) *58*:543-550.

[d] Charles and Suzuki, Biochem. Biophys. Res. Comm. (1965) *19*:686-690.

Table XVI
Adenosine-5'phosphosulfate reductase activity in CFE from thiosulfate grown *Pseudomonas aeruginosa*

CFE Fractionation	APS-Reductase Activity Electron Acceptors	
	Cytochrome c	$K_3Fe(CN)_6$
Crude cell-free extract (No dialyzed)	No activity (N.A.)	N.A.
Crude cell-free extract (Dialyzed)	N.A.	N.A.
50% $(NH_4)_2$ SO_4 supernatant (Dialyzed)	N.A.	N.A.
50% $(NH_4)_2$ SO_4 pellet (Dialyzed)	N.A.	N.A.
70% $(NH_4)_2$ SO_4 supernatant (Dialyzed)	N.A.	N.A.
70% $(NH_4)_2$ SO_4 pellet (Dialyzed)	N.A.	N.A.
Control (sulfite grown)	1.2	3.2

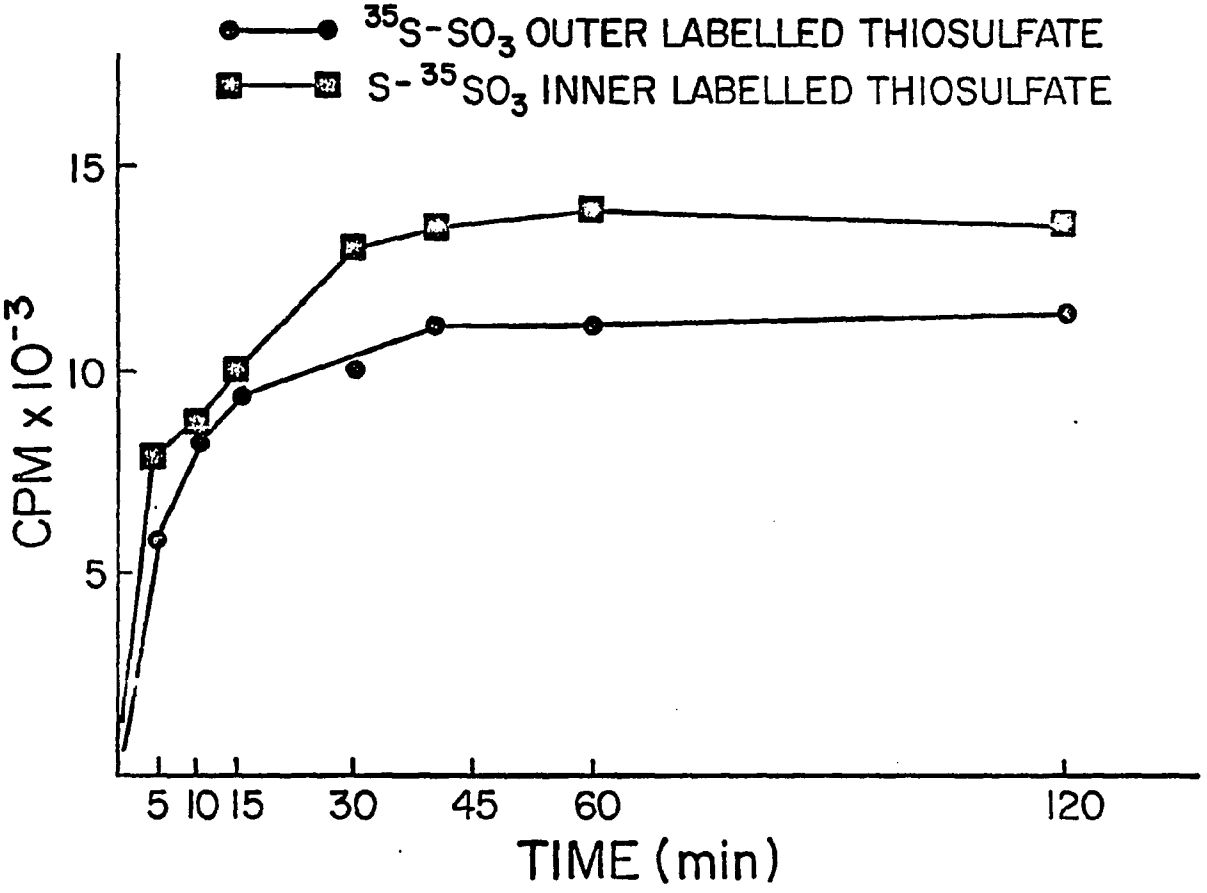
One unit of enzyme was defined as the amount reducing 1 µmole of electron acceptor per min. Enzyme activity was assayed in micro-Thunberg tubes under a nitrogen atmosphere for 5 min.

Table XVII
Effect of molybdenum on sulfite oxidase activity
in thiosulfite grown *Pseudomonas aeruginosa*

Growth Medium	Enzyme Activity[a]	Protein mg/ml	Specific Activity
1% w/v succinate-basal salts 4 µmol/ml Na_2SO_3 0.0 $Na_2MoO_4 \cdot 2H_2O$	0	4.7	0
1% s/v succinate-basal salts 4 µmol/ml Na_2SO_3 250 nmol/ml $Na_2MoO_4 \cdot 2H_2O$	0	3.1	0
1% w/v succinate-basal salts 4 µmol/ml Na_2SO_3 25 nmol/ml $Na_2MoO_4 \cdot 2H_2O$	0	6.5	0
1% w/v succinate-basal salts 4 µmol/ml Na_2SO_3 2.5 mol/ml $Na_2MoO_4 \cdot 2H_2O$	0	5.0	0
Control (sulfite grown)	8.0	2.5	3.2

[a] Following reduction of cytochrome c at 550 nm spectrophotometrically after 10 min.

Figure 10. Thiosulfate binding to resting cells of *P. aeruginosa*.


$^{35}S-SO_3$ OUTER LABELLED THIOSULFATE
$S-^{35}SO_3$ INNER LABELLED THIOSULFATE
CPM x 10^{-3}
15
10
5
5 10 15 30 45 60 120
TIME (min)

in time, suggesting a difference in metabolism rather than binding. Experiments using outer-labelled S^{35}-thiosulfate showed that uptake (per mg dry weight) appears to be constitutive since equal activity was observed for resting cells grown with either sulfate or thiosulfate. Washing experiments also showed binding to be of high affinity since less than 1% of outer labelled- and 2% of inner labelled-thiosulfate was removed after three successive washings. These findings are consistent with the work of Kelley *et al.* (31) using *T. ferrooxidans* where maximum uptake of outer labelled-thiosulfate occurred after 15 min and only 4% of the label was removed through washing.

The effect of thiosulfate concentration on uptake after 5 min by resting cells (Figure 11) showed that the kinetics are saturable at concentrations above 1 mM. A Lineweaver-Burk plot (see insert of Figure 11) resulted in a Km value of 0.5 mM for outer labelled S^{35}-thiosulfate which is consistent with the findings of Kelley *et al.* (31) using *T. ferrooxidans*.

Since facilitated and active transport both display saturable kinetics for a binding site, further experiments were performed to distinguish between these two types of substrate uptake. Inhibitor studies were performed with washed cells incubated for 5 min with the various compounds in basal salts before the addition of thiosulfate (0.5 mM) and then further incubated for 30 min. The effect of Group VI anions and inorganic sulfur compounds on thiosulfate uptake by washed cells is shown in Table XVIII. Both molybdate and tungstate were able to inhibit subsequent thiosulfate uptake probably through steric inhibition due to similar structures. Interestingly, both sulfate and cold thiosulfate had similar inhibitory patterns suggesting

Figure 11. Effect of thiosulfate concentration on uptake by resting cells of *P. aeruginosa*.

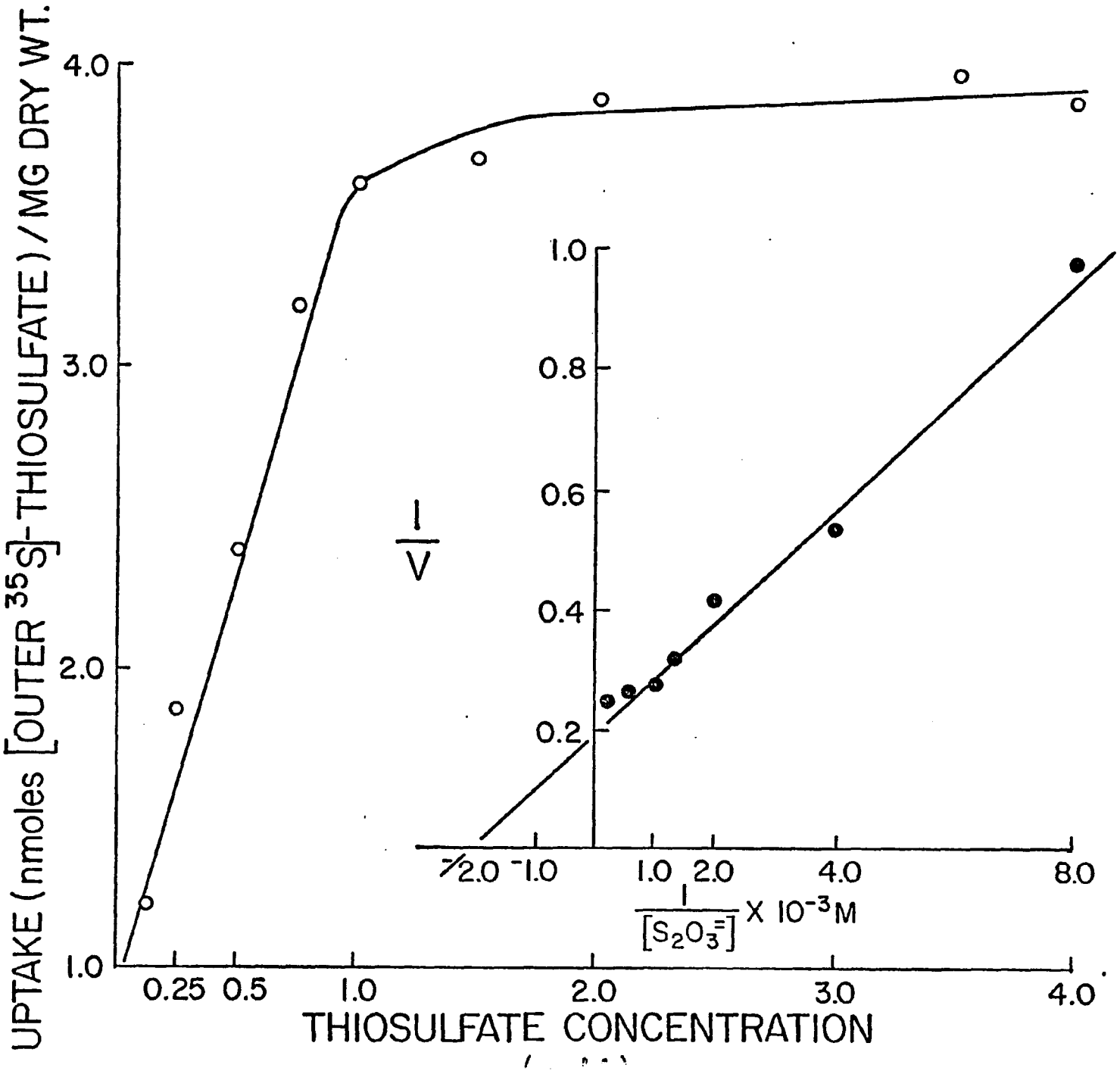

UPTAKE (nmoles [OUTER ^{35}S]- THIOSULFATE) / MG DRY WT.
4.0
3.0
2.0
1.0
0.25
0.5
1.0
2.0
3.0
4.0
THIOSULFATE CONCENTRATION
$\frac{1}{V}$
1.0
0.8
0.6
0.4
0.2
2.0
1.0
1.0
2.0
4.0
8.0
$\frac{1}{[S_2O_3^{=}]}$ X 10^{-3}M

Table XVIII
Effect of group VI anions and inorganic sulfur compounds on uptake of [outer ^{35}S]-thiosulfate by resting cells

Treatment of Washed Cells[a]	Final Concentration (mM)	Percent (%) Activity of Control
None	---	100
Molybdate	0.1	60
MoO_4	0.5	60
	1.0	40
Tungstate	0.5	49
WoO_4	1.0	33
	2.5	33
Sulfate	0.1	29
$SO_4^=$	0.5	23
	1.0	23
	5.0	9
Thiosulfate (cold)	0.1	21
$S_2O_3^=$	0.5	19
	1.0	20
Tetrathionate	0.1	97
$S_4O_6^=$	0.5	42
	1.0	33
Dithionate	0.1	62
$S_2O_6^=$	0.5	47
	1.0	33
Sulfite	0.1	95
$SO_3^=$	0.5	73
	1.0	73

[a] Cells were incubated for 5 min with the various compounds in basal salts at 37°C before addition of thiosulfate (0.5 mM of 1μCi/μmole) and then incubated further for 30 min.

that they may both have a common binding site, particularly since, as previously shown, growth with these two sulfur compounds yielded no difference in total thiosulfate bound. Tetrathionate and dithionate also affected thiosulfate uptake while sulfite appeared to have little inhibitory effect on subsequent thiosulfate uptake.

Unlike facilitated diffusion, active transport is enhanced by energy sources and is affected by inhibitors of energy production. The incubation of *P. aeruginosa* washed cells with an organic acid and a sugar as sources of energy showed uptake could be enhanced and therefore is indicative of active transport (Table XIX). Both succinate and glucose had a repressive effect on thiosulfate uptake at low levels but at substrate levels (40 mM) stimulated uptake. The repressive nature of low levels of carbon sources (0.1-1.0 mM) may be due to a selective transport of energy substrates over the non-energy sulfur source. Inhibitors of electron transport and energized membranes (Table XX) also suppressed thiosulfate uptake as would be expected if thiosulfate uptake is by active transport. Arsenate and 2,4-dinitrophenol were the most potent inhibitors whereas iodoacetic and cyanide were less effective.

G. Purification of Thiosulfate Oxidase

The previous examination of oxidase activities in different cell fractions (Table IX) showed that both the supernatant and pellet fractions had thiosulfate oxidase activity. In an attempt to further purify and characterize this enzyme, the crude CFE was subjected to ultracentrifugation in order to isolate the soluble form of the enzyme. The particulate (P_{150}) fraction probably represents either incorporation

Table XIX
Effect of carbon sources on uptake of
[outer ^{35}S]-thiosulfate

Treatment of Washed Cells[a]	Final Concentration (mM)	Percent (%) Activity of Control
None	---	100
Succinate	0.1	8
	0.5	22
	1.0	70
	5.0	70
	10.0	81
	40.0	125
Glucose	0.1	18
	0.5	20
	1.0	45
	5.0	50
	10.0	78
	40.0	110

[a] Cells were incubated for 5 min with the carbon source in basal salts at 37°C before addition of thiosulfate (0.5 mM of 1μCi/μmole) and then incubated further for 30 min.

Table XX
Effect of inhibitors of electron transport and energized membranes on uptake of [outer ^{35}S]-thiosulfate by resting cells

Treatment of Washed Cells[a]	Final Concentration (mM)	Percent (%) Activity of Control
None	---	100
Iodoacetic acid	0.1	86
	0.5	79
	1.0	35
Arsenate	0.1	50
	0.5	30
	1.0	25
Sodium azide	0.1	65
	0.5	44
	1.0	16
Potassium cyanide	0.5	93
	1.0	63
	2.5	61
2,4-dinitrophenol	0.1	44
	0.5	27
	1.0	29

[a] Cells were incubated for 5 min with inhibitor in basal salts at 37°C before addition of thiosulfate (0.5 mM of 1μCi/μmole), and then incubated further for 30 min.

of the soluble form into the cell membrane or oxidation of thiosulfate by membrane-associated cytochromes. Attempts to solubilize either thiosulfate oxidase or tetrathionate oxidase (Table XXI) by non-ionic (Triton X-100) or ionic (sodium deoxycholate) detergents were unsuccessful. However, no significant decrease in activity resulted from this procedure.

The next purification step was ultrafiltration of the S_{150} fraction. This was done since preliminary experiments had shown that ammonium sulfate precipitation on S_{150} fractions caused a reduction rather than an increase in specific activity. This toxic nature of ammonium sulfate (enzyme grade) on thiosulfate oxidase was also observed by Hall and Berk (26). The S_{150} fraction was filtered through membranes which either excluded proteins greater than 100,000 daltons and 300,000 daltons, respectively. The excluded portions were carefully removed from the filtration cell so as not to disturb any large residual particulate material associated with the membrane. Assays of the fractions revealed that 75% of the enzymatic activity was greater than 300,000 daltons, 15% between 100-300,000 daltons, and 10% less than 100,000 daltons. This distribution of enzymatic activity may have represented dissociation of a large enzyme complex into subunits or leakage of the membranes. The procedure gave a 70% yield with a 2.3-fold purification (Table XXII).

Aliquots of the ultrafiltration fraction that was greater than 300,000 daltons were dialyzed against 100 mM Tris-HCl from pH 7.5-9.0. The various pH fractions were added to DEAE-A-25 samples equilibrated at each of the different pH values tested. The samples were incubated at 4°C for 30 min, centrifuged, and the supernatants were assayed for thiosulfate activity. All activity was adsorbed to the beads and none

Table XXI
Solubilization of thiosulfate oxidase and tetrathionate oxidase

Fraction	Thiosulfate Oxidase units/ml	Tetrathionate Oxidase units/ml
$Supernatant_{150}$	21.0	21.0
$Pellet_{150}$	10.7	24.0
Buffer wash (P_{150})		
$Supernatant_{150}$	4.0	24.0
$Pellet_{150}$	10.0	19.2
Triton X-100 (2% v/v)		
$Supernatant_{150}$	3.0	0
$Pellet_{150}$	8.5	24.0
Deoxycholate (1 mg/mg protein)		
$Supernatant_{150}$	2.8	1.6
$Pellet_{150}$	9.0	21.0

Table XXII
Summary of purification of thiosulfate oxidase

Preparation	Volume (ml)	Protein (mg/ml)	Total Units	Units per mg Protein	Yield	Fold Purification
1. Crude CFE	75	7.0	9750	18.5	100%	1.0
2. Ultracentrifugation						
$Supernatant_{150}$	75	5.5	8250	20.0	85%	1.1
$Pellet_{150}$	10	7.0	756	10.8	8%	0.6
3. Ultrafiltration (XM300)						
> 300,000	13.75	10.0	5775	42.0	70%	2.3
4. DEAE-Sephadex						
Peak I	25	0.77	4385	227.0	45%	12.3
5. Sephacryl S-200	5	0.12	950	1575	9.7%	85.2

was detected in any of the supernatants above pH 7.5. Therefore, subsequent ionic exchange chromatography was done using 100 mM Tris-HCl (7.6). The A-25 type ion exchanger was chosen because the molecular weight of the enzyme appeared to be greater than 300,000 daltons from the ultrafiltration procedure.

A small (3 ml) column was made for determining the ionic strength at which thiosulfate oxidase was eluted from the DEAE-A-25 beads. After 30 min equilibration of the sample for adsorption onto the beads, 3 ml of NaCl with increasing ionic strength from 0.1 M to 1.0 M (increments of 0.1 M) were added (Figure 12). The eluted fractions were then assayed for both thiosulfate oxidase and tetrathionate oxidase activity. As shown (Figure 12) thiosulfate activity began eluting from the column at 0.1 M NaCL to a peak between 0.3 M and 0.7 M NaCl; tetrathionate activity followed a similar elution pattern except activity was also observed between 0.7 M and 0.9 M NaCl. Using the above information as a guide, a larger ion exchange column (2.5 cm x 25 cm) for further purification was prepared. The sample was prepared and applied as previously described and the column was then washed with equilibration buffer until all unadsorbed material was removed. Elution was then effected by stepwise addition of elution buffer plus 0.2 M, 0.5 M, and 0.8 M NaCl. The elution profile of thiosulfate oxidase and tetrathionate oxidase from a DEAE-Sephadex A-25 column is shown in Figure 13. The majority of the thiosulfate oxidase activity was eluted in the 0.2 M NaCl fractions. Particularly important was the low tetrathionate oxidase activity which was found associated with fractions 40 through 45. This pooled Peak I from the ion exchange column represented a yield of 45% with a 12.3-fold purification.

Figure 12. Elution profile of thiosulfate oxidase and tetrathionate oxidase from a DEAE-Sephadex A-25 column (0.1 - 1.0M NaCl step-wise gradients).

ION EXCHANGE CHROMATOGRAPHY DEAE SEPHADEX

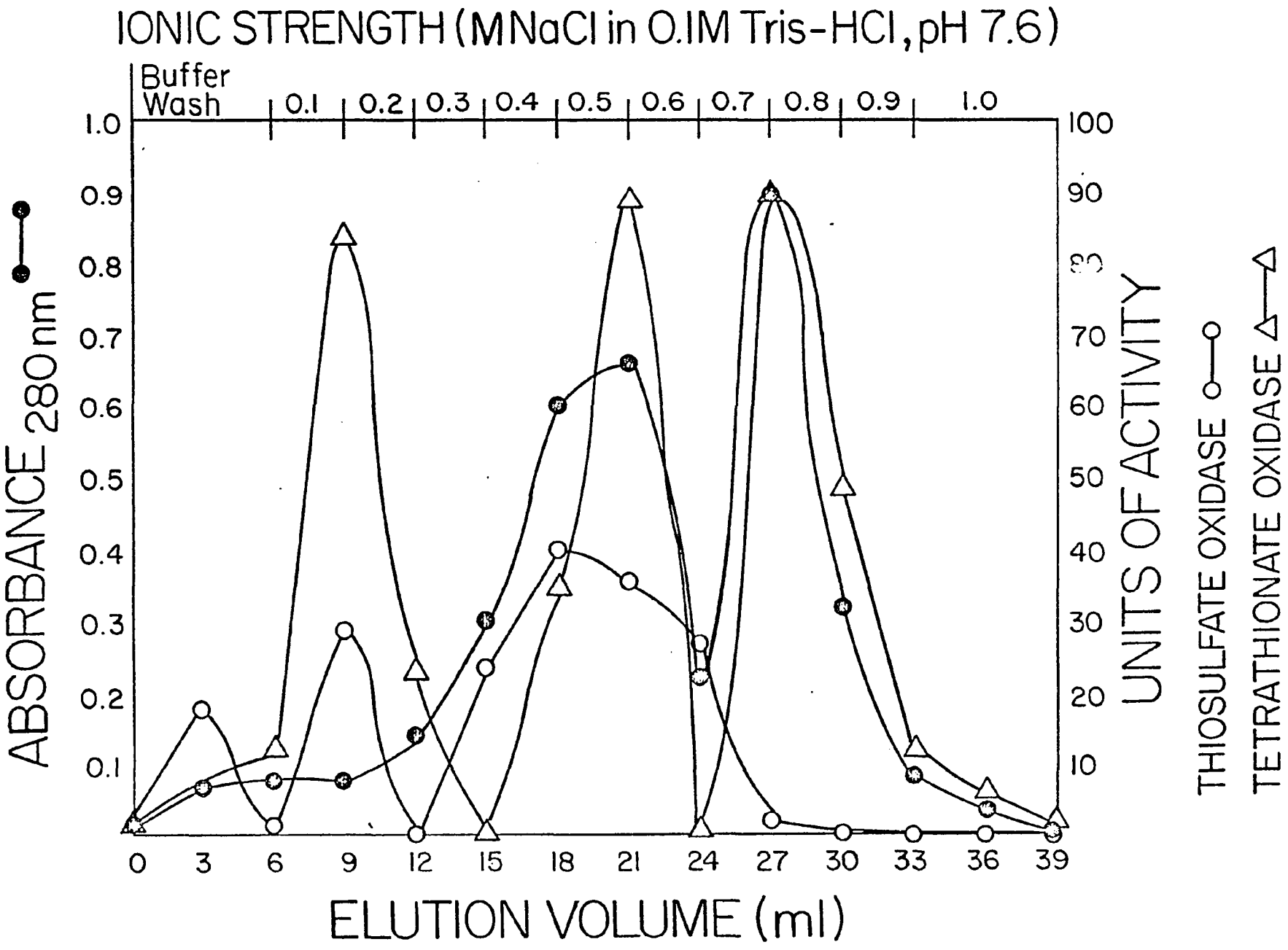
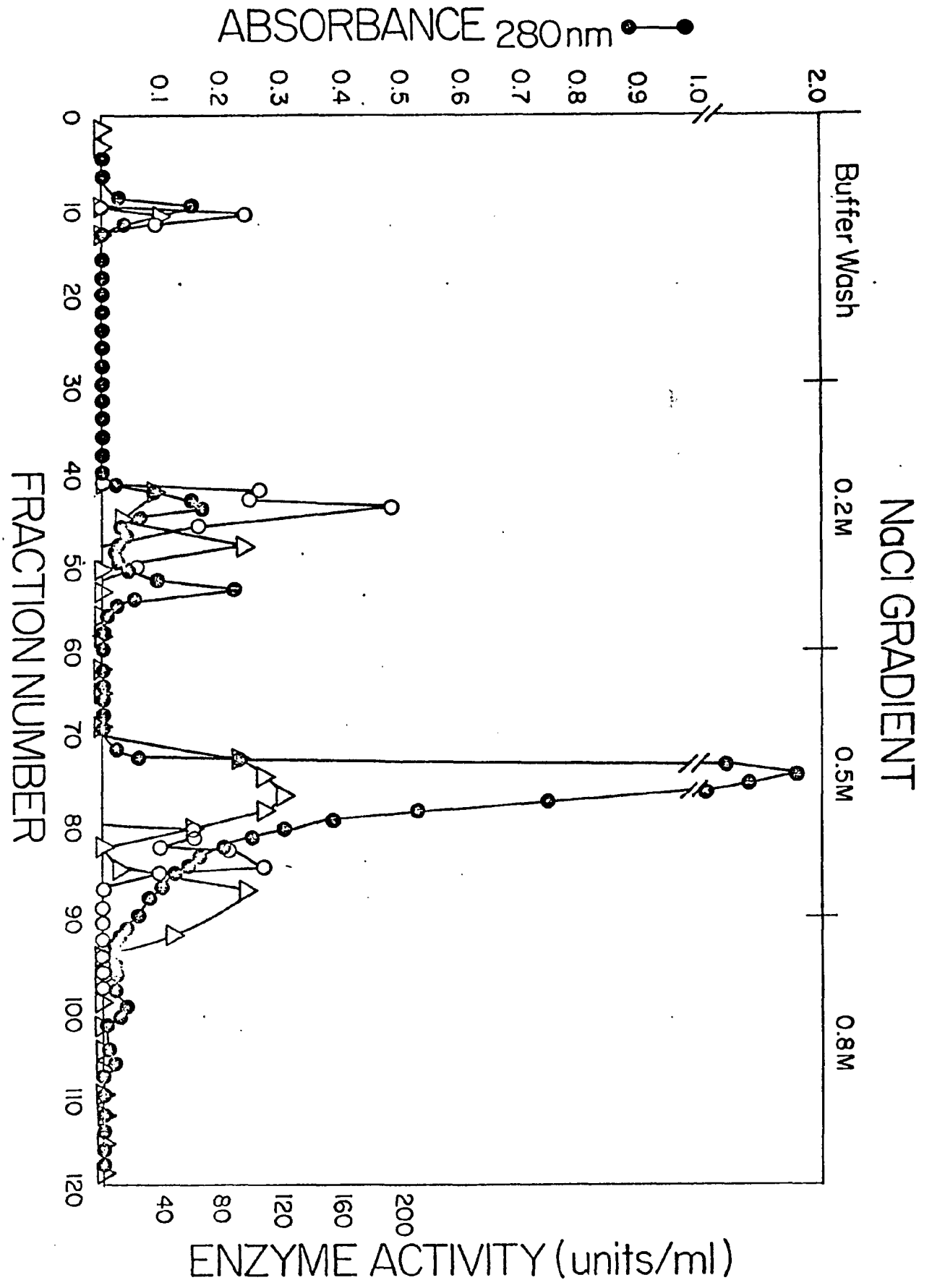


Figure 13. Elution profile of thiosulfate oxidase and tetrathionate oxidase from a DEAE-Sephadex A-25 column (0.2M - 0.8 NaCl step-wise gradients).


ABSORBANCE 280nm
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
2.0
Buffer Wash
0.2M
0.5M
0.8M
NaCl GRADIENT
FRACTION NUMBER
0
10
20
30
40
50
60
70
80
90
100
110
120
40
80
120
160
200
ENZYME ACTIVITY (units/ml)
THIOSULFATE OXIDASE
TETRATHIONATE OXIDASE

The pooled Peak I was concentrated and dialyzed against 100 mM phosphate buffer (6.2), and altered on a Sephacryl S-200 column. The elution profile of thiosulfate oxidase from the Sephacryl S-200 is shown in Figure 14. This column was able to separate the active fraction from the large amount of high molecular weight material. This step gave a 9.7% yield with a specific activity of 1575 representing a 85.2 fold purification.

H. Characterization of Thiosulfate Oxidase

Temperature Stability and Activity.

The crude CFE and various purified preparations were analyzed for stability during storage. All samples were stable when stored at -20°C and no loss of activity occurred up to 10 weeks. Enzymatic activity of these preparations declined when stored at 4°C, with only 50% activity after 3 days and 15% remaining after 14 days.

The effect of temperature on the activity of partially purified thiosulfate oxidase is shown in Figure 15. The Thunberg tubes were incubated for 5 min to allow equilibration of the assay mixtures at the described temperatures before addition of the substrate. The assays were done at the specified temperatures under standard assay conditions. Optimal activity was observed when assayed at 37°C with a sharp decline in activity occuring at temperatures below 20°C and above 50°C.

The stability of enzymatic activity was determined by incubating the partially purified enzyme for 30 min at the same temperatures used in determining activity. After the incubation period, the samples were assayed under standard conditions. No decrease in activity occurred if incubation was at temperatures below 40°C. Incubation at 50°C

Figure 14. Elution profile of thiosulfate oxidase from a Sephacryl S-200 column.

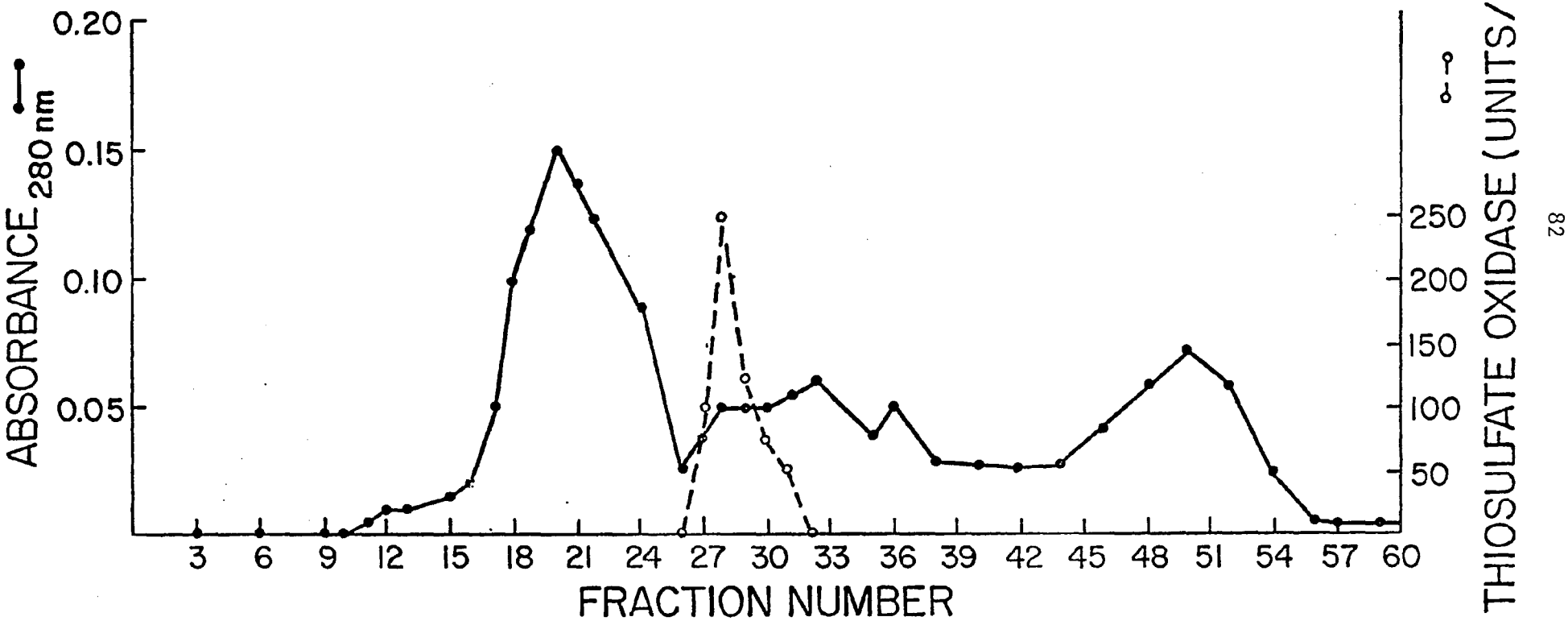
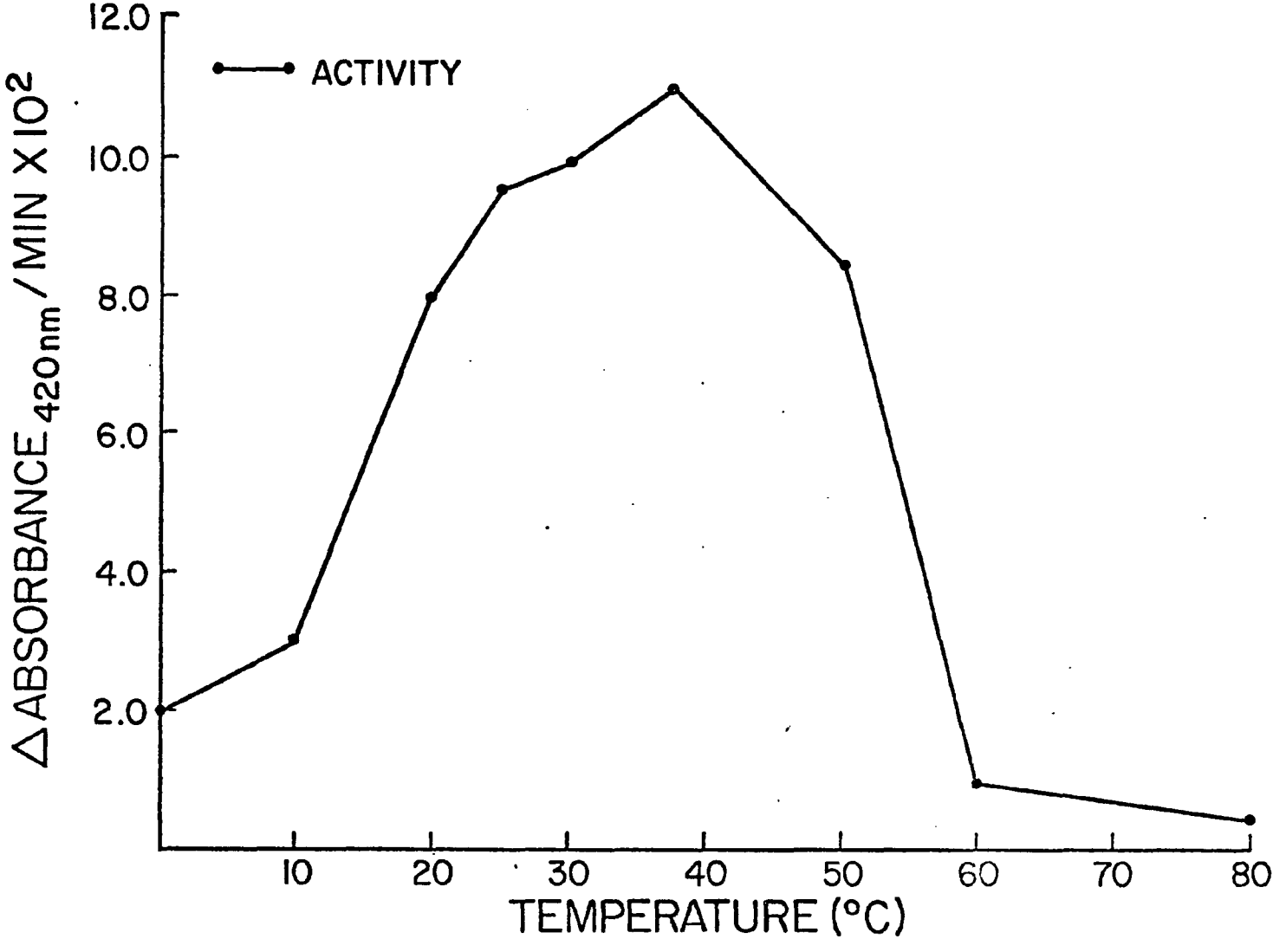

ABSORBANCE 280 nm
0.20
0.15
0.10
0.05
3 6 9 12 15 18 21 24 27 30 33 36 39 42 45 48 51 54 57 60
FRACTION NUMBER
THIOSULFATE OXIDASE (UNITS/ML)
250
200
150
100
50

Figure 15. Effect of temperature on thiosulfate oxidase activity.


ACTIVITY
12.0
10.0
8.0
6.0
4.0
2.0
ΔABSORBANCE 420nm / MIN X 10²
10
20
30
40
50
60
70
80
TEMPERATURE (°C)

caused a 50% decrease, at 60°C over 75% decrease, and at 80°C over 90% decrease in activity.

Effect of pH on Thiosulfate Oxidase Activity

The effect of pH on the enzymatic activity of partially purified thiosulfate oxidase was determined from pH 4.5 to 9.0 using potassium ferricyanide as the electron acceptor (Figure 16). The assays were performed under standard conditions using a final ionic strength of 0.05 for each buffer tested. Thiosulfate oxidase exhibited activity from pH 4.5 to 9.0 with optimal activity between pH 5.5 to 6.5 with citrate-phosphate buffer. Little activity was observed using Tris-HCl buffer from pH 7.5-9.0 while the phosphate buffered assays were optimal at pH 6.0 and 6.2 with decreased activity at increasing pH. Optimal activity was observed with citrate-phosphate. Since similar activity was observed between pH 6.0 and 6.5, the use of phosphate buffer (6.2) was continued in the further characterization of thiosulfate oxidase.

Specificity of Substrate and Electron Acceptor

The substrate specificity of the partially purified thiosulfate oxidase was determined by following the reduction of ferricyanide with various inorganic sulfur compounds. As shown in Table XXIII, only thiosulfate was oxidized. Dithionite, metabisulfite, tetrathionate, dithionate, sulfite, and sulfate did not yield a reduction of ferricyanide under these assay conditions. Also, neither AMP-dependent sulfite oxidase activity, nor rhodanese activity were detected in this partially purified preparation.

The effect of substrate concentration on thiosulfate oxidation by the partially purified enzyme was also determined (Figure 17). The

Figure 16. Effect of pH on thiosulfate oxidase activity.

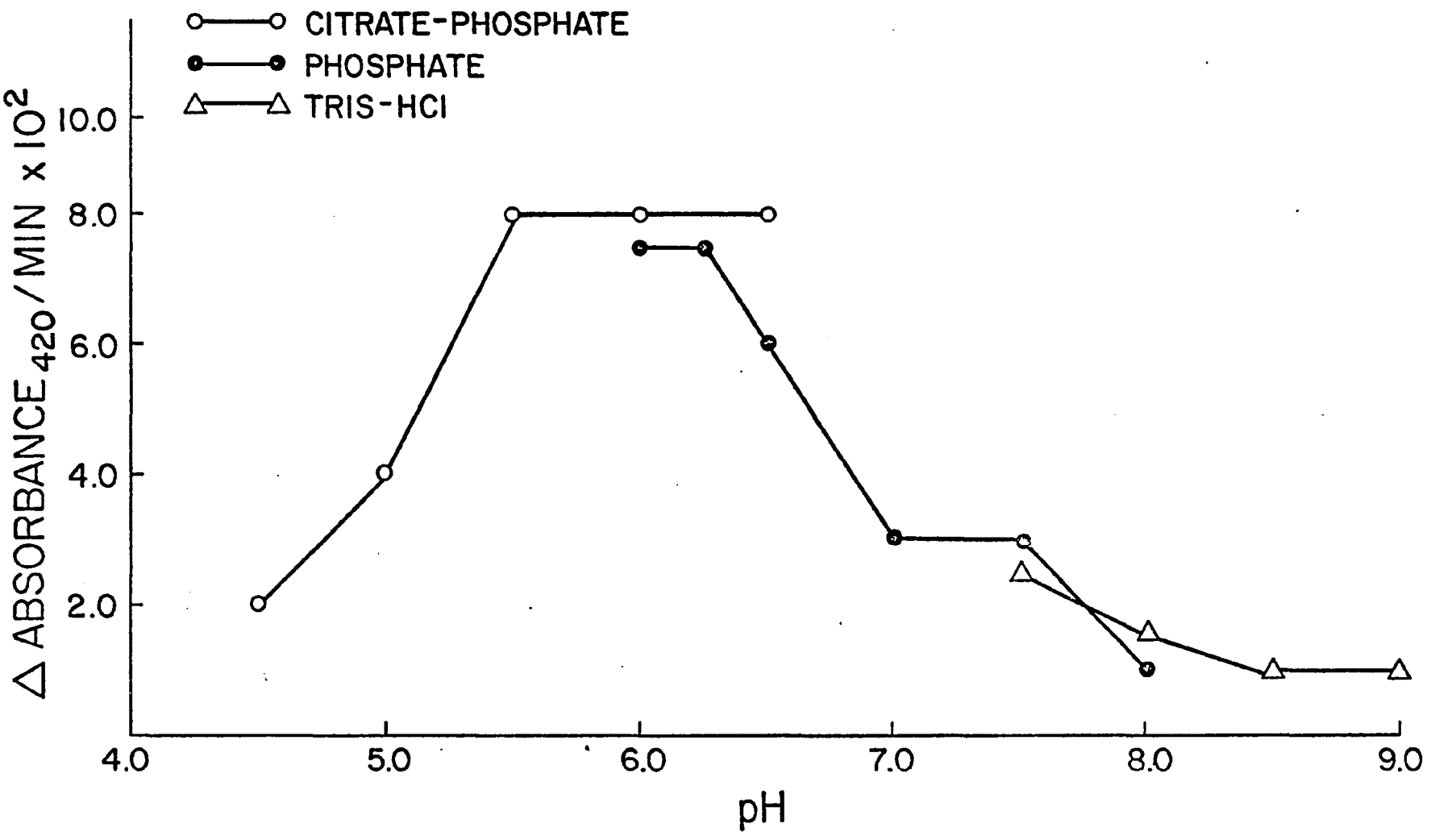

CITRATE-PHOSPHATE
PHOSPHATE
TRIS-HCl
Δ ABSORBANCE420/MIN x 10²
10.0
8.0
6.0
4.0
2.0
4.0
5.0
6.0
7.0
8.0
9.0
pH

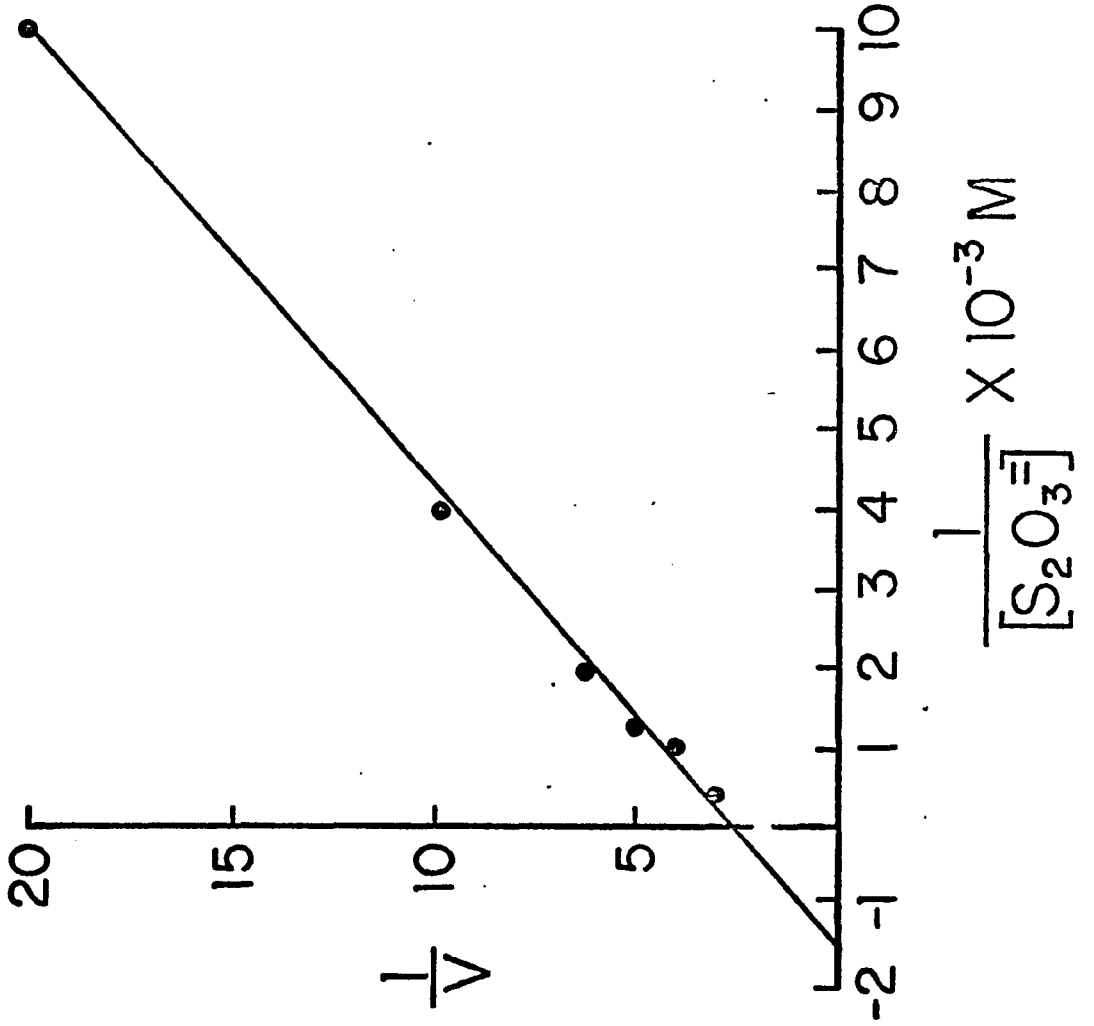
Table XXIII
Activity of thiosulfate oxidase on
various substrates

Substrate (5 mM)	Activity	Absorbance 420 nm/min x 10^2
Thiosulfate ($S_2O_3^=$)		14.5
Dithionite ($S_2O_4^=$)		0
Metabisulfite ($S_2O_5^=$)		0
Tetrathionate ($S_4O_6^=$)		0
Dithionate ($S_2O_6^=$)		0
Sulfite ($SO_3^=$)		0
Sulfite and AMP		0
Sulfate ($SO_4^=$)		0
Rhodanese[a]		0[b]

[a] Rhodanese assay, (Sörbo method) as described in Materials and Methods.

[b] Rhodanese units.

Figure 17. Lineweaver-Burk plot for thiosulfate oxidation by partially purified thiosulfate oxidase.


20
15
10
5
1/V
-2
-1
1
2
3
4
5
6
7
8
9
10
1/[S2O3=] X 10-3 M

velocity of the reaction was expressed as the change in absorbance at 420 nm per min for ferricyanide reduction. The assays were performed using 0.05 M phosphate buffer (6.2) with increasing amounts of substrate under standard assay conditions. The double-reciprocal Lineweaver-Burk plot yielded a straight line from which a Km for thiosulfate was determined to be 6.7×10^{-4}M.

Various electron acceptors were tested to determine whether they could couple with the thiosulfate oxidase reaction (Table XXIV). Potassium ferricyanide and cytochrome c (horse heart) both were reduced during thiosulfate oxidation with ferricyanide yielding a 3-fold greater sensitivity. Substitution of 2,6-dichlorophenol-indophenol, methylene blue, FAD, NAD, or NADP as the electron acceptor did not result in the specific reduction of these compounds during thiosulfate oxidation.

The effect of changes in potassium ferricyanide concentration on thiosulfate oxidation was measured. Assays were performed under standard conditions with increasing amounts of ferricyanide. The double-reciprocal plot of velocity against concentration of electron acceptor is shown in Figure 18. From this Lineweaver-Burk plot a Km for ferricyanide was determined to be 1.1×10^{-3}M.

Effect of Inhibitors on Thiosulfate Oxidase Activity

The effect of various inhibitors on enzyme activity is shown in Table XXV. Although tetrathionate and dithionate had very little effect on thiosulfate oxidase activity, sulfite and sulfate had some inhibitory affect. Sulfate at 1 mM inhibited 23%, while an equal concentration of sulfite inhibited 85%. Inhibitors affecting sulfhydryl groups, such as mercuric chloride, p-chloromercuribenzoate, and N-ethyl-

Table XXIV
Effect of various electron acceptors on thiosulfate oxidase activity

Electron Acceptor	Final Concentration (mM)	Assay Wavelength (nm)	Absorbance/min x 10^2
Potassium ferricyanide	1.0	420	15.0
	0.5		9.0
Cytochrome c	1.0	550	5.0
	0.5		2.5
2,6-dichlorophenol-indophenol	0.2	600	0.0
	0.1		0.0
Methylene blue	1.0	688	0.0
	0.5		0.0
Flavin adenine dinucleotide (FAD)	0.2	450	0.0
	0.1		0.0
Nicotinamide adenine dinucleotide (NAD)	1.0	340	0.0
	0.5		0.0
Nicotinamide adenine dinucleotide phosphate (NADP)	1.0	340	0.0
	0.5		0.0

Figure 18. Lineweaver-Burk plot for ferricyanide reduction by partially purified thiosulfate oxidase.

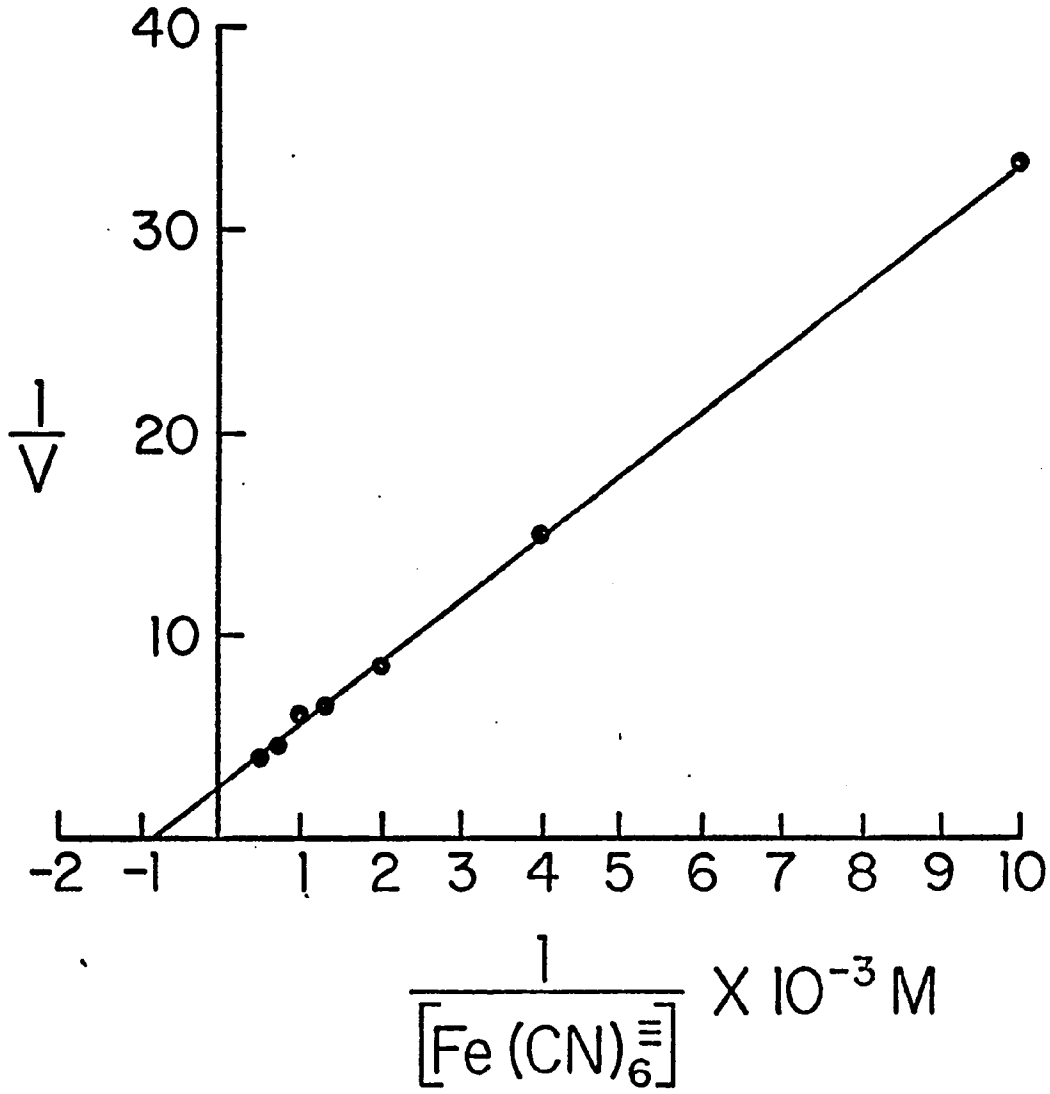

40
30
20
10
1/V
-2
-1
1
2
3
4
5
6
7
8
9
10
1 / [Fe(CN)6]3- X 10-3 M

Table XXV
Effect of various inhibitors on
thiosulfate oxidase activity

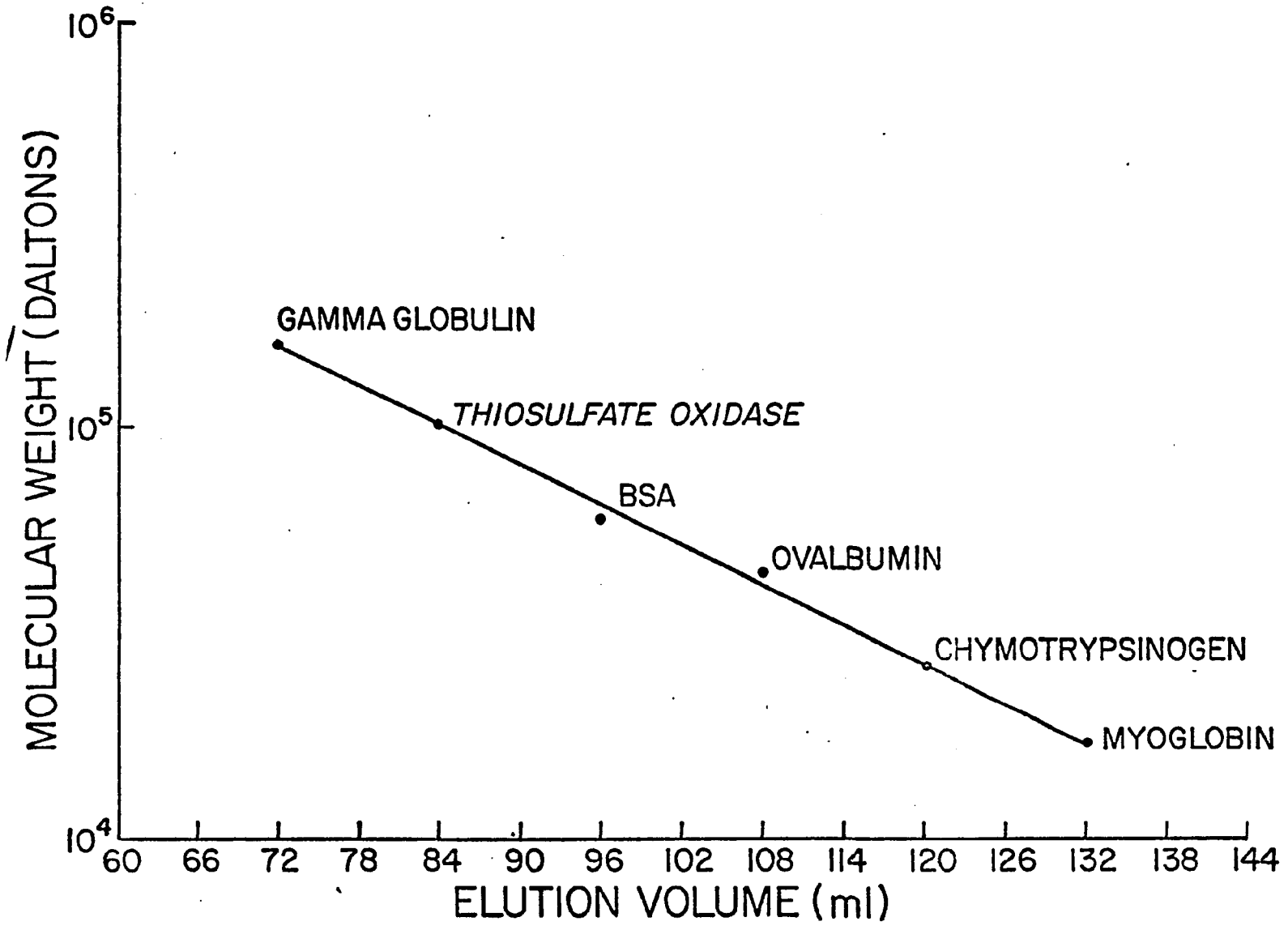
Inhibitor	Final Concentration (mM)	Activity (% of Control)
Tetrathionate	1.0	100
	0.1	100
	0.01	100
Dithionate	1.0	82
	0.1	85
	0.01	85
Sulfite	1.0	15
	0.1	52
	0.01	67
Sulfate	1.0	67
	0.1	75
	0.01	93
Mercuric chloride ($HgCl_2$)	1.0	0
	0.1	63
	0.01	87
p-chloromercuribenzoate (pCMB)	1.0	53
	0.1	93
	0.01	100
N-ethylmaleimide (NEM)	1.0	60
	0.1	75
	0.01	100
Potassium cyanide	1.0	100
	0.1	100
	0.01	100
Sodium azide	1.0	100
	0.1	100
	0.01	100
Hydroxylamine	1.0	100
	0.1	100
	0.01	100
Iodoacetate	1.0	100
	0.1	100
	0.01	100
EDTA	1.0	87
	0.1	97
	0.01	100
2,2'-dipyridyl	1.0	92
	0.1	100
	0.01	100
Diethyldithiocarbamate	1.0	100
	0.1	100
	0.01	100
Control		Activity 16.0×10^2

maleimide, significantly lowered activity. Particularly inhibitory was mercuric chloride with a concentration of 1 mM causing total inhibition of the reaction. No significant reduction in enzyme activity occurred if inhibitors of cytochrome c oxidase such as hydroxylamine, potassium cyanide, or sodium azide were used. Chelators of metals also appeared to have no affect on enzyme activity. Conversely, the addition of cations such as $FeCl_3 \cdot 6H_2O$, $MgCl_2$, $MnCl_2$, MoO_4, $CaCl_2$ at concentrations between 10^{-3}M to 10^{-5}M had no affect on enzyme activity.

Determinations of Molecular Weight

The pooled active fraction of thiosulfate oxidase obtained after Sephadex S-200 chromatography was filtered in order to estimate the molecular weight. By comparing the elution volume of the enzymatic activity to elution volumes of standard proteins, a molecular weight of approximately 100,000 was determined (Figure 19).

Figure 19. Determination of thiosulfate oxidase molecular weight on Sephacryl S-200 in 100 mM phosphate buffer (6.2).


MOLECULAR WEIGHT (DALTONS)
10^6
10^5
10^4
GAMMA GLOBULIN
THIOSULFATE OXIDASE
BSA
OVALBUMIN
CHYMOTRYPSINOGEN
MYOGLOBIN
60 66 72 78 84 90 96 102 108 114 120 126 132 138 144
ELUTION VOLUME (ml)

Discussion

Although a great deal of work has been done on the metabolism of sulfur amino acids by heterotrophs, little is known about their nutritional requirements and metabolic activities with respect to inorganic sulfur compounds. In addition, the control of inorganic sulfur oxidation in autotrophic thiobacilli is still unknown. Previous work in our laboratory (25, 27) had shown that a soil isolate *Alcaligenes* sp. (M1) was able to use mercaptosuccinate (MS) as a sole source of carbon, sulfur, and energy with the sulfur moiety oxidized to sulfate. Although thiosulfate oxidase, rhodanese, sulfite oxidase, and APS reductase were all detected in crude extracts, only thiosulfate oxidase was induced by growth of the organism on MS. Yet, *P. aeruginosa*, unlike the *Alcaligenes* soil isolate, could not utilize MS and required stimulation by other organic acids for growth. Attempts to aid utilization of MS by increasing the medium buffering capacity, adjusting pH, and increasing culture aeration were unsuccessful.

The ability of *P. aeruginosa* to utilize many inorganic sulfur compounds was surprising. Even more significant was the extensive oxidase system for these compounds. The growth of *P. aeruginosa* on sulfide, thiosulfate, tetrathionate, dithionite, metabisulfite, sulfite, and sulfate was characterized by a subsequent rise in pH and the accumulation of extracellular sulfate. At the present time, it is not known why dithionate did not support growth of the organism. Attempts to stimulate utilization of dithionate with various vitamins failed,

yet this compound had no toxic effect when added to growing cultures. Similar results for this substrate were also reported by London and Rittenberg (35) with *T. thioparus*. Since CFE were also inactive, one must rule out impermeability as the sole reason. As mentioned, extracellular sulfate accumulation occurred during growth and with CFE of *P. aeruginosa* with each sulfur source. Resting cells were incapable of oxidizing each compound to detectable amounts of sulfate. Manometrically, however, resting cells did show net oxygen uptake for each substrate except for dithionite and sulfite, but not stoichiometrically to sulfate (Table VII).

Evaluation of the ability of CFE to oxidize each inorganic substrate following the reduction of ferricyanide showed that thiosulfate oxidase was induced by growth on sulfide, thiosulfate, and tetrathionate. Thiosulfate-grown cells had no sulfite oxidase activity when assayed by several methods. These results are similar to those of Trudinger (74) in which his heterotrophic soil isolate could be induced to produce thiosulfate oxidase by either thiosulfate or tetrathionate. He also concluded that because growth was unaffected by increasing amounts of thiosulfate and because of the occurrence of an alkaline change in pH, the thiosulfate oxidizing enzyme does not appear to be involved in a detoxification or in the energy metabolism of the bacteria. Our studies also show that increasing amounts of thiosulfate in the growth medium does not stimulate additional growth. Tetrathionate, which was not studied by Trudinger, did, however, appear in our study to effect growth since the cell yield was proportional to the amount of tetrathionate present. Other studies (62) have shown that even with thiobacilli many variables can affect the outcome of thiosulfate

oxidation. Since thiosulfate oxidase was found in both the particulate and soluble fractions, its role in the organism's overall metabolism may be multifunctional. The particulate associated enzyme may be involved in the transfer of electrons through the cytochromes to molecular oxygen and generation of ATP or as a source of electrons for reduction of nitrate and nitrite (7, 58). The role of the soluble enzyme may be the same as postulated for the particulate fraction or perhaps may play a role in the oxidation of sulfur containing amino acids or reduced inorganic sulfur compounds. Rhodanese did not appear to be affected in any manner by the carbon or sulfur source used for growth, nor did *P. aeruginosa* appear to have sulfatase activity. The lack of any detectable sulfite oxidase, either AMP dependent and/or independent, was rather disturbing, since we could not account for a sequential oxidation of thiosulfate to sulfate bypassing sulfite as an intermediate. However, Trudinger (74) found sulfite not only inhibited thiosulfate oxidation by growing cells or extracts of thiosulfate grown cells but these preparations also did not oxidize sulfite. Tsang and Schiff (77) have suggested that sulfite may be a bound intermediate in the chain of reactions or perhaps the sulfite oxidizing enzyme(s) may be present as components of a thiosulfate-oxidizing enzyme complex rather than free enzymes (46). The ability of the organism to regulate free sulfite and sulfite oxidation would be extremely advantagious because sulfite has a strong affect on pseudomonas metabolism and growth (48, 49).

The significance of the oxidation of inorganic sulfur compounds by heterotrophic organisms is not clear, particularly since most studies have shown that thiosulfate oxidase is not directly involved in the energy production of the organism. Hall and Berk (25) showed that

resting cells of M1 oxidized thiosulfate to tetrathionate. Our results have already indicated that cell yield was proportional to the concentration of tetrathionate. Whether this increase in cell yield of *P. aeruginosa* is related to utilization of tetrathionate as a source of energy or whether tetrathionate has less toxic properties than other inorganic sulfur compounds is still not known. Whatever the case, the ability of pseudomonas to grow in the presence of reduced inorganic sulfur compounds is enhanced by the ability to oxidize these compounds. Particularly since thiosulfate oxidase was only induced by growth on sulfide, thiosulfate, or tetrathionate.

The presence of thiosulfate oxidase activity in both soluble and particulate (membrane associated) fractions is analogous to the nitrate reductase enzyme studied by MacGregor (40, 41). Using *Escherichia coli*, MacGregor (40), showed that the soluble form of the enzyme which was induced by anaerobic growth with nitrate, was incorporated into the membrane transport system. The nitrate reductase could be solubilized from the membrane fraction using Triton X-100 and was shown to be identical to the soluble form. Our attempts to solubilize the particulate form of thiosulfate oxidase with deoxycholate or Triton X-100 digestion were unsuccessful. Similar attempts by Oh and Suzuki (45, 46) to isolate a membrane-associated thiosulfate-oxidizing complex of *T. novellus* were also futile.

We were, however, able to partially purify the soluble form of the enzyme 85-fold through ultracentrifugation, ultrafiltration, ion exchange, chromatography and gel filtration. The properties of the enzyme with regards to size, substrate specificity, and susceptibility to inhibition are quite similar to the characterized thiosulfate oxi-

dases of the heterotroph M1 (25) and the autotrophs *T. novellus* (12), or *T. thioparus* (39). Roy and Trudinger (62) suggest that thiosulfate oxidase in the autotrophs represents a secondary pathway during utilization of high concentrations of thiosulfate which agrees with our findings that the soluble thiosulfate oxidase is responsible for regulating toxic reduced inorganic sulfur compounds.

Thiosulfate oxidase from *P. aeruginosa* was specific for thiosulfate since no activity was observed if other inorganic sulfur substrates were used. The ability of the partially purified thiosulfate oxidase to couple with mammalian cytochrome c also suggests a relationship of this enzyme to the membrane electron transport chain. This hypothesis is supported by Trudinger's (74) finding that endogenous cytochromes of the c-type were reduced when thiosulfate was added to extracts of induced but not uninduced heterotrophic bacteria. The relationship of the cytochromes is interesting since membrane-bound nitrate reductase of *E. coli* consists of three subunits one being the apoprotein of cytochrome b (40). The soluble form of the enzyme lacks this subunit and forms a complex on the membrane. Perhaps a similar incorporation of thiosulfate oxidase occurs in *P. aeruginosa*, Oh and Suzuki (45) have isolated from *T. novellus* a membrane-associated complex capable of oxidizing thiosulfate completely to sulfate. Included in this complex are cytochrome c oxidase, flavin, and cytochrome b components of the membrane electron transport.

The uptake of ^{35}S-thiosulfate by resting cells showed typical saturation kinetics and a Km value of 0.5 mM was determined. No difference was detected in total thiosulfate uptake per mg dry weight for thiosulfate or sulfate grown cells. This would suggest that

either the binding site for thiosulfate is not induced and is always present or both anions share a common binding site. The latter explanation is supported by the inhibitor studies which demonstrated sulfate to inhibit ^{35}S-thiosulfate uptake as strongly as unlabelled thiosulfate. Dreyfuss (19) studying sulfate transport in *Salmonella* sp. has shown that thiosulfate is also transported by the sulfate transport system. The binding of sulfate and thiosulfate to a common binding site in the mammalian kidney has also been shown (18). The binding was rapid and strong since successive washings of the cell suspensions failed to remove the label. The uptake was also affected by structurally similar anions such as tungstate and molybdate. Energy sources stimulated uptake and inhibitors of oxidative phosphorylation reduced uptake indicating that thiosulfate uptake is an active process. The active transport of thiosulfate may account for the inability of the resting cells to form sulfate whereas growing cells and CFE oxidized thiosulfate to sulfate. Additionally, the need for an energy source to transport thiosulfate may explain the low net oxygen uptake seen during the manometric studies using resting cells.

Besides sulfate, dithionate also inhibited thiosulfate uptake; sulfite did not appear to be a good inhibitor. The inability of sulfite to competively inhibit thiosulfate binding and probably the binding of sulfate to *P. aeruginosa* is significant since Palumbo (48, 49) has shown that sulfite regulates the production of secondary metabolites such as pigment and slime production in this organism. Hence, the organism may selectively bind thiosulfate or sulfate rather than sulfite. The results of the kinetics and inhibitor studies for ^{35}S-thiosulfate uptake by *P. aeruginosa* are similar to the findings of

Kelley *et al.* (31) using the chemolithotrophic *T. ferrooxidans* and Furusaka's (24) work with the autotroph *Desulfovibrio desulfuricans*.

The present investigation was initiated to further characterize the nutritional requirements and metabolic properties of *P. aeruginosa* when grown with inorganic sulfur compounds. The ability of *P. aeruginosa* to adapt to environments with high concentrations of reduced inorganic sulfur compounds was explained by the induction of an extensive oxidase system for these compounds. All compounds were oxidized to sulfate as the end product. Whether the soluble form of thiosulfate oxidase is incorporated into the organism's membrane and linked to oxidative phosphorylation could not be determined because of the inability to solubilize the particulate form. The inability to detect APS-reductase activity in CFE of *P. aeruginosa* grown on thiosulfate does suggest that no substrate level phosphorylation is linked to thiosulfate oxidation. Trudinger (74) has concluded that, because growth was unaffected by the concentration of thiosulfate, the thiosulfate oxidizing enzyme does not appear to be involved in energy production in the bacterium and that this pathway represents an incidental side reaction.

The role of soil heterotrophs in the sulfur cycle is still unknown. The ability of these organisms, such as pseudomonas, in adapting to different environments is widely recognized. Our results would suggest that Trudinger is correct in concluding that thiosulfate oxidation may not be linked to energy production but rather be an important means of detoxification allowing *P. aeruginosa* to exist in environments containing high concentrations of reduced sulfur compounds.

Richmond and Clarke (60) have shown interest in the evolutionary potential of *P. aeruginosa*, particularly the genetic flexibility of this organism. Of interest are the great similarities which exist between thiosulfate oxidase isolated from *Alcaligenes* sp. (25), *T. novellus* (12), *T. thioparus* (39), and the thiosulfate oxidase studied here. Jackson *et al.* (29) have shown using G + C % composition analysis that *T. novellus* (66%) and *T. thioparus* (66%) are closely related to *P. aeruginosa* (64-66%). The possibility that these organisms either have developed evolutionarily together or are capable of plasmid exchange should be considered with relation to their ability to oxidize reduced sulfur compounds. The significance of plasmids in the evolutionary development of *P. aeruginosa* is exciting since the organism has been shown to exchange plasmids with such soil organisms as rhodospirillum, rhodopseudomonas, rhizobium, azotobacter, and acinetobacter (16, 47).

Finally, the similarities between the heterotroph *P. aeruginosa* and soil organisms of the sulfur cycle should be further characterized with regards to genetic regulation and nutritional requirements. Through these further studies, the significance of such metabolic diversity by pseudomonas can be better understood.

Summary

Pseudomonas aeruginosa was grown on a succinate-basal salts medium supplemented with various inorganic sulfur compounds as their sole source of sulfur. The organism was able to grow on the sodium salts of either sulfide, thiosulfate, tetrathionate, dithionite, metabisulfite, sulfite or sulfate, but not on dithionate. Analyses of the culture media after 24 h growth showed the accumulation of sulfate from each of the inorganic sulfur sources other than sulfate. Manometric studies with resting cells obtained by growth on each of the individual sulfur sources yielded net oxygen uptake for each substrate except for sulfite and dithionate. Similar results were obtained with cell-free extracts from these cells using spectrophotometric techniques. Thiosulfate oxidase activity appeared to be induced by growth on sulfide, thiosulfate and tetrathionate, with little or no activity observed when cells were grown on inorganic sulfur sources of higher oxidative states. Metabisulfite oxidase appeared to be associated with growth on all inorganic sulfur compounds. Rhodanese activity appeared to be constitutively present and its activity appeared to be independent of the growth medium employed and was only observed in a soluble fraction. Thiosulfate and tetrathionate oxidase activities were studied in greater detail than the other sulfur oxidases and both were found to be distributed between particulate and soluble fractions.

The soluble form of thiosulfate oxidase was purified 85-fold through ultracentrifugation, ultrafiltration, ion exchange chromato-

graphy and gel filtration. Attempts to solubilize the particulate activity by deoxycholate or Triton X-100 treatment were unsuccessful. The partially purified enzyme had a molecular weight of approximately 100,000 with a pH optimum of 6.0-6.5 and an optimum temperature of 37°C when utilizing ferricyanide as electron acceptor. Cytochrome c was utilized as an electron acceptor whereas NAD, NADP, FAD, Methylene blue, and 2,6-dichlorophenol-indophenol, failed to replace ferricyanide. The Km values for thiosulfate and ferricyanide were 6.7×10^{-4}M and 1.1×10^{-3}M, respectively. Enzyme activity was inhibited 100% by 1 mM mercuric chloride and 53% by 1 mM pCMB. Sulfite also inhibited the oxidation of thiosulfate.

The uptake of ^{35}S-thiosulfate by resting cells showed typical saturation kinetics and a binding Km of 0.5 mM was determined. The uptake of ^{35}S-thiosulfate per mg dry weight was similar for both thiosulfate and sulfate grown cells. Uptake was stimulated by energy sources and was decreased by Group VI anions, sulfate, and inhibitors of oxidative phosphorylation.

Literature Cited

1. Adachi, K., and I. Suzuki. 1977. A study on the reaction mechanism of adenosine 5'phosphosulfate reductase from *Thiobacillus thioparus*, an iron-sulfur flavoprotein. Can. J. Biochem. *55*: 91-98.

2. Adachi, T., Y. Murooka, and T. Harada. 1975. Regulation of arylsulfatase synthesis by sulfur compounds in *Klebsiella aerogenes*. J. Bacteriol. *121*:29-35.

3. Adair, F. W., G. Geftie, and J. Gelzer. 1969. Resistance of *Pseudomonas* to quaternary ammonium compounds. Appl. Microbiol. *18*:299-302.

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

5. Aleem, M. I. H. 1965. Thiosulfate oxidation and electron transport in *Thiobacillus novellus*. J. Bacteriol. *90*:95-101.

6. Aminuddin, M., and D. J. D. Nicholas. 1974. An AMP-independent sulphite oxidase from *Thiobacillus denitrificans*: purification and properties. J. Gen. Microbiol. *82*:103-113.

7. Aminuddin, M., and D. J. D. Nicholas. 1974. Electron transfer during sulphide and sulphite oxidation in *Thiobacillus denitrificans*. J. Gen. Microbiol. *82*:115-123.

8. Ayliffe, G. A. J., K. M. Brightwell, B. J. Collins, and E. J. L. Lowbury. 1969. Varieties of a septic practice in hospital practice. Lancet 2:1117-1120.

9. Berglund, F., and B. H. Sörbo. 1960. Turbidimetric analysis of inorganic sulfate in serum, plasma, and urine. Scand. J. Clin. Lab. Invest. *12*:147-150.

10. Bowen, T. J., F. C. Happold, and B. F. Taylor. 1966. Studies on adenosine 5'-phosphosulfate reductase from *Thiobacillus denitrificans*. Biochim. Biophys. Acta *118*:566-576.

11. Brewer, J. M. 1974. Electrophoresis, p. 128-159. *In* J. M. Brewer, A. J. Pesce, and R. B. Ashworth (eds.), Experimental techniques in biochemistry. Prentice-Hall, New Jersey.

12. Charles, A. M., and I. Suzuki. 1966. Mechanism of thiosulphate oxidation by *Thiobacillus novellus*. Biochim. Biophys. Acta *128*:510-521.

13. Charles, A. M., and I. Suzuki. 1966. Purification and properties of sulphite:cytochrome c oxido-reductase from *Thiobacillus novellus*. Biochim. Biophys. Acta *128*:522-534.

14. Charles, A. M., and I. Suzuki. 1965. Sulphite oxidase of a facultative autotroph, *Thiobacillus novellus*. Biochem. Biophys. Res. Commun. *19*:686-690.

15. Cook, W. K. T. 1967. Aspects of dithionate metabolism in *Thiobacillus denitrificans*. Biochem. J. *102*:5p.

16. Datta, N., R. W. Hedges, E. J. Shaw, R. B. Sykes, and M. H. Richmond. 1971. Properties of an R factor from *Pseudomonas aeruginosa*. J. Bacteriol. *108*:1244-1249.

17. DeLey, J., and M. VanPoucke. 1961. The formation of sulphite during oxidation of thiosulphate by *Thiobacillus novellus*. Biochim. Biophys. Acta *50*:371-373.

18. Deyrup, I. J. 1964. Effect of kidney $S^{35}O_4$ uptake of compounds related to SO_4 transport and metabolism. Amer. J. Physiol. *207*:84-88.

19. Dreyfuss, J., and K. J. Monty. 1964. The biochemical characterization of cysteine-requiring mutants of *Salmonella typhimurium*. J. Biol. Chem. *238*:1019-1024.

20. Drozd, J. W. 1977. Energy conservation in *Thiobacillus neapolitanus* C: sulphide and sulphite oxidation. J. Gen. Microbiol. *98*: 309-312.

21. Fitzgerald, J. W., and P. C. Luschinski. 1977. Further studies on the formation of choline sulfate by bacteria. Can. J. Microbiol. *23*:483-490.

22. Fitzgerald, J. W., and L. C. Kight. 1977. Physiological control of alkylsulfatase synthesis in *Pseudomonas aeruginosa*: effects of glucose, glucose analogs, and sulfur. Can. J. Microbiol. *23*:1456-1464.

23. Fitzgerald, J. W. 1976. Sulfate ester formation and hydrolysis: a potentially important yet often ignored aspect of the sulfur cycle of aerobic soils. Bacteriol. Rev. *40*:698-721.

24. Furusaka, C. 1961. Sulphate transport and metabolism by *Desulphovibrio desulphuricans*. Nature (London) *192*:427-429.

25. Hall, M. R., and R. S. Berk. 1972. Thiosulfate oxidase from an *Alcaligenes* grown on mercaptosuccinate. Can. J. Microbiol. *18*:235-245.

26. Hall, M. R. 1971. Purification and characterization of thiosulfate oxidase from a soil heterotroph. Ph.D. dissertation, Wayne State University School of Medicine, Detroit, Michigan.

27. Hall, M. R., and R. S. Berk. 1968. Microbial growth on mercaptosuccinic acid. Can. J. Microbiol. *14*:515-523.

28. Imperato, T. J., C. G. Wong, L. J. Chen, and R. J. Bolt. 1977. Hydrolysis of lithocholate sulfate by *Pseudomonas aeruginosa*. J. Bacteriol. *130*:545-547.

29. Jackson, J. F., D. J. W. Moriarty, and D. J. D. Nicholas. 1968. Deoxyribonucleic acid base composition and taxonomy of thiobacilli and some nitrifying bacteria. J. Gen. Microbiol. *53*:53-60.

30. Johnson, J. L., and K. V. Rajagopalan. 1976. Purification and properties of sulfite oxidase from human liver. J. Clin. Invest. *58*:543-550.

31. Kelley, B. C., O. H. Tuovinen, and D. J. D. Nicholas. 1976. Utilization of ^{35}S-thiosulphate and an appraisal of the role of ATP-sulphurylase in the chemolithotrophic *Thiobacillus ferrooxidans*. Arch. Microbiol. *109*:205-208.

32. Kelly, D. P., and P. J. Syrett. 1966. [^{35}S] Thiosulfate oxidation by *Thiobacillus* strain C. Biochem. J. *98*:537-545.

33. Koch, A.L., and S. L. Putnam. 1971. Sensitive biuret method for determination of protein in an impure system such as whole bacteria. Anal Biochem. *44*:239-245.

34. LeJohn, H. B., L. VanCaeseele, and H. Lees. 1967. Catabolite repression in the facultative chemoautotroph *Thiobacillus novellus*. J. Bacteriol. *94*:1484-1491.

35. London, J., and S. C. Rittenberg. 1964. Path of sulfur in sulfide and thiosulfate oxidation by *Thiobacilli*. Proc. Natl. Acad. Sci. U.S.A. *52*:1183-1190.

36. Lowbury, E. J. L. 1957. Contamination of centrimide and other fluids with *Pseudomonas pyocyanea*. Brit. J. Industr. Med. *8*: 22-25.

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

38. Lyric, M. R., 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.

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

40. MacGregor, C. H. 1976. Biosynthesis of membrane-bound nitrate reductase in *Escherichia coli*: Evidence for a soluble precursor. J. Bacteriol. *126*:122-131.

41. MacGregor, C. H. 1975. Solubilization of *Escherichia coli* nitrate reductase by a membrane-bound protease. J. Bacteriol. *121*: 1102-1110.

42. Moriarty, D. J. W., and D. J. D. Nicholas. 1970. Electron transfer during sulphide and thiosulphate oxidation by *Thiobacillus concretivorus*. Biochim. Biophys. Acta *216*:130-138.

43. Murooka, V., T. Yamado, S. Tanabe, and T. Harada. 1977. Immunological study of the regulation of cellular arylsulfatase synthesis in *Klebsiella aerogenes*. J. Bacteriol. *132*:247-253.

44. Murphy, M. J., L. M. Siegel, S. R. Tore, and H. Kamin. 1974. Siroheme: a new prosthetic group participating in six-electron reduction reactions catalyzed by both sulfite and nitrate reductase. Proc. Natl. Acad. Sci. U.S.A. *71*:612-616.

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

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

47. Olsen, R. H., and P. Shipley. 1973. Host range and properties of the *Pseudomonas aeruginosa* R factor R1822. J. Bacteriol. *113*: 91-98.

48. Palumbo, S. A. 1973. Influence of sulfite on growth, slime, and fluorescent pigment formation by *Pseudomonas aeruginosa*. Can. J. Microbiol. *19*:505-511.

49. Palumbo, S. A. 1972. Role of iron and sulfur in pigment and slime formation by *Pseudomonas aeruginosa*. J. Bacteriol. *111*:430-436.

50. Pardee, A. B. 1968. Membrane transport proteins. Science *162*: 632-637.

51. Pardee, A. B., and L. S. Prestidge. 1966. Cell-free activity of a sulfate binding site involved in active transport. Proc. Natl. Acad. Sci. U.S.A. *55*:189-191.

52. Pardee, A. B., L. S. Prestidge, M. B. Whipple, and J. Dreyfuss. 1966. A binding site for sulfate and its relation to sulfate transport into *Salmonella typhimurium*. J. Biol. Chem. *241*: 3962-3969.

53. Pardee, A. B. 1966. Purification and properties of a sulfate-binding protein from *Salmonella typhimurium*. J. Biol. Chem. *241*:5886-5892.

54. Peck, H. D. 1974. The evolutionary significance of inorganic sulfur metabolism. Symp. Soc. Gen. Microbiol. *24*:241-262.

55. Peck, H. D. 1962. Comparative metabolism of inorganic sulfur compounds in microorganisms. Bacteriol. Rev. *26*:67-94.

56. Peck, H. D. 1960. Adenosine 5'-phosphosulphate as an intermediate in the oxidation of thiosulphate by *Thiobacillus thioparus*. Proc. Natl. Acad. Sci. U.S.A. *46*:1053-1057.

57. Pollock, M. R., and R. Knox. 1943. Bacterial reduction of tetrathionate. Biochem. J. *37*:476-481.

58. Radcliffe, B. C., and D. J. D. Nicholas. 1970. Some properties of a nitrate reductase from *Pseudomonas denitrificans*. Biochim. Biophys. Acta *205*:273-287.

59. Rammler, D. H., C. Grado, and L. R. Fowler. 1964. Sulfur metabolism of *Aerobacter aerogenes*. I. A repressible sulfatase. Biochem. *3*:224-230.

60. Richmond, M. H., and P. H. Clarke. 1975. Evolutionary prospects for *Pseudomonas* species, p. 341-358. *In* P. H. Clarke and M. H. Richmond (eds.), Genetics and biochemistry of *Pseudomonas aeruginosa*. J. Wiley and Sons, London.

61. Rogers, K. B. 1960. *Pseudomonas* infections in a children's hospital. J. Appl. Bacteriol. *23*:533-537.

62. Roy, A. B., and P. A. Trudinger. 1970. The biochemistry of inorganic compounds of sulfur. Cambridge University Press, London, England.

63. Santer, M., J. Boyer, and U. Santer. 1959. *Thiobacillus novellus*. I. Growth on organic and inorganic media. J. Bacteriol. *78*: 197-202.

64. Schedel, M., J. Legall, and J. Baldensperger. 1975. Sulfur metabolism in *Thiobacillus denitrificans*. Evidence for the prescence of a sulfite reductase activity. Arch. Microbiol. *105*:339-341.

65. Shih, V. E., I. F. Abroms, J. L. Johnson, M. Carney, R. Mandell, R. M. Robb, J. P. Cloherty, and K. V. Rajagopalan. 1977. Sulfite oxidase deficiency: Investigations of a hereditary metabolic disorder. New Engl. J. Med. *297*:1022-1028.

66. Siegel, L. M. 1965. A direct microdetermination for sulfide. Anal. Biochem. *11*:126-132.

67. Sinha, D. B., and C. C. Walden. 1966. Formation of polythionates and their relationships during oxidation of thiosulphate by *Thiobacillus ferrooxidans*. Can. J. Microbiol. *12*:1041-1054.

68. Silver, M., and D. G. Lundgren. 1968. The thiosulfate-oxidizing enzyme of *Ferrobacillus ferrooxidans (Thiobacillus ferrooxidans)*. Can. J. Biochem. *46*:1215-1220.

69. Sörbo, B. H. 1960. Rhodanese, p. 334-337. *In* S. P. Colowick and N. P. Kaplan (eds.), Methods in enzymology, vol. II. Academic Press, Inc., New York.

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

71. Suzuki, I., and C. H. Werkman. 1959. Glutathione and sulfur oxidation by *Thiobacillus thiooxidans*. Proc. Natl. Acad. Sci

U.S.A. *45*:239-254.

72. Tabita, R., M. Silver, and D. G. Lundgren. 1969. The rhodanese enzyme of *Ferrobacillus ferrooxidans (Thiobacillus ferrooxidans)*. Can. J. Biochem. *47*:1141-1145.

73. Trudinger, P. A. 1969. Assimilatory and dissimilatory metabolism of inorganic sulphur compounds by micro-organisms, p. 111-152. *In* A. H. Rose and J. F. Wilkinson (eds.), Advances in microbial physiology. Academic Press, Inc., New York.

74. Trudinger, P. A. 1967. Metabolism of thiosulfate and tetrathionate by heterotrophic bacteria from soil. J. Bacteriol. *93*: 550-559.

75. Trudinger, P. A. 1965. Effect of thiol-binding reagents on the metabolism of thiosulfate and tetrathionate by *Thiobacillus neapolitanus*. J. Bacteriol. *89*:617-625.

76. Trudinger, P. A. 1961. Thiosulfate oxidation and cytochromes in *Thiobacillus* X. II. Thiosulfate oxidizing enzyme. Biochem. *68*:680-686.

77. Tsang, M., and J. A. Schiff. 1976. Sulfate-reducing pathway in *Escherichia coli* involving bound intermediates. J. Bacteriol. *125*:923-933.

78. Tuovinen, O. H., B. C. Kelley, and D. J. D. Nicholas. 1976. Enzymatic comparisons of the inorganic sulfur metabolism in autotrophic and heterotrophic *Thiobacillus ferrooxidans*. Can. J. Microbiol. *22*:109-113.

79. Tuovinen, O. H., B. C. Kelley, and D. J. D. Nicholas. 1975. The uptake and assimilation of sulphate by *Thiobacillus ferrooxidans*. Arch. Microbiol. *105*:123-127.

80. Umbreit, W. W., R. H. Burris, and F. Stauffer. 1964. Manometric techniques, 4th edition. Burgess Publishing Co., Minneapolis, Minn.

81. Umbreit, W. W. 1962. Comparative physiology of autotrophic bacteria. Bacterial. Rev. *26*:145-150.

82. Villarejo, M., and J. Westley. 1966. Sulfur metabolism of *Bacillus subtilis*. Biochim. Biophys. Acta. *117*:209-216.

83. Vishniac, W. V. 1974. Organisms metabolizing sulfur and sulfur compounds, p. 456-461. *In* R. E. Buchanan and N. E. Gibbons (eds.), Bergey's manual of determinative bacteriology, 8th edition. Williams and Wilkins, Baltimore.

84. Vishniac, W. V., and P. A. Trudinger. 1962. Symposium on autotrophy. V. Carbon dioxide fixation and substrate oxidation in the chemosynthetic sulfur and hydrogen bacteria. Bacterial. Rev. *26*:168-175.

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

86. Vishniac, W. 1952. The metabolism of *Thiobacillus thioparus*. I. The oxidation of thiosulfate. J. Bacteriol. *64*:363-373.

87. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. *244*:4406-4412.

88. Westley, J. 1973. Rhodanese, p. 327-368. *In* A. Meister (ed.), Advances in enzymology, vol. 39. Academic Press, Inc., New York.

89. Youatt, J. B. 1954. Studies on the metabolism of *Thiobacillus thiocyanoxidans*. J. Gen. Microbiol. *11*:139-149.

Autobiographical Statement

NAME: Lawrence Byron Schook

BIRTH: December 13, 1949; Mt. Clemens, Michigan

MARITAL STATUS: Single

EDUCATION:

1972	B.A.	Albion College, Albion, Michigan Major: Biology
1975	M.S.	Wayne State University School of Medicine, Detroit, Michigan Major: Medical Microbiology Minor: Biochemistry
1978	Ph.D.	Wayne State University School of Medicine, Detroit, Michigan Major: Immunology and Microbiology Minor: Biochemistry
1972-1978		Graduate Assistantship Immunology and Microbiology Wayne State University School of Medicine, Detroit, Michigan

PROFESSIONAL SOCIETIES:

American Society for Microbiology (1973-present).

Detroit Physiological Society, Associate Member (1975-present).

Sigma Xi, Associate Member (1977-present).

National Registry of Microbiologists of the American Academy of Microbiology, Registered Microbiologist (1977-present).

PROFESSIONAL EXPERIENCE:

Instructor in Wayne State University High School Student Summer Research Program (1974).

Laboratory Assistant for 2nd Year Medical Student Unit in Immunology and Microbiology (1975, 1976).

Laboratory Assistant for Graduate Course in Bacterial Metabolism (1975).

Laboratory Assistant for Advanced Institute in Methods of Immunological Diagnosis (1977).

Instructor in Wayne State University School of Medicine Summer Enrichment Program (1977).

Consultant - Cartel Consulting Co. (1976-present).

PUBLICATIONS AND REPORTS:

1. *In vivo* studies of perorally induced infections of the murine lung and stomach by *Pseudomonas aeruginosa*. Schook, L. B., Carrick, L., Jr., and Berk, R. S. Abs. Annual Meeting, Amer. Soc. Microbiol. *74*:121.

2. Experimental studies with mice perorally administered *Pseudomonas aeruginosa*. Schook, L. B., Carrick, L., Jr., and Berk, R. S. Abs. Annual Meeting, Amer. Soc. Microbiol. *75*:22.

3. Murine gastrointestinal tract as a portal of entry in experimental *Pseudomonas aeruginosa* infections. Schook, L. B., Carrick, L., Jr., and Berk, R. S. 1976. Infect. and Immun. *14*:564-570.

4. Experimental pulmonary infection of mice by tracheal intubation of *Pseudomonas aeruginosa*. Schook, L. B., Carrick, L., Jr., and Berk, R. S. 1976. Internatl. Res. Commun. Med. Sci. *4*: 401.

5. Nutritional studies with *Pseudomonas aeruginosa* grown on inorganic sulfur sources. Schook, L. B. and Berk, R. S. Abs. Annual Meeting, Amer. Soc. Microbiol. *77*:220.

6. Experimental pulmonary infection of mice by tracheal intubation of *Pseudomonas aeruginosa*: The use of antineoplastic agents to overcome natural resistance. Schook, L. B., Carrick, L., Jr., and Berk, R. S. 1977. Can. J. Microbiol. *23*:823-826.

7. Nutritional studies with *Pseudomonas aeruginosa* grown on inorganic sulfur sources. Schook, L. B., and Berk, R. S. J. Bacteriol. (*In press*).