A FURTHER STUDY ON THE PURIFICATION OF THE SULFUR-OXIDIZING ENZYME FROM *Thiobacillus thiooxidans*

By

BHAG S. CHAHAL

A thesis

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ABSTRACT

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Thiobacillus thiooxidans was grown in a Starkey's medium supplemented with molybdate with elemental sulfur as energy source. The cell-free extracts were prepared by sonication of trypsin-treated cells and the sulfur-oxidizing enzyme activity was studied. The enzyme was purified by ultracentrifugation, pH 5 treatment, DEAE-cellulose chromatography and Sephadex G-100 chromatography. The crude extract had cytochromes *a*, *b* and *c* as well as flavin, but cytochromes *a* and *b* were absent in the 105,000 x *g* supernatant. A major flavin containing component was dissociated from the enzyme and was collected with 0.3 M Tris-Cl in DEAE-cellulose chromatography. The sulfur-oxidizing enzyme collected with 0.2 M Tris-Cl was further purified with Sephadex G-100 chromatography and was used for characterization. The purified enzyme was 80% pure, free of flavin and was an iron-sulfur protein containing one iron and one sulfur (Sulfide) per protein. The molecular weight was 46,000 consisting of two subunits with molecular weights of 21,000 and 26,000. The isoelectric point was pH 3.5. The apparent Km values for GSH and sulfur were 2 mM and 5.7 mM, respectively. GSH was the most effective cofactor among various compounds tested.

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ABBREVIATIONS

ADP - adenosine-5'-diphosphate

AMP - adenosine-5'-monophosphate

APS - adenosine phosphosulfate

ATP - adenosine-5'-triphosphate

BAL - British Anti Lewisite (2,3-dimercapto-1-propanol)

BSA - bovine serum albumin

Cyt - cytochrome

FAD, FADH - oxidized or reduced flavin adenine dinucleotide

FMN - flavin mononucleotide

GSH - reduced glutathione

GSSG - oxidized glutathione

NAD, NADH - oxidized or reduced nicotinamide adenine dinucleotide

NADP, NADPH - oxidized or reduced nicotinamide adenine dinucleotide phosphate

Tris - Tris (hydroxymethyl) aminomethane

INTRODUCTION

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The thiobacilli, a group of well known chemoautotrophic microorganisms, oxidize various inorganic sulfur compounds and use this energy to assimilate atmospheric carbon dioxide to synthesize cellular materials. These chemoautotrophs are further classified as obligate, faculative or mixotrophic depending upon the extent of utilization of organic compounds as a source of nutrition.

Thiobacillus thiooxidans is primarily an obligate chemo-autotrophic bacterium and can derive its energy through the oxidation of elemental sulfur to sulfuric acid. This bacterium is unique in its ability to withstand extremely acidic conditions, a pH less than 1.0. The organism lives freely in the soil or in water. In spite of the apparent simplicity of the nutritional requirements of T. thiooxidans, the organism is known to be very complex and sophisticated physiologically and biochemically.

There remains a considerable amount of controversy as to the pathway and mechanism of oxidation of the sulfur compounds and this subject has been a focus of a great deal of attention for the past several years. Earlier investgations with intact cells, cell-free extracts or purified individual enzymes have been able to elucidate only basic outlines of the mechanism of the sulfur oxidation.

The present investigation was undertake to purify and characterize the sulfur-oxidizing enzyme with a view to understand and clarify the nature of sulfur-oxidizing enzyme of T. thiooxidans.

HISTORICAL

HISTORICAL

Winogradsky in 1887 for the first time introduced the concept of autotrophic (=self-nourishing) bacteria based on the studies on a Beggiatoa Sp. Thiobacilli first discovered by Nathanson in 1902, are autotrophic bacteria which oxidize inorganic sulfur compounds as source of energy for growth. Waksman and Joffe in 1922 isolated Thiobacillus thiooxidans from soil. This bacterium was found to be an obligate chemo-autotroph deriving its energy and reducing power for growth from the oxidation of elemental sulfur and synthesize all the cellular carbon by assimilation of atmospheric CO_2. It is one of the most acidophilic organisms and can withstand an extremely low pH (less than 1.0), although the optimum pH for growth is near 3.5 (Vishniac and Santer, 1957). This organism has been found to be very complex physiologically and biochemically and has been the subject of a number of investigations to understand the general physiology and sulfur metabolism (Joffe, 1922; Waksman, 1922; Waksman and Starkey, 1922, 1923; Waksman et al, 1923; Lipman, 1923; Starkey, 1925). A number of reviews are also available in this area (Lee, 1953, 1960; Peck, 1962, 1968; Trudinger, 1967, 1969; Kelly, 1968; Roy and Trudinger, 1970; Suzuki, 1974; Aleem, 1975; Oh and Suzuki, 1980). However, in spite of intensive investigation, many aspects of sulfur metabolism have not been clearly delineated and remain to be vaguely understood, thus are still subject of further studies.

To start with, the mechanism of attack of elemental sulfur particles in the culture and their further mobilization to required enzyme system(s) by the cell remain to be understood. The direct contact between terminal fat globules of bacterial cells and sulfur particles was necessary in order to dissolve elemental sulfur for oxidation as reported by Umbreit et al (1941). In the electron microscopic studies, they failed to confirm the presence of such fat globules (Umbreit and Anderson, 1942) and a later work by Knaysi (1943) indicated that the fat globules actually consisted of volutin and sulfur. 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 cells (Schaeffer and Umbreit, 1963; Jones and Benson, 1965; Shively and Benson, 1967; Roy and Trudinger, 1970). The results of Vogler and Umbreit (1941) contradicted this theory, in that direct contact between the organism and sulfur was shown to be obligatory for oxidation. Microscopy has supported this latter theory by the fact that cells have been photographed clustered around eroded sulfur particles, 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). The mechanism based on this theory, however, appears to be a complex one. A further complication comes from the fact that whether the sulfur is oxidized directly at the outer

membrane surface or after its translocation into the cell is still unkown.

Sulfur oxidation

Although many thiobacilli are able to obtain energy for growth through the oxidation of elemental sulfur to sulfuric acid, the most intensive investigations on the mechanism of the sulfur oxidation have been conducted with sulfur-oxidizing system of T. thiooxidans (Waksman and Joffe, 1922). The early work with intact cells established that elemental sulfur is oxidized aerobically to sulfuric acid as follows: (Waksman, 1922; Waksman and Starkey, 1923; Starkey, 1925; Parker and Prisk, 1953).

$$S^{o} + 1\tfrac{1}{2}O_2 + H_2O \longrightarrow SO_4^{2-} + 2H^{+}$$

It is generally agreed that sulfite is the key intermediate in this oxidation (Peck, 1968; Suzuki, 1974).

$$S^{o} + O_2 + H_2O \longrightarrow SO_3^{2-} + 2H^{+}$$

$$SO_3^{2-} + \tfrac{1}{2}O_2 \longrightarrow SO_4^{2-}$$

Three types of sulfur-oxidizing systems have been prepared from the cell-free extracts of the organism (Suzuki, 1974).

(a) Sulfur-oxidizing enzyme (Sulfur: oxygen oxidoreductase E.C. 1.13.11.18) which catalyzes the oxidation of elemental sulfur to sulfite in the presence of a cytalytic amount of reduced glutathione (GSH) (Suzuki, 1965). It is found in the soluble fraction of cell-free extracts.

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

(b) A large cell wall-membrane complex which catalyzes the oxidation of elemental sulfur (presumably to sulfate) as intact cells (Adair, 1966; Taylor, 1968). The ability of thiol-binding agents to block this sulfur-oxidation indicate the presence of endogenous sulfhydryl groups.

(c) The sulfur oxidizing system which catalyzes the oxidation of sulfur to sulfate and requires both soluble and membrane fractions (Kodama, 1968, 1969).

However, the transformation of elemental sulfur to sulfate does take place in several steps. One suggested pathway of sulfur oxidation proposed the formation of the thiosulfate and polythionates as intermediates (Vishniac and Santer, 1957). Investigations by Suzuki and Werkman (1959) seemed to support this pathway. Cell-free extracts, prepared by Raytheon oscillation, were capable of oxidizing sulfur upon the addition of reduced glutathione as substrate. Polythionates and thiosulfates were detected to be the products. In 1937, Starkey noted the production of small amounts of hydrogen sulfide from sulfur by T. thiooxidans. He suggested that sulfide was formed from sulfur and sulfhydryl groups on the cells by a mechanism similar to the following non-enzymatic reaction proposed by Sluiter (1930).

$$S^{o} + 2GSH \longrightarrow H_2S + GSSG$$

Glutathione reductase is capable of regenerating the reduced form of glutathione from the oxidized form using NADPH. The presence of this enzyme has been detected in yeast (Meldrum and Tarr, 1935; Racher, 1955), plant tissues (Conn and

Vennesland, 1951; Mapson and Goddard, 1951), mammalian tissues (Rall and Lehninger, 1952; Racher, 1955) and bacterial sources (Asnis, 1955; Suzuki and Werkman, 1959). T. thiooxidans having glutathione reductase could regenerate GSH from any oxidized glutathione (GSSG) which might be formed during sulfur oxidation (Suzuki and Werkman, 1959):

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

Suzuki and Lees (1964) later prepared T. thiooxidans extracts which required only catalytic quantites of reduced glutathione for sulfur oxidation. The sulfur-oxidizing enzyme was partially purified (Suzuki, 1965) 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 found to be the end product of reaction. The mechanism of sulfur oxidation was revised to include glutathione polysulfide as an intermediate (Suzuki, 1965). Oxidation of sulfur 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. The following mechanism was proposed for the reaction catalyzed by partially purified sulfur-oxidizing enzyme:

$$S_n + GSH \longrightarrow GSS_nH$$

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

Where S_n represents a polymerized molecular state (S_8 ring structure) of elemental sulfur. Later, Suzuki and Silver (1966) found in T. thiooxidans and Thiobacillus

thioparus that the actual product of sulfur oxidation was sulfite not thiosulfate, identified by formaldehyde trapping. Thiosulfate was formed through a non-enzymatic condensation of sulfur and sulfite under assay conditions as follows:

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

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

In the cells sulfite would be further oxidized to sulfate via sulfite-oxidizing system as discussed later. In this reaction sulfur atoms can be converted successively to sulfite by oxidation while GSH would be regenerated for a further attack on S_n.

Suzuki (1965) tentatively identified the sulfur-oxidizing enzyme as an oxygenase based on the requirement for molecular oxygen and the incorporation of $^{18}O_2$ into thiosulfate during sulfur oxidation. The sulfur-oxidizing enzyme contains iron as cofactor apparently non-heme iron in nature and removal of the metal by 2,2' -dipyridyl results in relatively inactive enzyme suggesting that the iron is essential for the enzyme action (Suzuki and Silver, 1966).

Tano and Imai (1968) obtained a soluble sulfur-oxidizing complex from T. thiooxidans cells by the ultrasonic breakage followed by the centrifugation at 130,000 x g for 1 hour. Colloidal sulfur was metabolized without addition of cofactors. As glutathione reductase was detected in the soluble fraction, it could be possible to regenerate GSH. 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) may be responsible for the sulfur metabolism.

The sulfur-oxidizing enzyme was also isolated and characterized in T. thioparus, (Suzuki and Silver, 1966) Thiobacillus novellus (Charles and Suzuki, 1966) and Thiobacillus ferrooxidans (Silver and Lundgren, 1968) and the properties appear to be similar to that of T. thiooxidans. The enzyme has been resolved as one of the components for the membrane-bound thiosulfate-oxidizing complex of T. novellus (Oh and Suzuki, 1977).

The sulfur-oxidizing system which requires both soluble and membrane fractions has been prepared by sonication of T. thiooxidans cells under a nitrogen atmosphere (Kodama and Mori, 1968; Kodama, 1969). Addition of GSH 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 one hour, it was found that both the soluble and particulate fractions were required for sulfur oxidation, whereas the sulfite-oxidizing activity was recovered soley 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 former fraction could be replaced by NAD^+ or $NADP^+$, but not by cysteine or GSH (Kodama, 1969).

The soluble membrane-impermeable fraction was purified into components A and B (Takakuwa, 1975). A large component A was a non-heme iron protein with a molecular weight of

120,000 and an absorption at 410 nm in the oxidized form which shifted to 420 nm upon reduction. The smaller component B had an absorption maximum at 410 nm with a shoulder at 485 nm in the oxidized state. The shoulder disappeared upon reduction. The component B was identified as a flavo-protein containing non-heme iron with a molecular weight of 23,000.

Inhibition and spectrophotometric studies clearly indicated the involvement of the electron transport system containing a, b and c type cytochromes in the oxidation of sulfite to sulfate by the membrane fraction, while the nature of the electron transport in the oxidation of elemental sulfur to sulfite was not elucidated.

More recently the sulfur-oxidizing enzyme has been reinvestigated in our labotatory by measuring the oxygen uptake polarographically with a Clark oxygen electrode (Lukow, 1977). As compared to the Warburg manometric method originally used for the investigation (Suzuki, 1965; Suzuki and Silver, 1966), this method is more sensitive and rapid. Catalase and 2-2'- diphyridyl used to protect GSH from non-enzymatic oxidation are no longer required. The sulfur-oxidizing activity is found both in the soluble (66%) and membrane fractions (33%) whereas the sulfite-oxidizing activity is associated with the 105,000 x g membrane fraction. Presumably this membrane system catalyzes a complete oxidation of sulfur to sulfate since no thiosulfate is produced and both sulfur and sulfite are oxidized by the same 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. The thiol groups from polysulfide complex with the sulfur before its oxidation to sulfate through sulfite as an intermediate. Trudinger (1967) speculated that the sulfur-oxidizing enzyme, as described by Suzuki (1965), 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.

Sulfide Oxidation

The oxidation of sulfide to sulfate is a process requiring an 8-electron transfer. Sulfide is probably oxidized to sulfate through polysulfide and sulfite as intermediate as follows:

$$S^{2-} + \tfrac{1}{2}O_2 + 2H^+ \longrightarrow [S] + H_2O$$

$$[S] + O_2 + H_2O \longrightarrow SO_3^{2-} + 2H^+$$

$$SO_3^{2-} + \tfrac{1}{2}O_2 \longrightarrow SO_4^{2-}$$

$$S^{2-} + 2O_2 \longrightarrow SO_4^{2-}$$

Where [S] represents a polysulfide-sulfur. Thus the mechanism of sulfide oxidation in thiobacilli is analogous to that for the oxidation of colloidal sulfur (polysulfide) or elemental sulfur once sulfide is converted to the oxidation level of sulur.

Earlier studies related to sulfide oxidation in *T. thiooxidans* with both intact cells and crude extracts led

to the hypothesis that elemental sulfur, thiosulfate and polythionates were the intermediate products during oxidation of sulfide to sulfate (Parker, 1953; Vishniac, 1957; London, 1964). Studies on T. thiooxidans, Thiobacillus concretivorus and T. thioparus with intact cells as well as cell-free extracts, grown on elemental sulfur as an energy source, catalyse an enzymatic oxidation of sulfide (Moriarty and Nicholas, 1969, 1970) though oxidation of sulfide have been considered by some workers as a non-enzymatic process (Adair, 1966).

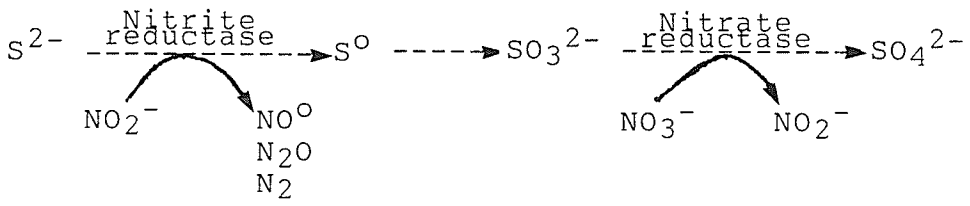
Sulfide oxidase enzyme responsible for sulfide oxidation to polysulfide level from the T. concretivorus extracts, prepared by a French pressure cell is associated with the membrane fraction and the following tentative electron transfer scheme has been presented for the oxidation (Moriarty and Nicholas, 1970):

$$S^{2-} \dashrightarrow \text{Cu protein} \dashrightarrow (\text{Flavin?}) \dashrightarrow \text{Cytochromes-}$$

$$\underline{b} \dashrightarrow \underline{c} \dashrightarrow \underline{d} \dashrightarrow O_2$$

A copper protein is implicated as a binding site for sulfide. These studies indicated that the membrane-bound sulfur polymer would be oxidized to sulfate by a sulfur-oxidizing enzyme and a sulfite-oxidizing system through a mechanism similar to those described by Suzuki and associates (Suzuki, 1965, 1966; Charles and Suzuki, 1966). A further study on the resolution and identification of individual enzymes involved is required before definite conclusions can be made.

In Thiobacillus denitrificans sulfide is oxidized by intact cells with either molecular oxygen or nitrate as the terminal electron acceptor (Peeters and Aleem, 1970). Aminuddin and Nicholas (1973) with intact cells and extracts of the organism showed that sulfide oxidation to be linked to the reduction of nitrite and nitrate. Cell suspensions reduced nitrite under anaerobic conditions to nitrogenous gases such as No, N_2O and N_2 with sulfide as electron donor. The work with extracts showed that sulfide was first oxidized to membrane-bound polysulfide, then to sulfite and sulfate as in T. concretivorus. A nitrate reductase, which catalysed the reduction of nitrate to nitrite with a concomitant oxidation of sulfite to sulfur was also located in the same membrane. The scheme for the mechanism of sulfide oxidation in this system is as follows:



Recently Sawhney and Nicholas (1978) reported the purification of sulfide-linked nitrite reductase from T. denitrificans. The enzyme contained c and d type cytochromes in the ratio of 1:1 and could also function as cytochrome oxidase. A possible scheme for the electron transfer during sulfide oxidation in this organism was proposed as follows:

$$S^{2-} \longrightarrow \text{Cyt } c\text{-551} \longrightarrow \underbrace{[\text{Cyt } c \text{ --- Cyt } d]}_{\text{Nitrite reductase}} \longrightarrow O_2 \text{ or } NO_2^-$$

Thus a native cytochrome c-551, stimulated the rate of

nitrite reduction or oxygen uptake by the enzyme with sulfide as the electron donor, while another native cytochrome c-554 or mammalian cytochrome c had no effect.

Sulfite Oxidation

In sulfur oxidation and also in thiosulfate oxidation sulfite is a key intermediate. Two pathways are basically functional in its oxidation.

(a) The APS reductase pathway involves adenosine phosphosulfate (APS) as an energy-rich phosphosulfate bond intermediate (Peck, 1960).

(b) The sulfite oxidase (Sulfite: cytochrome c oxidoreductase) pathway is a direct oxidation of sulfite to sulfate without involving APS (Charles and Suzuki, 1966). The former is coupled to substrate level phosphorylation and the latter to oxidative phosphorylation. Thus ATP synthesis linked to sulfite oxidation involves both of the two general methods of biological energy conservation. Peck (1960, 1962) working with T. thioparus, first proposed the formation of APS (adensine 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:

$$2SO_3^{2-} + 2AMP \xrightarrow{\text{APS reductase}} 2APS + 4e^-$$

$$2APS + Pi \xrightarrow{\text{ADP sulfurylase}} 2ADP + 2SO_4^{2-}$$

$$2ADP \xrightarrow{\text{Adenylate kinase}} AMP + ATP$$

$$2SO_3^{2-} + AMP + 2Pi \longrightarrow 2SO_4^{2-} + ATP + 4e^-$$

The pathway is apparently the same as the reversal of the dissimilatory sulfate-reducing system in species Desulfovibiro and Desulfotomaculum (Peck, 1961; Ishimoto, 1961;). APS reductases have been purified from Desulfovibiro vulgaris (Peck et al, 1965), T. dentrificans (Bowen et al, 1966), T. thioparus (Lyric and Suzuki, 1970) and photosynthetic sulfur bacteria such as Thiocapsa roseopersicina (Trüper and Rogers, 1971) and Chlorobium limicola (Kirchhoff and Trüper, 1974). All have a similar molecular weight (170,000-218,000) and contain one FAD, 4-13 non-heme irons and 4-12 labile sulfur per mole. A difference exists, however with respect to the specificity of enzymes towards different types of cytochrome c as electron acceptors.

Possible existence of an AMP-independent sulfite-oxidizing system was suggested by a rapid oxidation of sulfite catalysed by crude extracts of T. dentrificans without the addition of AMP (Milhaud et al, 1958). Similar results were obtained from extracts of T. thioparus after treatment with activated charcoal to eliminate endogenous AMP (London and Rittenberg, 1964).

Charles and Suzuki (1965, 1966) isolated and purified an AMP-independent sulfite oxidizing enzyme system, a sulfite: cytochrome c oxidoreductase (sulfite oxidase) from T. novellus, which neither needs AMP nor produces APS, functions as an additional mechanism of sulfite oxidation. The purified enzyme oxidize sulfite to sulfate with a concomitant reduction of cytochrome c.

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

The reduced cytochrome is then oxidized with molecular oxygen by cytochrome oxidase (cytochrome _c_: O_2 oxidoreductase).

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

Sulfite oxidase has been isolated from _T. intermedius_ (Charles, 1969), _T. thioparus_ (Lyric and Suzuki, 1970) and _T. ferrooxidans_ (Vestal and Lundgren, 1971). Recently both sulfite oxidase and cytochrome oxidase have been detected in the membrane-associated thiosulfate-oxidizing complex of _T. novellus_ (Oh and Suzuki, 1977).

A further study on the mechanism of APS reductase has been carried out by means of difference spectrum and stopped flow techniques (Adachi and Suzuki, 1977). The results of this study suggest the reduction of FAD to $FADH_2$ by sulfite followed by the partial reoxidation of $FADH_2$ to a red semiquinone (FADH) upon the addition of AMP with concomitant reduction of non-heme iron:

$$E\text{-}FAD\text{-}Fe^{3+} + SO_3^{2-} + H_2O \longrightarrow E\text{-}FADH_2\text{-}Fe^{3+}(SO_4^{2-})$$

$$E\text{-}FADH_2\text{-}Fe^{3+}(SO_4^{2-}) + AMP \longrightarrow E\text{-}FADH\text{-}Fe^{2+}(APS) + OH^-$$

This mechanism differs from a three step mechanism postulated for the _D. vulgaris_ system (Michaelis _et al_, 1970, 1971). In _T. denitrificans_ sulfite was oxidized both aerobically and anaerobically with nitrate through two different electron transport pathways involving cytochromes (Peeters and Aleem, 1970). As in _T. thioparus_ (Lyric and Suzuki, 1970) the

APS and sulfite oxidase systems 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.

An unusual sulfite-oxidizing system from T. neapolitanus was 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. Enzyme was probably intermediate to APS reductase and sulfite oxidase as it was stimulated by AMP but did not form APS. The properties of sulfite oxidase from thiobacilli were similar to those of liver sulfite oxidase (MacLeod, 1961; Howell, 1968) in that thiol-binding agents and anions such as phosphate and chloride were strongly inhibitory. The enzyme could couple with either horse heart cytochrome c or native cytochrome c as well as with ferricyanide as electron acceptor, but not with molecular oxygen.

It is interesting that from the bioenergetic point of view on evolution that so far APS reductase has not been detected in any facultative chemolithotrophs of thiobacilli, but strict chemolithotrophs contain both APS reductase and sulfite: cytochrome c oxidoreductase (Broda, 1970, 1975; Trüper, 1971).

Two similar membrane-bound sulfite-oxidizing systems from T. concretivorous and T. thiooxidans have been reported (Moriarty and Nicholas, 1970; Lukow, 1977). T. concretivorous system is associated with flavoprotein, ubiquinone and cytochromes of b, c and a types and oxidative phosphorylation

occurs during electron transfer via the cytochrome chain. In the T. thiooxidans system, the sulfite: cytochrome c oxidoreductase and cytochrome oxidase are also detected in the partially purified membrane fraction.

Another interesting sulfite-oxidizing membrane system has been obtained from T. thiooxidans (Kodama, 1968, 1970). The enzyme catalyses the oxidation of sulfite with either O_2 or bacterial cytochrome c-552 prepared from Pseudomonas stutzeri as electron acceptor. The presence of cytochromes of the a, b and c types in the membrane fraction and the study with respiratory inhibitors indicate the participation of a complete cytochrome system.

Sulfite oxidation by T. thiooxidans is at the preliminary stage of investigation. Presence of APS reductase pathway in this organism is controversial at present. As from Cooks report (quoted by Adair, 1966) the enzyme was absent from extracts, while on the other hand, Peck (1961, 1962) indicated its presence in this bacterium. In the membrane fractions of crude cell-free extracts (Adair, 1966; Kodama and Mori, 1968) sulfite oxidase pathway was detected. Two different kinds of terminal oxidases were reported in T. thiooxidans based on carbon-monoxide inhibition studies of sulfur and sulfite oxidations, sulfur oxidation being photo-irreversibly inhibited compared to photo-reversible sulfite oxidation (Twatsuka and Mori, 1960; Kodama and Mori, 1968). Kodama et al (1970) and Takakuma (1976) have also demonstrated the participation of membrane bound a, b and c type cytochromes and a flavoprotein in sulfite oxidation in T. thiooxidans.

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

Caution has to be exercised in interpreting some experimental results on aerobic sulfite oxidation since sulfite is oxidized by superoxide anion generated by some electron transport components such as flavin or non-heme iron (MacLeod, 1961; Fridovich, 1961; Nakamura, 1970).

Thiosulfate oxidation

For many thiobacilli growing around neutral pH conditions, thiosulfate has been widely used as a preferred growth substrate. Both sulfur atoms of thiosulfate are normally oxidized to sulfate as follows:

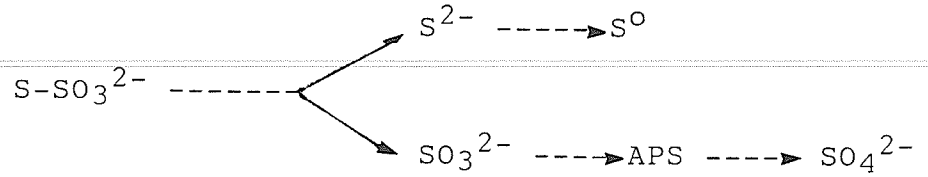
$$S_2O_3^{2-} + 2O_2 + H_2O \longrightarrow 2SO_4^{2-} + 2H^+$$

Sulfur and sulfite are well-documented intermediates. To elaborate the mechanism of thiosulfate oxidation, a great deal of attention has been focused and various theories have been proposed (Lees, 1960; Peck, 1962; Vishniac and Trudinger, 1962; Charles and Suzuki, 1966; Trudinger, 1967, 1969; Lyric and Suzuki, 1970; Suzuki, 1974; Oh and Suzuki, 1980). The first theory is that the oxidation of thiosulfate to sulfate involves tetrathionate and other polythionates as intermediates:

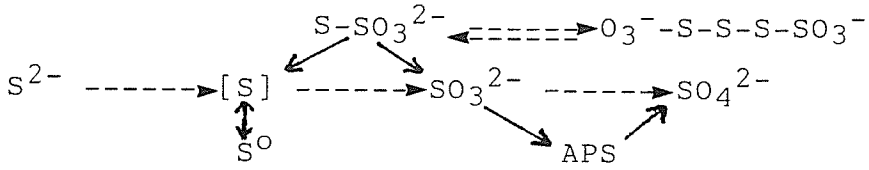
$$S_2O_3^{2-} \longrightarrow S_4O_6^{2-} \longrightarrow S_3O_6^{2-} \longrightarrow SO_3^{2-} \longrightarrow SO_4^{2-}$$

The oxidation is initiated by the thiosulfate-oxidizing enzyme converting thiosulfate to tetrathionate (Trudinger, 1961).

In the second theory thiosulfate is oxidized to sulfur and sulfate without formation of polythionates; a reductive cleavage of thiosulfate with GSH to sulfide and sulfite, followed by oxidation of sulfide to elemental sulfur and sulfite to sulfate via APS Pathway (Peck, 1962):



In third theory the thiosulfate oxidation is initiated by its cleavage to sulfur (colloidal sulfur), [S] and sulfite, followed by their oxidation through sulfur-oxidizing and sulfite-oxidizing systems:



In this reaction scheme when the rate of oxidation of sulfur is less than the rate of thiosulfate cleavage, elemental [S^o] accumulated and is excreted outside of bacterial cells T. thiopaurs . Once sulfite is produced tetrathionate formation is inhibited (Lyric and Suzuki, 1970), which is now oxidized after a reductive cleavage to thiosulfate, sulfur and sulfite (Suzuki, 1974).

The thiosulfate-oxidizing enzyme from T. neopolitanus was first isolated and characterized by Trudinger (1961, 1965). The native cytochrome c-553.5 is the natural electron acceptor. Other native c type cytochromes c-550 and c-557 do not react directly with the enzyme.

The enzyme has also been purified and characterized from T. ferrooxidans grown on sulfur (Silver and Lundgren, 1968) and T.thioparus (Lyric and Suzuki, 1970).

The first cell-free system from T. thioparus which oxidized thiosulfate to sulfate without tetrathionate formation was isolated by Peck (1960, 1962). In this system thiosulfate reductase requires a substrate quantity of GSH ($S_2O_3^{2-}$ + 2GSH -----► GSSG + H_2S + SO_3^{2-}) and elemental sulfur is produced from sulfide (H_2S + $\frac{1}{2}O_2$ ----►S^o + H_2O). Thiosulfate-reductase has yet to be purified and the specific activity of the enzyme is apparently very low (Peck. 1960).

The thiosulfate-cleaving enzyme (rhodanese) activity is normally associated with thiosulfate oxidation (Charles and Suzuki, 1966; Smith and Lascelle, 1966). According to the mechanism proposed by Suzuki (1974), thiosulfate is first cleaved to sulfur (which would form polysulfide by reacting with indigenous sulfhydryl groups) and sulfite by rhodanese. Sulfur derived from the sulfane group (outer position of thiosulfate) is oxidized to sulfite by sulfur oxidizing enzyme and sulfite is finally oxidized to sulfate by a sulfite oxidizing system (the sulfite oxidase pathway or the APS reductase pathway) as follows:

$$S_2O_3^{2-} \xrightarrow[\text{[rhodanese]}]{\text{Thiosulfate-cleaving enzyme}} [S] + SO_3^{2-}$$

$$[S] + O_2 + H_2O \xrightarrow{\text{Sulfur oxidizing enzyme}} SO_3^{2-} + 2H^+$$

$$2SO_3^{2-} + O_2 \xrightarrow{\text{Sulfite-oxidizing system}} 2SO_4^{2-}$$

$$S_2O_3^{2-} + 2O_2 + H_2O \longrightarrow 2SO_4^{2-} + 2H^+$$

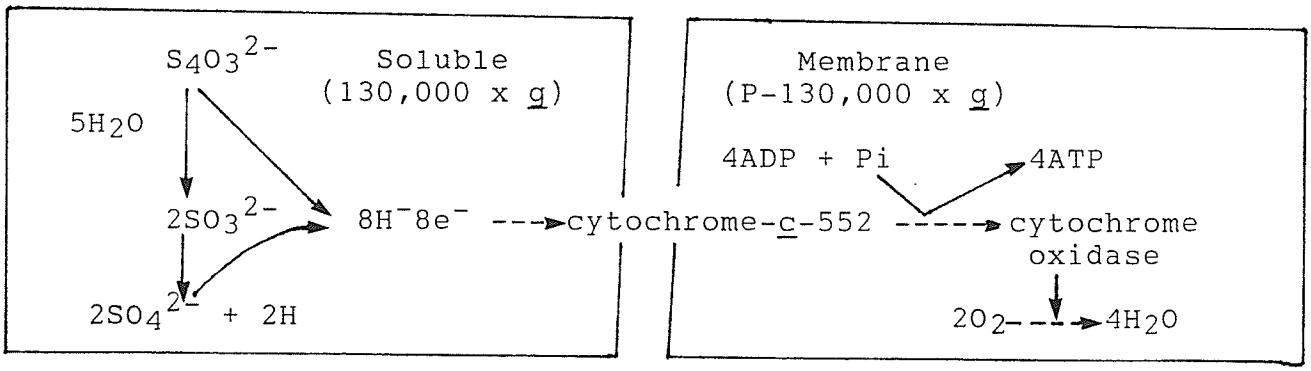
The thiosulfate-oxidizing system has been reported in T. novellus (Charles and Suzuki, 1970), T. intermedius (Charles, 1969) and T. thioparus (Lyric and Suzuki, 1970).

A membrane associated thiosulfate-oxidizing system has been isolated and characterized from T. novellus (Oh and Suzuki, 1977). The system required an initial reducing power which could be supplied by either sulfite, NADH, GSH or endogenous electron donor. A spectrophotometric study indicated the presence of flavin and cytochromes b, c, a and d. The isolated thiosulfate-oxidizing complex was phospholipoprotein in nature and contained rhodanese, sulfur-oxidizing enzyme, sulfite oxidase and cytochrome oxidase.

A recent work by Kelly (1982) on French pressure cell extracts from Thiobacillus A2 that effect stoichiometric oxidation of thiosulfate coupled to ATP synthesis:

$$Na_2S_2O_3 + 2O_2 + H_2O + 4ADP + Pi \dashrightarrow Na_2SO_4 + H_2SO_4 + 4ATP$$

Leads to another mechanism of thiosulfate oxidation to sulfate without the formation of intermediates, sulfur and sulfite. The scheme is as follows:



The factors present both in supernatant and pellet fractions are required for thiosulfate oxidation. By addition of

thiosulfate or sulfite to the supernatant no oxygen uptake occured, cytochrome c-552 but not cytochrome b was reduced. Addition of the pellet fraction resulted in cytochrome oxidation and oxygen uptake.

NADH and Succinate dehydrogenase transferring electrons to the membrane-associated cytochrome c-552 were present in the pellet while some factors linking the sulfite or thiosulfate oxidation to cytochrome reduction were only present in the supernatant fraction.

These results showed that while a limited thiosulfate oxidation could occur in the soluble fraction the oxygen uptake required the membrane associated cytochrome c and cytochrome oxidase.

The oxidation of thiosulfate to sulfate and coupled ATP synthesis did not involve the electron transport through cytochrome b in Thiobacillus A2. ATP synthesis did not involve substrate level phosphorylation in agreement with the apparent absence of APS reductase from the organism.

The present knowledge of sulfur metabolism of T. thiooxidans is very limited. A detailed knowledge of the molecular mechanism for this complex system is still missing. Studies regarding the nature of cell surface and mechanism of acidostability of this microorganism are at an earlier stage. Although, a, b, c and d type cytochromes have been detected (Kodama et al, 1970; Takakuwa, 1976), very little is known about the nature and physiological role of these electron transport components. With the exception of some c type cytochromes (Tano et al, 1968; Takakuwa, 1975), other

electron transport components have never been isolated and characterized. The energy coupling mechanism has to be studied further with respect to the number of potential energy-coupling sites, stoichiometry and possible roles of ATPase and membrane structure. More study is needed on the isolation, identification and characterization of respiratory components and on their role in the oxidation of sulfur compounds and in generation of energy and reducing power. Future studies on all these aspects of sulfur metabolism would eventually elucidate the complex mechanism of biological sulfur oxidation. In the present study an effort has been concentrated on the purification of the sulfur-oxidizing enzyme from T. thiooxidans.

MATERIALS AND METHODS

MATERIALS AND METHODS

Materials

All chemicals and reagents used were of analytical grade and were commercially obtained. Chemicals were obtained from the following sources: sulfur (precipitated) from British Drug House Lts. London, England; sodium sulfide, sodium sulfite and sodium thiosulfate from Fisher Scientific Company, Fairland, N.J., U.S.A.; GSH (reduced glutathione), GSSG (oxidized glutathione), catalase (liver, two time crystallized), AMP, ADP, riboflavin, FMN, FAD, Cytochrome c (type III, from heart), bovine serum albumin (crystalline and fraction V), lysozyme (egg white), lipase, trypsin (bovine pancreatic crystalline), trypsin inhibitor (from soybean), Tween-80, Triton X-100, Sodium deoxycholate, Sodium dithionite, Tris (Trizma base), British Anti Lewisite (BAL, 2,3-dimercapto-1-propanol), dithiothreitol, dithioerythritol, Dowex-50 (H^+), Dowex-1 (OH^-), ascorbate and cysteine were obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A.; DEAE-cellulose from Schleicher and Schuell Inc., Kenne, New Hamphshire; Silica gel coated sheets from Brinkmann Inst. Canada Ltd., Rexdale, Ontario, Canada; Sephadex G-100, blue dextran 2000 and molecular weight calibration kit were products of Pharmacia Fine Chemicals, Uppsala, Sweden; analytical electrofocusing materials were obtained from Bio-rad Laboratories (Canada) Ltd., Mississauga, Ontario, Canada.

Organism and Growth Conditions.

Thiobacillus thiooxidans (ATCC 8085) was used in this study. A pure culture of T. thiooxidans was grown under autotrophic conditions in Starkey's medium (1925), with the addition of molybdate which accelerated the growth of the organism (Takakuwa, 1977): 0.3g $(NH_4)_2SO_4$, 0.5g $MgSO_47H_2O$, 0.018g $FeSO_47H_2O$, 3.5g KH_2PO_4, 0.25g $CaCl_2$ and 0.75 mg $Na_2MoO_42H_2O$ per 1000 ml distilled water. The organism was grown in 3 litre Fernbach flasks,each containing one litre of the above mentioned Starkey's medium. The pH of medium was 4.5. After addition of 2.5-3% inoculum to the medium, flasks were swirled, enough powdered precipitated sulfur was then added to cover the surface of the medium. Sulfur was spread evenly to cover the surface of the medium by gently swirling flasks. Flasks were covered with tissue papers and incubated at 28°C for 6 days. During this time most of the sulfur remained floating on the surface. After incubation, flasks were shaken vigorously and 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 1.2 and 1.6. The cells were collected by a Sharples supercentrifuge fitted with a water cooling system (7°C) at 40,000 r.p.m. and were suspended in distilled water. The suspension was stirred overnight in distilled water at 4°C and washed two times with distilled water. The cell yield varied between 0.35-0.45g (wet weight) per litre of medium. The cells were stored at 4°C and used for preparing cell-free extract the same day. For

determination of growth during incubation, 5.0 ml of the culture medium was removed atappropriate intervals and separated from sulfur by filtration. The optical density of the filtrate was measured using a Klett-Summerson photoelectric colorimeter with a No. 66 filter. The pH was measured with a pH meter every day during growth.

Methods

Preparation of Sulfur Suspension.

The colloidal sulfur suspension used as substrate for the sulfur-oxidizing enzyme was prepared as follows: precipitated sulfur (36g) was suspended in 100 ml of distilled water containing 0.05% Tween-80. After vigorous stirring for 2hours, the suspension was sonicated for 30 minutes (10 times 3 minute intervals) at 4^{o}C with the large tip of the Insonater 1000 system at the maximum output (Ultrasonic system Inc.). The sonicated suspension was allowed to settle for a few minutes. The milky white colloidal sulfur was decanted off from the sonicated sulfur and was used as substrate for the sulfur-oxidizing enzyme. The sulfur concentration was determined by drying aliquots of the sulfur suspension at 65^{o}C and weighed after cooling to room temperature. The sulfur suspension was stored at 4^{o}C.

Preparation of Cell-Free Extract.

The cells were washed once more with 0.2 M Tris-Cl (pH 7.5) and suspended (100 mg wet weight cells per ml) in 0.05 M Tris-Cl buffer (pH 7.5). The cell suspension

was treated with trypsin (1.5 mg per gram wet weight of cells) for 15 minutes with gentle stirring at room temperature. The treatment was stopped by adding trypsin inhibitor (5mg trypsin inhibitor for every 1.5 mg trypsin). Trypsin-treated cells were sonicated for 15 minutes at 7°C in a 10 Kc/sec Raytheon sonic disintegrator. The sonicated cell suspension was centrifuged at 20,000 x g for 20 minutes to remove cell debris and unbroken cells. The resulting brownish-yellow supernatant was designated as cell-free extract. The cell-free extractcontained 15-18 mg of protein per ml and was used immediately or stored at 4°C until further use. The procedures described here were essentially those of Bhells (1981).

In some experiments the cell-free extract was prepared after the treatment of cells with ion-exchange resins according to Suzuki (1965a). Cells suspended in distilled water (100 mg/ml) were stirred for 15 minutes with 100 mg/ml each of Dowex 1 (OH^-) and Dowex 50 (H^+). The resins were removed by filtration through a cheese cloth. The cells collected by centrifugation were suspended in 0.1 M Tris-Cl (pH 7.5) at a concentration of 100 mg/ml. The suspension was sonicated for 15 minutes at 7°C in a 10 Kc/sec Raytheon sonic disintegrator. In another experiment the cells were suspended (100 mg/ml) in 0.05 M potassium phosphate buffer (pH 6.8) and sonicated without pre-treatment for 15 minutes at 7°C in a 10 Kc/sec. Raytheon sonic disintegrator.

Centrifugations.

Low speed centrifugations were performed in a Sorvall superspeed RC-2B automatic refrigerated centrifuge at 4°C. High speed centrifugations of cell-free extracts were carried out in a Beckman L3-50 refrigerated ultracentrifuge (4°C) using a 50 Ti or 60 Ti rotor at 150,000 x g for 2 hours to yield the supernatant and pellet fractions. The pellet was suspended in 1/10 the volume of extract in 50 mM Tris-Cl buffer (pH 7.5).

Sulfur-Oxidizing enzyme Assay.

Sulfur oxidation was assayed by measurement of oxygen consumption at 25°C 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 1.2 ml:

60 u moles potassium phosphate (pH 7.5)

0.5 mg sulfur (in 0.2 ml)

10.0 μ moles GSH

enzyme as indicated

The reaction was started by the addition of GSH to the rest of the reaction mixture. Enzymatic sulfur oxidation rates were always corrected for non-enzymatic oxidation rates of GSH and sulfur. One unit of enzyme was defined as the amount of enzyme that consumed 1.0 nmole of O_2 per minute under the standard conditions and the specific activity was defined as the units of enzyme per mg protein. In some

experiments, sulfur oxidation was measured manometrically at 30°C in a conventional Warburg apparatus as described by Suzuki and Silver (1966).

Sulfide and Sulfite Oxidation Assays.

Sulfide and sulfite oxidations were assayed by measuring oxygen consumption in Gilson oxygraph. In case of sulfide oxidation the reaction mixture contained unless otherwise indicated, the following in a total volume of 1.2 ml:

60 u moles potassium phosphate (pH 7.5)

2 μ moles Na_2S

enzyme as indicated.

In case of sulfite oxidation, the reaction mixture contained, unless otherwise indicated, the following in a total volume fo 1.2 ml:

60 μ moles potassium phosphate (pH 7.5)

2 μ moles Na_2SO_3

0.1 μ mole EDTA

enzyme as indicated.

The reaction was initiated by the addition of sulfide or sulfite-EDTA solution in microlitre quantity. EDTA served as an inhibitor of non-enzymatic oxidation of sulfite. The enzymatic rates were corrected for non-enzymatic rates of oxygen uptake as in sulfur oxidation.

Thiosulfate-Sulfur Transferase (Rhodanese) Assay.

Rhodanese activity was measured by the rate of thiocyanate

formation from thiosulfate and cyanide according to the method of Sörbo (1957) with minor modifications by Oh (1977a). The reaction mixture contained: 150 μ moles Tris-acetate (pH 7.5), 20 μ moles potassium phosphate (pH 7.5), 150 μ moles thiosulfate, 100 μ moles KCN, enzyme and water in a total volume of 2.5 ml and was incubated at 25°C for 10 minutes. The reaction was stopped by addition of 0.5 ml 38% (V/V) formaldehyde solution. After addition of 2.0 ml ferric nitrate reagent [20% (w/v) $Fe(NO_3)_3$ in 13% HNO_3], absorbance was measured with the Shimadzu MPS-50L spectrophotometer at 460 nm against a blank consisting of a complete system to which the formaldehyde solution had been added before KCN. One unit of enzyme was defined as that amount of enzyme which formed 1 μ mole of thiocyanate under the above standard conditions.

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 in a Warburg respirometer at 28°C with vigorous stirring. The components of the reaction mixture were similar to that of the Gilson oxygraph assays. The total volume of reaction mixture was 3.2 ml. The reaction was stopped by the addition of 0.5 ml of 1 M cadmium acetate. After centrifugation to remove sulfur, protein and GSH, the supernatant was used for the colorimetric determination of thiosulfate by the method as described by Sörbo (1957). To 1.0 ml sample containing 0.1 to 1.0 μ mole of thiosulfate, 1.1 ml of 0.2 M NH_4OH,

0.25 ml of 0.1 M KCN were added and after mixing 0.15 ml of 0.1 M $CuCl_2$ was added. The $CuCl_2$ was well mixed with the sample immediately after addition. To the mixture was then added 0.25 ml of ferric nitrate reagent (20% w/v $Fe(NO_3)_3 \quad 9H_2O$ in 13% HNO_3) and the mixture was well mixed. After 15 minutes of incubation, the optical density was measured in a Klett-Summerson colorimeter with a No. 42 blue filter. A blank reading was obtained by adding to the sample plus NH_4OH, first the ferric nitrate reagent followed by KCN and $CuCl_2$.

Protein Determination.

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

Determination of Iron in the Enzyme.

Iron was determined by the method of Massey (1957) and Rajagopalan and Handler (1964) with some modification as described by Suzuki and Silver (1966). To 1.0 ml sample was added 1.0 ml 0.1 M 2-2' bipyridyl. After one hour incubation in the dark 0.2 ml saturated ammonium acetate and 0.3 ml water were added. The mixture was incubated for an additional hour and the optical density at 520 nm was measured using a Shimadzu-MPS-50L spectrophotometer, using a reagent blank. The value obtained gave the ferrous iron content. Total iron was estimated by adding a small amount of sodium dithionite to the samples and incubating the mixture in the dark for one hour before measuring the

optical density as before.

Determination of Labile Sulfide in the Enzyme.

The sulfide content of the enzyme was determined by a modification of the method of Fogo and Popowsky (1949), essentially as described by Suzuki and Silver (1965). To a 0.65 ml sample containing 5-100 nmoles of sulfide, equal volume of 2% Zinc acetate was added and mixture was centrifuged. To the supernatant was added 2.5 ml of 0.1% p-aminodimethyl-aniline sulfate in 5.5 M HCL and 0.5 ml of 0.23 M $FeCl_3$ in 1.2 M HCL in a screw cap test tube and was shaken. After 30 minutes the intensity of methylene blue formed was measured at 670 nm in a Shimadzu MPS-50L spectrophotometer.

Identification of Flavin by Thin Layer Chromatography.

Thin layer chromatography on commercially obtained silica gel strips was carried out essentially as described by Fazekas and Kokai (1971). Extraction of flavin from different fractions was carried out by boiling the enzyme preparation in a hot water bath for 2-3 minutes and removing the denatured protein by centrifugation. Extracted supernatants along with riboflavin, FMN, FAD , AMP and ADP markers were spotted on the silica gel strips and ascending chromatography was performed using 5% Na_2HPO_4 $12H_2O$ as the solvent. This solvent was most suitable for quick separation of these compounds. After chromatography, plates were dried by means of a hair drier and examined under the UV light. Rf values of the compounds were measured and compared.

Spectrophotometry.

Absorption spectrum studies of various preparations were done with the Shimadzu MPS-50L or Beckman Du-8 spectrophotometer at room temperature using a cell with 1 cm light path length.

Disc Polyacrylamide Gel Electrophoresis.

Polyacrylamide gel electrophoresis under non-dissociating conditions was performed using the method described by Davis (1964). Samples containing 100-200 μg of protein were applied to gel tube and subjected to a current of 3-5 mA per tube. After termination of the run the gels were removed and protein was stained with coomassie blue (Colab) for 6-8 hours. The destaining was done with a 7% glacial acetic acid solution.

SDS-acrylamide Gel Electrophoresis.

Sodium dodecyl sulfate acrylamide gel electrophoresis under dissociating conditions was performed using the method described by Weber and Osborn (1969). SDS dissociates protein into their constituent polypeptide chains. Polyacrylamide gel electrophoresis in the presence of SDS separates polypeptide chains according to their molecular weights. Samples containing 100-200 μg protein were incubated for 2 minutes in a 100°C bath in the small plastic cap tube containing 1% SDS and 1% 2-mercaptoethanol, cooled to room temperature and used directly. The proteins were stained with coomassie blue for 10-12 hours. The destaining was done in a solution of 50 mlmethanol and 75 ml glacial acetic acid

per liter in water.

Molecular Weight Determination.

The molecular weight of purified enzyme was determined by gel filtration on a Sephadex G-100 column using a method similar to that reported by Andrew (1964). The Sephadex column (2.5 x 55 cm) was standardized using proteins from the Pharmacia molecular weight estimation kit. The purified enzyme was mixed with proteins from the estimation kit and applied to the column. Column effluents were collected in 3.0 ml fractions with a fraction collector. The enzyme activity was measured in an oxygraph and the spectrophotometric determination of proteins at 280 nm was done with a Shimadzu MPS-50L spectrophotometer for each fraction. The volume was plotted against the molecular weights of proteins.

Analytical Electrofocussing In Polyacrylamide Gel.

Analytical electrofocussing in horizontal polyacrylamide gel was carried out in an electrophoresis cell (Model 1415) from Bio-Rad Laboratories (Canada) Ltd. using a broad range ampholyte (pH range from pH 3-10). The samples were dialysed against water to remove salt contamination. The samples were then applied onto a piece of filter paper on the gel near the basic (negative) end. Cytochrome c was applied as a visible marker. A surface pH electrode was used to measure the gradient directly on an open faced gel. The protein bands were detected by staining the gel after fixing with a fixative solution which prevented the diffusion of all bands: a solution of 4% sulfosalicylic acid, 12.5%

trichloroacetic acid and 30% methanol for 30 minutes fixing and then a solution of 27% isopropanol, 10% acetic acid, 0.04% coomassie brilliant blue R-250 and 0.5% $CuSO_4$ for 2 hours staining. For destaining two solutions were used. The first destaining solution contained 12% isopropanol, 7% acetic acid and 0.5% $CuSO_4$. The gel was immersed in the solution, which was changed two or three times until the background was nearly clear. The second destaining solution contained 12% isopropanol and 7% acetic acid. The gel was immersed in the solution to remove the final traces of stain and $CuSO_4$.

RESULTS

RESULTS

General Physiology of Thiobacillus thiooxidans

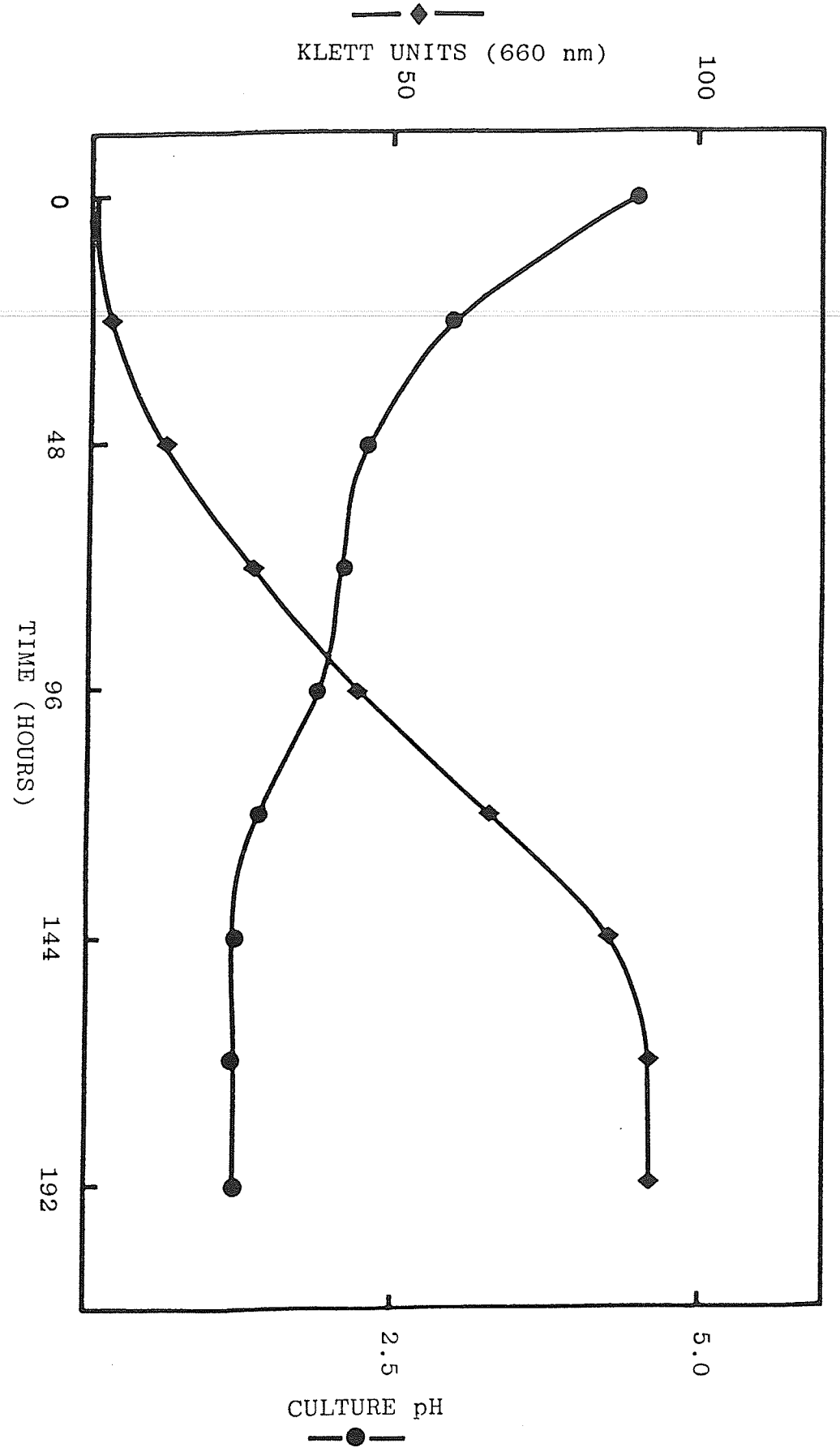
In spite of simplicity of the nutritional requirements, T. thiooxidans is known to be physiologically a very complex and sophisticated microorganism. Studies with the intact cells showed that T. thiooxidans readily oxidized sulfur, sulfide and sulfite without the requirement of any cofactor. Sulfur, sulfide and sulfite oxidising activities were also detected in the cell-free extracts. However, optimum pH values for the oxidation of these sulfur compounds shifted from an acidic pH (around 5.5) with cells to a neutral pH (around 7.5) with cell-free extracts. Cell-free extracts, however, required GSH for the sulfur oxidation. Cytochrome a, b and c types and flavin were also easily detectable spectrophotometrically in the cell-free extracts. After centrifugation of the extract at 150,000 x g for two hours, the sulfide and sulfite oxidizing activities were found in the pellet fraction. Approximately 65-75% sulfur-oxidizing activity was found in the supernatant and 30-35% in the pellet fraction. In the present study the sulfur-oxidizing enzyme, obtained in the soluble protein fraction (150,000 x g supernatant) was used for detailed investigation.

Growth of Thiobacillus thiooxidans

Growth of T. thiooxidans in the Starkey's medium with molybdate was accompanied by a drop in pH due to sulfuric acid production (Fig. 1). Growth after 5 to 6 days of incubation produced actively sulfur-oxidizing cells with good yield.

Fig. 1. Growth curve of Thiobacillus thiooxidans indicating change in the medium pH as a function of growth.

Cell density was measured with a Klett-summerson photoelectric colorimeter as described in Materials and Methods (—◆———◆—). Change in pH (—●———●—).


KLETT UNITS (660 nm)
50
100
0
48
96
144
192
TIME (HOURS)
2.5
5.0
CULTURE pH

Effect of Molybdate Supplementation on the Growth of T. thiooxidans and Distribution of Sulfur-Oxidizing Activity in Various Fractions of Cell Extract

T. thiooxidans was grown in the Starkey's medium with and without sodium molybdate supplementation (0.75 mg/l) for 6 days at 28°C. The cell yield in wet weight was found to be 0.5 g/l in molybdate supplemented medium and 0.42 g/l without molybdate. Cell-free extracts prepared from cells (100 mg/ml) obtained from the above mentioned two growth media showed similar absorption spectra and sulfur-oxidizing activities. Distribution of the sulfur-oxidizing activity in various fractions of cell-free extracts grown with or without molybdate is shown in Table 1. Since there was more growth and an improved recovery of the enzyme during purification from cells grown in molybdate supplemented medium, the organism was routinely grown in the medium containing molybdate.

Effect of Ion Exchange and Trypsin Treatment in Solubilizing the Sulfur Oxidizing Enzyme

The treatment of cells before breakage with ion-exchange resins, Dowex 50 (H^+) and Dowex 1 (OH^-), or trypsin (bovine pancreatic crystalline) as described in Materials and Methods was found to be almost equally effective in releasing the sulfur-oxidizing activity in the soluble form upon subsequent sonication. However, the resins treatment was more laborious and required care in filtration as cells adhered strongly to resins or filter cloth. The trypsin treatment, being simple and easy, was selected as standard

Table 1. Distribution of sulfur-oxidizing activity in various fractions of extracts from cells grown in medium with and without sodium molybdate supplementation.

Fractions[a]	Volume (ml)	Sulfur-oxidizing activity (unit)[b]	Recovery (%)
Cells with molybdate			
Extract	50	26,000	100
150,000 x g supernatant	50	12,250	46.7
Pellet	5	5,750	21.9
pH 5 supernatant	48	9,750	37.2
Precipitate	4.8	2,060	7.8
DEAE-cellulose fraction	7	8,750	33.4
Cells without molybdate			
Extract	50	25,700	100
150,000 x g supernatant	50	11,750	45.7
Pellet	5	5,500	21.4
pH 5 supernatant	48	8,020	31.2
Precipitate	4.8	3,120	12.1
DEAE-cellulose fraction	6	6,000	23.3

[a]Extracts were prepared from cell suspension (100 mg wet cells/ml) as described in Materials and Methods. Various fractions shown here are described in detail in the section on enzyme purification.

[b]Sulfur-oxidizing activity was determined as described in Materials and Methods. One unit of enzyme was defined as the amount of enzyme that consumed 1 n mole of O_2 per minute under the standard conditions.

procedure for the purification of sulfur-oxidizing enzyme. Lysozyme, lipase, glucosidase or detergent treatment of cells was unsuccessful in releasing the sulfur-oxidizing activity in a soluble form as mentioned by Bhella (1981).

Purification of Sulfur-Oxidizing Enzyme

For the purification of sulfur-oxidizing enzyme of T. thiooxidans the following steps were routinely performed. All the steps were carried out around 4°C.

(1) Cell-free extract. After washing, the cells were suspended at 100 mg wet cells per ml in 0.05 M Tris-Cl buffer (pH 7.5). The cell-free extracts were prepared by treating cells with trypsin then sonicating as described in Materials and Methods. The supernatant after centrifugation at 20,000 x g for 20 minutes was used as a cell-free extract. The reduced minus oxidized difference spectrum of extract suggested presence of cytochromes of a, b and c types and a flavin (Fig. 2).

(2) 150,000 x g centrifugation. Cell-free extract thus obtained were centrifuged at 150,000 x g for two hours and the supernatant was carefully removed with a syringe without disturbing the pellet. The reduced minus oxidized difference spectrum of supernatant suggested the presence of a c type cytochrome and a flavin (Fig. 3).

(3) Acidic treatment. The 150,000 x g supernatant fluid was adjusted to pH 5.0 by dropwise addition of 1M acetic acid and precipitated proteins were removed by centrifugation at 20,000 x g for 20 minutes. The resultant supernatant

was adjusted back to pH 7.5 with 2 M Tris-base solution. It was a bright yellow transparent fluid. The reduced minus oxidized spectrum (Fig. 4) was similar to that of the 150,000 x g supernatant.

(4) DEAE-cellulose columnn chromatography. The supernatant fluid from acidic treatment was applied to a 2.5 x 15 cm DEAE-cellulose column equilibrated with 0.05 M Tris-Cl (pH 7.5) buffer and the column was eluted with the same buffer using an elution rate of 25-30 ml per hour. A very light yellow fluid devoid of sulfur oxidizing activity showing an absorption peak at 422 nm passed through the column unabsorbed and a brownish yellow band could be seen absorbed to DEAE-cellulose at the top. The column was then washed with 200 ml of 50 mM Tris-Cl (pH 7.5). Dead volume of the column measured beforehand with dextran 2000 was 30 ml. After washing with 50 mM Tris-Cl the column was eluted with 30 ml each of 0.1 M, 0.15 M, 0.2 M, 0.25 M, and 0.3 M Tris-cl (pH 7.5) buffers unless otherwise specified. The fraction eluted with 0.1 M Tris-Cl was light yellow in color and devoid of sulfur-oxidizing activity. The fraction collected with 0.15 M Tris-Cl was yellow in color showing little sulfur-oxidizing activity and the spectrum revealed a peak at 450 nm. As the sulfur-oxidizing activity was eluted slowly in the 0.2 M Tris-Cl, a fraction of 35 ml was collected with this buffer. This fraction had a very faint yellow color and about 60% of the sulfur-oxidizing enzyme activity of the total activity applied to the column. The fraction

Fig. 2 Reduced minus oxidized difference spectrum of cell-free extract.

Preparation of cell-free extract was as described in Materials and Methods. The spectrum was recorded with a Beckman Du-8 spectrophotometer at room temperature using a cuvette with 1 cm light path. A few grains of $Na_2S_2O_4$ were added to the cuvette to reduce the extract (1.84 mg protein) in a total volume of 2 ml in 50 mM Tris-Cl (pH 7.5).

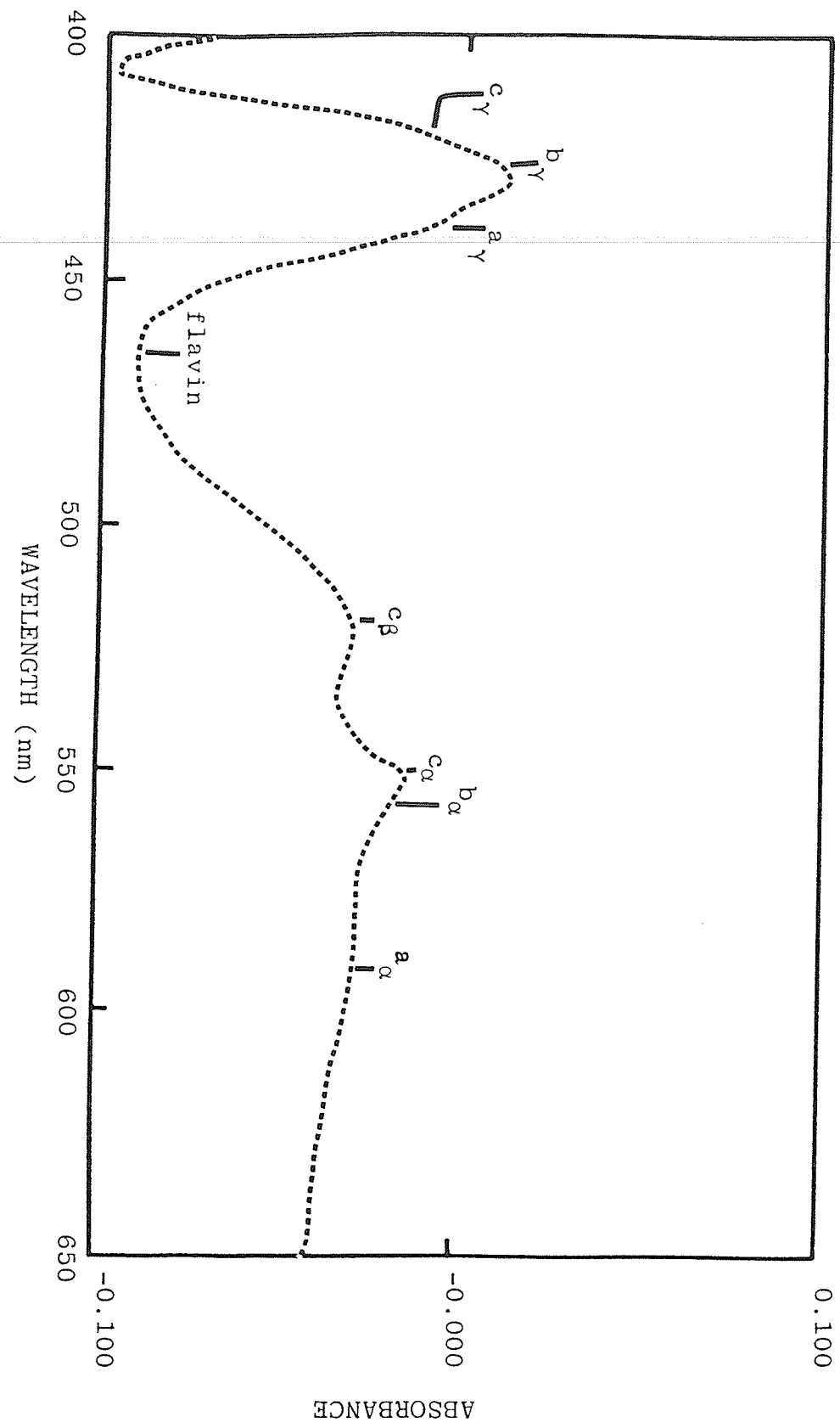

400
450
500
550
600
650
WAVELENGTH (nm)
-0.100
-0.000
0.100
ABSORBANCE
c_γ
b_γ
a_γ
flavin
c_β
c_α
b_α
a_α

Fig. 3. Reduced minus oxidized difference spectrum of the 150,000 x g supernatant.

Preparation of the 150,000 x g supernatant is described in the purification of sulfur-oxidizing enzyme. The spectrum was recorded with a Beckman Du-8 spectrophotometer at room temperature using a cuvette with a 1 cm light path. A few grains of $Na_2S_2O_4$ were added to the cuvette to reduce the 150,000 x g supernatant (1.92 mg protein) in a total volume of 2 ml in 50 mM Tris-Cl (pH 7.5).

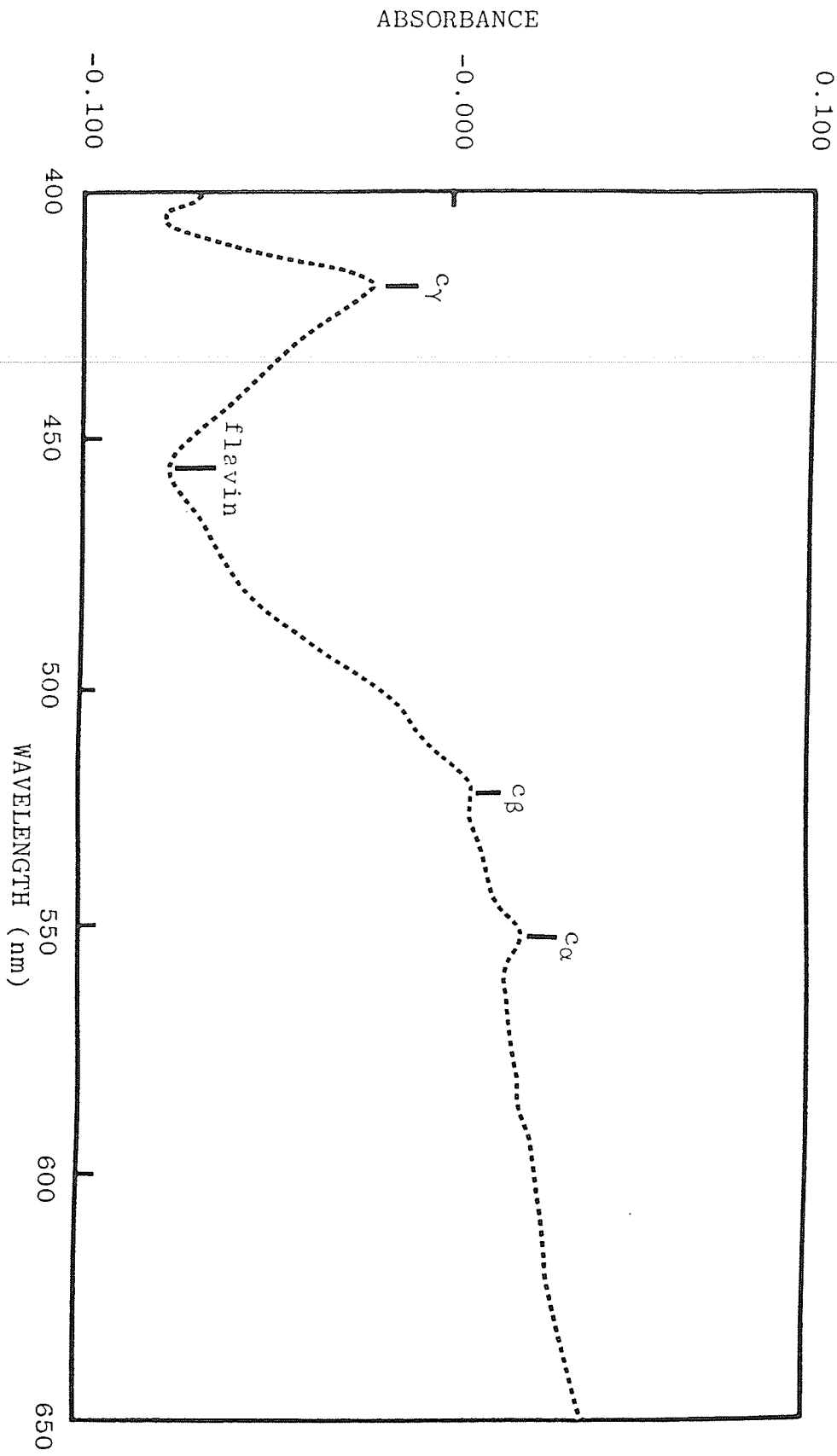

ABSORBANCE
0.100
-0.000
-0.100
400
450
500
550
600
650
WAVELENGTH (nm)
cγ
flavin
cβ
cα

Fig. 4. Reduced minus oxidized difference spectrum of pH 5 supernatant.

Preparation of pH 5 supernatant is described in the purification of sulfur-oxidizing enzyme. The spectrum was recorded with a Beckman Du-8 spectrophotometer at room temperature using a cuvette with 1 cm light path. A few grains of $Na_2S_2O_4$ were added to the cuvette to reduce the pH 5 supernatant (1.95 mg protein) in 50 mM Tris-Cl (pH 7.5).

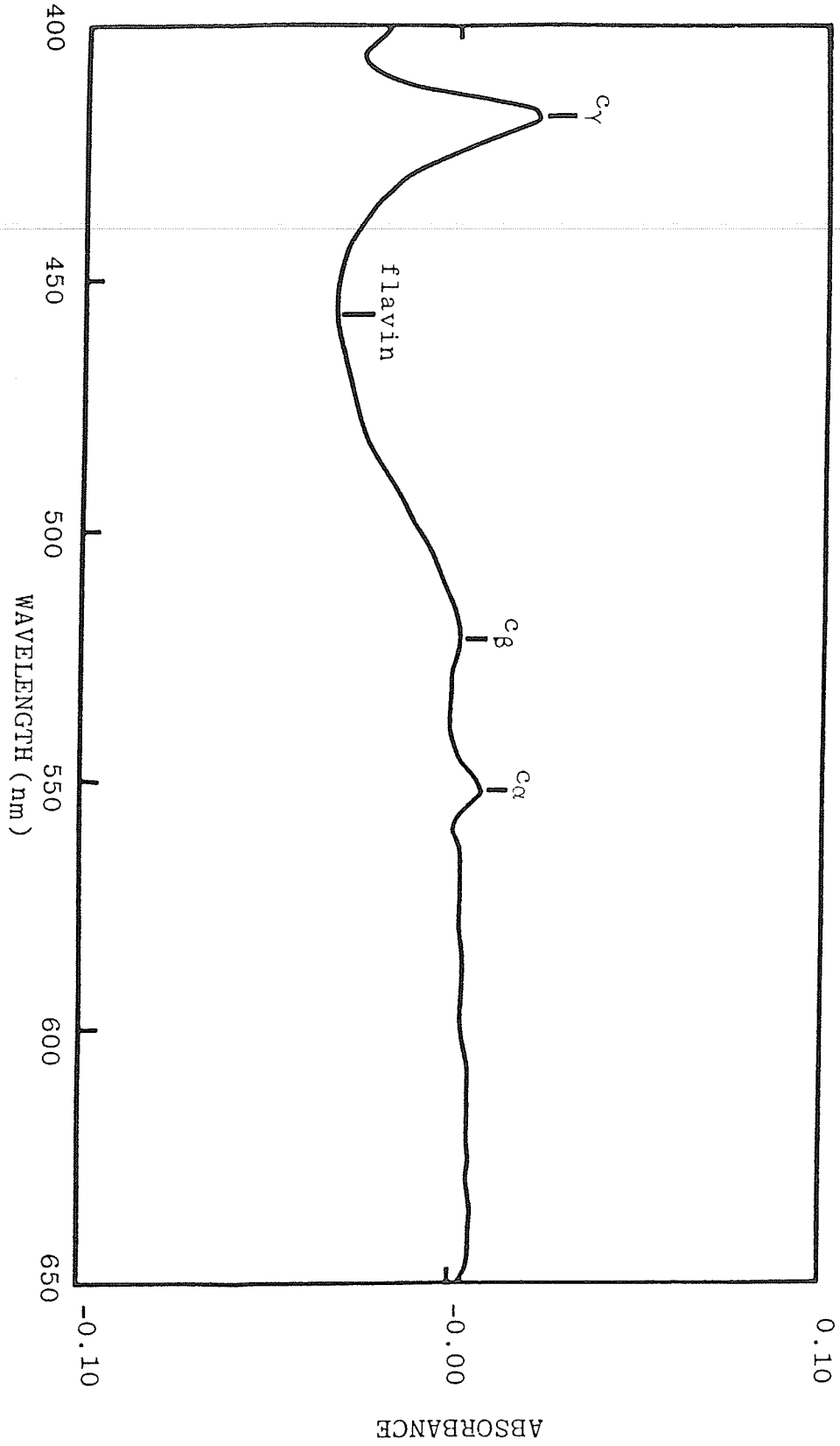
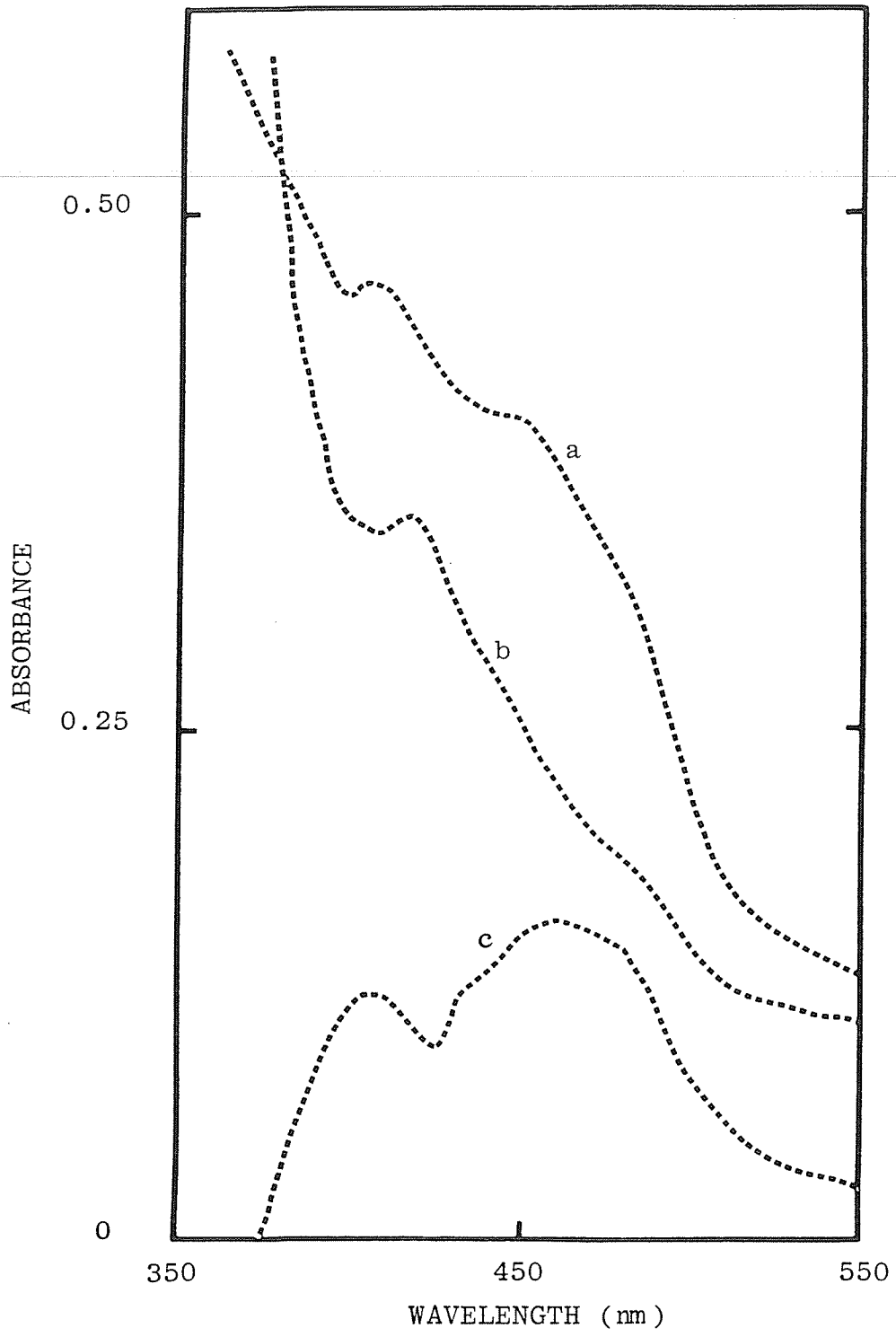

400
450
500
550
600
650
WAVELENGTH (nm)
-0.10
-0.00
0.10
ABSORBANCE
c_γ
flavin
c_β
c_α

Fig. 5. Oxidized and reduced spectra and oxidized minus reduced difference spectrum of 0.3 M Tris-Cl (pH 7.5) DEAE-cellulose fraction.

a - oxidized
b - dithionite reduced
c - oxidized minus dithionite reduced
Preparation of this fraction is described in the purification of sulfur-oxidizing enzyme (DEAE-cellulose column chromatography). The spectra were recorded with a Beckman Du-8 spectrophotometer at room temperature using a cuvette with 1 cm light path. A few grains of $Na_2S_2O_4$ were added to the cuvette to reduce the fraction (3.0 mg protein) in a total volume of 2 ml in 0.3 M Tris-Cl (pH 7.5).


0.50
0.25
0
ABSORBANCE
a
b
c
350
450
550
WAVELENGTH (nm)

collected with 0.25 M Tris-Cl was light yellow in color and had very little activity. Its spectrum was similar to that of the 0.15 M fraction. At the end of chromatography a bright yellow fraction moved as a single band in 3-4 ml with 0.3 M Tris-Cl. It had very little activity but contained most of the yellow color of the sample applied to the column. Its spectrum showed peaks at 410 nm and 450 nm and a shoulder at 475 nm (Fig. 5). The reduced spectrum (415-420 nm peak) and the difference spectrum (oxidized minus reduced) (450-460 nm peak) suggested the presence of a c type cytochrome and a flavin.

(5) Concentration on DEAE-cellulose. The 0.2 M Tris-Cl (pH 7.5) fraction collected from the previous DEAE-cellulose column was diluted four times with cold distilled water and was again applied to a small 2.0 x 12 cm DEAE-cellulose column equilibrated with 50 mM Tris-Cl (pH 7.5), using an elution rate of 20-25 ml per hour. A light yellow band was observed at the top of the column. The column was washed with 50 ml of 50 mM Tris-Cl (pH 7.5) and then with 50 ml of 0.1 M Tris-Cl (pH 7.5). The column was then eluted with 0.3 M Tris-Cl (pH 7.5). A light yellow fluid came out in a single 4-5 ml fraction. The sulfur-oxidizing activity was recovered with a yield of 65-70% in the concentrated fraction. The spectrum of this fraction showed a shoulder at 475 nm but no peak at 450 nm.

(6) Sephadex G-100 column chromatography. The concentrated enzyme fraction from the step 5 was applied to a sephadex G-100 column (2.5 x 55 cm) equiliberated with 50 mM Tris-Cl

Table 2. Purification of Sulfur-Oxidizing Enzyme

Fraction	Total volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein	Purification (folds)	% recovery
Extract (1)	80	1,472	24,000	16.3	1	100
150,000 x g (2)	80	768	15,200	19.8	1.2	63.3
pH 5 supernatant (3)	80	312	13,600	43.6	2.67	56.7
DEAE-cellulose						
0.1 M eluate	30	45	300	6.6	2.67	1.25
0.15 M eluate	30	45	1,200	26.6	1.63	5.0
0.2 M eluate (4)	35	58	8,400	145	9.0	35.0
0.2 M eluate (concentrated) (5)	5	33	5,500	167	10.2	23.0
0.25 M eluate	30	48	1,850	38.5	2.4	7.7
0.3 M eluate	3.5	21	648	30.8	1.9	2.7
Sephadex G-100 (6)	3.75	3.75	2,750	733	45	11.7

buffer (pH 7.5) and was eluted with the same buffer. Fractions of 3.0 ml were collected with a fraction collector and were examined for the sulfur-oxidizing enzyme activity with an oxygraph, using 50 µl of each fraction. The activity started appearing in the 6th fraction after the dead volume (45 ml). All the fractions showing activity were pooled together. The pooled fractions (37 ml) were colorless. Some light yellow color fluid came out in the first few fractions but had no sulfur-oxidizing activity. Elution rate of the sephadex column was found to be very critical in recovery of the active enzyme. If it was very slow (3-4 ml per hour), there was a considerable decrease in the specific activity of purified enzyme. The flow rate of column elution was maintained at 10-12 ml per hour. The pooled active fractions were subjected to concentration with solid ammonium sulfate (100% saturation). Enzyme precipitated was collected by centrifugation at 20,000 x g for 20 minutes and was resuspended in 1/10 volume of the original pooled fractions using 50 mM Tris-Cl buffer (pH 7.5). The suspension was dialysed against 50 mM Tris-Cl (pH 7.5) buffer for 8-10 hours with 4-5 changes of buffer at 4°C. The resultant clear fluid was considered as a purified sulfur-oxidizing enzyme preparation and was used for further study. The results of this purification is shown in Table 2.

Characteristics of Sulfur-Oxidizing Enzyme

Absorption spectrum. The absorption spectrum of purified enzyme showed a maximal absorption around 278 nm and shoulders

at 410 nm, 448 nm and 478 nm. Upon reduction with sodium dithionite shoulders at 448 nm and 478 nm disappeared and a shoulder at 410 nm shifted to 420 nm (Fig. 6). This shift suggests the presence of a small amount (less than 1% in protein) of a c-type cytochrome. The difference spectrum is characteristic of a non-heme iron protein (iron-sulfur protein), but not of a flavoprotein.

Purity of sulfur-oxidizing enzyme. The disc electrophoresis under non-dissociating conditions revealed one major and six very minor bands. From the intensity of the bands, the enzyme was considered to be 80% pure.

Molecular weight. Molecular weight of the enzyme was estimated by gel filtration on sephadex G-100 (Fig. 7) as described in Materials and Methods. A molecular weight of 46,000 ± 5% was estimated for the purified enzyme.

Sub-unit molecular weight. The disc SDS-polyacrylamide gel electrophoresis under dissociating conditions was preformed with purified enzyme as described in Materials and Methods. The gel showed two major bands, corresponding to molecular weights of 21,000 and 26,000 (Fig. 8).

Iron and labile sulfide content. Table 3 depicts the contents of iron and labile sulfide in the purified enzyme preparation determined by the methods as described in Materials and Methods. Considering the molecular weight of the enzyme as 46,000. Protein: total iron: labile sulfide was found to be in the molar ratio of 1:1:1 respectively. As shown

Fig. 6. Oxidized and reduced spectra and oxidized minus reduced difference spectrum of purified sulfur-oxidizing enzyme.

a and b - oxidized

c - dithionite reduced

d - oxidized minus dithionite reduced

Preparation of purified sulfur-oxidizing enzyme is as described in the purification of sulfur-oxidizing enzyme. The spectra in 50 mM Tris-Cl (pH 7.5) were recorded using a Shimadzu MPS-50L and a Beckman Du-8 spectrophotometer at room temperature using a cuvette with 1 cm light path. A few grains of $Na_2S_2O_4$ were added to the cuvette to reduce the purified sulfur-oxidizing enzyme (2.0 ml, 2.0 mg protein).

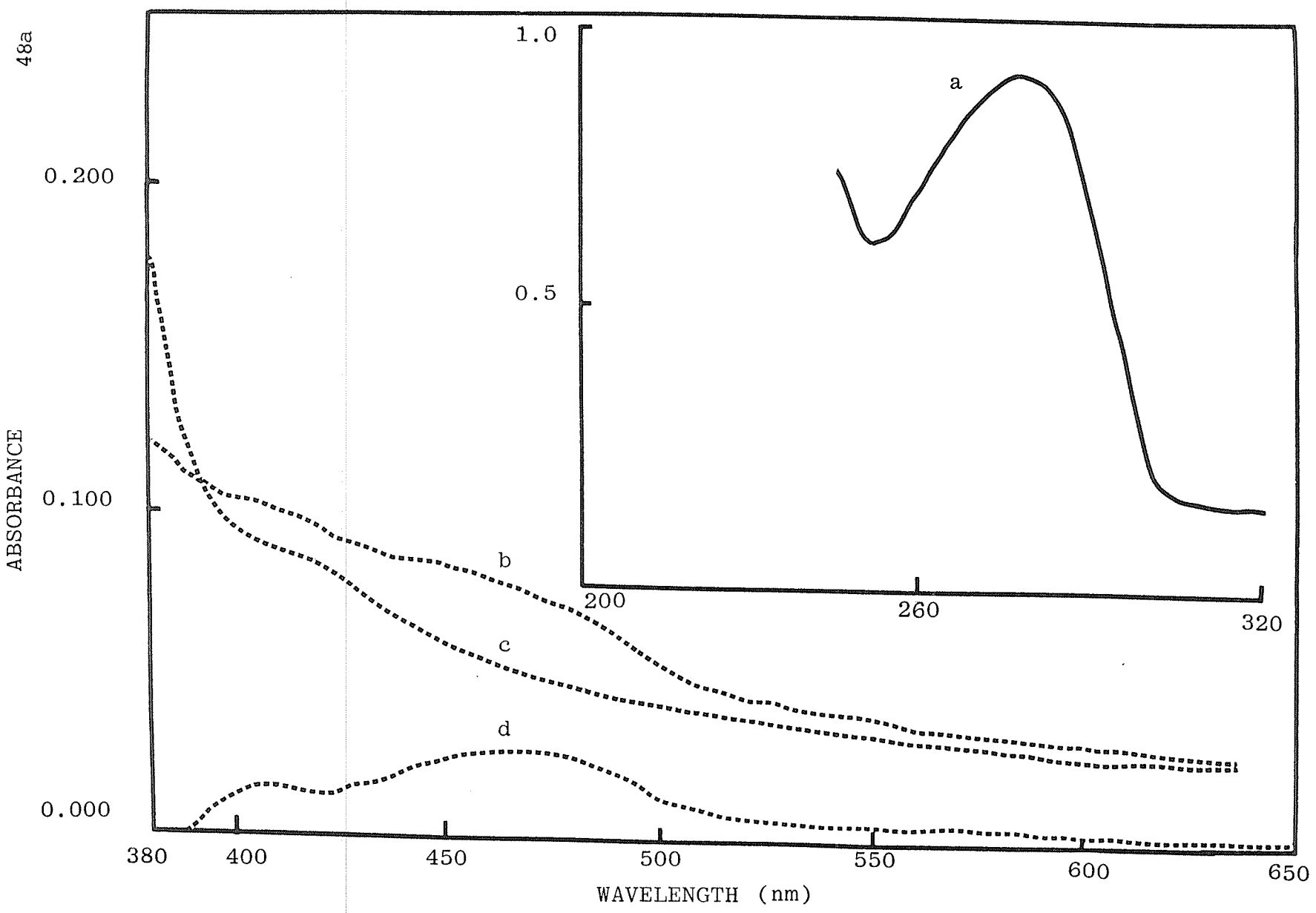

48a
1.0
0.5
a
200
260
320
0.200
0.100
0.000
ABSORBANCE
b
c
d
380
400
450
500
550
600
650
WAVELENGTH (nm)

Fig. 7. Molecular weight estimation of sulfur-oxidizing enzyme by sephadex G-100 column chromatography.

Molecular weight of purified enzyme was estimated by the gel filtration behaviour of proteins on sephadex G-100, using 50 mM Tris-Cl, pH 7.5 as eluant. The marker protein used were bovine serum albumin (Mol. wt. 68,000), ovalbumin (Mol. wt. 45,000); chymotrypsinogen (Mol. wt. 25,000) and horse heart cytochrome c (Mol. wt. 12,400).

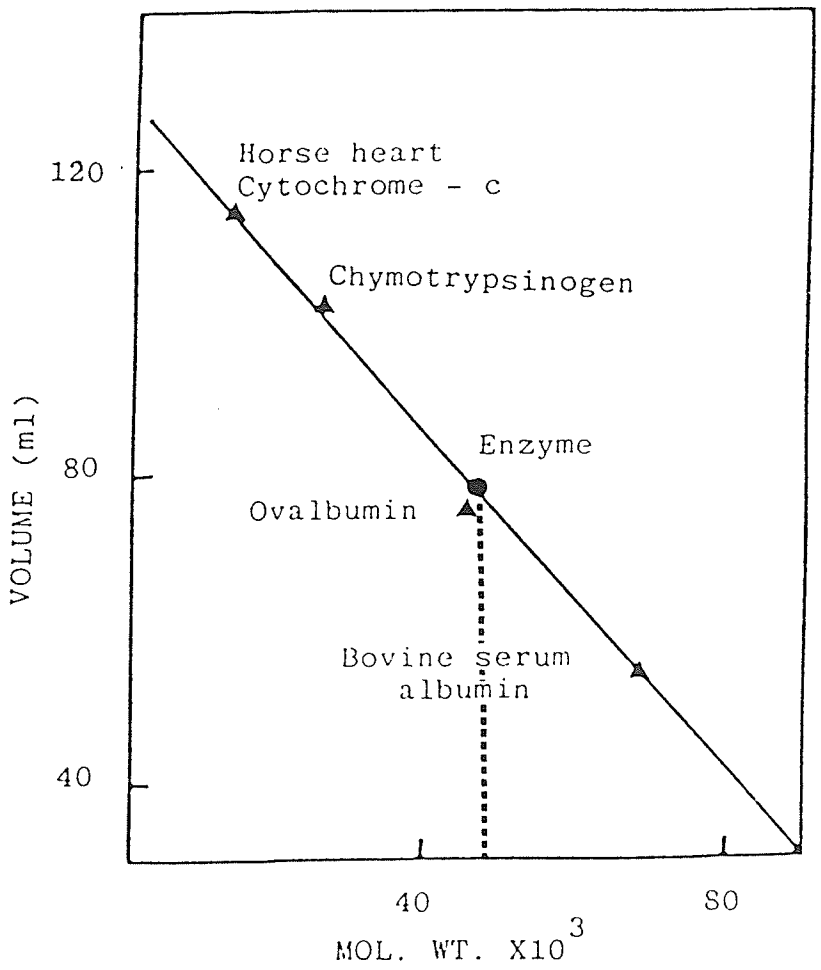

Horse heart
Cytochrome - c
Chymotrypsinogen
Enzyme
Ovalbumin
Bovine serum
albumin
120
80
40
VOLUME (ml)
40
80
MOL. WT. X10 3

Fig. 8. Measurement of sub-unit molecular weight of purified enzyme by SDS-polyacrylamide gel electrophoresis.

SDS-polyacrylamide gel electrophoresis under dissociating conditions was performed to measure the sub-unit molecular weight of purified enzyme as described in Materials and Methods. The marker proteins used were bovine serum albumin (Mol. wt. 68,000), catalase (Mol. wt. 58,000), ovalumin (Mol. wt. 43,000), G-3PD (Mol. wt. 36,000), carbonic anhydrase (Mol. wt. 29,000) and lysozyme (Mol. wt. 14,300).

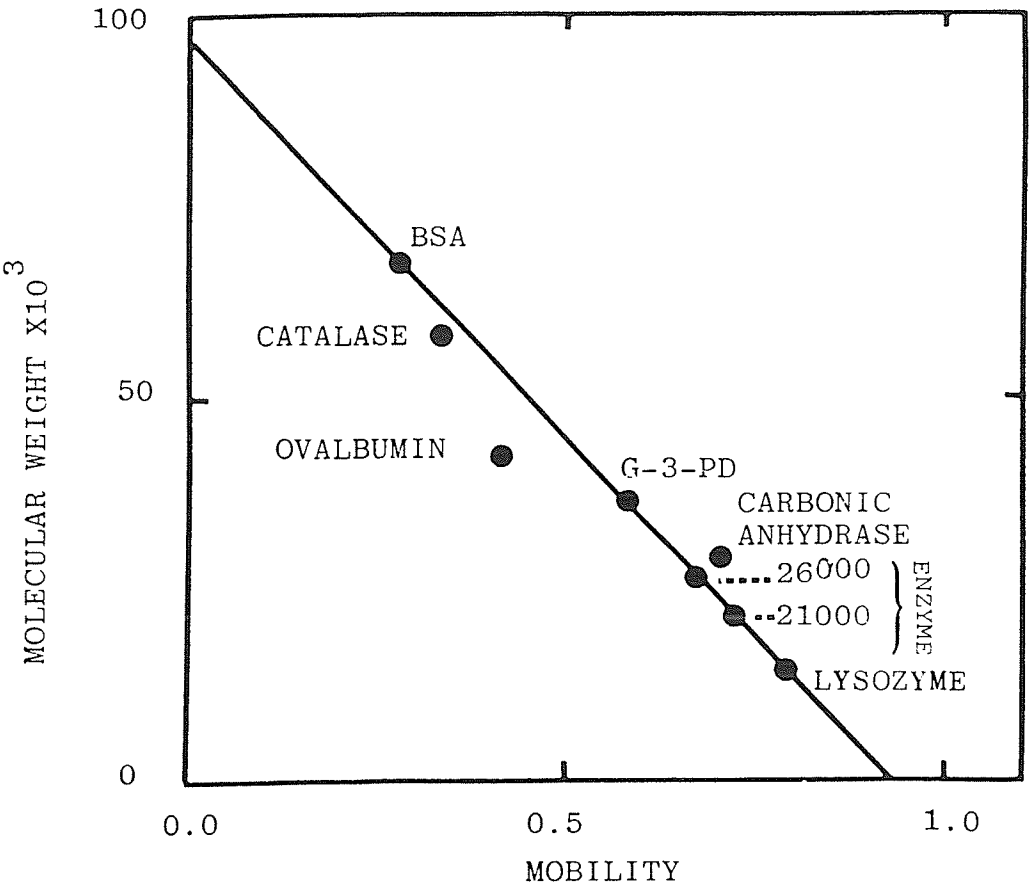

100
50
0
MOLECULAR WEIGHT X10^3
BSA
CATALASE
OVALBUMIN
G-3-PD
CARBONIC
ANHYDRASE
26000
21000
ENZYME
LYSOZYME
0.0
0.5
1.0
MOBILITY

Table 3. Determination of iron, sulfide and flavin content of the purified enzyme.

Protein (mg) (n moles)	Ferrous Iron (n moles)	Total Iron (n moles)	Sulfide (n moles)	Protein:Total:Sulfide Iron
1.0				
0.217	0.715	0.212	0.180	1 : 0.97 : 0.83

Fig. 9. Absorption spectra of riboflavin and boiled extracts of 0.3 M Tris-Cl (pH 7.5) DEAE-cellulose fraction and purified sulfur-oxidizing enzyme.

a - 0.3 M Tris-Cl (pH 7.5) DEAE-cellulose fraction

b - riboflavin (10 μM)

c - purified sulfur-oxidizing enzyme

The boiled extracts were obtained from 1.0 ml each of 0.3 M DEAE-cellulose fraction (6 mg protein) and purified sulfur-oxidizing enzyme (1.0 mg protein) as described in Materials and Methods. The specta were recorded using a Beckman Du-8 spectrophotometer.

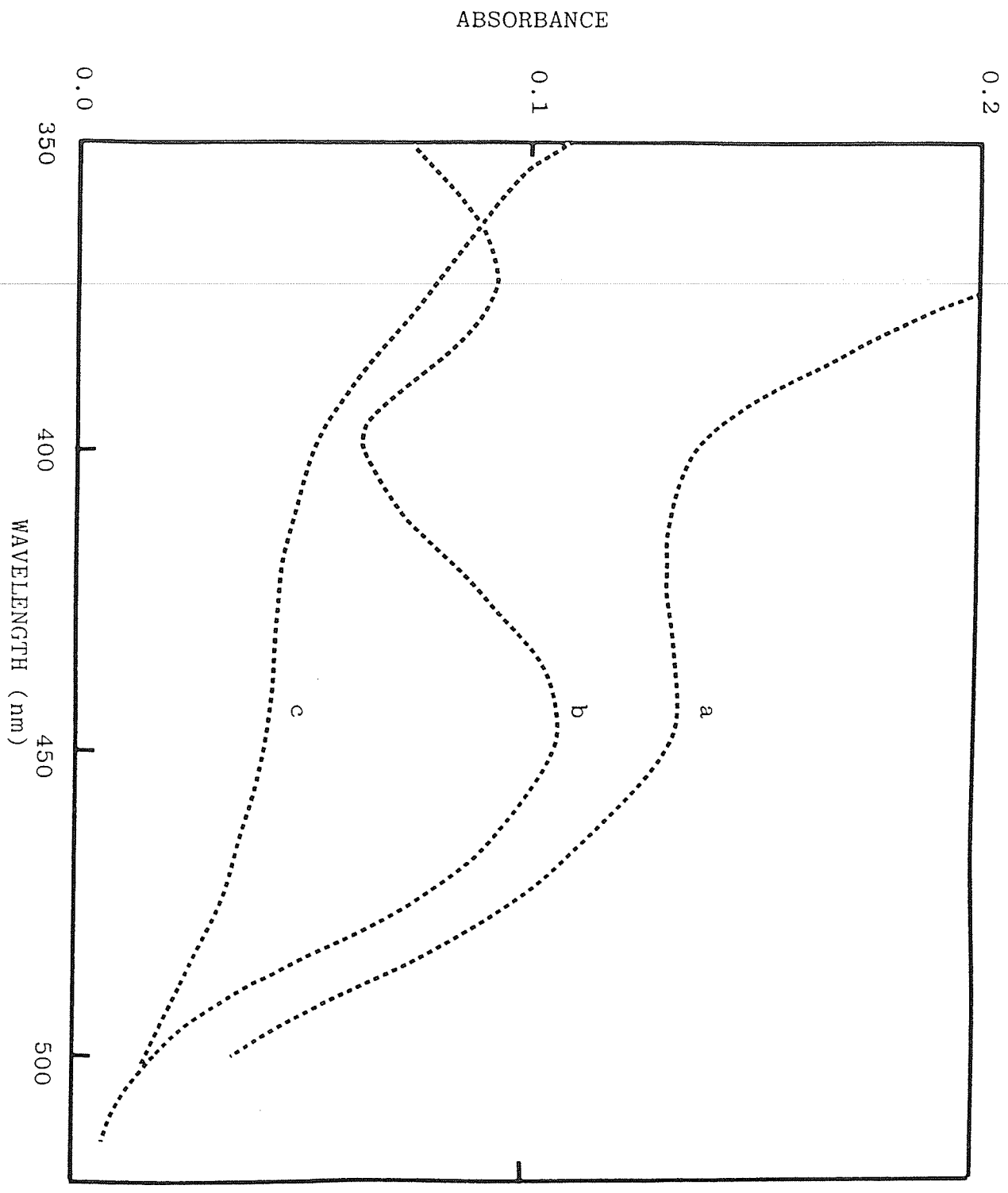

ABSORBANCE
0.0
0.1
0.2
350
400
450
500
WAVELENGTH (nm)
a
b
c

Fig. 10. Identification of flavin component by thin layer chromatography.

Thin layer chromatography was performed on silica gel coated plates as described in Materials and Methods. Rf values of the uv absorbing spots are shown on the figure. AMP, ADP, FMN, FAD, riboflavin and 0.3 M DEAE-cellulose fraction (5 µl of boiled supernatant) were used as marker with purified sulfur-oxidizing enzyme (20 µl of boiled supernatant). Yellow color of the spots is indicated as shaded area.

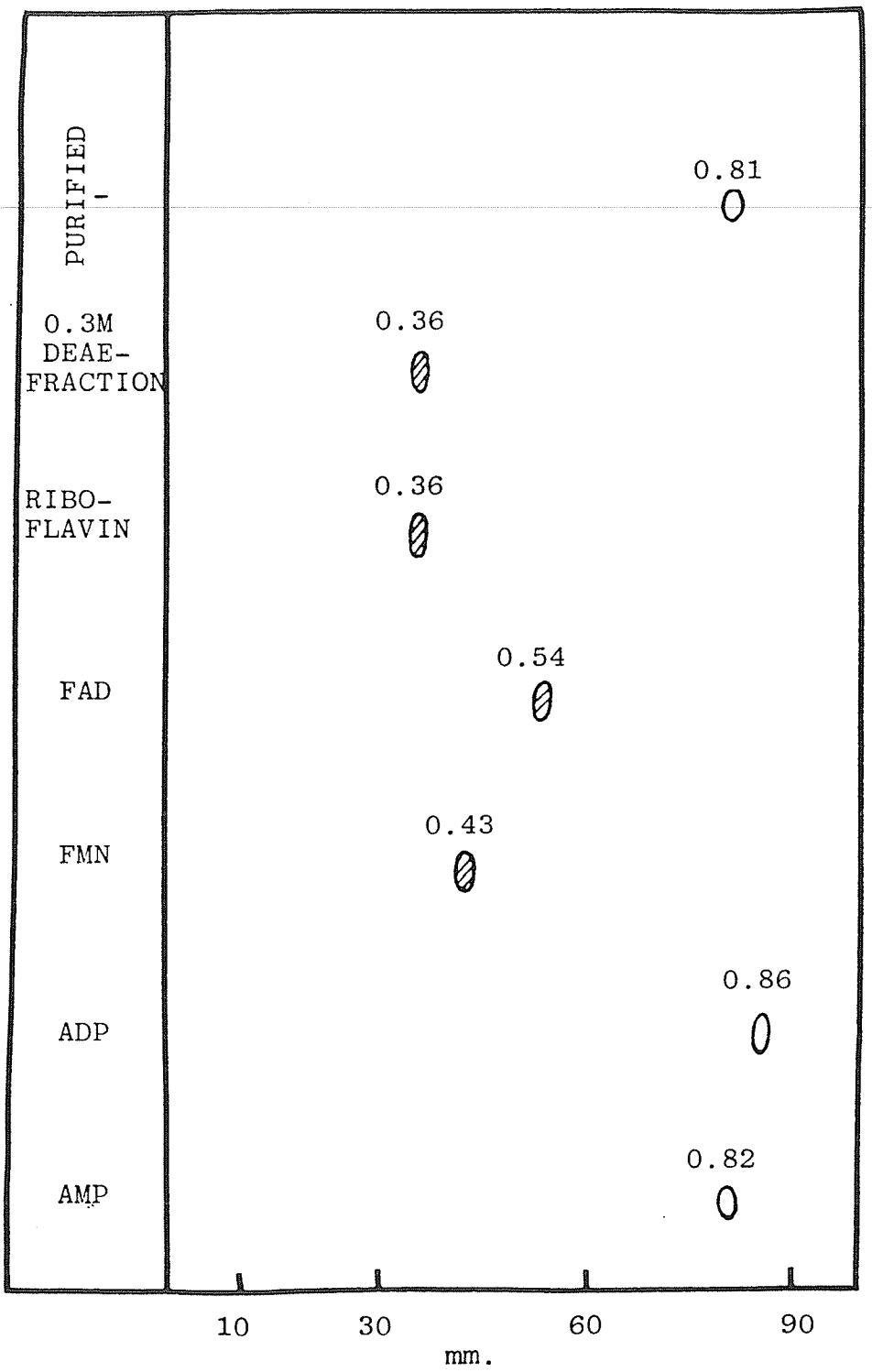
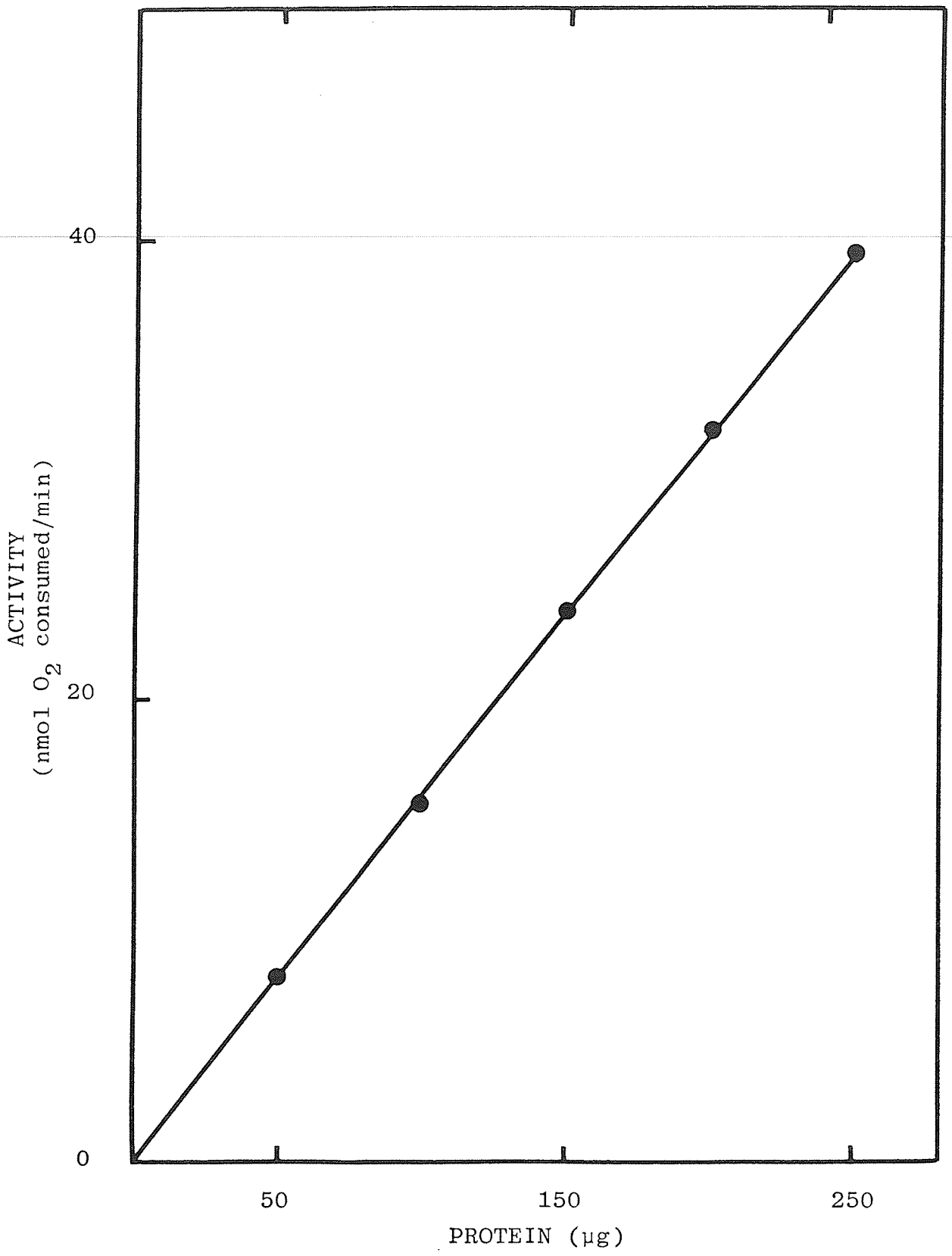

PURIFIED -
0.81
0.3M DEAE-FRACTION
0.36
RIBO-FLAVIN
0.36
FAD
0.54
FMN
0.43
ADP
0.86
AMP
0.82
10
30
60
90
mm.

Fig. 11. Sulfur-oxidizing activity of the enzyme as a function of protein concentration.

For estimating sulfur-oxidizing activity of the enzyme, DEAE-cellulose level purified enzyme was used. Protein concentration in assay mixture was varied from 50-250 ug.


40
20
0
ACTIVITY
(nmol O_2 consumed/min)
50
150
250
PROTEIN (µg)

in Figs. 9 and 10, no flavin was extracted from the purified enzyme, but riboflavin was extracted from the 0.3 M DEAE-cellulose fraction.

Iso-electric point. Analytical electrofocussing in poly-acrylamide gel was done with electrophoresis cells. The isoelectric point of purified enzyme was found to be pH 3.5. It showed a major strong band and a minor band after staining.

Enzyme storage and stability. Studies on storage and stability of enzyme revealed that the purified enzyme could be stored at -20^{o}C for a few weeks but a longer storage resulted in loss of activity. Repeated freezing and thawing caused a considerable loss of activity. Boiling the enzyme for one minute destroyed the activity.

Enzyme action. Sulfur-oxidizing activity of DEAE-cellulose level purified enzyme (0.2 M Tris-Cl eluate concentrated) was measured by increasing protein concentration in reaction mixture as shown in Fig. 11. It was found that there was a linear increase in enzyme activity with the increasing protein concentration in the reaction mixture.

The effect of pH (50 mM potassium phosphate buffer) on the sulfur-oxidizing activity of the purified enzyme is shown in Fig. 12. The enzyme had a broad optimal pH range from pH 7.5 to 10. The maximum activity of the enzyme was at 7.5 and this pH was routinely used to assay the enzyme.

The GSH concentration effect on sulfur-oxidizing activity

Fig. 12. Effect of pH on the sulfur-oxidizing activity of the enzyme.

Activity of the sulfur-oxidizing enzyme was determined as described in Materials and Methods. The reaction mixture contained in a total volume of 1.2 ml: 60 μ moles potassium phosphate, 0.5 mg sulfur, 10.0 μ moles GSH and 20 μg purified enzyme protein. The pH of potassium phosphate buffer was varied as indicated. Enzymatic rate (—●—●—) and non-enzymatic rate (—◆—◆—). The pH of potassium phosphate buffer adjusted beyond pH 8.0 (Bates and Bower, Analyt. Chem., 1322, 1956).

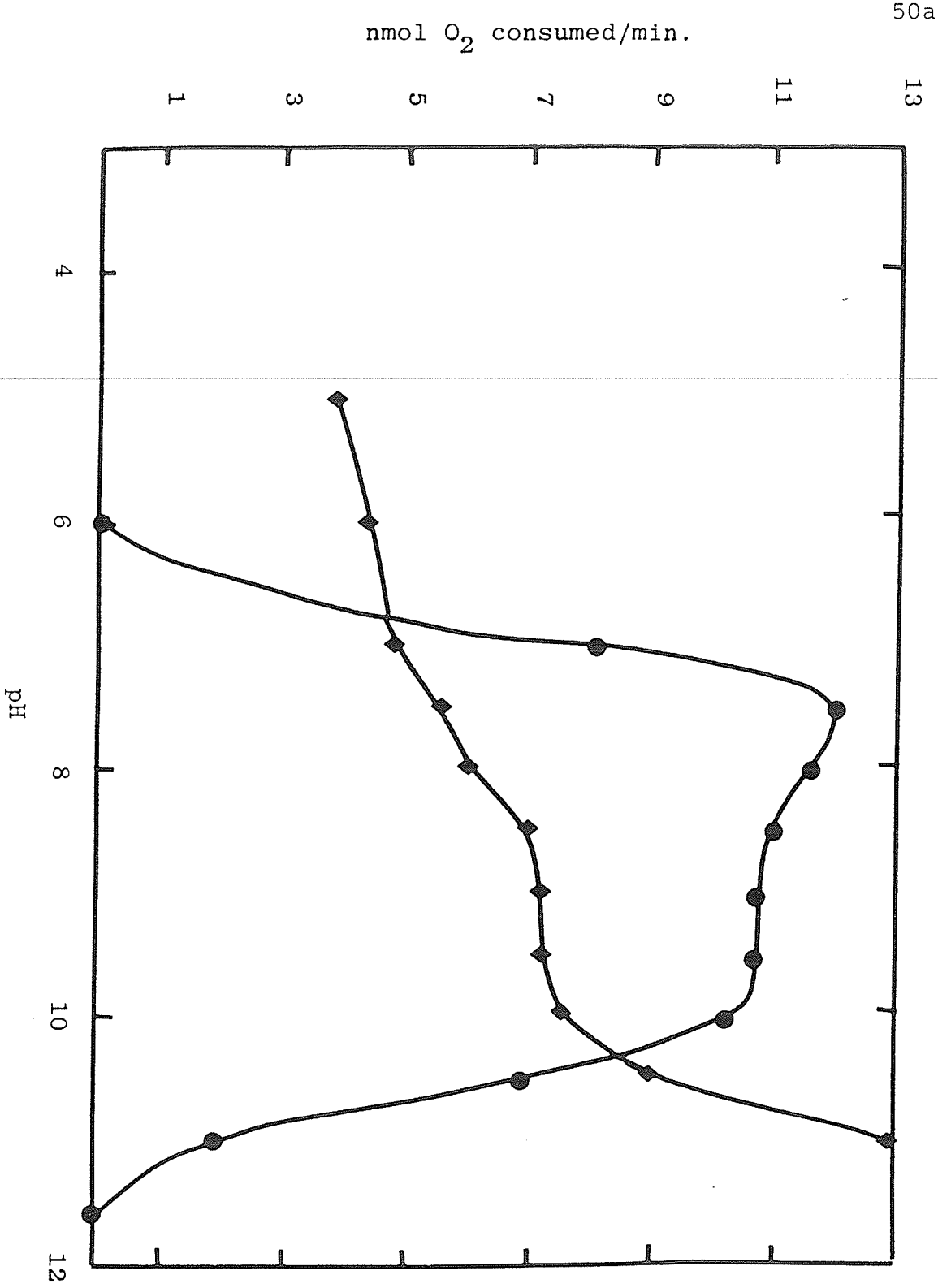

nmol O_2 consumed/min.
1
3
5
7
9
11
13
pH
4
6
8
10
12

Fig. 13. Effect of GSH concentration on the sulfur-oxidizing activity of the enzyme.

Sulfur-oxidizing activity was assayed as described in Materials and Methods, using 20 μg of purified enzyme and 0.5 mg sulfur.

The amount of GSH was varied as indicated.

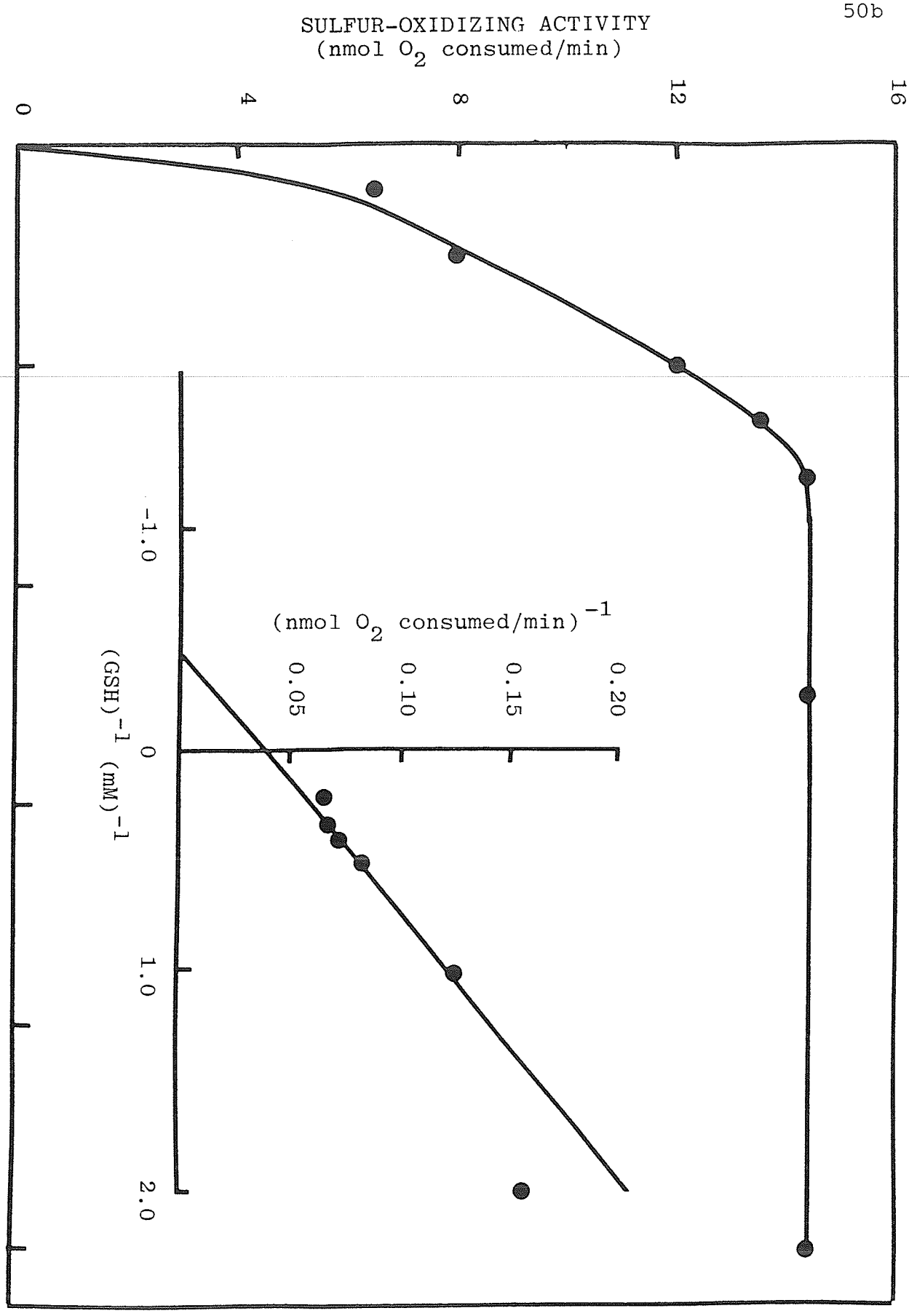
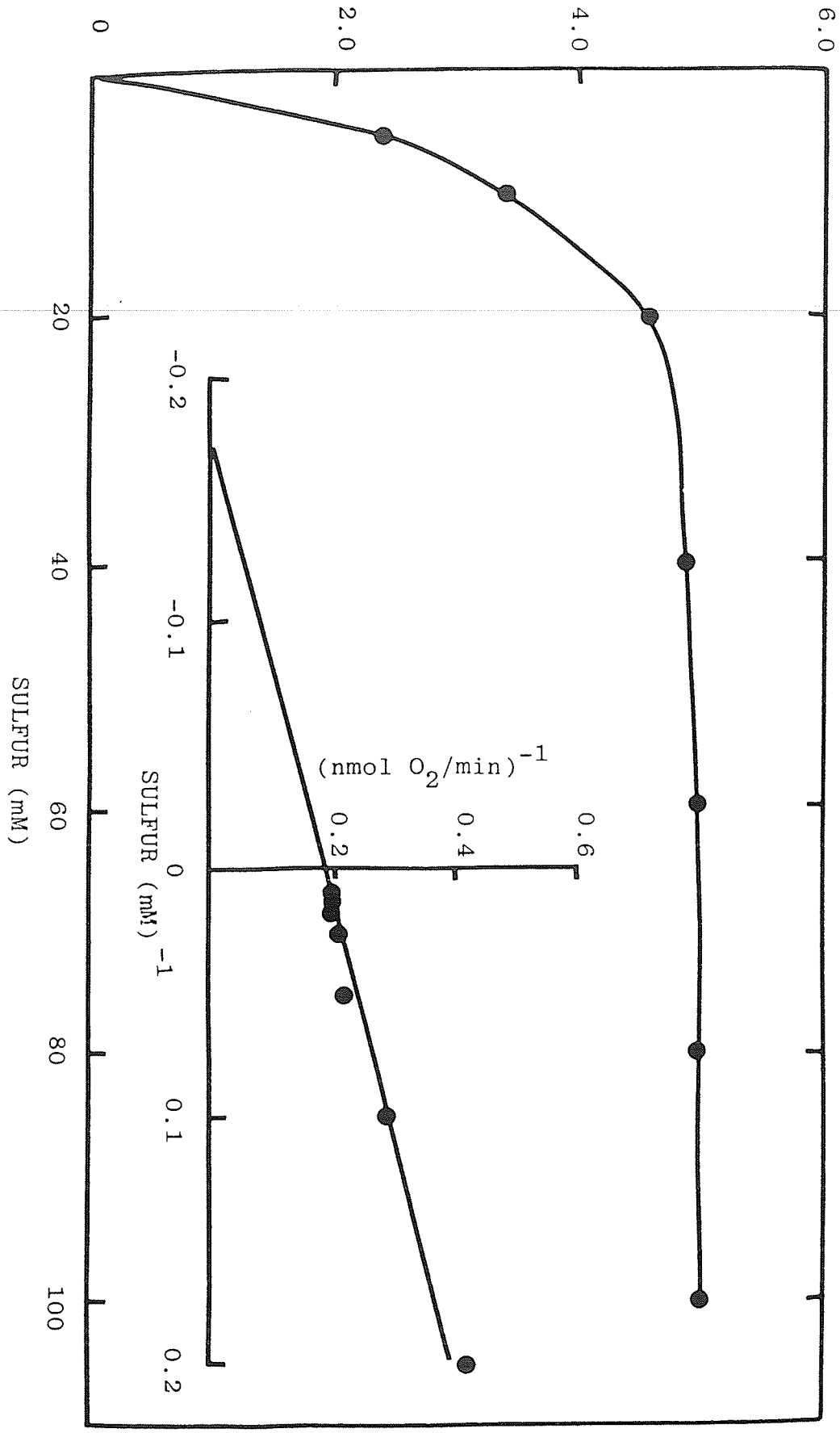

SULFUR-OXIDIZING ACTIVITY
(nmol O_2 consumed/min)
0
4
8
12
16
2
4
6
8
10
(nmol O_2 consumed/min)$^{-1}$
0.05
0.10
0.15
0.20
$(GSH)^{-1}$ $(mM)^{-1}$
-1.0
0
1.0
2.0

Fig. 14. Effect of sulfur concentration on sulfur-oxidizing activity of the purified enzyme.

Activity was measured as described in Materials and Methods, using 20 μg of purified enzyme, 10 μ moles GSH and varied concentration of sulfur as indicated.


ACTIVITY (nmol O_2 consumed/min)
0
2.0
4.0
6.0
SULFUR (mM)
20
40
60
80
100
$(nmol\ O_2/min)^{-1}$
-0.2
-0.1
0
0.2
0.4
0.6
SULFUR (mM) $^{-1}$
-0.2
-0.1
0
0.1
0.2

of purified enzyme is shown in Fig. 13. The concentration of GSH was varied from 0.05 mM to 10 mM. There was an increase in the enzyme activity with increasing GSH concentration before levelling off. The Km value was estimated as 2 mM.

The effect of sulfur concentration on the sulfur-oxidizing activity of the enzyme is shown in Fig. 14. The concentration of sulfur was varied from 5 mM to 100 mM. The apparent Km value was estimated as 5.7 mM (0.22 mg/1.2 ml).

Substrate specificity of the enzyme. The purified enzyme oxidized sulfur only in the presence of catalytic amounts of glutathione and did not have any sulfide, sulfite or thiosulfate oxidizing activity.

The rate of oxygen uptake with sulfur by the cell-free extract or purified enzyme was measured with other potential cofactors such as GSSG, ascorbate, sulfite, BAL, cysteine, dithiothreitol and dithioerythritol (Table 4). GSH had the highest net enzymatic activity of all these cofactors and was routinely used to measure the enzyme activity.

Rhodanase activity. Rhodanase activity of cell-free extract and purified enzyme was measured to compare with the sulfur and sulfite oxidizing activities of the enzyme (Table 5). It was found that there was no rhodanase and sulfite oxidizing activity in the purified enzyme preparation with a sulfur-oxidizing activity. Cell-free extracts had all of these activities.

Stoichiometry of reaction. The sulfur-oxidizing enzyme is expected to produce 1 mole of thiosulfate for every

Table 4. Cofactor requirement of the sulfur-oxidizing enzyme

Cofactor	Rate of oxygen uptake (n moles O_2 consumed/minute		
	Sulfur + Cofactor without enzyme	Sulfur + Cofactor with cell-free extracts	Sulfur + Cofactor with purified enzyme
GSH	6.0	22.0 *(16.0)	21.0 *(15.0)
GSSG	0.0	0.0 (0.0)	0.0 (0.0)
Ascorbate	3.0	3.0 (0.0)	2.0 (0.0)
Sulfite	0.0	6.0 (6.0)	0.0 (0.0)
BAL	31.0	70.0 (24.0)	39.0 (8.0)
Cysteine	10.0	**(i)34.0 (24.0) **(ii)10.0 (0.0)	9.0 (0.0)
Dithiothreitol	11.6	36.0 (25.2)	18.0 (6.4)
Dithioerythritol	11.0	42.0 (31.0)	15.0 (4.0)

Rate of oxygen uptake with potential cofactors was measured without enzyme, with cell-free extracts and purified enzyme as described in Materials and Methods with 0.5 mg sulfur. Ten μ moles of cofactor were used per assay except for sodium sulfite and BAL, where 0.1 and 1.0 μ mole were used respectively. Each assay for cell-free extract contained 1.84 mg protein and for purified enzyme contained 25 μg protein.

* Net enzymatic activity.

** (i) Initial fast phase.

(ii) Second slow phase.

Table. 5 Comparison of rhodanase activity to sulfur and sulfite-oxidizing activities

System	Sulfur-oxidizing activity (n moles O_2/min)	Sulfite-oxidizing activity (n moles O_2/min)	Rhodanase activity (n moles SCN^-/min)
Cell-free extract	30.0	6.4	510.0
Purified enzyme	11.0	0.0	0.0

For cell-free extracts 1.84 mg protein was used per assay.

For purified enzyme 15 µg enzyme protein was used per assay.

mole of oxygen consumed. Table 6 shows that the cell-free extract oxidized sulfur producing nearly stoichiometric amounts of thiosulfate. The purified enzyme preparation produced a slightly lower stoichiometry for thiosulfate possibly because of its instability during the long incubation period.

Table 6. Relationship of oxygen consumption to thiosulfate formation during sulfur oxidation

Preparation	Incubation time (min.)	O_2 (μ moles)	$S_2O_3^{2-}$ (μ moles)
Cell-free extract	90	6.8	6.0
Purified enzyme	180	8.2	5.5

O_2 consumption was measured manometrically at 30°C in Warburg apparatus. Reaction mixture contained in a total volume of 3.2 ml: 600 μ moles Tris-Cl (pH 7.5), 48 mg sulfur, 3.3 mg catalase, 0.4 μ mole 2,2' dipyridyl, 5 μ moles GSH, enzyme (9 mg cell-free extract or 1.0 mg purified enzyme protein) and water. The reaction was stopped by adding 0.1 ml of 1.0 M cadmium acetate solution and thiosulfate was determined as described in Materials and Methods.

DISCUSSION

DISCUSSION

Earlier studies directed at investigating the metabolism of sulfur by Thiobacillus thiooxidans have been carried out with intact cells, cell-free extracts and partially purified enzyme preparations. However, to understand the intermediate steps of sulfur metabolism, it is important to start with a purified preparation of the enzyme. Therefore, in the present investigation an attempt has been made to purify and characterize the enzyme involved in the metabolism of sulfur in T. thiooxidans. Sulfur metabolism was studied by measuring the oxygen uptake during sulfur oxidation using a Clark oxygen electrode attached to a Gilson oxygraph. This method was preferred to Warburg method because it was found to be more convenient, rapid, reliable and sensitive.

T. thiooxidans grown in Starkey's medium supplemented with molybdate (0.75 mg/liter) produced more cells and higher recovery of the enzyme during purification. Therefore, cells were routinely grown in the presence of molybdate. This finding on the effect of molybdate on growth is in accordance with the report of Takakuwa et al (1977).

Active cell-free extracts were prepared by aerobic sonication of whole cells. Prior to sonication cells were treated with trypsin to facilitate the release of sulfur-oxidizing activity in the soluble form upon subsequent sonication. On the other hand, glucosidase or detergent was unsuccesful in releasing the sulfur-oxidizing activity in a soluble form as has been mentioned by Bhella (1981).

It has been reported that T. thiooxidans cell wall lacks a typical peptidoglycan layer and is mostly composed of protein (Marunouchi and Mori, 1968; Noguchi et al, 1977). Mild trypsin treatment probably disintegrates the protein layers, making the cells vulnerable to sonication. Thus, sonication releases the sulfur-oxidizing enzyme from partially disintegrated cell envelope.

The oxidized and reduced spectra of cell-free extracts indicated the presence of cytochromes a , b, and c and also flavin, whereas the spectra of 105,000 x g supernatant suggested the presence of a c type cytochrome and a flavin. These results show that cytochromes a and b being membrane bound settled down in the precipitate. The spectra of enzyme preparations following acidic treatment and DEAE-cellulose chromatography remained similar to that of the 105,000 x g supernatant. Suzuki (1965) reported that his purified sulfur-oxidizing enzyme was uncontaminated by cytochromes. Further concentration on DEAE-cellulose and column chromatography using Sephadex G-100 of the enzyme indicated that the sulfur-oxidizing activity was mainly in the clear fluid devoid of any flavin moiety. This was confirmed by comparing spectra of the purified enzyme preparation, the 0.3 M Tris-Cl DEAE-cellulose eluant and riboflavin (Figs. 5, 6 and 9). In addition, thin layer chromatography of the preparation using markers such as FMN, FAD, ADP and AMP also indicated that purified enzyme contained no flavin, wheras 0.3 M Tris-Cl DEAE-cellulose eluant contained riboflavin. This purified enzyme preparation

was used in further studies to investigate the characteristics.

The purity of sulfur-oxidizing enzyme was studied using discpolyacrylamide gel electrophoresis under non-dissociating conditions. This revealed one major and six minor bands, and from the intensity of the bands the enzyme was estimated to be 80% pure. (The enzyme constituted about 2 percent of the total cell protein). Molecular weight of the enzyme as determined by gel filtration was 46,000 $\pm$ 5%. The SDS-electrophoresis revealed that the enzyme was made up of two polypeptide chains with molecular weights of 21,000 and 26,000. The observed spectral shift in the shoulder from 410 nm to 420 nm upon reduction suggested the presence of a small amount (less than 1% in protein) of a c-type cytochrome. The difference spectrum is characteristic of a non-heme iron protein (iron-sulfur protein) but not of a flavoprotein.

This enzyme was found to be a non-heme iron protein, containing non-heme iron, labile sulfide and protein in 1:1:1 ratio. It was of interest to note that no flavin as such was extracted from the purified enzyme. The iso-electric point of purified enzyme was found to be pH 3.5, indicating the acidic nature of the enzyme, The apparent Km values for sulfur and GSH were found to be 5.7 mM and 2 mM respectively. Similar Km values for GSH have been reported by Suzuki (1965).

The spectral properties of this enzyme have some resemblance to those of component B of Takakuwa (1975)

who reported this component to be a flavoprotein containing non-heme iron and showing an absorption shoulder around 485 nm. However, in the spectrum analysis of Takakuwa's report no flavin was identified and no evidence was given for the requirement or participation of any flavin in sulfur oxidation.

The activity of the enzyme was found to be maximal at pH 7.5. GSH from exogenous sources was required for the enzymatic activity and there was a linear increase in the enzyme activity with increasing GSH concentration before levelling off. Among other potential cofactors tested such as GSSG, ascorbate, sulfide, BAL, cysteine, dithiothreitol and dithioerythritol, GSH had the highest effect on the net enzymatic activity. Also, comparison of rhodanase activity and sulfur and sulfite oxidizing activities revealed that the sulfur-oxidizing activity was the only activity associated with the purified preparation of the enzyme.

The purified enzyme preparation produced thiosulfate from sulfur in the presence of GSH with the thiosulfate: O_2 ratio of around 0.7. The stoichiometry expected from the equations:

$S + O_2 + H_2O \longrightarrow H_2SO_3$ and $H_2SO_3 + S \longrightarrow H_2S_2O_3$ is the ratio of 1. Considering the instability of the purified enzyme the results are considered to be in agreement with the theoretical stoichiometry.

In conclusion, the sulfur-oxidizing enzyme of T. thiooxidans was found to be a non-flavoprotein containing non-heme iron

and labile sulfide. Flavin as such does not play any role in the enzymatic reaction of this enzyme.

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