PURIFICATION AND CHARACTERIZATION OF SULFUR-OXIDISING ENZYME OF *Thiobacillus thiooxidans*

By

RESHAM S. BHELLA

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ABSTRACT

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Sulfur metabolism of Thiobacillus thiooxidans was studied with specific emphasis on the purification and characterization of sulfur-oxidising enzyme. A mild trypsin treatment of the cells prior to sonication was found to be a very effective method for solubilizing the sulfur-oxidising activity. The purified enzyme had a bright yellow color and a molecular weight of about 40,000. It showed absorption peaks at 272nm, 410nm, 448nm and a shoulder at 478nm. On reduction with dithionite, absorption peak at 448nm and a shoulder at 478nm disappeared and 410nm peak diminished to some extent. Enzyme was found to be flavoprotein containing flavin, non-heme iron and labile sulfide in 1:1:1:1 molar ratio with protein. Flavin was tentatively identified as riboflavin along with AMP. Riboflavin and FMN but not FAD were shown to stimulate the catalytic activity of flavin-free sulfur-oxidising enzyme. Reconstitution of inactive flavin free enzyme with riboflavin and AMP was also attempted to provide further evidence for the participation of flavin in sulfur oxidation.

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ABBREVIATIONS

ADP - adenosine-5'-diphosphate

AMP - adenosine-5'-monophosphate

ATP - adenosine-5'-triphosphate

DEAE - diethylaminoethyl

FAD - flavin adenine dinucleotide

FMN - flavin mononucleotide

GSH - reduced glutathione

GSSG - oxidised glutathione

NAD, NADH - oxidised or reduced nicotinamide adenine dinucleotide

NADP, NADPH - oxidised or reduced nicotinamide adenine dinucleotide phosphate

Tris - Tris (hydroxymethyl) aminomethane

INTRODUCTION

INTRODUCTION

The thiobacilli are chemoautotrophic microorganisms that derive energy and reducing power for their growth from the oxidation of inorganic sulfur compounds and assimilate atmospheric carbon dioxide to synthesize cellular carbon. Depending upon the degree by which organic compounds supplement the nutrition, these sulfur bacteria can be classified as obligate, facultative or mixotrophic chemoautotrophs.

Thiobacillus thiooxidans, an obligate chemoautotrophic bacterium, can utilize various reduced or partially reduced sulfur compounds including elemental sulfur, sulfide, thiosulphate, polythionates and sulfite, although sulfur seems to be the preferred substrate for optimal growth. Its ability to withstand extremely acidic conditions (lower than ph 1.0), distinguishes this organism from other thiobacilli.

In spite of the simplicity of the nutritional requirements. T. thiooxidans is known to be a very complex and sophisticated microorganism, physiologically and biochemically. Sulfur metabolism of this organism has been a focus of great deal of attention for the past several years. Based on the studies with intact cells, cell free extracts or purified individual enzymes, only a basic outline of the mechanism of sulfur oxidation has been elucidated. There have been various divergent reports regarding the sulfur oxidising system. In this present study, an attempt was made to purify and characterize the sulfur-oxidising enzyme, a key enzyme in sulfur metabolism, with an aim to better understand and clarify the nature and characteristics of sulfur oxidation in general and sulfur-oxidising enzyme in particular.

HISTORICAL

HISTORICAL

Thiobacillus thiooxidans, first discovered by Waksman and Joffe (1922), is an obligate chemoautotrophic organism which derives its energy and reducing power for growth from the oxidation of inorganic sulfur compounds. It is one of the most acidostable organisms since the bacterium, although the optimum pH for growth is near 3.5, can metabolize elementary sulfur at pH values less than 1 and even survive pH values close to 0 (Vishniac and Santer, 1957). Its ability to withstand extreme acidic conditions distinguishes this organism from the other thiobacilli. Immediately following the discovery and isolation of this organism, intensive investigations were carried out on the general physiology and sulfur metabolism (Joffe,1922: Waksman, 1922; Waksman et al, 1923; Waksman and Strakey, 1922, 1923; Lipman, 1923; Starkey, 1925). The current status of these biochemical aspects has been thoroughly reviewed (Lees, 1955, 1960; Peck, 1962, 1968; Trudinger, 1967, 1969; Kelly, 1968; Roy and Trudinger, 1970; Suzuki, 1974; Aleem, 1975; Oh and Suzuki, 1980). In spite of intensive investigation, many aspects of sulfur metabolism of this organism continue to be vaguely understood.

(1) Sulfur Oxidation

Elemental sulfur is in key position in the oxidations of various other reduced sulfur compounds and to understand the mechanism of sulfur oxidation, sulfur-oxidising system of T. thiooxidans has been subject of most intensive studies. The early work with intact cells established that elemental sulfur is oxidised aerobically to sulfuric acid as follows (Waksman, 1922; Waksman and Starkey, 1923; Starkey, 1925; Parker and Prisk, 1953):

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

However this transformation does take place in several steps. One suggested pathway of sulfur oxidation proposed the formation of thiosulfate and polythionates as intermediates (Vishniac and Santer, 1957). Initially, investigation by Suzuki and Werkman (1959) seemed to support this pathway. Cell-free extracts, prepared by Raytheon oscillation, were capable of oxidising sulfur upon the addition of substrate quantities of reduced glutathione. Polythionates and thiosulfate were detected to be the products. On the basis of production of hydrogen sulfide from sulfur by T. thiooxidans (Starkey, 1937) and inhibition of sulfur oxidation by whole cells on addition of thiol-binding reagents (Vogler, 1942; Vogler et al; 1942), Suzuki and Werkman (1959) proposed that sulfur was initially reduced to sulfide by the following non-enzymatic reaction:

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

GSH (reduced glutathione) can be regenerated by the glutathione reductase present in the extracts (Suzuki and Werkman, 1960). However later on Suzuki and Lees (1964) prepared T. thiooxidans extracts which required only catalytic quantities of GSH for sulfur oxidation. The partially purified sulfur-oxidising enzyme was found to be devoid of glutathione reductase and sulfide oxidising activity (Suzuki, 1965), an indication that formation of free sulfide did not occur during sulfur oxidation. Thiosulfate was found to be the end product of the reaction. Subsequently, the mechanism of sulfur oxidation was revised to include glutathione polysulfide as an intermediate (Suzuki, 1965). 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 oxidised by the sulfur-oxidising enzyme as follows:

$$S_n + GSH \longrightarrow GSS_nH$$

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

Later, it was found out that the actual product of the sulfur oxidation was sulfite rather than thiosulfate (Suzuki and Silver, 1966). Thiosulfate was produced from the non-enzymatic condensation of sulfur with sulfite as follows:

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

$$SO_3^{2-} + S \longrightarrow S_2O_3^{2-}$$

The sulfur-oxidising enzyme has been tentatively identified as non-heme iron containing oxygenase (Suzuki, 1965; Suzuki and Silver, 1966).

Besides the above-mentioned sulfur-oxidising system requiring catalytic amounts of GSH, two other sulfur-oxidising systems have been prepared from cell-free extracts of T. thiooxidans.

(a) A large cell wall-membrane complex which catalyses the oxidation of elemental sulfur (presumably to sulfate) without the addition of GSH (Adair, 1966; Taylor, 1968). The ability of thiol-binding agents to block this sulfur oxidation indicates the presence of endogenous sulfhydryl groups.

(b) The sulfur-oxidising system which catalyses the oxidation of elemental sulfur to sulfate and requires both soluble and membrane fractions (Kodama and Mori, 1968; Kodama, 1964).

The soluble fraction was further separated into collodion membrane-

permeable and -impermeable components. In reconstitution of the sulfur-oxidising system, the function of the former component was shown to be replaceable by NAD or NADP, but not by cysteine or reduced glutathione (Kodama, 1964). Additional purification and characterization of the soluble system was performed by Takakuwa (1975). It 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 diethyldithiorcarbamate treatment decreased the enzyme activity, showing that non-heme iron was essential to sulfur oxidation. Inhibition by certain known inhibitors of flavoprotein was taken to indicate the involvement of flavin as well in sulfur oxidation.

Sulfur-oxidising enzymes have also been isolated and characterized from T. thioparus (Suzuki and Silver, 1966), T. novellus (Charles and Suzuki, 1966) and T. ferrooxidans (Silver and Lundgren, 1968).

Despite intensive investigation of the mechanism of sulfur oxidation, very little information is available in regard to the mechanism of attack on the elemental sulfur particles by bacterial cells and the mobilization of this insoluble substrate to the essential enzyme systems. Umbreit et al (1942) reported that direct contact between terminal fat globules of bacterial cells and sulfur particles was necessary in order to dissolve the elemental sulfur for oxidation. However they were unable to confirm the fat globules by microscopic studies and later Knaysi (1943) demonstrated that so-called "fat globules" actually consisted of volutin and sulfur.

Another concept is that both the attachment of the organisms to sulfur and solubilization of the sulfur particles by phospholipids and other extracellular compounds released by the cells are necessary for the oxidation (Schaeffer and Umbreit, 1963 and Jones and Benson, 1965; Shively

and Benson, 1967; Roy and Trudinger, 1970). There is some experimental evidence for this theory that cells have been photographed clustered around eroded sulfur particles and a brief stationary phase after inoculation of a culture appears to be necessary for the production of phospholipids or other cellular materials required for adhesion (Cook, 1964).

Recently Takakuwa and his co-workers (1979) have done extensive studies on cell-sulfur adhesion and have reported that cell-sulfur adhesion is inhibited by thiol-binding reagents indicating that thiol groups which may be present in the cell envelope, are essential for cell-sulfur adhesion process. Both the adhesion process and oxygen uptake activity were shown to be significantly inhibited by some heavy metal chelators, electron transport chain inhibitors and anoxia indicating that adhesion process must be energy dependent.

As a whole, our present knowledge on the oxidation of sulfur seems to indicate that thiol groups on the bacterial cell envelope form polysulfide complex with the sulfur before its oxidation to sulfate through sulfite as an intermediate. Sulfur-oxidising enzyme is probably located on or near the cell envelope in the vicinity of thiol groups. Different sulfur-oxidising systems reported by various investigators are simply caused by the presence or absence of membranous thiol groups in the preparations.

(2) Sulfite Oxidation

Sulfite is a key intermediate in the oxidation of all inorganic sulfur compounds. Two different pathways, namely APS reductase (adenosine-phosphosulphate reductase) and sulfite oxidase (sulfite: cytochrome c oxidoreductase), are basically functional in the oxidation of sulfite.

APS reductase pathway was first proposed by Peck (1960, 1962). Based on his studies with T. thioparus, he 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:

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

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

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

APS reductases have been purified from Desulfovibrio vulgaris (Peck, et al, 1965), T. denitrificans (Brown et al, 1966), T. thioparus (Lyric and Suzuki, 1970) and Thicocapsa roseopersicina (Trüper and Rogers, 1971).

The finding of a sulfite : cytochrome c oxidoreductase in T. novellus distinct from the APS reductase has led to the proposal that an AMP independent system, which neither needs AMP nor produces APS, functions as an additional mechanism of sulfite oxidation (Charles and Suzuki, 1965, 1966). The purified enzyme oxidises sulfite to sulfate with a concomitant reduction of cytochrome c:

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

The reduced cytochrome is then oxidised with molecular oxygen by cytochrome oxidase (cytochrome c : O_2 oxidoreductase):

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

Sulfite oxidase (sulfite : cytochrome c oxidoreductase) 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-oxidising complex of T. novellus (Oh and Suzuki, 1977). An unusual sulfite-oxidising enzyme from T. neopolitanus has been isolated and characterised (Hempfling et al, 1967). 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.

Sulfite oxidation by T. thiooxidans is at the preliminary stage of investigation. Presence of APS reductase pathway in this organism is controversial at present. Peck (1961, 1962) has reported its presence while Adair (1966) has indicated otherwise. However sulfite oxidase pathway has been detected in the membrane fractions of crude cell-free extracts (Adair, 1966; Kodama and Mori, 1968). Two different kinds of terminal oxidases have been reported in T. thiooxidans based on carbon-monoxide inhibition studies of sulfur and sulfite oxidations. Sulfur oxidation was photo-irreversibly inhibited compared to photo-reversible sulfite oxidation (Swatsuka and Mori, 1960; Kodama and Mori, 1968). However care should be used in interpreting the carbon-monoxide inhibition studies until spectrophotometric evidence of carbon-monoxide binding to terminal oxidases is available. Kodama et al (1970) and Takakuwa (1976) have also demonstrated the participation of membrane bound a, b and c type cytochromes and a flavoprotein in sulfite oxidation in T. thiooxidans.

(3) Sulfide Oxidation

Sulfide is probably oxidised to sulfate through polysulfide and sulfite

sulfide as intermediate as follows:

$$S^{2-} + 1/2\ O_2 + 2H^+ \longrightarrow [S] + H_2O$$
$$[S] + O_2 + H_2O \longrightarrow SO_3^{2-} + 2H^+$$
$$SO_3^{2-} + 1/2\ O_2 \longrightarrow SO_4^{2-}$$

where [S] represents a polysulfide-sulfur. Once sulfide is converted to polysulfide, it can be further oxidised by a mechanism similar to elemental sulfur oxidation.

Intact cells as well as cell-free extracts of T. thiooxidans, T. concretivorus and T. thioparus grown on elemental sulfur as energy source catalyse an enzymatic oxidation of sulfide (Moriarty and Nicholas, 1969, 1970) though oxidation of sulfide was considered by some workers as a non-enzymatic process (Adair, 1966). Sulfide oxidase enzyme responsible for sulfide oxidation to polysulfide level has been reported to be membrane bound in T. concretivorus and the following tentative electron transfer scheme has been presented for sulfide oxidation to polysulfide level (Moriarty and Nicholas, 1970).

$$S^{2-} \longrightarrow \text{Cu-protein} \longrightarrow \text{(Flavin?)} \longrightarrow \text{Cytochromes-}$$
$$\underline{b} \longrightarrow \underline{c} \longrightarrow \underline{d} \longrightarrow O_2$$

In T. denitrificans sulfide is oxidised by intact cells with either molecular oxygen or nitrate as the terminal electron acceptor (Peeters and Aleem, 1970). Recently Sawhney and Nicholas (1978) reported the purification of sulfide-linked nitrite reductase from T. denitrificans. 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 follow:

$$S^{2-} \longrightarrow \text{Cyt } \underline{c}\text{-551} \longrightarrow [\text{Cyt } \underline{c} \longrightarrow \text{Cyt } \underline{d}] \longrightarrow O_2 \text{ or } NO_2^{2-}$$

nitrite reductase

(4) Thiosulfate Oxidation

Thiosulfate is the preferred substrate of many thiobacilli that grow around neutral pH conditions. Both sulfur atoms of thiosulfate are normally oxidised to sulfate as follows:

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

where sulfur and sulfite are well-documented intermediates.

A great deal of attention has been focused on the elucidation of mechanism of thiosulfate oxidation and various theories have been proposed (Lees, 1960; Peck, 1962; Vishniac and Trudinger, 1962; charles and Suzuki, 1966a; Trudinger, 1967,1969; Lyric and Suzuki, 1970; Suzuki, 1974; Oh and Suzuki, 1980).

At present, very little is known about thiosulfate oxidation by T. thiooxidans. Thiosulfate-oxidising activity has been shown to be present in this organism (London and Rittenberg, 1964). The rhodanese activity, normally associated with thiosulfate oxidation (Charles and Suzuki, 1966a; Smith and Lascelles, 1966) is also found in this organism (Lukow, 1977) and probably functions in thiosulfate oxidation by a similar mechanism as proposed by Suzuki (1974). According to this mechanism, thiosulfate is first cleaved to sulfur (which would form polysulfide by reacting with indigenous sulfhydryl groups) and sulfite by thiosulfate-cleaving enzyme (rhodanese). Sulfur derived

from the sulfane group (outer position of thiosulfate) is oxidised to sulfite by sulfur-oxidising enzyme and sulfite is finally oxidised to sulfate by sulfite-oxidising system as follows:

$$S_2O_3^{2-} \longrightarrow S + SO_3^{2-}$$

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

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

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

In conclusion, we can say that our present knowledge of sulfur metabolism of T thiooxidans is very limited. Studies regarding the nature of cell surface and mechanism of acidostability of this microorganism are at a primitive stage. Although a, b, c and o 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, 1975a), other electron transport components have never been isolated and characterized. Mechanisms of energy coupling and reducing power generation remain to be unraveled. Hopefully, in future all these aspects of sulfur metabolism would be clarified.

MATERIALS AND METHODS

MATERIALS AND METHODS

Materials

All chemicals and reagents used were of analytical grade and commercially obtained. Sulfur (precipitated) was obtained from British Drug Houses Ltd., London, England. Sodium sulfide, sodium thiosulfate and sodium sulfite were the products of Fisher Scientific Company, Fairlawn, N.J., U.S.A. Catalase (liver, 2 times crystallized), GSH, GSSG, AMP, ADP, riboflavin, FMN, FAD, Cytochrome c (type III, from horse 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) were obtained from Sigma Chemical Co. St. Louis, Missouri. DEAE-cellulose was from Schleicher & Schuell Inc., Keene, New Hampshire. Silica gel coated sheets for thin layer chromatography were obtained from Brinkmann Inst. Canada Ltd., Rexdale, Ont. The gel chromatography media, Sephadex G-100, Sephadex G-25, Blue Dextran 2000 and molecular weight calibration kit were products of Pharmacia Fine Chemicals, Uppsula, Sweden.

All the reagents including buffers were prepared in glass distilled water.

Organism and Growth Conditions. A pure culture of Thiobacillus thiooxidans (ATTCC 8085) was grown under autotrophic conditions in Starkey's medium (1925) which contained 0.3 g $(NH_4)_2 SO_4$, 0.5 g $MgSO_4 7H_2O$, 0.018 g $FeSO_4 7H_2O$, 3.5 g KH_2PO_4 and 0.25 g $CaCl_2$ per one l distilled water. The organism was grown in 3-l Fernbach flasks, each containing one l of above mentioned Starkey's medium (pH 4.5). After addition of 2-2.5% inoculum,

10-12 g powdered precipitated sulfur was added and spread around to cover the surface of the medium by gently swirling the flask. Flasks were covered with tissue papers and incubated at 28°C for four days. 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 1.2 and 1.8. The cells were collected by a Sharples super centrifuge fitted with a water cooling system (7°C) at 40,000 r.p.m. and washed three times with distilled water. The cell yield varied between 0.2 - 0.4 g (wet weight) per 1 of the medium. The cells were maintained (about 10 mg wet wt. cells per ml water) at 4°C and normally used within 1 to 2 days for preparing cell-free extracts.

Methods

Preparation of Sulfur Suspension. The sulfur suspension which was used as a substrate for the sulfur-oxidising enzyme was prepared by suspending 20 g precipitated sulfur in 100 ml of 0.05% Tween-80. After stirring the sulfur suspension vigorously for six hours, it was sonicated for one hour in a 10 kc/sec. Raytheon sonic disintegrator. The milky white colloidal sulfur was decanted off from the sonicated sulfur and was dialyzed for 24 hours in 0.05% Tween-80 to remove contaminating ions. The sulfur concentration was determined by drying aliquots of sulfur suspension at 65°C and weighing after cooling to room temperature.

Preparation of Cell-Free Extract. The cell suspension was centrifuged and washed once more with 0.2 M Tris-Cl buffer (pH 7.5) and resuspended (100-140 mg wet weight cells per ml) in 0.05 M Tris -Cl buffer (pH 7.5). This cell suspension was treated with trypsin (1.5 ug per mg wet weight cells) for 15 minutes with gentle stirring at room temperature. The treatment was stopped by adding trypsin inhibitor. Trypsin treated cells

were sonicated for 10 minutes at 7°C in a 10 kc/sec. Raytheon sonic disintigrator. The sonicated cell suspension was centrifuged at 22,000 xg for 15 minutes to remove cell debris and unbroken cells. The resulting supernatant was designated as cell-free extract. The cell-free extract contained 8-12 mg of protein per ml and was used immediately or stored at 4°C until further use.

In some experiments the preparation of crude extract was performed without trypsin treatment and by sonicating the cells for 20 minutes.

Protein Determination. Protein contents were determined according to 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 Rajagopalan and Handler (1964) and Massey (1957) with some modification as described by Suzuki and Silver (1966). To a 1.0 ml sample was added 1.0 saturated aqueous 2,2'-bipyridyl. After 1 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 determined 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 sample and incubating the mixture in the dark for 1 hour before determining the O.D. 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 Popowski (1949), essentially as described by Suzuki and Silver (1965). To a 0.65 ml sample containing 5-100 n moles of sulfide, equal volume of 2% zinc acetate was added and mixture was centrifuged. To the supernatant, 2.5 ml of 0.1% p-aminodimethylaniline sulfate in 5-5 M HCl and 0.5 ml of 0.23 M $FeCl_3$ in 1.2 M HCl were added in a screw cap test tube and shaken.

After 30 minutes, the intensity of methylene blue formed was measured at 670 nm in a Gilford-2400 spectrophotometer.

Determination of Thiosulfate. Thiosulfate was determined according to a colorimetric procedure as described by Sorbo (1957). To a 2.0 ml sample containing 0.1 to 1.0 u mole of thiosulfate, 2.2 ml of 0.2 M NH_4OH, 0.5 ml of 0.1M KCN were added and after mixing 0.3ml of 0.1M $CuCl_2$ was added. The $CuCl_2$ was well mixed with the sample immediately after addition. To the mixture was then added 0.5 ml of ferric nitrate reagent (20% w/v $Fe(NO_3)_3 \cdot 9H_2O$ in 13% HNO_3) and the mixture was well mixed. After 15 minutes 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, first the ferric nitrate reagent followed by KCN and $CuCl_2$.

Centrifugations. Low speed centrifugations were performed in a Sorvall superspeed RC-2B auto-refrigerated centrifuge at 4^oC. High speed centrifugations were carried out in a Beckman L3-50 refrigerated ultracentrifuge using a 50 Ti or 60 Ti rotor at 4^oC.

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). Flavin was extracted by boiling the enzyme protein in hot water bath for 2-3 minutes and removing the denatured protein by centrifugation. Extracted flavin along with riboflavin, FMN and FAD markers was spotted on the silica gel strips and ascending chromatography was performed using 5% $Na_2HPO_4 \cdot 12H_2O$ as solvent. After chromatography flavins were detected under UV light.

Identification and Quantitation of Enzyme Flavin. Enzyme flavin was extracted by boiling the enzyme in water bath and identified by comparing

the UV and visible range spectra to standards of riboflavin, FMN and FAD. For quantitative analysis, an extinction-coefficient of riboflavin ($10 \times 10^3 M^{-1} Cm^{-1}$) at 445 nm in 0.1 M Tris-Cl (pH 7.5) was used.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis under non-dissociating conditions was performed according to Davis (1964).

Spectrophotometery. Absorption spectrum studies of various preparations were done with the Shimadzu MPS-50L at room temperature using a cell with 1 cm light path. A Gilford-2400 spectrophotometer was also frequently used for estimation of color intensity or protein contents.

Preparation of the Flavin Free Enzyme. The flavin free enzyme was prepared by the method of Massey and Curty (1966). An enzyme sample (2-3 mg protein) in 2 ml volume was dialyzed against 0.2 M Tris-Cl buffer containing 1 M KBr (pH 2.5 unless indicated otherwise). The buffer was changed several times over a period of 24-36 hours. During this period resolution of flavin was monitored by loss of yellow color of the enzyme and when no yellow color was visible, dialysis bag containing flavin free enzyme was removed and further dialyzed in 0.2 M Tris-Cl (pH 7.5) to readjust the pH and remove KBr from the enzyme protein.

Molecular Weight Determination. Molecular weight of purified enzyme was determined by gel filtration on Sephadex G-100 column using a method similar to that reported by Andrew (1964). Sephadex column was standardized using proteins from Pharmacia molecular weight estimation kit.

Sulfur-Oxidising 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). The reaction mixture contained, unless indicated otherwise, the following in a total volume of 1.2 ml:

140 μ moles Tris-Cl (pH 7.5)

0.8 mg sulfur (in the form of suspension in 0.05% Tween-80)

5 μ moles GSH

Enzyme as indicated

The reaction was started by the addition of GSH. Enzymatic sulfur oxidation rates were always corrected for endogenous oxygen uptake rates an non-enzymatic oxidation rates of GSH and sulfur with or without other cofactors. One unit of enzyme was defined as the amount of enzyme that consumed 1 n mole O_2 per minute under the standard conditions and specific activity was defined as the units of enzyme per mg protein.

In the case of whole cells, unless otherwise indicated, the reaction mixture contained water or 140 μ moles potassium phosphate (pH 5.5), 0.8mg sulfur and about 3.75mg wet weight cells in total volume of 1.2 ml. Sulfur oxidation rates of whole cells were similar in potassium phosphate(pH 5.5) or water.

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

Sulfide and Sulfite Ozidising Enzyme Assay. Sulfide and sulfite oxidations were assayed by measuring oxygen consumption with an oxygraph as described in the essay of sulfur-oxydising enzyme. In the case of sulfide oxidation, reaction mixture contained unless indicated otherwise, the following in a total volume of 1.2 ml:

140 μ moles Tris-Cl (pH 7.5)

2 μ moles Na_2S

Enzyme as indicated

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

140 μ moles Tris-cl (pH 7.5)

2.0 μ moles Na_2SO_3

0.1 μ mole EDTA

Enzyme as indicated.

The reaction was initiated by addition of sulfide or sulfite in microliter quantity and enzymatic rates were corrected for endogenous and non-enzymatic rates as in sulfur oxidation.

RESULTS

RESULTS

General Physiology of Thiobacillus thiooxidans

The whole cells of T. thiooxidans readily oxidised sulfur, sulfide, and sulfite without any cofactor requirement. Sulfur, sulfide and sulfite oxidising activities were also detected in cell-free abstracts. However cell-free extracts required GSH for sulfur oxidation and the optimum pH values for oxidation of these sulfur compounds shifted from acidic (around 5.5) pH with cells to neutral (around 7.5) in case of cell-free extract. Cytochromes of a, b and c types were also easily detectable in the cell-free extracts. Sulfur-oxidising enzyme was chosen for detailed study.

Effectiveness of Mild Trypsin Treatment in Solubilizing the Sulfur-Oxidising Enzyme

During the purification of sulfur-oxidising enzyme of Thiobacillus thiooxidans from normal cell-free extracts (extracts prepared without any treatment of cells before sonication as described in the Material and Methods section), most of the activity remained in the pellet fraction after centrifugation of the extract at 150,000 xg. Even the activity remaining in the supernatant fraction was eluted within the void volume of G-150 sephadex and could not be eluted from a DEAE column at all where it appeared to be physically stuck indicating the membranous nature of the enzyme.

Lysozyme, lipase, glucosidase or detergent treatment of cells or extracts was unsuccessful in releasing the sulfur oxidising activity in the soluble form. However mild trypsin treatment of cells before breakage (as described in Material and Methods) was found to be very effective in releasing the sulfur oxidising activity in the solube form. Cell-free

Table 1. Effect of trypsin treatment of cells on the distribution of sulfur-oxidising activity in various fractions of extracts.

[a]Preparation	Volume (ml)	[b]Sulfur-oxidising activity (unit)	Recovery (%)
Untreated Cells			
Cells	20	86,400	
Extract	20	7,800	100
150,000 xg supernatant	18	4,680	60
precipitate	5	3,100	40
pH 5 supernatant	18	3,690	47
precipitate	5	1,000	13
Trypsin Treated Cells			
Cells	20	86,400	
Extract	20	8,800	100
150,000xg supernatant	19	8,360	95
precipitate	5	500	5
pH5 supernatant	19	7,695	87
precipitate	5	650	7

[a]Various fractions shown here are described in detail in the following section on the enzyme purification.

[b]Sulfur-oxidising 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 O_2 per minute under the standard conditions.

extracts prepared by this method were clearer and pale yellowish in appearance compared to extracts without this treatment. Distribution of the Sulfur Oxidising activity in various fractions of normal and trypsin treated cell extracts is shown in Table 1. Since the trypsin treatment increased the degree of solubilization of the enzyme activity and improved the recovery of the enzyme during purification, this method was used for the standard purification of the sulfur-oxidising enzyme.

Purification of Sulfur-Oxidising Enzyme

The following steps were routinely used in the purification of sulfur-oxidising enzyme of T. thiooxidans. All the steps were carried out around $4^{o}C$.

1. Cell-free extract. Cell-free extracts for this purification were always prepared by treating cells with trypsin as described in Materials and Methods.

2. 150,000 xg centrifugation. Cell-free extracts (80-100 ml pH 7.5) were centrifuged at 150,000 xg for two hours and the supernatant was carefully removed with a syringe without disturbing the pellet.

3. Acidic treatment. The 150,000 xg 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 xg for 20 minutes. The resultant supernatant was adjusted back to pH 7.5 with 2 M tris-base solution.

4. DEAE cellulose column chromatography I. The bright yellow transparent supernatant fluid was now applied to a 2.5 x 15 cm DEAE cellulose column equilibrated with 0.05 M Tris-Cl (pH 7.5) buffer using 20-25 ml 1 hour elution rate. Light yellow fluid devoid of sulfur oxidising activity and showing the spectrum of Cytochrome C-552 passed through the column

unadsorbed and a brownish yellow band could be seen adsorbed to DEAE column at the top. The column was washed with 200 ml of 0.1 M Tris-Cl (pH 7.5) followed by 200 ml of 0.15 M Tris-Cl (pH 7.5). Fractions collected had no sulfur oxidising activity. Finally the brownish yellow band was eluted with 0.3 M Tris-Cl (pH 7.5) as a single band and collected in 8-10 ml volume. Most of the activity applied to the column was found in this fraction.

5. G-100 Sephadex column chromatography I. The DEAE-cellulose eluate was passed through G-100 sephadex column (2.5 x 55 cm) equilibrated with 50 mM Tris-Cl buffer (pH 7.5) using the same buffer for elution. Fractions of 3.4 ml were collected and examined for activity. All the fractions showing activity were pooled together. Elution rate of the sephadex column was found to be very critical in recovery of the active enzyme. With very slow elution rate (less than 3-4 ml/hour) there was considerable decrease in specific activity of sephadex purified enzyme along with the decrease of intensity of the yellow color. To avoid the loss of activity during this step a fast flow rate (12-15 ml/hour) was routinely used.

6. DEAE-cellulose column chromatography II. All the active fractions from step 5 were pooled together and subjected to DEAE-cellulose column chromatography as described in step 4.

7. Sephadex G-100 column chromatography II. The enzyme eluted from DEAE-cellulose column at step 6 was once more passed through a sephadex G-100 column (2.5 x 55 cm) as in step 5 and active fractions were pooled together and concentrated by a DEAE-cellulose column as in step 4. This concentrated enzyme was used as the purified enzyme. Results of a typical purification procedure are shown in Table 2 and Figures 1 and 2.

Table 2. Purification of Sulfur-Oxidising Enzyme.

Fraction	Total Protein (mg)	Total Activity (unit)	Specific Activity (unit/mg protein)	Purification (fold)	% Recovery
Cell free extract	1,058	16,399	15.5	1	100%
150,000 X g	492	15,990	32.5	2.1	97.5
pH 5 Supernatant	400	15,600	39.0	2.5	95.1
DEAE Eluate I	66	8,448	128	8.3	51.5
Sephadex G-100 I	23.0	6,923	301	19.4	42.2
DEAE Eluate II	8.0	3,256	407	26.3	19.9
Sephadex G-100 II	4.0	1,644	411	26.5	10

Enzyme activity was measured as described in Materials and Methods section.

Fig. 1. Sephadex G-100 I Elution Profile.

Sephadex G-100 column chromatography I was carried out as described in Results section. Fractions were collected in 3.4 ml volume and activity was assayed as outlined in Materials and Methods using 0.5 ml of each fraction.

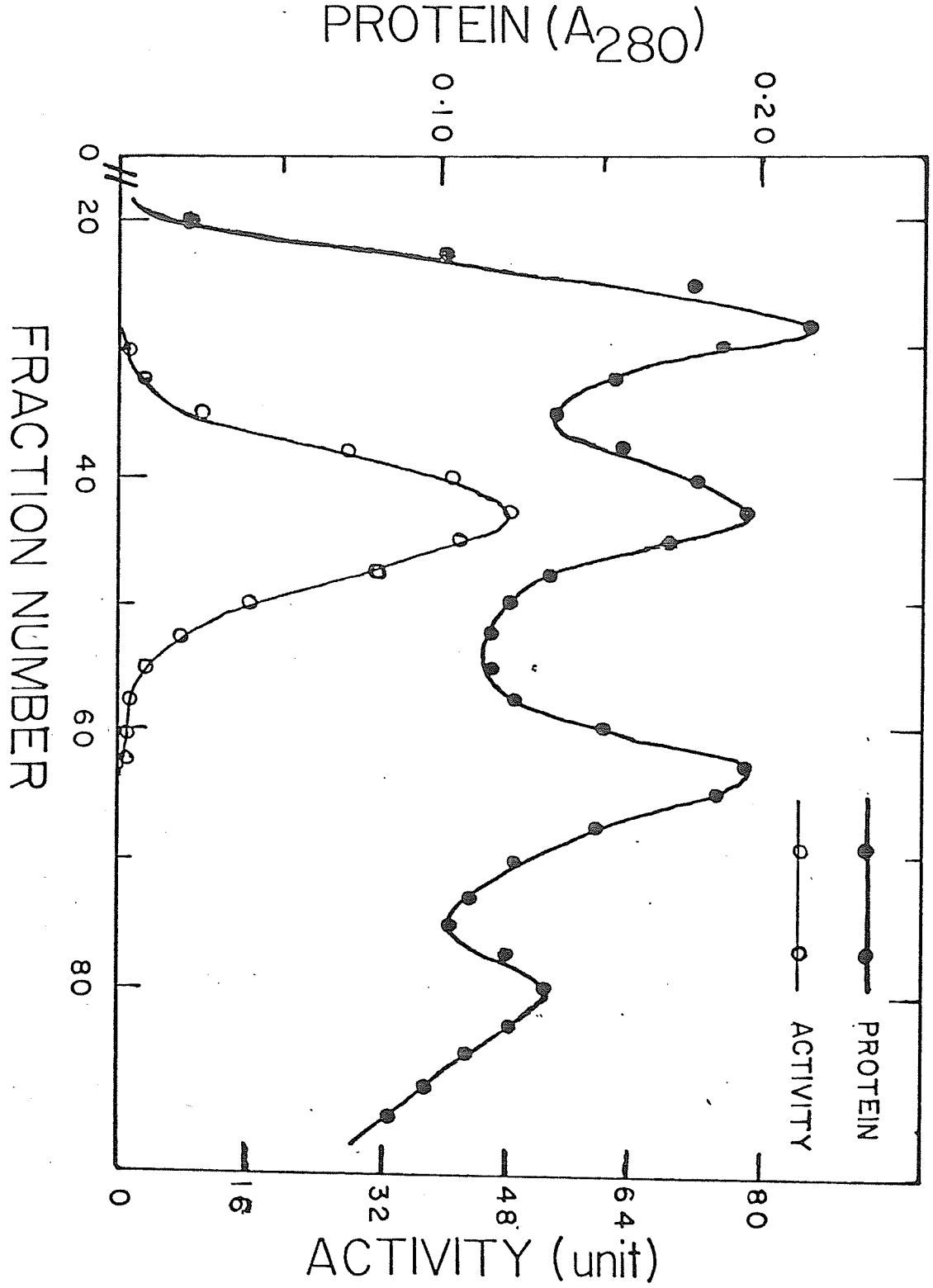
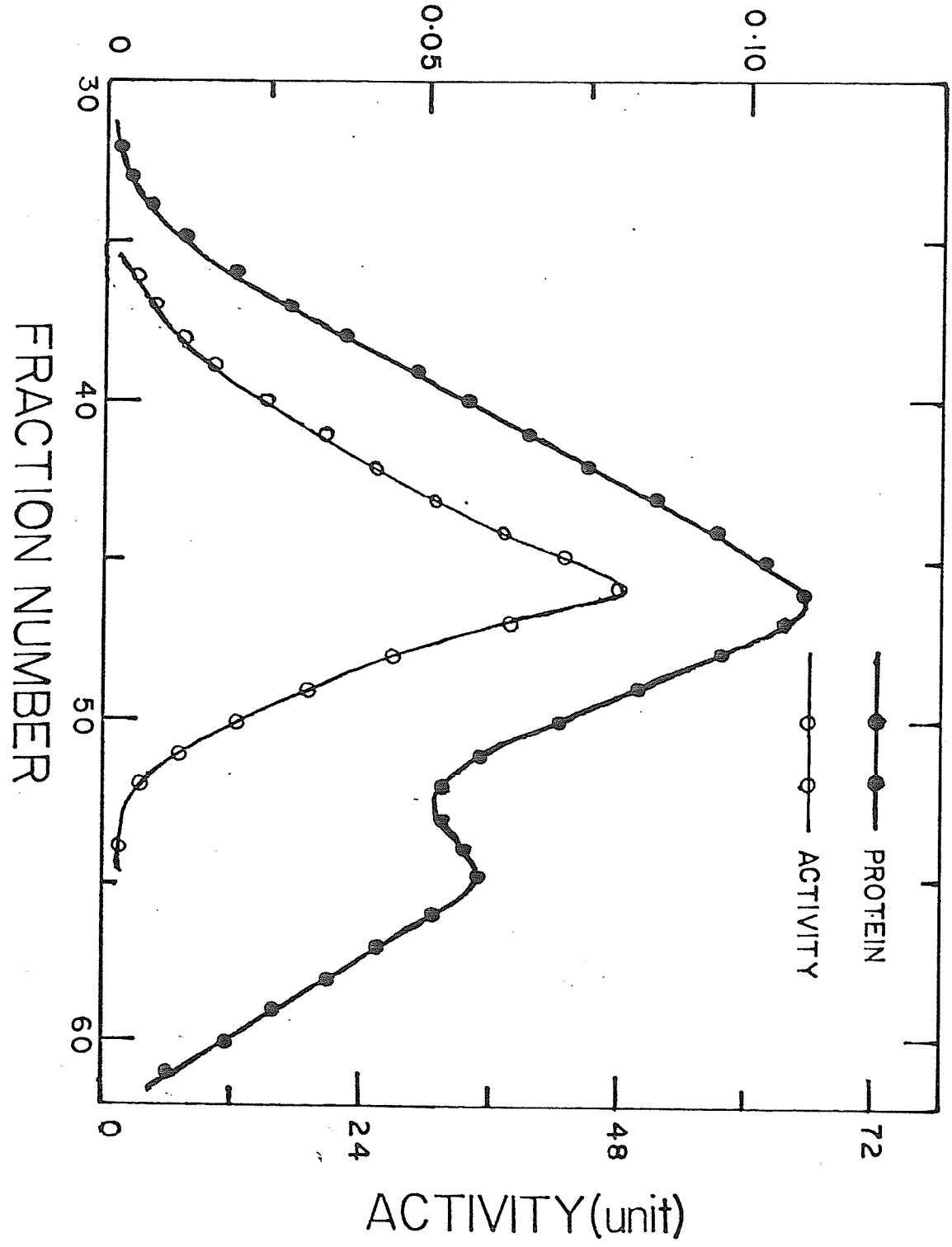

PROTEIN (A_{280})
0·10
0·20
0
20
40
60
80
FRACTION NUMBER
PROTEIN
ACTIVITY
0
16
32
48
64
80
ACTIVITY (unit)

Fig. 2. Sephadex G-100 II Elution Profile.

Sephadex G-100 column chromatography II was carried out as described in Results section. Fractions were collected in 3.4 ml volume and activity was measured as described in Materials and Methods using 0.5 ml of each fraction.


PROTEIN (A280)
0
0·05
0·10
30
40
50
60
FRACTION NUMBER
PROTEIN
ACTIVITY
0
24
48
72
ACTIVITY (unit)

Characteristics of the Purified Sulfur-Oxidising Enzyme

Molecular Weight. A molecular weight of 40,000 ± 5% was estimated for the purified enzyme by gel filtration on sephadex G-100 (Figure 3.) as described in Materials and Methods section.

Absorption spectrum. The purified enzyme showed absorption maxima around 272, 410, 448nm and a shoulder at 478nm. Upon reduction with sodium dithionite, an absorption maximum around 448nm and a shoulder at 478 nm disappeared and absorption of 410 nm was diminished to some extent (Figure 4.).

Iron and labile sulfide content. Iron and labile sulfide contents of enzyme were determined as described in Materials and Methods. The results of the determinations are shown in Table 3. Considering the molecular weight of the enzyme as 40,000, non-heme iron, labile sulfide and protein were found to be in the molar ratio of 1:1:1 respectively.

Purity of Sulfur-Oxidising Enzyme. Disc polyacrylamide gel electrophoresis under non-dissociating conditions revealed one major and a minor band. From the intensity of the bands, the enzyme was considered to be almost homogenous.

Enzyme Storage and Stability. Enzyme could be stored at -20°C or 4°C for several weeks without loss of any activity. Repeated freezing and thawing resulted in considerable loss of activity. Boiling the enzyme for 1 minute resulted in the loss of 95% activity and remaining 5% activity was probably due to chemical reaction of free flavin with sulfur and GSH.

Substrate Specificity of the Enzyme. The purified enzyme only oxidised sulfur in the presence of catalytic amounts of glutathione and did not

Fig. 3. Molecular Weight Estimation of Sulfur-Oxidising Enzyme by Sephadex G-100 in 50mM Tris-Cl, pH 7.5.

The marker proteins used were bovine serum albumin (mol. wt. 68, 000), Ovalbumin (mol. wt. 45, 000), chymotrypsinogen-A (mol. wt. 25, 000) and horse heart cytochrome C (mol. wt. 12400).

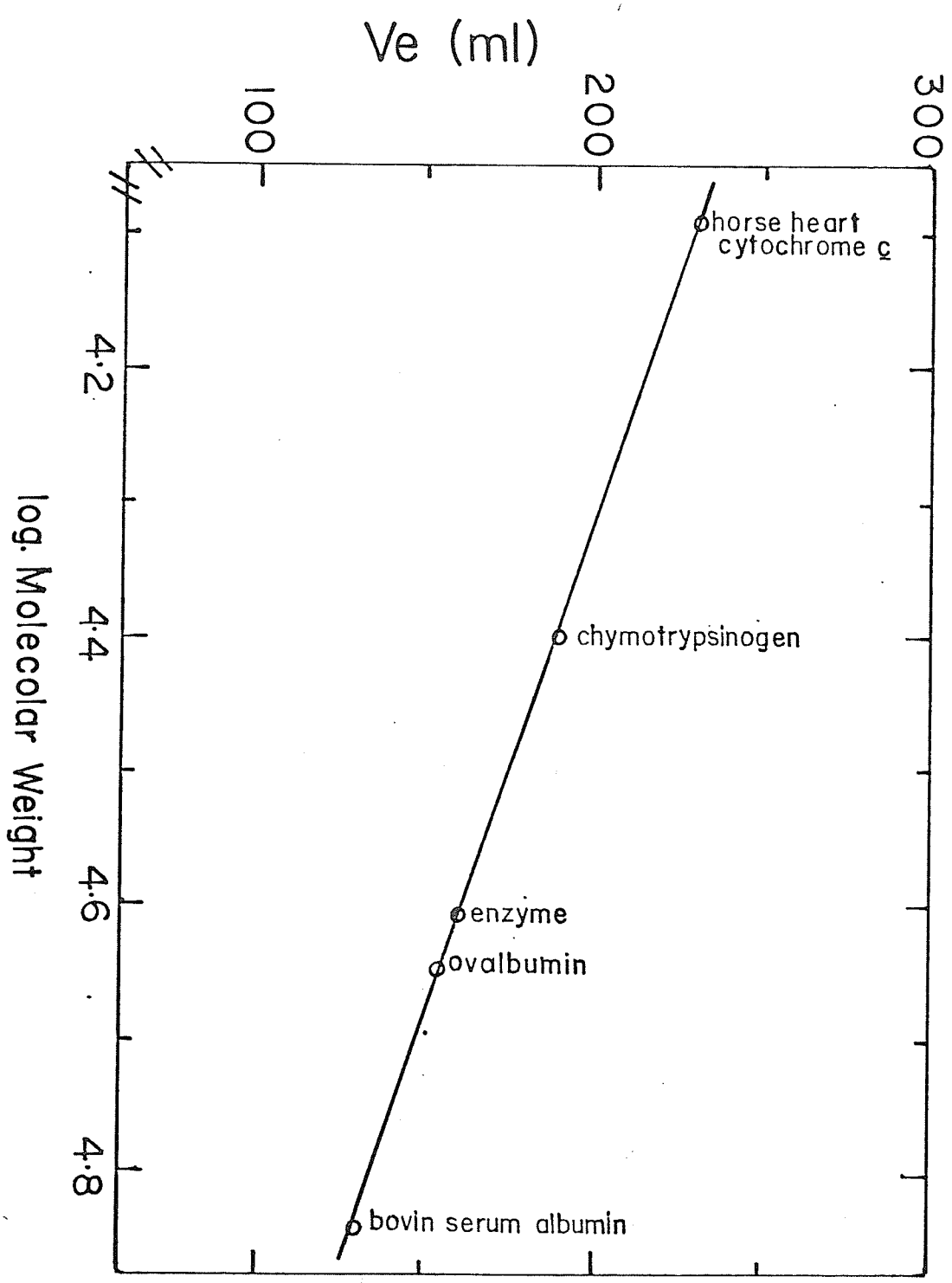

Ve (ml)
100
200
300
log. Molecolar Weight
4.2
4.4
4.6
4.8
horse heart cytochrome c
chymotrypsinogen
enzyme
ovalbumin
bovin serum albumin

Fig. 4. Oxidised and oxidised-reduced difference spectra of sulfur-oxidising enzyme in 0.2M Tris-Cl (pH 7.5).

a and b - oxidised

(0.5 mg and 1.5 mg enzyme protein was used respectively)

c - oxidised - dithionite reduced

(2.4 mg protein was used)

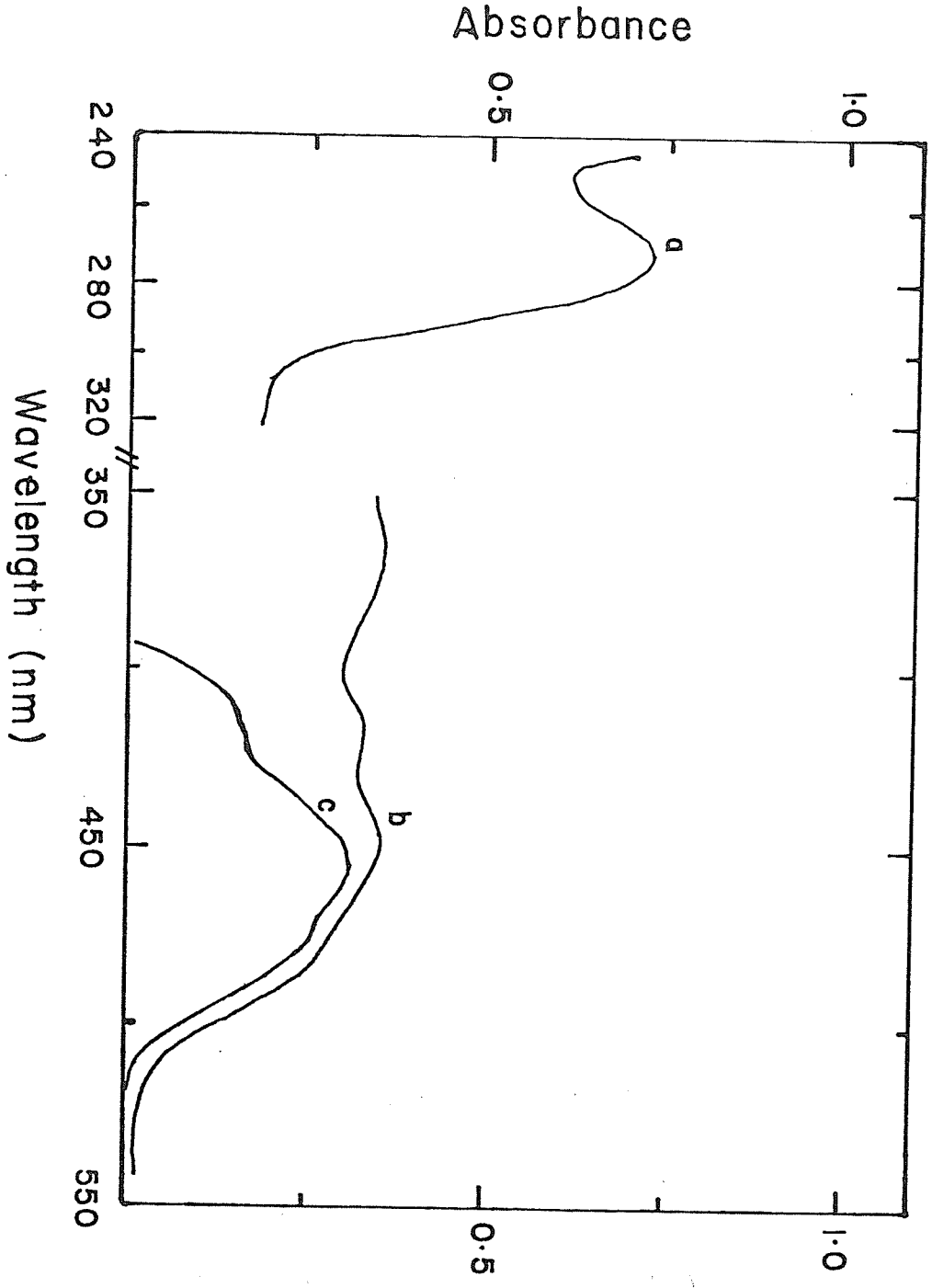

Absorbance
0·5
1·0
240
280
320
350
450
550
Wavelength (nm)
a
b
c
0·5
1·0

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

Sample	Protein (mg)	Protein (moles)	Ferrors Iron (moles)	Total Iron (moles)	Sulfide (moles)	Flavin (moles)	Protein:Total Iron:Sulfide:Flavin
1	1.6	$4.0X10^{-8}$	$3.8X10^{-8}$	$4.2X10^{-8}$	$3.6X10^{-8}$	$3.5X10^{-8}$	1: 0.95: 0.90: 0.88
2	1.6	$4.0X10^{-8}$	$4.1X10^{-8}$	$5.0X10^{-8}$	$3.9X10^{-8}$	$4.2X10^{-8}$	1: 1.02: 0.98: 1.05

have any sulfide, sulfite or thiosulfate oxidising activity.

Identification of Flavin Component of Sulfur-Oxidising Enzyme

Flavin component of purified enzyme was identified as riboflavin by spectrophotometry and thin layer chromatography as described in Materials and Methods.

Spectrophotometric analysis. Spectra of native enzyme flavin, extracted flavin (flavin was extracted from the enzyme protein by boiling the protein in water bath for 2-3 minutes and separating the denatured protein by centrifugation) and riboflavin are shown in Figure 5. Extracted flavin showed a spectrum very similar to riboflavin spectrum with absorption maxima around 266, 370, 445nm and a shoulder around 475nm. Absorption at 266nm by extracted flavin was much higher than that expected from the quantity of flavin used for analysis indicating presence of some nucleotides (identified as AMP by thin layer chrometography as described below). In the case of native enzyme there was 2-3nm shift of absorption peak around 445nm and 475nm shoulder towards longer wavelength possibly caused by binding of flavin to the protein.

Identification of Flavin by Thin layer Chromatography. Thin layer chromatography was performed as described in Materials and Methods. Extracted flavin sample (prepared by boiling the enzyme protein in water bath for 2-3 minutes and separating the protein by centrifugation) was spotted on a silica gel strip along with standards riboflavin, FMN, FAD, AMP and ADP. The results of the chromatography are shown in figure 6. Flavin from enzyme was identified as Riboflavin, but the enzyme extract contained another U.V. absorbing component identified as AMP.

Fig. 5. Absorption spectra of riboflavin and extracted flavin in 0.2M Tris-Cl (pH 7.5).

a - riboflavin (20 uM)

b - extracted flavin

c - riboflavin (10 uM)

Flavin was extracted from 0.6 mg native enzyme protein as described in Materials and Methods.

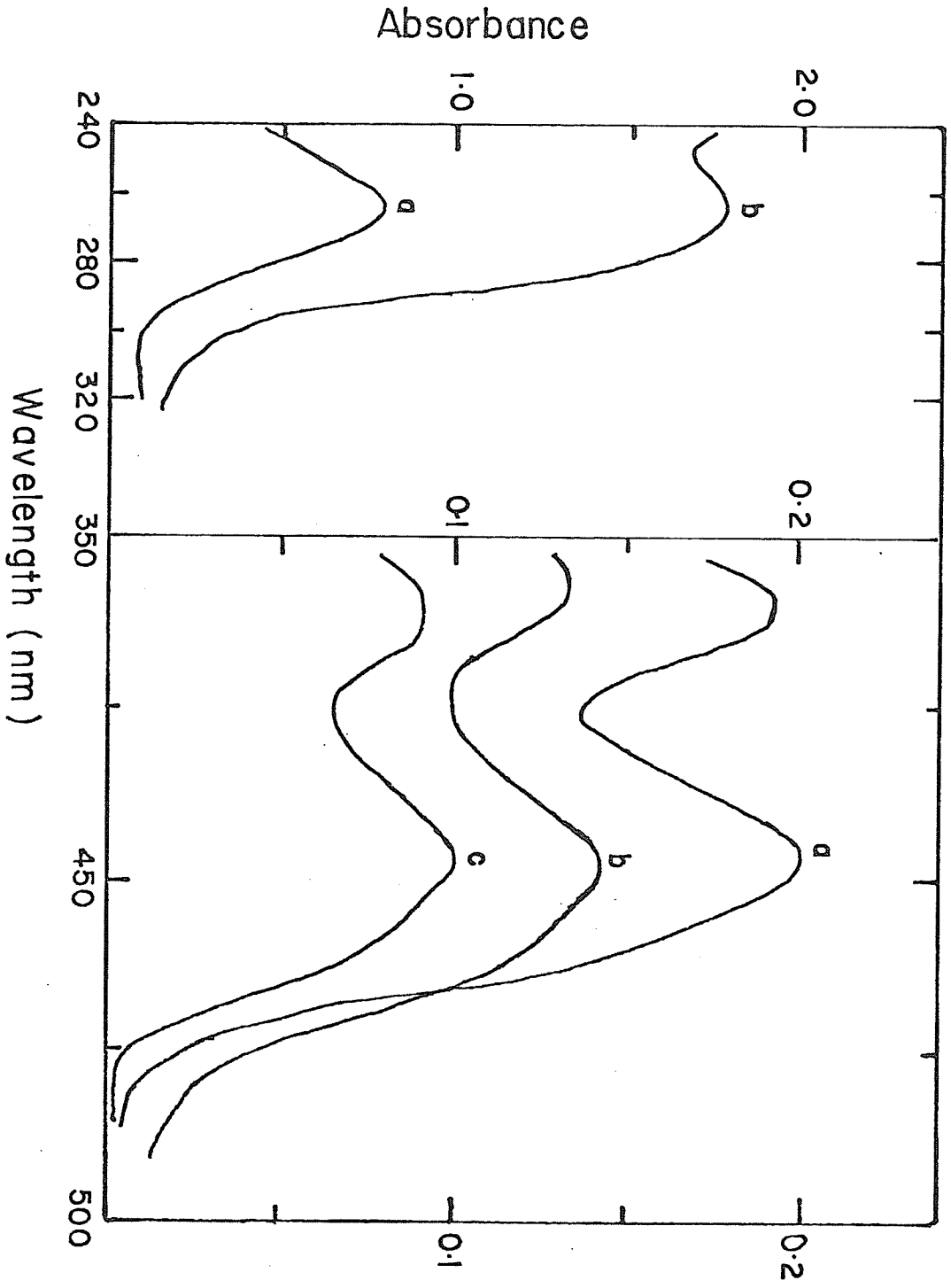
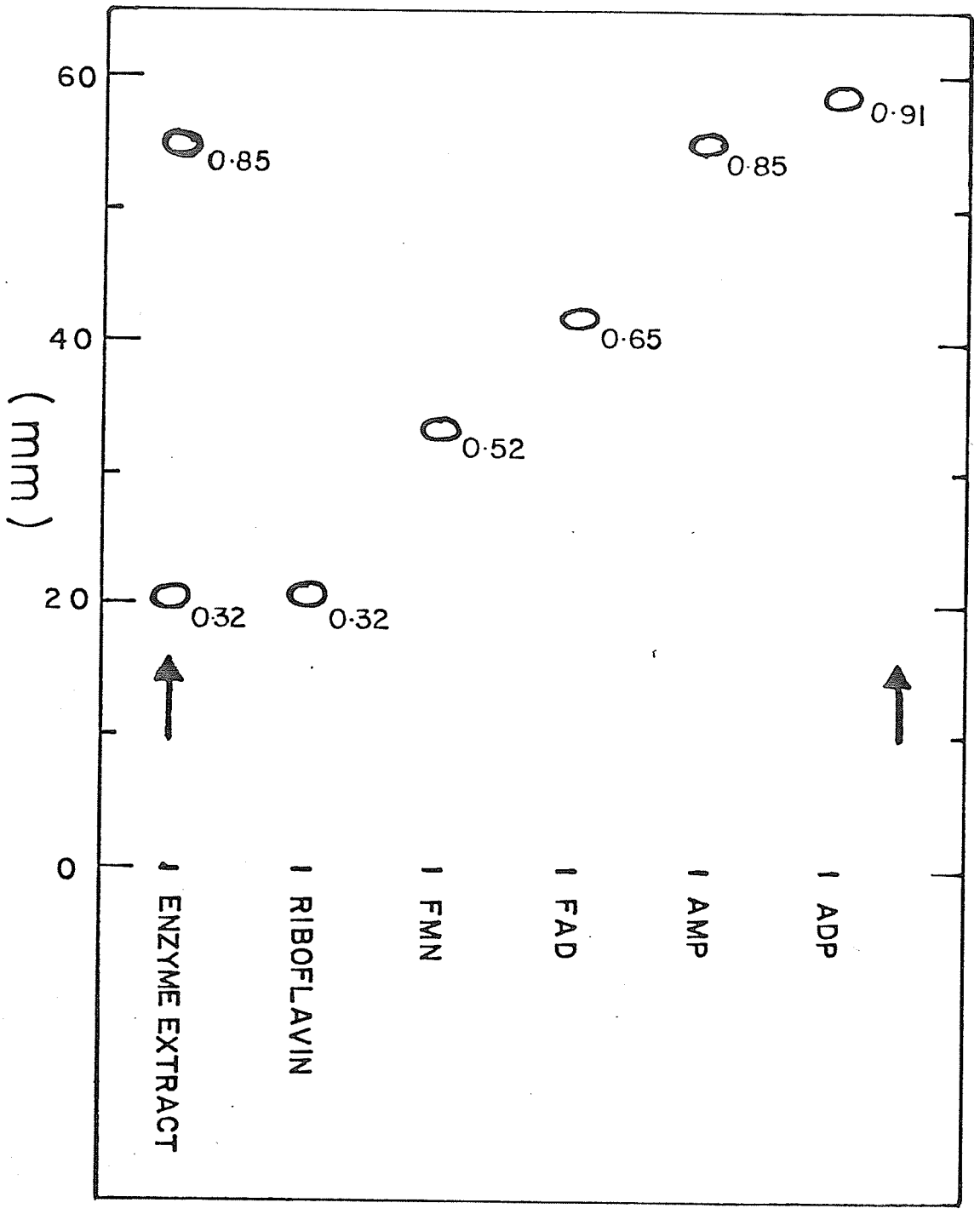

Absorbance
2·0
1·0
0·2
0·1
240
280
320
350
450
500
Wavelength (nm)
a
b
c

Fig. 6. Identification of flavin component of Sulfur-Oxidising Enzyme by thin layer Chromatography.

Chromatography was done as described in Materials and Methods. Rf values of the U.V. absorbing spots are also shown on the figure.


60
40
20
0
(mm)
0·85
0·91
0·85
0·65
0·52
0·32
0·32
ENZYME EXTRACT
RIBOFLAVIN
FMN
FAD
AMP
ADP

Requirement of Flavin for sulfur-oxidising enzyme activity

Since the purified sulfur-oxidising enzyme contained riboflavin and AMP, further studies were done to determine the requirement of Flavin for sulfur oxidation by this enzyme. Basically following three approches were taken:

I. Effect of removal of Flavin on the activity of sulfur-oxidising enzyme,

II. Effect of Flavins on the activity of Flavin-free sulfur-oxidising enzyme,

III. reconstitution of Flavin-free enzyme.

I. Effect of removal of Flavin on the activity of sulfur-oxidising enzyme. Purified enzyme was dialyzed in 1 ml aliquots (1.5 mg protein) for different time periods against 0.2M Tris-Cl (pH 7.5) for 5-6 hours with 2-3 changes of the buffer. Enzyme activity was assayed and iron, sulfide and flavin contents were determined as described in Materials and Methods. Results of this study are shown in Table 4.

II. Effect of flavins and nucleotides on the activity of the flavin-free sulfur-oxidising enzyme. The purified sulfur-oxidising enzyme (3.2mg protein) was dialyzed in 2ml volume against 0.2M Tris-cl containing 1M KBr buffer (pH 2.5) for 24 hours. Then KBr was removed from the enzyme sample by dialyzing it against 0.2M Tris-Cl pH 7.5 for 6 hours with 3 changes of this buffer. Denatured protein was removed by centrifugation. The colorless enzyme obtained after this treatment was found to be devoid of flavin and sulfur-oxidising activity. The effect of various

Table 4. Effect of removal of flavin by dialysis, on the activity of sulfur-oxidising enzyme.

Dialysis time (hour)	Activity (%)	Flavin (%)	Iron (%)	Sulfide (%)
0	100	100	100	100
12	88	62	100	100
18	55	46	100	not determined
24	40	38	100	not determined

Purified enzyme was dialyzed in 1 ml (1.5 mg Protein) aliquots for different time periods as indicated against 0.2M Tris-Cl (pH 7.5) containing 1M KBr and activity was assayed as outlined in Materials and Methods. About 150 ug enzyme protein was used per assay. Flavin, iron and sulfide contents were determined as described in Materials and Methods.

flavins and nucleotides on the sulfur-oxidising activity of this flavin-free enzyme was studied.

Flavins and nucleotides did not have any effect on the sulfur-oxidising activity of the enzyme without incubation of the enzyme with flavins for a minimum period of ½ hour before assay. Incubation of flavins and enzyme in Tris-Cl (pH 7.5) did not stimulate the activity to any considerable extent. However incubation of the enzyme with FMN or riboflavin in the presence of a phosphate buffer (pH 7.5) was found to be very effective in restoring the activity of flavin-free inactive sulfur-oxidising enzyme. AMP had stimulatory effect on the activity of the enzyme incubated with riboflavin but not with FMN. Normally incubation of the enzyme with flavins was done for 18 hours in the presence of 0.1 M phosphate ions at pH 7.5. The results of this study are shown in Table 5.

III. Reconstitution of the inactive flavin-free sulfur-oxidising enzyme with riboflavin and AMP. As purified sulfur oxidising enzyme was found to have riboflavin and AMP components, an attempt was made to reconstitute the flavin and nucleotide-free enzyme with riboflavin and AMP. The flavin-free enzyme preparation (obtained as described in II above) was incubated for 18 hours in 2 ml volume (∿1 mg protein) with 50 nmoles of riboflavin and 100 nmoles of AMP in 0.1 M phosphate buffer (pH 7.5). This preparation was applied to DEAE-cellulose column (1.0 x 3.0 cm) and unbound flavin and AMP were eluted by washing the column with 0.1 M Tris-Cl (pH 7.5) and sulfur-oxidising enzyme was finally eluted with 0.3 M Tris-Cl (pH 7.5).

The sulfur-oxidising activity and flavin content of this 0.3 M Tris-Cl DEAE eluate was examined and results of this study are shown in Table 6 and Figure 7.

Table 5. Effect of flavins and nucleotides on the activity of flavin-free sulfur-oxidising enzyme.

Reaction mixture contained in volume of 1.2 ml: 120 umoles potassium phosphate (pH 7.5), 1.2 mg S^{o}, 5 umoles GSH, 100 ug enzyme protein and additions as shown in the table.

Flavin free enzyme (prepared as described in Materials and Methods) was preincubated with all additions in presence of 100 umoles phosphate buffer (pH 7.5) in a total volume of 1 ml for 18 hours.

Table 5. Effect of flavins and nucleotides on the activity of flavin-free sulfur-oxidising enzyme.

Preparation	Addition	*Sulfur-oxidising activity (unit)	Activity (%)
Native Enzyme	-	40.5	100
	50 nmoles riboflavin	33.6	75.5
	50 nmoles FMN	33.6	75.5
Flavin free Enzyme	-	2.3	5.6
	25 nmoles riboflavin	20.6	50.8
	25 nmoles riboflavin + 25 nmoles AMP	26.4	65.2
	25 nmoles riboflavin + 50 nmoles AMP	26.4	65.2
	25 nmoles riboflavin + 25 nmoles ADP	20.0	49.4
	50 nmoles riboflavin	23.0	50.5
	50 nmoles riboflavin + 50 nmoles AMP	28.5	70.4
	25 nmoles FMN	25.0	61.7
	25 nmoles riboflavin + 25 nmoles AMP	25.2	62.2
	25 nmoles riboflavin + 25 nmoles ADP	25.0	61.7
	50 nmoles riboflavin	27.1	69.9
	50 nmoles FAD	2.5	6.1
	50 nmoles AMP	2.4	5.4

Enzymatic rates shown in this table have been corrected for non-enzymatic rates: 7.1 without addition, 9.7 with 25 nmoles flavin and 11.4 with 50nmoles flavin in nmoles O_2/minute. Riboflavin, FMN and FAD had identical non-enzymatic rates and AMP had no effect.

* Sulfur-oxidising activity - as described in Matereals and Methods.

Table 6. Reconstitution of flavin free sulfur-oxidising enzyme.

Preparation	Total Protein (mg)	*Total Activity (unit)	*Specific Activity (unit/mg)	Flavin:Protein
Native enzyme	3.0	1215	405	1: 1
Flavin-free enzyme	1.0	13	13	0: 1
Reconstituted enzyme	0.60	219.6	366	0.9: 1

* as described in Materials and Methods

Enzyme reconstitution was carried out as described under 'Reconstitution of the flavin free enzyme' in the Text and enzyme activity was assayed as described in Materials and Methods: 100 ug enzyme protein was used per assay.

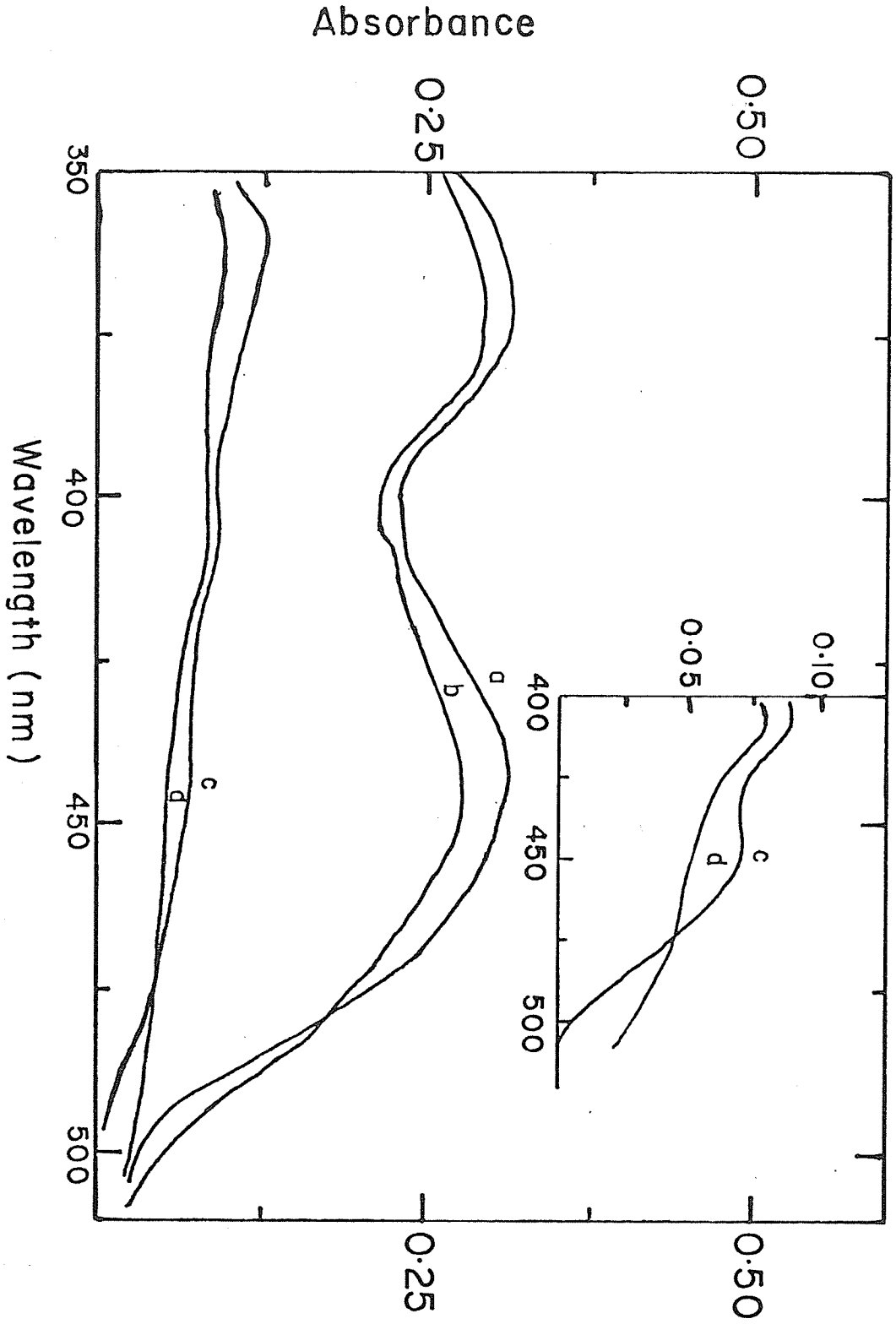
Fig. 7. Absorption spectra of flavin free and reconstituted sulfur-oxidising enzyme in 0.1M potassium phosphate (pH 7.5).

a - riboflavin (30 uM)

b - flavin free enzyme + riboflavin + AMP (0.5 mg enzyme protein was incubated with 25 nmoles of riboflavin, 50 nmoles of AMP and 100 umoles of potassium phosphate (pH 7.5) in 1 ml volume, for 18 hours)

c - reconstituted enzyme
(reconstitution was carried out as described in Results section and 0.3 mg of reconstituted enzyme protein was used)

d - flavin free enzyme
(0.5 mg protein was used)


Absorbance
0·25
0·50
350
400
450
500
Wavelength (nm)
a
b
c
d
0·05
0·10
400
450
500
d
c
0·25
0·50

General Properties of Sulfur-Oxidising Enzyme

DEAE cillulose I level purified enzyme was used to study general properties of the sulfur-oxidising enzyme. The enzyme showed maximum activity between pH 7.5 and 7.6 with sharp decrease of activity below pH 7.0 or above 8.0 (figure 11). Activity was routinely assayed at pH 7.5. Effect of protein concentration on the activity is shown in figure 8.

On the basis of the lineweaver-Burk plot of the reaction velocity versus the concentration of sulfur (figure 9), the apparent K_m of sulfur was calculated to be 5.7mM and from a similar study with GSH (figure 10), the apparent K_m of GSH was determined to be 2mM. Enzyme produced thiosulfate in 1:1 ratio with O_2 (table 7).

Fig. 8. Effect of protein concentrations on sulfur-oxidising activity of the enzyme.

Sulfur-oxidising activity was measured as described in Materials and Methods. DEAE-cellulose I level purified enzyme was used in this study with varied protein concentration as indicated.

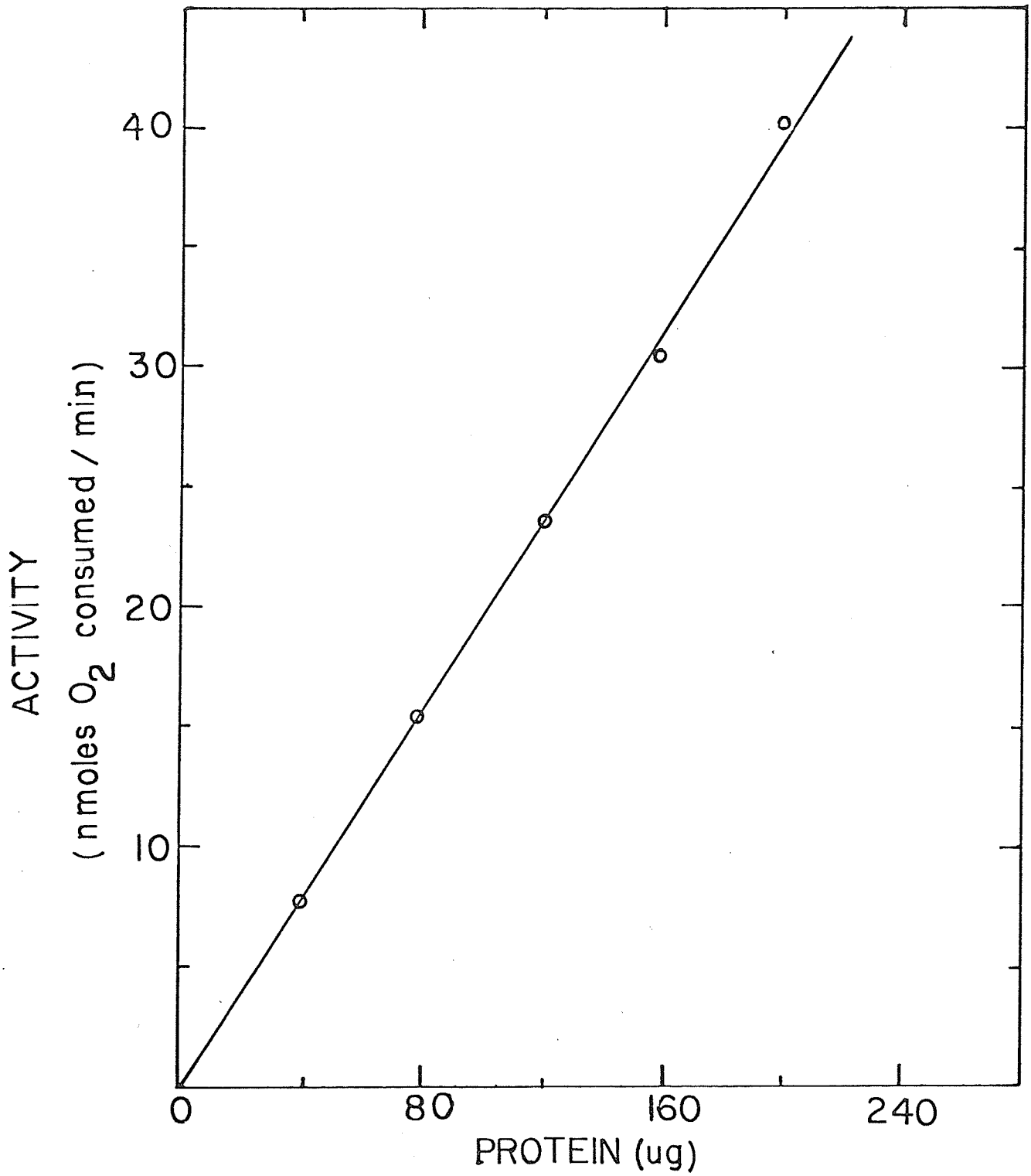

ACTIVITY
(n moles O_2 consumed / min)
40
30
20
10
0
80
160
240
PROTEIN (ug)

Fig. 9. Effect of sulfur concentration on sulfur-oxidising activity of the enzyme.

Activity was assayed as described in Materials and Methods using 100 ug of DEAE-cellulose I purified enzyme, 5 umoles GSH and varied concentration of sulfur as indicated.

ACTIVITY

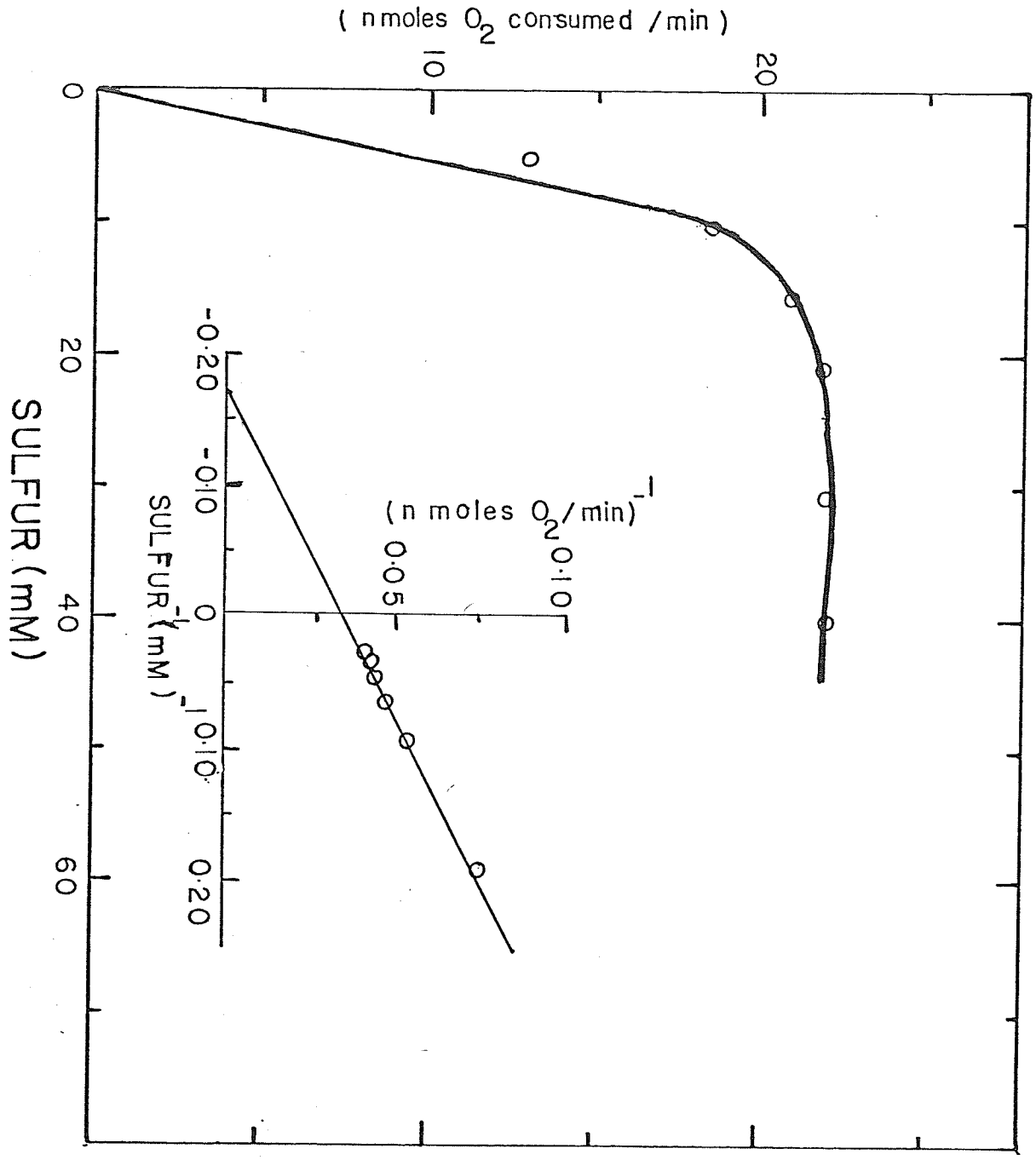

(n moles O_2 consumed / min)
0
10
20
SULFUR (mM)
0
20
40
60
0
-0·20
-0·10
0
0·10
0·20
SULFUR^{-1} (mM)$^{-1}$
(n moles O_2 / min)$^{-1}$
0·05
0·10

Fig. 10. Effect of GSH concentration on sulfur-oxidising activity of the enzyme.

Activity was measured as described in Materials and Methods using 100 ug of DEAE-I purified enzyme, 0.8 mg sulfur and varied amount of GSH as indicated.

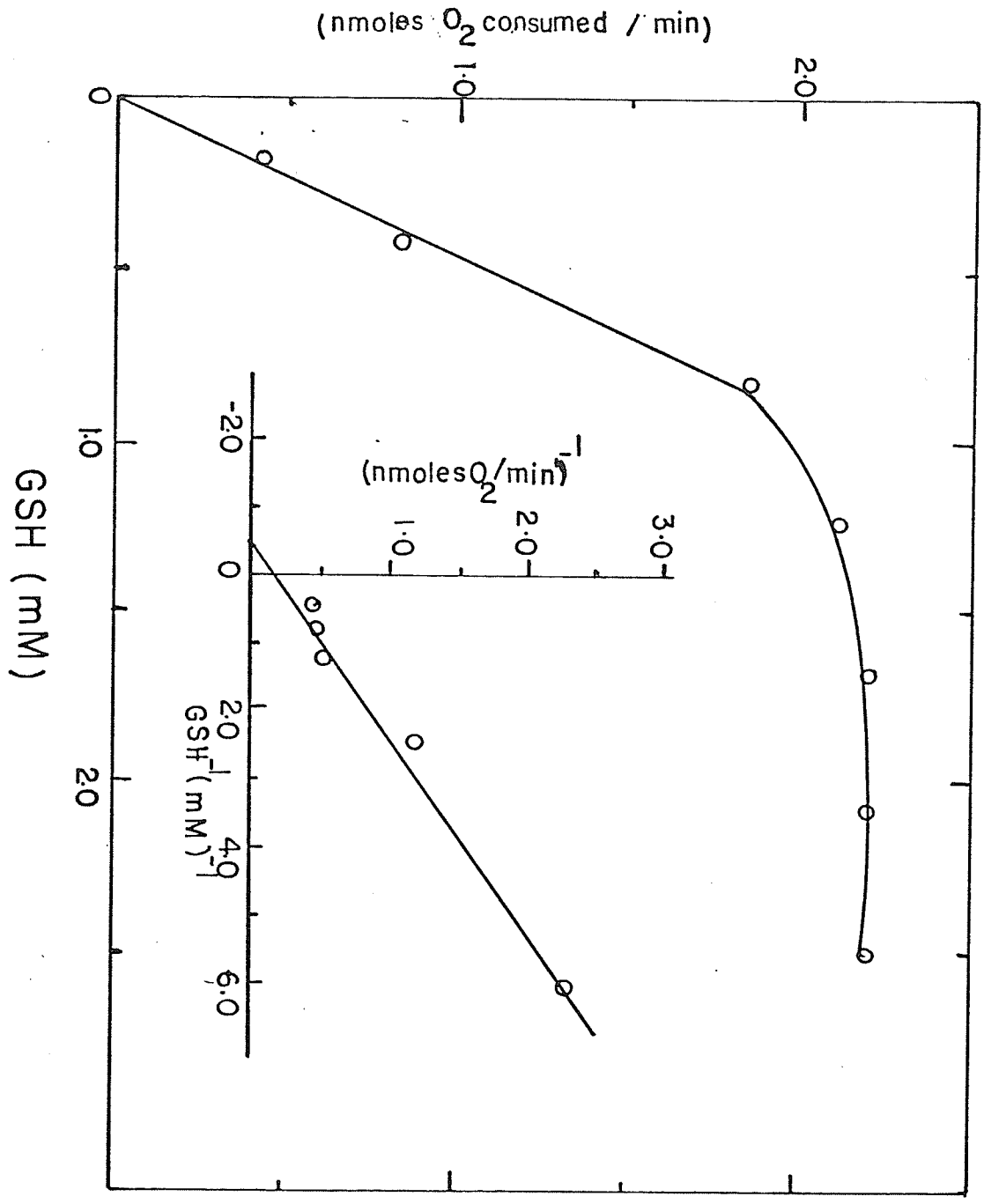

ACTIVITY
(nmoles O_2 consumed / min)
0
1·0
2·0
GSH (mM)
0
1·0
2·0
-2·0
0
2·0
4·0
6·0
GSH^{-1} $(mM)^{-1}$
$(nmoles O_2/min)^{-1}$
1·0
2·0
3·0

Fig. 11. Effect of pH on the sulfur-oxidising activity of the enzyme.

Activity was determined as described in Materials and Methods. Reaction mixture contained the following, in total volume of 1.2 ml: 140 umoles Tris-Cl, 0.8 mg sulfur, 5.0 umoles GSH, and 100 ug DEAE-cellulose I purified enzyme protein.

The pH of Tris-Cl buffer was varied as indicated.

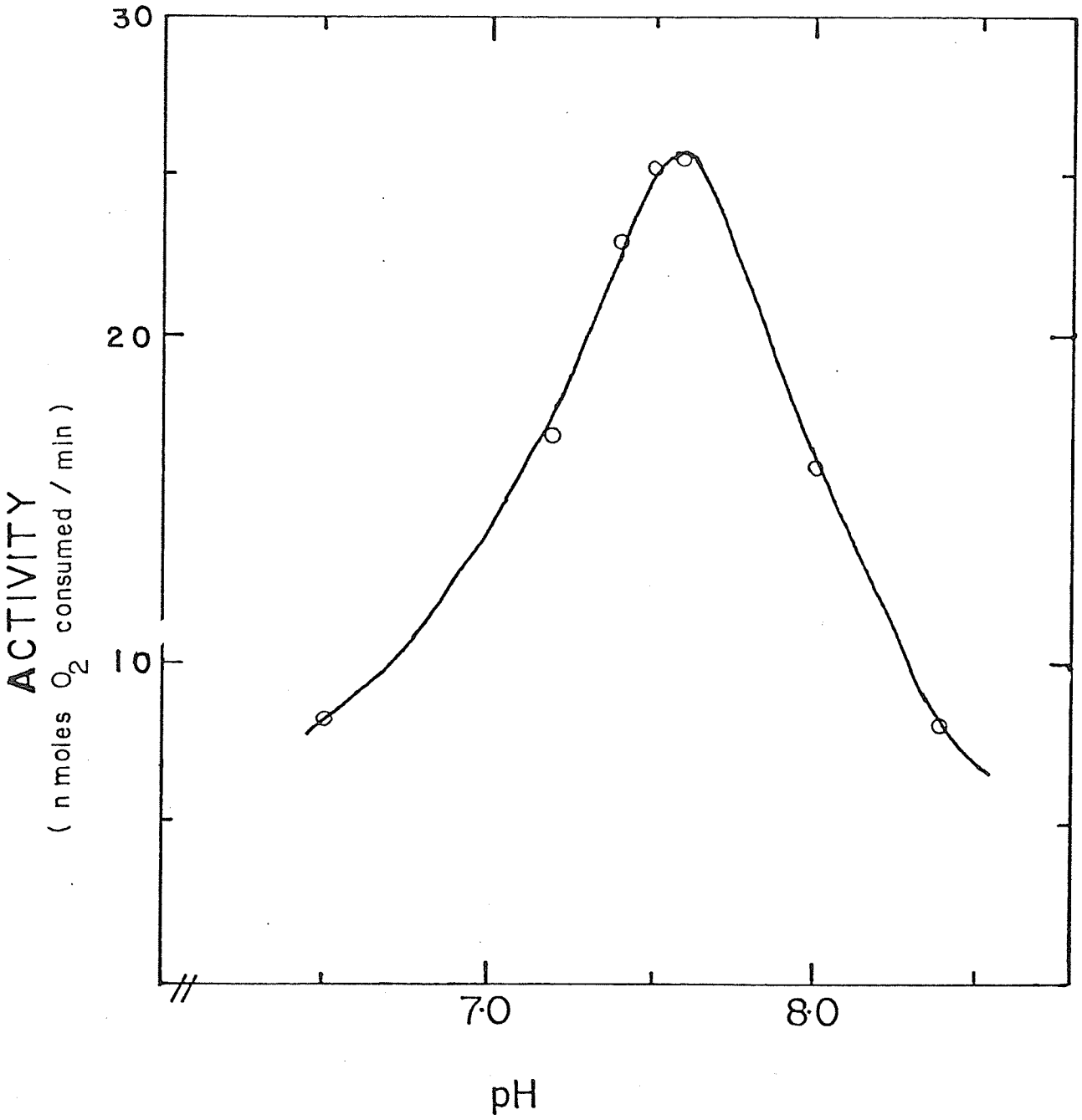

30
20
10
ACTIVITY
(n moles O_2 consumed / min)
7·0
8·0
pH

Table 7. Relationship of oxygen consumption to thiosulfate formation during sulfur oxidation.

Preparation	Incubation time (min)	O_2 (umoles)	$S_2O_3^{2-}$ (umoles)
Cell free extract	100	7.7	7.1
DEAE-cellulose purified enzyme	225	9.7	7.0

O_2 consumption was measured manometrically at 30°C in Warburg apparatus. Reaction mixture contained in a total volume of 3.2 ml: 600 umoles Tris-Cl (pH 7.5), 48 mg sulfur, 3.3 mg catalase, 0.4 umole 2, 2' dipyridyl, 5 unoles GSH, enzyme (7 mg cell free extract or 3 mg DEAE purified enzyme protein) and water. The reaction was stopped by adding 0.1 ml of 1.0M cadmium acetate solution and thiosulfate was determined as described in Materials and Methods.

DISCUSSION

DISCUSSION

Sulfur metabolism of Thiobacillus thiooxidans was studied by measuring the oxygen uptake during sulfur oxidation using a Clark oxygen electrode attached to a Gilson oxygraph. This assay method was found to be very convenient, rapid and reliable, with the added advantage that it had a much higher sensitivity compared to the conventional Warburg method. In the past, studies have been carried out with intact cells, cell-free extracts and partially purified enzyme preparations. To understand the exact nature and the products of the intermediate steps of sulfur metabolism, it is almost imperative that individual enzymes should be purified and characterized. In this present study, although intact cells and cell-free extracts were given some consideration, most emphasis was placed on purification and characterization of the sulfur-oxidising enzyme.

Active cell-free extracts were prepared by aerobic sonication of whole cells. Sulfur oxidation by the extracts exhibited an absolute requirement for thiol or sulfhydryl groups. The requirement for thiol groups could be met by GSH but not by mercaptoethandl or cysteine, in agreement with the findings of Suzuki (1965) and Lukow (1977). According to Kodama and Mori (1968), sonication under a nitrogen atmosphere was essential for the preparation of an active sulfur-oxidising system due to its labile nature under oxygen. It should be mentioned that their cell-free sulfur-oxidising system did not require any addition of thiol groups for sulfur oxidation. The fact that its sulfur oxidation was inhibited by thiol binding agents, indicates that indigenous thiol groups were however, present in their preparation. Probably, native or indigenous sulfhydryl groups are very labile and are easily destroyed during aerobic sonication of the

cells thus necessitating the addition of sulfhydryl groups in the form of GSH for sulfur oxidation. Polysulfide formed by the interaction of GSH and sulfur has been shown to be the actual substrate for sulfur-oxidising enzyme (Suzuki, 1965). Since intact cells oxidise sulfur without the addition of GSH, these must possess thiol groups to form polysulfide with sulfur. Recently Takakuwa et al (1979) have shown that cell surface has thiol groups on it and these are required for sulfur-cell adhesion process and sulfur oxidation. From these findings it seems that sulfhydryl groups on the cell surface, attack sulfur and form polysulfide which is then oxidised by the sulfur-oxidising enzyme as proposed by Suzuki (1965).

During the purification of sulfur-oxidising enzyme, a problem was faced in that the most of the activity remained in particulate fractions of cell free extract. Adair (1966), Taylor (1968) and Lukow (1977) have also reported similar findings. A mild treatment of cells with trypsin prior to sonication was found to be very effective in releasing the sulfur-oxidising activity into soluble fractions. Some workers have reported that T. thiooxidans cell wall lacks a typical peptidoglycan layer and is mostly composed of protein (Noguchi etal., 1977; Marunouchi and Mori, 1968). Probably, protein layers of cell wall, make cells resistant to breakage and a mild trypsin treatment disintegrates the protein layers, making the cells vulnerable to sonication. Sonication enhances the release of sulfur-oxidising enzyme from partially disintegrated cell envelope. It's interesting to note that Suzuki (1965) was able to get most of the activity in the soluble fractions of cell free extract, by treating the cells with ion exchange resins prior to sonication. It is possible that such a

deionization treatment made the outer protein layer of cells more susceptible to disintegration upon sonication.

Sulfur-oxidising enzyme was purified to almost electrophoretic homogeneity. It was estimated that the enzyme constituted 2-3% of the total cell protein. Molecular weight of the purified enzyme was determined to be 40,000 $\pm$ 2,000 by gel filtration. The enzyme had a bright yellow color and its spectrum showed absorption maxima around 272, 410, 448nm and a shoulder at 478nm. Enzyme oxidised sulfur with GSH and was devoid of sulfide, thiosulfate or sulfite-oxidising activity. The apparent k_m values of sulfur and GSH were found to be 5.7mM and 2mM respectively. Similar k_m value for GSH has been reported by Suzuki (1965). The optimum pH for activity was between 7.5 and 7.6. Thiosulfate was determined to be the reaction product of this enzyme. Thiosulfate formed and oxygen consumed were found to be in 1:1 ratio in agreement with the characteristics of the purified enzyme of Suzuki and Silver (1966).

The enzyme was found to be a non-heme iron flavoprotein, containing non-heme iron, labile sulfide, flavin and protein in 1.1.1.1 ratio. Extracted flavin showed a spectrum similar to FMN and riboflavin with absorption maxima around 264,365,445nm and a shoulder at 475nm. In the case of the native enzyme absorption maxima around 448nm and 478nm were shifted by 3-5nm towards longer wavelength. Flavin component of the enzyme was tentatively identified as riboflavin by thin layer chromatography. AMP was also found in the enzyme extract. Flavin seemed to be very loosely associated with the enzyme. Passage of the enzyme through sephadex columns with slow elution rates resulted in the loss of the yellow color, absorption around 448nm and activity. This problem was

eliminated by using fast elution rates.

Takakuwa (1975b) also reported a flavoprotein containing non-heme iron and showing an absorption shoulder around 485nm, as a component of his sulfur-oxidising system. Suzuki's (1965) enzyme preparation also had non-heme iron and labile sulfide. Evidence for the participation of non-heme iron in sulfur oxidation was provided by these workers (Suzuki, 1965; Takakuwa, 1975b). Although Takakuwa (1975b) reported the spectrum of the component of his sulfur-oxidising system, flavin was not identified and no evidence was given for the requirement or participation of any flavin in sulfur oxidation. In the present study, not only flavin was identified as riboflavin, an attempt was also made to establish its requirement for sulfur oxidation. The removal of flavin from the enzyme by dialysis resulted in corresponding loss of the sulfur-oxidising activity, which could be restored after incubation of protein for half an hour or more with FMN or riboflavin. A Tris-Cl buffer (pH 7.5) was not very effective in restoring the catalytic activity especially in the case of riboflavin. In a phosphate buffer (pH 7.5) however, a significant amount of activity was restored with both riboflavin and FMN. AMP but not ADP or ATP further increased the riboflavin-stimulated activity of the enzyme. The stimulatory effect of AMP on the sulfur-oxidising enzyme activity was also reported by Suzuki (1965). Addition of flavins to the native enzyme resulted in a considerable inhibition of the activity. Probably an excess flavin interfered in the action of the enzyme. This also explains why some workers found flavins as inhibitory to sulfur oxidation (Suzuki, 1965; Taylor, 1968; Vogler etal, 1942).

To provide further evidence for the participation of flavin in sulfur oxidation, a flavin containing active enzyme was reconstituted

from a flavin-free inactive enzyme and riboflavin plus AMP and was isolated free of excess flavin and AMP by DEAE-cellulose chromatography. Riboflavin bound to the reconstituted enzyme was estimated to be about 90% of its original flavin content, with reappearance of corresponding catalytic activity. The spectrum of reconstituted enzyme was very similar to that of the native enzyme except a shoulder at 475nm instead of 478nm on thus resembling a free flavin spectrum rather than that of native enzyme flavin. The result may indicate that in reconstituted enzyme, the flavin binding to protein was not exactly the same as in the native enzyme.

From this study, it appears that both riboflavin and FMN can interact with the enzyme protein to generate the catalytic activity, although riboflavin is found in the native enzyme. Massey and Curti (1966) have proposed that the flavin interaction with a protein leads to a change of protein conformation which is followed by appearance of the catalytic activity. In this study, the interaction seems to be a slow process and phosphate ions probably enhance or stabilize the change in the protein conformation. Similar conditions have been reported for reconstitution of lipoamide dehydrogenase from the apoprotein and FAD (Visser and Veeger, 1970).

In conclusion, the sulfur-oxidising enzyme of T. thiooxidans was found to be a flavoprotein containing non-heme iron and labile sulfide. Although riboflavin was the true component of the native enzyme, FMN could also interact with the enzyme protein to give it catalytic activity. AMP was also found associated with the purified enzyme and had a stimulatory effect on the catalytic activity of the riboflavin-reconstituted enzyme. I have presented the evidence for the flavin participation in sulfur oxidation but the exact mechanism of sulfur

oxidation involving flavin remains to be elucidated. It is haped that a further research in this area would add significantly to our knowledge of sulfur metabolism of T. thiooxidans.

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