OXIDATION OF ELEMENTAL SULFUR BY THE SULFUR OXIDIZING ENZYME OF *THIOBACILLUS THIOPARUS*

by

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To my grandparents,

Jack and Minnie Weinberg.

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ABSTRACT

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An enzyme that oxidizes elemental sulfur to thiosulfate in the presence of catalytic amounts of reduced glutathione was partially purified from Thiobacillus thioparus. The Michaelis constant for GSH, which could not be replaced by other sulfhydryl compounds, oxidized glutathione or ascorbic acid, was found to be 6.25 mM. Catalase, and catalase and low concentrations of 2:2'-dipyridyl stimulated the reaction, but high concentrations of 2:2'-dipyridyl were inhibitory. Flavine nucleotides, copper, zinc and cobalt were also inhibitory. The enzyme was found to contain iron and was dependent upon this for its activity. When iron was removed, the enzyme was inactivated.

Sulfite was found to be the initial product of the reaction catalyzed by this enzyme. Thiosulfate was formed by a secondary non-enzymatic reaction between sulfur and sulfite.

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INTRODUCTION

INTRODUCTION

Winogradsky established the fundamental principles of autotrophy in bacteria when he published reports on sulfur bacteria in 1887 and iron bacteria in 1888. Autotrophs, by his definition, were organisms capable of the synthesis of all organic compounds needed for growth without the aid of sunlight. The energy requirements of these bacteria would be satisfied completely by the energy liberated from the oxidation of reduced or incompletely oxidized inorganic compounds. Atmospheric CO_2 alone would be used as the source of carbon, and organic carbon compounds would be utilized poorly if at all. There has been much progress in solving the mysteries of autotrophy since Winogradsky discovered this phenomenon. Subsequently this manner of growth has been demonstrated in many more organisms.

Thiobacillus thioparus is a strict autotroph and is the type species of the family Thiobacillaceae. The bacteria in this family derive their energy from the oxidation of elemental sulfur and inorganic sulfur compounds. The individual members of this family are distinguished from one another on the basis of their obligate requirement for CO_2 as the source of carbon, the utilization of atmospheric oxygen as the final electron acceptor and their tolerance to acid. T. thioparus is an obligate aerobe that requires CO_2 and grows optimally at neutral or slightly alkaline conditions.

Although the end product of sulfur and thiosulfate oxidation has been known to be sulfate, the actual mechanism of the complete oxidation

sequence is still not known. Recently sulfite was shown to be oxidized to sulfate either through adenosine phosphosulfate by Peck or through the cytochrome system by Charles and Suzuki. These discoveries would explain the formation of sulfate from the inner moiety of thiosulfate, but at present little evidence has been presented to explain the formation of sulfate from either elemental sulfur or the outer moiety of thiosulfate. It is possible that these forms of sulfur are oxidized by a reaction catalyzed by the sulfur oxidizing enzyme. With this possibility in mind, the characterization of this enzyme and the study of the reactions involved were undertaken.

HISTORICAL

HISTORICAL

Thiobacillus thioparus, a chemoautotrophic bacterium, was discovered by Nathansohn (1902) and isolated by Beijerinck (1904) on a medium containing thiosulfate as the sole energy source. This organism is an obligate aerobe that grows in a neutral environment using reduced sulfur compounds as the source of energy and CO_2 as the source of carbon. With thiosulfate as the energy source, the organism frequently deposits large amounts of elemental sulfur in the medium. Jacobsen (1912) found that this organism was able to use elemental sulfur as the sole energy source. The end product of the oxidation of sulfur and its derivatives has been shown to be sulfate, but very little is known of the mechanism of this reaction.

Nathansohn (1902) initially considered two alternative pathways for the oxidation of thiosulfate. The first, which included elemental sulfur as a key product, was rejected because no stoichiometry could be established between thiosulfate disappearance and sulfur and sulfate formation. The second involved polythionates as intermediates, and was proposed because tetrathionate was observed to accumulate in the culture media. Considerable evidence has been obtained in support of both views and the mechanisms may not be mutually exclusive.

Most of the research on the thiobacilli has been carried out on T. thioparus and Thiobacillus thiooxidans. The latter is distinguished from the former by growing best below pH 5, and its ability to oxidize elemental sulfur at a rate comparable to its oxidation of thiosulfate. In contrast, T. thioparus oxidizes elemental sulfur more slowly than thiosulfate.

A number of ingenious hypotheses have been advanced on the mechanism of bacterial attack on elemental sulfur. Vogler and Umbreit (1941), using a dialyzing membrane, showed that direct contact between the sulfur particles and the bacterial cells was required for oxidation to proceed. They found a direct relationship between sulfur oxidation and the surface area of the sulfur particles. At each end of the cells, they found a droplet of highly unsaturated fat which was responsible for the dipolar staining character of the cells. The sulfur was thought to be dissolved in this fat globule upon contact and taken into the cell for oxidation in this way (Umbreit, Vogel and Vogler, 1942). Knaysi (1943) suggested that the "fat globules" were vacuoles containing volutin which could dissolve sulfur. In this state, the sulfur would possess similar staining qualities and would react similarly in iodine absorption as lipids. Barker and Kornberg (1954) found large amounts of easily hydrolyzed polyphosphates, which were probably responsible for the staining characteristics of volutin in T. thiooxidans. Umbreit and Anderson (1942), in a study with the electron microscope, could not show the presence of bipolar fat bodies.

If intimate contact between the sulfur and the cells is required, one would expect that sulfur oxidation would proceed most rapidly in stationary cultures. However, Starkey, Jones and Frederick (1956) and Newburgh (1954) found an increase in the rate of sulfur oxidation by T. thiooxidans with vigorous agitation. In addition, Knaysi (1943) observed a capsule which, he thought, would not allow close contact between the sulfur particles and the bacteria, but the frequency with which this capsule occurs is in doubt.

Diffusion and reduction to sulfide have both been suggested as mechanisms for transport of sulfur into the cells. Vishniac and Santer (1957)

state that diffusion is unlikely due to the insolubility of sulfur in water.

Starkey (1937) considered it unlikely that elemental sulfur undergoes hydrogenation before entering the cell due to the fact that little sulfide was detected either with T. thiooxidans (Starkey, 1935) or T. thioparus (Starkey, 1936). He thought that the energy required for this reduction would be greater than the energy that could be obtained from the oxidation of H_2S to sulfate. He suggested, however, that the sulfide might be formed through a mechanism similar to that proposed by Sluiter (1930). She found a reduction of elemental sulfur by reduced glutathione, forming sulfide and oxidized glutathione according to the following equation:

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

Glutathione, a tripeptide with a sulfhydryl group that can reduce sulfur, was discovered in yeast, animals and plants, and was investigated by Hopkins (1921, 1929). Callow and Robinson (1925) discovered this compound in bacterial cells. The oxidized glutathione can then be reduced by a TPNH-linked glutathione reductase according to the following equation:

$$TPNH + H + GSSG \longrightarrow TPN^+ + 2GSH$$

This enzyme has been found in yeast (Meldrum and Tarr, 1935; Racker, 1955), plant tissues (Mapson and Goddard, 1951; Conn and Vennesland, 1951), mammalian tissues, (Rall and Lehninger, 1952; Racker, 1955), E. coli (Asnis, 1955) and T. thiooxidans (Suzuki and Werkman, 1959).

Suzuki and Werkman (1959) showed that cell-free extracts of T. thiooxidans oxidized elemental sulfur only in the presence of substrate quantities of GSH. Since these extracts also oxidized sulfide, it was suggested that elemental sulfur is first reduced according to the reaction:

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

after which the sulfide formed was oxidized to thiosulfate by a reaction postulated by Baxter and van Reen (1958) as follows:

$$2H_2S + 2O_2 \longrightarrow H_2S_2O_3 + H_2O$$

Glutathione reductase (Suzuki and Werkman, 1959) would then convert GSSG to GSH.

Peck (1960) and Peck and Fisher (1962), working with T. thioparus grown on thiosulfate, demonstrated a requirement for GSH in the cell-free oxidation of thiosulfate. They postulated a reductive cleavage of thiosulfate to sulfite and sulfide by thiosulfate reductase as the initial step in the oxidation of thiosulfate by the organism. The formation of sulfite and sulfide from thiosulfate by such a mechanism had been observed by Kaji and McElroy (1959) to take place in yeast.

Using whole cells and thiosulfate labelled with radioactive sulfur either at the inner sulfur atom ($SS^{35}O_3^{=}$) or at the outer sulfur atom ($S^{35}SO_3^{=}$), Santer, et al (1960) and Peck and Fisher (1962) demonstrated the ability of T. thioparus to oxidize both sulfur atoms to sulfate.

Suzuki and Lees (1964) and Suzuki (1965) reinvestigated the sulfur oxidation in T. thiooxidans, and showed the requirement for catalytic rather than substrate quantities of GSH. If sulfide was formed from elemental sulfur, and this was oxidized to thiosulfate according to the reaction:

$$2S + 4GSH + 2O_2 \longrightarrow 2GSSG + H_2S_2O_3 + H_2O$$

then 1.25 μmoles of thiosulfate would be formed from 5 μmoles of GSH with the uptake of 2.5 μmoles of O_2. Suzuki (1965) found that in actual experiments, over 20 times more than the predicted amount of thiosulfate was formed. The oxygen uptake was always approximately equal to the thiosulfate

formed. The equation of this reaction is as follows:

$$2S + O_2 + H_2O \xrightarrow{GSH} H_2S_2O_3$$

From results of O^{18} experiments, Suzuki (1965a) tentatively identified the sulfur oxidizing enzyme as an oxygenase. According to the definition by Hayaishi (1962), the enzyme seems to be a true oxygenase rather than a hydroxylase because both atoms of the oxygen molecule are incorporated into the substrate, and GSH is a cofactor and not an electron donor.

Suzuki (1965a) postulated a series of reactions in which a polysulfide of GSH (GSS_nH) is the initial intermediate between sulfur (S_n) and GSH. This intermediate could take up oxygen to form thiosulfate. The series of reactions postulated are as follows:

$$S_n + GSH \longrightarrow GSS_nH$$

$$GSS_nH + O_2 \longrightarrow GSS_{n-1}SO_2H$$

$$GSS_{n-1}SO_2H + H_2O \longrightarrow GSS_{n-2}H + H_2S_2O_3$$

It was suggested that sulfite might be the initial product of the reaction (Suzuki, 1965). This would be converted to thiosulfate when incubated with sulfur under the experimental conditions according to the following equation:

$$S + H_2SO_3 \longrightarrow H_2S_2O_3$$

The sulfur oxidizing enzyme has been demonstrated in both T. thiooxidans and T. thioparus and seems, therefore, to play an important role in the oxidation of both sulfur and thiosulfate.

MATERIALS AND METHODS

MATERIALS AND METHODS

Organism and Growth of Organism

Thiobacillus thioparus (ATCC #8158) was used in this investigation. The stock culture and inocula were grown in 500 ml Erlenmeyer flasks on a rotary shaker with 200 ml Starkey's medium No. 2 (Starkey, 1934). Large scale growth was carried out in 10 and 15 litre aerated carboys with the same medium. The composition of this medium per litre is as follows:

1.0 g	$Na_2S_2O_3 \cdot 5H_2O$
4.0 g	KH_2PO_4
4.0 g	K_2HPO_4
0.05-0.1 g	$CaCl_2$
0.3 g	$(NH_4)_2SO_4$
0.02 g	$FeCl_3 \cdot 6H_2O$
0.02 g	$MnSO_4 \cdot 2H_2O$
0.3 ml	Phenol red (2%)

The pH was adjusted to pH 7.0-7.5 with 10% K_2CO_3 and the volume was made up to 1000 ml with water.

For growth of stock culture and inocula, the medium, minus thiosulfate, was sterilized at 120°C for 30 min. Thiosulfate was sterilized separately by filtration of a 10% solution and added to the cooled medium. No sterilization was carried out on the medium in the carboys. After 5-7 days growth at room temperature with periodic neutralization with 10% K_2CO_3, the cells were harvested in a Sharples centrifuge.

Chemicals

The following chemicals were obtained from commercial sources:

Glutathione, reduced form (GSH) - Sigma Chemical Company

Catalase from bovine liver, twice crystallized, 20 mg/ml,
- Sigma Chemical Company

2:2'-dipyridyl - The British Drug Houses Limited

Sodium diethyldithiocarbamate - Fisher Scientific Company

Flavine adenine dinucleotide (FAD) - Sigma Chemical Company

Flavine mononucleotide (FMN) - Sigma Chemical Company

Cleland's reagent (dithiothreitol) - California Corporation for
Biochemical Research

Sulfur - J. T. Baker Chemical Company

Atebrin (quinacrine dihydrochloride) - Mann Research Laboratories, Inc.

All reagents and buffers were prepared in glass distilled water. The elemental sulfur was suspended in water containing 0.05% Tween 80 to make a final concentration of 240 mg/ml. This suspension was sonicated for 30 min in a 10 kc/sec Raytheon sonic oscillator and dialyzed overnight in 0.05% Tween 80 to remove any contaminating ions.

Preparation of Cell-Free Extract

After harvesting, the cells were washed and decanted twice with distilled water to remove residual sulfur. Cells were collected by centrifugation of the decanted liquid, washed in 0.2 M Tris-HCl buffer (pH 7.8), and centrifuged again. This was repeated until little or no sulfur remained with the cells. The cells were stored in the centrifuge tubes at -20°C until needed. Yield was 0.50-0.75 g wet weight of cells per litre.

Cell-free extracts were prepared by sonication of the cells. A 10-15% weight/volume suspension was made by stirring 3-7 g of frozen cells with 25-50 ml of 0.2 M Tris-HCl buffer (pH 7.4). Disintegration was carried out in a 10 kc/sec Raytheon sonic oscillator for 20 min under a N_2 atmosphere at 7°C. Cell debris was removed by centrifugation at 23,500 x g for 20 min.

Enzyme Purification Procedure

The enzyme was purified by a method similar to that used in the purification of the sulfur oxidizing enzyme of T. thiooxidans (Suzuki, 1965a). The cell-free extract was adjusted to pH 5.0 with N acetic acid and the precipitate removed by centrifugation at 23,500 x g for 20 min. Absolute ethanol was added to the supernatant to a final concentration of 15% with care being taken to keep the temperature near -10°C. After two hours storage at this temperature, the precipitate was removed by centrifugation at -10°C at 23,500 x g for 20 min and discarded. Additional absolute ethanol was added to the supernatant to a final concentration of 30%, and this was allowed to stand for 6 hours or overnight at -10°C. The precipitate was collected by centrifugation at -10°C at 23,500 x g for 20 min and suspended in 0.05 M or 0.2 M Tris-HCl buffer (pH 7.8). It was this fraction that was used for the greater part of these experiments.

Further purification was achieved by adsorption of the enzyme on DEAE-cellulose and subsequent elution with Tris-HCl buffer (pH 7.5). The 15-30% ethanol precipitate in 0.05 M Tris-HCl buffer (pH 7.8) was stirred for 30 min at 4°C with an equal volume of DEAE-cellulose in 0.05 M Tris-HCl buffer (pH 7.5). After centrifugation at 23,500 x g for 20 min at 0°C,

the DEAE-cellulose-enzyme suspension was washed once with 0.05 M Tris-HCl buffer (pH 7.5), centrifuged, washed once with 0.10 M Tris-HCl buffer (pH 7.5), and centrifuged again. Washing was carried out each time by stirring the suspension for 30 min at 4°C with a volume of buffer equal to the initial volume of the 15-30% ethanol fraction treated. The enzyme was then eluted by repeating this process with 0.25 M Tris-HCl buffer (pH 7.5). Protein was determined by the method of Lowry et al (1951).

Enzyme Assay Procedure

All manometric experiments were carried out at 30°C in a conventional Warburg apparatus with air as the gas phase. Unless otherwise indicated, the reaction mixture contained the following in a total volume of 2.0 ml:

500 µmoles	Tris-HCl buffer (pH 7.8)
48 mg	Sulfur
5 µmoles	GSH
250 µg	Catalase
0.2 µmole	2:2'-dipyridyl
	Enzyme as indicated

The reaction was started by tipping the GSH from one of the side arms into the main flask. After incubation for 210 min, unless otherwise indicated, thiosulfate was determined by the methods of Goldman and Yagoda (1934) and/or Sörbo (1957, 1958). Where applicable, sulfite was determined by the methods of Goldman and Yagoda (1934) and/or Trüper and Schlegel (1964).

Determination of Thiosulfate and Sulfite by Iodine Titration

Thiosulfate and sulfite were measured by a modification of the method of Goldman and Yagoda (1934). A 1.0 ml portion of the reaction mixture was treated with 1.0 ml of 0.8% uranyl acetate to precipitate glutathione and protein and centrifuged. The supernatant was incubated with 0.1 ml of 40% formaldehyde for 5 min to bind the sulfite. The mixture was then titrated to a blue end point with 0.01 N iodine with a starch indicator. Five ml of sodium carbonate buffer (80 g sodium carbonate dissolved in 500 ml of water, after addition of 20 ml of glacial acetic acid) was then added to break the formaldehyde-bisulfite complex and the solution was again titrated to a blue end point with iodine. The first titration value gave the amount of thiosulfate, the second value gave the amount of sulfite.

Colorimetric Determination of Thiosulfate

Thiosulfate was determined colorimetrically by the method of Sörbo (1957, 1958). A portion containing 1-2 µmoles of thiosulfate was made up to 1.0 ml with water and 0.1 ml of M cadmium acetate was added to precipitate GSH and protein, which were then centrifuged off. The following reagents were added in this order:

2.0 ml	0.2 N NH_4OH
1.2 ml	Water
0.5 ml	0.1 M KCN
0.3 ml	0.1 M $CuCl_2$
0.5 ml	Ferric nitrate reagent (20% $Fe(NO_3)_3$ in 3 N HNO_3)

The intensity of the colour was measured on a Klett-Summerson colorimeter with a 42 KS (blue) filter and compared to a standard. If formaldehyde were present, the sample was frozen and stored at -20°C for a few hours. This treatment would polymerize formaldehyde to trioxymethylene, which would not cause interference.

Determination of Sulfite with Acid Fuchsin

Sulfite was determined by a modification of the method of Trüper and Schlegel (1964). The sample, 3.0 ml containing 0.1-0.3 µmoles of sulfite was deproteinized with 1.0 ml of 1% zinc acetate and centrifuged. To break the formaldehyde-bisulfite complex, 0.1 ml of N NaOH was incubated with the supernatant at room temperature for 30 min. The volume was adjusted to 4.0 ml with water and 0.5 ml of acid fuchsin reagent (40 mg acid fuchsin per 100 ml of 12.5% H_2SO_4) was added and shaken. After 10 min, 0.05 ml of 40% formaldehyde was added. After another 20 min, the optical density was measured at 570 mµ on a Unicam SP-700 spectrophotometer. The blank used was an identical mixture with no sulfite. Standards of 0.1 and 0.2 µmoles of sulfite were used in every series of determinations.

Determination of Iron in the Enzyme

The iron in the enzyme preparation was determined by measuring the intensity of the ferrous-2:2'-dipyridyl complex by a modification of methods described by Rajagopalan and Handler (1964) and Massey (1957).

A 0.5 ml portion of the enzyme eluted from DEAE-cellulose was treated with 0.05 ml of 50% trichloroacetic acid to release the iron from the enzyme.

Denatured protein was removed by centrifugation. The supernatant was added to 1.5 ml of water and 0.2 ml of saturated ammonium acetate was then added. If total iron, rather than ferrous iron was to be measured, the ferric iron was reduced by 0.1 ml of freshly prepared 2% sodium dithionite. After the addition of 0.1 ml of 0.01 M 2:2'-dipyridyl, the optical density was measured at 520 mμ on a Unicam SP-700 spectrophotometer.

Determination of Copper in the Enzyme

The copper in the enzyme was determined by two methods. The first of these was the spectrophotometric measurement of the copper-diethyldithiocarbamate complex. The sample, 2.0 ml, was treated with 0.1 ml of 10^{-3} M sodium diethyldithiocarbamate. The intensity of the yellow-brown colour was measured immediately at 436 mμ on a Unicam SP-700 spectrophotometer and compared to standards of 0.1 and 0.2 μmole.

The second method of analysis was carried out by Dr. K. Ramlal, Geology Department, University of Manitoba, on a Perkin Elmer Model 303 Atomic Absorption Flame Spectrophotometer. Absorption by the enzyme was measured at 325 mμ and compared to standards of 1, 3, 6 and 8 p.p.m.

Determination of Labile Sulfide in the Enzyme

The labile sulfide in the enzyme was determined by a modification of the method of Fogo and Popowsky (1949). The sample, 0.65 ml containing 0.05-0.4 μmole of sulfide, was deproteinized with an equal volume of 2% zinc acetate and centrifuged. To the supernatant, 2.5 ml of 0.1% p-aminodimethylaniline sulfate in 5.5 M HCl and 0.5 ml of 0.023 M $FeCl_3$ in 1.2 M

HCl were added in a screw cap test tube and shaken. After 30 min, the intensity of the methylene blue dye that was formed was measured at 670 mμ on a Unicam SP-700 spectrophotometer and compared to a standard curve.

RESULTS

RESULTS

Enzyme Purification

Results of the enzyme purification are shown in Table 1. In this purification procedure, specific activity is expressed as the number of μmoles of thiosulfate formed in 210 min per milligram of protein.

The crude extracts of T. thioparus had relatively little activity, probably due to some interfering substance which was largely removed in the first purification step. The 15-30% ethanol precipitate fraction, which was used in all the experiments unless otherwise indicated, was purified approximately 24 fold compared to the cell-free extract. This calculation was made on the assumption that the total activity of the cell-free extract was equal to that of the pH 5 supernatant. This degree of purification was consistent. Upon centrifugation of this fraction at 144,000 x g for 60 min, all activity remained in the supernatant. This fraction contained a small amount of thiosulfate reductase activity and moderate catalase activity.

All fractions could be stored at -20°C at pH 7.5-8.0 for at least two weeks without noticeable loss of activity with the exception of the DEAE-cellulose-treated fraction. With this fraction, loss of activity was detected when the preparation was frozen and thawed. Storage at -20°C for longer than 7 days greatly inactivated the enzyme. No fraction was stable to freezing at pH 5. Neither the cell-free extract nor the 15-30%

TABLE 1

Purification of the sulfur oxidizing enzyme from Thiobacillus thioparus

Fraction	Total protein (mg)	Specific activity
Cell-free extract	2650	0.43
pH 5 supernatant	188	16.6
15-30% ethanol precipitate	150	34.2
DEAE-cellulose-treated fraction	100	53.5

The reaction mixtures for the determination of enzyme activity contained in a total volume of 2.0 ml: 500 μmoles of Tris-HCl (pH 7.8), 250 μg of catalase, 0.2 μmole of 2:2'-dipyridyl, 48 mg of sulfur, 5 μmoles of GSH, and enzyme. The amount of protein used was as follows: crude cell-free extract, 15 mg; pH 5 supernatant, 1.1 mg; 15-30% ethanol precipitate, 0.6 mg; or DEAE-cellulose-treated enzyme, eluted between 0.1-0.25 M Tris-HCl (pH 7.5), 0.4 mg.

ethanol precipitate fraction was stable at 4°C; both showed a loss of approximately half the activity when kept at this temperature overnight.

Half the protein could be removed by heating the 15-30% ethanol precipitate fraction at 50°C for 5 min. This treatment did not raise the specific activity due to some inactivation during this procedure. Little protection was afforded by the addition of sulfur and GSH, thiosulfate, or sulfite and EDTA. The activity was almost totally destroyed by heating this enzyme preparation at 60°C for 5 min.

Attempts to purify the enzyme with ammonium sulfate precipitation were unsuccessful due to severe loss of activity.

Linear gradient chromatography (0.05-0.20 M Tris-HCl (pH 7.5)) through a DEAE-cellulose column of the 15-30% ethanol precipitate fraction which had been centrifuged at 27,500 x g for 60 min did not raise the specific activity of this preparation due to considerable inactivation during this procedure. GSH, GSH and sulfite, sulfite and EDTA, thiosulfate, 2-mercaptoethanol, gelatin, or Cleland's reagent (dithiothreitol) gave little protection to the enzyme in this treatment.

Effect of Enzyme Concentration

The relationship between the amount of thiosulfate formed and the amount of enzyme used in the reaction mixture is shown in Fig. 1. The relationship was linear at low concentrations of enzyme. At high concentrations, however, the linear relationship was lost due to the limiting concentration of GSH.

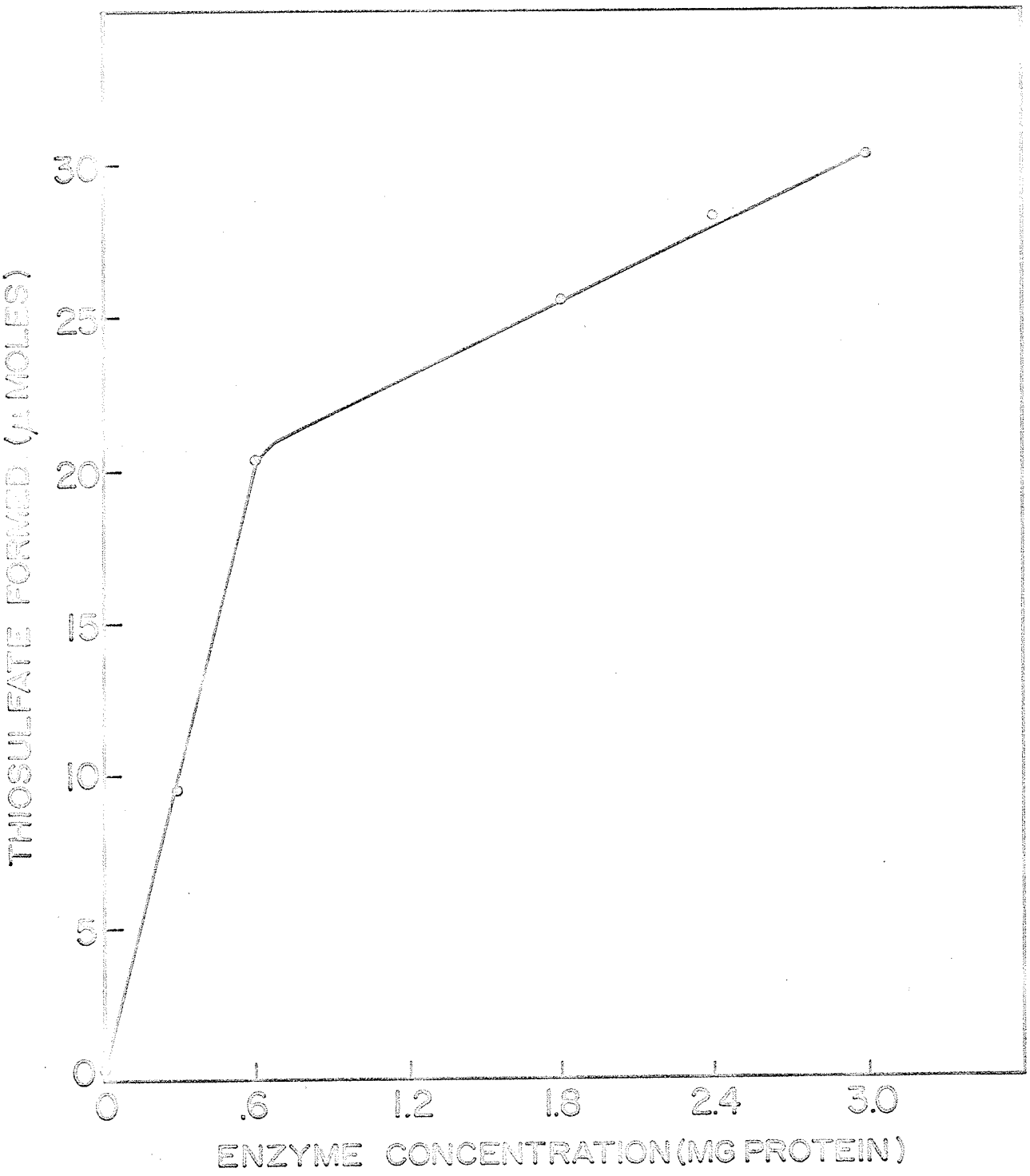

FIG. 1 EFFECT OF ENZYME CONCENTRATION ON SULFUR OXIDIZING ACTIVITY.

Effect of GSH Concentration on the Enzymatic and Non-Enzymatic Oxidation of Sulfur to Thiosulfate

The effect of GSH concentration on both the enzymatic and non-enzymatic oxidation is shown in Table 2 and Fig. 2. It should be noted that the ratio of thiosulfate formed to the oxygen consumed was always approximately 1.

Although the optimum concentration of GSH was higher, most of the experiments hereafter described were carried out at a concentration of 2.5×10^{-3} M (5 μmoles/2 ml) in order to avoid complications caused by the non-enzymatic reaction.

The apparent K_m of the enzyme was calculated to be 6.25 mM on the basis of the Lineweaver-Burk plot of the reaction velocity versus the concentration of GSH (Fig. 3).

Specificity for GSH as Cofactor

The requirement for GSH is specific, as shown in Table 3. Cysteine, 2-mercaptoethanol, 2,3-dimercaptopropanol (B.A.L.), sulfide, GSSG, and ascorbic acid did not replace GSH in the reaction mixture.

Effect of 2:2'-Dipyridyl Concentration

Both ferrous and ferric ions were found to be inhibitory, the former being more so than the latter. This inhibition occurred only after prolonged incubation periods. This was apparently due to the removal of GSH through oxidation to GSSG, because with the subsequent addition of GSH, the

TABLE 2

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

	Non-enzymatic		Enzymatic*	
GSH μmoles	O_2 uptake μmoles	$S_2O_3^=$ formation μmoles	O_2 uptake μmoles	$S_2O_3^=$ formation μmoles
0	0.0	0.0	0.0	0.14
1	0.0	0.4	4.0	3.85
3	0.0	0.7	12.0	14.2
5	0.0	0.9	16.4	16.0
10	1.5	1.5	22.2	22.2
20	3.3	3.6	38.0	36.0

The reaction mixtures contained in a total volume of 2.0 ml: 500 μmoles of Tris-HCl (pH 7.8), 250 μg of catalase, 0.2 μmole of 2:2'-dipyridyl, 48 mg of sulfur, GSH as indicated, and 3.0 mg of enzyme where indicated(*).

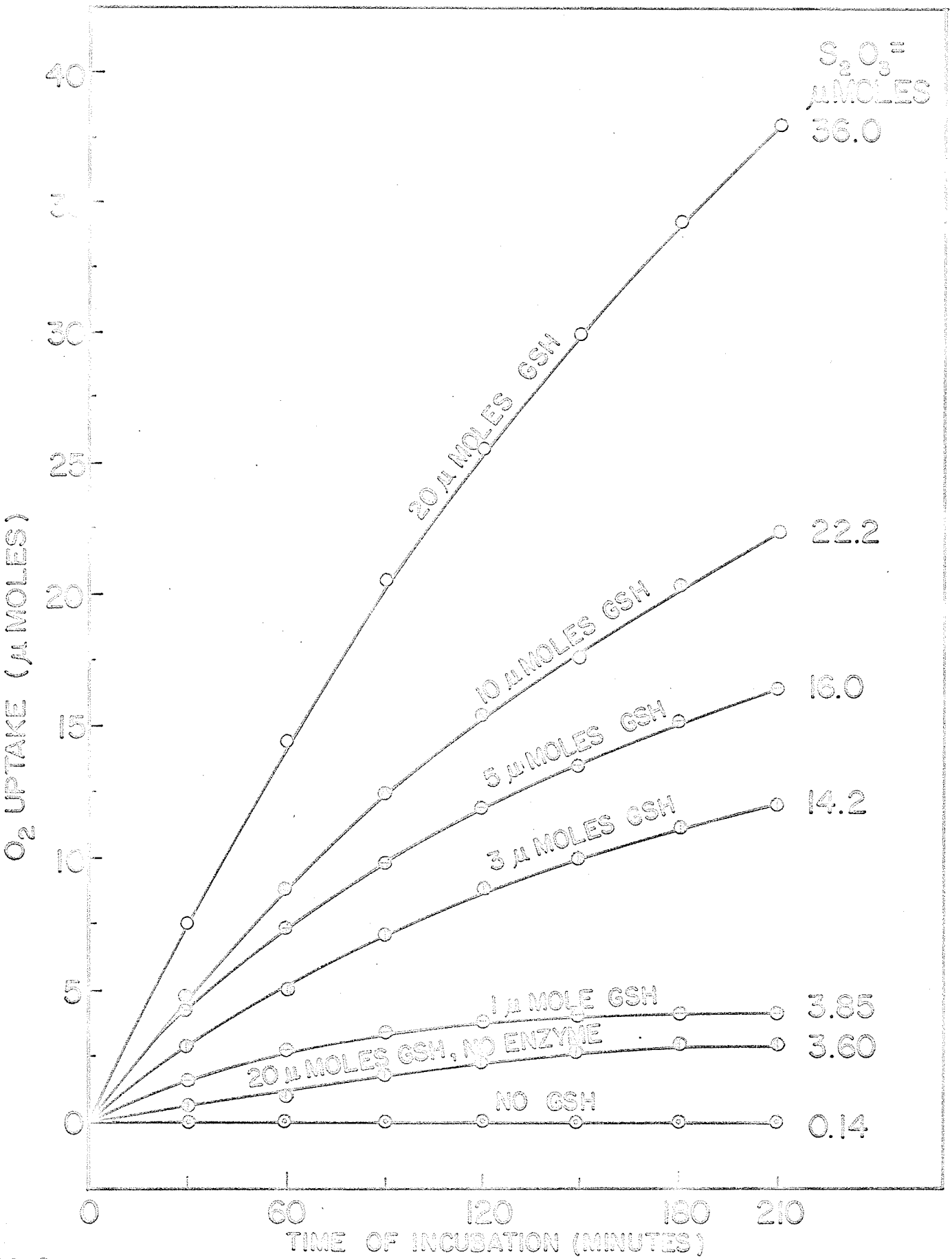

FIG. 2 EFFECT OF GSH CONCENTRATION ON THE ENZYMATIC AND NON-ENZYMATIC OXIDATION OF SULFUR TO THIOSULFATE.

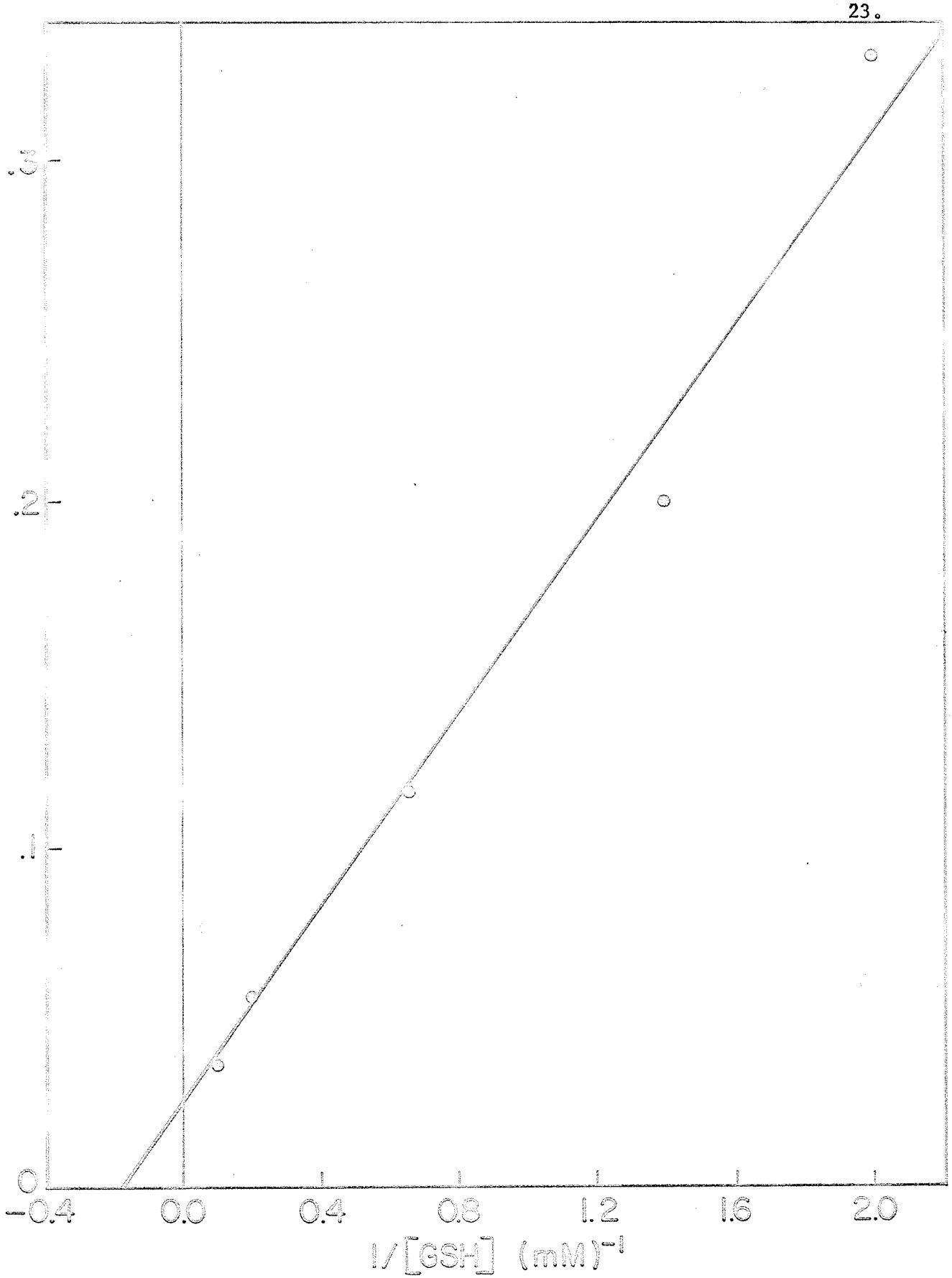

FIG. 3 LINEWEAVER-BURK PLOT OF THE EFFECT OF GSH CONCENTRATION.

TABLE 3

Specificity for GSH as cofactor for the sulfur oxidizing enzyme

Additions	Non-enzymatic		Enzymatic*	
	O_2 uptake μmoles	$S_2O_3^=$ formation μmoles	O_2 uptake μmoles	$S_2O_3^=$ formation μmoles
GSH	3.3	3.6	38.0	36.0
Cysteine	0.9	0.4	1.5	0.5
2-mercaptoethanol	1.9	0.5	4.0	1.2
Na_2S	4.3	2.2	3.2	2.5
GSSG	---	---	0.0	1.3
Ascorbate	0.0	0.2	0.0	0.2
B.A.L.	0.9	0.9	1.1	1.4

The reaction mixtures contained in a total volume of 2.0 ml: 500 μmoles of Tris-HCl (pH 7.8), 250 μg of catalase, 0.2 μmole of 2:2'-dipyridyl, 48 mg of sulfur, 20 μmoles of cofactor, and 3.0 mg of enzyme where indicated(*).

reaction resumed its initial rapid rate of oxidation. The inhibition by iron could be reversed by the addition of 10^{-4} M 2:2'-dipyridyl. In fact, 2:2'-dipyridyl at low concentration stimulated the reaction by removing extraneous iron present in the enzyme preparation.

The effect of increasing concentrations of 2:2'-dipyridyl is shown in Table 4 and Fig. 4. After 210 min, another 5 μmoles of GSH was added to the reaction mixture, and the reaction was allowed to proceed for another 90 min. The GSH caused the reactions to resume the initial rates, showing that 2:2'-dipyridyl protects GSH from oxidation to GSSG by the iron in the enzyme preparation. The optimal concentration of 2:2'-dipyridyl is shown to be 10^{-4} M. The enzyme is inhibited by 2:2'-dipyridyl at higher concentrations apparently due to the removal of iron bound to the enzyme.

Specificity for 2:2'-Dipyridyl as Chelator

The most effective chelating agents were 2:2'-dipyridyl and ortho-phenanthroline, both specific binders of iron. Versene (EDTA) and sodium diethyldithiocarbamate were inhibitory to the reaction, as shown in Fig. 5. All chelating agents were present in the reaction mixture at 10^{-4} M final concentration.

Reactivation of DEAE-Cellulose-Treated Fraction With Iron

The enzyme was stirred for 30 min at 4°C with 2:2'-dipyridyl (10^{-1} M final concentration) in an effort to remove iron from the enzyme. The 2:2'-dipyridyl was separated from the enzyme preparation by treatment with DEAE-

TABLE 4

Effect of 2:2'-dipyridyl concentration on sulfur oxidation

Concentration of 2:2'-dipyridyl	O_2 uptake μmoles	$S_2O_3^=$ formation μmoles
nil	25.2	22.9
5×10^{-5} M	34.0	34.1
10^{-4} M	34.6	34.8
5×10^{-4} M	32.4	33.3
10^{-3} M	29.0	32.4
10^{-2} M	22.0	21.4

The reaction mixture contained in a total volume of 2.0 ml: 500 μmoles of Tris-HCl (pH 7.8), 250 μg of catalase, 48 mg of sulfur, 5 μmoles of GSH, 3.0 mg of enzyme, and 2:2'-dipyridyl as indicated.

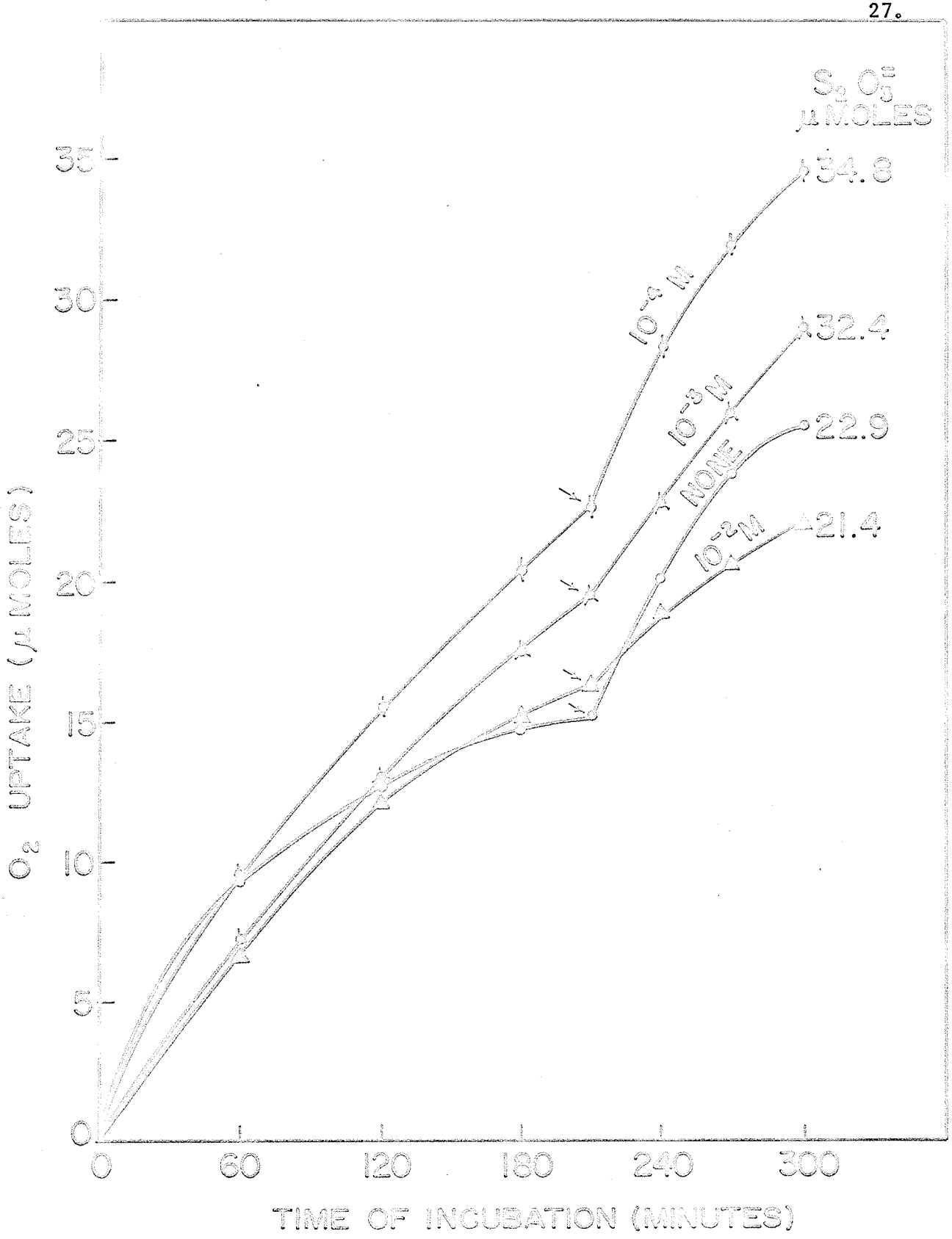

FIG. 4 EFFECT OF 2:2'-DIPYRIDYL CONCENTRATION ON SULFUR OXIDIZING ACTIVITY.

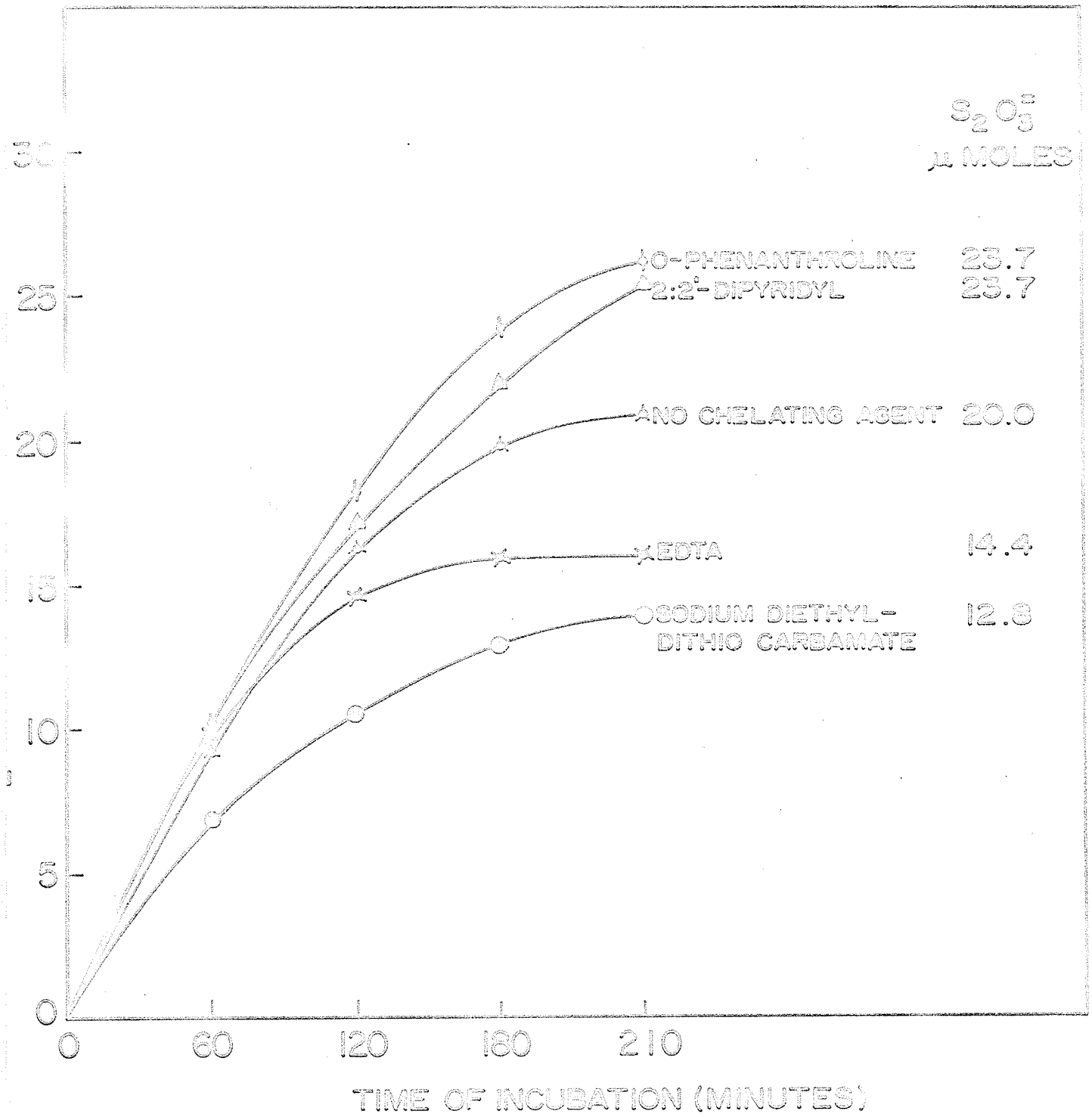

FIG.5 EFFECT OF DIFFERENT CHELATING AGENTS ON SULFUR OXIDIZING ACTIVITY.

cellulose as described in the enzyme purification procedure of the materials and methods section. The DEAE-cellulose-enzyme suspension was washed with 40 ml of 0.05 M Tris-HCl buffer (pH 7.5) and 40 ml of 0.10 M Tris-HCl buffer (pH 7.5) during the purification procedure. The enzyme, eluted with 0.25 M Tris-HCl buffer (pH 7.5), could be stimulated by ferrous iron at a final concentration of 10^{-5} M, and inhibited at a final concentration of 10^{-4} M. The results of these reactions are shown in Table 5.

Enzyme treated similarly with sodium diethyldithiocarbamate, a chelating agent specific for copper, could not be stimulated by the addition of cupric ions. Iron was strongly inhibitory to the enzyme treated in this way.

Iron, Copper and Labile Sulfide in the Enzyme

The iron in the DEAE-cellulose-treated fraction was determined as previously described. It was found that each milligram of protein contained 0.087 µmole of iron, of which 0.055 µmole was in the reduced ferrous state.

Iron could be removed from the 15-30% ethanol precipitate fraction which had been incubated with 2:2'-dipyridyl at a final concentration of 10^{-4} M for 30 min at 4°C by passage through a column of Sephadex G-25. The sulfur-oxidizing activity was totally lost. This activity could not be restored by the addition of iron to the reaction mixture at a final concentration of 10^{-4} M.

The spectrum of the DEAE-cellulose-treated fraction showed one absorption peak only, that of the 280 mµ peak of protein. No haeme iron was shown to be in the enzyme preparation.

TABLE 5

Reactivation of the DEAE-cellulose fraction with iron

Final concentration of Fe^{++}	O_2 uptake μmoles	$S_2O_3^{=}$ formation μmoles
nil	13.0	12.5
10^{-5} M	14.4	14.1
10^{-4} M	10.5	10.3

The reaction mixtures contained in a total volume of 2.0 ml: 500 μmoles of Tris-HCl (pH 7.8), 250 μg of catalase, 48 mg of sulfur, 5 μmoles of GSH, ferrous iron to the final concentration as shown, and 0.4 mg of enzyme. The enzyme was treated as follows: 2.0 ml of the 15-30% ethanol precipitate fraction was treated with 2:2'-dipyridyl at a final concentration of 10^{-1} M for 30 min, after which it was treated with DEAE-cellulose as described in the text.

Copper could not be detected by either of the methods described in the materials and methods section.

The labile sulfide was measured by a modification of the methylene blue method (Fogo and Popowsky, 1949) which was described in the materials and methods section. The DEAE-cellulose-treated fraction contained 0.018 μmole of labile sulfide per mg of protein. This was very similar to the concentration of labile sulfide formed in the ethanol precipitate fraction.

Effect of Catalase Concentration

Although moderate catalase activity was present in the cell-free extract, pH 5 supernatant, and 15-30% ethanol precipitation fraction, additional catalase was shown to be stimulatory. Fig. 6 shows that this stimulation is more pronounced after long incubation periods.

Role of Catalase in the Stimulation of Sulfur Oxidation

In order to investigate the role of catalase in the stimulation of enzyme activity, the reactions were carried out with 10 μmoles of H_2O_2, catalase, and a second addition of 5 μmoles of GSH added at various times. All the flasks initially contained the standard reaction mixture of buffer, sulfur, catalase, 2:2'-dipyridyl, enzyme and GSH as described in the materials and methods section unless otherwise indicated.

The reactions were carried out as follows:

Flask 1: Control reaction; additional GSH was added after 120 min.

Flask 2: Control reaction; no additions.

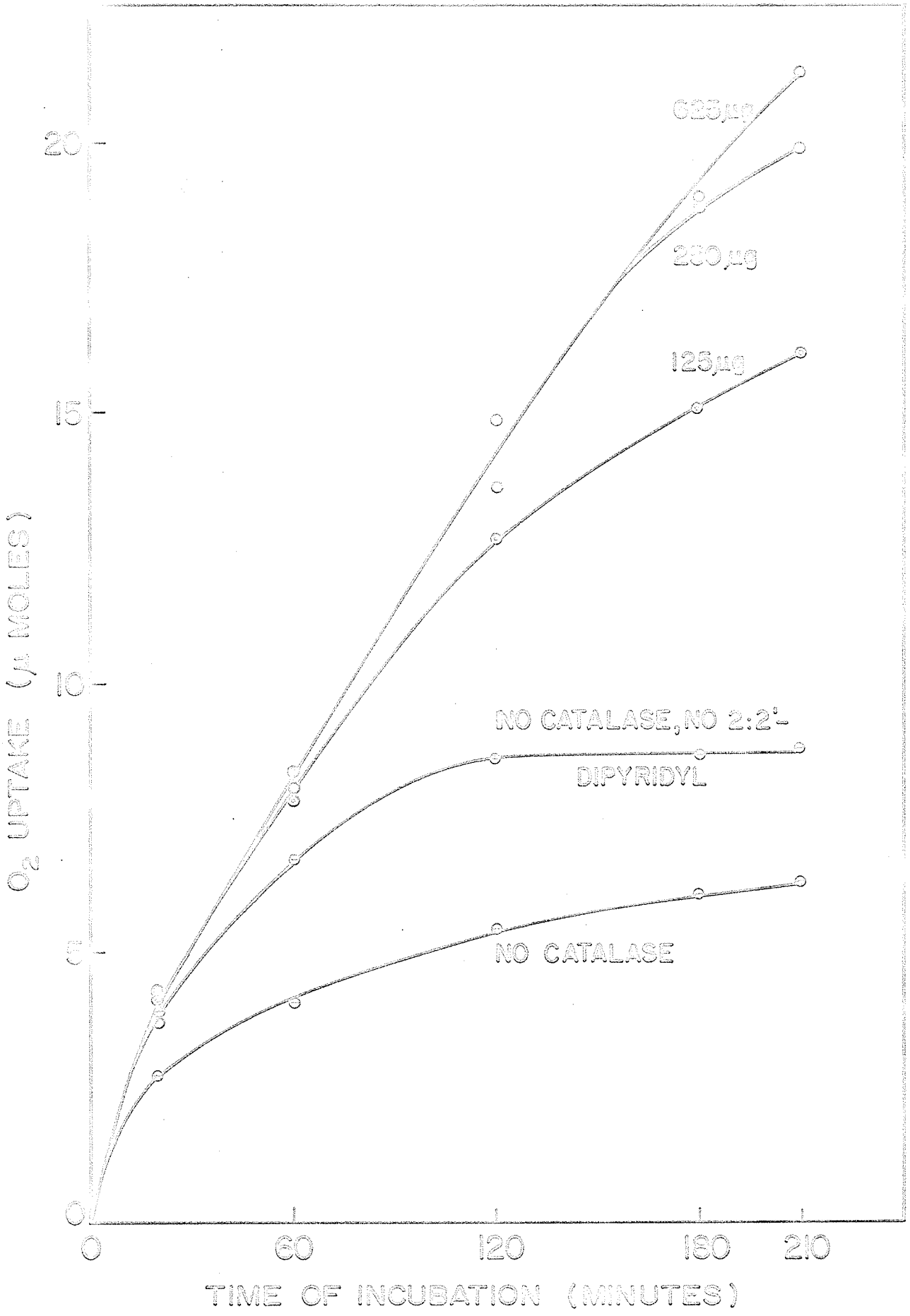

FIG.6 EFFECT OF CATALASE CONCENTRATION ON SULFUR OXIDIZING ACTIVITY

Flask 3: Catalase was not present initially. H_2O_2 was added after 60 min and catalase was added after 90 min. Additional GSH was added after 120 min.

Flask 4: H_2O_2, but not catalase was added initially. Catalase was added after 90 min, and additional GSH was added after 120 min.

Flask 5: Non-enzymatic control reaction; catalase was not present initially. H_2O_2 was added after 60 min, catalase was added after 90 min, and additional GSH was added after 120 min.

Flask 6: H_2O_2 was added initially, catalase was added after 90 min, and additional GSH was added after 120 min.

Oxygen uptake by the reactions in flasks 1, 2, 3 and 4 are plotted graphically in Fig. 7. The oxygen uptake of flask 6 was virtually identical to that of flask 4, indicating immediate oxidation of GSH by H_2O_2 even in the presence of catalase. Flask 5 was used to determine the volume of oxygen evolved from the destruction of H_2O_2, which was subtracted from the readings where necessary.

Additional Inhibition Studies

Flavine nucleotides were inhibitory, FMN being more so than FAD. Atabrine was only slightly inhibitory.

The inhibition of the reaction by iron has been discussed at length previously. Zinc, cobalt and copper also appear to inhibit the reaction,

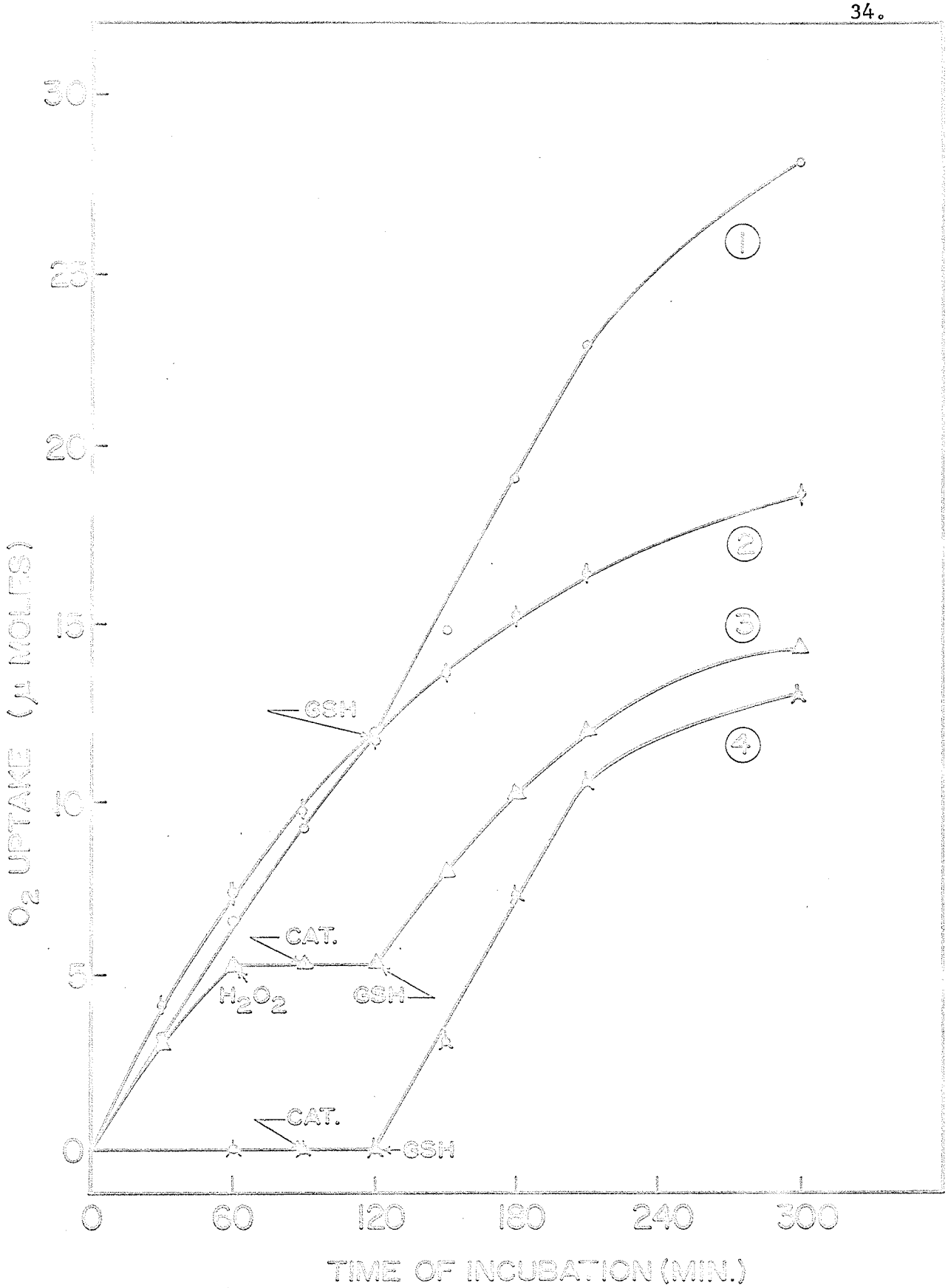

FIG. 7 ROLE OF CATALASE IN THE STIMULATION OF THE OXIDATION OF SULFUR.

not only by the removal of GSH, but also by destruction of the enzyme. This is shown by the inability of additional GSH which was added after 210 min to reinitiate the reaction.

Magnesium and manganese ions were neither stimulatory nor inhibitory.

Initial Product of the Reaction

In order to determine whether thiosulfate or sulfite is the initial product of sulfur oxidation, the reaction was carried out in the presence of formaldehyde. Under these conditions, sulfite would be trapped as the formaldehyde-bisulfite complex. As shown in Table 6, there was a definite trapping of sulfite, which became more efficient with increasing concentrations of formaldehyde. As manifested by the oxygen uptake, little inhibition was caused by formaldehyde below the concentration of 5×10^{-2} M (100 μmoles). Similar results were obtained with the sulfur-oxidizing enzyme of T. thiooxidans. In all experiments, the oxygen consumption equalled the sum of thiosulfate and sulfite produced.

Thiosulfate was determined by iodometric titration (method A) and by the method of Sörbo (1957, 1958) (method B). Sulfite was determined by iodometric titration (method A) and by the method of Trüper and Schlegel (1964) (method B). All have been described previously in the materials and methods section.

Control experiments were carried out; the results of these are shown in Table 7.

TABLE 6

Effect of formaldehyde on products of the sulfur oxidizing system

System	O_2 uptake µmoles	$S_2O_3^=$ µmoles		$SO_3^=$ µmoles	
		A	B	A	B
T. thioparus enzyme					
S + GSH	29.1	28.9	29.6	0.5	0.5
S + GSH + HCHO (10*)	26.5	20.0	21.8	5.5	5.8
S + GSH + HCHO (50*)	25.6	13.3	15.9	10.5	12.3
S + GSH + HCHO (100*)	22.4	8.0	8.7	14.7	16.4
S + GSH + HCHO (200*)	13.7	1.6	1.4	13.5	13.6
T. thiooxidans enzyme					
S + GSH	11.2	12.4	12.5	0.2	0.5
S + GSH + HCHO (50*)	11.4	8.8	7.0	2.6	2.2
S + GSH + HCHO (100*)	10.7	6.6	5.3	5.2	4.6

* Micromoles.

The reaction mixtures contained in a total volume of 2.0 ml: 500 µmoles of Tris-HCl (pH 7.8), 250 µg of catalase, 0.2 µmole of 2:2'-dipyridyl, 48 mg of sulfur, 5 µmoles of GSH, 3.0 mg of T. thioparus enzyme or 2.5 mg of T. thiooxidans enzyme, and other additions as indicated. Thiosulfate and sulfite were determined by the methods A or B, as described in the text.

TABLE 7

Control experiments carried out on the sulfur oxidizing-formaldehyde system

System	O_2 uptake μmoles	$S_2O_3^=$ μmoles		$SO_3^=$ μmoles	
		A	B	A	B
T. thioparus enzyme					
S	0.0	0.0	0.0	0.0	0.0
S + HCHO (50*)	0.0	0.0	0.0	0.0	0.0
$Na_2S_2O_3$ (20*) + GSH	2.5	19.4	19.2	0.5	0.5
$Na_2S_2O_3$ (20*) + GSH + HCHO (50*)	2.0	19.6	18.6	2.7	2.6
GSH	2.0	0.8	0.6	0.0	0.0
non-enzymatic					
S + GSH	0.5	1.8	1.1	0.0	0.0
S + GSH + HCHO (50*)	0.4	0.8	0.8	1.0	1.1
$Na_2S_2O_3$ (20*) + GSH	1.5	20.0	19.4	0.5	0.5
$Na_2S_2O_3$ (20*) + GSH + HCHO (50*)	0.5	20.0	19.8	1.5	1.4
S + Na_2SO_3 (10*)	---	10.0	9.6	0.0	0.0
S + Na_2SO_3 (10*) + GSH	---	10.4	9.6	0.0	0.0

* Micromoles.

The reaction mixtures contained in a total volume of 2.0 ml: 500 μmoles of Tris-HCl (pH 7.8), 250 μg of catalase, 0.2 μmole of 2:2'-dipyridyl. Where indicated, 48 mg of sulfur, 5 μmoles of GSH, 50 μmoles of formaldehyde, 20 μmoles of $Na_2S_2O_3$, 10 μmoles of Na_2SO_3, and 3.0 mg of T. thioparus enzyme were also included. Thiosulfate and sulfite were determined by the methods A and B, as described in the text.

DISCUSSION

DISCUSSION

The results obtained in the course of this investigation show that extracts of T. thioparus contain an enzyme which oxidizes elemental sulfur in the presence of GSH. GSH is required in catalytic and not substrate quantities, and could not be replaced by oxidized glutathione (GSSG) or other compounds containing free sulfhydryl groups. The sulfur-oxidizing activity of this organism and T. thiooxidans (Suzuki, 1965) could be completely separated from glutathione reductase and sulfide-oxidizing activities.

The enzyme responsible for the oxidation of sulfur appears to be a true oxygenase (Hayaishi, 1962) containing non-haeme iron and labile sulfide. It was difficult to estimate the number of iron atoms or labile sulfide atoms bound to each molecule of enzyme because the enzyme was only partially purified. Iron is apparently tightly bound to the protein since it was only partially removed at 10^{-1} M 2:2'-dipyridyl. The removal of iron largely destroyed the enzyme activity which was only slightly restored by the addition of ferrous iron. Similar properties have been reported for other oxygenases with non-haeme iron, such as 3,4-dihydroxyphenylacetate 2,3-oxygenase (Kita, 1965), pyrocatechase (Mehler, 1962) and steroid 11β-hydroxylase complex (Suzuki and Kimura, 1965). The latter is also reported to contain labile sulfide.

Iron was shown to be the only required metal component. Others tested either had no effect, or were inhibitory. When some of the iron was removed from the enzyme, some activity was lost. This activity could be regained partially with the addition of 10^{-5} M ferrous iron, but 10^{-4} M was inhibitory. Conrad et al (1965) observed a similar phenomenon with keto-lactonase, a mixed function oxygenase.

The spectrum of the most purified preparation of the enzyme showed one absorption peak only, that of the 280 mμ peak of protein. Haeme iron, flavins and cytochromes were not found to be present in the enzyme.

Flavins were found to be inhibitory to the sulfur-oxidizing enzyme in T. thioparus during the course of this investigation and in T. thiooxidans by Suzuki (1965). These findings are interesting, since Vogler et al (1942) reported that 10 μg/ml of riboflavin inhibited both the growth and sulfur oxidation of T. thiooxidans.

Treatment with ammonium sulfate inactivated the sulfur-oxidizing enzyme completely. Unlike p-hydroxyphenylpyruvate hydroxylase (Goswami, 1964), this inactivation could not be reversed with GSH.

When catalase was present, low concentrations of 2:2'-dipyridyl stimulated the enzyme activity, but concentrations of 10^{-2} M or higher were inhibitory. The stimulation is believed to be due to the protection of GSH from oxidation by iron (Boyer, 1959). This is shown by the following observations. In the absence of 2:2'-dipyridyl, the oxidation reaction levelled off after a long incubation period, but with the addition of GSH, the original rapid rate was resumed. At low concentrations of 2:2'-dipyridyl,

the reaction continued linearly for a longer time, but the initial velocity and the resumed velocity after the addition of GSH was unchanged. At higher concentrations, 2:2'-dipyridyl inhibited the enzyme by reducing the initial velocity, probably by binding the iron in the protein. The subsequent addition of GSH did not result in as rapid a rate of oxidation as in the case of low concentrations or complete absence of 2:2'-dipyridyl. The inhibition of the activity by 2:2'-dipyridyl, even at low concentrations, in the absence of catalase cannot be explained at present.

GSH is oxidized in air in the presence of copper and iron ions (Slater, 1952 and Boyer, 1959). The initial reaction in this oxidation is a slow formation of GSSG and H_2O_2 according to the following equation:

$$2GSH + O_2 \longrightarrow GSSG + H_2O_2$$

The H_2O_2 produced will cause a rapid secondary oxidation of GSH as follows:

$$2GSH + H_2O_2 \longrightarrow GSSG + 2H_2O$$

When catalase is present, the H_2O_2 will be destroyed, and this rapid secondary oxidation of GSH will not take place.

Suzuki (1965) suggested that the substrate for this enzyme might be a sulfur-GSH complex, possibly GSSH or GSS_nH. Elemental sulfur is considered to be a stable cyclic molecule with eight sulfur atoms (Donohue, 1961). The action of GSH might be to open up this ring by nucleophilic attack to form a linear polysulfide chain. This is similar to reactions of elemental sulfur with various nucleophilic reagents (Parker and Kharasch, 1959; Foss, 1961; and Van der Heijde, 1961). The intermediary formation of similar compounds in biological reactions has been proposed for mercaptopyruvate transsulfury-

lase (Hylin and Wood, 1959), cysteamine oxidation to hypotaurine (Cavallini, Scandurra, and DeMarco, 1963) and cysteine decomposition (Flavin, 1962). An enzyme-bound polysulfide or polythiosulfate complex has been postulated by Lees (1960) as an intermediate of thiosulfate oxidation by thiobacilli in order to explain the formation of polythionates and sulfur during the oxidation. The following scheme was proposed by Suzuki (1965) to explain the oxidation of sulfur:

$$S_n + GS^- \longrightarrow GSS_n^-$$

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

Glutathione polysulfide (GSS_n^-), formed by the nucleophilic attack on the S_8 sulfur ring, is oxidized by the enzyme to thiosulfate and glutathione polysulfide with a shortened sulfur chain length. This oxidation is repeated until all the polysulfide sulfur is oxidized. The compound remaining, GSH, is then free to attack another sulfur ring. Glutathione polysulfide is apparently decomposed non-enzymatically under aerobic conditions to oxidized glutathione and inorganic polysulfide according to the reaction:

$$2GSS_n^- \longrightarrow GSSG + S_{2n}^=$$

The polysulfide thus formed will be oxidized to elemental sulfur and thiosulfate by a poorly understood reaction (Van der Heijde, 1961).

Suzuki (1965) suggested that the initial end product of the oxidation of sulfur might be sulfite. When the reaction was carried out with a smaller amount of sulfur in the reaction mixture, he found sulfate formed by the oxidation of sulfur in the presence of GSH by crude extracts of T. thiooxidans. Some of the sulfite could have been oxidized enzymatically to sulfate, while some could be trapped by any remaining sulfur to form thiosulfate.

Sulfite was indeed shown to be the initial product of the enzymatic oxidation of sulfur in both T. thioparus and T. thiooxidans during the course of this investigation. The sulfite formed was trapped by formaldehyde to form a formaldehyde-bisulfite complex (Donnelly, 1943; and Goldman and Yagoda, 1943). Because of this finding, the following scheme for the oxidation of elemental sulfur is advanced:

$$S_n + GSH \longrightarrow GSS_nH$$

$$GSS_nH + O_2 \longrightarrow GSS_{n-1}SO_2H$$

$$GSS_{n-1}SO_2H + H_2O \longrightarrow GSS_{n-1}H + H_2SO_3$$

The overall reaction would be as follows:

$$S + O_2 + H_2O \xrightarrow[\text{Enzyme}]{GSH} H_2SO_3$$

Sulfite trapped as the formaldehyde-bisulfite complex could not have come from the secondary decomposition of thiosulfate either chemically or enzymatically through the action of thiosulfate reductase (Kaji and McElroy, 1959) since the amount of sulfite formed under experimental conditions was quantitatively much smaller. A small amount of sulfite formed during the incubation of thiosulfite and GSH was probably due partly to a non-enzymatic reaction between the two compounds (Kaji and McElroy, 1959) and partly to thiosulfate reductase activity present in the sulfur-oxidizing enzyme preparation as well as in the crude extracts as reported by Peck (1960).

When incubated with sulfur under the experimental conditions, sulfite was completely converted to thiosulfate according to the following equation:

$$S^o + H_2SO_3 \longrightarrow H_2S_2O_3$$

It is possible that the sulfite formed is not released from the enzyme as free sulfite, but trapped directly by sulfur to form thiosulfate. Formaldehyde can also trap the sulfite, forming the formaldehyde-bisulfite complex which is very stable at the experimental conditions.

The discovery that sulfite rather than thiosulfate is the initial product of sulfur oxidation suggests that the oxidation of sulfur to sulfate by the thiobacilli may be explained by the combination of a sulfur-oxidizing system and a sulfite-oxidizing system, either through adenosine-5'-phosphosulfate (APS) (Peck et al, 1965) or directly through a cytochrome system (Charles and Suzuki, 1965).

The presence of an active sulfur-oxidizing enzyme in T. thioparus may signify a possible role of the enzyme in the oxidation of the outer sulfur of the thiosulfate molecule observed with whole cells (Santer et al, 1960; Peck, 1960). Further work is required on the splitting of the thiosulfate molecule and the subsequent reactions of the cleaved products before the entire pathway is determined.

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* Not read in the original.