STUDIES ON THE SULFUR METABOLISM OF

THIOBACILLUS THIOOXIDANS

BY

ODEAN M. LUKOW

A Thesis

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In partial fulfillment

of the requirements for the degree

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TO MY FAMILY

ABSTRACT

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Sulfur and sulfite oxidation of intact cells and cell-free preparations of _Thiobacillus thiooxidans_ was studied by means of oxygen uptake with the Gilson Oxygraph. Aerobic sonication of whole cells produced enzymatically active extracts. Sulfite-oxidizing activity was associated solely with the 152,000 x g membrane fraction whereas sulfur-oxidizing activity was distributed between both the soluble and particulate fractions. The K_m of sulfite in the membrane fraction was found to be 0.15 mM as compared to 10.0 mM for whole cells.

Cell-free preparations required the addition of small amounts of reduced sulfhydryl compounds for sulfur oxidation. The requirement was met equally by reduced glutathione (GSH), dithiothreitol or dithioerythritol.

The sulfur-oxidizing enzyme (sulfur dioxygenase EC 1.13.11.18) was purified 18-fold. Rhodanese was found associated with the enzyme. The K_m of GSH for purified enzyme fractions was 61-65 μM. Thiosulfate was determined as the end product of sulfur oxidation by the 152,000 x g supernatant.

Preliminary spectrophotometric studies revealed the presence of cytochromes in the Sepharose 4B purified enzyme and the acid pellet fractions. Sulfite: cytochrome _c_ and cytochrome oxidase were also detected in the acid pellet fraction.

ACKNOWLEDGEMENTS

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INTRODUCTION

INTRODUCTION

The concept of autotrophic bacteria was first introduced in 1887 by Winogradsky. Although his theories were based on studies on a Beggiatoa sp., which remain unsubstantiated, the idea of an autotrophic mode of life was essentially correct. However, no generally accepted definition of a chemoautotrophic bacterium has been proposed to date. Taken literally, the word autotroph refers to an organism that is self-sufficient. It is an organism able to live, grow and multiply in an environment totally free from organic compounds (Woods and Lascelles, 1954). Chemoautotrophic bacteria derive their energy and reducing power from the oxidation of inorganic substances whereas cellular carbon is totally supplied by carbon dioxide. The degree by which organic compounds supplement the nutrition of the microorganism determines its classification as an obligate, facultative, or mixotrophic chemoautotroph.

Thiobacillus thiooxidans, an obligate chemoautotrophic bacterium, oxidizes reduced or partially reduced sulfur compounds including elemental sulfur, sulfides, thiosulfate, polythionates, and sulfite. Its ability to withstand acid conditions lower than pH 1.0 distinguish this microorganism from other thiobacilli.

The simplicity of the nutritional requirements of Thiobacillus thiooxidans belies its biochemical complexity, particularly in the area of sulfur oxidation. Only a basic outline of the mechanism of sulfur oxidation has been elucidated. In the past, the problem

posed by sulfur oxidation has been approached in three ways: whole cell studies, examination of cell-free preparations, and purification of individual enzymes. This study encompassed all of these processes of enquiry, specifically the characterization of the sulfur- and sulfite- oxidizing systems of whole cells and subcellular fractions, and the partial purification of the sulfur-oxidizing enzyme (sulfur dioxygenase EC 1.13.11.18). The aim was to acquire further insight into the relationship between the enzymes involved in sulfur oxidation and a greater understanding of the complete sulfur oxidation pathway of *Thiobacillus thiooxidans*.

HISTORICAL

HISTORICAL

Although the thiobacilli were discovered in 1902 by Nathansohn, it was not until 1921 (a,b) that Waksman and Joffe succeeded in isolating an unusual chemoautotrophic sulfur-oxidizing bacterium from soil, later named *Thiobacillus thiooxidans* (Waksman and Joffe, 1922). The discovery was quickly followed by numerous studies on the physiology of the microorganism (Lipman *et al*., 1921; Joffe, 1922 a,b,c; Waksman, 1922 a,b,c; Waksman *et al*., 1923; Waksman and Starkey, 1922, 1923; Lipman, 1923; Starkey, 1925 a,b) together with an examination of sulfur metabolism. The current status of these biochemical aspects has been reviewed thoroughly (Lees, 1955, 1960; Peck, 1962, 1968; Trudinger, 1967, 1969; Kelly, 1968; Roy and Trudinger, 1970; Suzuki, 1974; Aleem, 1975). In spite of intensive investigation, however, the process of sulfur oxidation by chemoautotrophic bacteria has continued to be an enigma for many scientists.

One major unsolved problem involves the mechanism of attack of the bacterial cell on the sulfur particle and the mobilization of this insoluble substrate to the essential enzyme systems. Phospholipids and other extracellular compounds released by the cells have been postulated to react with and solubilize the sulfur prior to its entrance into the cell (Schaeffer and Umbreit, 1963; Jones and Benson, 1965; Shively and Benson, 1967; Roy and Trudinger, 1970). The results of Vogler and Umbreit (1941) contradict this theory in that direct contact between the

organism and sulfur was shown to be obligatory for oxidation. Furthermore, as washed whole cells oxidized sulfur (Suzuki, 1965 a), an extracellular sulfur-oxidizing enzyme was discounted.

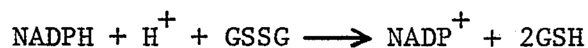
Microscopy has revealed a firm attachment of the cells to sulfur particles. Cells have been photographed clustered around eroded sulfur crystals, indicating more than a transient attachment (Waksman, 1932; Schaeffer *et al.*, 1963). A brief stationary phase after inoculation of a culture appears to be necessary for the production of phospholipids or other materials required for adhesion (Cook, 1964).

Whether the sulfur is metabolized directly at the outer membrane surface or after its translocation into the cell is still unknown. Fat globules, described by Umbreit *et al.* (1942), were thought to be instrumental in dissolving sulfur in *Thiobacillus thiooxidans*. However, besides an inadequate explanation as to how the sulfur may be transported across the enclosing membrane, the fat globules were not confirmed in electron microscopic studies (Umbreit and Anderson, 1942). In addition, Knaysi (1943) demonstrated that the "fat" globules actually consisted of volutin and sulfur.

In 1937, Starkey noted the production of small amounts of hydrogen sulfide from sulfur by *Thiobacillus thiooxidans*. A mechanism for hydrogenation of sulfur by sulfhydryl groups, similar to the proposal of Sluiter (1930) involving reduced glutathione (GSH), was suggested as follows:

$$S + 2\ GSH \longrightarrow H_2S + GSSG$$

Nevertheless, Starkey considered that sulfur reduction prior to its cellular entrance was untenable, since the energy required for sulfhydryl synthesis would be greater than that gained from sulfide oxidation. In reality, complete sulfhydryl synthesis is unwarranted. Glutathione reductase is capable of regenerating the reduced form of glutathione from the oxidized form and NADPH. The presence of the enzyme has been detected in yeast (Meldrum and Tarr 1935; Racker, 1955), plant tissues (Conn and Vennesland, 1951; Mapson and Goddard, 1951), mammalian tissues (Rall and Lehninger, 1952; Racker, 1955), and bacterial sources (Asnis, 1955; Suzuki and Werkman, 1959 b). Oxidized glutathione is reduced according to the following equation:



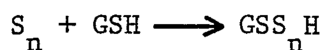
$$NADPH + H^{+} + GSSG \longrightarrow NADP^{+} + 2GSH$$

Moreover, sulfur oxidation by whole cells was inhibited by thiol-binding reagents which suggests that native sulfhydryl groups play an active part in sulfur oxidation (Vogler, 1942; Vogler et al., 1942; Iwatsuka et al., 1962; Adair,1966; Kodama and Mori, 1968 b). As the nature of the reaction occurring at the intact cell surface remains unresolved, cell-free preparations have been investigated in greater detail.

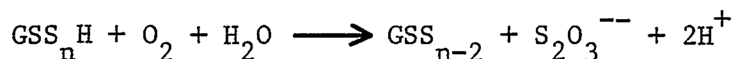
Studies on Thiobacillus thiooxidans have shown that sulfur is converted to sulfuric acid by whole cells without the accumulation of intermediate products (Lipman et al., 1921; Waksman, 1922 a; Waksman and Starkey, 1923; Starkey, 1925; Parker and Prisk, 1953). However, the transformation does take place in several steps. One suggested

pathway of sulfur oxidation proposed the temporary formation of thiosulfate and polythionates (Vishniac and Santer, 1957). Initially, research by Suzuki and Werkman (1959 a) on a cell-free sulfur-oxidizing system appeared to support this pathway. Cell-free extracts, prepared by Raytheon oscillation, were capable of oxidizing sulfur upon the addition of substrate quantities of reduced glutathione (GSH). Polythionates and thiosulfate were detected in the spent reaction mixture. It was suggested that sulfur was reduced to hydrogen sulfide prior to its further oxidation within the cell. Parker and Prisk (1953) observed hydrogen sulfide oxidation by _Thiobacillus thiooxidans_ and this finding was reconfirmed in whole cells and cell-free extracts (Suzuki and Werkman, 1959 a).

Suzuki and Lees (1964) later prepared _Thiobacillus thiooxidans_ extracts which required only catalytic quantities of reduced glutathione (GSH) for sulfur oxidation. The sulfur-oxidizing enzyme was partially purified (Suzuki, 1965 a) and found to be devoid of glutathione reductase and sulfide-oxidizing activity, an indication that the intermediate formation of free sulfide did not occur during sulfur oxidation. Thiosulfate was determined as the end product of the reaction. Subsequently, the mechanism of sulfur oxidation was revised to include glutathione polysulfide as an intermediate (Suzuki, 1965 a). Sulfur oxidation was proposed to be initiated by nucleophilic attack of a sulfhydryl compound on the S_8 ring resulting in the formation of a linear polysulfide chain. Such a compound could be oxidized by the sulfur-oxidizing enzyme as follows:



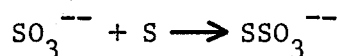
$$S_n + GSH \longrightarrow GSS_nH$$



$$GSS_nH + O_2 + H_2O \longrightarrow GSS_{n-2} + S_2O_3^{--} + 2H^+$$

Research on the sulfur-oxidizing enzymes of Thiobacillus thioparus and Thiobacillus thiooxidans (Suzuki and Silver, 1966) revealed that the actual product of the enzyme was not thiosulfate but rather sulfite. Thiosulfate was produced from the non-enzymatic condensation of sulfur with sulfite as follows:

$$S + O_2 + H_2O \xrightarrow{GSH} SO_3^{--} + 2H^+$$



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

Some discepancy exists in that Imai et al. (1962) reported that the latter reaction was carried out enzymatically by Thiobacillus thiooxidans. This observation has not been confirmed.

Suzuki (1965 b) identified the sulfur-oxidizing enzyme as an oxygenase based on the absolute requirement for molecular oxygen and the incorporation of $^{18}O_2$ into thiosulfate during sulfur oxidation.

Adair (1966) prepared a particulate enzyme system from Thiobacillus thiooxidans by French Pressure Cell that was capable of oxidizing sulfur or sulfite to sulfate, but was ineffective with thiosulfate. Addition of a sulfhydryl compound, such as reduced glutathione, was not required for sulfur oxidation. The ability of thiol-binding agents to inhibit the reaction illustrated the participation of indigenous

acceptors. The particulate system probably arose from the bacterial cell wall and may represent a coordinated enzyme system like that found in whole cells. The supernatant fraction (30,000 x g) from this treatment oxidized sulfur only in the presence of reduced glutathione and compares with the system of Suzuki (1965 a). Adair also subjected cells to Raytheon sonic oscillation and found that, in this case, membrane fragments were unable to oxidize sulfur. Whether or not sulfur oxidation with the addition of reduced glutathione was attempted was not indicated.

Tano and Imai (1968) obtained a soluble sulfur-oxidizing complex from Thiobacillus thiooxidans by ultrasonic oscillation and centrifugation at 130,000 x g for 1 hour. Colloidal sulfur was metabolized without the addition of cofactors. As glutathione reductase was detected in the soluble fraction, it would be possible to regenerate reduced glutathione. Evidence for the presence of active sulfhydryl groups was shown in the inhibition of sulfur-oxidizing activity by p-chloromercuribenzoate and acetate monoiodide. Therefore, the sulfur-oxidizing enzyme as described by Suzuki (1965 a) may be responsible for the sulfur metabolism.

Recently, extensive studies on the sulfur-oxidizing systems of Thiobacillus thiooxidans were carried out (Iwatsuka and Mori, 1960; Kodama and Mori, 1968 a,b; Kodama, 1969; Takakuwa, 1975 a). Whole cells were disrupted by sonic oscillation under a nitrogen atmosphere. Even though the resulting cell-free extract oxidized sulfur, it possessed only a small proportion of whole cell activity (Kodama and Mori, 1968 b)

unlike Suzuki's (1965 a) preparation which retained 60-70% of the original activity. Addition of reduced glutathione to the reaction mixture had no effect on the activity. When the cell-free extract was fractionated by centrifugation at 105,000 x g for 1 hour, it was found that both the soluble and particulate fractions were required for sulfur oxidation, whereas sulfite-oxidizing activity was recovered solely in the pellet (Kodama, 1969). The soluble fraction was separated into collodion membrane-permeable and -impermeable components. In reconstitution of the sulfur-oxidizing system, the function of the latter fraction could be replaced by NAD or NADP, but not by cysteine or reduced glutathione (Kodama, 1969). Additional purification and characterization of the soluble system was performed by Takakuwa(1975 a). By means of ammonium sulfate fractionation, Amberlite CG-50, DEAE cellulose column chromatography and Sephadex gel filtration, the soluble fraction was resolved into two components: a non-heme iron protein of molecular weight 120,000 and a flavoprotein containing non-heme iron with a molecular weight of 23,000. Removal of iron by KCN or diethyldithiocarbamate treatment decreased the enzyme activity, showing that non-heme iron was essential to sulfur oxidation. This finding substantiated the claim of Suzuki and Silver (1966) that the purified sulfur-oxidizing enzyme of Thiobacillus thioparus contained tightly bound non-heme iron as well as labile sulfide. Inhibition by certain nucleotides, known inhibitors of flavoprotein, support the idea of flavin involvement in sulfur oxidation. The presence of labile sulfide was not investigated.

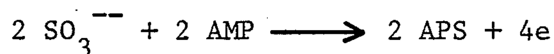
Sulfur-oxidizing enzymes have been isolated from a number of thiobacilli, including *Thiobacillus thioparus* (Suzuki and Silver, 1966), *Thiobacillus novellus* (Charles and Suzuki, 1966, a, b), and *Thiobacillus ferroxidans* (Silver and Lundgren, 1968). In terms of thiosulfate oxidation, the enzyme appears to be significant in the oxidation of the outer sulfur moiety, after the cleavage of thiosulfate to sulfur and sulfite (Suzuki and Silver, 1966). Resolution of the membrane-associated thiosulfate-oxidizing complex of *Thiobacillus novellus* showed that the sulfur-oxidizing enzyme was one of the components (Oh and Suzuki, 1977 a,b). The mechanism of thiosulfate oxidation by *Thiobacillus intermedius* also involves the sulfur-oxidizing enzyme (Charles, 1969). However, activity was demonstrated only with whole cells, possibly because of the labile nature of the enzyme in extracts.

Taylor (1968) reported that a complete oxidation of sulfur to sulfate, without addition of cofactors, was catalyzed by a soluble fraction from *Thiobacillus neapolitanus*, prepared by French Pressure Cell. Because of inhibition of sulfur oxidation by thiol-binding reagents and sodium azide, native sulfhydryl compounds and the electron transport system were implicated, respectively. The preparation likely contained membrane particles with an intact sulfur-oxidizing system similar to the one prepared by Adair (1966), as the "soluble" supernatant fraction was produced from 30,000 x g centrifugation.

The mechanism of sulfide oxidation in Thiobacillus concretivorous was investigated by Moriarty and Nicholas (1969, 1970). The membrane fraction, again prepared by French Pressure Cell, oxidized sulfide to sulfur which remained membrane bound as a polysulfide and was further oxidized to sulfate upon the addition of dialyzed crude extract. In this system, it appears that the sulfur-oxidizing enzyme resided in the soluble fraction.

As a whole, experimental evidence upholds the suggestion of Vishniac and Santer (1957) that thiol groups on the bacterial cell membrane function in sulfur metabolism. Trudinger (1967) speculated that the sulfur-oxidizing enzyme, as described by Suzuki (1965 a), may be located at or near the cell membrane, and that a membrane-bound thiol, rather than reduced glutathione may be the functional cofactor. Such a proposal is attractive in the light of data already presented.

Sulfite is a key intermediate not only in sulfur oxidation but also in thiosulfate oxidation. There are basically two mechanisms by which thiobacilli can oxidize sulfite to sulfate: APS reductase (adenosine-phosphosulfate reductase) and sulfite oxidase (sulfite: cytochrome c oxidoreductase). Peck (1960, 1962), working with Thiobacillus thioparus, first proposed the intermediary formation of APS (adenosine phosphosulfate) from sulfite and AMP by APS reductase. During substrate-level phosphorylation, APS is converted to ADP and sulfate by ADP sulfurylase. ATP and AMP are produced by means of adenylate kinase as follows:



$$2\ SO_3^{--} + 2\ AMP \longrightarrow 2\ APS + 4e$$

$$2\ APS + 2\ Pi \longrightarrow 2\ ADP + 2SO_4^{--}$$

$$2\ ADP \longrightarrow AMP + ATP$$

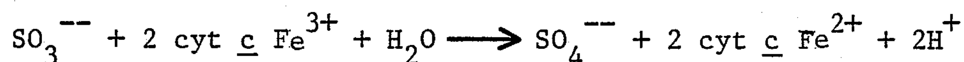
The validity of the initial hypothesis has been confirmed (Peck, 1961 a,b; Peck and Fisher, 1962; Peck and Stulberg, 1962; Peck *et al.*, 1965).

APS reductases have been purified from *Desulfovibrio vulgaris* (Peck *et al.*, 1965), *Thiobacillus denitrificans* (Bowen *et al.*, 1966), *Thiobacillus thioparus* (Lyric and Suzuki, 1970 b), and *Thiocapsa roseopersicina* (Trüper and Rogers, 1971). All have a similar molecular weight (170,000 - 218,500) and contain one FAD, 4-13 non-heme irons, and 4-12 labile sulfurs per mole. Either ferricyanide or cytochrome *c* can act as electron acceptor. The reaction mechanism of the *Thiobacillus thioparus* enzyme was investigated by initial velocity and product inhibition patterns (Lyric and Suzuki, 1970), and characterization of short-life intermediates (Adachi and Suzuki, 1977). Such studies as these have served to point out the existing differences between the APS reductases from various microorganisms, differences that perhaps may be attributed to diverse physiological functions.

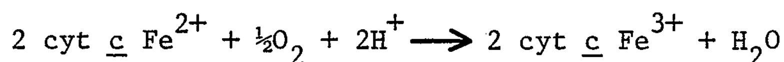
Possible existence of an AMP-independent sulfite-oxidizing system was suggested by a rapid oxidation of sulfite catalyzed by crude extracts *Thiobacillus denitrificans*, without the addition of AMP (Milhaud *et al.*, 1958). Similar results were obtained from extracts

of Thiobacillus thioparus after treatment with activated charcoal to eliminate endogenous AMP (London and Rittenberg, 1964).

Charles and Suzuki (1965, 1966 a,b) isolated and purified an AMP-independent sulfite-oxidizing enzyme from Thiobacillus novellus. It was concluded that sulfite oxidation was composed of two reactions: the oxidation of sulfite to sulfate with the concomittant reduction of cytochrome c by sulfite: cytochrome c oxidoreductase, and the regeneration of oxidized cytochrome c by cytochrome oxidase as follows:



$$SO_3^{--} + 2\ \text{cyt}\ \underline{c}\ Fe^{3+} + H_2O \longrightarrow SO_4^{--} + 2\ \text{cyt}\ \underline{c}\ Fe^{2+} + 2H^+$$



$$2\ \text{cyt}\ \underline{c}\ Fe^{2+} + \tfrac{1}{2}O_2 + 2H^+ \longrightarrow 2\ \text{cyt}\ \underline{c}\ Fe^{3+} + H_2O$$

Sulfite: cytochrome c oxidoreductase and cytochrome c oxidase have both been identified from the membrane-associated thiosulfate-oxidizing complex of Thiobacillus novellus (Oh and Suzuki, 1977 a,b). Sulfite oxidase has been isolated from Thiobacillus intermedius (Charles, 1969), Thiobacillus thioparus (Lyric and Suzuki, 1970 a), and Thiobacillus ferrooxidans (Vestal and Lundgren, 1971).

In Thiobacillus denitrificans, sulfite was oxidized both anaerobically with nitrate and aerobically, through two different electron transport pathways involving cytochromes (Peeters and Aleem, 1970). The fact that the APS and sulfite oxidase systems are not mutually exclusive is apparent, as both pathways are present in this bacterium (Aminuddin and Nicholas, 1973). Sulfite oxidase was associated with the membrane fraction, whereas APS reductase resided in the soluble portion. Purification of the former enzyme and study of the electron transfer

was carried out (Aminuddin and Nicholas, 1974, a,b).

An unusual sulfite-oxidizing enzyme from Thiobacillus neapolitanus has been isolated and characterized (Hempfling et al., 1967). The enzyme was stimulated by AMP and reacted directly with either ferricyanide or oxygen, but did not reduce native or horse-heart cytochrome c. Hempfling et al. proposed that a single enzyme was responsible for both AMP-dependent and -independent activities since the relative activities remained fairly constant throughout the course or purification. However, the formation of APS was not shown with the purified enzyme.

Sulfite oxidation by Thiobacillus thiooxidans is at the preliminary stage of investigation. The uncertainty of the APS reductase system is exemplified by Cook's report (quoted by Adair, 1966) that the enzyme was absent from extracts, while on the other hand, Peck (1961 a, 1962) indicated its presence in this bacterium. The examination of crude cell-free preparations has shown that, like Thiobacillus concretivorus (Moriarty and Nicholas, 1970), a sulfite-oxidizing system exists and is found in the membrane fraction (Adair, 1966; Kodama and Mori, 1968 b). Particulate associated sulfite oxidation has been probed in detail by a number of workers (Kodama and Mori, 1968 b; Kodama et al., 1970; Takakuwa, 1976). The sulfite-oxidizing system was obtained from Thiobacillus thiooxidans by sonication followed by high speed centrifugation (at 105,000 x g for 1 hour). Sulfite oxidation was catalyzed by the membrane fraction with oxygen or bacterial cytochrome c-552 prepared from Pseudomonas stutzeri as

electron acceptor (Kodama *et al.*, 1970). Unlike sulfur oxidation previously discussed, sulfhydryl inhibitors and metal chelating agents did not affect the oxidation of sulfite (Kodama and Mori, 1968 b).

Early studies on the sensitivity of sulfur oxidation to known inhibitors of the cytochrome system led to the proposal that electron transport is coupled via a cytochrome chain to the oxidation of sulfur compounds by thiobacilli (Vogler *et al.*, 1942). Numerous *c*-type cytochromes have been purified and characterized (Klimek *et al.*, 1956; Skarzynski *et al.*, 1956; Aubert *et al.*, 1958; Milhaud *et al.*, 1958; Trudinger, 1961 a,b; Charles and Suzuki, 1966; Moriarty and Nicholas, 1969; Yamanaka *et al.*, 1971; Yamanaka and Kimura, 1974). Cytochromes of the *b*-type have been found in *Thiobacillus neapolitanus* (Trudinger, 1961 a) and *Thiobacillus denitrificans* (Peeters and Aleem, 1970). The terminal oxidase of individual thiobacilli, however, is relatively unknown. For the most part, available data has been derived from inhibition and spectrophotometric studies. The a- or a_3-type cytochromes have been demonstrated in *Thiobacillus novellus* (Aleem, 1965) and *Thiobacillus denitrificans* (Peeters and Aleem, 1970). Furthermore, *Thiobacillus denitrificans* appears to contain an *o*- and a *d*- type cytochrome (Peeters and Aleem, 1970; Aminuddin and Nicholas, 1974 b).

Although Szczepkowski and Skarzynski (1952) reported that *Thiobacillus thiooxidans* was devoid of cytochromes, many in fact have been shown to occur (Cook and Umbreit, 1963; London, 1963) thus confirming Emoto's (1933) earlier observation. The cytochrome system has been examined recently with renewed interest. C-type cytochromes of thiobacilli, including *Thiobacillus thiooxidans*, share similar physiochemical

properties with both mammalian and plant cytochrome _c_ (Tano _et al_., 1968; Takakuwa, 1975 b). Cytochrome oxidase was indicated through inhibition of sulfur oxidation by cyanide and azide (Vogler _et al_., 1942; Iwatsuka and Mori, 1960; Adair, 1966; Tano and Imai, 1968). It differed from classical cytochrome oxidase in that carbon monoxide inhibition was not reversed by light (Iwatsuka and Mori, 1960; Kodama and Mori, 1968 b). In contrast, the binding of carbon monoxide to the terminal oxidase in sulfite oxidation was photoreversible (Kodama and Mori, 1968 b). These data supported the view that the electron transport mechanisms operating in sulfur oxidation distinctly differ from those in sulfite oxidation. In addition, Kodama _et al_., (1970) and Takakuwa (1976) demonstrated that particulate-bound _a_, _b_, and _c_-type cytochromes and flavoprotein participated in sulfite oxidation.

In conclusion, it is apparent from this brief account, that the extent of existing knowledge on the oxidation of inorganic sulfur compounds by thiobacilli is greatly restricted. Clearly, there is a need for further research to investigate and clarify the mechanism of sulfur metabolism by these physiologically unique and sophisticated microorganisms.

MATERIALS AND METHODS

MATERIALS AND METHODS

MATERIALS

All chemicals and reagents used were of analytical grade. Chemicals were obtained from the following sources:

Sulfur (precipitated) - British Drug Houses Ltd.

Glutathione, reduced form (GSH) - Sigma Chemical Company

Sodium sulfite - Fisher Scientific Company

Sodium thiosulfate - Fisher Scientific Company

Cytochrome c (type III, horse heart) - Sigma Chemical Company

British Anti Lewisite (B.A.L.) - Sigma Chemical Company

Dithiothreitol - Sigma Chemical Company

Dithioerytritol - Sigma Chemical Company

Potassium cyanide - J.T. Baker Chemical Company

Catalase (bovine liver, twice crystallized) - Sigma Chemical Company

Aquacide 1-A - Calbiochem

The gel chromatography media, Activated Thiol-Sepharose 4B, Sepharose 4B, and Blue Dextran 2000 were products of Pharmacia.

All reagents and buffers were prepared in glass distilled water.

The sulfur suspension which was used as substrate for the sulfur-oxidizing enzyme was prepared by suspending 36 g precipitated sulfur in 100 ml of 0.04% Tween 80. After the mixture was vigorously stirred for two hours, it was sonicated for 30 min in a 10 kc/sec Raytheon sonic oscillator. The sonicated suspension was allowed to settle for a few

minutes and the supernatant decanted. After repeating the decanting procedure, the white sulfur supernatant was dialyzed 48 hours in 0.04% Tween 80 to remove any contaminating ions. The sulfur concentration was determined by drying aliquots of sulfur suspension at 65°C and weighing, after cooling to room temperature.

ORGANISM AND GROWTH CONDITIONS

Thiobacillus thiooxidans (ATCC 8085) was used in this study. The organism was grown in 3-1 Fernbach flasks containing a 1 each of Starkey's medium (1925): 0.3 g $(NH_4)_2SO_4$, 0.5 g $M_gSO_4 \cdot 7H_2O$, 0.018 g $FeSO_4 \cdot 7H_2O$, 3.5 g KH_2PO_4, 0.25 g $CaCl_2$, and 1000 ml distilled water. The pH of the medium was 4.5. The medium, minus sulfur, was sterilized at 120°C for 20 min. Sterilization of the precipitated sulfur was not necessary. After addition of 3% inoculum, enough powdered precipitated sulfur was added to cover the surface of the medium and the flask was gently swirled. Flasks were cotton-plugged very loosely. As it was found that covering the flasks with only tissue paper promoted extremely rapid growth and low enzyme activity, such practise was discontinued. The flasks were incubated for five days at 28°C and during this time most of the sulfur remained floating on the surface. After incubation, sulfur was removed by filtration through Whatman No. 1 filter paper under suction. At the time of harvesting, the pH of the culture was between 1.4 and 1.6. The cells were collected by a Sharples super centrifuge fitted with a water cooling system (7°C) at 50,000 r.p.m.

and washed 3 times with distilled water. The yield varied between 0.06 - 0.70 g wet weight cells per 1 of medium. Cells were maintained in suspension (about 5 mg wet wt cells per ml water) at 4°c until used for preparing cell-free extract.

For determination of growth during incubation, 10.0 ml of the culture medium was removed at appropriate intervals and separated from sulfur by filtration. The optical density of the filtrate was measured using a Klett-type photometer with a No. 66 filter. Changes of pH with bacterial growth were also monitored.

PREPARATION OF CELL-FREE EXTRACT

The cell suspension was centrifuged and washed once more in 0.05M potassium phosphate buffer (pH 6.8). About 100 mg wet weight cells were resuspended per ml of the same buffer. For whole cell studies, aliquots of this suspension were used. The remaining cell suspension was sonicated with the microtip of the Insonator 1000 system (Ultrasonic Systems Inc.) for up to 60 - 10 second bursts at 20 KH_z and 0°C.

Cell debris was removed by three successive centrifugations at 3,600 x g for 15 min. The resulting supernatant was designated as cell-free extract. The cell-free extract contained 3.0 to 10.0 mg of protein per ml and was used immediately or stored at 4°C until further use.

CENTRIFUGATIONS

Low speed centrifugations were performed in a Sorvall Superspeed RC-2B automatic refrigerated centrifuge at 4°C. High speed centrifugation

of cell-free extract was carried out in a Beckman L3-50 refrigerated ultracentrifuge using a 50 Ti or 60 Ti rotor at a maximum force of 152,000 x g for 1 hr at 4°C to yield the supernatant and pellet fractions. The pellet was resuspended in a volume of 0.05M potassium phosphate buffer (pH 6.8).

SULFUR-OXIDIZING ENZYME PURIFICATION PROCEDURE

The cell-free extract was adjusted to pH 5.0 with N acetic acid at 0° and the precipitate removed by centrifugation at 23,500 x g for 20 min. The acid supernatant fraction could be concentrated 3-fold by dialysis against Aquacide at 4°C. The acid pellet fraction was resuspended in a volume of 0.1M potassium phosphate buffer (pH 7.5) equivalent to that of the acid supernatant fraction.

The acid supernatant (1.0 ml) was applied to a column of Sepharose 4B (6.0 x 0.5 cm) that had been equilibrated with 0.1M potassium phosphate buffer (pH 7.5) at 4°C. The sample was allowed to equilibrate for 15 min before eluting with the same buffer. One ml fractions were collected and the sulfur-oxidizing activity measured in each. The flow rate was about 4.0 ml per hour. The sulfur-oxidizing activity was recovered in fractions immediately following the void volume of 2.0 ml.

Affinity chromatography was attempted with Thiol-Sepharose 4B and Glutathione-Sepharose 4B under the above conditions with one exception. The equilibrating and eluting buffer was 0.1M potassium phosphate containing 1.0 mM EDTA and 0.1 M NaCl.

SULFUR AND SULFITE ENZYME ASSAY PROCEDURE

Sulfur and sulfite oxidations were assayed by measurement of oxygen consumption in a thermostated vessel equipped with a Teflon-covered Clark oxygen electrode (a Gilson Oxygraph). Unless otherwise indicated, the reaction mixture for sulfur oxidation, contained the following in a total volume of 1.5 ml:

118.0 μmoles potassium phosphate (pH 7.5)
0.24 mg sulfur (in the form of 0.1 ml sulfur-Tween 80 suspension)
10.0 μmoles GSH
enzyme as indicated.

In the case of whole cells, the reaction was started by the addition of sulfur suspension. For cell-free fractions, GSH initiated the reaction after the addition of sulfur. The endogenous rates of oxygen uptake due to enzyme plus buffer were routinely measured and subtracted from the enzymatic rates of sulfur oxidation. Similarly, the rate of non-enzymatic oxidation of GSH or other cofactors was measured in the presence of buffer and sulfur, and was used to calculate the net rate of enzymatic sulfur oxidation. Sulfur alone did not display non-enzymatic oxidation.

Unless otherwise indicated, the reaction mixture for sulfite oxidation contained in a total volume of 1.5 ml:

135.0 μmoles potassium phosphate (pH 7.5)
2.5 μmoles Na_2SO_3
2.5 μmoles EDTA
enzyme as indicated.

The reaction was initiated by the addition of sodium sulfite - EDTA solution in microliter quantity. EDTA served to completely inhibit non-enzymatic oxidation of sodium sulfite. The enzymatic rates of sulfite oxidation were corrected for endogenous rates of oxygen uptake as in sulfur oxidation.

All assays were carried out at 30°C.

THIOSULFATE-SULFUR TRANSFERASE (RHODANESE) ASSAY PROCEDURE

Rhodanese activity was measured by the rate of thiocyanate formation from thiosulfate and cyanide according to the method of Sörbo (1959) with minor modifications by Oh (1977 a). One unit of enzyme was defined as that amount of enzyme which formed 1 μmole of thiocyanate under the standard conditions.

SULFITE-CYTOCHROME c OXIDOREDUCTASE ASSAY PROCEDURE

The enzyme activity of the cell-free extract was measured by following the rate of reduction of cytochrome _c_ as described by Lyric and Suzuki (1970 a). For the acid pellet fraction, the reaction mixture contained in a total volume of 0.7 ml:

50.0 μmoles potassium phosphate (pH 7.5)

0.5 μmole Na_2SO_3

0.5 μmole EDTA

0.2 μmole cytochrome _c_ (type III, horse heart)

1.7 mg protein.

The rate of cytochrome c reduction was followed at 550 nm spectrophotometrically.

CYTOCHROME OXIDASE ASSAY PROCEDURE

Activity was assayed according to the method of Oh (1977 a).

THIOSULFATE - OXIDIZING ENZYME ASSAY PROCEDURE

Activity was assayed by coupling to cytochrome c reduction according to the method of Lyric and Suzuki (1970 c).

THIOSULFATE DETERMINATION

The product of the sulfur-oxidizing system was investigated by assaying for thiosulfate at the completion of the reaction. Sulfur oxidation was carried out at room temperature in 5.0 ml serum bottles with vigorous stirring. The components of the reaction mixture were identical to that of the Gilson Oxygraph assays. After incubation, the reaction was stopped by the addition of 0.25 ml of 1M cadmium acetate. After centrifugation to remove sulfur, protein and GSH, 1.0 ml of the supernatant was used for the colorimetric determination of thiosulfate by the method of Sörbo (1957, 1958).

PROTEIN DETERMINATION

Protein was determined by the colorimetric method of Lowry *et al.* (1951). Crystalline bovine serum albumin was used as the reference protein.

SPECTROPHOTOMETRY

The Unicam SP-700 spectrophotometer and the Shimadzu multi-purpose recording (MPS-50L) spectrophotometer were used to record the absorption spectra of enzyme preparations. The Gilford 2400 automatic recording spectrophotometer was used for assays of enzyme activities.

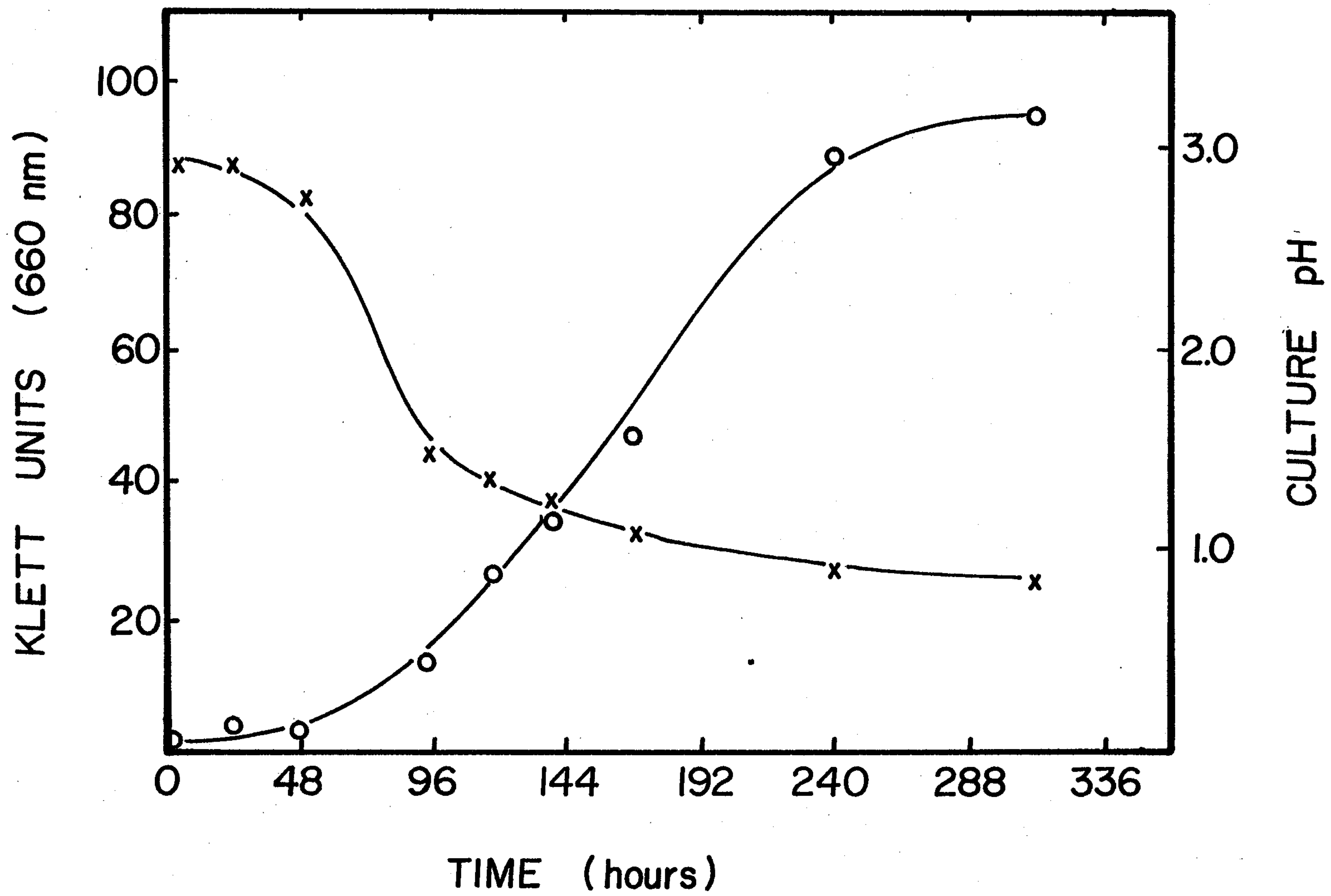
RESULTS

RESULTS

GROWTH OF THIOBACILLUS THIOOXIDANS

To obtain physiologically active cells, low yields of up to 0.1g wet wt cells/1 medium were required. Extremely rapid growth or delayed harvesting resulted in high cell yields and distinct changes in cell morphology and enzyme activity. In addition to cells becoming thicker, elongated, and heavily capsulated, the specific activities of sulfur oxidation enzymes sharply declined. The growth curve (Fig. 1), derived from culture turbidity, was not an adequate indication of the exponential growth phase of the organism. It is probable that not all the cells attached to sulfur crystals were removed during filtration of young culture samples, thereby resulting in an underestimation of cell density. The sharp drop in pH (Fig. 1) was subsequently taken as an indication of rapid growth and the cultures were harvested at that time (120 hours).

Absence of heterotrophic contamination was periodically shown by lack of growth on nutrient agar plates. The *Thiobacillus thiooxidans* culture could be purified by streaking on to thiosulfate agar plates in which 1.0% thiosulfate replaced sulfur in the medium detailed in *Materials and Methods*. Within one week at 28°C, pale yellow-green colonies of 0.3 mm diameter were visible. Addition of 0.02% yeast extract to the thiosulfate agar had no effect on the growth of the organism.


KLETT UNITS (660 nm)
100
80
60
40
20
0
48
96
144
192
240
288
336
TIME (hours)
3.0
2.0
1.0
CULTURE pH

ASSAY SYSTEM

The use of the Gilson Oxygraph with an oxygen electrode proved to be a convenient method for assaying the enzymes involved in sulfur oxidation. The sulfur suspension or sulfhydryl compounds caused little interference with the oxygen probe response. Small non-enzymatic rates of oxidation could be determined and used to calculate net enzymatic rates of oxidation. The choice of sulfur, however, was limited to the white sulfur suspension as prepared in Materials and Methods. In general, 0.1 ml sulfur suspension equivalent to 0.24 mg sulfur was used per assay of sulfur-oxidizing enzyme. Larger amounts of sulfur could cause clogging of the oxygen probe area and a poor response.

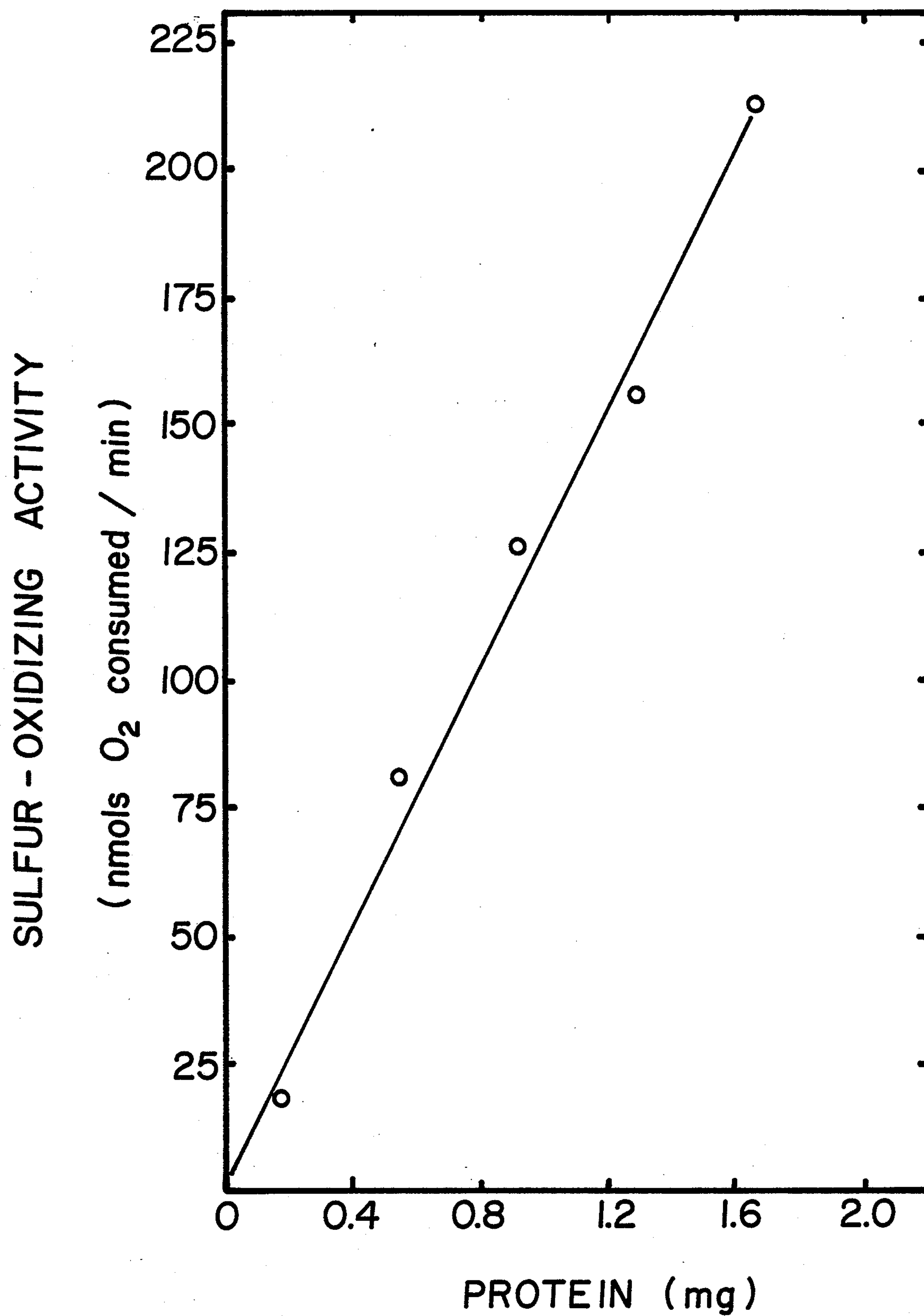
The linear relationships between sulfur- and sulfite-oxidizing activities and protein concentration for whole cells are shown in Fig. 2 and Fig. 3.

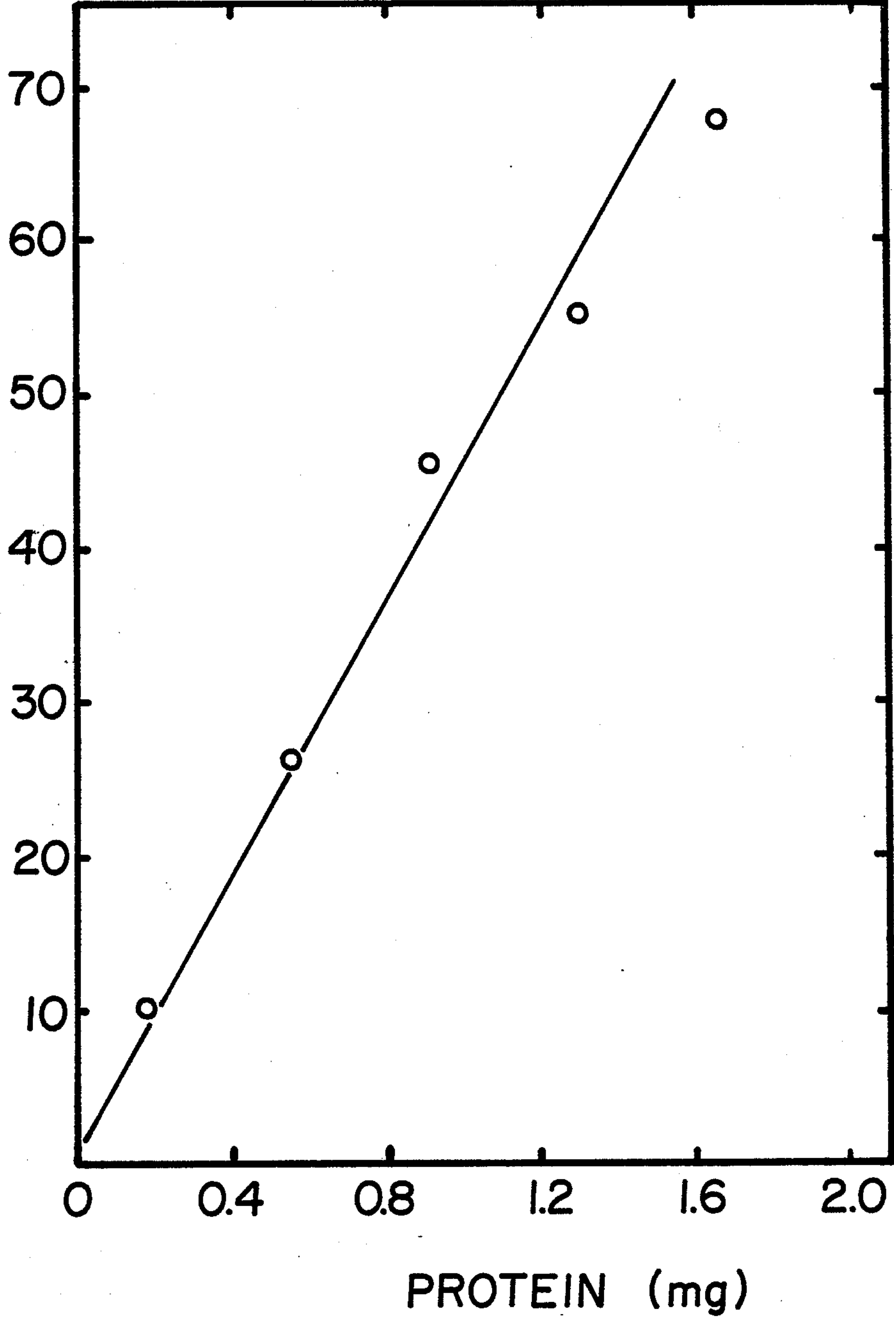
WHOLE CELL CHARACTERISTICS

(1) Oxidation of Sulfur

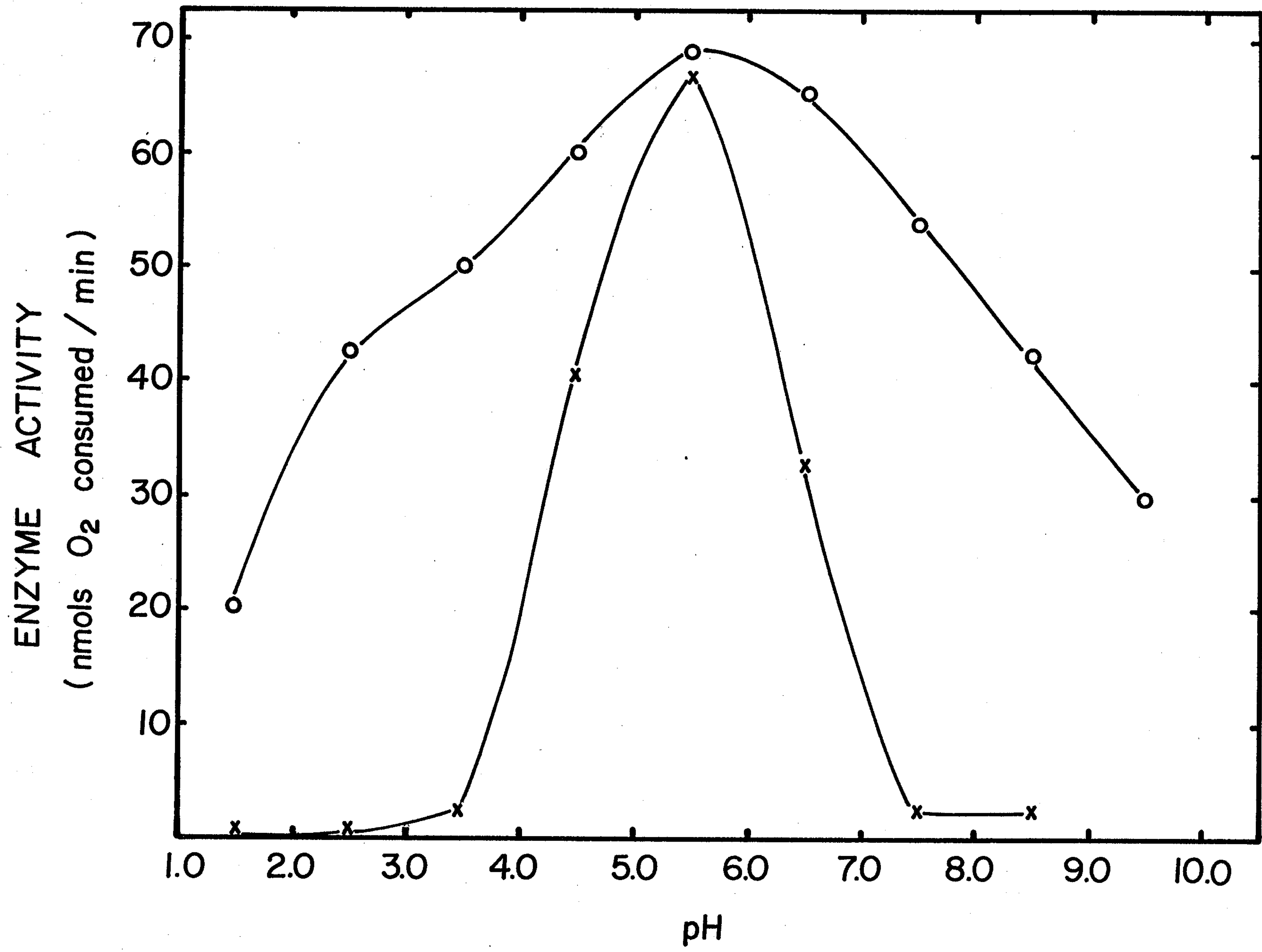
Whole cells showed sulfur-oxidizing activity over a broad pH range, with a maximum at pH values between 4.5 and 6.5 (Fig. 4). At pH values less than 4.5, initial lags in sulfur oxidation lasting up to five minutes were noted. Cells were routinely assayed at pH 5.5 as an indication of relative activity from culture to culture.

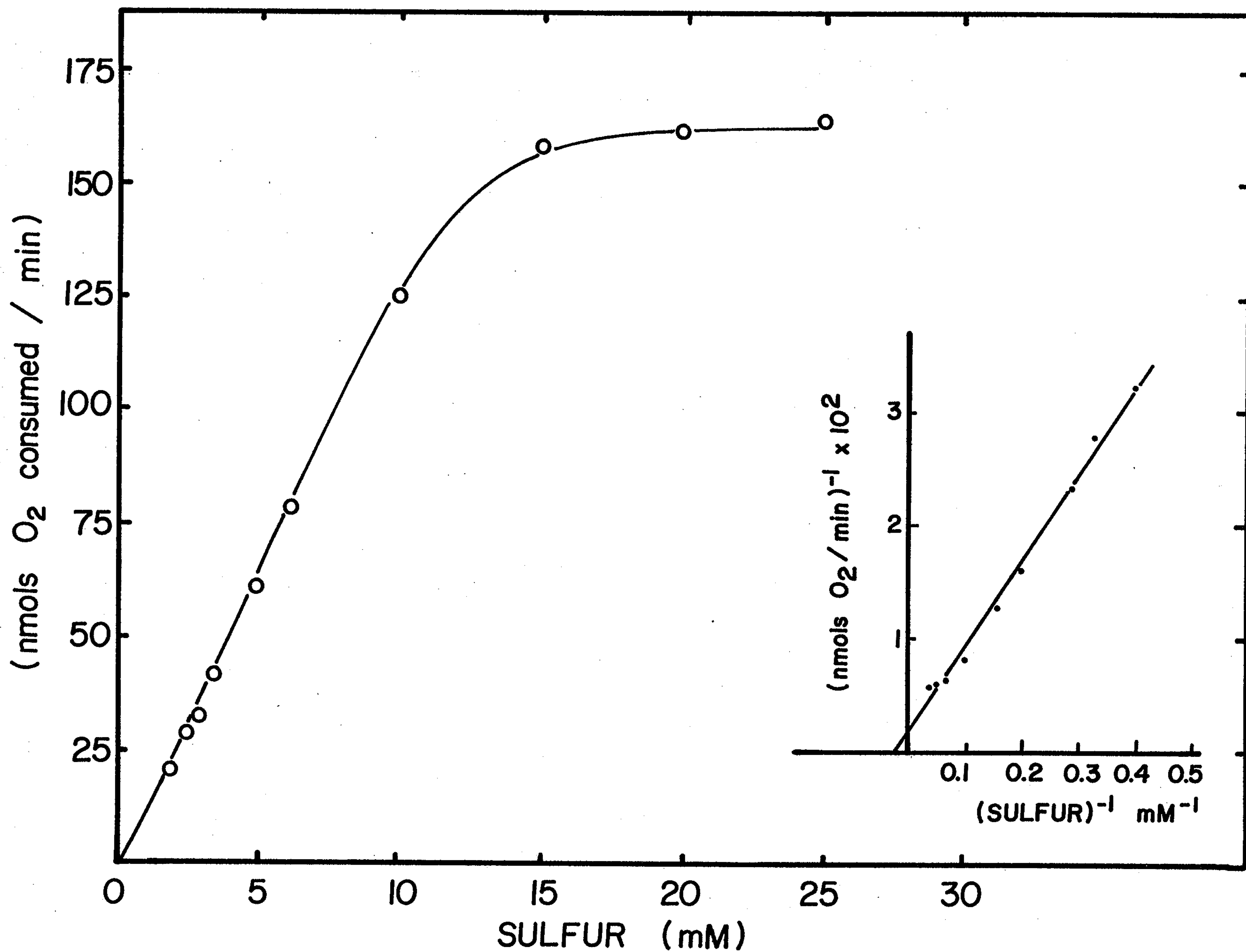
On the basis of the Lineweaver-Burk plot of the reaction velocity versus the concentration of sulfur (Fig. 5), the apparent K_m of sulfur


SULFUR - OXIDIZING ACTIVITY
(nmols O_2 consumed / min)
225
200
175
150
125
100
75
50
25
0
0.4
0.8
1.2
1.6
2.0
PROTEIN (mg)



SULFITE - OXIDIZING ACTIVITY
(nmols O_2 consumed / min)
10
20
30
40
50
60
70
0
0.4
0.8
1.2
1.6
2.0
PROTEIN (mg)


ENZYME ACTIVITY
(nmols O2 consumed / min)
70
60
50
40
30
20
10
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
pH


SULFUR - OXIDIZING ACTIVITY
(nmols O_2 consumed / min)
175
150
125
100
75
50
25
0
5
10
15
20
25
30
SULFUR (mM)
(nmols O_2 / min)$^{-1}$ x 10^2
3
2
1
0.1
0.2
0.3
0.4
0.5
(SULFUR)$^{-1}$ mM^{-1}

was calculated to be 50.0 mM. Enzyme activities at extremely low or high concentrations of sulfur were unreliable due to the nature of the sulfur suspension as previously outlined. A typical assay contained 5.0 mM sulfur.

Whole cells retained 75% of the sulfur-oxidizing activity after storage at 4°C in a buffer suspension for 5 days. Hence, after harvesting, cells were processed within two days to retain 100% activity.

Freezing cell suspensions for 16 to 25 days at -20°C produced a sharp drop in the sulfur-oxidizing activity as compared to freshly harvested cells. When either physiologically active or inactive cells were freeze-thawed, up to 61% of the original activity was lost. Reduced glutathione (GSH) caused approximately 37% inhibition of sulfur oxidation of freshly harvested young cells at pH 5.5, 7.5 or 9.5 (Table 1). When the same cells were freeze-thawed, GSH again inhibited the residual activity throughout the pH range assayed (Table 2). A rapidly growing culture produced cells that reacted differently to GSH after freeze-thawing. Sulfur oxidation was inhibited at and below pH 7.5 whereas activity was restored at pH 8.5 and 9.5 by GSH (Table 3).

(2) Oxidation of Sulfite

The sulfite-oxidizing activity of whole cells was maximal at pH 5.5 (Fig. 4). As compared to the sulfur-oxidizing activity, whole cells oxidized sulfite in a narrow optimum pH range. From Fig. 6, the apparent K_m of the enzyme was calculated to be 10.0 mM on the basis of the Lineweaver-Burk plot of the reaction velocity versus the concentration of sodium sulfite. This high apparent K_m value was a characteristic of enzymatically active cells. Relatively inactive cells in two separate

TABLE 1

Effect of GSH on sulfur oxidation of freshly harvested whole cells.

pH	Sulfur-oxidizing activity*	Sulfur-GSH-oxidizing activity*	Inhibition (%)
5.5	100.4	60.2	40.0
7.5	82.8	52.7	36.4
9.5	56.3	36.4	35.4

*nmole O_2 consumed per min. Oxidation rates have been corrected for endogenous respiration and non-enzymatic GSH oxidation.

The reaction mixture contained in a total volume of 1.5 ml:

127.0 µmoles potassium phosphate
0.24 mg sulfur
10.0 µmoles GSH
1.3 mg protein

Enzyme activity was measured as outlined in Materials and Methods.

TABLE 2

Effect of GSH on sulfur oxidation of freeze-thawed whole cells. Experiment #1

pH	Sulfur-oxidizing activity*	Sulfur-GSH-oxidizing activity*	Inhibition (%)
5.5	13.8	1.2	91.3
6.5	30.1	17.5	41.9
7.5	17.5	3.7	78.9
8.5	4.9	0.0	100.0

* as in Table 1

The reaction mixture was as described in Table 1. Enzyme activity was measured as outlined in Materials and Methods.

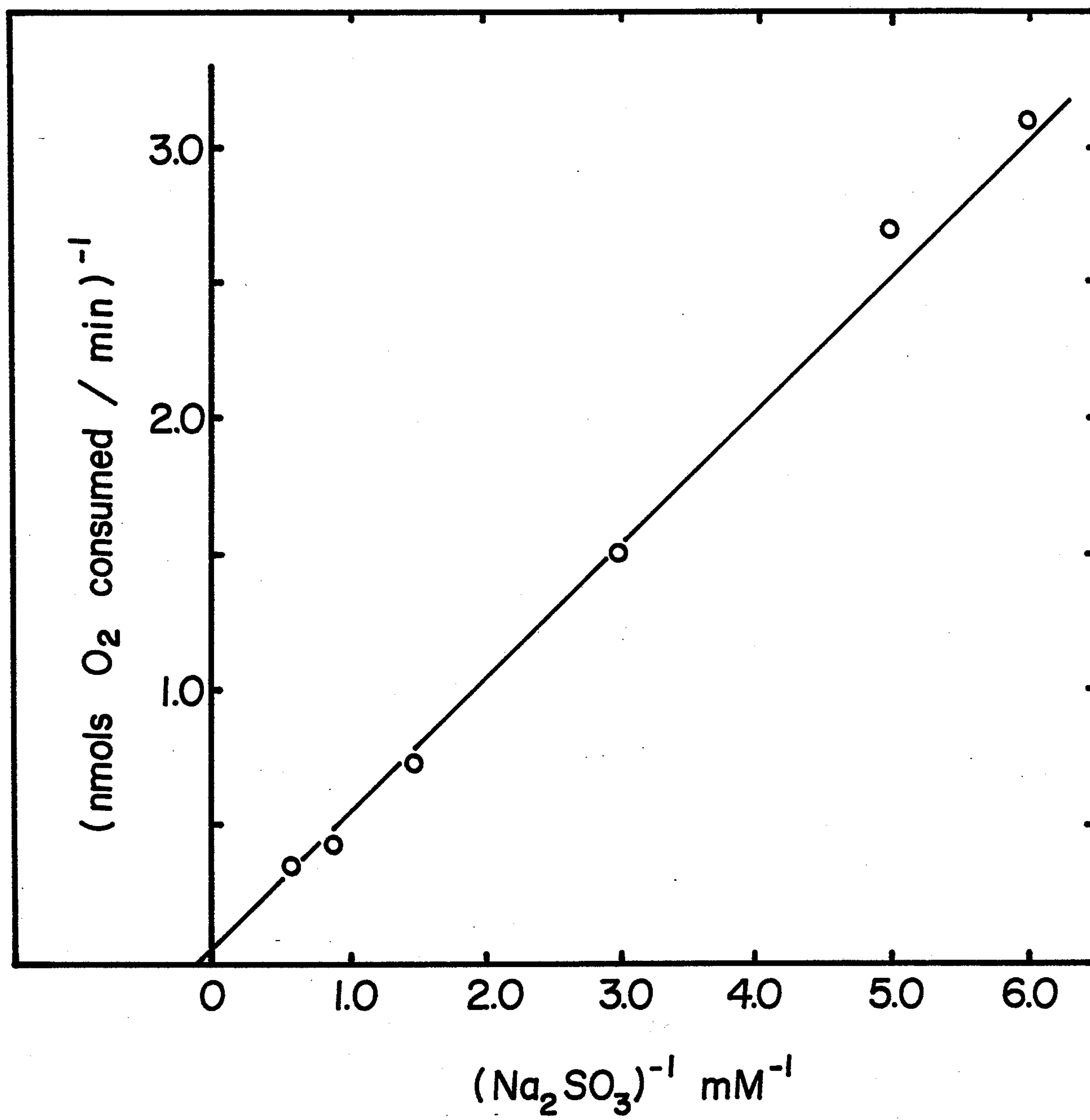
TABLE 3

Effect of GSH on sulfur oxidation of freeze-thawed whole cells. Experiment #2

pH	Sulfur-oxidizing activity*	Sulfur-GSH-oxidizing activity*	Activation or Inhibition (%)	
5.5	22.6	0.6	97.4	Inhibition
6.5	33.8	17.5	48.2	Inhibition
7.5	17.5	16.2	7.4	Inhibition
8.5	2.5	20.0	87.5	Activation
9.5	2.5	16.3	84.7	Activation

*as in Table 1.

The reaction mixture was as described in Table 1 except 1.1 mg protein were used per assay. Enzyme activity was measured as outlined in Materials and Methods.


3.0
2.0
1.0
(nmols O_2 consumed / min)$^{-1}$
0
1.0
2.0
3.0
4.0
5.0
6.0
$(Na_2SO_3)^{-1}$ mM^{-1}

cases yielded K_m values of 2.3 and 0.6 mM.

In terms of sulfite-oxidizing activity, cell suspensions were stable at 4°C for up to 3 days. However, freezing at -20°C for 25 days raised the optimum pH for sulfite oxidation from 5.5 to 7.5. The specific activity increased 2 fold over freshly harvested cells.

(3) Additional Whole Cell Characteristics

Whole cells were unable to oxidize thiosulfate under conditions identical to those used for the assay of sulfite-oxidizing activity, with the exception of the substitution of thiosulfate for sulfite. The K_m values for molecular oxygen in the whole cell sulfur-and sulfite-oxidizing systems could not be determined accurately. The time course of the decrease of dissolved oxygen concentration by both systems, as measured by the Clark oxygen electrode, was linear to almost zero oxygen concentration. Similar observations were noted for sulfur and sulfite oxidation in cell-free fractions. The approximate K_m of molecular oxygen for any of these systems was 10 μM or less.

OXIDATION OF SULFUR AND SULFITE BY CELL-FREE EXTRACT AND SUBCELLULAR FRACTIONS

(1) Cell Free Extract

Recovery of the sulfur-oxidizing enzyme in the cell-free extract varied between 3 and 17% of the whole cell activity. In addition, treating cells prior to sonication with a mixture of cationic and anionic resin as described by Suzuki and Werkman (1958)

and preparing cell-free extract in 0.1 M Tris-HCl buffer of pH 7.5 did not result in greater enzyme recovery.

Sulfur oxidation by the turbid, pale yellow cell-free extract revealed an absolute requirement for activation by sulfhydryl compounds. Reduced glutathione (GSH) was found to be an effective cofactor in sulfur oxidation (Fig. 7) and was routinely used in assays. The effect of GSH concentration on the enzymatic and non-enzymatic oxidation of sulfur is shown in Table 4. As the optimal concentration of GSH was 6.7×10^{-3}M (10 µmoles/1.5 ml), most of the experiments were carried out at this concentration and the enzymatic rate of sulfur oxidation was corrected for non-enzymatic activity. Catalase (300 µg) had no effect on sulfur oxidation. Optimal pH of the GSH-stimulated sulfur-oxidizing enzyme was 7.5 (Fig. 8). A plateau in the activity in the area of pH 8.5 - 9.5 may be attributed to sulfide formation as discussed at a later time.

The possibility of a replacement for GSH in the sulfur-oxidizing enzyme assay was explored (Table 5). Oxidized glutathione, ascorbate and sodium sulfite were completely ineffective as activators. Whereas cysteine and B.A.L. were unstable and only slightly activated sulfur oxidation, dithiothreitol and dithioerythritol produced 96.5 and 85.2% the activation by GSH, respectively.

The K_m values of GSH, dithiothreitol and dithioerythritol were compared as a further indication of sulfydryl specificity in sulfur oxidation. The apparent K_m of GSH was calculated to be 0.18 mM from the Lineweaver-Burk plot of reaction velocity versus substrate concentration (Fig. 9). Similar plots for dithiothreitol and dithioerythritol yielded

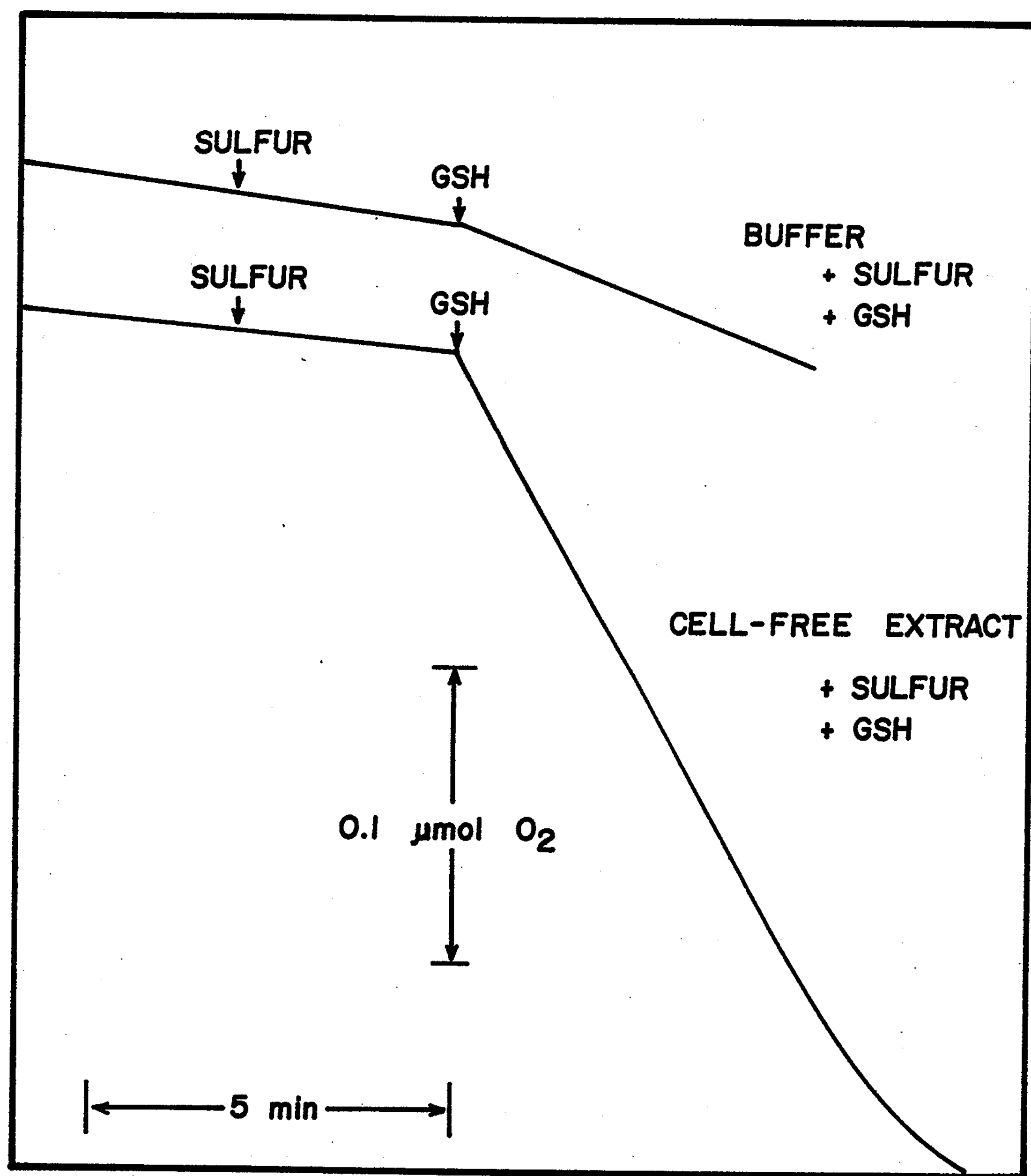

SULFUR
GSH
BUFFER
+ SULFUR
+ GSH
SULFUR
GSH
CELL-FREE EXTRACT
+ SULFUR
+ GSH
0.1 μmol O_2
5 min

TABLE 4

Effect of GSH concentration on the enzymatic and non-enzymatic oxidation of sulfur.

GSH µmoles	Non-enzymatic rate (nmole O_2 consumed per min)	Enzymatic rate (nmole O_2 consumed per min)
0.3	0.0	16.3
5.0	2.5	26.4
10.0	7.6	33.8
20.0	10.1	30.1

Rates of oxidation were measured as described in Materials and Methods. The enzymatic assay contained 1.2 mg protein in the form of cell-free extract.

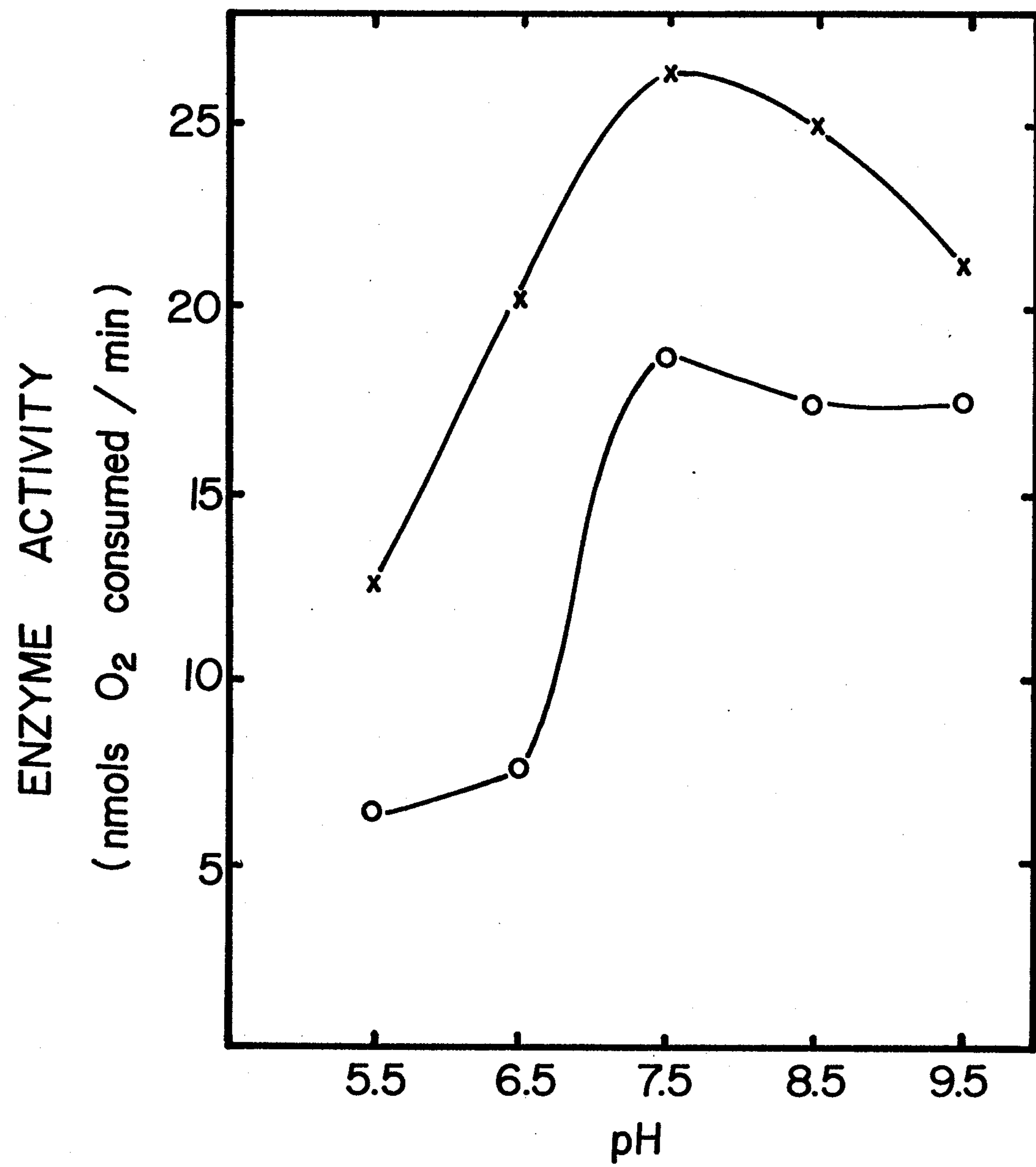

ENZYME ACTIVITY
(nmols O_2 consumed / min)
25
20
15
10
5
5.5
6.5
7.5
8.5
9.5
pH

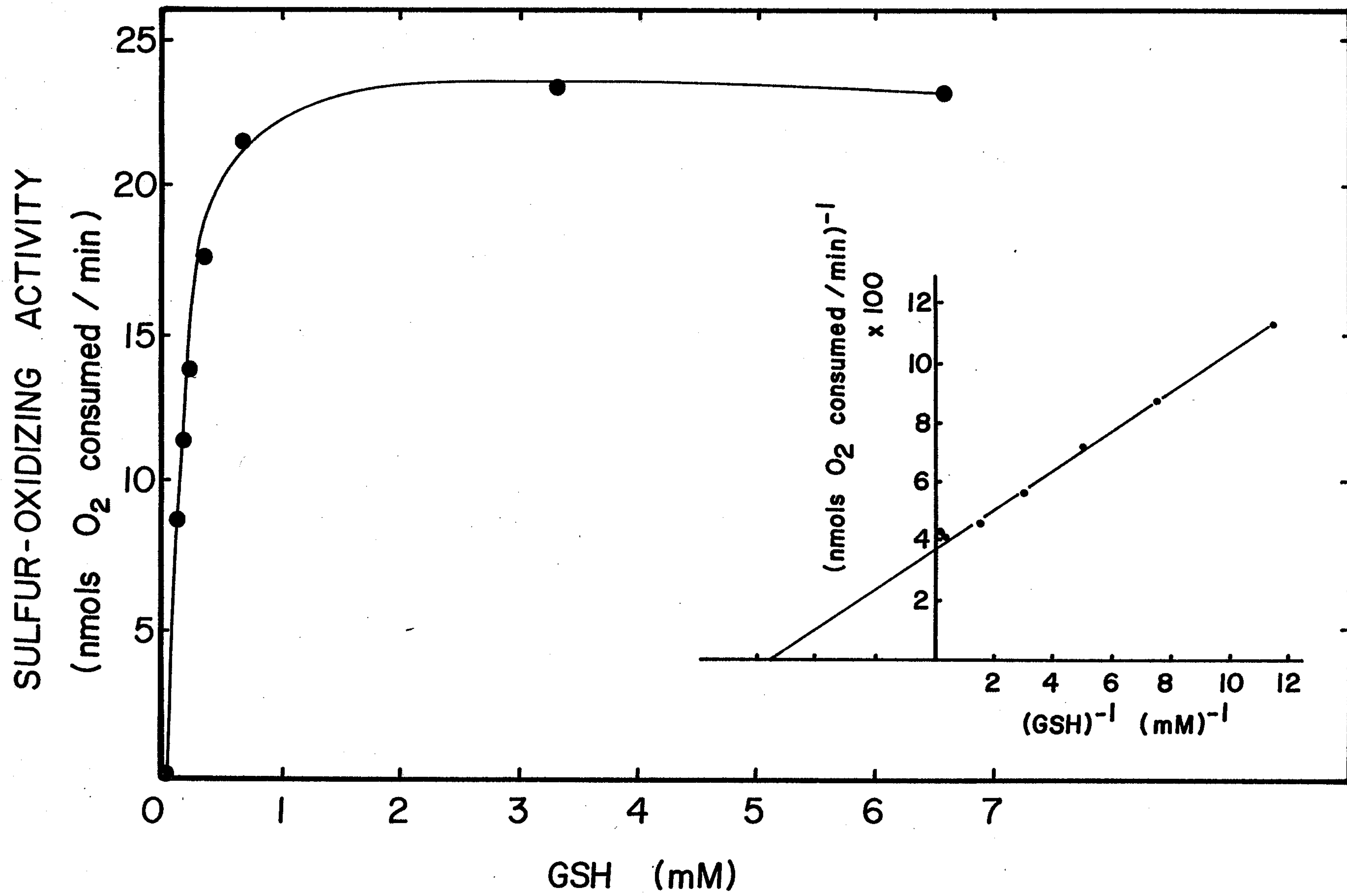
TABLE 5

Cofactor requirement of the sulfur-oxidizing enzyme.

Cofactor	Rate of oxidation (nmole O_2 consumed per min)		
	Sulfur + Cofactor activity*	Cofactor non-enzymatic activity	Net Sulfur + Cofactor activity
GSH	41.4	7.6	33.8
GSSG	0.0	0.0	0.0
ascorbate	6.2	15.1	0.0
sodium sulfite	0.0	0.0	0.0
B.A.L.	40.2	38.9	1.3
cysteine	46.4	35.2	11.2
dithiothreitol	45.1	12.5	32.6
dithioerythritol	40.1	11.3	28.8

*corrected for endogenous respiration

Rates of oxidation were measured as described in Materials and Methods. Ten µmoles of cofactor were used per assay except for sodium sulfite and B.A.L. where 0.1 and 1.0 µmole were used, respectively. Each assay contained 1.8 mg protein in the form of cell-free extract.



SULFUR-OXIDIZING ACTIVITY
(nmols O_2 consumed / min)
25
20
15
10
5
0
1
2
3
4
5
6
7
GSH (mM)
(nmols O_2 consumed / min)$^{-1}$
x 100
12
10
8
6
4
2
2
4
6
8
10
12
$(GSH)^{-1}$ $(mM)^{-1}$

apparent K_m values of 0.11 mM for both compounds.

Freezing cell-free extract at $-20^{o}C$ often resulted in severe loss of sulfur-(and sulfite-) oxidizing activity. In addition, storage at $4^{o}C$ was followed by a loss of up to 80% activity within 6 days. Cell-free extract was used within 2 days of preparation to maintain 100% enzyme activity.

Recovery of sulfite-oxidizing activity in the cell-free extract varied between 8 and 28% of the whole cell activity. The optimum pH for sulfite oxidation was 7.5 (Fig. 8). From the Lineweaver-Burk plot of reaction velocity versus substrate concentration (Fig. 10), the apparent K_m of sulfite was determined as 0.20 mM.

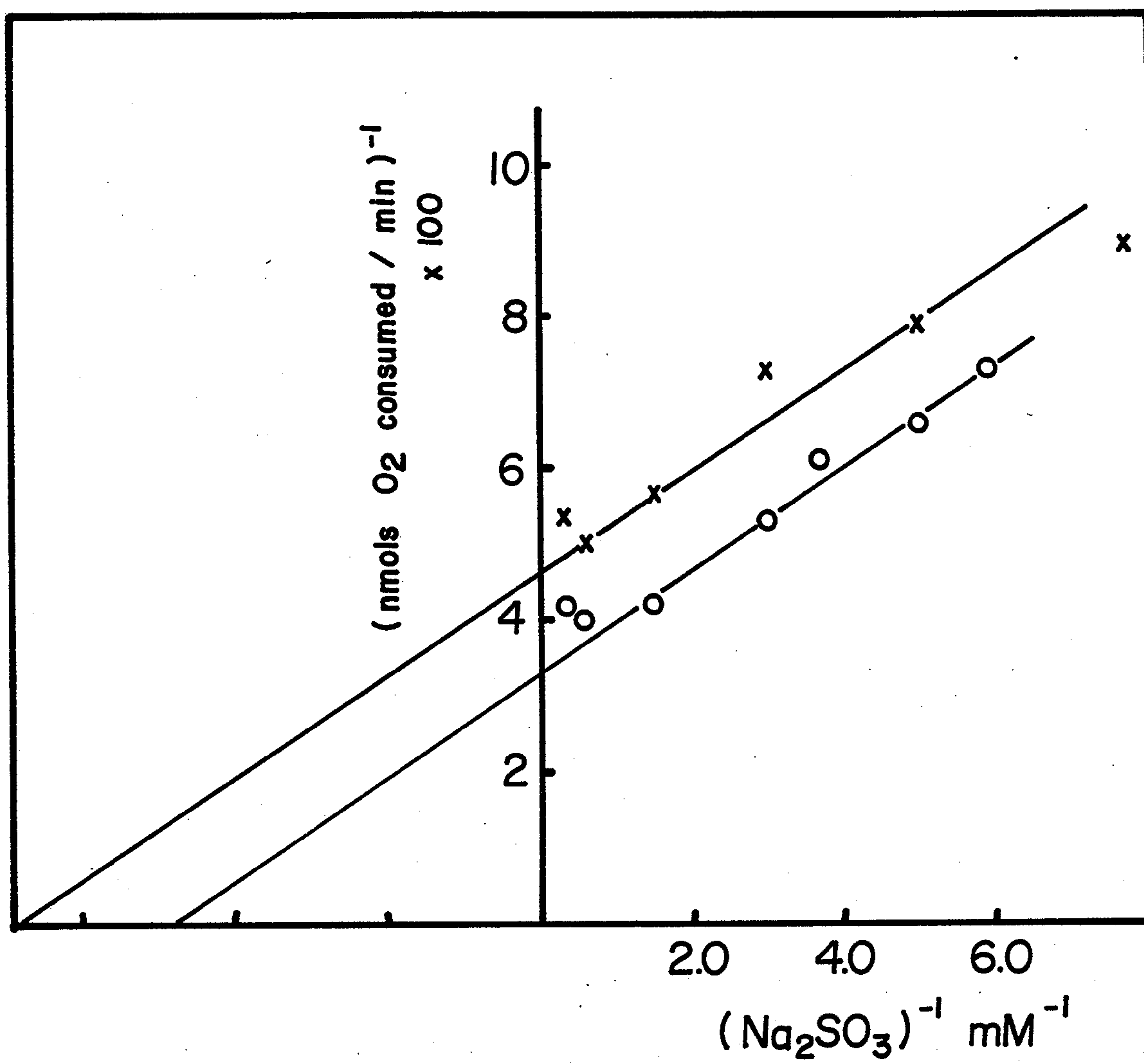
(2) <u>Fractionation of Cell-Free Extract</u>

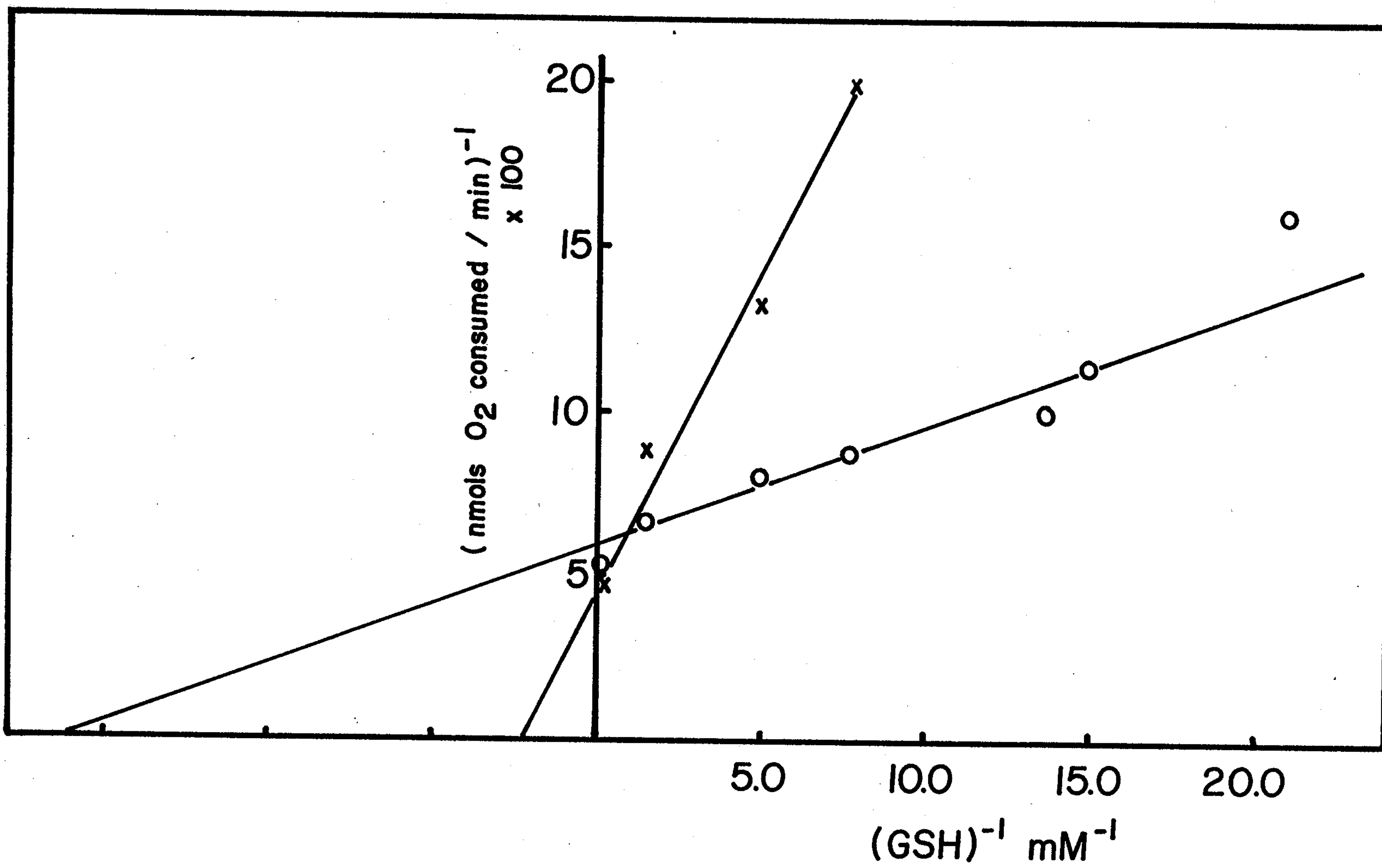
a) <u>Ultracentrifugation</u>

Ultracentrifugation at 152,000 x g for 1 hour resulted in distribution of the sulfur-oxidizing system between the supernatant and pellet fractions. Whereas the 152,000 x g supernatant was clear, pale yellow, the pellet resuspension was turbid with only a slight yellow color. Optimum pH for sulfur oxidation was 7.5 for both fractions but K_m values of GSH differed. These were calculated from Fig. 11 to be 61.0 μM and 0.44 mM for the supernatant and pellet fractions, respectively.

The sulfite-oxidizing system was found entirely in the pellet fraction. Maximum enzyme activity occurred at pH 7.5. From Fig. 10, the apparent K_m of sulfite was calculated to be 0.15 mM.

Ultracentrifugation increased the specific activity of the sulfur-oxidizing enzyme in the supernatant and that of sulfite in the pellet, together with an increase in total activity in both systems (Table 6,7).


(nmols O_2 consumed / min)$^{-1}$
x 100
10
8
6
4
2
2.0
4.0
6.0
$(Na_2SO_3)^{-1}$ mM^{-1}



20
15
10
5
(nmols O_2 consumed / min)$^{-1}$ x 100
5.0
10.0
15.0
20.0
$(GSH)^{-1}$ mM^{-1}

TABLE 6

Distribution of sulfur-oxidizing activity.

System	Sulfur-oxidizing activity*	Total volume ml	Total activity*	Specific activity**
whole cells	99.2	14.4	20407.0	75.7
cell-free extract	17.5	10.0	700.0	18.9
152,000 x g supernatant	17.4	9.4	657.0	29.1
152,000 x g pellet	38.9	2.6	398.3	16.3
acid supernatant	46.4	9.2	1709.4	240.8
acid pellet	7.5	12.7	382.9	18.6
15% ethanol supernatant	16.3	10.2	664.2	203.8
15% ethanol pellet	22.5	10.8	975.6	310.3
30% ethanol supernatant	1.2	11.2	53.8	25.0
30% ethanol pellet	2.4	6.8	64.9	19.0

*Activity is expressed as nmole O_2 consumed per min.

**Specific activity is expressed as nmole O_2 consumed per min per mg protein.

For whole cells, the reaction mixture contained in a total volume of 1.5 ml:

137.0 µmoles potassium phosphate (pH5.5)
0.24 mg sulfur
0.07 ml cell suspension

The remaining enzyme assays were done as described in Materials and Methods. Sulfur-oxidizing activity is expressed per 0.25 ml sample.

TABLE 7

Distribution of sulfite-oxidizing activity.

System	Sulfite-oxidizing activity*	Total volume ml	Total activity*	Specific activity**
whole cells	145.6	14.4	29952.0	111.2
cell-free extract	59.0	10.0	2360.0	63.4
152,000 x g supernatant	0.0	9.4	-	-
152,000 x g pellet	321.3	2.6	3342.0	135.0

*, ** as in Table 6

For whole cells, the reaction mixture contained in a total volume of 1.5 ml:

144.0 µmoles potassium phosphate
2.5 µmoles Na_2SO_3
2.5 µmoles EDTA
0.07 ml cell suspension

The remaining enzyme assays were done as described in Materials and Methods. Each assay was carried out on 0.25 ml sample.

After the oxidation of sulfur, the presence of thiosulfate was assayed as an indication of the product of oxidation. The oxygen to thiosulfate ratio was virtually 1:1 for the supernatant fraction. In contrast, no thiosulfate was produced by the pellet fraction (Table 8).

b) <u>Acid and Ethanol Fractionation</u>

Acidification of the 152,000 x g supernatant increased the specific activity of the sulfur-oxidizing enzyme in the acid supernatant fraction with a concomitant increase in total activity (Table 6). It was possible to omit the ultracentrifugation step in enzyme purification and proceed directly to acid fractionation with the cell-free extract. In terms of sulfite oxidation, activity was totally recovered in the acid pellet fraction as in the 152,000 x g pellet fraction.

Acid supernatant with low sulfur-oxidizing activity was concentrated in dialysis tubing against Aquacide at $4^{o}C$ without loss in enzyme activity.

Ethanol fractionation of the sulfur-oxidizing enzyme was unsuccessful. Addition of 15% absolute ethanol to the acid supernatant resulted in partial precipitation of the enzyme activity. Increasing the ethanol concentration led to substantial loss of activity (Table 6). The enzyme was unstable after ethanol fractionation and rapidly lost activity (within 24 hours).

a) <u>Sepharose 4B Fractionation</u>

Affinity chromatography was not an effective means of purifying the sulfur-oxidizing enzyme. The enzyme did not bind to either the

TABLE 8

Relationship of oxygen consumption to thiosulfate formation during sulfur oxidation.

System		Incubation time min.	GSH µmoles	O_2 µmoles	$S_2O_3^{--}$ µmoles	$O_2:S_2O_3^{--}$
152,000 x g supernatant	(1)	30	5.0	1.73	1.80	0.96
	(2)	100	10.0	0.62	0.56	1.10
152,000 x g pellet	(3)	100	10.0	0.62	0.02	-

Oxygen consumption and thiosulfate formation were assayed as described in Materials and Methods. Both values have been corrected for endogenous and non-enzymatic rates. Reaction mixture (1) contained 0.43 mg protein per assay whereas reaction mixtures (2) and (3) contained 0.39 mg protein per assay.

thiol or glutathione derivative of Sepharose 4B but was purified nevertheless. Subsequently, gel filtration with Sepharose 4B was used as a segment of the purification procedure. The final purification scheme is shown in Table 9. Sulfur-oxidizing enzyme was purified 18 fold.

The clear, bright yellow Sepharose 4B fraction was eluted slightly after the void volume of the column. From the Lineweaver-Burk plot of reaction velocity versus substrate concentration, the K_m of GSH was calculated to be 65.0 μM.

EFFECT OF POTASSIUM CYANIDE ON SULFUR AND SULFITE OXIDATION

Potassium cyanide (0.1 mM) inhibited sulfite oxidation by 48%, but had no effect on sulfur oxidation in the cell-free extract. In the acid pellet fraction, 1mM KCN inhibited sulfur and sulfite oxidation 81% and 62%, respectively. Sulfur oxidation by the Sepharose 4B fraction was not inhibited by 1 mM KCN.

ADDITIONAL ENZYMES INVOLVED WITH SULFUR OXIDATION

The enzymes, thiosulfate oxidase, sulfite-cytochrome _c_ oxidoreductase, and cytochrome oxidase were not detected in cell-free extract under the standard conditions used. However, the presence of sulfite-cytochrome _c_ oxidoreductase was indicated by very slow reduction of horse-heart cytochrome _c_ when a large amount (1.7 mg) of acid pellet fraction was assayed in the Shimadzu spectrophotometer. Upon shaking of the reaction mixture with air, no reoxidation of horse-heart cytochrome _c_ occurred.

TABLE 9

Final purification of sulfur-oxidizing enzyme.

System	Sulfur-oxidizing activity *	Total volume ml	Total activity*	Specific activity**
whole cells	55.2	8.8	6916.0	49.9
cell-free extract	20.2	6.7	537.3	8.6
acid supernatant	26.4	6.1	646.3	107.8
acid pellet	23.8	6.7	633.1	13.0
Sepharose 4B fraction	21.2	6.7	563.9	154.2

*,** as in Table 6.

Enzyme activities were assayed according to Table 6.

Strong rhodanese activity was found associated with the cell-free extract and the Sepharose 4B fraction (Table 10).

PRELIMINARY SPECTROPHOTOMETRIC STUDIES

(1) Acid Pellet Fraction

No absorption bands were observed when 3.4 mg. of the untreated acid pellet fraction was analyzed in a Shimadzu spectrophotometer with 0.5 ml silica cuvettes of a 1 cm light path. Addition of 5.0 μmoles Na_2SO_3 or 0.5 μmole KCN produced absorption at 443 nm, characteristic of the Soret band of cytochrome a. The absorption spectrum taken after chemical reduction by a few grains of hydrosulfite indicated the following cytochromes: cytochrome a (443 nm and 610 nm), cytochrome c (420 nm and 550 nm), and cytochrome b (432 nm).

The reduced minus oxidized difference spectra of the acid pellet were analyzed after separate reductions by sulfite and hydrosulfite (Fig. 12). With the addition of sulfite, characteristic Soret- and α-bands of cytochrome a appeared at 445 nm and 612 nm. Absorption peaks at 527, 563 and 554 nm correspond to β- and an α- band of cytochrome c, respectively. After one-half hour and repeated shaking with air, no reoxidation of the cytochromes occurred due to the presence of excess sulfite. Reduction with hydrosulfite resulted in a similar difference spectrum with additional shoulders at 433 nm and 457 nm (Fig. 12). The former is indicative of the Soret-band of cytochrome b and/or c whereas the latter may be attributed to the Soret-band of cytochrome d.

TABLE 10

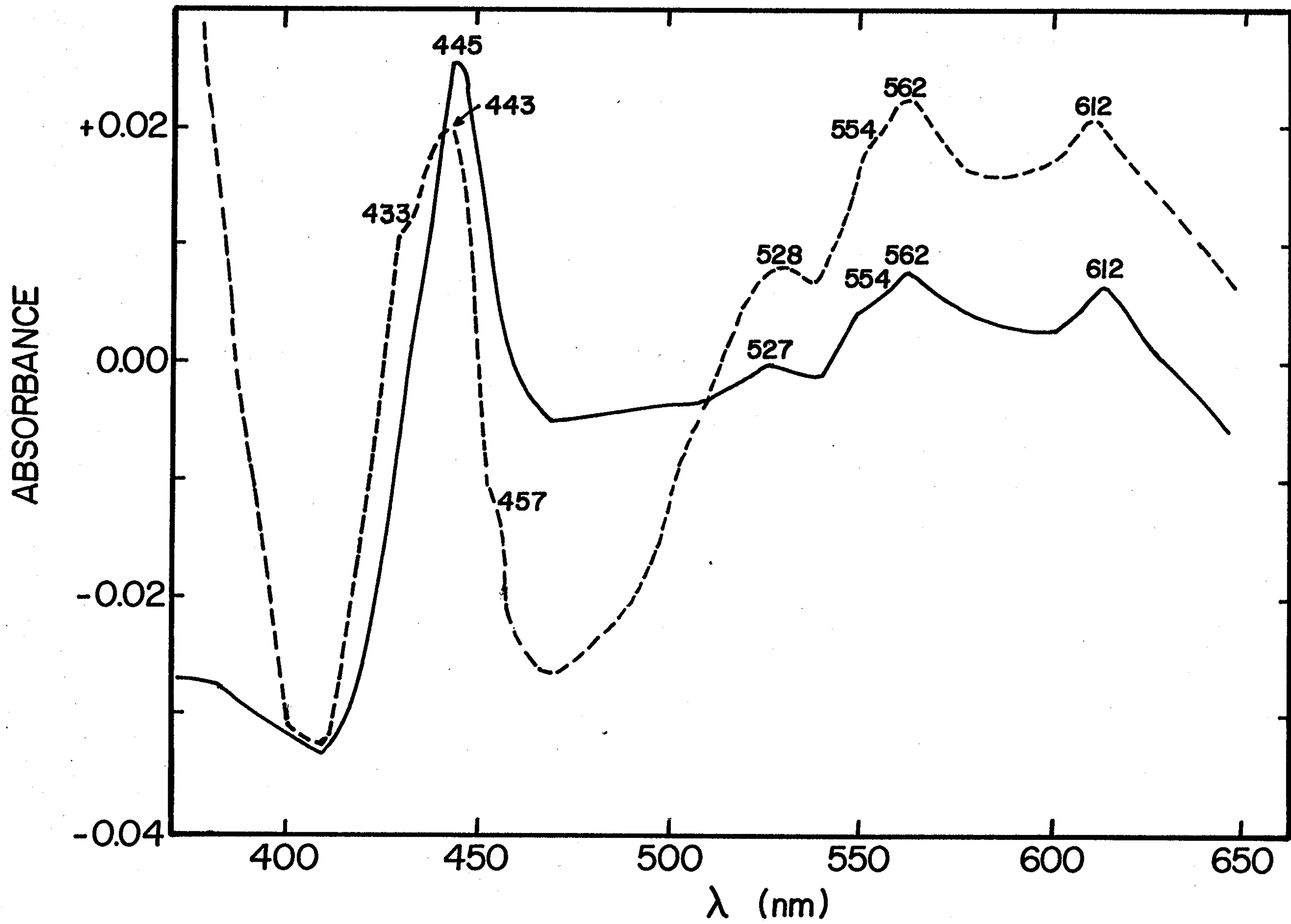
Comparison of rhodanese activity to sulfur- and sulfite- oxidizing activities.

System	Sulfur-oxidizing activity*	Sulfite-oxidizing activity*	Rhodanese activity**
Cell-free extract	31.5	308.8	210.0
Sepharose 4B fraction	12.5	0.0	150.0

* nmole O_2 consumed per min

** nmole thiocyanate produced per min

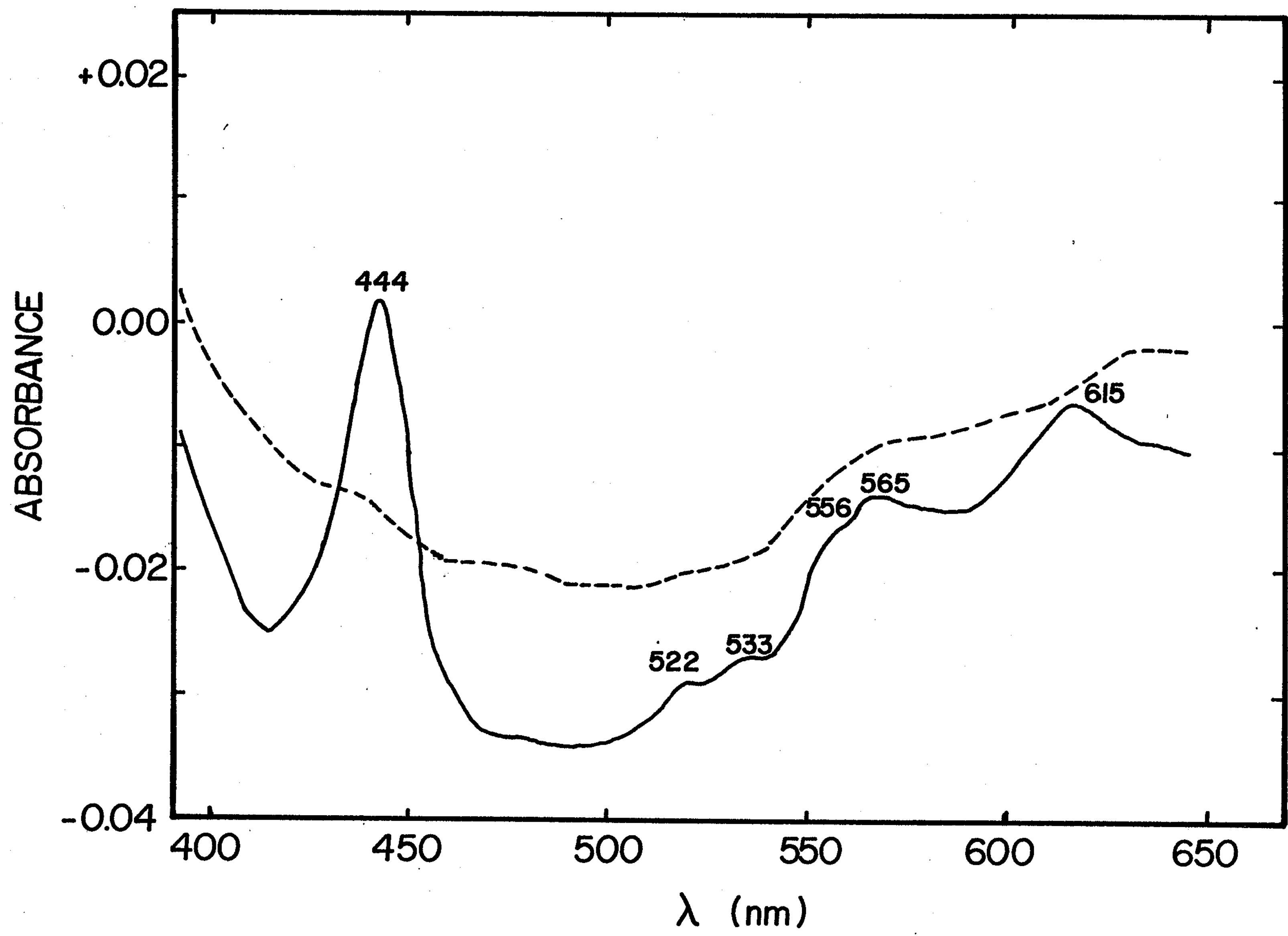
For the cell-free extract and the Sepharose 4B fraction 2.5 and 0.22 mg protein were used per assay, respectively.

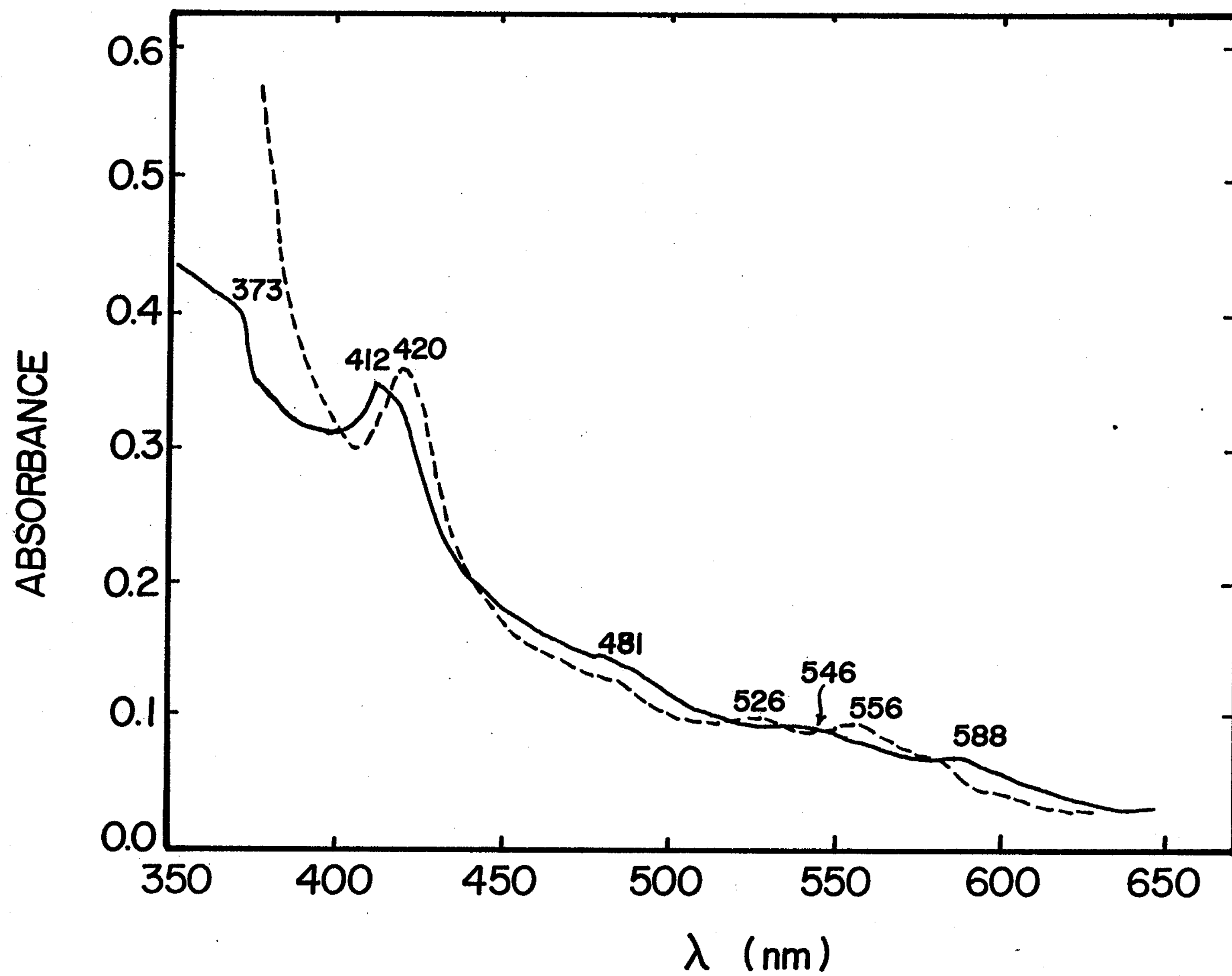

445
443
433
457
528
527
554
562
612
554
562
612
+0.02
0.00
-0.02
-0.04
ABSORBANCE
400
450
500
550
600
650
λ (nm)

Reduction of the acid pellet fraction with a smaller amount of sulfite (Fig. 13) resulted in the appearance of additional β-bands of cytochrome c and b (522 nm and 533 nm). After vigorous shaking of the reaction mixture with air, all cytochromes were oxidized (Fig. 13).

(2) Sepharose 4B Fraction

The spectra of the untreated and hydrosulfite reduced Sepharose 4B fraction are shown in Fig. 14. Absorption bands of the reduced spectrum occurred at 420, 526 and 556 nm which are characteristic Soret, β- and α- bands of cytochrome c. The absorption at 526 nm could also be attributed to the β-band of cytochrome b. Shoulders at 373 nm and 481 nm in the untreated spectrum were not identified. The spectra of the concentrated and untreated acid supernatant fractions were qualitatively similar to that of the Sepharose 4B fraction.


444
522
533
556
565
615
+0.02
0.00
-0.02
-0.04
ABSORBANCE
400
450
500
550
600
650
λ (nm)


0.6
0.5
0.4
0.3
0.2
0.1
0.0
ABSORBANCE
350
400
450
500
550
600
650
λ (nm)
373
412
420
481
526
546
556
588

DISCUSSION

DISCUSSION

Sulfur metabolism by whole cells and subcellular fractions of *Thiobacillus thiooxidans* was measured accurately by the Clark oxygen electrode of the Gilson Oxygraph. As compared to the Warburg method originally used for investigation, this assay was reliable, sensitive and most importantly, it was rapid. Measurement of the initial enzyme velocity gave a more precise indication of the prevalent reaction than the determination of oxygen uptake over a number of hours with the Warburg assay. Unlike the long term assay, (Suzuki and Silver, 1966) catalase or 2:2'-dipyridyl was not required to protect reduced glutathione from non-enzymatic oxidation.

The pH optimum of whole cells for both sulfur and sulfite oxidation was 5.5. The broad distribution of sulfur-oxidizing activity had been noted previously (Iwatsuka and Mori, 1960; Adair, 1966; Kodama and Mori, 1968a; Noguchi *et al*., 1977). Possibly the acidic environment surrounding the cell is retained after washing and remains relatively unaffected by the pH of the reaction mixture during the short experimental periods. That being the case, the outer cell membrane and immediate external region are implicated in sulfur oxidation by the intact cell. It should be remembered, however, that the organism cannot grow above pH 5.0 and that the cells lose the sulfur-oxidizing activity after prolonged incubation at a higher pH (Suzuki, 1965 a).

Whole cells oxidized sulfur without the addition of reduced glutathione (GSH). In fact, glutathione inhibited the oxidation of sulfur by active cells. Glutathione may have reacted with active native sulfhydryl groups on the cells and prevented the access of elemental sulfur to the sulfur-oxidizing complex as suggested by Suzuki (1974). The inhibition was stronger with freeze-thawed cells, indicating a greater accessibility of the active sulfhydryl groups to glutathione. Less active freeze-thawed cells might have suffered from destruction or alteration of vital membrane units containing the active sulfhydryl groups and required GSH for sulfur oxidation under alkaline conditions similar to cell-free systems.

The apparent K_m values of sulfur and sulfite for active whole cells were found to be 50.0 mM and 10.0 mM, respectively. The velocity of sulfite oxidation by active intact cells appears to be limited by the diffusion of the substrate into the cell, as evidenced by the relatively high sulfite K_m as compared to less active cells. This conclusion is further supported by the increased enzyme activity of freeze-thawed whole cells. In these cases, damaged cellular membranes may have approximated the cell-free state which had a K_m of 0.2 mM for sulfite.

Active cell-free extract was prepared by aerobic sonication of whole cells. According to Kodama and Mori (1968 b), sonication under a nitrogen atmosphere was compulsory for the preparation of an active sulfur-oxidizing system due to its labile nature under oxygen. The result was not applicable to the strain of <u>Thiobacillus thiooxidans</u>

used in this study.

Only small percentages of whole cell sulfur- and sulfite-oxidizing activities were recovered in the cell-free extract. Kodama and Mori (1968 b) recovered even less sulfur-oxidizing activity (5%). On the other hand, by treating cells with ionic resins before sonication, Suzuki (1965 a) found 60-70% of whole cell sulfur-oxidizing activity in the enzyme preparation. When cells of the present investigation were treated in this manner prior to sonication, no effect on enzyme recovery was noted, implying that the amount and intensity of sonication was insufficient to liberate the sulfur-oxidizing enzyme from the membrane. As a consequence, it appears from the elution profile on Sepharose 4B, that even the partially purified enzyme fraction consisted of small membrane fragments. The partial precipitation of the sulfur-oxidizing enzyme by ethanol also may indicate its membrane involvement. Other treatments, such as drying with acetone, grinding with alumina, and lyophilization are known to produce inactive cell-free extracts from Thiobacillus thiooxidans (Iwatsuka and Mori, 1960) and were not attempted.

Sulfur-oxidation by cell-free fractions exhibited an absolute requirement for reduced thiol groups. The requirement for catalytic quantities of GSH was also met by dithiothreitol or dithioerythritol. Affinity of the sulfur-oxidizing enzyme for all three compounds was essentially equivalent in the cell-free extract.

It should be mentioned that the results obtained strongly resemble those of Suzuki (1965 a,b), and Suzuki and Silver (1966) in terms

of sulfhydryl requirements for cell-free sulfur oxidation. Adair (1966) also prepared a 30,000 x g supernatant of _Thiobacillus thiooxidans_ that required GSH and which may be analogous to the cell-free extract of the present study.

Optimum pH for both the sulfur- and sulfite- oxidizing systems of the cell-free preparations was 7.5. In addition, freeze-thawing shifted the optimum pH of whole cell sulfur- and sulfite- oxidizing activity from pH 5.5 to pH 6.5 and 7.5, respectively. The process may have damaged cell membranes causing the shift towards the optimum pH of a cell-free system. Numerous reports of a shift of optimum activity to neutrality levels in subcellular systems confirm this finding (Suzuki, 1965 a; Adair, 1966; Tano and Imai, 1968; Kodama, 1969). In the absence of intact cell membranes, the sulfur-oxidizing system functions only around neutrality instead of the natural (pH 1-2) levels in the growth medium.

Although a more detailed study is required, the high sulfur-oxidizing activity of the cell-free extract at pH 8.5 and 9.5 may be partially due to sulfide oxidation. Formation of polysulfide from glutathione attack on the S_8 ring proceeds at a faster rate under alkaline conditions. Polysulfide decomposes to sulfide which may be oxidized by the extract to sulfur and thiosulfate (Suzuki and Werkman, 1959 a). Moreover, the non-enzymatic oxidation of GSH is relatively high at pH 8.5 and 9.5, and is stimulated by the cell-free extract, thereby contributing to an inaccurate impression of sulfur oxidation.

The apparent K_m values of GSH for the 152,000 x g supernatant and

the purified Sepharose 4B fractions were essentially equivalent, being 61-65 μM, while the value for the cell-free extract was 3-fold higher. The apparent K_m of GSH determined by Suzuki and Silver (1966) for the purified sulfur-oxidizing enzyme was approximately 100-fold greater. The discrepancy could be a function of enzyme affinities for the glutathione-polysulfide complex or of the assay procedure employed. Suzuki and Silver (1966) followed the reaction by measuring the total amount of thiosulfate produced or oxygen consumed during a long incubation time rather than the initial oxygen uptake.

Fractionation of the cell-free extract into the 152,000 x g supernatant and pellet resulted in division of the sulfur-oxidizing activity between the two fractions. Removal of unidentified inhibiting substances increased the total activity with each step of purification. With the addition of reduced glutathione, both the supernatant and pellet fractions were capable of independent oxidation of sulfur. The K_m of GSH for the pellet fraction was greater than that of the supernatant, perhaps indicating that the glutathione-polysulfide complex was not as accessible to the sulfur-oxidizing enzyme. Sulfite-oxidizing activity was recovered in the particulate fraction as found by Adair (1966), and Kodama and Mori(1968 b). In terms of sulfur oxidation, the system prepared in this study differs in certain features from others that have been described. Koadma and Mori (1968 b) found that both soluble and particulate fractions were required for sulfur oxidation. The 152,000 x g pellet was similar to the membrane fragments

described by Adair (1966) with the exception of the GSH requirement. Presumably, the indigenous sulfhydryl acceptors were intact and functional in the preparations of Adair, and Kodama and Mori. It is reasonable to conclude that in the 152,000 x g pellet fraction, sulfur was oxidized to sulfate since complete sulfur- and sulfite-oxidizing systems were detected and thiosulfate was not an end product. The sulfur-oxidizing enzyme of the 152,000 x g supernatant produced thiosulfate in the ratio of 1:1 with oxygen in agreement with the characteristics of the purified enzyme of Suzuki and Silver (1966). Sulfite, the initial product of the reaction, combines non-enzymatically with sulfur to form thiosulfate. In the whole cell system, sulfite may act as a key intermediate in sulfur oxidation.

The particulate fraction of Kodama *et al*. (1970) showed a 10-fold decrease in the K_m for sulfite as compared to intact cells, 0.4 mM from 4 mM. The 152,000 x g pellet fraction in this study showed an even more dramatic decrease, 0.15 mM from 10 mM.

The sulfur-oxidizing enzyme was purified 18-fold. The value is within the range reported by other workers in the purification of the enzyme: *Thiobacillus thiooxidans*, 12-fold (Suzuki, 1965 a), *Thiobacillus thioparus*, 24-fold (Suzuki and Silver, 1966), and *Ferrobacillus ferrooxidans*, 15-fold (Silver and Lundgren, 1968). The final purified enzyme fraction was probably composed of small membrane fragments as discussed earlier.

Rhodanese was found to be associated with the sulfur-oxidizing enzyme, a circumstance also occurring in Thiobacillus thioparus extracts (Charles and Suzuki, 1966 a). The enzyme may have significance in terms of oxidation and growth of thiobacilli on thiosulfate (Charles and Suzuki, 1966 a; Charles, 1969). Thiosulfate was not oxidized by intact cells or cell-free extracts of Thiobacillus thiooxidans. Other reports (Kodama and Mori, 1968 a) have indicated that glutathione, cysteine or thioglycolate is needed to stimulate thiosulfate oxidation. This possibility was not investigated.

The yellow pigment associated with the sulfur-oxidizing enzyme was especially evident in the purified enzyme fraction. Takakuwa (1975), upon fractionation of the 105,000 x g supernatant derived from Thiobacillus thiooxidans, also found a yellow component with an absorption shoulder at 485 nm in the oxidized state. As with the Sepharose 4B fraction, the absorption band was diminished substantially after reduction of the sample. He identified the component as a flavoprotein containing non-heme iron.

Cytochromes of the b and c-type were indicated in the absorption spectra of the purified Sepharose 4B and the acid supernatant fractions. Kodama et al. (1970) also obtained reduced absorption bands at 419, 523, and 552 nm, characteristic of cytochrome c, from the 105,000 x g supernatant of Thiobacillus thiooxidans. Suzuki (1965 a) found that his purified sulfur-oxidizing enzyme was uncontaminated by cytochromes.

In keeping with the previous observations made on the similarities of the sulfur-oxidizing enzyme of this work with Suzuki's enzyme, cytochromes might be nonfunctional in the purified fractions. Takakuwa (1975) proposed the participation of flavin and non-heme iron in sulfur oxidation in his reconstituted system.

Sulfite partially reduced cytochromes a, b, and c in the acid pellet (membrane) fraction. Dithionite further reduced the cytochromes and produced additional shoulders at 433 and 457 nm. The 433 nm absorption band also appeared in the spectrum of the membrane fraction of Kodama et al. (1970). Cytochromes of the acid pellet, previously reduced with a small amount of sulfite, were completely reoxidized by shaking with air. The phenomenon was most apparent with cytochrome oxidase (cytochrome a) at 444 nm and may indicate a complete electron transport system coupled to oxygen. Furthermore, KCN inhibition of sulfur and sulfite oxidation points to metal-enzyme involvement, presumably cytochrome oxidase. In addition, the involvement of flavin in the sulfur and sulfite oxidation reactions of the pellet fraction cannot be ruled out as Takakuwa (1976) presented evidence to this effect in the sulfite-oxidizing system.

The presence of sulfite: cytochrome c oxidoreductase was demonstrated in the membrane fraction by the slow sulfite-dependent reduction of horse-heart cytochrome c. Reoxidation did not occur with shaking, suggesting the inability of horse-heart cytochrome c to couple with native cytochrome oxidase. Even though Kodama et al. (1970) also

found that the particulate fraction coupled sulfite with mammalian cytochrome _c_, the reduction was much slower than with _Pseudomonas stutzeri_ cytochrome _c_-552. The illustration of sulfite:cytochrome _c_ oxidoreductase and cytochrome oxidase in the acid pellet fraction is suggestive of the AMP-independent oxidation of sulfite to sulfate. The presence of APS-reductase was not investigated, but may conceivably serve as an alternate sulfite-oxidizing pathway.

In closing, this preliminary study has endeavored to clarify the biochemical aspects of sulfur metabolism by _Thiobacillus thiooxidans_. Rather than speculation only on discrete enzymes and characteristics, the ultimate solution to sulfur oxidation will depend on consideration of a number of interrelated factors. Future research hopefully will reconcile divergent reports and produce a plausible explanation of the phenomenon.

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