ISOLATION, CHARACTERIZATION AND RESOLUTION OF THE MEMBRANE-ASSOCIATED THIOSULFATE-OXIDIZING SYSTEM OF *THIOBACILLUS NOVELLUS*

BY

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TO MY PARENTS AND MY WIFE

ABSTRACT

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The thiosulfate-oxidizing system of Thiobacillus novellus grown on thiosulfate was studied using intact cells, crude cell-free extracts and isolated membrane preparations. All active preparations oxidized thiosulfate to sulfate without the intermediary formation of polythionates consuming two moles of molecular oxygen for every mole of thiosulfate oxidized:

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

On the other hand, less active crude extracts and isolated membrane vesicles prepared from less active cells, old cells or stored frozen cells required GSH, NADH or sulfite for a complete oxidation of thiosulfate to sulfate.

The active membrane-associated thiosulfate-oxidizing system from crude cell-free extracts was partially purified by differential centrifugation, Sepharose 4B column chromatography or sucrose density gradient centrifugation. The properties of the purified multi-enzyme complex system as well as crude extracts were further investigated. These cell-free systems were highly sensitive to oxygen, storage at various temperature and freezing. The thiosulfate-oxidizing system was also

sensitive to various inhibitors such as metal-binding agents, mercaptide-forming agents, electron-transport chain inhibitors and oxidative phosphorylation inhibitors. Spectrophotometric study showed the membrane-associated thiosulfate-oxidizing system contained flavin and cytochromes b, c, a and d. Although cytochromes of c and a types are definitely involved in the sulfite-oxidizing system, functions of flavin and cytochrome b and d remain unclear at the present time. The cell-free thiosulfate-oxidizing system had the optimal pH and temperature at pH 7.5 and 25°C with an apparent Km for thiosulfate of 1.2×10^{-4}M. The Km value with intact cells was 2×10^{-5}M. The isolated thiosulfate-oxidizing complex system exhibited high substrate specificity to thiosulfate while both intact cells and crude extracts could oxidize sulfite or tetrathionate as well as thiosulfate. The enzyme that oxidizes thiosulfate to tetrathionate (thiosulfate-oxidizing enzyme) could not be detected in any cell-free preparations and sulfide was not oxidized by these preparations.

The studies with treatment of proteolytic enzymes, phospholipases, detergents or lysolecithin indicated that the thiosulfate-oxidizing complex system was phospholipoprotein in nature.

Electron micrographs of active crude extracts and isolated membrane complex after negative staining showed

the presence of unique spherical structures with a diameter of about 100 to 400 nm. The ultra-thin section of the membrane complex revealed that the large spherical particles observed in the negatively stained preparations were aggregated structures consisting of smaller vesicles of 20 to 50 nm in diameter.

The thiosulfate-oxidizing multienzyme complex could be resolved into the intermediary enzymes known to be involved in the oxidation of thiosulfate: thiosulfate-cleaving enzyme (rhodanese), sulfur-oxidizing enzyme, sulfite: cytochrome c oxidoreductase and cytochrome c oxidase. However, all attempts to reconstitute the active complex system with either the dissociated components or purified intermediary enzymes were unsuccessful.

A possible scheme for thiosulfate oxidation by T. novellus based on results obtained in the present study and informations reported by previous workers (Charles and Suzuki, 1966a) has been proposed.

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ABBREVIATIONS

ADP	- adenosine-5'-diphosphate
AMP	- adenosine-5'-monophosphate
APS	- adenosine-5'-phosphosulfate
ATP	- adenosine-5'-triphosphate
cyclic AMP	- adenosine-3'5'-cyclic phosphate
deoxy AMP	- deoxy adenosine-5'-monophosphate
DCPIP	- 2'6-dichlorophenol indophenol
DTT	- dithiothreitol
EDTA	- ethylenediamine tetraacetic acid
FAD	- flavin adenine dinucleotide
FMN	- flavin mononucleotide
GSH	- reduced glutathione
G-S-S-G	- oxidized glutathione
GTP	- guanosine-5'-triphosphate
NAD, NADH	- oxidized or reduced nicotinamide adenine dinucleotide
NADP, NADPH	- oxidized or reduced nicotinamide adenine dinucleotide phosphate
Tricine	- N-tris (hydroxymethyl) methylglycine
Tris	- tris (hydroxymethyl) aminomethane

INTRODUCTION

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Thiobacilli are microorganisms that have ability to utilize for growth the energy which is released from the oxidation of reduced inorganic sulfur compounds. Thiobacillus novellus, a facultative chemolithotroph, is able to switch from the chemolithotrophic mode of life to a heterotrophic way, viz., deriving its energy and carbon sources from organic compounds.

When the organism grows on a mineral medium containing thiosulfate as energy source, the end product of thiosulfate oxidation is normally sulfate (Starkey, 1934a; Parker and Prisk, 1953). However, under certain growth condition tetrathionate along with sulfur and sulfate are produced in the growing cultures (Vishniac and Trudinger, 1962) as in other thiobacilli.

Various mechanisms have been proposed in thiobacilli for the oxidations of thiosulfate and intermediates of thiosulfate oxidation such as sulfur and sulfite (Lees, 1960; Peck, 1962, 1968; Vishniac and Trudinger, 1962; Trudinger, 1967, 1969; Suzuki, 1974). While new theories have been advanced and old ones reevaluated, at least three distinct theories of thiosulfate oxidation in thiobacilli have emerged. The first one is that the oxidation of thiosulfate to sulfate involves polythionates as intermediates (Vishniac and Santer, 1957; London and

Rittenberg, 1964; Trudinger, 1964a, b, 1967, 1969). The significance of this theory has been widely questioned since there is general lack of information of specific enzymes capable of further oxidation of tetrathionate or polythionates as pointed out by Peck(1968).

The second theory is that thiosulfate is oxidized to sulfate and sulfur without formation of polythionates (Peck, 1960, 1962; Peck and Fisher, 1962). They have postulated that a reductive cleavage of thiosulfate with glutathione by thiosulfate reductase to yield sulfide and sulfite is the initial step and the resulting sulfite is oxidized to sulfate via an APS pathway. Sulfide is oxidized to elemental sulfur by a sulfide oxidase. In this theory, the pathway of thiosulfate oxidation seems to be incomplete because the further oxidation of elemental sulfur to sulfate has been overlooked. Lyric and Suzuki (1970c) suggested that the sulfur would be oxidized further to sulfate since T. thioparus cells contained strong sulfur-oxidizing enzyme and sulfite-oxidizing activities. In fact, the sulfur-oxidizing enzyme was isolated and purified in T. thioparus (Suzuki and Silver, 1966). In addition, the thiosulfate reductase has not yet been purified and the specific activity of the enzyme appears to be very low.

The third alternate concept is that a cleavage of thiosulfate to sulfur and sulfite by rhodanese is directly

coupled to sulfur-oxidizing enzyme system and sulfite-oxidizing system. It has been shown in T. novellus that reduced glutathione is not required for the oxidation of thiosulfate to sulfate and that rhodanese (thiosulfate-cleaving enzyme), sulfur-oxidizing enzyme, sulfite: cytochrome c oxidoreductase and cytochrome c oxidase are involved in the thiosulfate oxidation (Charles and Suzuki, 1966a).

Although the individual enzymatic steps involved in the oxidation of thiosulfate have been elucidated relatively well, the thiosulfate-oxidizing complex system containing known intermediary enzymes has not been studied extensively, especially in the cell-free system. During the course of further study of the mechanism of thiosulfate oxidation in T. novellus it was found that a membrane-associated thiosulfate-oxidizing system was responsible for the oxidation of thiosulfate to sulfate. As pointed out by Suzuki(1974), the study of such system is very important in bridging a gap between the study of intact cells and that of isolated enzymes.

In the present study an attempt was made to better understand the characteristics of partially purified thiosulfate-oxidizing complex as well as cell-free extracts and intact cells. A resolution of the membrane-associated thiosulfate-oxidizing system was also undertaken in order to provide further knowledge on the mechanism of the

oxidation of thiosulfate in T. novellus.

HISTORICAL

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General Physiology and Characteristics of a Facultative Chemolithotrophic Bacterium, Thiobacillus novellus

In 1887-1888, S. N. Winogradsky, on the basis of his studies on the genus Beggiatoa and Leptothrix, classically defined chemoautotrophic organisms as microorganisms which derived their energy for all metabolic requirements from the oxidation of reduced inorganic compounds and utilized it for the fixation of carbon dioxide. This process was termed biological inorganic oxidation.

Nathansohn(1902) isolated autotrophic microorganisms which were minute, non-motile rods from the silts in the Bay of Naples. Bacteria, described by him, derived their energy from the oxidation of thiosulfate to tetrathionate and sulfuric acid.

$$6Na_2S_2O_3 + 5O_2 \longrightarrow 2Na_2S_4O_6 + 4Na_2SO_4$$

In the further studies, Nathansohn observed that the presence of atmospheric carbon dioxide or of carbonates was absolutely required when these bacteria were growing in mineral media containing thiosulfate.

In 1904, bacteria, similarly oxidizing sulfur compounds, but physiologically clearly distinct from both the filamentous and the purple sulfur bacteria, were isolated in pure culture by Beijerinck from fresh water, silts of the Delft canals, and sea water. On the basis of morphological and physiological properties, Beijerinck designated these bacteria as members of the genus Thiobacillus. Subsequently, a number of organisms included in this genus have been described by many other workers.

Thiobacillus novellus which is a facultative chemolithotrophic bacterial species was first isolated from soil and described by Starkey (1934 and 1935). He found that it was a small, Gram-negative, non-motile, and non-sporulating rod of 0.6 x 1.2 μ in size and it grew very well on nutrient broth and could also grow on a mineral medium containing thiosulfate. In the latter case, the only oxidation products were sulfates and sulfuric acid (Starkey, 1935).

$$Na_2S_2O_3 + 2O_2 + H_2O \longrightarrow Na_2SO_4 + H_2SO_4$$

Starkey also observed no growth of T. novellus under anaerobic conditions in the presence of nitrates.

From a later study on the growth of T. novellus under chemically defined conditions, Santer and co-workers (1959) observed that bacteria grown heterotrophically required a lag phase before the growth on a thiosulfate medium, whereas bacteria grown under autotrophic conditions started growing

immediately upon transfer to an organic substrate medium. This adaptive manner of growth on the autotrophic and heterotrophic media was limited to a few substrates and glutamate, of various carbon compounds tested, appeared to be the best substrate supporting growth.

LeJohn et al.,(1967), working with T. novellus, reported that simple organic substrates served as carbon and energy sources even in the presence of thiosulfate. All intermediate enzymes believed to be involved in thiosulfate oxidation, except thiosulfate-oxidizing enzyme, were completely supressed by glucose, lactate, glycerol, lactose, ribose, and pyruvate. The catabolic repression could be relieved by transferring the culture to a thiosulfate-mineral salts medium. From the experimental data, it was suggested that the repressor could be ATP which is the sole common by-product derived directly from metabolism of inorganic sulfur and organic compounds. Meanwhile, glutamic acid, citric acid and succinic acid had little or no effect on the thiosulfate-oxidizing system. Presumably under these conditions, thiosulfate also served as an energy source. Both NAD and NADP specific glutamate dehydrogenases from T. novellus cells grown on a glutamate-thiosulfate mineral salts medium were isolated, purified about 250-fold, and the physical properties were characterized (LeJohn and McCrea, 1968). The NAD-linked enzyme was fully inducible and allosteric. AMP and ADP were

positive effectors which regulated the catalytic behavior of the enzyme in favor of breakdown of glutamate. Later, the kinetic properties of the two enzymes were further studied and a possible control mechanism of biosynthetic and catabolic functions were proposed (LeJohn et al.,1968).

Aleem and Huang(1965) reported that the level of ribulose-diphosphate carboxylase(carboxydismutase) activity in the crude extract of autotrophically grown T. novellus cells was high and its level in glutamate-grown cells was less than 1 % of the value in autotrophic cells. However, the level of phosphoenolpyruvate carboxylase remained unchanged under autotrophic or heterotrophic growth conditions.

Charles(1966) observed that the ribulose diphosphate pathway was repressed and the phosphoenol-pyruvate pathway was the only pathway formed in extracts of glucose-grown cells. Isocitric dehydrogenese from T. novellus was studied by Charles(1970). An NADP-specific enzyme was isolated, partially purified, and characterized from cells grown on acetate as source of energy and carbon. Both the crude extract and purified enzyme(12-fold) lacked NAD-specific isocitrate dehydrogenese activity although transhydrogenase activity was found in crude extracts of both autotrophically and acetate-grown cells. Unfortunately, the enzyme properties of the NADP-specific

isocitric dehydrogenase from autotrophic cells were not given and the physiological significance of this enzyme in autotrophic and heterotrophic cells remains obscure. Charles(1971) also studied the changes in the level of enzymes involved in the tricarboxylic and glyoxylic acid cycles in T. novellus under both autotrophic and heterotrophic growth conditions. He noticed that the specific activities of the enzymes of both cycles were usually lowest in extracts from autotrophic cells and highest in extracts from acetate-grown cells. He observed that the acetate-grown cells or their crude extracts did not oxidize thiosulfate although washed autotrophic cells readily oxidized succinate, glucose, and acetate without a lag period. The autotrophically grown cells did not oxidize glutamate.

Taylor and Hoare(1969) had also observed the inability of the organism to grow on glutamate. Other investigator, however, reported that this organism was capable of growing on glutamate(LeJohn et al., 1967). Thus, conflicting views exist as to a heterotrophic metabolism in T. novellus and this point has not yet been settled.

A fatty acid spectrum of both autotrophic and heterotrophic cells of T. novellus was reported under various cultural conditions(Levin, 1972). Almost all of

the lipid material extracted from organism was phospholipid and predominant fatty acids were C_{16}:0, C_{18}:0, C_{18}:1 and C_{19} cyclopropane.

When the bacterial growth was switched from the autotrophic to the heterotrophic condition, the content of saturated fatty acids, particularly the C_{16} and C_{18} acids decreased and that of the specific unsaturated fatty acid (C_{18}:1) increased. Temperature, culture agitation, phosphate concentration, and addition of branched precursors had no significant effect on cellular fatty acids.

In the course of a comprehensive survey of marine bacteria in the oceanic invironments surrounding the Hawaiian Islands, an obligate halophile was discovered and classified as a marine strain of T. novellus (Adair and Gunderson, 1966a). The physiological characterization of this organism also has been carried out by the same group (Adair and Gunderson, 1969b). The whole cells grown autotrophically oxidized thiosulfate to sulfate with the concomittant production of energy which was utilized for $^{14}CO_2$-fixation. The uncoupling agent, 2,4-dinitrophenol almost completely inhibited $^{14}CO_2$ fixation at a final concentration of 5×10^{-4}M. The thiosulfate-oxidizing system in this strain appeared to be inducible as was reported earlier in T. novellus (LeJohn et al., 1967).

While conducting an investigation of enrichment culture for anaerobic thiobacilli, Taylor and Hoare(1969) isolated a new facultative autotrophic Thiobacillus in pure culture which was designated by the name of Thiobacillus A2 and described the general physiological characteristics of the isolate, with particular emphasis on its gross physiology as a versatile autotroph. The new organism closely resembled T. novellus in its lack of motility, pH tolerance, metabolism of inorganic sulfur compounds, and ability to grow well on organic compounds in the absence of reduced sulfur compounds. However, it grew at a faster rate than T. novellus on all substrates and could be transferred readily between autotrophic and heterotrophic conditions. It also grew anaerobically with nitrate as the terminal electron acceptor on a number of organic compounds, but not on thiosulfate. A comparative study of the citrate synthases from T. novellus and T. A2 was reported by Taylor(1970). The citrate synthases were not inhibited by α- ketoglutarate. The enzyme from T. A2 which resembled that from other Gram-negative bacteria was inhibited by NADH whereas that of T. novellus was not. This difference was considered responsible for the better heterotrophic potential of T. A2 compared with T. novellus. Peeters and Aleem(1970) reported a quantitative survey of the enzymes involved in the TCA cycle and the glyoxylate pathway in T. A2.

The cell-free extracts from cells grown on succinate, aerobically or anaerobically, and on glutamate aerobically showed both α-ketoglutarate dehydrogenase and succinyl CoA synthetase activities, while the extracts of the organism grown under autotrophic conditions on thiosulfate lacked these enzymes.

London(1963) while working on a comprehensive study of the Thiobacillus thiooxidans and Thiobacillus thioparus from muds, isolated and characterized an unusual facultative autotroph which he named Thiobacillus intermedius. The new isolate differed from T. novellus in its properties which required the addition of organic substrates to autotrophic medium for optimum development and lowered the final pH to between 1.9 and 2.2. He also studied the effects of organic matter on thiosulfate oxidation and $^{14}CO_2$-fixation, and attempted to delineate the role of the organic material in the development of the bacterium. He concluded that the organism could not grow on single organic compounds without the presence of thiosulfate and the availability of organic matter eliminated the autotrophic assimilatory mechanism of the organism but not its autotrophic energy-generating system.
Attempts to reisolate this organism, however, had been unsuccessful although they had resulted in the isolation of Thiobacillus permetabolis nov. sp., a non-autotrophic

Thiobacillus which unlike T. intermedius, did not grow autotrophically(London and Rittenberg, 1967).

Matin and Rittenberg(1970a and 1970b) studied an enzymatic analyses concerning of regulation of glucose metabolism to delineate the role of glucose in the growth of the organism. The Entner-Doudoroff pathway was major route of glucose metabolism in cells grown on glucose-casein hydrolysate or glucose-yeast extract broth and the enzymes essential for this pathway were induced or repressed by the presence of glucose or thiosulfate. Both thiosulfate and glucose inhibited the synthesis of the TCA cycle enzymes. Most recently, a study on regulation of glucose uptake system by thiosulfate was reported by Romano et al.,(1975). They showed the evidence, by using a non-metabolizable analogue U-^{14}C 2-deoxy-D-glucose, that T. intermedius had an inducible, non-phosphorylative, active transport system for glucose that was formed only under conditions where glucose was used as a source of energy, and that thiosulfate regulated the transport system both by repression and by inhibition of its activity. However, the mechanism of the inhibition of glucose transport system by thiosulfate has not been elucidated yet.

There is another unusual facultative autotroph, T. coproliticus, isolated from Triassic coprolite of

Arizona(Lipman and McLees, 1940); however, there seems to be no further report dealing with the reisolation, successful cultivation and studies associated with the physiological characterization and classification of this bacterium.

A new thermophilic non-spore-forming strain of facultative autotrophic Thiobacillus was isolated in pure culture from hot spring waters in the Yellowstone National Park in U. S. A. and the physiology of the organism was studied(Williams and Hoare, 1972). The organism grew on nutrient broth, but not on single organic compounds. The α-ketoglutarate dehydrogenase was absent in the cell-free extract and the incorporation of acetate into amino acids took place via the glyoxylate cycle to bypass the block in the TCA cycle. The mean base composition of the bacterial DNA fell within the range for the genus, close to that of T. novellus(Jackson et al.,1968) and phospholipids positively identified were the same as those in all other thiobacilli(Barridge and Shively, 1968).

In 1968, Kocur and his colleagues(Kocur et al., 1968) reported an ultrastructual study of the T. novellus. They found that the cell envelopes were similar to the other Gram-negative organisms when the bacterium was grown autotrophically. A large vacuole enclosed in a triplet membrane and electron dense inclusion bodies were in the cytoplasm of the organism.

From a comprehensive study of the ultrastructure of the selected chemolithotrophic bacteria, Van Caeseele et al.,(1969) observed that the cell envelope of autotrophically grown T. novellus did not have a middle electron-dense layer although the structure of the cell envelope grown on 1% glucose appeared to be typical of normal heterotrophic Gram-negative bacteria.
The cytoplasm of heterotrophic cells contained large electron transparent globules which appeared to be polysaccharides. The similar observation was reported by Shively and his co-workers(Shively et al., 1970).
The ultrastructure of T. A2 grown autotrophically and harvested in the logarithmic phase appeared to be similar to T. novellus and other Gram-negative bacteria(Taylor and Hoare, 1969).

Mechanism of Inorganic Sulfur Oxidation

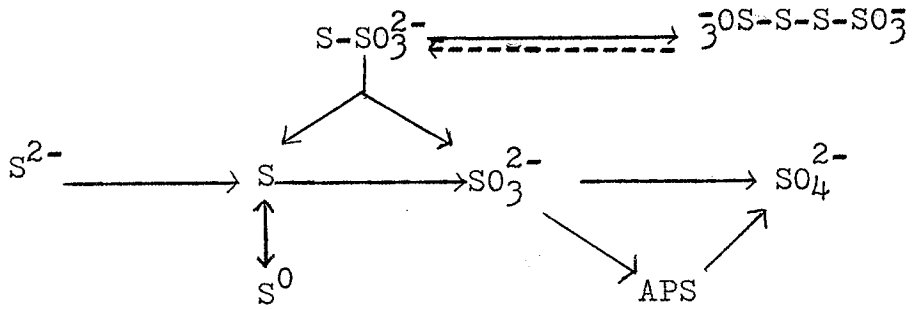
Thiobacillus species are chemolithotrophic microorganisms that depend on the oxidation of reduced inorganic sulfur compounds as a source of all energy for growth under autotrophic conditions.

Ever since the discovery of thiobacilli by Nathansohn in 1902, the mechanism of oxidation of inorganic sulfur compounds by these organisms in relation to their

unique mode of life has been studied by many scientists with considerable interest, but has always been a controversial subject. Although substantial progress has been made in the past two decades in elucidating the mechanism of the individual intermediate enzymes involving thiosulfate oxidation, even in recent years there was no general agreement on many aspects of the mode of its oxidation(Vishniac and Santer, 1957; Lees, 1960; Peck, 1962; Vishniac and Trudinger, 1962; Trudinger, 1967; Peck, 1968; Trudinger, 1969; Roy and Trudinger, 1970).

However, in a more recent review, Suzuki(1974) has presented a general scheme of inorganic sulfur oxidation based on most of the experimental results in the past and, significantly, based on enzymes, most of which have been isolated and characterized from the extracts of various thiobacilli.

The new scheme is as follows:



In order to let the reader understand this scheme in

greater details, a brief history on the mechanism of oxidation of individual inorganic sulfur compounds will be described in the following sections.

(1) Sulfur Oxidation

Although many thiobacilli have ability to grow through the oxidation of sulfur or colloidal sulfur to sulfuric acid, most studies on the oxidation of elemental sulfur were performed extensively in Thiobacillus thiooxidans which was first isolated by Waksman and Joffe in 1922. It has been known for a long time that the intact cells of T. thiooxidans oxidize elemental sulfur to sulfuric acid, according to the following equation:

$$S^0 + 1\tfrac{1}{2}O_2 + H_2O \longrightarrow SO_4^{2-} + 2H^+$$

In earlier work, Starkey(1937) observed that T. thiooxidans cells produced a small amount of hydrogen sulfide from elemental sulfur and suggested that the mechanism could be similar to that for a non-enzymatic reduction of elemental sulfur by reduced glutathione(GSH) as follow:

$$S + 2GSH \longrightarrow H_2S + G\text{-}S\text{-}S\text{-}G$$

Umbreit and his co-workers investigated the mechanism

of the bacterial attack on elemental sulfur in the culture of T. thiooxidans(Vogler and Umbreit, 1942; Umbreit, et al., 1942; Umbreit and Anderson, 1942). They observed that direct contact between terminal fat globules of bacterial cells and sulfur particles was necessary for sulfur oxidation. They, however, failed to confirm the presence of such fat globules under an electron microscope. This observation was also questioned by Starkey et al.,(1956), who found an increase in the rate of sulfur oxidation on rapidly shaking cultures instead of stationary cultures of T. thiooxidans. Later findings from experiments carried out on whole cells indicated that elemental sulfur could be oxidized via thiosulfate as a possible intermediate and hydrogen sulfide could be oxidized to sulfuric acid in several sulfur-oxidizing organisms(Vishniac,1952; Parker and Prisk, 1953). However, in spite of many interesting observations on the sulfur oxidation by T. thiooxidans, the actual mechanism of sulfur oxidation was totally unknown until Suzuki and Werkman(1959) discovered a cell-free sulfur-oxidizing system from the organism. Catalytic amounts of reduced gluthione were required for activity of the first soluble sulfur-oxidizing enzyme system and thiosulfate and polythionates were products in the reaction mixtures. The cell-free extract also oxidized sulfides to elemental

sulfur and thiosulfate. They demonstrated that the same extract had glutathione reductase which would convert oxidized glutathione to the reduced form during sulfur oxidation according to the following equation:

$$NADPH + H^+ + G\text{-}S\text{-}S\text{-}G \longrightarrow 2GSH + NADP^+$$

Subsequently, the sulfur-oxidizing enzyme was partially purified from the cell-free sonicates of T. thiooxidans and the purified enzyme was free of both sulfide-oxidizing activity and glutathione reductase(Suzuki, 1965a). From this study a possible reaction mechanism of sulfur oxidation involving glutathione polysulfide as intermediate was proposed as follows:

$$Sn + GSH \longrightarrow GSSnH$$

$$GSSnH + O_2 + H_2O \xrightarrow{\text{sulfur-oxidizing enzyme}} GSSn\text{-}2H + S_2O_3^{2-} + 2H^+$$

Direct support of an oxygenase nature of the sulfur-oxidizing enzyme was given by Suzuki(1965b), who employed $^{18}O_2$ to show that the oxygen utilized in the oxidation of elemental sulfur was derived from molecular oxygen.

In further investigation, the initial product of sulfur-oxidizing enzyme of T. thiooxidans was confired as sulfite, rather than thiosulfate(Suzuki and Silver, 1966). Thiosulfate was subsequently formed through a

non-enzymatic condensation of sulfur and sulfite according to the following equation:

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

$$SO_3^{2-} + 2H^+ + S \xrightarrow{\text{non-enzymatic reaction}} S_2O_3^{2-} + 2H^+$$

$$2S + O_2 + H_2O \xrightarrow[GSH]{\text{enzyme}} S_2O_3^{2-} + 2H^+$$

The purified sulfur-oxidizing enzyme contained iron as cofactor, apparently non-heme iron in nature and removal of the metal with 2,2′ dipyridyl from the purified enzyme resulted in relatively inactive enzyme, suggesting that the iron was essential for the enzyme action.

The sulfur-oxidizing enzyme has also been isolated in T. thioparus(Suzuki and Silver, 1966), T. novellus (Charles and Suzuki,1966), and Thiobacillus ferrooxidans (Silver and Lundgren, 1968) and the enzyme property found to be similar to that of T. thiooxidans. These findings would seem to elevate elemental sulfur or colloidal sulfur to an important position in the thiosulfate oxidation by thiobacilli. Recently, a cell-free system prepared by a sonic disruption of T. thiooxidans oxidizing colloidal sulfur as substrate were found in a soluble fraction (the supernatant of centrifugation at 130,000 x g for 1 hour) and the reaction products were sulfide and thiosulfate

(Tano and Imai, 1968). The activity was inhibited by p-hydroxymercuribenzoate, acetate monoiodide, and potassium cyanide, indicating that the enzyme might contain essential active sulfhydryl groups or disulfide bridges. Since they were able to detect the glutathione reductase activity, it is possible that this system contains probably the sulfur-oxidizing enzyme described by Suzuki (1965) plus glutathione reductase and a catalytic amount of glutathione and NADPH for the generation of reduced glutathione.

A number of cell-free membrane-bound systems which oxidized elemental sulfur to sulfate had been reported by several workers. A sulfur-oxidizing system isolated by Adair(1966) from T. thiooxidans did not require GSH but was inhibited strongly by sulfhydryl inhibitors. Since Adairs system appears to be a large fragment of cell wall-membrane complex, it is conceivable that his system may have protein-bound active sulfhydryl groups which may substitute for GSH requirement of the soluble sulfur-oxidizing enzyme. Although he suggested that the particulate,sulfur-oxidizing system prepared by a French pressure cell could be linked to oxygen via a cytochrome chain since cyanide and azide inhibited the system, the precise mechanism involved is not known. The cytochrome chain is of course involved in the oxidation of sulfite (the product of the sulfur-oxidizing enzyme) to sulfate

(the product of his oxidation system) as described in the later section and cyanide and azide would inhibit the sulfite oxidation.

Adair(1968) also reported that the inhibition in sulfur-oxidizing activity of T. thiooxidans on exposure to ultraviolet(UV) irradiation was accompanied by a parallel decrease in cellular ubiquinone. The ubiquinone was found to be a component of the cell wall-membrane complex. However, the question of electron transfer during the elemental sulfur oxidation still remains obscure.

In 1968, Taylor reported a soluble cell-free system from Thiobacillus neapolitanus which catalyzed the oxidation of elemental sulfur to sulfate with a concomitant uptake of oxygen. The preparation obtained by a French Pressure cell did not require the addition of reduced glutathione to initiate the attack on the elemental sulfur. Under certain assay conditions, GSH, cysteine and β- mercaptoethanol were inhibitory and the nature of these inhibitors remained unclear. Since the soluble fraction was prepared at a low speed centrifugation(30,000 x g for 45 minutes), the cell-free system could contain membrane or particulate particles.

Comparative studies on properties of sulfur- and sulfite-oxidizing systems in a strain of T. thiooxidans were investigated by Kodama and Mori(1968). The cell-free extract prepared by sonication of cells under a nitrogen

atmosphere oxidized both sulfur and sulfite, while the particulate fraction oxidized only sulfite. The sulfur-oxidizing system required both the particulate(membrane) and soluble fractions. Later the system was resolved into three subfractions and reconstituted from these fractions (Kodama,1969). The soluble fraction was further fractionated into two subfractions, the collodion membrane-permeable and impermeable fractions, and the former was identified as NAD or NADP, but not cysteine or GSH. Either NAD or NADP in oxidized or reduced forms was equally effective in the reconstituted sulfur-oxidizing system. It was noticed that the particulate fraction also was able to oxidize both NADH and NADPH.

Perhaps for reconstitution studies which are based on the restoration of catalytic activity from the individual components a more useful goal would be highly purified components uncontaminated with the other constituents of the sulfur-oxidizing system.

(2) Sulfide Oxidation

The mechanism of sulfide oxidation seems to be analogous to that for the oxidation of colloidal sulfur (polysulfide) or elemental sulfur once sulfide is oxidized to the level of sulfur.

In early days, Parker and Prisk(1953) noticed that some thiobacilli were able to grow on the mineral medium containing sulfide as an energy source. Elemental sulfur was proposed as an intermediate during the oxidation of sulfide. A similar observation was described in the cell-free extracts of T. thiooxidans by Suzuki and Werkman (1959).

However, Vishniac and Santer(1957), Peck and Fisher (1962), and London and Rittenberg(1964) proposed that thiosulfate and polythionates were the intermediate products in the oxidation of sulfide to sulfate. Although cell-free extracts of T. thiooxidans were shown to oxidize sulfide with a concomitant reduction of cytochrome(Poucke, 1962 and London, 1963), Adair(1966) suggested that the oxidation of sulfide was a non-enzymatic process and cytochromes were not involved.

Moriarty and Nicholas(1969) found an enzymatic process of sulfide oxidation in whole cells and cell-free extracts of Thiobacillus concretivorus, T. thiooxidans and T. thioparus grown on the elemental sulfur as an energy source. The sulfide-oxidizing enzyme from the cell-free extracts prepared by repeated treatment in a French Pressure Cell was further fractionated and proved to be associated with the membrane fraction by a high speed centrifugation(the Pellet of 144,000 x g for 1 hour). The sulfide was oxidized to an membrane bound polysulfur

intermediate as early stable intermediate through the cytochrome system by oxygen. The first rapid step of sulfide oxidation with its concomitant O_2 consumption was inhibited by sodium diethyldithiocarbamate and Tris-HCl, but not by CO and sodium azide.

The second step which was slow and produced the conjugated polysulfides consequently was inhibited by CO. They identified a native cytochrome C-550 which was purified 120-fold as the CO-binding site for the inhibition of the polysulfide formation and an ubiquinone-8 as a heat-stable and acetone-soluble factor, essential for sulfide oxidation.

Sulfate was the only product of the oxidation of sulfide as well as the bound sulfur intermediate in the dialyzed cell-free extracts and a possible mechanism was tentatively proposed for the enzymatic oxidation of sulfide to sulfate via a bound sulfur polymer as shown in the following equations(Moriarty and Nicholas, 1970):

$$SH^- \xrightarrow{\text{sulfide oxidase}} (S) + H^+ + 2e^-$$

$$2(S) \longrightarrow (S\text{-}S)$$

$$(S\text{-}S) + SH^- \longrightarrow {}^-S\text{-}S\text{-}SH$$

$${}^-S\text{-}S\text{-}SH + X^+ \longrightarrow X\text{-}S\text{-}S\text{-}SH$$

$$2(X\text{-}S\text{-}S\text{-}SH) \xrightarrow{\text{polysulfide oxidase}} X\text{-}S_6\text{-}X$$

In the above equations, X represents the group linking the polysulfide chain to the lipoprotein membrane fraction.

They assumed that the bound sulfur polymer would be subsequently oxidized to sulfate by a sulfur-oxidizing enzyme and a sulfite oxidase via a mechanism similar to those described by Suzuki and his co-workers(Suzuki, 1965; Suzuki and Silver, 1966; Charles and Suzuki, 1966).

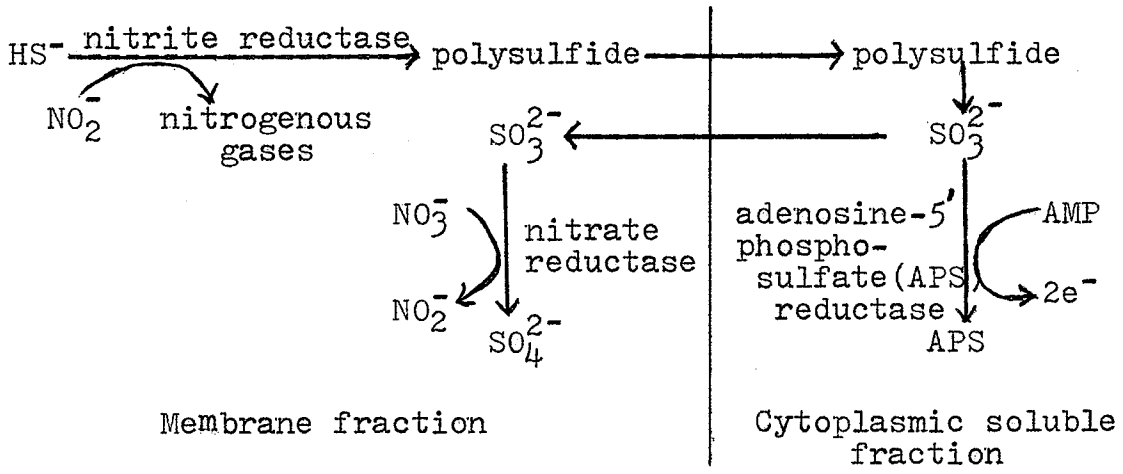
A study on the electron transfer during oxidation of sulfide in T. concretivorus was reported by the same workers(Moriarty and Nicholas, 1970). Cytochromes of the b, c, a and d types, reduced immediately on the addition of sulfide, were reoxidized by oxygen and oxidative phosphorylation occurred concomitantly with sulfide oxidation. A copper protein was implicated in the sulfide oxidation as a binding site for sulfide and both flavin and ubiquinone were also suggested for the possible components of the electron transfer chain. From the data obtained previously and those reported in this work, a tentative scheme was presented as follows:

$$\begin{matrix} S^{2-} \\ \downarrow \\ S^{o} \end{matrix} \xrightarrow{2e^-} \text{Cu protein} \longrightarrow (\text{Flavin?}) \longrightarrow \text{cytochrome b} \longrightarrow \text{cytochrome c} \longrightarrow \text{cytochrome d} \longrightarrow O_2$$

A study on the mechanism of sulfide oxidation, involving an electron transfer chain, was also reported in a facultative anaerobe, Thiobacillus denitrificans (Peeters and Aleem, 1970).

Under aerobic and anaerobic conditions, the oxidation of sulfide was sensitive to the inhibitors of the flavoprotein and cyanide and azide. However, the products of the sulfide-oxidizing system were not identified in their experiments.

More recently, Aminuddin and Nicholas(1973), working with whole cells and crude extracts of T. denitrificans reported a sulfide-oxidizing system linked to the reduction of nitrite and nitrate. These workers found that the oxidation of sulfide to polysulfide was linked to nitrite reduction and the complete oxidation of sulfide to sulfate was linked to nitrate reduction via sulfite as an intermediate. Under anaerobic conditions and in the presence of nitrite, sulfide was oxidized to polysulfide only. The following mechanism was proposed for the sulfide oxidation in T. denitrificans:



In this system, however, these soluble and membrane fractions were not further fractionated and the specific

enzymes involved were not identified completely.

(3) Sulfite Oxidation

Since sulfite is a key intermediate in the oxidation of reduced inorganic sulfur compounds, intensive studies have been made in recent years on the mechanism of sulfite oxidation in various thiobacilli. The oxidation of sulfite to sulfate in thiobacilli is the terminal stage of the oxidation for all inorganic sulfur compounds and two basic types of reaction mechanism have been advanced concerning the oxidation of sulfite. The first of those, proposed in 1960 by Peck, involved APS(adenosinephosphosulfate) as intermediate; the second pathway, which Suzuki proposed in 1966, was the direct oxidation of sulfite to sulfate without involving APS.

Although APS reductase was first found as an enzyme involved in the reduction of sulfate to sulfite in Desulfovibrio by Ishimoto and Fujimoto(1959 and 1961), the reversal of the reaction catalyzed by APS reductase and its associated enzymes in thiobacilli was largely studied by Peck and his colleagues(Peck, 1960 and 1961; Peck and Fisher, 1962). A possible role of these enzymes on the mechanism of sulfite oxidation was reviewed in detail by Peck(1962). The proposed reactions involving APS reductase, ADP sulfurylase and adenylate kinase for

sulfite oxidation in T. thioparus were summarized as follows:

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

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

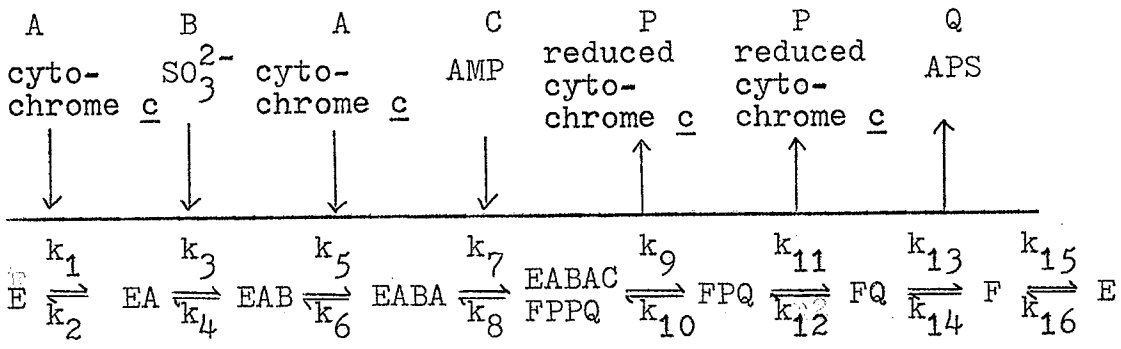
$$2ADP \xrightarrow{\text{adenylate kinase}} AMP + ATP$$

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

The oxidation of sulfite to sulfate is accompanied by a generation of energy-rich phosphate bond by the substrate level phosphorylation during conversion of APS to sulfate and ADP. This pathway was supported by the work of another group, who was able to purify and characterize the APS reductase from T. denitrificans(Bowen, et al., 1966). The properties of this purified enzyme appeared to be similar to those of the enzyme from Desulfovibrio desulfuricans(Peck, et al., 1965). Recently, using T. thioparus, used by Peck in his original work, Lyric and Suzuki(1970) succeded in purifying APS reductase from the crude extracts and studied the enzyme properties extensively. They were able to show that the purified enzyme contained acid-labile sulfide in addition to FAD and non-heme iron and the enzyme could couple with a Candida krusei cytochrome c as well as ferricyanide as

the electron acceptor. Although the activity coupled with the yeast cytochrome c was much lower than that with ferricyanide, the affinity for sulfite as substrate was over a 100-fold higher with the cytochrome and the pH optimum shifted from 7.4(ferricyanide) to 9.5(cytochrome). Since 3% of the cell protein in T. thioparus and 4-5% in T. denitrificans(Bowen, et al., 1966) were in the form of APS reductase and the cytochrome was a physiological electron acceptor, the APS pathway for sulfite oxidation was considered to be important in the energy metabolism of these organisms via substrate-level and oxidative phosphorylation.

In the later paper, Lyric and Suzuki(1970) proposed an ordered Quad Ter reaction as a possible reaction mechanism for APS reductase from the kinetic studies involving both initial velocity and product inhibition experiments and presented the following reaction scheme to explain the kinetic results.



One of the most interesting aspects of the APS

reductase in the mechanism of sulfite oxidation is the question of how the flavin-moiety as prosthetic group of the enzyme functions during the catalytic reaction. Contrary to earlier findings, however, recent evidence suggests that the APS pathway is not the sole, or possibly even the main, mechanism of sulfite oxidation in some thiobacilli.

The discovery of a sulfite; cytochrome c oxidoreductase in Thiobacillus novellus distinct from the APS reductase(Charles and Suzuki, 1965 and 1966) led to the suggestion that an AMP-independent system functioned as an additional mechanism of sulfite oxidation.
The purified enzyme oxidized sulfite to sulfate with a concomitant reduction of cytochrome c as shown by the following reaction:

$$SO_3^{2-} + 2\text{cytochrome } c(Fe^{3+}) + H_2O \xrightarrow{\substack{\text{sulfite: cytochrome } c \\ \text{oxidoreductase}}} SO_4^{2-} + 2\text{cytochrome } c(Fe^{2+}) + 2H^+$$

The reduced cytochrome c was then reoxidized with O_2 by cytochrome oxidase as follows:

$$2 \text{ cytochrome } c(Fe^{2+}) + \tfrac{1}{2}O_2 + 2H^+ \xrightarrow{\text{cytochrome oxidase}} 2 \text{ cytochrome } c(Fe^{3+}) + H_2O$$

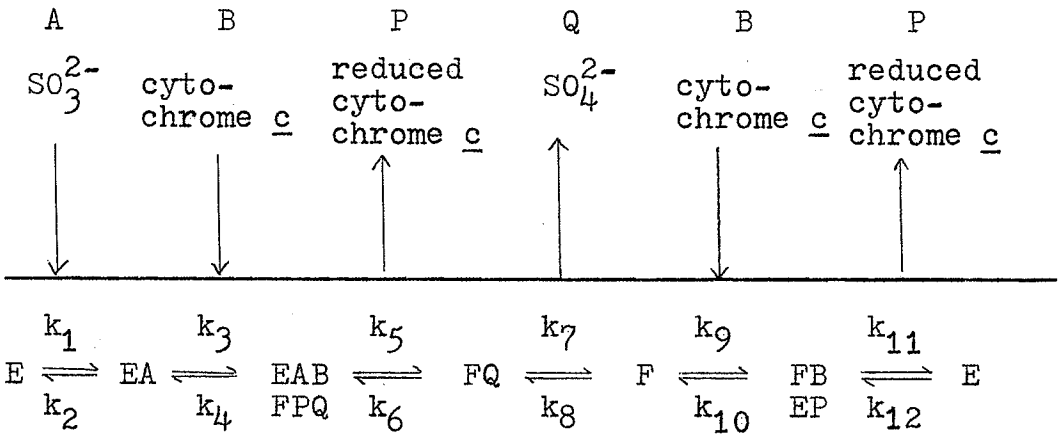
The properties of the enzyme were similar to those of hepatic sulfite oxidase from mammalian liver(MacLeod

et al., 1961). The T. novellus enzyme did require AMP as a cofactor either in the crude or purified state and could couple with horse heart or native cytochrome c as well as with ferricyanide as electron acceptor, but not with oxygen. A similar enzyme was isolated from another facultative chemolithotroph, T. intermedius(Charles, 1969). The enzyme APS reductase was absent.

The sulfite: cytochrome c oxidoreductase was also purified and characterized in T. thioparus and Thiobacillus ferrooxidans(Lyric and Suzuki, 1970a; Vestal and Lundgren, 1971). The properties of the enzyme were found to be similar to those of the T. novellus enzyme(Charles and Suzuki, 1966) in that sulfhydryl reagents and anions such as phosphate and chloride were strongly inhibitory.

In T. thioparus, the enzyme was purified 160-fold and the properties were studied in detail(Lyric and Suzuki, 1970a). The molecular weight of the purified enzyme was estimated to be 54,000 dalton and the enzyme contained non-heme iron and acid-labile sulfide as an essential component. The pH had a marked effect on Km value for sulfite; 4×10^{-5} M at pH 8.0 and 2×10^{-6} M at pH 6.0.

From the initial velocity and product inhibition studies a Bi Bi Uni Uni Ping Pong mechanism for the enzyme reaction was proposed as follows(Lyric and Suzuki, 1970d):



Since a non-heme iron was essential for the enzyme activity, they suggested that the iron could be a mediator of two electrons transferred from sulfite to cytochrome c. This kinetic mechanism differs from a Ping Pong mechanism proposed for bovine liver sulfite oxidase by Howell and Fridovich(1968).

Vestal and Lungren(1971) reported the sulfite: cytochrome c oxidoreductase from T. ferrooxidans grown on colloidal sulfur. The properties of the partially purified enzyme showed a striking likeness to the enzyme described for T. novellus(Charles and Suzuki, 1966) and T. thioparus(Lyric and Suzuki, 1970). However, the apparent Km for sulfite was about 10 times higher than those of T. novellus and T. thioparus enzymes.

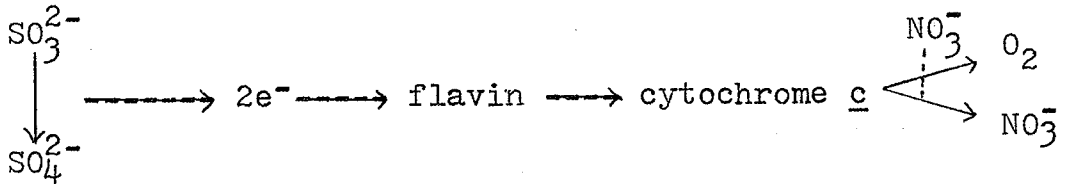
Milhaud et al.(1958) observed a sulfite-oxidizing system in T. denitrificans which was independent of AMP, but was stimulated by the addition of hypoxanthine.

In many aspects this system mimics the aerobic oxidation of sulfite in mammalian liver which is known to be a free radical chain reaction and is readily initiated by reactive superoxide anions, O_2^-(Fridovich and Handler, 1956 and 1961; MacLeod et al., 1961). Recently, McCord and Fridovich(1969a and 1969b) further demonstrated that erythrocuprein, a potent superoxide dismutase which catalyzed the disproportionation of the superoxide anions ($O_2^- + O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$), strongly inhibited the autoxidation of sulfite in the presence of 5×10^{-6} M EDTA.

A further work concerning the sulfite-oxidizing system in T. denitrificans was studied by Peeters and Aleem(1970). Sulfite was oxidized both aerobically and anaerobically with nitrate by intact cells. The anaerobic oxidation of sulfite by nitrate was more sensitive to cyanide, azide and the inhibitors of the flavoproteins compared to the aerobic sulfite oxidation. Adams et al. (1971) isolated a particulate, sulfite-dependent nitrate reductase from T. denitrificans which utilized sulfite for the reduction of nitrate to nitrite. Kinetic studies with the sulfite-linked nitrate reductase indicated a Ping Pong mechanism and Km values of 7.2×10^{-5} M for nitrate and 2.5×10^{-4} M for sulfite.

Recently, Aminuddin and Nicholas(1973 and 1974a) reported the isolation, purification and properties of

an AMP-independent sulfite oxidase from T. denitrificans. They showed that the membrane-bound sulfite oxidase used nitrate, oxygen or ferricyanide as an electron acceptor and AMP had no effect on the enzyme. The 50-fold purified enzyme coupled only with ferricyanide, but not with either nitrate or oxygen. The enzyme was strongly inhibited by thiol-binding reagents and the inhibition was completely reversed by dithiothreitol indicating the involvement of active sulfhydryl groups for catalysis. Unlike the sulfite: cytochrome c oxidoreductase from T. novellus(Charles and Suzuki, 1966) and T. thioparus(Lyric and Suzuki, 1970a), the T. denitrificans enzyme was not inhibited by phosphate and chloride ions at concentration of up to 7.5×10^{-2} M. In the later paper Aminuddin and Nicholas(1974b) suggested that flavin and cytochromes of the b and c types and possibly a cytochrome oxidase were involved in the membrane-bound sulfite oxidase linked to nitrate reduction. From data on the electron transfer system during sulfite oxidation in the same organism, a tentative scheme was proposed as follows:

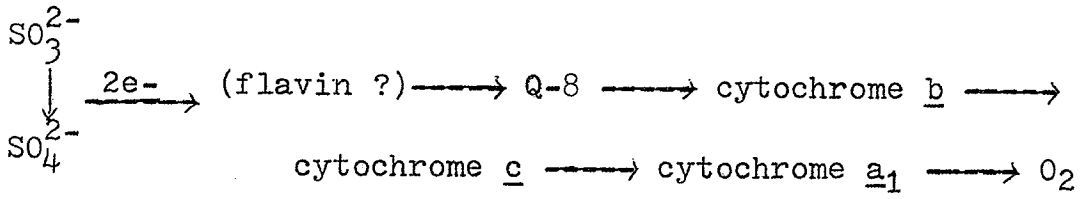


In this system, nitrate inhibited the uptake of oxygen during sulfite oxidation.

Another interesting observation made by Kodama et al.(1970) was a cell-free sulfite-oxidizing system obtained from a strain of T. thiooxidans by sonication followed by fractionation with a high speed centrifugation(at 105,000 x g for 1 hour).

The enzyme which was tightly bound to the membrane catalyzed oxidation of sulfite with oxygen or bacterial cytochrome c-552 prepared from Pseudomonas stutzeri as electron acceptor and cytochromes of the a, b and c types were contained in the particulate fraction. Since Antimycin A as well as cyanide, azide, carbon monoxide and 1,10-phenanthroline markedly inhibited the sulfite-oxidizing system with either oxygen or cytochrome c-552, cytochrome of b type was suggested to be participating in the sulfite oxidation.

A similar membrane-bound sulfite oxidase prepared by a French Pressure Cell from T. concretivorus was reported by Moriaty and Nicholas(1970). An electron-transfer system which involved cytochromes b, c and a was associated with the sulfite oxidase and oxidative phosphorylation occurred during electron transfer via the cytochrome chain. Inhibitor studies showed that flavin and ubiquinone were also required for the sulfite-oxidizing system. A tentative scheme to account for the experimental results was presented as follows:



These observations differed from those of Adair(1966), working with a different strain of T. thiooxidans and Charles and Suzuki(1966) in T. novellus. Therefore, whether or not differences exist between electron transport mechanisms of sulfite oxidation in different species of thiobacilli remains uncertain.

An unusual sulfite-oxidizing enzyme which reacted with either ferricyanide or oxygen was isolated and purified 100-fold from extracts of T. neapolitanus(Hempfling, 1964; Hempfling et al., 1967). The properties of the enzyme differed markedly from that obtained from T. novellus (Charles and Suzuki, 1965) in that the enzyme transferred reducing equivalents from sulfite directly to oxygen, did not catalyze the reduction of native or horse heart cytochrome c by sulfite, was relatively heat stable, was stimulated 1,5 - 2 fold by the addition of AMP and was not inhibited by phosphate. Since the ratio of AMP-dependent and AMP-indenpendent activities remained fairly constant throughout the purification and GSH inhibited activity and prevented the AMP-dependent stimulation, they suggested that a single enzyme was responsible, and that an enzyme-

sulfite complex was formed which reacted either with AMP to form APS or with water to form sulfite. It should be noted, however, that a very high sulfite concentration (10^{-2} M) was required for the assay and the formation of APS was not shown with the purified enzyme. At such high sulfite concentration, there would be an oxidation of sulfite due to a chain reaction initiated by free radicals as discussed before. Although we do not know what the physiological meaning of this type of enzyme is at the present time, this aspect of sulfite oxidation was well documented in recent review by Fridovich(Fridovich, 1974).

(4) Thiosulfate Oxidation

It is well documented that most of members of the genus Thiobacillus are capable of metabolizing thiosulfate (Trudinger, 1967 and Roy and Trudinger, 1970). Thiosulfate, very soluble and reasonably stable at neutral pH, has widely been used as a preferred growth substrate in the routine cultivation of thiobacilli which grow at neutral pH values, and a great deal of research has been carried out on the mechanism of thiosulfate oxidation in these microorganisms.

In early studies, there were three basically different observations unique to the mechanism of thiosulfate oxidation: first, polythionates consisting mainly

of tetrathionate along with sulfur and sulfate were produced during thiosulfate oxidation by the growing cultures or intact cells of T. thioparus(Nathansohn, 1902; Vishniac, 1952; Vishniac, 1957; Pratt, 1958; Woolley et al., 1959; Santer et al., 1960; Jones and Happold, 1961; Vishniac and Trudinger, 1962; London and Rittenberg, 1964; Pankhurst, 1964), T. neapolitanus(Parker and Prisk, 1953; Trudinger, 1959; Trudinger, 1964), Thiobacillus strain C (Kelly and Syrett, 1966), T. novellus(Vishniac and Trudinger, 1962), T. denitrificans(Baalsrud and Baalsrud, 1954), T. thiooxidans(Parker and Prisk, 1953; London and Rittenberg, 1964), T. concretivorous(Parker and Prisk, 1953), and T. thiocyanoxidans(Pratt, 1958; Pankhurst, 1964). Studies by the above investigators using polarographic, manometric, and chromatographic techniques, sometimes combined with the use of radioactive substrates had established that the first product of thiosulfate was tetrathionate,whereas sulfur and sulfate appeared during further chemical and biological transformations of the tetrathionate. Vishniac and Santer(1957) expresses the initial oxidative step of thiosulfate to tetrathionate as follows:

$$2S_2O_3^{2-} + \tfrac{1}{2}O_2 + H_2O \longrightarrow {}^{-}_{3}OSSSSO_3^{-} + 2OH^{-}$$

Second, in the cultures of T. thioparus and related organims Starkey(1935), Parker and Prisk(1953), Skarzynski et al.(1957),

Skarzynski and Szczepkowski(1959), and Peck(1962) observed only sulfate with large amounts of elemental sulfur, but no detectable quantities of polythionates. It was assumed that the thiosulfate oxidation proceeded as follows:

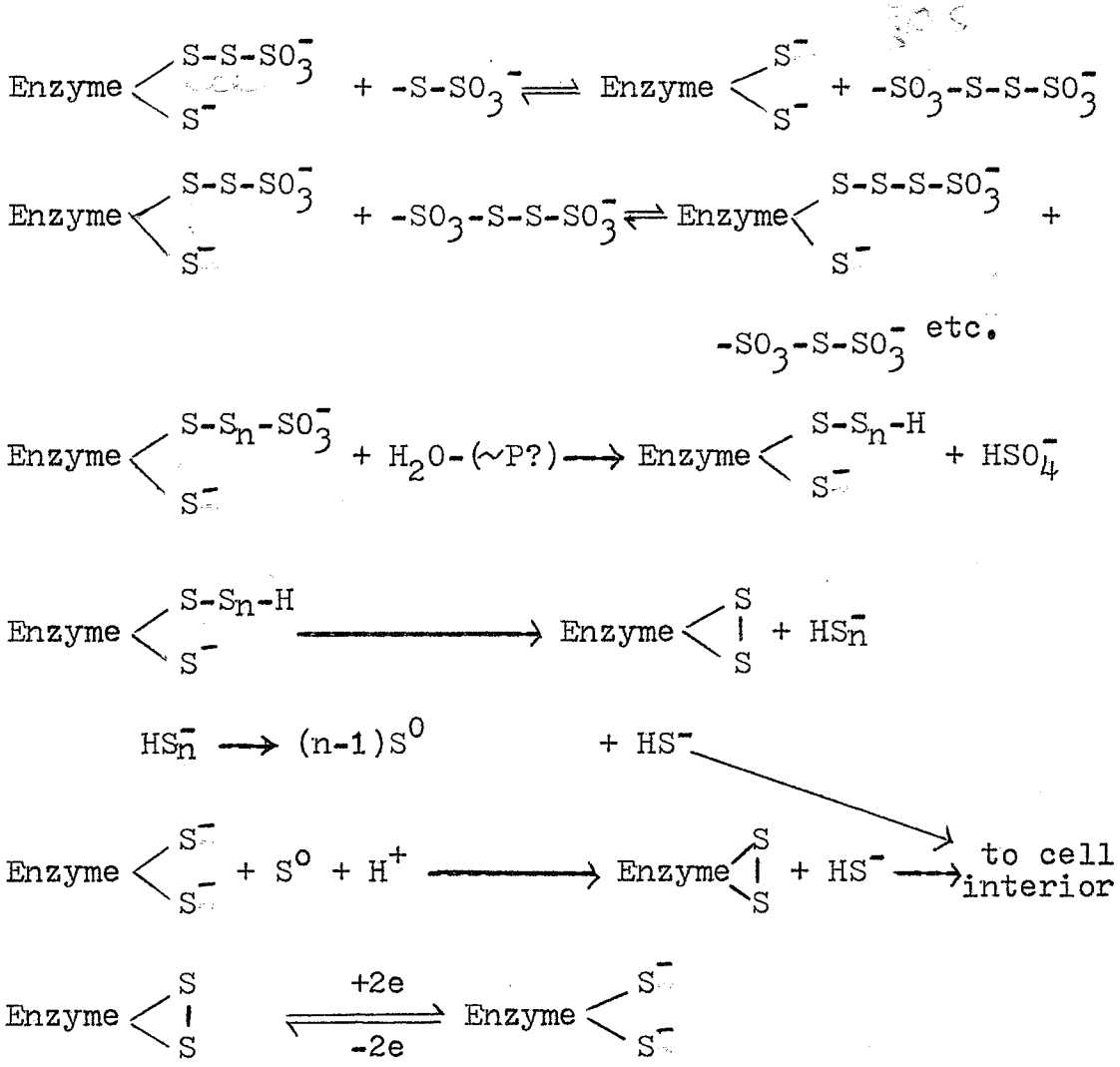
$$5Na_2S_2O_3 + 4O_2 + H_2O \longrightarrow 5Na_2SO_4 + H_2SO_4 + 4S$$

Third, the culture of T. novellus(Starkey, 1934a and 1934b; Parker and Prisk, 1953), grown on thiosulfate, oxidized thiosulfate completely to sulfate according to the following equation:

$$Na_2S_2O_3 + 2O_2 + H_2O \longrightarrow Na_2SO_4 + H_2SO_4$$

In an attempt to coordinate the previous diverse findings based largely on whole cell studies, Lees(1960) advanced a speculative working hypothesis for the mechanism of thiosulfate oxidation. He proceeded from assumption that there was an enzyme system capable of thiosulfate cleavage and polythionate formation on the cell surface. The postulated scheme is outlined in the following equations:

$$\text{Enzyme} < \begin{matrix} S \\ | \\ S \end{matrix} + \text{-S-SO}_3^- \rightleftharpoons \text{Enzyme} < \begin{matrix} \text{S-S-SO}_3^- \\ S^- \end{matrix}$$



*~P indicates high energy phosphate bond.

A dithiol group of the enzyme would react with thiosulfate to form sulfenyl thiosulfate($-S-S-SO_3^-$) which could produce polythionates with another thiosulfate molecule. When the sulfenyl thiosulfates reacted with the polythionates, the formation of polysulfenyl thiosulfate($-Sn-SO_3^-$) chains would occur and a hydrolysis of the chains would result in

splitting off the terminal sulfonic acid($-SO_3^-$) as sulfate. According to this theory, the behavior of different thiobacilli with regard to the formation of various end-product such as polythionates, elemental sulfur and sulfate could be explained by assuming that they could split the polysulfenyl thiosulfate chain at different lengths. However, this scheme overlooked the intracellular oxidation of the sulfhydryl groups(SH^-) arising from the outer sulfur groups(-S-) of thiosulfate and did not take into account the function of phosphorous compounds which had been detected in this process by other investigators (Vishniac and Santer, 1957; Santer, 1959; Santer et al., 1960).

A first cell-free system prepared by a French Pressure Cell from T. neapolitanus capable of catalysing thiosulfate quantitatively to tetrathionate was isolated and partially purified by Trudinger(1961a) and the properties of the thiosulfate-oxidizing enzyme were studied (Trudinger, 1961b; 1965). The enzyme catalyzed the following reaction: $2S_2O_3^{2-} \longrightarrow {}^-_3OSSSSO_3^- + 2e^-$ The cytochrome c-553.5 separated from the crude extracts was shown to be a natural electron acceptor although the enzyme coupled well with ferricyanide. Mammalian cytochrome c-550 also was reduced slowly by the enzyme. However, other native cytochromes such as c-550 and c-557 did not react directly with the enzyme. This enzyme has been isolated, purified, and characterized from T. ferro-

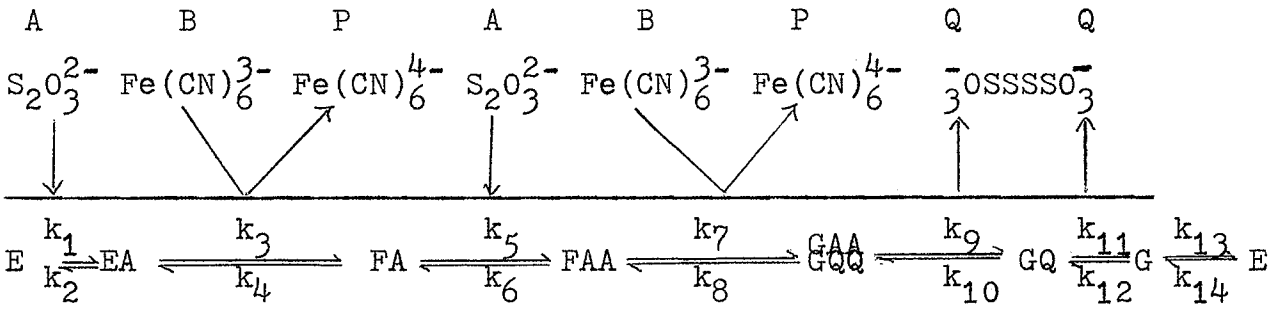
oxidans(Silver and Lundgren, 1968) grown on sulfur and *T. thioparus*(Lyric and Suzuki, 1970c).

The *T. ferrooxidans* enzyme, unlike the thiosulfate-oxidizing enzyme of other thiobacilli, catalyzed the oxidation of thiosulfate to tetrathionate only with ferricyanide as an electron acceptor, but not with either native or mammalian cytochrome c-550. The Km value for thiosulfate was 9×10^{-4} M and very little inhibition was observed with various sulfhydryl inhibitors, except $HgCl_2$.

Most recently, *T. thioparus* enzyme was purified from crude extracts nearly 160-fold and the properties were studied by Lyric and Suzuki(1970c). The enzyme contained non-heme iron and showed a wide range of optimum pH(4.5-10.0). Although ferricyanide was considerably better electron acceptor than horse heart cytochrome *c*, the Km for thiosulfate in the cytochrome coupled reaction was lowered significantly from 10^{-4} M to 5×10^{-6} M and the pH optimum shifted from 5.0 to 7.0.

The enzyme was sensitive to sulfhydryl inhibitors and sulfite was shown to be a strong inhibitor, causing a loss of 50% of the activity in 5 minutes at the final concentration of 5×10^{-6} M. This sulfite inhibition of the thiosulfate-oxidizing enzyme was time-dependent and irreversible. From kinetic studies involving both initial velocity and product inhibition experiments using ferricyanide as an electron acceptor Lyric and Suzuki(1970d) proposed

a Theorell-Chance reaction mechanism as shown in the following scheme:



Aleem(1965) reported a soluble thiosulfate: cytochrome c reductase prepared by sonication of T. novellus cells catalysing the reduction of cytochrome c with thiosulfate. He claimed that the enzyme was purified 215-fold from the sonicated crude extract and was inhibited by metal-binding reagents such as 1,10-phenanthroline and diethyl-thiocarbamate but not by thiol-binding reagents such as p-hydroxymercuribenzoate. Although the end-product of the thiosulfate:cytochrome c reductase was not reported, this enzyme presumably oxidizes thiosulfate to tetrathionate. Since in the absence of cyanide, little or no activity was detected at low concentration of thiosulfate(3.5 to 7×10^{-3} M) and the enzyme showed a marked increase in affinity for thiosulfate in the presence of cyanide(Km, approximately 2×10^{-1} M without cyanide $\longrightarrow$ 10^{-3} M with cyanide), it is also quite possible to conceive the existence of a mixture consisting of thiosulfate sulfurtransferase(rhodanese) and

sulfite: cytochrome c oxidoreductase.

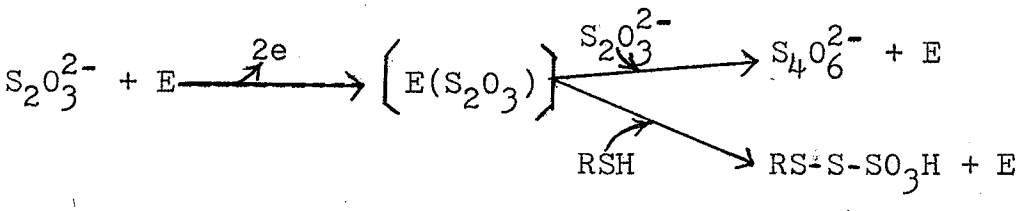
The quantitative oxidation of thiosulfate to tetrathionate was also reported in untreated crude extracts of T. thioparus(Peck and Fisher, 1962; London and Rittenberg, 1964), T. thiooxidans(London and Rittenberg, 1964; Okuzumi and Kita(1965); Tano et al., 1968), T. concretivorus (Moriarty and Nicholas, 1968) and T. novellus(Vishniac and Trudinger, 1962).

London and Rittenberg(1964) reported that cell-free extracts of T. thioparus treated with activated charcoal and dialyzed against polyethylene glycol oxidized completely thiosulfate to sulfate via transient formations of tetrathionate and trithionate. They claimed that this extensive treatment removed an inhibitor present in the crude extracts prepared by a sonication. However, the nature of the unidentified inhibitor is still left unsolved.

Vishniac and Trudinger(1962) and Trudinger(1964; 1965) observed that both intact cells and soluble thiosulfate-oxidizing enzyme of the T. neapolitanus oxidized thiosulfate to tetrathionate at linear rates in the presence of thiol-binding reagents or 100 % oxygen in the gas phase which could cause the oxidation of all the active thiols in the enzyme to disulfides. Under these conditions the oxidations of thiosulfate and polythionate to sulfate was markedly inhibited.

The soluble thiosulfate-oxidizing system from

T. novellus(Aleem, 1965) was also insensitive to thiol-binding reagents. Since these facts based on mainly inhibition studies were not easily reconciled with Lees' theory, a modified hypothetical mechanism for thiosulfate oxidation was deduced by Vishniac and Trudinger(1962) and Trudinger(1967) to account for these observations as shown below:



E : thiosulfate-oxidizing enzyme

according to this scheme, the true function of the thiosulfate-oxidizing enzyme is to transfer thiosulfate molecule to a thiol group within the cell to form a sulfenyl thiosulfate which is then further oxidized to sulfate. Tetrathionate could arise from an enzyme-bound intermediate capable of reacting with a molecule of thiosulfate and this process was thought to be due to a non-specific secondary reaction. Similar observation was reported for tetrathionate formation by a photosynthetic purple sulfur bacterium, Chromatium strain D(Smith, 1966b).

Sinha and Walden(1966) reported that thiosulfate in the presence of thiol-binding reagents was oxidized to sulfate and trithionate by T. ferrooxidans through tetra-

thionate as an intermediate which was identified from the chromatographic analysis. The accumulation of polythionates during thiosulfate oxidation by a cell-free particulate preparation of T. thiooxidans was reported by Tano et al.(1968). Both tetrathionate and trithionate were formed and thiol-binding reagents were not inhibitory.

In spite of the evidence discussed above to support the polythionate pathway, the proposal is still suffering from the lack of direct confirmation from experimental evidence and it is still not clear how this pathway involving polythionates as intermediates operates for the overall oxidation of thiosulfate to sulfate. Furthermore, it has been widely questioned because of the following facts:

(1) There is general lack of knowledge of specific enzymes responsible for thiosulfate oxidation via this pathway expect the thiosulfate-oxidizing enzyme.

(2) The formation of polythionates from thiosulfate by growing cultures, resting intact cells, and cell-free preparations shows very conflicting results, viz,often stopping at tetrathionate or trithionate. Some reasons for such variations obtained by different workers have been discussed by Trudinger(1967 and 1969):

These are strain differences, the composition of growth medium, growth conditions(i.e. aeration, pH, cell density and temperature etc.), the different stages of growth and experimental methods or conditions used. However, the

discrepancies apparently arising from the experimental results remain to be established.

(3) A number of workers have reported that intact cells or cell-free extracts of some thiobacilli oxidized thiosulfate completely to sulfate without polythionate formation and were unable to metabolize polythionates(Charles and Suzuki, 1966 Peck, 1962;1968; Trudinger, 1969).

(4) From a study on the catabolic repression of all enzymes involved in thiosulfate oxidation in T. novellus the thiosulfate-oxidizing enzyme has been shown to be not simultaneously induced and repressed, suggesting that tetrathionate may not be a necessary intermediate in the thiosulfate oxidation(LeJohn et al., 1967).

Therefore, the full significance of this pathway has yet to be critically explored and evaluated in thiobacilli.

Correlating previous worker's experimental data obtained from growing culture and intact cells(Skarzynski et al., 1957; Santer, 1959) with the results of his cell-free enzymatic studies, Peck(1960; 1962) proposed a scheme concerning the enzymatic mechanism involved in thiosulfate oxidation in T. thioparus. The first stage of thiosulfate oxidation was a reductive process catalyzed by an enzyme, designated thiosulfate reductase which was first reported by Kaji and McElroy(1959) in yeast and this reductive cleavage required absolutely substrate amounts of reduced glutathione.

$$S_2O_3^{2-} + 2e^- + 2H^+ \xrightarrow{\text{thiosulfate reductase}} SO_3^{2-} + H_2S$$

Sulfide was further oxidized to sulfur by a sulfide oxidase.

$$2H_2S + O_2 \xrightarrow{\text{sulfide oxidase}} 2S^o + 2H_2O$$

Sulfite was oxidized to sulfate via APS pathway as the mechanism previously described in sulfite oxidation.

ATP was produced by a substrate-level phosphorylation in the APS pathway of sulfite oxidation.

In overall reaction, the thiosulfate was oxidized to sulfur and sulfate according to the following reaction.

$$2S_2O_3^{2-} + O_2 + AMP + 2Pi + 4H^+ \longrightarrow 2S^o + 2SO_4^{2-} + ATP + 2H^+$$

Although he suggested a possible recycling mechanism from elemental sulfur to thiosulfate in the presence of GSH, the further oxidation of the elemental sulfur to sulfate was not studied(Peck, 1962). The types of reactions envisaged were:

$$S^o + 2GSH \longrightarrow H_2S + G\text{-}S\text{-}S\text{-}G$$

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

Hempfling(1964) observed a result conflicting with the proposal of Peck, in that the oxygen uptate with GSH plus thiosulfate in extracts of T. neopolitanus was greater

than that with thiosulfate alone, the reaction was AMP-independent, and tetrathionate instead of sulfate as a product was formed. He interpreted his results by the following mechanism where thiosulfate was first oxidized to tetrathionate and then regenerated by the chemical reduction of tetrathionate by GSH.

$$2S_2O_3^{2-} \text{----------} S_4O_6^{2-} + 2e^-$$

$$S_4O_6^{2-} + 2GSH \xrightarrow{\text{non-enzymatic}} 2S_2O_3^{2-} + G\text{-}S\text{-}S\text{-}G + 2H^+$$

It seems that Hempfling was only dealing with the thiosulfate-oxidizing enzyme and not the complete oxidation of thiosulfate to sulfate as Peck was doing. Since the sulfur-oxidizing enzyme was isolated and studied in T. thioparus (Suzuki and Silver, 1966), it should be emphasized that the elemental sulfur would be further oxidized to sulfate by sulfur-oxidizing enzyme and sulfite oxidase or APS reductase as suggested by Lyric and Suzuki(1970).

While attempting to study the enzymes involved in the oxidative mechanism of thiosulfate, sulfur and sulfite in T. novellus, Charles and Suzuki (1965; 1966) observed that GSH was not required for the oxidation of thiosulfate to sulfate and thiosulfate sulfurtransferase (rhodanese), sulfur-oxidizing enzyme, sulfite-cytochrome c oxidoreductase and cytochrome oxidase were present, but not thiosulfate-

oxidizing enzyme and APS-reductase.

Contradictory to earlier findings in T. thioparus and T. neapolitanus, the discovery in T. novellus of rhodanese and sulfite oxidase rather than the thiosulfate-oxidizing enzyme and APS reductase led to a suggestion of an alternative mechanism for thiosulfate oxidation as the following reaction sequences:

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

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

$$2SO_3^{2-} + 4\text{cyt. } \underline{c}\ Fe^{3+} + 2H_2O \xrightarrow[\text{oxidase}]{\text{sulfite}} 2SO_4^{2-} + 4\text{cyt. } \underline{c}\ Fe^{2+} + 4H^+$$

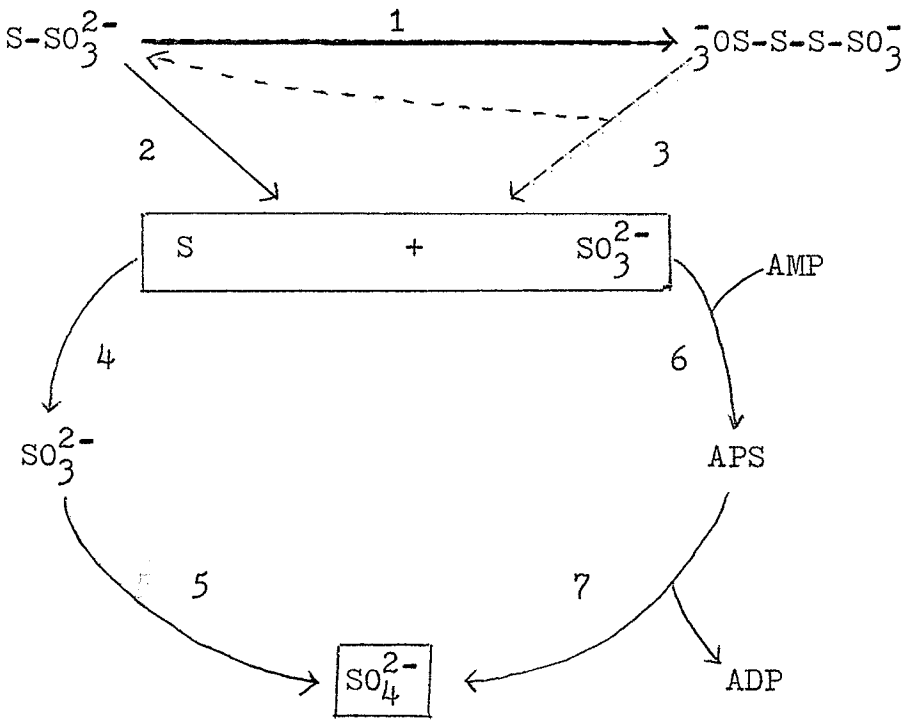
$$4\text{cyt. } \underline{c}Fe^{2+} + O_2 + 4H^+ \xrightarrow[\text{oxidase}]{\text{cytochrome}} 4\text{cyte. } \underline{c}\ Fe^{3+} + 2H_2O$$

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

In the above scheme, thiosulfate was cleaved to sulfur and sulfite, sulfur derived from the sulfane group of thiosulfate was oxidized to sulfite, and sulfite was finally oxidized to sulfate.

ATP was produced by an oxidative phosphorylation at the level of sulfite oxidation. The proposal has received further support from the study of Charles(1969) on the mechanism of thiosulfate oxidation in autotrophically grown T. intermedius, a recently isolated facultative autotroph.

During the course of further enzymatic studies concerned with mechanism of thiosulfate oxidation in T. thioparus it was found that the thiosulfate-oxidizing enzyme was present and sulfite was further oxidized to sulfate either by sulfite oxidase or APS reductase(Lyric and Suzuki, 1970a,b,c and d). Therefore, on the basis of informations obtained by studies of T. novellus and T. thioparus an alternative scheme was proposed by Lyric and Suzuki(1970) as follows:



1. Thiosulfate-oxidizing enzyme
2. Thiosulfate-splitting enzyme(Rhodanese)
3. Enzyme(s) unknown
4. Sulfur-oxidizing enzyme
5. Sulfite oxidase

6. APS reductase

7. ADP sulfurylase

In the above scheme, the oxidation of thiosulfate in thiobacilli can be initiated either by thiosulfate-splitting enzyme or thiosulfate-oxidizing enzyme:

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

or $$2SSO_3^{2-} \longrightarrow {}^{-}_{3}OS\text{-}S\text{-}S\text{-}SO_3^{-} + 2e^{-}$$

The tetrathionate formed can be converted to thiosulfate, sulfur and sulfite by a reductive cleavage under anaerobic conditions, but when there is an unlimited supply of thiosulfate available to the organisms, tetrathionate is expected to accumulate and be metabolized later through a reductive splitting process. However, once thiosulfate is cleaved, the thiosulfate-oxidizing enzyme system will be inhibited by sulfite produced.

Sulfur will be oxidized to sulfite by a sulfur-oxidizing enzyme or will be released into outside of cells as elemental sulfur when the sulfur-oxidizing enzyme system is disturbed. Sulfite will be oxidized to sulfate by one of the two sulfite-oxidizing systems: sulfite oxidase pathway or APS reductase pathway, as noted in the foregoing section. This explanation is attractive because it is based upon known properties of enzymes involved in the

thiosulfate oxidation which have been isolated and characterized from various species of Thiobacillus.
Thiosulfate sulfurtransferase(rhodanese), an apparently ubiquitous enzyme, is present at high activity levels in most mammalian tissues, plants and a number of bacteria, where it probably serves a variety of functions related to its capacity as a general sulfane transferase by catalytic double-displacement mechanism.

Lang(1933) discovered the enzyme, rhodanese, which catalyzes the following reaction:

$$S_2O_3^{2-} + CN^- \longrightarrow SCN^- + SO_3^{2-}$$

The enzyme is first charged with the donor sulfur(the sulfane group of thiosulfate) and then discharged by the acceptor(cyanide).

The mammalian enzyme is particularly rich in liver and kidney and is mainly associated with the mitochondria. Since the enzyme is readily purified and crystallized to pure form, intensive studies into the mechanism of action of this enzyme have been made in the last 40 years.
As a result the molecular mechanism of the enzyme has been fairly well elucidated. The current states of our knowledge concerning this enzyme has been reviewed with particular emphasis on the distribution, properties, mechanism of action, and biological function of the catalytic activity

(Roy and Trudinger, 1970; Westley, 1973).
In studies of the mechanism of thiosulfate binding and the catalytic site of rhodanese, it has been implicated that thiol, aromatic(tryptophan group), cationic and electrophilic groups are involved in the active site of the enzyme (Sörbo, 1962; Davidson and Westley, 1965; Wang and Volini, 1967; Mintel and Westley, 1966a and b).

The mammalian rhodanese appears to be involved in the intracellular turnover of reduced sulfur although the diverse biological functions of rhodanese in mammalian system have been proposed. These proposals are cyanide-detoxifying role(Lang, 1933 and Sörbo, 1957), the disposal of sulfide produced from cysteine by cystathionase action (Cavallini, et al.,1962; Flavin, 1962; Szczepkowski and Wood, 1967) and its thiosulfate reductase activity to fulfill a catalytic role in the transfer of reduced sulfur (Koj, 1967; Sido and Koj, 1972).

Torii and Bandurski(1967) also suggested a synthetic function of rhodanse for the formation of cysteine in micro-organisms and plants having access to sulfur as sulfate.

Recently, a possible role for bovine rhodanese in forming the "labile sulfide" of nonheme-iron proteins from thiosulfate was proposed by Finazzi Agro et al.(1971). They showed that treatment with rhodanese and thiosulfate resulted in partial reactivation of a ferrodoxin preparation which had lost its activity upon removal of the labile sulfides.

More recently they found that rhodanese, crystallized from beef kidney, was able to accept the cleaved sulfur in the forms of persulfide groups(R-S-SH) in the active site of enzyme(Finazzi Agro et al.,1972). Furthermore, it has been confirmed that the absorbancy exhibited by rhodanese with cyanide and with thiosulfate in the area of 335 nm has to be related to the presence of transferable sulfur loosely bound to the enzyme possibly in the form of a persulfide group(Cannella et al., 1974).

Nevertheless, the precise biological function of rhodanese has not yet been fully explained and it appears that by serving as a general sulfane transferase, rhodanese may fulfil a variety of functions in different particular situations.

The physiological function suggested for rhodanese in the photosynthetic and chemosynthetic sulfur bacteria was the role of the enzyme in the oxidation of thiosulfate to sulfate in Chromatium(Smith and Lascelles, 1966) and in Thiobacillus(Charles and Suzuki, 1966 and Charles, 1969).

McChesney(1958) detected first the rhodanese activity in species of Thiobacillus.

The enzyme has been isolated and the properties have been studied T. denitrificans(Bowen et al.,1965a and b) and T. ferrooxidans(Tabita et al., 1969).

The purified monomeric form of the T. denitrificans enzyme with a molecular weight of about 38,000 daltons was

similar to that of the dimeric mammalian rhodanese. The optimum pH was between 8 and 9 and copper was apparently not present. The enzyme was strongly inhibited by thiol-binding reagents and the activity of rhodanese was enhanced by reduced glutathione and dihydrolipoate at concentrations of 10^{-3} M and 10^{-2} M.

The T. ferrooxidans enzyme was purified about 40-fold and was shown to have a wide pH optimum(pH 7.5-9.0). Optimum temperature was 25°C and the apparent Km values for thiosulfate and cyanide were 5.8×10^{-4} M and 1.1×10^{-2} M. This enzyme was also inhibited by thiol-binding reagents.

Rhodanese from Chromatium studied by Smith and Lascelles(1966) was partly purified and two active fractions were obtained from DEAE-cellulose chromatography. The enzyme showed the optimum pH at 8.7 and the Km values for thiosulfate and cyanide were 6×10^{-4} M and 2×10^{-2} M which were close to the values for T. ferrooxidans enzymes. The Chromatium rhodanese was not inducible by thiosulfate and the level of activity was in the same order as that of the non-sulfur photosynthetic bacterium, Rhodospirillum rubrum.

LeJohn et al.(1967), however, reported that the rhodanese was apparently induced by thiosulfate in the facultative chemolithotroph, T. novellus, during the change from heterotrophic to autotrophic growth conditions. This result may implicate the role of rhodanese in thiosulfate

oxidation of thiobacilli. These conflicting views have not yet been settled. It is interesting to note that two distinct enzymes appear to be responsible for the cyanolysis of thiosulfate and the oxidation of thiosulfate to tetrathionate by Chromatium (Smith and Lascelles, 1966a and b). The enzymatic activities of thiosulfate reductase and mercaptopyruvate sulfurtransferase are closely related to the activity of rhodanese.

Villarejo and Westley (1963a and b) reported that crystalline beef liver rhodanese had ability to catalyze the reduction of thiosulfate to sulfide and sulfite in the presence of several electron donors such as borohydride, dithionate, 2,3-mercaptopropanol, dihydrolipoate and dihydrolipoamide. Cysteine, reduced glutathione, mercaptoethanol, NADH and NADPH were not effective electron donors for the reduction. From the experimental result, they proposed a mechanism for the rhodanese-catalyzed thiosulfate-lipoate reaction as follow:

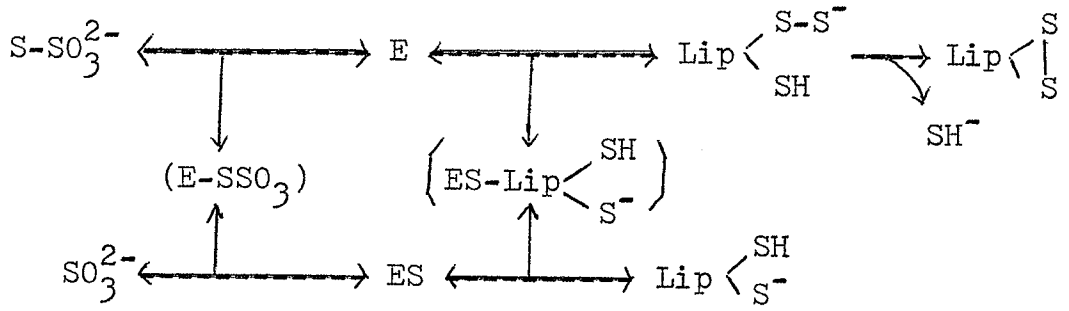
$$\text{Enzyme} + 2SSO_3^{2-} \rightleftharpoons \text{Enzyme} - S_2 + 2SO_3^{2-}$$

$$\text{Enzyme} - S_2 + 2\ \text{lip}\begin{matrix}\diagup \text{SH} \\ \diagdown \text{SH}\end{matrix} \longrightarrow \text{Enzyme} + 2\ \text{lip}\begin{matrix}\diagup \text{S-SH} \\ \diagdown \text{SH}\end{matrix}$$

$$2\ \text{lip}\begin{matrix}\diagup \text{S-SH} \\ \diagdown \text{SH}\end{matrix} \longrightarrow 2\ \text{lip}\begin{matrix}\diagup \text{S} \\ \diagdown \text{S}\end{matrix} + 2H_2S$$

Later, from the background of the kinetic analysis, Volini

and Westley(1966) presented a complete scheme where the sulfur-substituted rhodanese formed a kinetically significant complex with lipoate:



Rhodanese from Bacillus subtilis has been shown to catalyze both the cyanolysis and reduction by thiosulfate (Villarejo and Westley, 1966).

Sörbo(1964) and Koj(1968 and 1972) have argued, however, that rhodanese and thiosulfate reductase are different enzymes. Thiosulfate reductase was principally responsible for thiosulfate reduction in liver and cysteine and reduced glutathione were active electron donors. This enzyme was separated from rhodanese by Koj (1968) and shown to have a different pH optimum and different substrate specificity.

Bacterial thiosulfate reductase has also been shown to be separate from rhodanese in Desulfutomaculum nigrificans(Burtner and Akagi, 1971). Neverthless, it is not possible at this stage to state with any certainty whether the rhodanese and thiosulfate reductase are different. Although rhodanese has ability to catalyze the same reaction with electron donor(dihydrolipoate), the

thiosulfate reductase is not capable of transferring thiosulfate sulfur to cyanide(Westley, 1973).

A reductive cleavage of thiosulfate by thiosulfate reductase to sulfide(SH) and sulfite(SO_3^{2-}) has been proposed as an initial step of the oxidative metabolism of thiosulfate in T. thioparus(Peck, 1962). But, the specific activity was very low and no one has yet been able to purify and characterize the enzyme from cell-free extracts of thiobacilli. Furthermore, the thiosulfate reductase has not been reported in other thiobacilli. The thiosulfate oxidation by cell-free extracts of T. novellus was strongly inhibited by reduced glutathione, whereas, if thiosulfate reductase was involved, glutathione should be required for activity(Charles and Suzuki, 1966a).
The significance of this enzyme for thiosulfate oxidation in the thiobacilli, therefore, remains to be established.

Mercaptopyruvate sulfurtransferase which catalyzes a conversion of 3-mercaptopyruvate to pyruvate and elemental sulfur closely resembles rhodanese in many properties and the metabolic function in animals and bacteria of this enzyme has been considered to involve the interconversions of sulfur compounds, i.e. desulfuration and transsulfuration (Roy and Trudinger, 1970).

The enzyme has been intensively studied by several workers and a mechanism involving the formation of a sulfur-substituted enzyme as a persulfide(R-SSH) and the elimination

of pyruvate was proposed. The persulfide groups in the sulfur-enzyme complex have been shown to be non-dialyzable and subsequently to react with either with cyanide to form thiocyanide or to be decomposed to enzyme and elemental sulfur(Kun and Fanshier, 1959; Hylin and Wood, 1959; Fanshier and Kun, 1962; Kun, 1969; Vachek and Wood, 1972). It is probable that a similar mechanism operates in thiosulfate metabolism, but, the enzyme activity has not yet been detected in thiobacilli grown on thiosulfate.

Also Although little direct evidence is yet available to substantiate or refute the proposed mechanism of thiosulfate cleavage in thiobacilli, it is possible that these types of sulfurtransferases are responsible for the early stages of thiosulfate oxidation as pointed out by Suzuki (1974). The precise roles of the sulfurtransferases for thiosulfate oxidation in thiobacilli, however, obviously await further elucidation.

(5) Polythionates Oxidations

Little information is yet available on the mechanism of polythionates oxidations.

In early studies it was shown that resting bacterial suspensions of some thiobacilli could oxidize both tetrathionate and trithionate to sulfate(Vishniac, 1952, Parker and Prisk; 1953; Jones and Happold, 1961). Later, Trudinger

(1964a and 1964c) reported that tetrathionate was metabolized anaerobically by intact cells of T. neapolitanus and thiosulfate, elemental sulfur and sulfate were formed as reaction products. From the quantitative relationship between the amounts of tetrathionate metabolized and the products he suggested an initial reaction for an asymmetric hydrolysis of tetrathionate according to either one of the following reactions:

$$^{-}_{3}OS\text{-}S\text{-}S\text{-}SO^{-}_{3} + OH^{-} \xrightarrow{\text{enzymatic}} SSO^{2-}_{3} + S^{o} + HSO^{-}_{4}$$

or

$$^{-}_{3}OS\text{-}S\text{-}S\text{-}SO^{-}_{3} + 2OH^{-} \xrightarrow{\text{enzymatic}} SSO^{2-}_{3} + HO\text{-}S\text{-}OH + SO^{2-}_{3}$$

In the latter equation, anaerobically the sulfate formation was explained by the following chemical and enzymatic reactions:

$$^{-}_{3}OS\text{-}S\text{-}S\text{-}SO^{-}_{3} + SO^{2-}_{3} \xrightarrow{\text{chemical}} SSO^{2-}_{3} + {}^{-}_{3}OS\text{-}S\text{-}SO^{-}_{3}$$

$$^{-}_{3}OS\text{-}S\text{-}SO^{-}_{3} + H_2O \xrightarrow{\text{enzymatic}} S_2O^{2-}_{3} + SO^{2-}_{4} + 2H^{+}$$

However, it was observed that the rates of tetrathionate and trithionate oxidations were very low compared with the rates of thiosulfate oxidation and a high cell concentration was required for the oxidations. Furthermore, it was pointed out that the rates of oxidations were markedly influenced by a number of experimental conditions: i.e., dissolved oxygen concentration, the presence of

thiosulfate and the previous growth-history of the cells. The oxidation of these compounds was inhibited by 100 % oxygen. Since both aerobic and anaerobic oxidations of polythionates by T. neapolitanus were extremly sensitive to thiol-binding reagents, Trudinger(1965) suggested that active thiol groups are necessary and a low oxygen tension is required for generation of reduced thiol groups for polythionate oxidation. At both high cell concentrations and a low oxygen concentration, a sparking of tetrathionate oxidation by the addition of a catalytic amount of thiosulfate was observed.

Although some possible implications of these observations on the metabolism of anaerobic polythionate metabolism by the organism was discussed, no enzymatic conversion of polythionate to sulfate could be demonstrated in the cell-free systems.

London and Rittenberg(1964), however, showed that the cell-free extracts obtained by Hughes Press of T. thioparus with prior dialysis or Norite A treatment and sonically prepared cell-free extracts of T. thiooxidans catalyzed the complete oxidation of tetra-and trithionate to sulfate. Tetra-and trithionate were shown to be intermediates during thiosulfate oxidation by cell-free extracts of both organisms with radioautographs of reaction samples during the course of complete oxidation.

Despite these observations, since a precise pathway and

individual enzymes involved in polythionate oxidation have not been reported, the significance of polythionate metabolism in thiobacilli is somewhat doubtful.

The detailed discussion of previous works on the metabolism of polythionates has been described by Trudinger(1967 and 1969).

Recently, an alternative interpretation for the mechanism of polythionate metabolism was presented by Suzuki(1964), based on previously observed results from whole cells of T. neapolitanus(Trudinger, 1964a, 1964b and 1964c) and cell-free extracts of T. thioparus (London and Rittenberg, 1964). This proposal is outlined as follows: A reductive cleavage, requiring $2e^-$, of tetrathionate linked to sulfite oxidation would yield thiosulfate, elemental sulfur and sulfate as anaerobic products according to the following reactions:

$$^-_3OS\text{-}S\text{-}S\text{-}SO^-_3 + 2e^- \longrightarrow SO^{2-}_3 + S + SSO^{2-}_3$$

$$SO^{2-}_3 + H_2O \longrightarrow SO^{2-}_4 + 2H^+ + 2e^-$$

$$\text{Overall : } ^-_3OS\text{-}S\text{-}S\text{-}SO^-_3 + H_2O \longrightarrow SSO^{2-}_3 + S + SO^{2-}_4 + 2H^+$$

Under aerobic conditions, the initial scission of the tetrathionate molecule would be hindered by a shortage of electrons required for the cleavage and under anaerobic conditions, the oxidation of tetrathionate could be sparked

by supply of electrons from thiosulfate. It has been shown that c-types of cytochrome, sulfite oxidase and thiosulfate-oxidizing enzyme are present in both T. neapolitanus and T. thioparus(Trudinger, 1961; Trudinger, 1964; Lyric and Suzuki, 1970c).

When the rate of sulfite formation is faster than that of sulfite oxidation, the formation of trithionate would occur chemically formation a mixture of sulfite and tetrathionate as follows:

$$\bar{3}OS\text{-}S\text{-}S\text{-}SO_3^{2-} + SO_3^{2-} \longrightarrow S\text{-}SO_3^{2-} + \bar{3}OS\text{-}S\text{-}SO_3^{-}$$

The trithionate could be oxidized further to thiosulfate and sulfate by a reductive cleavage similar to that for tetrathionate. The scheme is as follows:

$$\bar{3}OS\text{-}S\text{-}SO_3^{-} + 2e^{-} \longrightarrow S_2O_3^{2-} + SO_3^{2-}$$

$$SO_3^{2-} + H_2O \longrightarrow SO_4^{2-} + 2H^{+} + 2e^{-}$$

$$\text{Overall : } \bar{3}OS\text{-}S\text{-}SO_3^{-} + H_2O \longrightarrow S_2O_3^{2-} + SO_4^{2-} + 2H^{+}$$

Imai et al.(1962) proposed such a reductive cleavage of trithionate in T. thiooxidans. Later, Okuzumi(1966b) suggested that both the reductive cleavage and hydrolysis of trithionate were catalyzed by the same organism.

This interpretation is more attractive because the

proposed scheme is consistent with most observations and data in Trudinger's work as well as in other literatures. Nevertheless, the foregoing reaction mechanism of polythionate metabolism is purely speculative one and as yet there is little direct evidence for this postulate.

Summarizing, it would appear that despite a large number of studies by many noted investigators, we still have much to unravel before we shall truly understand the mechanism of inorganic sulfur oxidation by thiobacilli.

MATERIALS AND METHODS

MATERIALS AND METHODS

MATERIALS

All chemicals and reagents used were of analytical grade and commercially obtained. Labeled sodium thiosulfate(inner tagged and outer tagged $Na_2^{35}S_2O_3$), labeled sulfate($Na_2^{35}SO_4$) and Protosol were purchased from New England Nuclear, Boston, Mass., U.S.A.; sodium thiosulfate, sodium sulfite, sodium sulfide, sodium sulfate, hydroxylamine hydrochloride and sodium diethyldithiocarbamate from Fisher Scientific Company, Fairlawn, N.J., U.S.A.; sodium tetrathionate from K & K Laboratories Inc., Plainview, New York, U.S.A. and atabrine(atebrin; quinacrine dihydrochloride) from Mann Research Labs. Inc., New York, U.S.A. Catalase(liver, 2 times crystallized), GSH, FAD, FMN, cytochrome c(type III, from horse heart), AMP, ADP, ATP, GTP, deoxy AMP, 3';5'-cyclic AMP, NADPH, bovine serum albumin(crystalline and fraction V), spermine(tetrahydrochloride), spermidine, lysozyme(egg white), phospholipases A, C and D, lipase, trypsin(bovine pancreatic crystalline), deoxyribonuclease(pancreatic), ribonuclease, 2,2'-dipyridyl, 1,10-phenanthroline(monohydrate), bathocuproin disulfonate (Na salt), valinomydin(crystalline), oligomycin(crystalline), 2,4-dinitrophenol, dicoumarol(bishydroxycoumarine), amytal (amobarbital; 5-ethyl-5-isoamylbarbituric acid), B.A.L.

(2,3-dimercaptopropanol), rotenone, TTFA(2-thenoyltrifluoroacetone), HQNO(2-heptyl-4-hydroxyquinoline-N-oxide), p-HMB(p-hydroxymercuribenzoate), NEM(n-ethylmaleimide), sodium iodoacetate, DTNB(5,5'-dithiobis-(2-nitrobenzoate)) and DMSO(dimethyl sulfoxide) were obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A. Pronase (B grade), Aquacide 1-A, Antimycin A, CCCP(carbonylcyanide-m-chlorophenyl hydrazone), DTT(dithiothreital) and P-NBT (p-nitroblue tetrazolium) were from Calbiochem., Los Angeles, California, U.S.A.; precipitated sulfur powder, potassium cyanide, silver nitrate, mercuric chloride, sodium arsenite, sodium arsenate and phosphotungstic acid from J.T. Baker Chem. Co., N.J., U.S.A. and sodium azide, EDTA(ethylenediamine tetraacetic acid, Na salt), TMPD (N:N:N':N'-tetramethyl-p-phenylene-diamine dihydrochloride) and uranyl acetate from the British Drug Houses Ltd., London, England. NADH(nicotinamide adenine dinucleotide, reduced, disodium salt) was obtained from P.L. Biochemicals Inc., Wisconsin, U.S.A. and nitrogen gas cylinder from Union Carbide Co., Canada. The gel chromatography media, Sephadex G-25 and Sepharose 4B, and Blue Dextran 2000 were products of Pharmacia Uppsala, Sweden. The colloidal sulfur which was used as substrate for sulfur-oxidizing enzyme was prepared by the method of Suzuki(1965).

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

ORGANISMS AND GROWTH CONDITIONS

Thiobacillus novellus.

A pure culture of Thiobacillus novellus(ATCC 8093) originated from Dr.R. L. Starkey was grown under autotrophic condition in a modified Starkey's No. 3 medium (Starkey, 1934) which contained the following: 10.0 g $Na_2S_2O_3 \cdot 5H_2O$, 4.0 g K_2HPO_4, 1.5 g K_2HPO_4, 0.3 g $(NH_4)_2SO_4$, 0.05 g $MgSO_4$, 0.014 g $CaCl_2$, 0.014 g $MnSO_4 \cdot H_2O$, 0.8 mg chelated iron in EDTA, 8 mg phenol red, and glass distilled water to make the final volume of 1 liter. Solutions of $Na_2S_2O_3 \cdot 5H_2O$, $MgSO_4$, $CaCl_2$, $MnSO_4 \cdot H_2O$, and chelated iron were autoclaved separately. The stock solution of chelated iron was prepared by dissolving 20 mg of $FeCl_3$ in 100 ml of glass distilled water containing 40 mg of EDTA-Na_2, and 4 ml of the stock solution were used in a liter of the medium. All components were combined aseptically after cooling. The final pH of the medium was 7.5.

Stock cultures and inocula were grown in 250 ml flasks containing 100 ml of the above medium for 4 days at 26°C on a rotary shaker(New Brunswick Scientific Co.) at 120 r.p.m. During growth of the organism the pH of the cultures was maintained at 7.0-7.5 by adjusting with a sterile 10%(w/v) potassium carbonate solution as required.

Large scale cultures of the organism were grown in 5 gallon

glass carboys(Quickfit^{R}, England) which contained 20 liters of the autoclaved media. The culture carboy was fitted with a pH control unit on the top and with two sintered glass spargers to the bottom of the medium.
A pH electrode(Radiometer GK 2303 C) was sterilized separately by immersion for 4 hours in a solution of 175 ml of absolute ethanol, 1.75 ml of conc-H_2SO_4, and 75 ml of sterile distilled water and rinsed gently twice with 250 ml of sterile distilled water. The sterile pH electrode was stored in a column tube which contained a sterile standard buffer(pH 6.5) prior to use. The bubbling of air through the carboys was started 4 hours before they were inoculated with 10 %(v/v) fresh cultures. Carboys were incubated at 26°C or 28°C with forced aeration through a sterilized cotton wool air filter and then through the spargers.
The pH was controlled during growth by automatically titrating the culture with a sterile 10%(w/v) potassium carbonate solution by means of a Radiometer pH-stat unit (Model 28 pH meter, Model GK 2303 C combination electrode, and Model TTT11 titrator. All from Radiometer, Copenhagen). The pH of the culture was checked once daily by independent measurements on a pH meter.

After 4½ days, the cells which were in the late phase of logarithmic growth were collected by a Sharples super-centrifuge fitted with a water cooling system(7°C) at 50,000 r.p.m. The harvested cells were washed three

times in 0.04 M potassium phosphate buffer(pH 7.0), suspended in the same buffer(50 mg wet weight cells/ml) and stirred gently at 4°C overnight to reduce endogenous metabolism. The cells collected by centrifugation at 2,000 x g for 20 minutes were washed once more in 0.1 M Tris acetate-0.02 M potassium phosphate buffer(pH 7.5) and resuspended in the same buffer to give a final cell concentration of 200 mg wet weight per ml. The cell suspension thus prepared was stored at 4°C and used within 3 to 4 days for preparing crude cell-free extracts.

In some growth experiments, cells were grown autotrophically in 1 l flasks containing 500 ml of autoclaved media on a rotary shaker(120 r.p.m.). Components for Starkey's(No. 3) medium and Charles' medium used for these experiments in addition to the modified Starkey's medium described earlier in this section were the same as described by Starkey(1934) and Charles and Suzuki(1966).

The pH of cultures was maintained at 7.0 to 7.5 as growth progressed by adding a sterile 10%(w/v) Na_2CO_3 solution. After the cultures were incubated at 26°C for $4\frac{1}{2}$ days, the cells were collected by centrifugation at 2,000 x g for 20 minutes and washed three times in 0.04 M potassium phosphate buffer(pH 7.0). Washed cells were suspended in the same buffer(50 mg wet weight/ml), stirred at 5°C for 6 hours and used for the experiments.

Thiobacillus thioparus

The culture of T. thioparus(ATCC 8158) used in this work was grown on Starkey's medium No. 2(Starkey, 1934b) as previously described by Lyric and Suzuki(1970) with a slight modification. The medium composition was: $Na_2S_2O_3 \cdot 5H_2O$, 10.0 g; K_2HPO_4, 4.0 g; KH_2PO_4, 4.0 g; $(NH_4)_2SO_4$, 0.3 g; $CaCl_2$, 0.014 g; $MgSO_4$, 0.05 g; $MnSO_4 \cdot H_2O$, 0.014 g; $FeCl_3 \cdot 6H_2O$, 0.02 g; in 1 liter glass distilled water. A solution of $Na_2S_2O_3 \cdot 5H_2O$ was autoclaved separately and added aseptically just prior to inoculation.

The maintenance of the stock culture, the growth conditions of large-scale cultivation, the harvesting of cells, and the removal of elemental sulfur were as described previously by Lyric and Suzuki(1970).

Thiobacillus thiooxidans

T. thiooxidans(ATCC 8085) was grown and the cell suspension was used immediately for the preparation of cell-free extracts as described by Suzuki(1965).

METHODS

Standard Methods for the Preparations of Crude Cell-free Extracts and Membrane Vesicles

(1) Crude Cell-free Extracts

The resting cells were washed once and were resuspended(150 to 200 mg wet weight cells per ml) in 0.1 M Tris acetate-0.02 M potassium phosphate buffer(pH 7.5) unless otherwise indicated. The cell suspension was sonicated for 15 minutes at 7°C under a stream of nitrogen using a Raytheon sonic disintegrator. The sonicated extract was treated with approximately 1 μg of deoxyribonuclease and ribonuclease and 10 mM $MgCl_2$ and the treated extract was then centrifuged at 2,000 x g for 20 minutes to remove any unbroken cells. The resulting supernatant was carefully removed with a syringe and was designated as "crude cell-free extract". The extract thus obtained contained 16 to 24 mg of protein per ml and was used immediately or stored at 4°C until further used.

In some experiments the preparation of the crude cell-free extract was performed using the same procedure as described above in the absence of nitrogen gas. All the experimental procedures were carried out at 4°C unless otherwise indicated.

(2) Membrane Vesicles

The membrane vesicles were prepared from the crude cell-free extracts by either differential centrifugation

(105,000 x g for 90 minutes) or gel filtration through a column of Sepharose 4B. For differential centrifugation method, the extract(5 to 10 ml) was centrifuged at 105,000 x g for 90 minutes. The supernatant fraction was carefully aspirated by means of a syringe and the particulate fraction, "membrane vesicle", was resuspended in 0.1 M Tris acetate-0.02 M potassium phosphate buffer (pH 7.5) to give an approximate protein concentration of 6 to 12 mg per ml. For gel filtration method, the crude cell-free extract(5 ml) was applied to a column of Sepharose 4B(2.5 x 37.5 cm) that had been equilibriated with 0.1 M Tris acetate-0.02 M potassium phosphate(pH 7.5). The column was then eluted with the same buffer and each fraction(2.5 ml) was collected in a test tube. The flow rate was about 50 ml per hour. When the fractions were measured for their thiosulfate-oxidizing activities as described in other section, the membrane vesicles were recovered in tubes of the void volume(68 ml) of the column as shown in the results. The membrane vesicles thus prepared were used immediately or stored at -74°C in 0.5 ml of aliquots after concentration with Aquacide 1-A (Calbiochem.) to high protein concentrations(6 to 12 mg of protein per ml). All the procedures were carried out at 4°C.

Other Methods for Cell Disruption

(1) Freezing and Thawing

The cell suspension(200 mg wet weight cells per ml) in 0.1 M Tris acetate-0.02 M potassium phosphate buffer(pH 7.5) was frozen at -15°C and thawed twice. The mixture was then centrifuged at 2,000 x g for 20 minutes and the resulting supernatant was removed to obtain "2,000 x g supernatant". The pellet was washed once with the same buffer(pH 7.5) and suspended in the original volume of the same buffer(2,000 x g pellet).

(2) Treatment with lysozyme and EDTA

Spheroplasts were prepared by the method of Suzuki and Kwok(1969) with a slight modification as follows: the bacterial cells were washed with 0.04 potassium phosphate(pH 7.4) and the washed cells(1 g wet weight) were suspended to 50 ml in the 0.5 M sucrose-0.1 M Tris acetate-0.02 M potassium phosphate(pH 7.5) to which had been added 2 x 10^{-3}M EDTA and 100 mg lysozyme(Grade I, 3 times crystallized). The suspension was incubated at 28°C for 2 hours with gentle shaking and then centrifuged at 2,000 x g for 20 minutes at 4°C to collect spheroplasts. The spheroplasts thus obtained were washed twice in 0.5 M sucrose-0.1 M Tris acetate-0.02 M potassium phosphate buffer(pH 7.5) and suspended in 5 ml of the same buffer.

(3) Osmotic Shock

In order to prepare osmotic shock fractionations, both intact cells and spheroplasts were used. For the spheroplast, the method of Rees and Nason(1965) was followed as described below. The washed spheroplast solution(5 ml) was diluted to 40 ml with the 0.005 potassium phosphate(pH 7.5) containing 10^{-3}M $MgCl_2$ and 40 µg of deoxyribonuclease. The mixture was incubated with gentle swirling for 2 hours at 28°C and centrifuged at 2,000 x g for 20 minutes at 4°C to remove unbroken spheroplasts. The turbid supernatant fraction which was designated as "spheroplast lysate" was centrifuged at 10,000 x g for 20 minutes at 4°C. The resulting supernatant fluid was removed and concentrated with Aquacide I-A (Calbiochem) in a dialysing tube to the original volume. The pellet was washed twice in 0.1 M Tris acetate-0.02 M potassium phosphate(pH 7.5) and suspended in 5 ml of the same buffer.

For the intact cells, the method of Neu and Heppel(1965) was used as described below. The cells were washed twice with 0.04 M potassium phosphate buffer(pH 7.4) and the washed cells(1 g wet weight) were suspended in 80 ml of 0.5 M sucrose-0.1 M Tris acetate-0.02 M potassium phosphate buffer(pH 7.5). The cell suspension was treated with EDTA to give a concentration of 10^{-3}M and incubated

in a flask on a rotary shaker for 2 hours with a rotation speed of 180 r.p.m. at 28°C. The mixture was centrifuged at 10,000 x g for 20 minutes at 4°C and the supernatant was removed. The well-drained cells were rapidly resuspended in 80 ml of cold 10^{-3}M $MgCl_2$ solution containing 80 ug of deoxyribonuclease. After gentle stirring for 30 minutes at 4°C, the suspension was centrifuged at 10,000 x g for 20 minutes at 4°C. The shock fluid thus obtained was placed in a dialysing tube and concentrated with Aquacide powders to 15 ml in a final volume. The pellet was resuspended in 5 ml of 0.1 M Tris acetate-0.02 M potassium phosphate buffer(pH 7.5).

(4) French Pressure Cell

For preparation of crude cell-free extract, 5 ml of cell suspension(200 mg wet weight cells per ml) were passed through an Aminco French Pressure Cell at 18,000 p.s.i. and the resulting extract was centrifuged at 2,000 x g for 20 minutes at 4°C. The sedimented pellet was discarded and the cell-free supernatant was used as the source of the crude cell-free extract. In some experiments, the cell suspensions were disrupted in the presence of bovine serum albumin(Fraction V, 20 mg per ml), with approximately 1 µg of deoxyribonuclease and 10 mM $MgCl_2$ or under nitrogen atmosphere.

Enzyme Assay Procedures

(1) Thiosulfate-oxidizing Activity

The thiosulfate oxidation was routinely assayed by measurement of oxygen consumption in a thermostated vessel equipped with a Teflon-covered Clark Oxygen Electrode(a Gilson Oxygraph, G.M.E., Wisconsin, U.S.A.) with an appropriate polarization circuitry. The reaction mixture contained, unless otherwise stated, Tris acetate buffer(pH 7.5), 150 μmoles; potassium phosphate buffer (pH 7.5), 20 μmoles; thiosulfate, 1.0 μmole; enzyme and water to make a final volume of 1.5 ml. The reaction was started by the addition of thiosulfate solution in microliter quantity by using a Hamilton microliter syringe equipped with a long needle to a standard mixture at 25°C. The reaction mixture was constantly stirred with a small magnetic stirring bar. The volumes of enzyme used for assays were 0.2 ml of crude cell-free extract (4.2 mg of protein per ml) or 0.25 ml of isolated membrane vesicles(1.5 mg of protein per ml) unless otherwise mentioned. The activity was expressed in nmoles or μmoles O_2 consumed per minute under the above standard conditions.

(2) Tetrathionate-oxidizing Activity

The tetrathionate-oxidizing activity was assayed by following the measurement of oxygen consumption polarographically with an oxygen electrode as described in the assay of thiosulfate-oxidizing activity with the exception that tetrathionate(1.0 µmole) was used. The activity was expressed in µmoles O_2 consumed per minute under the standard conditions.

(3) Sulfite-oxidizing Activity

The enzyme activity was assayed by measuring the rate of sulfite oxidation using an oxygraph as described in the assay of thiosulfate-oxidizing activity except that sulfite(0.3 µmole) was used as a substrate. When mammalian cytochrome c was required for assay, 0.3 µmole of horse heart cytochrome c(type III) was used.

(4) Thiosulfate-sulfur-transferase(Rhodanese) Activity

Thiosulfatesulfurtransferase activity was measured by the rate of thiocyanate formation from thiosulfate and cyanide according to the method of Sörbo(1959) with minor modifications. A test tube containing 150 µmole of Tris acetate buffer(pH 7.5), 20 µmoles of potassium phosphate buffer(pH 7.5), 150 µmoles of thiosulfate, 100 µmoles of potassium cyanide, enzyme and water in a total volume of

2.5 ml was incubated at 25°C for 10 minutes. The reaction was stopped by addition of 0.5 ml of 38% of formaldehyde solution. After addition of 2.0 ml of 20%(w/v) $Fe(NO_3)_3$ reagent, the absorbance at 460 nm was measured with a Unicam SP 500 spectrophotometer, reading against a blank consisting of a complete system but to which the formaldehyde solution had been added before cyanide. A calibration curve was prepared with known amounts of KCNS. One unit of enzyme was defined as amounts of enzyme which formed 1 µmole of thiocyanate under the above standard conditions.

(5) Sulfur-oxidizing Enzyme Activity

The rate of sulfur oxidation was measured manometrically at 30°C in a conventional Warburg apparatus as described by Suzuki and Silver(1966). The standard reaction mixture contained the following: 500 µmoles of Tris HCl buffer(pH 7.8), 48 mg of sulfur, 5 µmoles of GSH, 250 µg of catalase(E.C.1. 11. 1. 6), 0.2 µmole of 2,2'-dipyridyl, enzyme(0.6 mg of protein) and water to make a total volume of 2.0 ml. The reaction was initiated by tipping a GSH solution from the side-arm of Warburg vessels. The flasks were incubated with constant shaking for 2 hours and the reaction was stopped by the addition of 0.1 ml of 1 M cadmium acetate. After centrifugation at 12,000 x g for

20 minutes, the resulting supernatant was analyzed for thiosulfate by a modification of the method of Sörbo (1959) as described in a later section.

(6) Sulfite: Cytochrome c Oxidoreductase Activity

The enzyme activity was measured by following the rate of reduction of cytochrome c as described by Lyric and Suzuki(1970). The assay system contained the following: 20 µmoles of Tricine-NaOH buffer(pH 8.5), 0.12 µmole of horse heart cytochrome c(type III), 0.3 µmole of sodium sulfite, enzyme and water to a total volume of 3.0 ml. The reaction was started by adding sulfite. The rate of cytochrome c reduction was followed at 550 nm spectrophotometrically. The activity was expressed in terms of µmoles of cytochrome c reduced per minute using the above assay system.

(7) Cytochrome c Oxidase Activity

The spectrophotometric assay of cytochrome c oxidase was carried out at 25°C in cuvettes of 1.0-cm light path by measuring the initial rate of oxidation of reduced cytochrome c(horse heart, type III) in the presence of excess oxygen. The reaction mixture contained 300 µmoles of Tris acetate(pH 7.5), 40 µmoles of potassium

phosphate(pH 7.5), 0.12 µmole of reduced cytochrome c, enzyme and distilled water to a final volume of 3.0 ml. The reaction was initiated with the addition of 5 µl to 20 µl of enzyme preparations(crude cell-free extracts or membrane vesicles). The activity of the cytochrome c oxidase was expressed as the number of µmoles of cytochrome c oxidized per minute under the above conditions.

(8) Thiosulfate-oxidizing Enzyme Activity

The enzyme activity was measured by following either the reduction of ferricyanide or the reduction of cytochrome c as described by Lyric and Suzuki(1970). For the ferricyanide coupled assay, the reaction mixture contained the following: 3 µmoles of potassium ferricyanide, 3 µmoles of sodium thiosulfate, 150 µmoles of potassium phosphate buffer(pH 7.4), enzyme and water to a total volume of 3.0 ml. The reaction was initiated by adding enzyme. The rate of ferricyanide reduction was followed at 420 nm spectrophotometrically.

For the cytochrome c coupled assay, the conditions were identical with the exception that ferricyanide was replaced with 1.8 µmoles of cytochrome c(horse heart, type III) and the rate of cytochrome c reduction was followed at 550 nm spectrophotometrically. The activity was expressed as the number of µmoles of ferricyanide or

cytochrome c reduced per minute under the above conditions.

Centrifugations

Low speed centrifugations were performed in a Sorvall Superspeed RC-2B automatic refrigerated centrifuge at 4°C. High speed centrifugation were carried out in a Beckman model L2-65 B refrigerated ultracentrifuge using a 50 Ti or 60 Ti rotor at 4°C.

Sucrose Density Gradient Centrifugation

Linear sucrose gradients were generated from 15 and 40%(w/v) sucrose solutions in 0.1 M Tris acetate-0.02 M potassium phosphate buffer, pH 7.5 with a 50%(w/v) sucrose cushion. The sixty ml of density gradients were made in cellulose nitrate tubes and stored in the cold room(4°C) for 3 hours prior to use. Enzyme samples were contained in volume of 2 ml and layered gently on the top of the gradient. Tubes were placed in a SW 25.2 swinging bucket rotor and then centrifuged at 60,000 x g(22,000 r.p.m.) for 12 hours at 4°C in a Beckman Model L2-65 B ultracentrifuge.

Fractions of 1.5 ml were collected from bottom of punctured tubes and the protein content in each fraction

was determined by the method of Lowry et al. (1951) as described in a later section. The peaks(I, II and III - see Results) were pooled, dialyzed against the same buffer for 4 hours at 4°C and assayed for thiosulfate-oxidizing activity as described in a previous section.

Since sucrose was inhibitory to the activity and the enzyme system was also inactivated in sucrose, control enzyme samples were kept at 4°C in 15% sucrose solution in the same buffer for the same length of time as required for the above procedures and were then dialyzed to remove sucrose. The thiosulfate oxidizing activities of the treated control samples were determined.

Thin-layer Chromatography and Radioautography of Inorganic Sulfur Compounds

An one-dimensional ascending thin-layer chromatography on sheets of Gelman Instant Thin-Layer Chromatography (I.T.L.C.) media, type SA(20 x 20 cm and 5 x 20 cm) was used to separate and identify thiosulfate, tetrathionate and sulfate as described by Kelly(1970).

Sodium thiosulfate(0.1 μmole) labelled with S-35 in the inner($-SO_3H$) and outer(-S-) positions were added into the reaction mixtures in microliter volumes by using Hamilton microsyringes equipped with long needles and oxygen consumptions were measured in a Gilson Oxygraph

as described previously. After completion of thiosulfate oxidations, aliquots of 10 µl with markers were applied to ITLC media and subjected to chromatography in a solvent system consisting of 5%(v/v) water in 1-butanol using a Gelman ITLC chamber at room temperature(22 to 25°C). Markers were either S-35 labelled or cold thiosulfate and tetrathionate. Sulfate labelled with S-35 was also used as a marker compound. Tetrathionate labelled with S-35 was prepared by treating S-35 labelled thiosulfate with iodine as described by Trudinger(1964).

The chromatograms were air-dried and rechromatographed to the same direction in a second solvent system composed of methanol and 1-propanol in the proportion of 1:1. After air drying, radioactive compounds on the chromatograms were detected by autoradiography after exposure of the ITLC sheets to Kodak X-ray film(No Screen) for 2 days. Radioactive areas were outlined on the chromatograms by tracing over the developed film. In some experiments, the spots of the marker compounds were directly located by spraying the chromatograms with ammonial silver nitrate as described by Trudinger(1965). After the second run, thiosulfate, tetrathionate, and sulfate were easily separated from each other, giving spots with Rf= 0, Rf = 0.38 and Rf = 0.8. These values were in full agreement with the values for these compounds reported by Kelly(1970).

After identification of the radioactive compounds, the radioactive areas were removed by cutting with a blade and were ground to powder. The ground samples were transferred to scintillation vials and suspended in 0.5 ml of distilled water and then mixed with 1 ml of Protosol^{R}(New England Nuclear). In some experiments, radioactive compounds which were identified from their positions on the chromatograms were eluted with water and lyophilized. The lyophilized samples were redissolved in 0.5 ml of distilled water and a part of the solution was transferred to scintillation vials.

After addition of 10 ml of Bray's fluid(Bray, 1960), the radioactivity was determined using a Parkard Tri-Carb liquid scintillation spectrometer. All counts were corrected for background.

Treatments of Thiosulfate-oxidizing Systems with Various Agents

(1) Proteolytic Enzymes

For the whole cells, 1.0 ml of the resting cell suspension(2 mg wet weight cells per ml) in 0.1 M Tris acetate-0.02 M potassium phosphate(pH 7.5) and 10 mM $MgSO_4$ was incubated at room temperature with either 100 or 200 μg of trypsin. A 1.0 ml of the same cell suspen-

sion was incubated without trypsin as a control. After the desired incubation periods, proteolytic action was stopped by addition of 250 μg of soybean trypsin inhibitor in the same buffer. The mixture was diluted with 5 ml of the same cold buffer and centrifuged at 2,000 x g for 20 min. The cells obtained in the pellet was suspended in the buffer to a final volume of 1.0 ml. Thiosulfate-oxidizing activity and sulfur-oxidizing activity were measured using 1.0 ml of the suspension as described in other sections. Pronase treatment was done exactly as described above except that the amount of pronase was either 100 or 200 μg per ml of cell suspension(4.5 or 9 PUK units) and inhibitor was not used.

To study the influence of proteolytic digestion on thiosulfate-oxidizing activity of the cell-free extract and the membrane vesicles, the preparations were incubated(1.5 mg protein per ml) at room temperature in 0.1 M Tris acetate-0.02 M potassium phosphate(pH 7.5) containing 1 to 2 mg of bovine pancreatic trypsin per ml and 10 mM $MgSO_4$. A 0.5 ml aliquot was withdrawn before the addition of trypsin and served as a control. At various times 0.5 ml aliquots of the mixture were taken and pipetted into 5 ml of ice-cold 0.1 M Tris acetate-0.02 M potassium phosphate buffer(pH 7.5) containing 3 mg of soybean trypsin inhibitor. The mixtrues

were stirred by a mixer and were then immediately sedimented by centrifugation at 105,000 x g for 120 minutes. The supernatant solution was removed and the pellet suspended in 0.5 ml of the standard buffer containing 0.5 mg of trypsin inhibitor. The resulting supernatant and resuspended membrane vesicles were assayed for thiosulfate-oxidizing activity and sulfur-oxidizing activity using the same procedures as described in other sections. In some experiments, the trypsin treatment was not terminated by adding trypsin inhibitor and aliquots of the mixtures were withdrawn directly for measurement of activities without centrifugation as noted in the figure legends.

The experimental procedures of pronase treatments were the same as those described above for trypsin except that the amount of pronase was 100 or 200 μg in 1.0 ml of the preparations and the inhibitor was not used.

(2) Lipase, Phospholipases and Lysozyme

The treatment of either intact cells(2 mg wet weight cells) or membrane vesicles(1.55 mg protein per ml) was carried out by preincubation with various enzymes at room temperature(25°C) for 20 minutes in 0.1 M Tris acetate-0.02 M potassium phosphate buffer(pH 7.5) containing 2 mM $CaCl_2$.

Lipase and phospholipase A were dissolved in the same buffer at a concentration of 1 mg per ml, phospholipase C and D were dissolved in the buffer at a concentration of 5 mg per ml, and lysozyme was dissolved in water at a concentration of 100 mg per ml. These enzymes were used without further purification and added in microliter volumes. The concentrations of these enzymes were varied as required.

After incubation, the thiosulfate-oxidizing activity was assayed in a Gilson Oxygraph as described in other section.

(3) Ionic and Nonionic Detergents

Either crude cell-free extracts or membrane vesicles were prepared from fresh cells as described in other sections.

The preparations, approximately 3.0 mg of protein, in 0.1 M Tris acetate-0.02 M potassium phosphate buffer (pH 7.5) were divided into two equal parts(1 ml each). All detergents were added slowly to a final concentrations as required in microliter volumes and the mixtures were stirred gently. After incubation at 4°C for 30 minutes, one part was immediately assayed for the thiosulfate-oxidizing activity as described in other sections. The other part was dialyzed against the same buffer for

3 hours at 4°C and was assayed for the thiosulfate-oxidizing activity in a Gilson Oxygraph.

(4) Lysolecithin

A solution of lysolecithin(30 mg per ml in water) was added to the crude cell-free extract(24 mg of protein per ml) at a ratio of 0.8 ml per ml of extract. The mixture was then incubated at 25°C and the thiosulfate-oxidizing activity was measured by withdrawing 0.2 ml aliquots from the reaction mixture at various time intervals.

Purification of Sulfur-oxidizing Enzyme

Sulfur-oxidizing enzyme of T. thiooxidans was partially purified according to the procedure of Suzuki (1965). The enzyme was also purified as described by Silver and Suzuki(1966) using T. thioparus cells. The purified enzyme was stored at -74°C and was used within one week.

Purification of Sulfite: Cytochrome c Oxidoreductase

Sulfite: cytochrome c oxidoreductase from T. novellus cells was highly purified as described by Lyric

and Suzuki(1970a) and was stored at -20°C until further used.

Preparation of Ferrocytochrome c-550

Reduced cytochrome c was prepared by a modified method of Wharton and Tzagoloff(1967). A fresh solution of ascorbic acid(50 mg/ml) was prepared in 0.01 M potassium phosphate buffer(pH 7.0) containing 1 mM EDTA saturated with nitrogen gas and was neutralized to pH 7.0 with potassium hydroxide. The ferrocytochrome c was prepared by dissolving 25 mg of lyophilized cytochrome c (type III, from horse heart) in 1 ml of the ascorbate solution at room temperature. The excess amount of ascorbate was removed by placing the solution on a column (1 x 15 cm) of Sephadex G-25(fine-grain bead) which had previously been equilibrated with the 0.01 M potassium phosphate buffer(pH 7.0) containing 1 mM EDTA saturated with nitrogen, and eluting with the same buffer at room temperature. The gel filtration was carried out under a minimal hydrostatic pressure to obtain a slow rate which resulted in a maximal separation of reduced cytochrome c from exess ascorbate and its oxidation product. The middle three-fifth(about 1.5 ml) of the pink-red fraction was collected and the concentration of ferrocytochrome c-550 in the elute was determined spectro-

photometrically. The solution was appropriately diluted with the same buffer to obtain 1.2 mM ferrocytochrome c-550. Cytochrome c so prepared was usually more than 95% reduced.

Chemical Determinations

(1) Thiosulfate and Polythionates

Thiosulfate and polythionates were estimated according to a colorimetric procedure as described by Sörbo(1957). The method is based on the formation of thiocyanate when thiosulfate is mixed with cyanide in the presence of catalytic amounts of copper ions and when polythionates are mixed with cyanide in the absence of copper ions. To 2.0 ml of the sample containing 0.1 to 2.0 µmole of thiosulfate, 2,2 ml of 0.2 N NH_4OH, 0.5 ml of 0.1 M KCN and, after mixing, 0.3 ml of 0.1 M $CuCl_2$ were 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 $Fe(NO_3)_2 \cdot 9H_2O$ 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 $CuCl_2$.

Polythionates were determined as above except that sample was preincubated with NH_4OH and KCN for 30 minutes at room temperature before the addition of ferric nitrate reagent. After the mixture was incubated further for 15 minutes at room temperature, 0.3 ml of 0.1 M $CuCl_2$ was added and the optical density was measured. It was corrected for a "blank" reading as described above.

When proteins and GSH interfered with the determination, the thiols were precipitated by addition of cadmium ions.

(2) Protein

Protein was determined by a colorimetric method of Lowry et al.(1951), using crystalline bovine albumin as a standard.

Electron Microscopic Studies

All the electron microscopic examinations were carried out on an AEI(Associated Electrical Industries Ltd., England) electron microscope Model EM 6Boperated at 60 Kv.

(1) Reagents Used

(i) Fixative Hess method(Hess, 1966). Ten ml of fixative were prepared fresh for every experiment by mixing the following reagents:

Gluteraldehyde (25%, K & K Lab., U.S.A.)..... 1.2 ml
Acrolein (Eastman Kodak & Co., N.Y., U.S.A.)..... 0.3 ml
Distilled Water..... 3.5 ml
0.2 M Cacodylate buffer (City Chem., N.Y., U.S.A.)..... 5.0 ml

(ii) Uranyl acetate (B.D.H. Chem., England). 0.5% aqu. sol.

(iii) Osmium tetroxide (Steven's Met. Corp., U.S.A.). 1% aqu. sol.

(iv) Ethyl alcohol. 50%, 70%, 90% & absolute

(v) Embedding mixture. Methyl methacrylate (Matheson Coleman & Bell, U.S.A.) 15 ml, butyl methacrylate (same as above) 85 ml, divinyl benzene (5%, Dow Chemicals) 5 ml and benzoyl peroxide (Matheson, Coleman & Bell) 1 g. These chemicals were mixed and allowed to age at $4^{o}C$ for at least 24 hours before use.

(vi) Stains. Lead citrate stain was prepared according to Reynolds method(Reynold, 1963) as follows:

A mixture of 1.33 g of lead nitrate and 1.76 g of sodium citrate was suspended in 30 ml of distilled water (CO_2-free) in a 50 ml volumetric flask. The suspension was allowed to stand at room temperature

for 30 minutes with intermittent shaking. Eight ml of 1 N NaOH were then added to the flask and the contents were mixed well. The volume was made up to 50 ml with distilled water (CO_2-free). Upon mixing by inversion, lead citrate dissolved completely and if there was any turbidity remained, the solution was centrifuged to remove it.

Potassium phosphotungstate for negative staining was prepared as follows:

One per cent solution of phosphotungstic acid (J.T. Baker & Co., U.S.A.) was prepared in distilled water and the pH of the solution was adjusted to 7.4 with concentrated KOH solution.

(2) Methods

(i) Thin Sections. Crude cell-free extract or membrane vesicles (105,000 x g pellet) were embedded in 2% agar. The gelled agar was cut into tiny square cubes. The cubes were washed with four changes of 0.1 M cacodylate buffer(pH 7.5), post-fixed with 1% OsO_4 solution for 2 hours at 4°C, washed again with four changes of the same buffer and were then allowed to stand in 0.5% uranyl acetate solution overnight at room temperature. On the following day, the agar cubes were washed once with the cacodylate buffer and dehydrated by allowing them to stand at room temperature for 15

minutes each in 50, 70 and 90% ethanol and finally in absolute alchol for 1 hour with one change of alcohol at 30 minutes interval.

The dehydrated agar cubes were placed in gelatin capsules (Parke-Davis & Co., Canada) - one cube per capsule- containing a few drops of the embedding mixture and allowed to stand at room temperature for at least 2 hours. The capsules were then filled with the embedding mixture keeping the agar cube in the center with a long needle and the capsules were kept in a vacuum oven at 50°C for 16 to 24 hours to polymerize the resin to clear plastic blocks.

Ultra-thin sections(60-90 nm) were cut on an LKB type 4802 A (LKB Productur-Sweden) or a Reichert Om U2 (Reichert, Austria) ultratome with a glass knife prepared by using an LKB type 7801 A Knife-Maker. The sections, mounted on a carbon-coated copper grids (Ladd Research Corporation, U.S.A.), were stained as described below and examined on the electron microscope.

(ii) Staining of the Ultra-thin Sections. Thin sections mounted on copper grids as described above were stained with a mixture of 0.5% uranyl acetate and 1% $KMnO_4$ (2:1, v/v) at room temperature for 1 hour. The grids were then allowed to stand for 30 seconds in a bleaching solution which was prepared by mixing equal volumes of

5% oxalic acid and 5% sodium sulfite solutions. The grids were then washed twice with 0.1 M cacodylate buffer(pH 7.5) and post-stained with lead citrate for 10 minutes at room temperature, followed by washing with 0.02 N NaOH for 10 to 15 seconds and then with 0.1 M cacodylate buffer (pH 7.5) and allowed to air dry before examination.

(iii) Negative Staining Procedure

The samples (crude cell-free extract and membrane-vesicles prepared by Sepharose 4B column chromatography) were diluted 1:60 in 0.1 M Tris acetate-0.02 M potassium phosphate buffer (pH 7.5), thinly spread on carbon-coated copper grids (Ladd Research Corporation, U.S.A.), and allowed to stand at room temperature for 15 minutes. The grids were then negatively stained for 10 seconds with a freshly prepared solution of 1% potassium phosphotungstate, washed with 0.1 M cacodylate buffer (pH 7.5), air dried and examined.

RESULTS

RESULTS

General Conditions for the Preparation of Active Cell-free Thiosulfate-oxidizing System

(1) Effect of Different Media on the Growth and Thiosulfate-oxidizing Activity

Since very active intact cells of T. novellus were essential in order to prepare active cell-free extracts for the thiosulfate-oxidizing activity, a comparative study concerning the development of an improved medium for the organism which would yield the physiologically active cells was examined.

The cell yields and the specific activity of thiosulfate-oxidizing system in intact cell suspensions grown in the different media are given in Table 1. The levels of the specific and total activities of the thiosulfate-oxidizing system in cells grown in Charles and modified media were much higher than those of Starkey's medium(No. 3), whereas no profound differences in the final cell yields were observed among the different media. The modified medium where all of the constituents were soluble and which contained a chelated iron(0.8 mg Fe/l) yielded consistently the most active

Table 1. Comparisons of media for growth and thiosulfate-oxidizing activity of T. novellus.

Medium	Wet weight of cells (mg)	Total protein (mg)	Total activity (nmole O_2 consumed /min)	Specific activity (nmole O_2 consumed /min/2mg of wet cells)
Starkey's medium (No. 3)	41	3.6	1,209	59
Charles' medium	49	4.9	1,906	80
Modified medium	50	4.8	3,150	126

The cultures(500 ml of medium in 1-liter flask) were grown in the various media as indicated, according to the same procedure as described in Materials and Methods. Each culture was maintained for 5 transfers in the same medium and used for the experiment. The measurements of thiosulfate-oxidizing activity and protein were as described in Materials and Methods.

cells and the color of the cells was deep red, possibly due to the presence of high concentration of cytochrome components.

In contrast to these cells, the intact cells obtained from either Starkey's medium or Charles' medium showed variable rates in the oxidation of thiosulfate and the colors of cells varied from orange to pink. Therefore, the modified medium was used in all subsequent studies.

(2) Effect of Temperature and Phosphate Concentration on the Growth and Thiosulfate-oxidizing Activity

Results of preliminary growth studies with T. novellus grown in the modified medium showed the optimum temperature to be 26°C, viz., the cells grown at 26°C exhibited the highest specific activity(Table 2). When the cultures were grown at 28°C and 30°C, the cell yields were slightly increased, but the specific activities were considerably lower than that of cells grown at 26°C. A much slower growth was observed at 22°C. Buffering the media with phosphate at concentrations ranging from 0.4 to 0.6%(w/v) in the basal salts of the modified medium had little effect upon growth. However, decreasing the concentration of phosphate ions to 1 g per liter(or less) resulted in severely limited growth.

Table 2. Effect of temperature on the growth and the specific activity of thiosulfate-oxidizing system of T. novellus.

Temperature (°C)	Wet weight of cells (mg)	Total protein (mg)	Total activity (nmole O_2 consumed /min)	Specific activity (nmole O_2 consumed /min/2 mg of wet cells)
22	19	1.4	1,026	108
26	49	4.8	2,989	122
28	54	5.3	2,349	87
30	58	5.6	1,769	61

The cultures(500 ml of modified medium in 1-liter flask) were grown at various temperatures as indicated with constant rotary shaking as described in Materials and Methods.

The measurements of thiosulfate-oxidizing activity and proteins were as described in Materials and Methods.

Tolerance to high phosphate concentrations(0.6 to 2%, w/v) for the growth of T. novellus was also reported by Starkey(1934b).

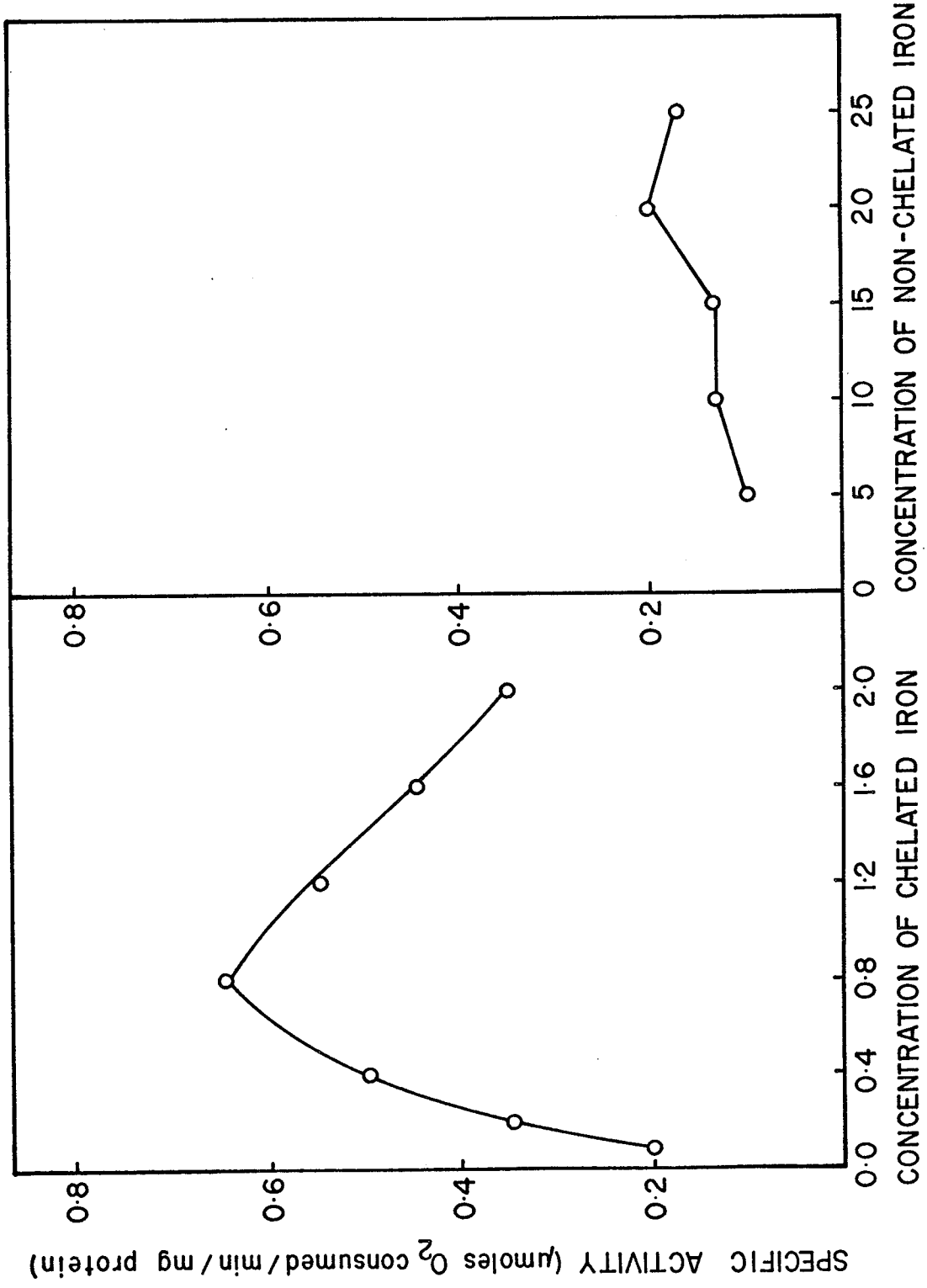
Although results are not shown here, thiosulfate ions as an energy source at a concentration of 0.8 to 1.0%(w/v) appeared to be optimal for the most rapid production of high yields of cells with active thiosulfate-oxidizing activity.

Replacement of calcium chloride with calcium carbonate or the supplemental addition to the medium of calcium carbonate(0.05 to 0.5 g per liter of medium) had no effect on yield of cells and specific activity of the thiosulfate-oxidizing activity.

(3) Effect of Iron on the Growth and Thiosulfate-Activity

The effect of concentrations of iron(0.1 to 20 mg Fe/l medium) was tested in the absence and presence of EDTA(0.25 to 50 mg of disodium salt of EDTA/l medium). The results are illustrated in Fig. 1, in which are plotted the average results of two parallel cultures. Best growth and highest specific activity occured with 0.8 to 1.2 mg iron in 2 to 3.0 mg of EDTA. Both cell yield and the specific activity for thiosulfate-oxidizing increased substantially when cells were grown

Fig. 1. Effect of iron on the growth and thiosulfate-oxidizing activity of T. novellus.
The cultures(500 ml of medium in 1-liter-flask) were grown with a chelated ferric iron(Fe-EDTA, $\frac{1}{2}$ molar ratio of Fe/EDTA) or a non-chelated iron($FeCl_3$) as indicated at 28° C with constant rotary shaking as described in Materials and Methods.
The cultures were maintained for 5 transfers in the same conditions of medium and used for present experiments.
The measurements of thiosulfate-oxidizing activity and proteins were the same as described in Materials and Methods.


SPECIFIC ACTIVITY (μmoles O_2 consumed / min / mg protein)
0·2
0·4
0·6
0·8
0·0
0·4
0·8
1·2
1·6
2·0
CONCENTRATION OF CHELATED IRON
(mg / l medium)
0
5
10
15
20
25
CONCENTRATION OF NON-CHELATED IRON
(mg / l medium)

on a chelated iron rather than on a non-chelated iron ($FeCl_3$) medium and cells with deep red color were obtained. This effect may be due to better availability of iron to the cells.

(4) Growth and Thiosulfate-oxidizing Activity in the Batch Culture of T. novellus

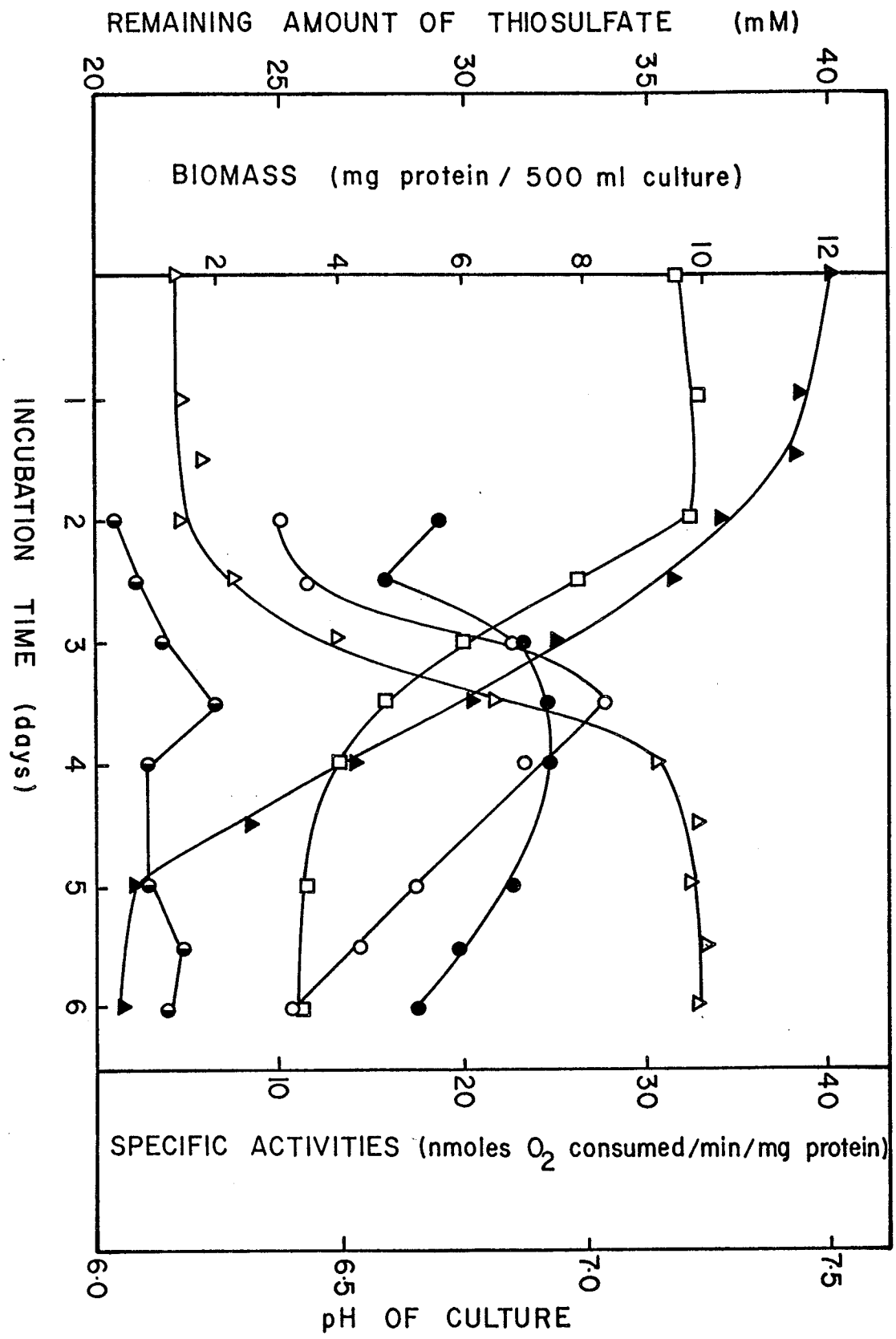
As background for the main studies on mechanism of thiosulfate oxidation, experiments were carried out initially to measure the growth characteristic of T. novellus, and to see whether the thiosulfate-oxidizing activity remains constant during all portions of the growth cycle.

Fig. 2 presents the results of one such experiment which shows the characteristic changes in growth parameters(pH, thiosulfate utilization and biomass) as a function of incubation time for the batch culture of T. novellus.

At zero time, a 10% inoculum of culture was transferred into an autotrophic medium and the culture was incubated with a low aeration(0.9 l air/minute) for one day and then with a constant aeration(1.8 l air/minute) for the rest incubation time at 28°C. It was observed that vigorous aeration of the culture of T. novellus at the beginning of the growth period prolonged the lag

Fig. 2. Growth experiment and changes in the specific activities of thiosulfate-oxidizing activity and sulfite-oxidizing activity during the growth cycle in the batch culture of T. novellus. The culture(20 l medium in a 25 l glass carboy) was grown as described in Materials and Methods. The culture samples(500 ml) were taken at various time intervals as indicated, and the pH was measured with a pH meter. They were centrifuged 12,100 x g for 20 minutes and the resulting supernatants were used for the determinations of thiosulfate and polythionate according to Sörbo(1957) as described in Materials and Methods. The pellets were washed three times with 0.1 M Tris acetate-0.02 M potassium phosphate buffer(pH 7.5), suspended in 1 ml of the same buffer and used for measurements of thiosulfate-oxidizing activity, sulfite-oxidizing activity and proteins. The measurements were the same as described in Materials and Methods.

Remaining amount of thiosulfate(mM), ▲—▲ ; pH change, □—□ ; thiosulfate-oxidizing activity, ○—○ ; sulfite-oxidizing activity, ●—● ; endogenous activity, ◑—◑ and biomass(mg of protein 1500 ml of culture), △—△ .


REMAINING AMOUNT OF THIOSULFATE (mM)
20
25
30
35
40
BIOMASS (mg protein / 500 ml culture)
2
4
6
8
10
12
INCUBATION TIME (days)
1
2
3
4
5
6
10
20
30
40
SPECIFIC ACTIVITIES (nmoles O_2 consumed/min/mg protein)
6.0
6.5
7.0
7.5
pH OF CULTURE

phase. After a lag phase of about 2 days, the protein content of the culture increased exponentially with a doubling time of 12 hours; this exponential phase lasted for 2 days, after which growth slowed down and then stopped.

As growth continued, the pH starting from about 7.5 and thiosulfate concentration in the medium decreased steadily until about pH 6.6 was reached after 4 to 5 days, whereupon a little change in pH was seen after this period. When changes in specific activity of thiosulfate-oxidizing activity were followed during the growth period, it was observed that the activity increased gradually until early stationary phase($3\frac{1}{2}$ days) was approached and decreased gradually one late stationary phase(5 to $5\frac{1}{2}$ days) was reached, respectively. However, there were no dramatic changes in specific activity of sulfite-oxidizing activity during the growth cycle, although the pattern was similar to that of thiosulfate-oxidizing activity.

(5) Growth Experiments in the pH-controlled Culture of T. novellus

Since there were too many parameters changing in the previous experiment, a batch culture cultivation of organism was performed at a constant pH(pH 7.2) with

constant aeration(1.8 l air/minute) and temperature ($28^{o}C$) and the specific activity was followed at various phases of growth in the autotrophic medium.

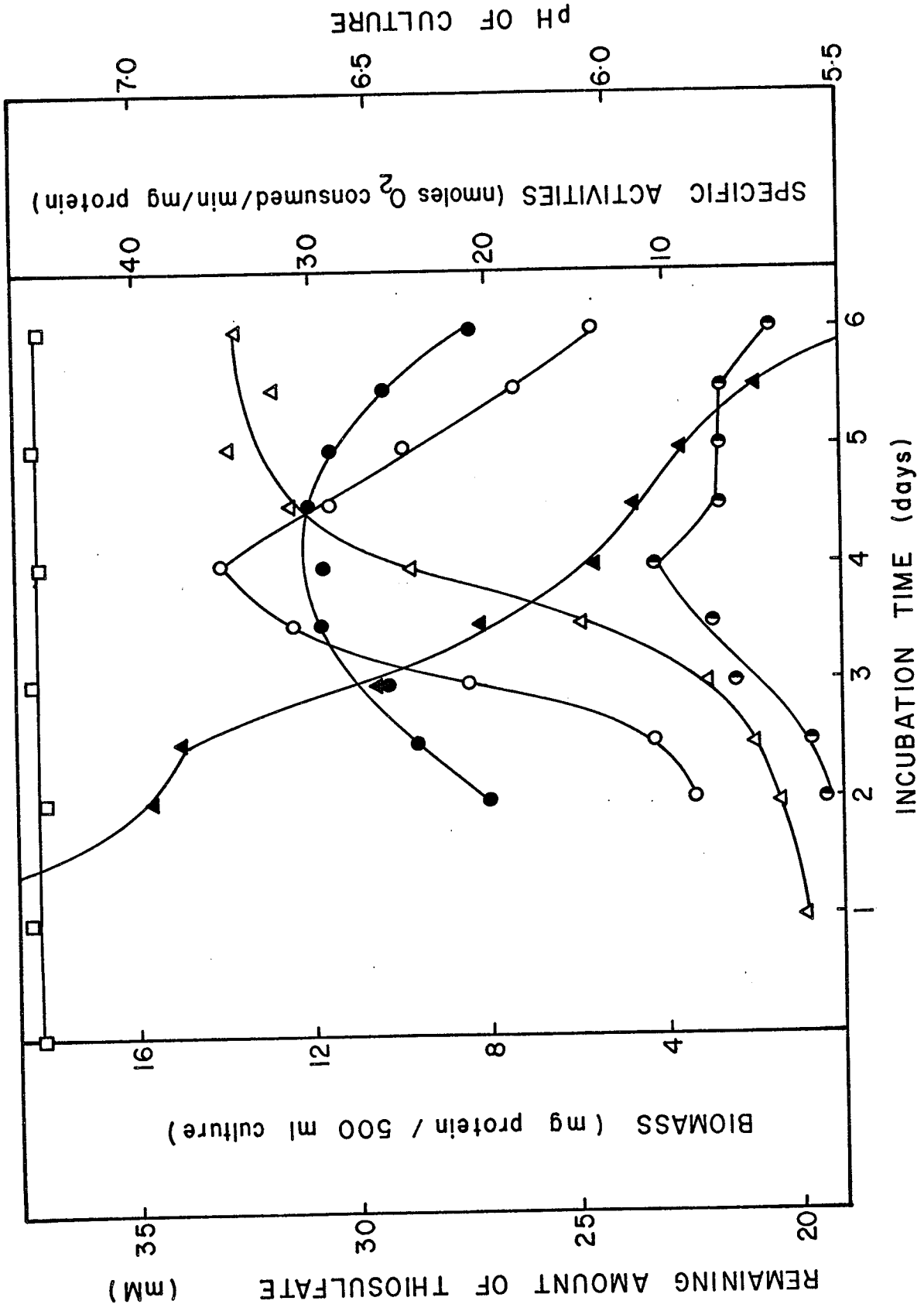
The cultures started growing 2 to $2\frac{1}{2}$ days following inoculation and grew exponentially for about the same period of time. A mean generation time in this experiment was about 12 hours. However, the growth slowed down and ceased at stationary phase. Since about 40% of thiosulfate still remained in the medium, different amounts of Mg^{2+} Ca^{2+} Mn^{2+} and chelated-iron were added into the culture to see whether further growth occured beyond the stationary phase. It was observed that there was sometimes a further increase of the bimass(protein) after these additions, but the specific activity of thiosulfate-oxidizing activity was markedly decreased.
With most cultures this took place within $4\frac{1}{2}$ days. Tetrathionate was also determined during the growth cycle, but the amount was always negligible within the limit of experimental error.

A typical experiment is shown in Fig. 3. The thiosulfate-oxidizing activity in the pH-controlled batch cultures was present at much higher levels of activity than those of the non-controlled cultures (Fig. 2 and Fig. 3) and remained relatively constant throughout the growth cycle although its specific

Fig. 3. Growth experiment and changes in the specific activities of thiosulfate-oxidizing activity and sulfite-oxidizing activity during the growth cycle in the pH-controlled culture of T. novellus.

The experimental conditions were the same as those described in Fig. 2, except that pH was kept constant at 7.2.

Remaining amount of thiosulfate(mM), ▲——▲ ; pH change, □——□ ; thiosulfate-oxidizing activity, ○——○ ; sulfite-oxidizing zctivity, ●——● ; endogenous activity, ◑——◑ and biomass(mg of protein/500 ml of culture), △——△ .


REMAINING AMOUNT OF THIOSULFATE (mM)
BIOMASS (mg protein / 500 ml culture)
SPECIFIC ACTIVITIES (nmoles O_2 consumed/min/mg protein)
pH OF CULTURE
INCUBATION TIME (days)

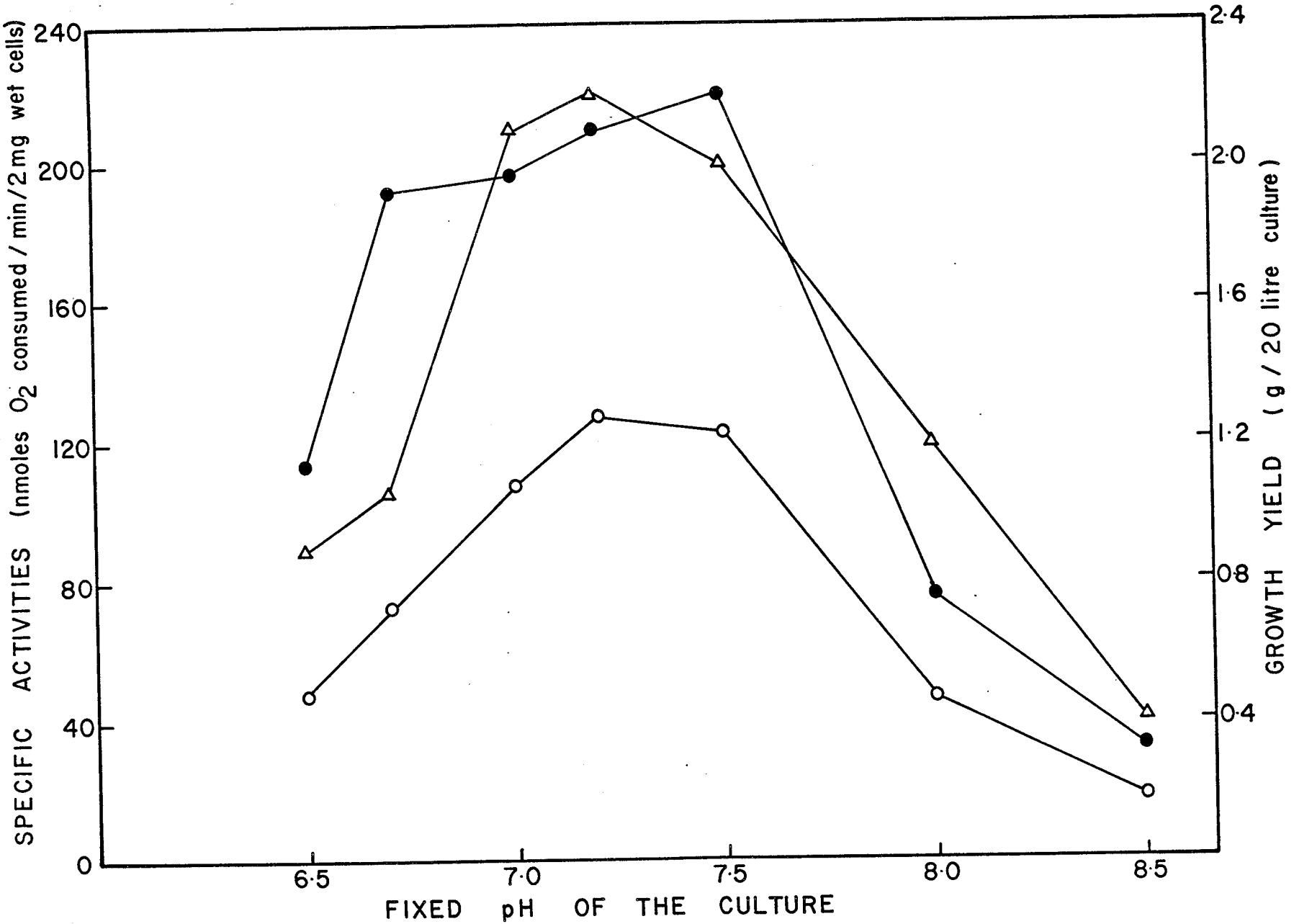
activity was somewhat higher at mid-exponential phase and was slightly lower at stationary phase. The sulfite-oxidizing activity followed a similar pattern during the growth cycle, although the change was less marked. It was also noted that the level of thiosulfate-oxidizing activity rose in conjunction with the increase in activity of the endogenous substrate oxidation.

The growth and specific activity of thiosulfate-oxidizing activity of T. novellus cells at various controlled hydrogen ion concentrations was also investigated. The pH values involved ranged from 6.5 to 8.5. When the fixed pH of the growing culture was varied, some changes in the specific activity of thiosulfate-oxidizing activity were observed (Fig. 4).

When the pH of the medium was held constant at pH 7.0 to 7.5, results similar to that obtained at pH 7.2 (A typical growth condition to obtain active cells of T. novellus) were observed. However, keeping the pH above 7.5 or below 7.0 resulted in dramatic decreases of the levels of thiosulfate-oxidizing activity and of cell yields as well as the level of sulfite-oxidizing activity. It was also observed that the growth at the pH above 7.5 fluctuated frequently because of lysis of cells.

Fig. 4. Changes in cell yield and specific activities at different pH values in the pH-controlled culture of T. novellus.

The experimental conditions were the same as those described in Fig. 3 expect that the cells were harvested after incubation for $4\frac{1}{2}$ days. Thiosulfate-oxidizing activity, ●——● ; Sulfite-oxidizing activity, o——o and growth yield(gm/ 20 l culture), Δ——Δ .


SPECIFIC ACTIVITIES (nmoles O_2 consumed / min/2mg wet cells)
GROWTH YIELD (g / 20 litre culture)
FIXED pH OF THE CULTURE
0
40
80
120
160
200
240
0·4
0·8
1·2
1·6
2·0
2·4
6·5
7·0
7·5
8·0
8·5

(6) Various Methods for Extraction of Active Cell-free Preparation from T. novellus

Several cell disruption methods were tested to find the best condition for the extraction of the active cell-free system from the active whole cells of T. novellus, i.e., freezing and thawing, sonic oscillation, French Pressure Cell disruption, treatment with lysozyme and EDTA, osmotic shock, and a number of combinations of the above methods. The results of a comparative study are shown in Table 3.

Attempts to prepare active extracts by freeze-thaw treatment, lysis of the cells, either lysozyme-EDTA treatment or in conjunction with the osmotic shock procedure, were unsuccessful, only 1 to 2% of the activity of the whole cells being recovered in the extracts. An effort to disrupt intact cells by an osmotic shock treatment was unsuccessful.

When the spheroplasts prepared from T. novellus were examined in a phase contrast microscope, it was very difficult to distinguish them from whole cells. However, when 20-fold dilution of the spheroplast preparation was made in distilled water, a slight decrease in 540 nm absorbance over 30 minutes resulted, indicating some lysis of the spheroplasts. Under the same condition there was no lysis of the intact cells.

Table 3. Methods for the extraction of active cell-free thiosulfate-oxidizing system from T. novellus.

Method of cell disruption	Total protein released (mg)	Total enzyme activity detected (umole O_2 consumed /min)	Specific activity (umole O_2 consumed /min/mg protein)	Recovery of activity (%)
1. Intact cells	106.0	60.0	0.566	100.0
2. Freezing and thawing				
a. 2,000 x g supernatant	42.0	1.2	0.028	2.0
b. 2,000 x g pellet	61.6	20.8	0.320	34.9
3. Lysozyme and EDTA(spheroplast)	101.0	1.4	0.014	2.0
4. 3 plus osmotic shock				
a. fluid	20.5	0.0	0.000	0.0
b. pellet	69.5	0.0	0.000	0.0
5. Osmotic shock				
a. fluid	15.7	1.2	0.076	2.0
b. pellet	88.2	24.1	0.273	40.1
6. French Pressure cell 2,000 x g supernatant				
a. Single passage	66.5	2.4	0.038	4.1
b. Two passages	87.1	5.5	0.063	9.2
c. Three passages	89.6	3.1	0.036	5.2
d. b under N_2 atmosphere	80.4	5.2	0.065	8.8
e. b plus DNase, RNase and Mg^{2+}	82.9	5.4	0.066	8.9

Table 3. continued

7. Sonic treatment 2,000 x g supernatant				
a. 10 min	65.0	2.8	0.043	4.6
b. 15 min	91.5	4.1	0.046	6.7
c. 20 min	94.1	2.9	0.030	4.8
d. 30 min	97.3	2.0	0.020	3.1
e. 10 min under N_2 atmosphere	62.4	5.1	0.081	8.6
f. 15 min under N_2 atmosphere	90.5	8.2	0.092	13.6
g. 20 min under N_2 atmosphere	93.6	5.9	0.063	9.8
h. 30 min under N_2 atmosphere	95.5	5.5	0.057	9.2
i. f plus DNase, RNase and Mg^{2+}	91.6	8.4	0.092	14.0
8. Combination of 6b and 7i	98.7	3.1	0.031	5.1
9. 2b plus 7i	57.1	1.7	0.030	2.8
10. 5b plus 7i	80.2	3.4	0.042	5.6

In all experiments, 5 ml of cell suspensions(200 mg wet cells per ml) was used for the extractions of cell-free system. The treated samples were centrifuged at 2,000 x g for 20 minutes and the resulting supernatants were used for measurements of the thiosulfate-oxidizing activity and protein as described in Materials and Methods.

This spheroplast preparation oxidized thiosulfate and sulfite at low rate and the addition of spermine(2.0 mM), bovine serum albumin(20 mg/ml), $MgCl_2$(10 mM) on the reaction mixture or preincubation with the compounds did not increase the rate of thiosulfate oxidation. These spheroplasts had no sulfur-oxidizing activity. It was found, however, that the reasonably active cell-free extracts were obtained either by sonic disruption or passage through a French Pressure Cell. The best and consistent results were obtained either by combining Mg^{2+}(10 mM), deoxyribonuclease(2 µg of enzyme per ml of the whole cell suspension) and ribonuclease(1 µg of enzyme per ml) treatments with the sonication of the active whole cells under a nitrogen atmosphere or by using sonication alone under a nitrogen atmosphere. Nitrogen atmosphere prevented the loss of activity during sonic treatment of the cell suspension.

Extracts of intact cells prepared either by osmotic shock or in the French Pressure Cell were extremely viscous and were readily clarified by addition of deoxyribonuclease, ribonuclease and Mg^{2+}. When the cells were disrupted sonically the extracts were less viscous, but small amounts of deoxyribonuclease, ribonuclease and Mg^{2+} were routinely added to break down nucleic acids since these enzymes had no

effect on thiosulfate-oxidizing activity.

When the cell-free extracts were prepared by sonication for 15 minutes under a nitrogen atmosphere, the activity in the crude extracts reached 9 to 14% of that of whole cell suspension. However, a longer treatment resulted in a fall of activity although the concentration of soluble protein in the extracts increased.

The effect of cell concentration on the thiosulfate-oxidizing activity during sonication is shown in Fig. 5.

If high cell concentrations(120 mg to 200 mg wet cells per ml) were used for sonic treatments the specific activities of thiosulfate-oxidizing activity in crude cell-free extracts were high; however, the specific activities were decreased markedly at low-cell concentrations(20 to 80 mg wet cells per ml). There appeared to be a critical concentration below which enzyme inactivation occured.

Distributions of Thiosulfate-oxidizing Activity in Various Fractions Prepared by Sonication of T. novellus Cells

A summary of the distribution of thiosulfate-oxidizing activity in various fractions of T. novellus cells is tabulated in Table 4.

Fig. 5. Effect of cell concentration on the thiosulfate-oxidizing activity of T. novellus extracts prepared by sonic treatment.

The experimental conditions were the same as described in Materials and Methods except that the cell concentrations were varied as indicated.

The measurements of thiosulfate-oxidizing activity and protein were as described in Materials and Methods.

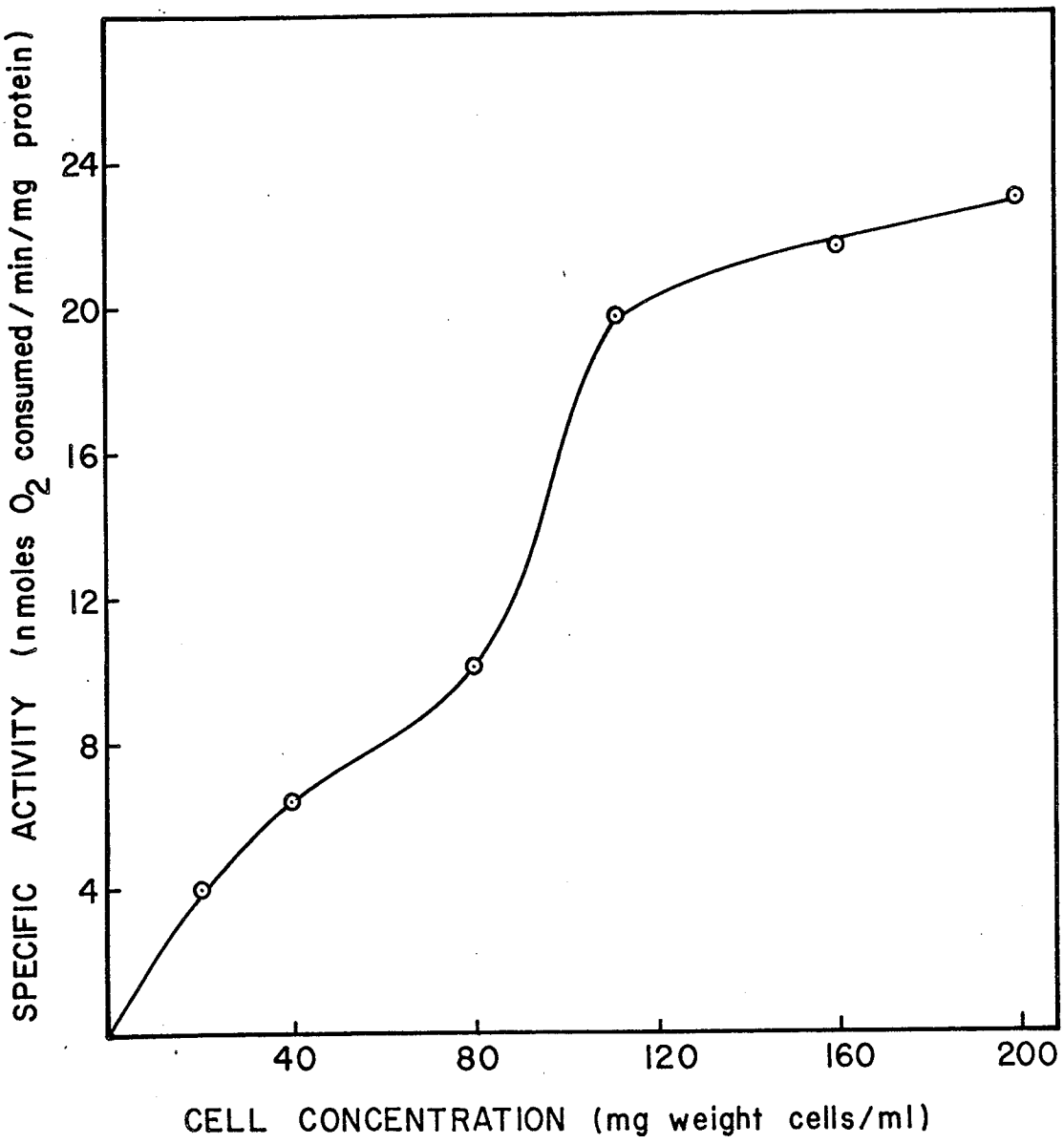

SPECIFIC ACTIVITY (n moles O_2 consumed / min / mg protein)
24
20
16
12
8
4
40
80
120
160
200
CELL CONCENTRATION (mg weight cells/ml)

Table 4. Distributions of thiosulfate-oxidizing activity in various fractions prepared by sonication of T. novellus cells.

Cell fraction	Total protein (mg)	Total enzyme activity (μmole O_2 consumed /min)	Specific activity (μmole O_2 consumed /min/mg protein)	% recovery from intact cells
Intact cells	248.0	120.0	0.479	100.0
Crude extract (supernatant fraction from 2,000 x g for 20 min)	291.0	16.8	0.072	14.0
Soluble fraction (supernatant fraction from 105,000 x g for 90 min)	99.5	0.0	0.000	0.0
Particulate fraction (pellet from 105,000 x g for 90 min)	117.6	16.0	0.136	13.3
Sepharose 4B fraction(eluate from Sepharose 4B column)	54.9	8.2	0.147	6.9

Ten milliliters of intact cell suspension(200 mg wet weight per ml) were disrupted by sonication and the various fractions were prepared as described in Materials and Methods. Measurements of protein and thiosulfate-oxidizing activity were the same as described in Materials and Methods.

When cells were disrupted by the sonic treatment under nitrogen atmosphere, approximately 6 to 14% of the thiosulfate-oxidizing activity of intact cells was found in the subsequent supernatant fraction after centrifugation at 2,000 x g for 20 minutes. This crude extract was very turbid and deep red in color. The suspended 2,000 x g pellet fraction showed some activity which was probably due to large cellular fragments and unbroken cells.

After centrifugation of the crude extract at 105,000 x g for 90 minutes, most of the thiosulfate-oxidizing activity remained in the pellet fraction, which represented approximately 95 to 100% of the total enzyme activity in the crude cell-free extract respectively.

In the earlier work, thiosulfate-oxidizing system prepared by a sonication has been reported to be a soluble enzyme in T. novellus(Aleem, 1965). At present work, it was found, however, that there was no activity in the 105,000 x g supernatant fraction and the addition of the supernatant fraction to the pellet suspension did not increase the thiosulfate-oxidizing activity.

The crude cell-free extracts prepared by passing through a French Pressure Cell showed a similar distribution of the thiosulfate-oxidizing activity(result

not shown).

On the other hand, sulfite-oxidizing system was detected in neither pellet nor supernatant fraction after centrifugation of the crude extract at 105,000 x g for 90 minutes. This clearly confirmed the previous observation initially made by Charles and Suzuki(1966). In contrast, the sulfite-oxidizing system in the cell-free extracts prepared by sonic disruption of T. thiooxidans was catalyzed by the particular fraction alone(Kodama and Mori, 1968).

Preparation of Active Vesicular Membrane Fractions

Several methods based on techniques of differential centrifugation, Sepharose 4B column chromatography or sucrose density gradient centrifugation were investigated for the isolation of active membrane vesicles for thiosulfate oxidation from crude cell-free extracts. The active preparations of thiosulfate-oxidizing system which oxidized thiosulfate to sulfate was obtained by either differential centrifugation technique or column chromatographic method, while the sucrose density gradient centrifugation was not as effective as these two methods. A rapid procedure for the isolation of active thiosulfate-oxidizing system was essential for investigations on this membrane-bound complex system, where the age of the

preparation was critical for the thiosulfate-oxidizing activity.

(1) Differential Centrifugation

As described earlier, approximately 95 to 100% of the cell-free extract activity were recovered in the pellet after ultracentrifugation at 105,000 x g for 90 minutes(Table 5).

The resulting pellets were suspended in 0.1 M Tris acetate-0.02 M potassium phosphate buffer(pH 7.5) to give a final protein concentration of 6 to 12 mg per ml and the preparation thus isolated was designated as active membrane vesicles.

In many experiments, the active membrane vesicles were obtained routinely by this method and the preparations were used immediately or stored frozen at -74°C prior to use. It is, however, possible that such isolated membrane vesicles obtained by one ultracentrifugation are contaminated with soluble proteins.

The addition of the supernatant to the vesicle preparation, however, had no effect on the activity.

The active membrane vesicles obtained by the differential centrifugation of the crude cell-free extracts had also NADH oxidase and cytochrome c oxidase activities, whereas almost all the activity of soluble sulfite: cytochrome c

Table 5. Activities of enzymes in the various fractions prepared by ultracentrifugation of crude cell-free extracts of T. novellus.

Preparation	Total protein (mg)	Total thiosulfate-oxidizing activity (μmole O_2 consumed /min)	Total sulfite-oxidizing activity (μmole O_2 consumed /min)	Total NADH oxidase (μmole O_2 consumed /min)	Total sulfite: cytochrome c oxidoreductase (μmole cytochrome c reduced /min)	Total cytochrome c oxidase (μmole cytochrome c oxidized /min)
1. Crude extract	206 (100)	16.2 (100)	15.7 (100)	32.7 (100)	62.1 (100)	64.0 (100)
2. Pellet fraction	102 (49.5)	16.0 (98.7)	0.0 (0.0)	31.8 (97.2)	0.0 (0.0)	62.8 (98.1)
3. Soluble fraction	97 (47.1)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	58.5 (94)	0.0 (0.0)
4. A combination of 2 and 3	NT	16.5 (101.1)	0.0 (0.0)	32.0 (97.4)	NT	NT
5. 4 plus cytochrome C-550 (horse heart, type III)	NT	16.1 (99.9)	15.3 (97.5)	NT	NT	NT

A sample containing 206 mg of protein in 10 ml of active crude cell-free extract was centrifuged at 105,000 x g for 90 minutes as described in Materials and Methods. All enzyme activities and protein were measured as described in Materials and Methods.

NT : Not tested.

( ) : % of cell-free extract.

oxidoreductase was present in the supernatant fraction (Table 5).

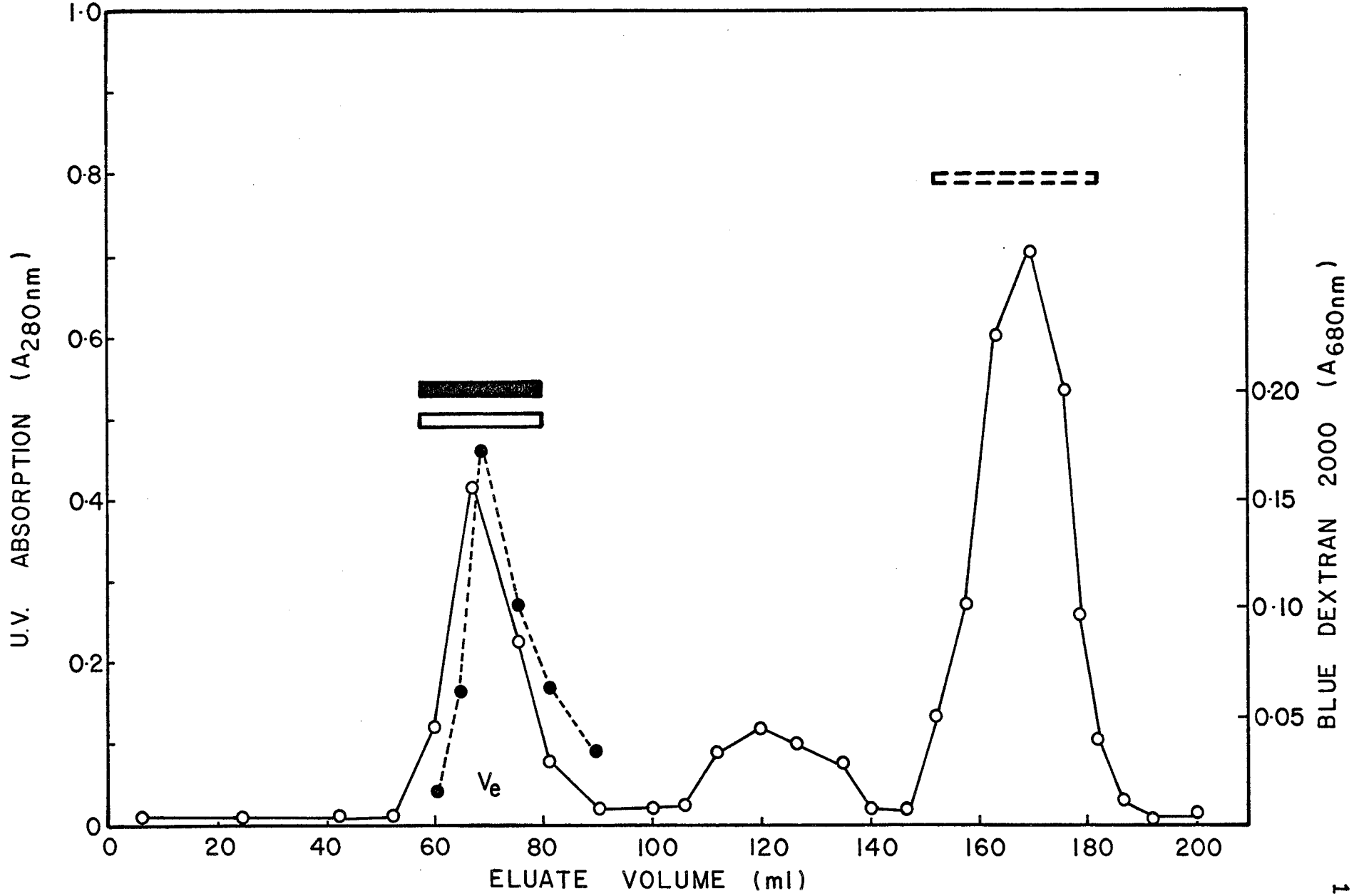
(2) Column Chromatography on Sepharose 4B

The active preparations of thiosulfate-oxidizing membrane system were also obtained by Sepharose 4B column chromatography of crude extracts. A typical elution profile of the various enzyme activities and protein from a Sepharose 4B column(2.5 x 37.5 cm; column bed volume, 196 ml) is shown in Fig. 6.

In the eluate three distinct fractions were found: the first turbid one which was eluted at a volume of 68 ml(void volume), the second colorless peak eluting at 117 to 135 ml and the third pinkish red one which was eluted in a symmetrical peak between 153 and 182 ml. All the activity of thiosulfate-oxidizing system emerged at the void volume of the column. This first fraction was the basis for its identification as the membrane vesicles fraction. Therefore, the active thiosulfate-oxidizing system thus purified was also designated as active membrane vesicles. The pooled active fractions were used immediately or concentrated by either Aquacide® or ultracentrifugation(105,000 x g for 2 hours) and stored frozen at -74°C at a high protein concentration (6 to 12 mg per ml).

Fig. 6. The elution profile of thiosulfate-oxidizing activity and associated enzyme activities during Sepharose 4B chromatography of T. novellus extracts. A sample containing 109 mg of protein in 5 ml active crude cell-free extract was subjected to chromatography in the conditions described in Materials and Methods. Thiosulfate-oxidizing activity(▬▬), cytochrome oxidase(═══) and sulfite: cytochrome c oxidoreductase were assayed as described in Materials and Methods.

Blue Dextran 2000 was chromatographed in a successive experiment and its absorption read at 680 nm(●—●). The Ve was considered to be the volume at the midpoint of peak height. Protein was presented by absorptions at 280 nm (o—o).


1·0
0·8
0·6
0·4
0·2
0
U.V. ABSORPTION (A_{280nm})
0·20
0·15
0·10
0·05
BLUE DEXTRAN 2000 (A_{680nm})
0
20
40
60
80
100
120
140
160
180
200
ELUATE VOLUME (ml)
V_e

All the preparation procedures should be carried out as quickly as possible because the thiosulfate-oxidizing activity was lost rapidly on storage at 4°C. The purification of the thiosulfate-oxidizing activity from crude cell-free extracts reported here resulted in a two-fold increase in the specific activity of the system with a recovery of 50% of the original activity.

As given in Table 6, the active membrane vesicles contained also NADH oxidase and cytochrome c oxidase activities at levels comparable to those of the ultra-centrifuged membrane vesicles(Table 5), but were not active in sulfite oxidation and NADPH oxidation.

The second fraction contained cytoplasmic soluble proteins, presumably enzymes which are responsible for the fixation of carbon dioxide.

Nearly all the activity of sulfite: cytochrome c oxido-reductase was located in the third fraction which also contained all soluble cytochromes.

(3) Sucrose Density Gradient Sedimentation

When the crude cell-free extract was directly subjected to centrifugation through a 15 to 40%(w/v) linear sucrose density gradient with 50%(w/v) sucrose cushion, three distinct peaks were found, viz., a turbid membrane fraction which contained all the thiosulfate-

Table 6. Enzyme activities in the isolated membrane vesicles and soluble preparations by chromatography of crude cell-free extracts on Sepharose 4B column.

Preparation	Total protein (mg)	Total thio-sulfate-oxidizing activity (μmole O_2 consumed /min)	Total sulfite-oxidizing activity (μmole O_2 consumed /min)	Total NADH oxidase (μmole O_2 consumed /min)	Total sulfite: cyto-chrome c oxido-reductase (μmole of cyto-chrome c reduced /min)	Total cyto-chrome c oxidase (μmole of cyto-chrome c oxidized /min)
1. Crude extract	198 (100)	15.2 (100)	14.8 (100)	34.6 (100)	59.1 (100)	61.9 (100)
2. Membrane fraction (1st peak)	51 (25.8)	7.4 (48.7)	0.0 (0.0)	32.1 (92.7)	0.0 (0.0)	59.6 (96.3)
3. Soluble fraction (2nd peak)	21 (11.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
4. Soluble fraction (3rd peak)	123 (62.1)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	58.2 (98.5)	0.0 (0.0)
5. A combination of 2 and 4	NT	7.6 (49.8)	0.0 (0.0)	NT	NT	NT
6. 5 plus cytochrome c-550(horse heart, type III)	NT	7.6 (49.8)	14.4 (97.2)	NT	NT	NT

A sample containing 99 mg of protein in 5 ml of active crude cell-free extract was subjected to chromatography as described in Materials and Methods. Measurements of various enzyme activities and protein content were the same as described in Materials and Methods. In order to make a comparison with the results presented in Table 5, all data obtained were multiplied by 2.

NT : Not tested.

( ) : % of cell-free extract.

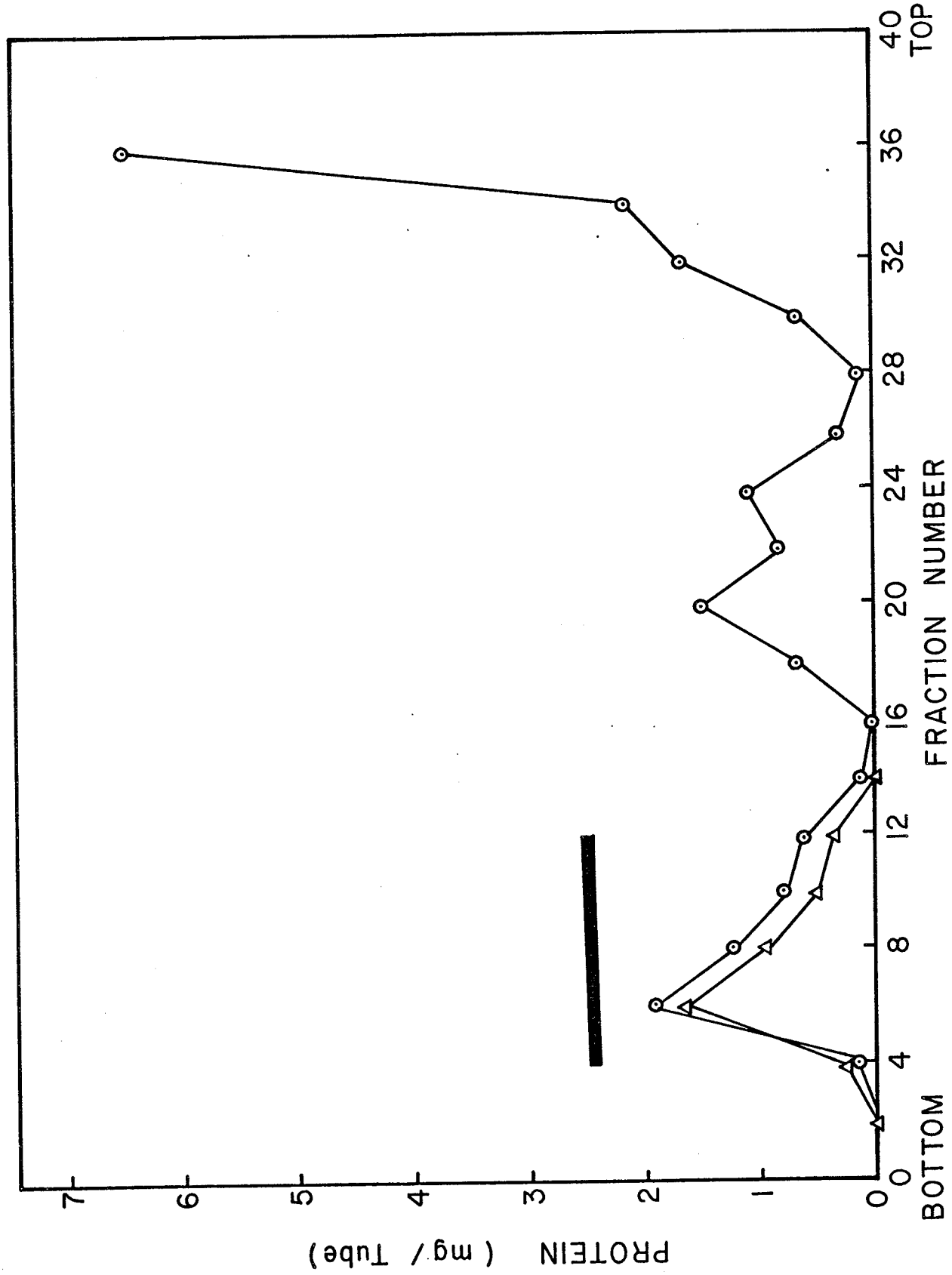
oxidizing activity, a colorless protein fraction and a pinkish red fraction, remaining on the top of the sucrose gradient. Therefore, no significant differences in the patterns of the fractions were observed when these results were compared with those from Sepharose 4B column chromatography. The Fig. 7 illustrates the results obtained in a typical experiment. In order to confirm the identity of the results, a preparation of active membrane vesicles obtained by Sepharose 4B column chromatography of crude cell-free extract was subjected to the sucrose density gradient centrifugation. As shown in Fig. 7 there was only one turbid fraction which was sedimented at the identical position as the membrane fraction from the crude cell-free turbid extract subjected to the centrifugation. This turbid fraction contained over 90% of the protein of vesicles applied on the sucrose gradient and also showed the thiosulfate-oxidizing activity.

However, the sucrose gradient procedure resulted in a great loss of thiosulfate-oxidizing activity and only about 10% of the input activity was recovered. The reason for this loss was an inhibitory effect of sucrose in thiosulfate-oxidizing system as mentioned later.

Stability of the Thiosulfate-oxidizing System

Aliquots of thiosulfate-oxidizing preparations were

Fig. 7. The fraction profile of thiosulfate-oxidizing activity and protein in the crude cell-free extracts and isolated membrane vesicles sedimented on sucrose density gradient centrifugation. Either crude cell-free extract(19.8 mg of protein in 2 ml of sample) or active membrane vesicles(4.2 mg of protein in 2 ml of sample) isolated from Sepharose 4B column chromatography were directly subjected to centrifugation through parallel separate sucrose gradients. Details of enzyme preparation, assay and sucrose gradient centrifugation were given under Materials and Methods.
The direction of sedimentation was from right to left. Protein was determined by the method of Lowry et al.(1951) and thiosulfate-oxidizing activity(▬▬) was measured as described in Materials and Methods.
Protein content from the crude cell-free extract, ⊙—⊙ and protein content from the isolated membrane vesicles, Δ—Δ.


PROTEIN (mg/ Tube)
0
1
2
3
4
5
6
7
BOTTOM
0
4
8
12
16
20
24
28
32
36
40
TOP
FRACTION NUMBER

tested for activity after storage at various temperatures for various periods.

At 4°C the activity of whole cell suspension(50 mg wet cells/ml) was stable for at least several days, but it lost more than 80% of the initial activity after storage at 25°C for 1 day. When the whole cells were frozen and stored at -20°C and -74°C, the thiosulfate-oxidizing activity declined progressively with increasing time of storage(Fig. 8).

Studies with the cell-free extracts and the membrane vesicles indicated that preparations were extremely unstable, especially in the presence of oxygen, losing most of the activity after storage at 25°C and 4°C for 6 to 7 hours and 10 to 12 hours, respectively. Storage of the cell-free preparations at 4°C and pH 7.5 (0.1 M Tris acetate-0.02 M potassium phosphate) resulted in a gradual decrease in the thiosulfate-oxidizing activity(Fig. 9). The rate of decay was higher at the lower the protein concentration, but was independent of the pH. The membrane vesicles lost the activity more rapidly than the crude cell-free extracts.

When either cell-free extract or membrane vesicles were stored at -20°C and a protein concentration of about 6 mg of protein per ml, the thiosulfate-oxidizing activity was lost up to 68% of it's original levels during the first 2 days. About 80% of it's activity was lost after

Fig. 8. Effect of storage on thiosulfate-oxidizing activity in whole cells of T. novellus.
The whole cell suspensions(50 mg wet weight per ml) were stored at 4^{o} C and 25^{o} C in the 0.1 M Tris acetate-0.02 M potassium phosphate buffer (pH 7.5). Samples were withdrawn at the times indicated and assayed for thiosulfate-oxidizing activity at 25^{o} C by the standard procedure as described in Materials and Methods. To test effect of storage on the thiosulfate-oxidizing activity at -20^{o} C and -74^{o} C, the experimental procedure was the same as outlined in above with the exception that intact cells without the buffer were stored, thawed rapidly, and suspended in the standard buffer(pH 7.5) before assay. Stored at 25^{o} C, o—o ; stored at 4^{o} C, ●—● ; stored at -20^{o} C, Δ—Δ ; and stored at -74^{o} C, □—□ .

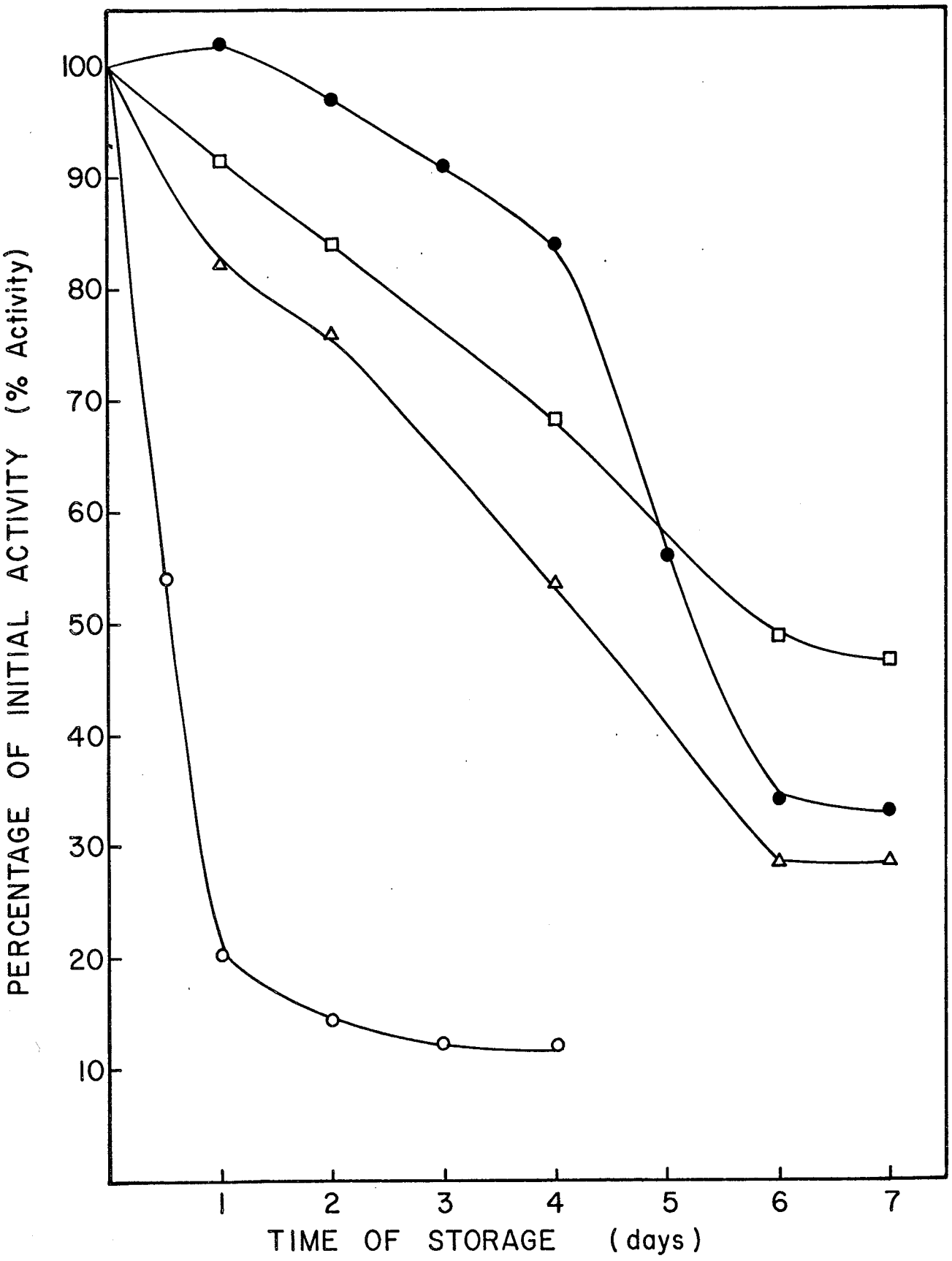
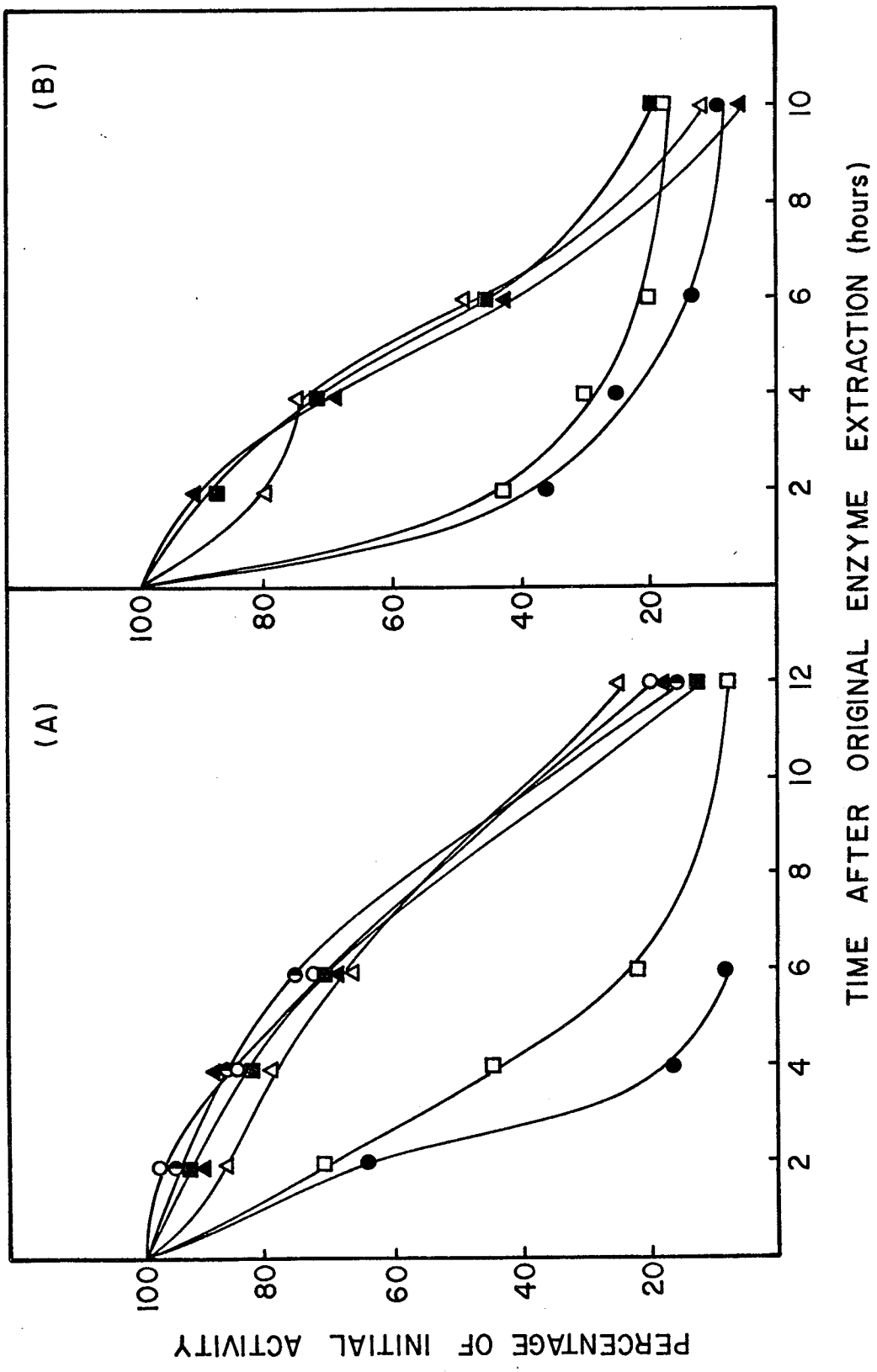

PERCENTAGE OF INITIAL ACTIVITY (% Activity)
100
90
80
70
60
50
40
30
20
10
1
2
3
4
5
6
7
TIME OF STORAGE (days)

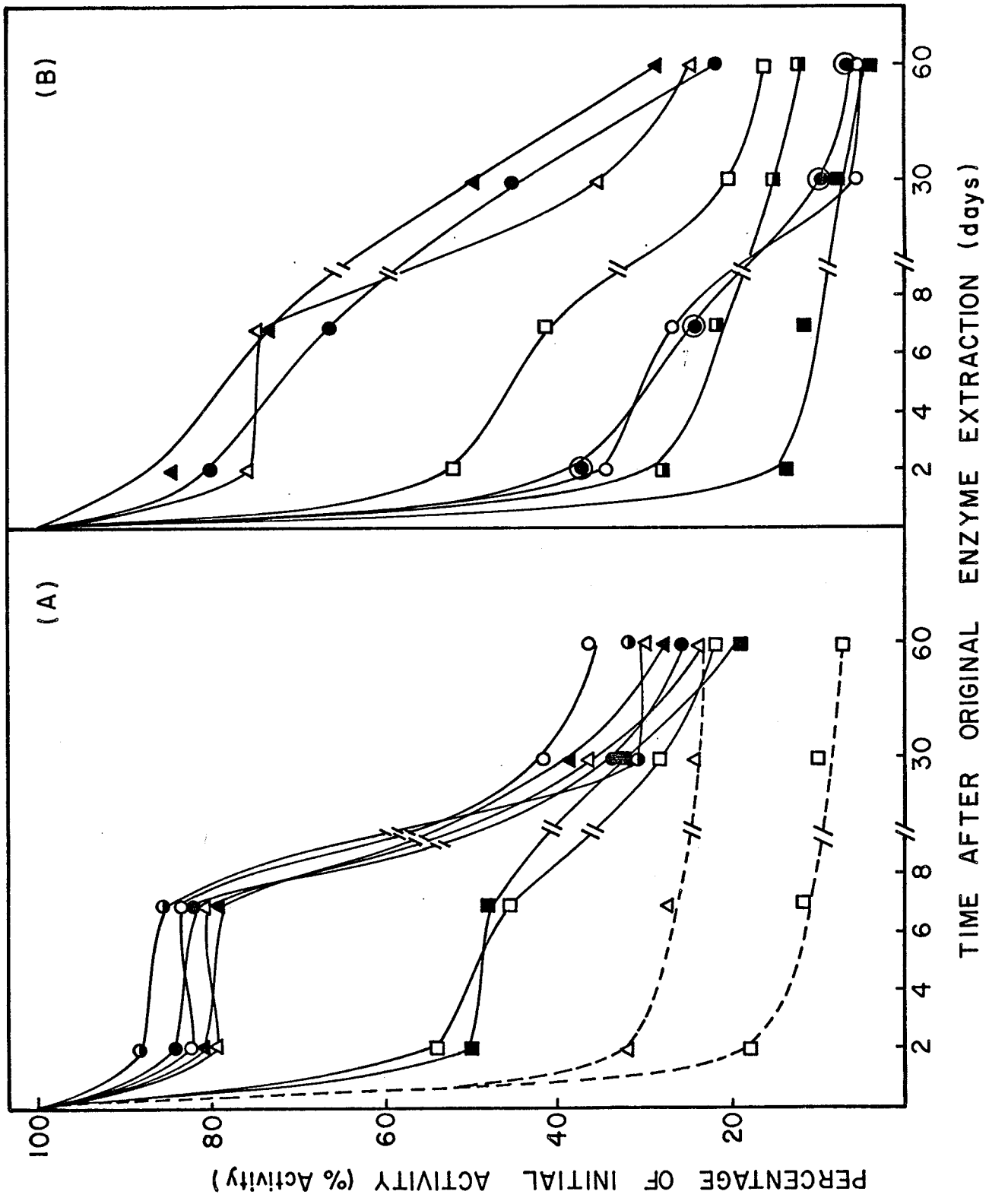
Fig. 9. Effects of protein concentration and pH on the decay of thiosulfate-oxidizing activity in crude cell-free extracts(A) and isolated membrane vesicles(B) at 4° and 25° C.
The crude cell-free extracts active membrane vesicles were prepared as described in Materials and Methods.
The preparations were stored under various protein concentration and pH at 4°C and 25° C in the 0.1 M Tris acetate-0.02 M potassium phosphate. Samples were withdrawn at the times indicated and assayed for thiosulfate-oxidizing activity at 25° C as described in Materials and Methods. The enzyme preparations were stored at 4° C with a protein concentration of 24 mg per ml(◐—◐), 12 mg per ml(○—○), 6 mg per ml (■—■) and 3 mg per ml(□—□) at pH 7.5.
The preparations were also stored at 4° C with a 6 mg of protein per ml at pH 6.5(▲—▲) and pH 8.5(△—△). ●—● indicated the rate of decay in thiosulfate-oxidizing activity when the preparations were stored at 25° C with a 6 mg of protein per ml at pH 7.5.


(A)
(B)
100
80
60
40
20
2
4
6
8
10
12
PERCENTAGE OF INITIAL ACTIVITY
TIME AFTER ORIGINAL ENZYME EXTRACTION (hours)

2 months at -20°C in the standard buffer. The degree of decrease in activity occurred during storage of enzymes was dependent on the protein concentration, but was independent on the various pH as presented in Fig. 10. Only when the cell-free preparations were frozen rapidly, were kept at -74°C in the small aliquots of standard buffer(0.5 ml in the serological test tubes), and thawed slowly before use, the thiosulfate-oxidizing activity was relatively stable for at least one week at high protein concentrations(e.g., at 6 to 12 mg protein per ml). The preparations lost 30 to 50% of the initial activity, however, after 2 months at -74°C in the standard buffer. Thus, the loss of activity in samples stored at -74°C was much less than that of samples stored at -20°C.
When the membrane vesicles obtained by either differential centrifugation(105,000 x g for 90 minutes) or Sepharose 4B column chromatography stored in the standard buffer(pH 7.5) containing bovine serum albumin(12 mg per ml), the thiosulfate-oxidizing activity was not protected against the inactivation during storage at various temperatures.

In general, it was observed that the thiosulfate-oxidizing activity of frozen cell-free preparation was activated by reduced glutathione, but not by cysteine, DTT, bovine serum albumin, spermidine, sulfite and NADH and the thiosulfate-oxidizing activity of the preparations was protected against inactivation for longer periods

Fig. 10. Effects of protein concentration and pH on the storage of thiosulfate-oxidizing activity in the crude cell-free extracts(A) and membrane vesicles (B) at -20° and -74°C. The experimental conditions were the same as those described in Fig. 9 except that the temperatures for storage were -20° and -74°C. The sample solutions were frozen rapidly and thawed slowly before assays. In (A), ◒——◒ , enzyme preparation stored at -74°C with a protein concentration of 24 mg per ml(pH 7.5); ○——○ , stored at -74°C(12 mg protein per ml, pH 7.5); △——△ , stored at -74°C(6 mg protein per ml, pH 7.5); □——□ , stored at -74°C(3 mg protein per ml, pH 7.5); ▲——▲ , stored at -74°C (6 mg protein per ml, pH 6.5); ●——● , stored at -74°C(6 mg protein per ml, pH 8.5); ■——■ , stored at -74°C(3 mg protein and 12 mg of bovine serum albumin per ml, pH 7.5); △---△ , stored at -20°C(6 mg protein per ml, pH 7.5) and□---□ , stored at -20°C(3 mg protein per ml, pH 7.5). In (B), all symbols were the same as described in (A) except the followings; ◉——◉ , stored at -74°C(1.5 mg protein and 12 mg of BSA per ml, pH 7.5); ○——○ , stored at -74°C(1.5 mg protein per ml, pH 7.5); ◘——◘ , stored at -20°C(6 mg protein per ml, pH 7.5) and ■——■ , stored at -20°C(3 mg protein per ml, pH 7.5).


(A)
(B)
PERCENTAGE OF INITIAL ACTIVITY (% Activity)
100
80
60
40
20
2
4
6
8
30
60
TIME AFTER ORIGINAL ENZYME EXTRACTION (days)

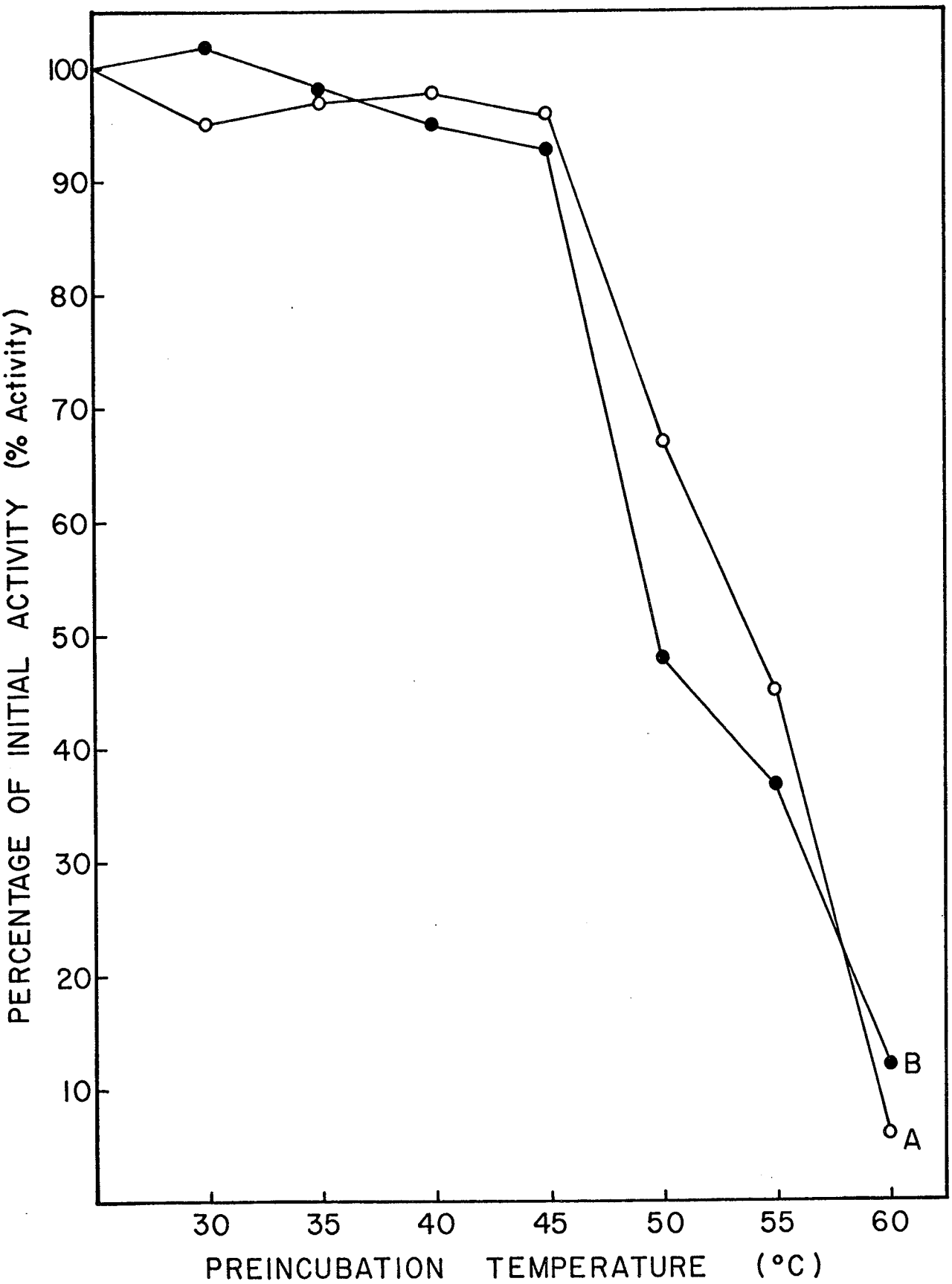
during storage under nitrogen atmosphere at the various temperatures(result not shown). Addition of mercapto-ethanol, GSH, spermidine, NADH, glycerol, or DTT did not appreciably increase the stability of the thiosulfate-oxidizing system.

Incubation of cell-free extracts and membrane vesicles at temperature higher than 45°C resulted in inactivation of the membrane-bound thiosulfate-oxidizing activity, while little changes in enzymatic activity were found after incubation for 5 minutes at below 45°C. Fig. 11 illustrates thermal inactivation curves for thiosulfate-oxidizing system incubated in 0.1 M Tris acetate-0.02 M potassium phosphate buffer(pH 7.5). An incubation for 5 minutes at 55°C caused a 58 to 62% decrease in the activity, whereas the most enzyme activity was destroyed by heating at 65°C for 5 minutes. Heating at 60°C for longer periods resulted in protein precipitation with simultaneous activity losses. The thiosulfate-oxidizing system was completely inactivated after boiling for 2 minutes.

Substrate Specificity

The active membrane vesicles purified by either differential centrifugation or Sepharose 4B column

Fig. 11. Effect of thermal inactivation on the thiosulfate-oxidizing activity of crude cell-free extracts(A) and membrane vesicles(B). The crude cell-free extracts and active membrane vesicles were prepared as described in Materials and Methods and were pre-incubated at various temperatures for 15 minutes. After incubations, the preparations were placed in an ice-bath to cool and assayed for thiosulfate-oxidizing activity as described in Materials and Methods.


PERCENTAGE OF INITIAL ACTIVITY (% Activity)
100
90
80
70
60
50
40
30
20
10
30
35
40
45
50
55
60
PREINCUBATION TEMPERATURE (°C)
B
A

chromatography oxidized only thiosulfate as substrate, indicating that the thiosulfate-oxidizing system was specific for thiosulfate. It was not possible to replace thiosulfate by tetrathionate, sulfide, sulfite, colloidal sulfur, reduced glutathione or cysteine. When thiosulfate-oxidizing enzyme activity($2S_2O_3^{2-} + 2e^- \longrightarrow S_4O_6^{2-}$) was assayed with either ferricyanide or cytochrome c(horse heart, type III) as described in Materials and Methods, no activity could be detected in the crude cell-free extracts or partially purified membrane vesicles, whereas both preparations exhibited rhodanese activity. The active membrane vesicles showed strong NADH oxidase activity and almost no NADPH oxidase activity.

On the other hand, both intact cells and crude cell-free extracts oxidized thiosulfate, sulfite or tetrathionate and a negligible activity was noted for sulfide, reduced glutathione or cysteine in both preparations. Considering the above information, a question arises as to whether the two enzyme systems for the oxidations of thiosulfate and tetrathionate are identical.

Stoichiometry of Thiosulfate Oxidation

(1) Oxygen Consumption and Thiosulfate Disappearance During Thiosulfate Oxidation

Both active intact cells and active crude cell-free extracts consumed oxygen at a rapid and constant rate, giving a 2:1 stoichiometry of oxygen utilization and thiosulfate disappearance from the oxidative reaction of thiosulfate. As shown in Table 7, periodic analyses of reaction mixtures showed that thiosulfate disappearance paralleled oxygen consumption and that no polythionates were formed during the oxidation. The amount of oxygen consumed due to thiosulfate oxidation corresponded to the following equation:

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

These preparations also catalyzed a complete oxidation of sulfite to sulfate according to the following equation:

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

These results are in full agreement with those reported by Charles and Suzuki(1966a). They used relatively high concentrations of enzymes(10 mg wet weight of intact cells and 6 mg of cell-free extract) and substrate(5 to 10 μmoles of thiosulfate) for their manometric experiments.

Table 7. Stoichiometry of thiosulfate oxidation by intact cells, crude cell-free extracts and isolated membrane vesicles of T. novellus.

Reaction system	Initial concentration of thiosulfate (μmole)	Thiosulfate oxidized (μmole)	Oxygen consumed (μmole)	Polythionate formed (μmole)	Ratio of O_2 consumed: thiosulfate oxidized
1. Intact cells	0.1	0.10	0.2	0	2.00
	1.0	0.10	0.2	0	2.00
	1.0	0.15	0.3	0	2.00
	1.0	0.22	0.4	0	1.82
	2.5	0.21	0.4	0	1.91
2. Crude cell-free extract	0.1	0.10	0.2	0	2.00
	1.0	0.11	0.2	0	1.82
	1.0	0.16	0.3	0	1.87
3. Membrane vesicles	0.1	0.10	0.15	0	1.50
	2.5	0.16	0.3	0	1.87

The intact cells(2 mg wet weight), crude cell-free extracts(4 mg of protein) or isolated membrane vesicles (1.55 mg of protein) obtained by Sepharose 4B column chromatography was freshly prepared as described in Materials and Methods and used for the experiments. Measurements of thiosulfate, tetrathionate and oxygen consumption were the same as described in Materials and Methods.

On the other hand, the membrane vesicles isolated by Sepharose 4B column chromatography showed a bi-phasic oxygen uptake in thiosulfate oxidation. The first stage of thiosulfate oxidation at a uniform and high rate ceased at consumption of 0.25 to 0.5 μmoles oxygen per μmole of total thiosulfate added. The second stage of thiosulfate oxidation proceeded at a slower rate and the reaction stopped with an oxygen consumption of about 75 to 90 per cent of that required for the stoichiometric amount of sulfate production. It was, however, observed that no polythionates were detected in the reaction mixture throughout the both stages of the thiosulfate oxidation and the stoichiometries calculated from the oxygen uptake and the thiosulfate disappeared in both stages approached the anticipated theoretical ratio of 2. In contrast to the biphasic oxidations observed with the isolated membrane vesicles from Sepharose 4B column chromatography, the membrane vesicles prepared by differential centrifugation(105,000 x g for 90 minutes) of the crude extracts sometimes oxidized thiosulfate at linear and high rates comparable to those of crude extracts when very active extracts were used for preparations of the membrane vesicles.

(2) Identification of the Reaction Product for the Thiosulfate-oxidizing System

An attempt was made to obtain further evidence by a product identification as a sulfate with active preparations of intact cells and crude cell-free extracts. The stoichiometry data are summarized in Table 8. In the complete system, the end product of the reaction was demonstrated to be sulfate and all of the added S-35 labelled thiosulfate could be almost entirely accounted for as sulfates. There was no appreciable quantities of sulfate formation in the absence of thiosulfate-oxidizing system and polythionates were not detected in the complete system (Fig. 12).

When the experiment was carried out with thiosulfate labelled in either inner or outer positions, similar results were obtained, indicating that these preparations oxidized both atoms of sulfur in a molecule of thiosulfate.

Effect of Substrate Concentration on Thiosulfate Oxidation

The effect of increasing thiosulfate concentrations on the thiosulfate-oxidizing activity of the intact cells is shown in Fig. 13.

The velocity plot with varying thiosulfate concentration was a standard rectangular hyperbola and from double-reciprocal plots of the data, the apparent Km for thiosulfate at pH 7.5 was estimated to be 2×10^{-5}M.

Table 8. Stoichiometry of radioactive thiosulfate oxidation by intact cells and crude cell-free extracts of T. novellus.

Reaction system	Positions of S-35 labelled thio-sulfate	Thio-sulfate oxidized (nmole)	Thio-sulfate remained (nmole)	Oxygen con-sumed (nmole)	S^{35}-sulfate formed (nmole)	Tetra-thionate formed (nmole)
1. Intact cells	inner ($S\text{-}S^{35}O_3^{2-}$)	100	0	210	96	0
	outer ($S^{35}\text{-}SO_3^{2-}$)	100	0	200	87	0
2. Crude cell-free extract	inner ($S\text{-}S^{35}O_3^{2-}$)	96	4	200	92	0
	outer ($S^{35}\text{-}SO_3^{2-}$)	100	0	200	91	0

The intact cells(2mg wet weight) and the cell-free extracts(4.2mg of protein) were prepared and used, and the reaction was carried out in a Gilson Oxygraph as described in Materials and Methods. Under these conditions the radioactive thiosulfate(0.1 μmole) was oxidized completely to sulfate in 5 minutes. The experimental details were given in Materials and Methods.

Fig. 12. Identification of the reaction product of thiosulfate-oxidizing system.

The intact cells(2 mg wet weight) and freshly prepared cell-free extracts(4.2 mg of protein) were used. The reaction was carried out in a Gilson Oxygraph using thiosulfate labelled in outer positions(S^{35}-SO_3^{2-}).

The experimental procedures for thin-layer chromatography and identification of inorganic sulfur compounds were the same as described in Materials and Methods.

Radioactive thiosulfate(0.1 μmole) was oxidized completely in 5 minutes.

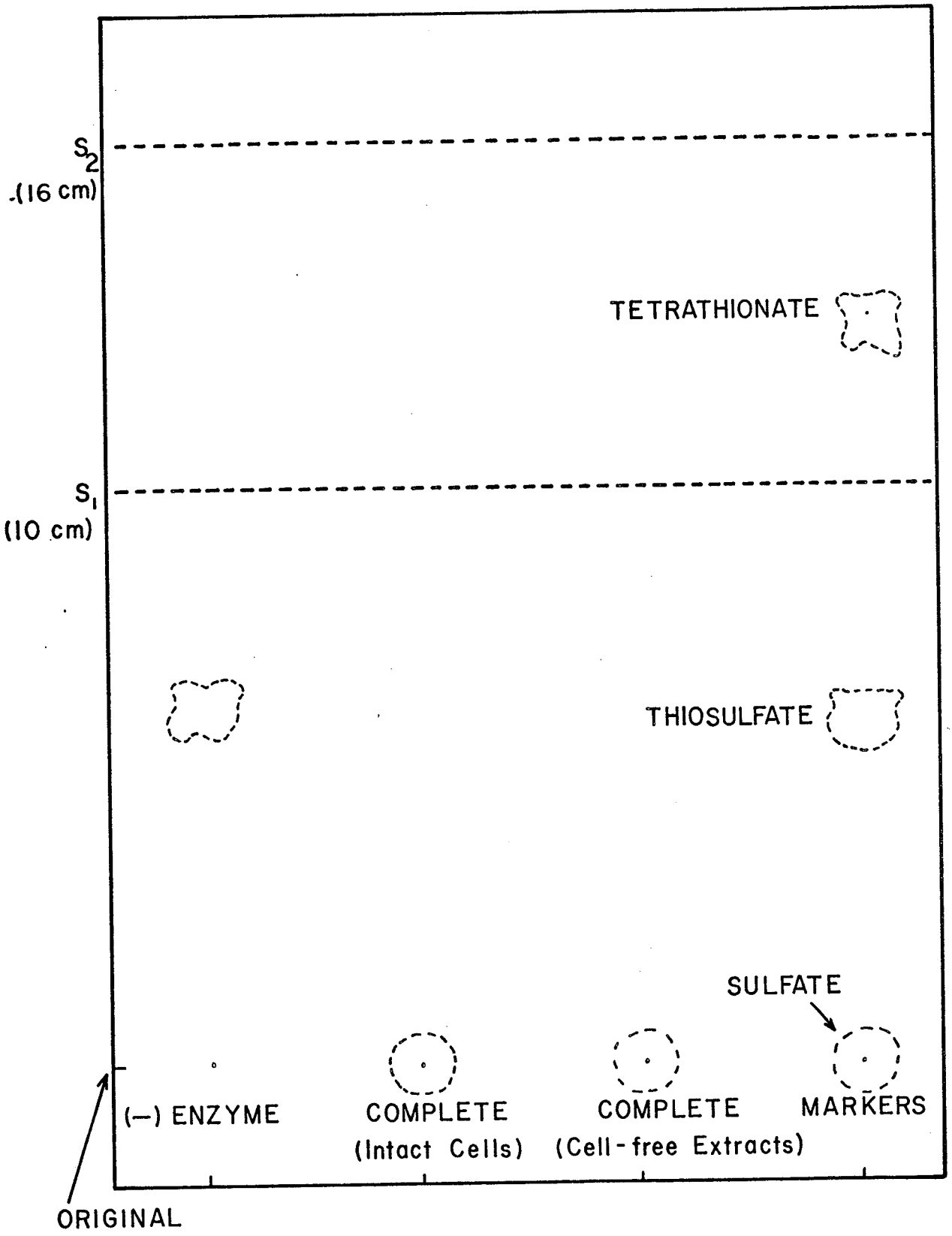
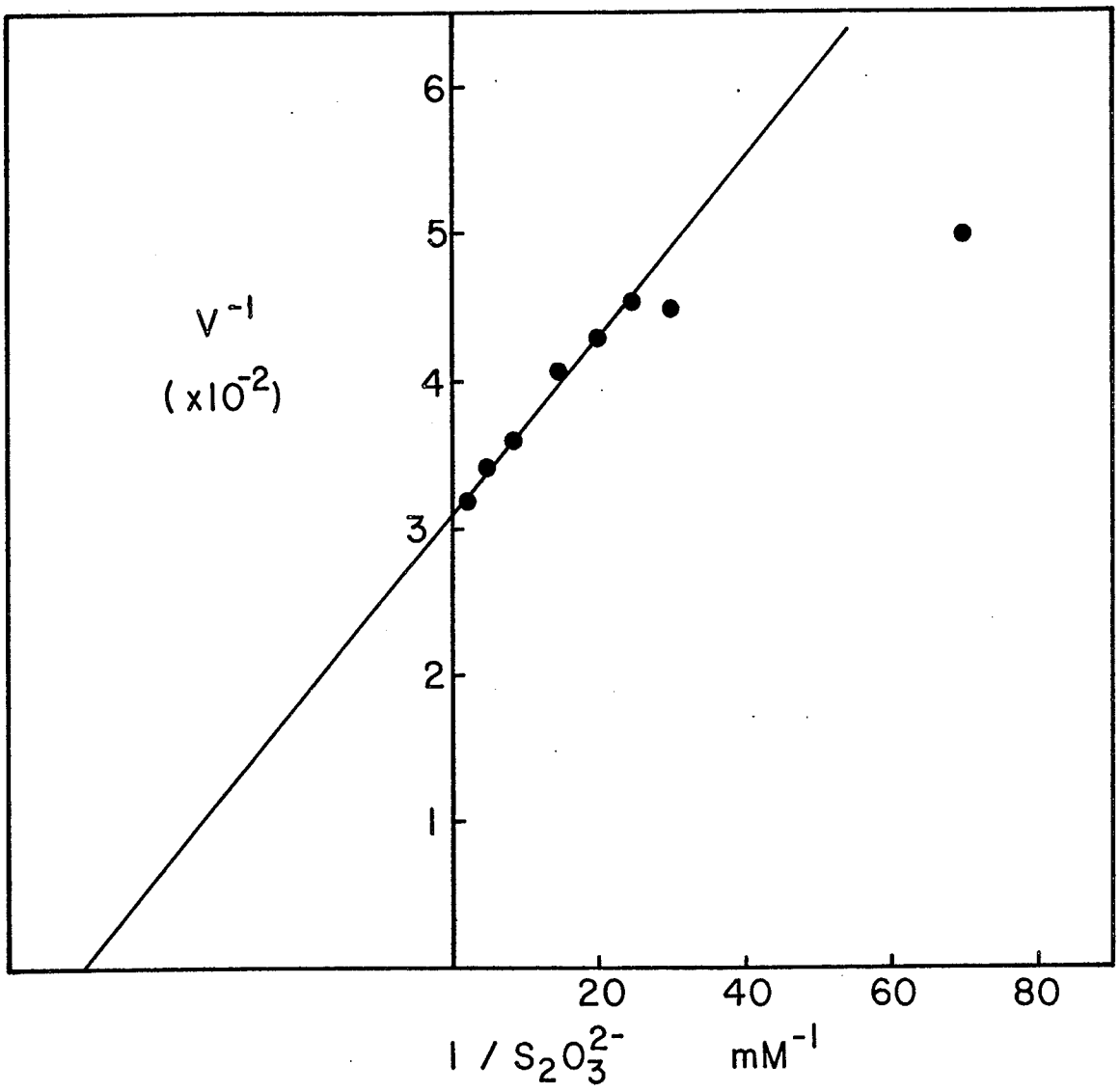

S2
(16 cm)
S1
(10 cm)
TETRATHIONATE
THIOSULFATE
SULFATE
(–) ENZYME
COMPLETE
(Intact Cells)
COMPLETE
(Cell-free Extracts)
MARKERS
ORIGINAL

Fig. 13. Effect of thiosulfate concentration on the rate of thiosulfate oxidation by intact cells. The activity was measured in an oxygraph at 25^{0} C as described in the Materials and Methods with the exception that thiosulfate concentration was used as indicated. For assays 2 mg wet weight of whole cells were used.


6
5
4
3
2
1
V^{-1}
$(x10^{-2})$
20
40
60
80
$1 / S_2O_3^{2-}$ mM^{-1}

It should be noted, however, that the determination of Km value with very active intact cells was almost impossible because of the low concentration of thiosulfate required for determination. The Km value for isolated membrane vesicles for thiosulfate was 1.2×10^{-4}M(Fig. 14). Of particular interest was the fact that a soluble enzyme, thiosulfate-cytochrome c reductase which was isolated and purified from T. novellus had about 1,000 times higher Km than that of this enzyme, although author did not determine the Km value for thiosulfate oxidase which was another soluble enzyme involved in thiosulfate oxidation (Aleem, 1965). From his data it was calculated that the apparent Km for thiosulfate in the absence of cyanide was approximately 2×10^{-1}M and the Km value in the presence of cyanide was about 10^{-3}M.

Effect of pH on Thiosulfate Oxidation

The optimum hydrogen ion concentration for the oxidation of thiosulfate by the membrane vesicles was determined by using 0.1 M Tris acetate-0.02 M potassium phosphate buffer (pH 5.5-10.0), Tricine buffer (pH 7.5-8.5) and Bicine buffer (pH 7.7-8.7). The optimal pH for thiosulfate-oxidizing activity was 7.5 with 0.1 M Tris acetate-0.02 M potassium phosphate buffer and Tricine buffer, while with Bicine buffer,

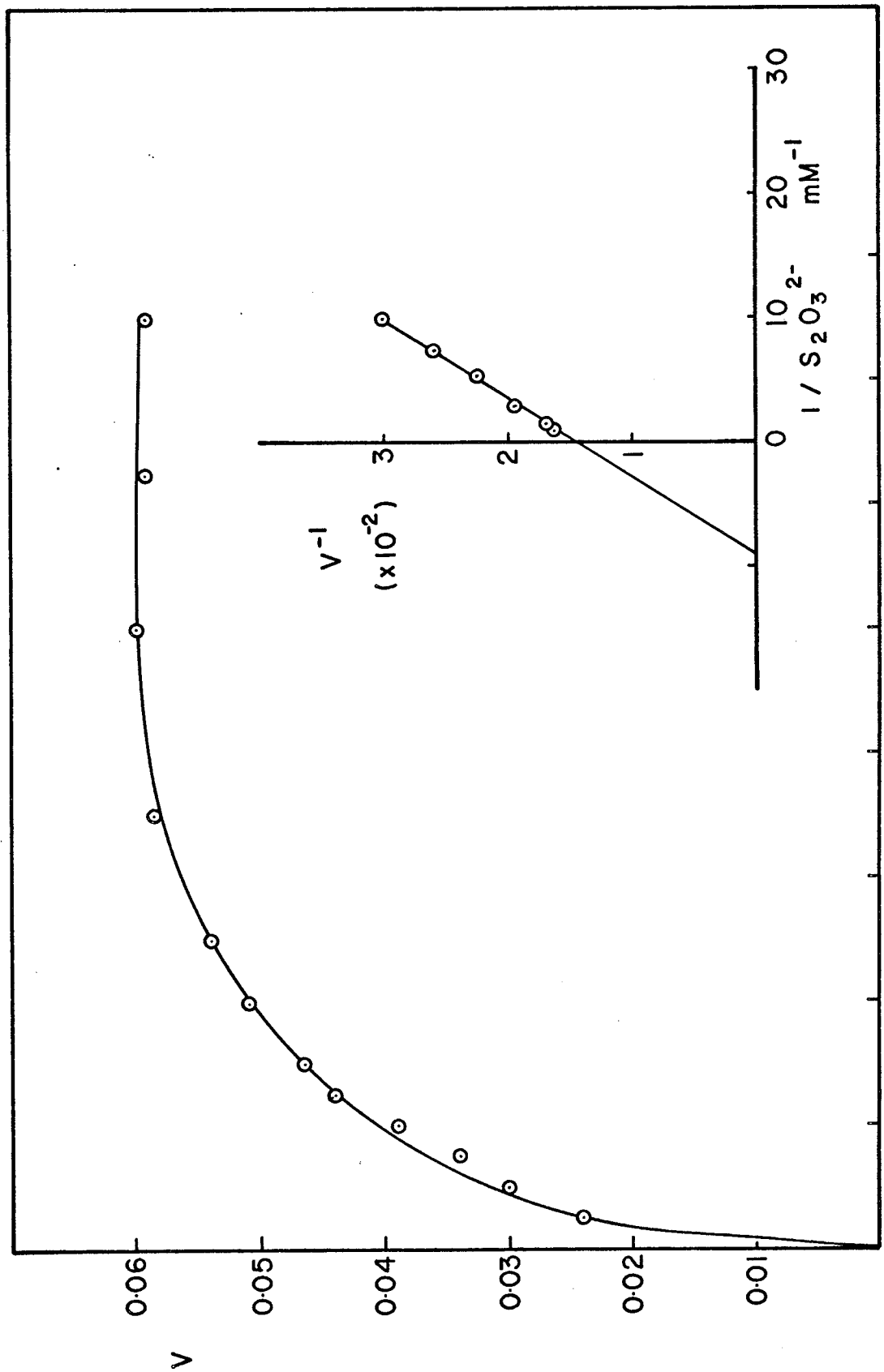

V
0·01
0·02
0·03
0·04
0·05
0·06
0·0
0·2
0·4
0·6
0·8
1·0
1·2
1·4
1·6
$S_2O_3^{2-}$ (mM)
V^{-1} ($\times 10^{-2}$)
1
2
3
0
10
20
30
$1 / S_2O_3^{2-}$ mM^{-1}

Fig. 14. Effect of thiosulfate concentration on the thiosulfate-oxidizing activity in the membrane vesicles. The membrane vesicles were prepared by a Sepharose 4B column chromatography and the thiosulfate-oxidizing activity was measured as described in the Materials and Methods with the exception that thiosulfate concentration was used as indicated.

About 250 μg of protein was used in each assay.

the pH optimum appeared to be 8.4(Fig. 15). When Bicine buffer was used, the activity was slightly lower than those with two other buffers.

A sharp drop in activity was observed either below pH 6.5 or above pH 8.5 and there was essentially no measurable activity below pH 5.5 or above pH 10.

The optimum of intact cells for thiosulfate oxidation occured around pH 7.5 agreeing with the optimum pH of the membrane vesicles(not shown).

Effect of Protein Concentration on Thiosulfate Oxidation.

The relationship between protein concentration and thiosulfate-oxidizing activity was examined with isolated membrane vesicles. From Fig. 16 it could be seen that the enzyme activity was linear with protein concentration from 180 to 600 μg. The enzyme appeared to be inactivated at protein concentration below 180 μg.

Effect of Phosphate on Thiosulfate Oxidation

When the crude extracts were prepared by sonication in 0.1 M Tris-acetate buffer(pH 7.5), 0.1 M Tris-HCl buffer (pH 7.5), 0.1 M potassium phosphate buffer(pH 7.5), 0.04 M potassium phosphate buffer(pH 7.5) or 0.1 M HEPES(N-2-

Fig. 15. Effect of hydrogen ion concentration on thiosulfate-oxidizing activity of membrane vesicles.

The rate of thiosulfate-oxidizing activity was measured as described in the Materials and Methods except that 0.1 M Tris acetate-0.02 M potassium phosphate buffer was used from pH 5.5 to pH 10.0(o—o); 0.1 M Tricine-NaOH buffer containing 0.02 M potassium phosphate was used from pH 7.0 to pH 8.5(Δ—Δ) and Bicine-NaOH buffer containing 0.02 M potassium phosphate was used from pH 7.7 to pH 8.7(□—□).

About 250 ug of enzyme protein from the membrane vesicle suspensions obtained by a Sepharose 4B column chromatography was used in each assay.

The pH of each reaction mixture was determined after the measurement of the rate of thiosulfate-oxidizing activity.

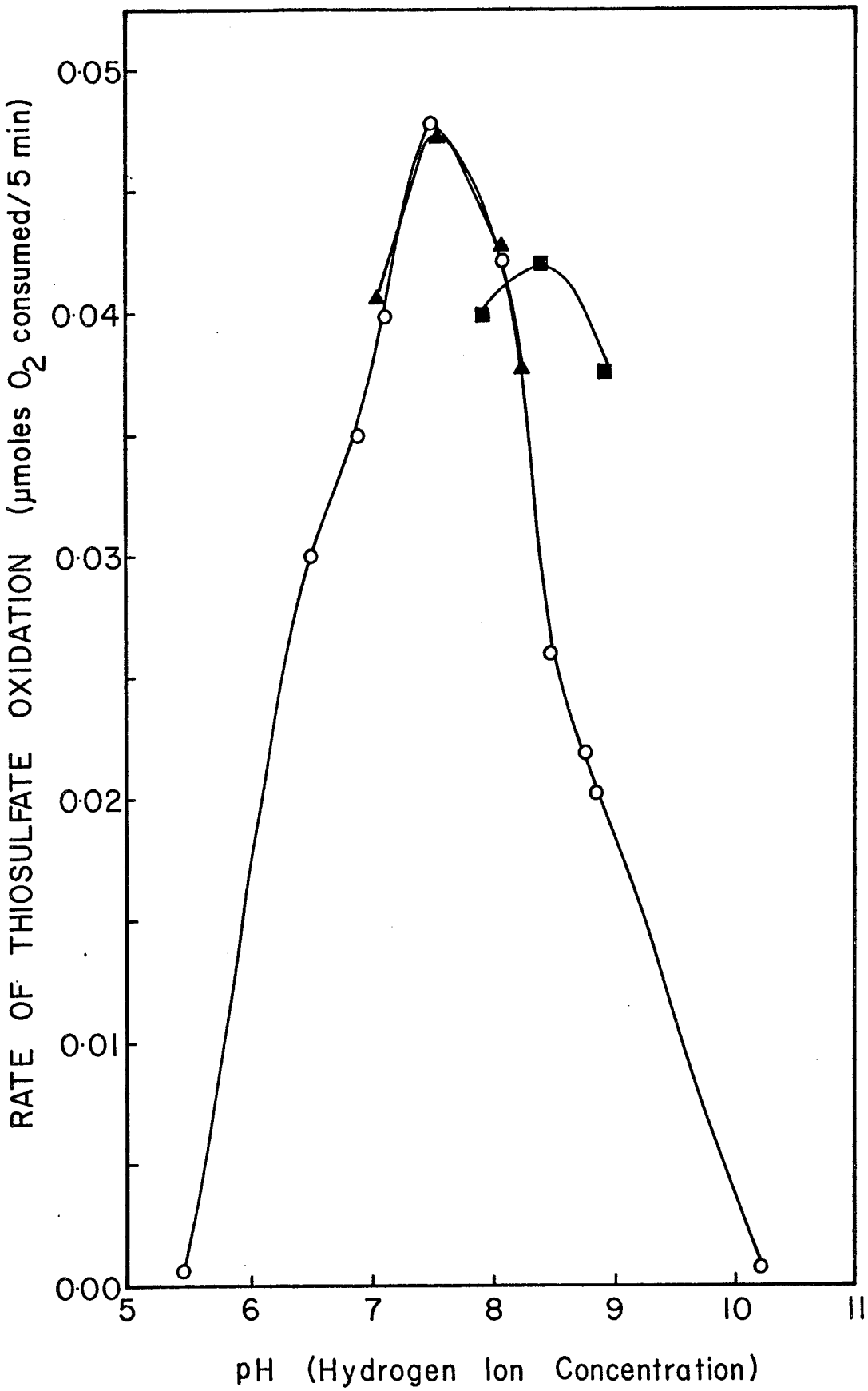
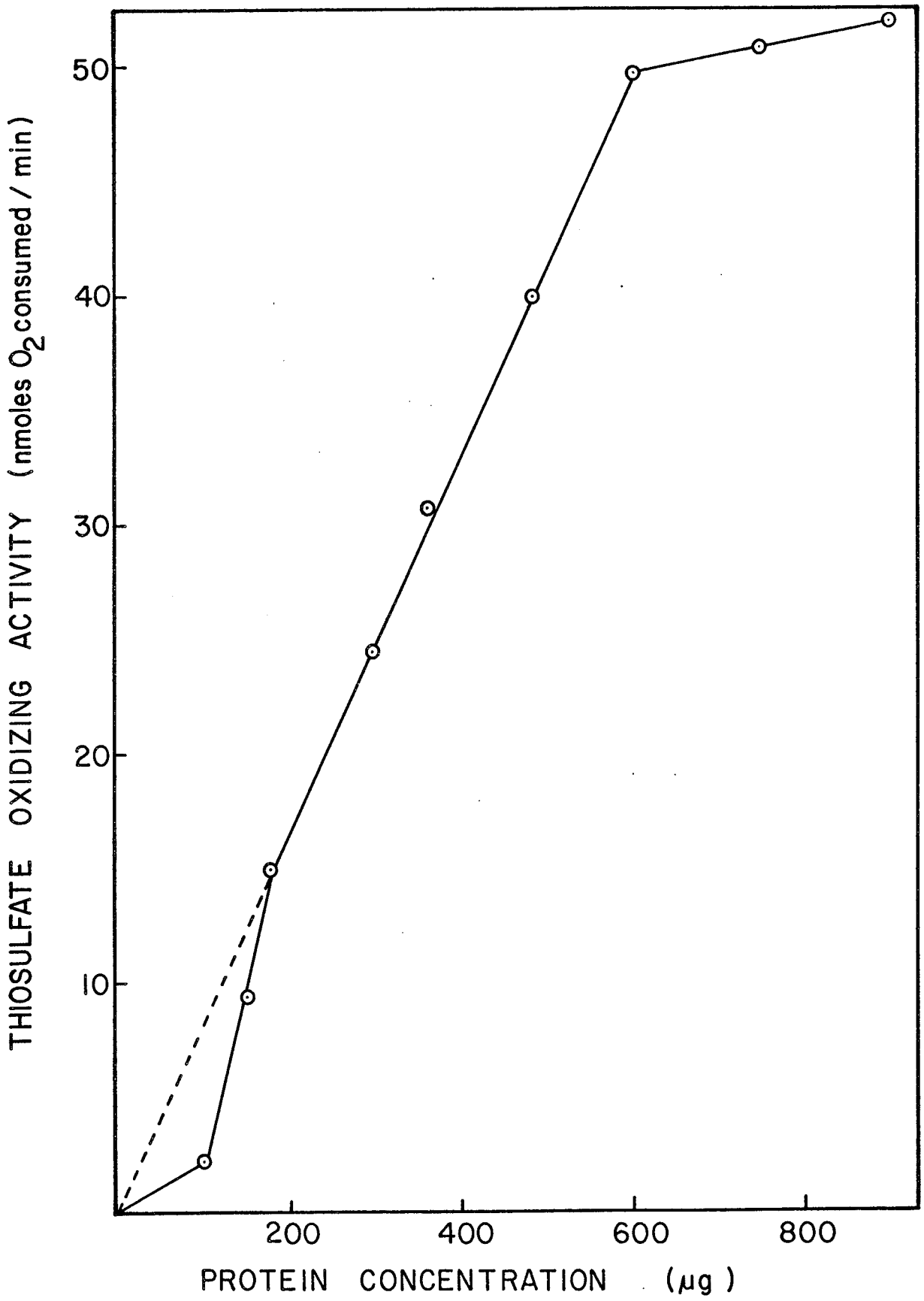

RATE OF THIOSULFATE OXIDATION (μmoles O_2 consumed/5 min)
0·05
0·04
0·03
0·02
0·01
0·00
5
6
7
8
9
10
11
pH (Hydrogen Ion Concentration)

Fig. 16. Effect of protein concentration on thiosulfate-oxidizing activity of isolated membrane vesicles. The active membrane vesicles were prepared from crude cell-free extracts by a Sepharose 4B column chromatography and thiosulfate-oxidizing activity was assayed as described in Materials and Methods except that the protein concentrations were varied as indicated.


THIOSULFATE OXIDIZING ACTIVITY (nmoles O_2 consumed / min)
50
40
30
20
10
200
400
600
800
PROTEIN CONCENTRATION (µg)

hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer (pH 7.5) and tested in the same buffers, the thiosulfate-oxidizing activities were lower (30 to 49%) than that prepared in 0.1 M Tris acetate-0.02 M potassium phosphate buffer (pH 7.5). However, if these extracts (0.1 ml of each preparation) were assayed in the 0.1 M Tris acetate-0.02 M potassium phosphate buffer, the activities equal to that of 0.1 M Tris acetate-0.02 M potassium phosphate buffer (pH 7.5) were obtained suggesting a requirement of appropriate phosphate ions to obtain a maximal activity. In addition, when the crude extracts were prepared in 0.1 M Tris acetate-0.02 M potassium phosphate buffer (pH 7.5) and tested in 0.1 M Tris acetate-0.1 M potassium phosphate buffer (pH 7.5), the thiosulfate-oxidizing activity was 69% that in system buffered with 0.1 M Tris acetate-0.02 M potassium phosphate (pH 7.5), indicating that high concentration of phosphate ions inhibited the oxidation of thiosulfate.

The saturating phosphate concentration for inhibitory effect on the thiosulfate-oxidizing activity was 0.1 M, and no additional effect was observed up to 0.25 M phosphate. The thiosulfate-oxidizing system exhibited maximum activity at the final phosphate concentrations of 0.02 M to 0.04 M in 0.1 M Tris-acetate buffer (pH 7.5).

Effect of Temperature on Thiosulfate Oxidation

The rate of thiosulfate oxidation was studied as a function of temperature under otherwise identical conditions. Fig. 17 shows the effect of temperature on the thiosulfate-oxidizing activity in the crude cell-free extracts and purified membrane vesicles from 10 to 35°C. The optimal temperature for the maximal velocity of the reaction was found to occur at 25°C. At 30°C and 35°C, the activities were approximately 65% and 53% of that observed at 25°C.

A Arrhenius plot constructed from the results obtained by the crude cell-free extracts at the temperature between 10 to 25°C was linear over the entire temperature range, indicating that no temperature induced conformational changes in the thiosulfate-oxidizing system seemed to have occurred (Fig. 18).

Effect of Reduced Glutathione, NADH and Sulfite on the Thiosulfate-oxidizing System

In order to prepare active cell-free extracts and membrane vesicles, it was essential to start with very active intact cells which were obtained from well controlled cultures, viz., controlled aeration, temperature, pH and incubation time etc.

Fig. 17. Effect of temperature on the oxidation of thiosulfate by crude cell-free extracts and purified membrane vesicles of T. novellus. The preparations of crude cell-free extracts and membrane vesicles(differential centrifugation method) and the experimental procedure for the measurement of thiosulfate-oxidizing activity in a Gilson Oxygraph at various temperatures as indicated, were the same as those described in Materials and Methods.

The actual amount of oxygen consumed in each measurement was corrected for both endogenous respiration and the solubility of oxygen at various temperatures. In the endogenous respiration, it was assumed that substrate (thiosulfate) had no effect on the rate of endogenous activity.

Experiment with crude cell-free extracts(4 mg of protein in each assay), ▲—▲ and experiment with purified membrane vesicles(1.55 mg of protein in each assay), ●—● .

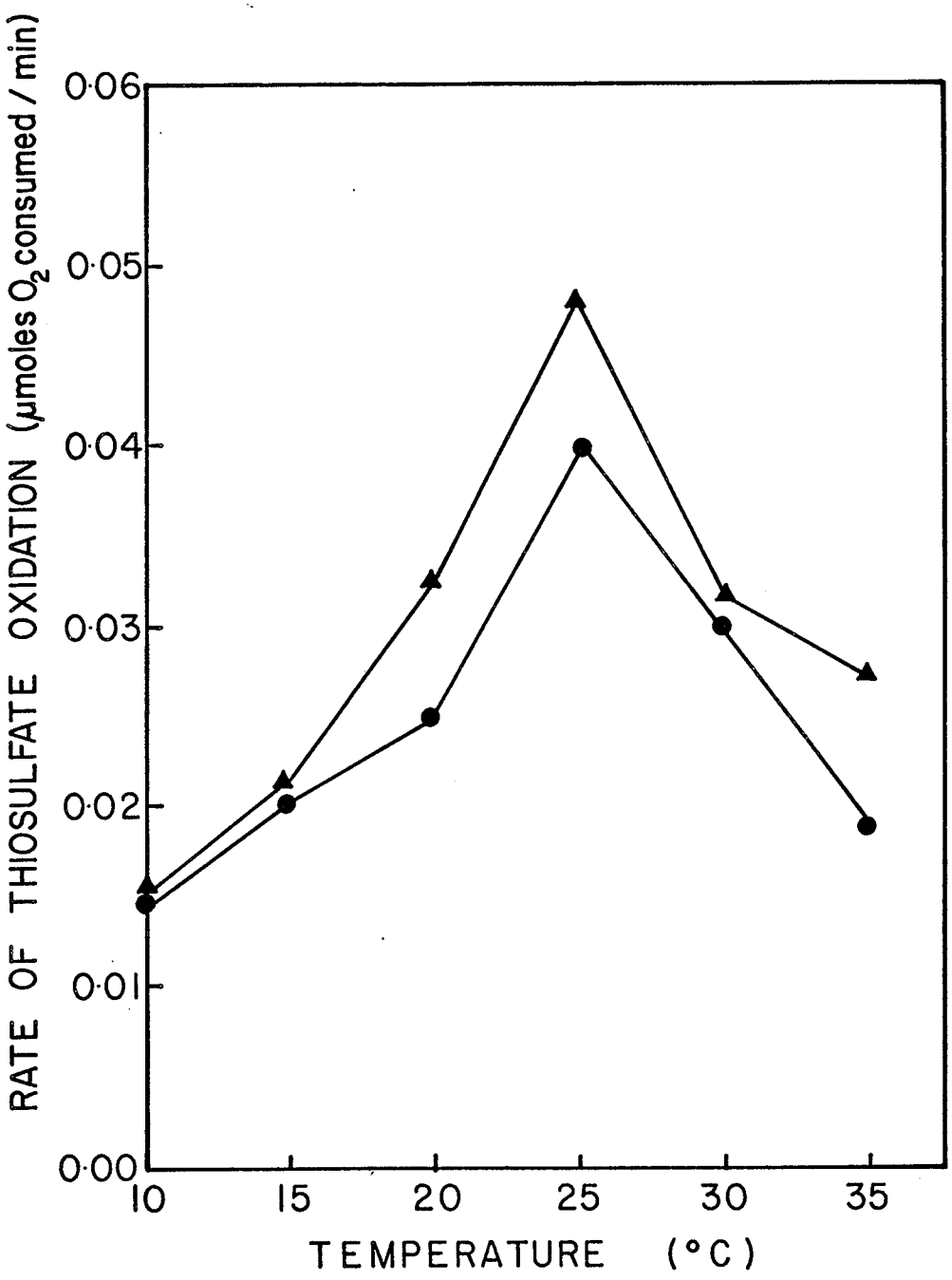
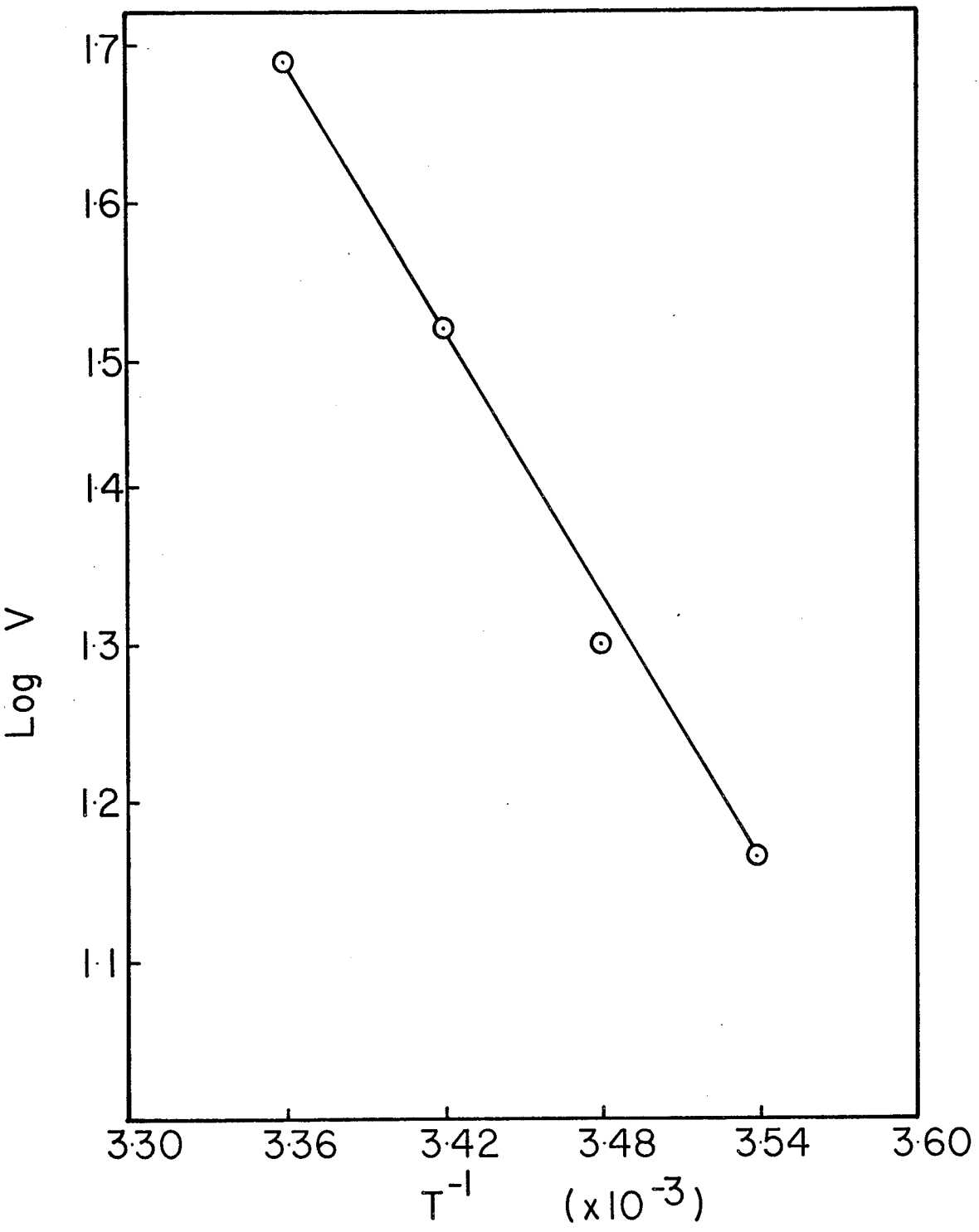

RATE OF THIOSULFATE OXIDATION (μmoles O_2 consumed / min)
0·06
0·05
0·04
0·03
0·02
0·01
0·00
10
15
20
25
30
35
TEMPERATURE (°C)

Fig. 18. Arrhenius plot for thiosulfate-oxidizing activity in the crude cell-free extracts of T. novellus.

The experimental procedure was the same as described in Fig. 17.

Log V refers to the log 10 of the reaction rate in terms of nmoles of oxygen consumed per minutes per 4 mg of protein.

T indicates absolute temperature.


1·7
1·6
1·5
1·4
1·3
1·2
1·1
Log V
3·30
3·36
3·42
3·48
3·54
3·60
T⁻¹ (x10⁻³)

However, in a poorly controlled culture, the whole cells were less active and thiosulfate-oxidizing activity was much lower when the crude cell-free extract was prepared by sonication. The activity of cell-free extracts could be stimulated by additions of small amount of GSH, NADH or sulfite(Table 9). These activators had similar stimulative effects on active cell-free extracts after some loss of activity during storage at $4^{o}C$ or cell-free extracts prepared from old cells which had been stored at $4^{o}C$ for 5 days.

Contrast to the above observation, when the cell-free extracts were prepared from the active intact cells which had been frozen at -20^{o} or $-74^{o}C$, the addition of GSH activated the thiosulfate-oxidiaing activity and the activity could not be stimulated by either NADH or sulfite. A similar result was observed when the crude cell-free extract was obtained by sonicating the active intact cells for 30 minutes instead of the normal 15 minutes under N_2 atmosphere or breaking the cells without N_2 gas.

These results indicated that the cell-free extracts damaged during the preparation by physical means were different from those from less active cells and required GSH for the oxidation of thiosulfate. NADH or sulfite could not replace GSH for activation of the thiosulfate-oxidizing system.

All activators described above had similar activation

Table 9. Stimulative effects of GSH, NADH and sulfite on the thiosulfate-oxidizing system.

Preparation	Rate of thiosulfate oxidation (nmole O_2 consumed/min)			
	None	+GSH	+NADH	+sulfite
1. Active cell-free extract	90	89	89	90
2. Less active cell-free extract				
a. Prepared from less active cells	18	80	76	75
b. Prepared from old cells (at 4°C for 5 days)	44	78	74	76
c. 1 stored at 4°C for 12 hours	29	61	56	58
d. Prepared from frozen(-20°C for 4 days) and thawed cells	28	71	31	28
e. Prepared from frozen (-74°C for 7 days) and thawed cells	36	83	39	37
f. Prepared by sonication for 30 min under N_2 atmosphere	57	85	59	57
g. Prepared by sonication for 30 min without N_2 gas	20	71	22	NT
3. Membrane vesicles				
a. Prepared from 1	49	54	51	49
b. 3a stored at 4°C for 6 hours	21	38	35	NT
c. 3a stored at -74°C for 30 days	24	31	NT	NT

The crude cell-free extracts were prepared from various sources of intact cells and the membrane vesicles were prepared by a Sepharose 4B column chromatography as described in Materials and Methods. All additions were made last in microliter volumes. The thiosulfate-oxidizing activity was assayed as described in Materials and Methods. Final concentrations of the additions were: thiosulfate, 1.0 μmole; GSH, 0.5 μmole; NADH 0.05 μmole; sulfite, 0.05 μmole.

effects on the thiosulfate-oxidizing activity of isolated membrane vesicles. In all cases of activations described above, the amounts of oxygen consumed were much more than that required for the oxidation of NADH or sulfite and the preparations did not oxidize GSH at the concentrations tested. The actual amount of oxygen consumed due to thiosulfate oxidation in the presence of all activators were over 90% of those expected from the reaction systems (Table 10). A typical experiment with GSH is shown in Fig. 19.

As shown in Table 11, the ratio of GSH and thiosulfate to obtain a maximal activation for the oxidation of thiosulfate was 1:2.

At the high concentration, the reduced glutathione had slightly inhibitory effect on the thiosulfate-oxidizing activity.

Bovine serum albumin(20 mg/ml, Fraction V), Mg^{2+} (10^{-2}M), $CuCl_2$(10^{-4}M), polyamine(3 x 10^{-4}M spermine or spermidine), glycerol(15%, v/v), sucrose(15%, w/v), dithiothreitol(10^{-3}M) and mercaptoethanol(10^{-3}M) were without effect in the activation of these less active preparations.

Effect of Metal Ions on the Thiosulfate Oxidation

The additions of the metal ions such as Fe^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Cu^{+}, Cu^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+} and Ca^{2+} at a

Table 10. The increased rate of oxygen consumptions by GSH, NADH and sulfite on the oxidation of thiosulfate in the crude cell-free extracts of T. novellus.

Reaction system	Total O_2 consumed (μmole)	O_2 consumed due to thiosulfate (μmole)
1. Cell-free extract	0.036	-
2. Cell-free extract + thiosulfate	0.054	-
3. Cell-free extract + GSH	0.038	-
4. Cell-free extract + GSH + thiosulfate	0.296	0.231
5. Cell-free extract + NADH	0.023	-
6. Cell-free extract + thiosulfate + NADH	0.212	0.189
7. Cell-free extract + sulfite	0.024	-
8. Cell-free extract + thiosulfate + sulfite	0.217	0.193

The crude cell-free extract was prepared from less active cells and thiosulfate-oxidizing activity was measured as described in Materials and Methods.
Final concentrations of additions were : thiosulfate, 0.1 μmole; GSH, 0.05 μmole; NADH, 0.05 μmole; sulfite, 0.05 μmole.

Fig. 19. Time-course activation of the thiosulfate-oxidizing activity in crude cell-free extract of T. novellus by GSH.

The crude cell-free extract was prepared from less active cells and the thiosulfate-oxidizing activity was assayed as described in Materials and Methods.

At the times shown by arrows, either 0.1 umole of thiosulfate or 0.05 μmole of GSH were added.

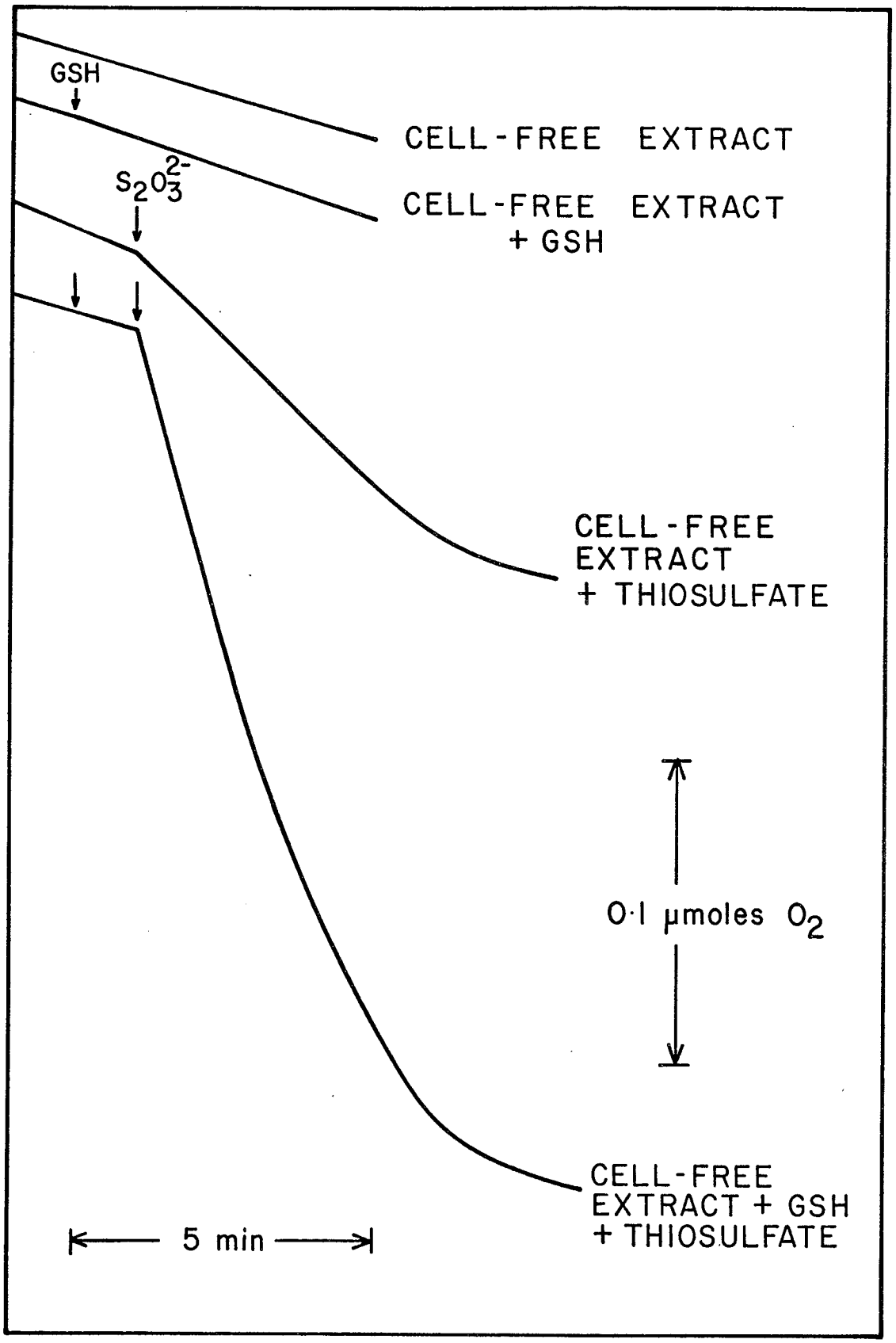

GSH
CELL-FREE EXTRACT
$S_2O_3^{2-}$
CELL-FREE EXTRACT + GSH
CELL-FREE EXTRACT + THIOSULFATE
0·1 μmoles O_2
CELL-FREE EXTRACT + GSH + THIOSULFATE
5 min

Table 11. Effect of concentration of GSH on the thiosulfate-oxidizing activity in crude cell-free extract of T. novellus.

Reaction system	Concentration of GSH(μmole)	% activity
1. Cell-free extract + thiosulfate	0	28
2. Cell-free extract + GSH + thiosulfate	0.05	42
3. Cell-free extract + GSH + thiosulfate	0.1	60
4. Cell-free extract + GSH + thiosulfate	0.25	72
5. Cell-free extract + GSH + thiosulfate	0.5	100
6. Cell-free extract + GSH + thiosulfate	1.0	90
7. Cell-free extract + GSH + thiosulfate	2.5	81
8. Cell-free extract + GSH + thiosulfate	5.0	76

The crude cell-free extract was prepared by sonicating the active cells for 30 minutes and thiosulfate-oxidizing activity was assayed as described in Materials and Methods. The concentration of thiosulfate for assay was 1 μmole.

final concentration of 10^{-3} M to the reaction mixture or preincubations with these metal ions did not stimulate thiosulfate-oxidizing activity. These results indicated that external metal ions were not required for the oxidation of thiosulfate.

Effect of Various Inhibitors on the Thiosulfate-oxidizing System.

The effect of metal-binding reagents on the thiosulfate-oxidizing activity in various preparations is shown in Table 12. Potassium cyanide and sodium azide were very potent inhibitors of thiosulfate-oxidizing activity in intact cells, crude cell-free extracts and isolated membrane vesicles of T. novellus. The inhibitory effect of thiosulfate oxidation by potassium cyanide was time-dependent(Fig. 20). The thiosulfate-oxidizing activity in all preparations was completely inhibited by potassium cyanide at a final concentration of 10^{-4} M in 5 minutes. On the other hand, there was no complete inhibition by sodium azide. Even at a high concentration (5×10^{-3}) only a partial inhibition(about 50%) of the activity in all preparations by this compound was observed (Table 12). The potassium cyanide inhibition of thiosulfate oxidation by intact cells was not reversed either by removal of KCN by washing or by the addition of metal

Table 12. Inhibitory effect of metal-binding reagents on the thiosulfate-oxidizing system of T. novellus.

Thiosulfate-oxidizing system	Inhibitor	Concentration (M)	Activity (%)
I. Intact cells	None	-	100
	Potassium cyanide	10^{-4}	0(2)[a]
		$5x10^{-5}$	25(27)[a]
		10^{-5}	48(54)[a]
		$5x10^{-6}$	76
		10^{-6}	100
	Diethyldithiocarbamate	10^{-4}	73
		$5x10^{-5}$	88
		10^{-5}	100
	1,10-orthophenanthroline	$5x10^{-3}$	98
		10^{-3}	100
	2,2'-dipyridyl[c]	10^{-3}	93
		$5x10^{-4}$	100
	EDTA	$5x10^{-3}$	92
		10^{-3}	97
		$5x10^{-4}$	100
	Sodium azide	$5x10^{-3}$	50
		10^{-3}	67
		$5x10^{-4}$	83
		10^{-4}	100
	Sodium sulfide	10^{-4}	100
	Bathocuproine disulfonate[d]	10^{-4}	92
		$5x10^{-5}$	100
II. Crude cell-free extract	None	-	100
	Potassium cyanide	10^{-4}	0(3)[a]
		$5x10^{-5}$	1(7)[a]
		10^{-5}	6(9)[a]
		$5x10^{-6}$	24
		10^{-6}	42

Table 12. continued

	Diethyldithiocarbamate	10^{-4}	67
		$5x10^{-5}$	85
		10^{-5}	100
	1,10-orthophenanthroline	10^{-3}	125[b]
		$5x10^{-4}$	128[b]
		10^{-4}	135[b]
	2,2'-dipyridyl[c]	10^{-3}	91
		$5x10^{-4}$	100
	EDTA	$5x10^{-3}$	96
		10^{-3}	100
	Sodium azide	$5x10^{-3}$	50
		10^{-3}	67
		$5x10^{-4}$	83
		10^{-4}	100
	Sodium sulfide	10^{-4}	100
	Bathocuproine disulfonate[d]	10^{-4}	92
		$5x10^{-5}$	100
III. Membrane vesicles	None	-	100
	Potassium cyanide	10^{-4}	0(6)[a]
		$5x10^{-5}$	2(11)[a]
		10^{-5}	8
		$5x10^{-6}$	22
		10^{-6}	48
	Diethydithiocarbamate	10^{-4}	75
		$5x10^{-5}$	92
		10^{-5}	100
	1,10-orthophenanthroline	10^{-3}	105[b]
		$5x10^{-4}$	117[b]
		10^{-4}	129[b]
	2,2'-dipyridyl[c]	10^{-3}	86
		$5x10^{-4}$	95
		10^{-4}	100
	EDTA	$5x10^{-3}$	93
		10^{-3}	100

Table 12. continued

Sodium azide	$5x10^{-3}$	37
	10^{-3}	50
	$5x10^{-4}$	69
	10^{-4}	86
Sodium sulfide	10^{-4}	96
Bathocuproine disulfonate[d]	10^{-4}	100

The reaction mixtures contained either 2 mg wet weight of intact cells or 1.53 mg of protein of the crude cell-free extract and isolated membrane vesicles, inhibitors as indicated and 0.1 M Tris acetate-0.02 M potassium phosphate (pH 7.5) to 1.5 ml.

The effect of inhibitors was examined by preincubating the reaction mixtures for 5 minutes at 25°C and by measuring the thiosulfate-oxidizing activity as described in Materials and Methods.

All inhibitors were dissolved in the same standard buffer(pH 7.5) except otherwise indicated, and added in microliter volumes before preincubations. The concentrations of all inhibitors presented the final concentration.

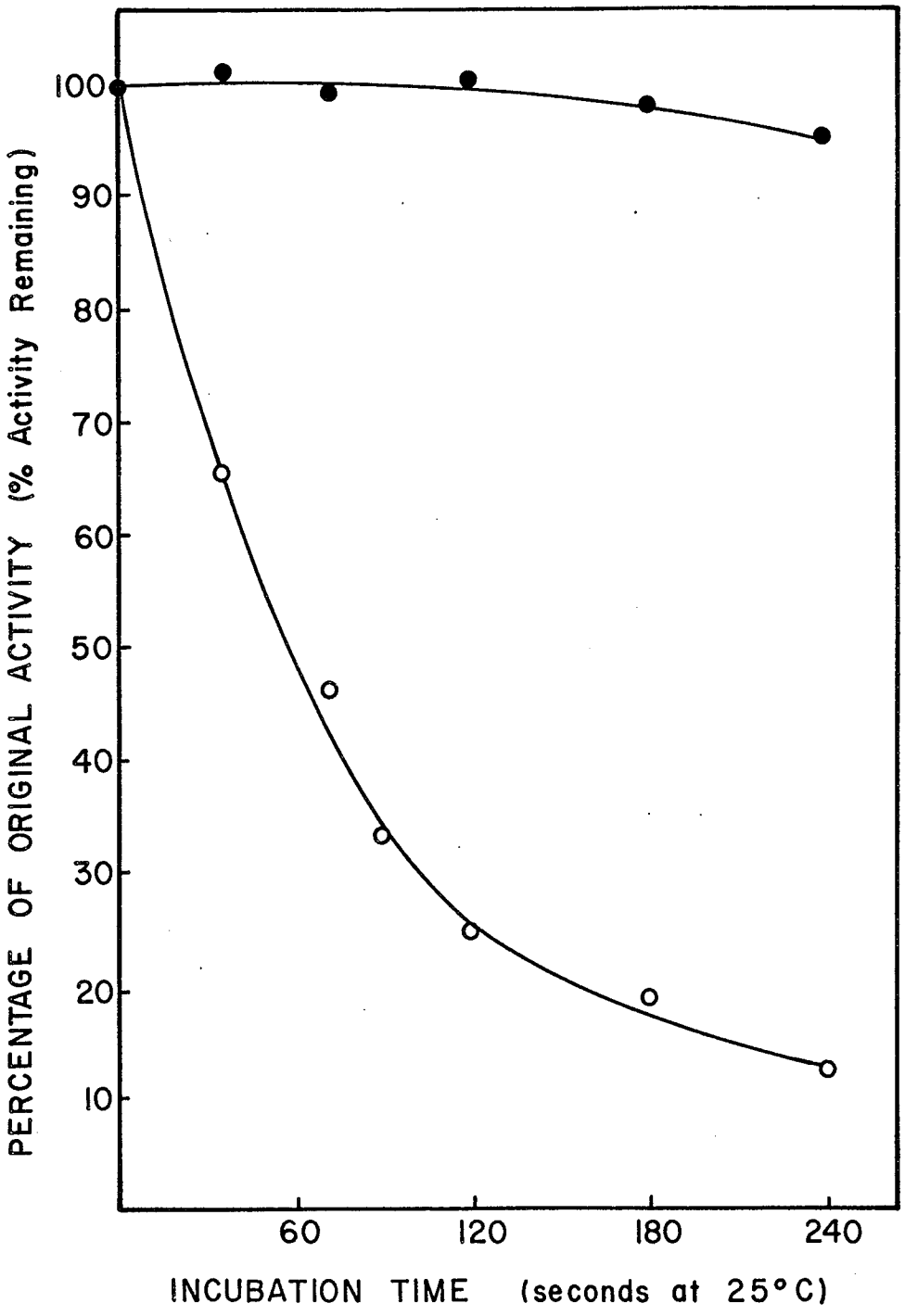
To test reversibility of inhibition in experiments with intact cells, the cells(2 mg wet weight) were preincubated with potassium cyanide as described in above. Cells were then sedimented by centrifugation at 2,000 x g for 20 minutes and resuspended in the same buffer. This washing procedure was repeated once more prior to measurement for thiosulfate-oxidizing activity.

For either crude extract or isolated membrane, the treated preparations were dialyzed at 4°C for 3 hours against the same standard buffer and the dialysates were used for assay of thiosulfate-oxidizing activity.

a: activity after reversal of inhibition.
b: activation
c: dissolved in dimethylsulfoxide(DMSO).
d: dissolved in 95% ethanol.

The final concentrations of DMSO and ethanol were never more than 0.2%(v/v) when inhibitors were added to reaction mixture. Under such conditions the organic solvents had no effect on the thiosulfate-oxidizing activity.

Fig. 20. The time-course inactivation of the thiosulfate-oxidizing activity by potassium cyanide. The reaction mixtures contained 1.53 mg of protein of isolated membrane vesicles. Experimental conditions were identical with those of Table 12 except that the concentration of potassium cyanide was 10^{-4} M at a final concentration and the incubation times in the preincubation medium were varied as indicated. Control(membrane vesicles), ● — ● and membrane vesicles plus 10^{-4} M potassium cyanide, ○ — ○ .


PERCENTAGE OF ORIGINAL ACTIVITY (% Activity Remaining)
100
90
80
70
60
50
40
30
20
10
60
120
180
240
INCUBATION TIME (seconds at 25°C)

ions such as $CuCl_2$, $FeCl_2$, $FeCl_3$, $CoCl_2$ and $NiCl_3$. Similar results were obtained from the crude cell-free extracts and isolated membrane vesicles: when either crude cell-free extracts or membrane vesicles treated with KCN were dialyzed at 4^oC for 3 hours against 0.1 M Tris acetate-0.02 M potassium phosphate buffer(pH 7.5) with one change of the buffer to remove the inhibitor, only less than 10% of the original activity was recovered, and if dialysis was continued more thiosulfate-oxidizing activity was lost rapidly(Table 12).

An attempt to remove the inhibitor by Sephadex G-25 gel filtration also resulted in a similar partial recovery of thiosulfate-oxidizing activity.

Other metal-binding reagents such as diethyldithiocarbamate, 1, 10-orthophenanthroline and 2, 2'-dipyridyl were considerably less inhibitory. Diethyldithiocarbamate caused about 30% inhibition of the activity at a final concentration of 10^{-4} M.
In either crude cell-free extract or membrane vesicles 1, 10-orthophenanthroline activated 30-35% of the thiosulfate-oxidizing activity at a final concentration of 10^{-4} M. At high concentration(10^{-3}M) the stimulative effect was lower.

Bathocuproine disulfate(disodium salt), a copper-binding reagent, was not inhibitory and sodium sulfide as a non-specific metal-binding agent had little or no

effect on the thiosulfate-oxidizing activity in all preparations.

The action of sulfhydryl(-SH) inhibitors have been tested with the thiosulfate-oxidizing system of T. novellus. The thiosulfate-oxidizing activity was inhibited markedly when intact cells, crude cell-free extract and isolated membrane vesicles were preincubated with various sulfhydryl inhibitors, suggesting the possibility that active sulfhydryl groups were necessary for oxidation of thiosulfate(Table 13).

Mercaptide-forming agents such as p-hydroxymercuribenzoate, Hg^{2+} and Ag^{+} were very effective inhibitors which inhibited 100% of the thiosulfate-oxidizing activity in all preparations at a concentration of 10^{-3} M.

As can be seen from data presented in Table 13, the degree of inhibition of thiosulfate oxidation was increased by increasing the concentration of the inhibitors.

By contrast, with N-ethylmaleimide, and particularly with iodoacetate, higher concentrations were needed and these alkylating agents were considerably less inhibitory, possibly due to inaccesability of the sulfhydryl groups. A 10^{-4} M concentration of 5,5'-dithiobis-(2-nitro-benzoate), an oxidizing agent, exhibited a slight inactivation of thiosulfate-oxidizing activity(20 to 30% inhibition) in all preparations.

Arsenite, a reagent for dithiol groups, showed

Table 13. Effect of sulfhydryl inhibitors on the thiosulfate-oxidizing system of *T. novellus*.

Thiosulfate-oxidizing system	Inhibitor	Concentration (M)	Activity (%)
I. Intact cells	None	-	100
	Mercuric chloride	10^{-3}	0
		$5x10^{-4}$	7
		10^{-4}	15
		10^{-5}	20
		10^{-6}	28
	Silver nitrate	10^{-4}	17
	P-hydroxymercuribenzoate (p-HMB)	$5x10^{-4}$	0
		10^{-4}	30
		10^{-5}	4
	N-ethylmaleimide (NEM)	10^{-3}	26
		$5x10^{-4}$	39
		10^{-4}	56
	Sodium iodoacetate	10^{-3}	73
		$5x10^{-4}$	77
	5,5'-dithiobis-(2-nitro benzoate) (DTNB)	10^{-3}	55
		10^{-4}	71
	Sodium arsenite	$5x10^{-4}$	49
		10^{-4}	72
II. Crude cell-free extract	None	-	100
	Mercuric chloride	10^{-3}	0
		$5x10^{-4}$	46
		10^{-4}	55
		10^{-5}	83
		10^{-6}	96
	Silver nitrate	10^{-4}	58

Table 13. continued

	P-hydroxymercuribenzoate	10^{-3}	1
		10^{-4}	86
	N-ethylmaleimide	10^{-4}	72
		$5x10^{-5}$	80
	Sodium iodoacetate	10^{-3}	52
		$5x10^{-4}$	60
		10^{-4}	80
	5,5'-dithiobis-(2-nitrobenzoate)	10^{-4}	75
	Sodium arsenite	$5x10^{-4}$	49
		10^{-4}	74
III. Membrane vesicles	None	-	100
	Mercuric chloride	10^{-3}	0
		10^{-4}	61
	Silver nitrate	10^{-3}	0
		10^{-4}	65
	P-hydroxymercuribenzoate	10^{-3}	0
		$5x10^{-4}$	67
		10^{-4}	83
	N-ethylmaleimide	$5x10^{-4}$	78
		10^{-4}	82
	Sodium iodoacetate	10^{-3}	60
		$5x10^{-4}$	64
		10^{-4}	83
	5,5'-dithiobis-(2-nitrobenzoate)	10^{-4}	79
	Sodium arsenite	10^{-4}	76

Experimental conditions were exactly same as described in Table 12.

also some inhibitory effect, indicating a possible involvement of dithiol groups in the thiosulfate-oxidizing system.

Characteristics of the electron transport chain involved in the thiosulfate-oxidizing system were studied by the effect of respiratory-chain inhibitors on the activity(Table 14).

The thiosulfate-oxidizing activity was inhibited strongly by electron transport chain inhibitors of cytochrome c oxidase such as potassium cyanide, sodium azide and hydroxylamine. Hydroxylamine at $3x10^{-3}$M inactivated over 90% of the activity in all preparations. Hydroxylamine is a well-known reagent which reacts with carbonyl groups of proteins and forms a complex with heme iron. Thus, this reagent may inhibit the thiosulfate-oxidizing activity, probably by chelating heme iron as reported in particulate cytochrome oxidase of mitochondrial system(Takemori et al., 1960).

As listed in Table 14, inhibitors of the electron transport chain between cytochromes b and c were less effective than the cytochrome oxidase inhibitors and caused only 10 to 20% inactivation of the thiosulfate-oxidizing activity in all preparations at the concentrations tested. A higher inhibitory effect(15 to 25%) was observed with inhibitors of the early portion of the electron transport sequence at the same concentration.

Table 14. Effect of electron transport chain inhibitors on thiosulfate-oxidizing system in T. novellus.

Thiosulfate-oxidizing system	Inhibitor	Concentration (M)	Activity (%)
1. Intact cells	None	-	100
	Atabrine(Atebrin)	10^{-3}	50
		$5x10^{-4}$	83
		10^{-4}	100
	Amobarbital[a](Amytal)	$5x10^{-4}$	86
		10^{-4}	93
	Rotenone[a]	$5x10^{-4}$	94
	2-thenoyltrifluoroacetone (TTFA)[a]	10^{-3}	78
	2-Heptyl-4-hydroxy-quinoline-N-oxide (HQNO)[a]	25 µg	98
	P-nitroblue terazolium (p-NBT)	10^{-3}	100
	Antimycin A[b]	10^{-3}	90
	N:N:N':N'-tetramethyl-p-phenylene-diamine (TMPD)	10^{-4}	100
	Potassium cyanide	10^{-4}	0
	Sodium azide	10^{-3}	67
	Hydroxylamine	$3x10^{-3}$	8
		10^{-3}	51
II. Crude cell-free extract	None	-	100
	Atabrine	10^{-3}	79
		10^{-4}	92
	Amytal	$5x10^{-3}$	64
		$5x10^{-4}$	82
		10^{-4}	88
	Rotenone[a]	$5x10^{-4}$	80

Table 14. continued

		1.25×10^{-4}	85
	TTFA[a]	10^{-3}	76
		10^{-4}	100
	HQNO[a]	25 µg	95
	p-NBT	10^{-3}	78
	Antimycin A[b]	5×10^{-4}	86
		10^{-4}	91
	TMPD	10^{-4}	100
	Potassium cyanide	10^{-4}	0
	Sodium azide	10^{-3}	64
	Hydroxylamine	3×10^{-3}	6
		10^{-3}	43
III. Membrane vesicles	None	-	100
	Atabrine	10^{-3}	82
		10^{-4}	96
	B.A.L. (British Anti-Lewisite)	10^{-4}	97
	Rotenone[a]	5×10^{-4}	80
	TTFA[a]	10^{-3}	73
		10^{-4}	100
	HQNO[a]	25 ug	92
	p-NBT	10^{-3}	86
		10^{-4}	100
	Antimycin A[b]	5×10^{-4}	81
		10^{-4}	84
	TMPD	10^{-4}	100
	Potassium cyanide	10^{-4}	0
	Sodium azide	10^{-3}	50
	Hydroxylamine	3×10^{-3}	4
		10^{-3}	42

The experimental conditions were identical with those of Table 12.
a: Dissolved in DMSO.
b: Dissolved in a mixture of ethanol and 0.1% serum albumin in the standard buffer(1:9).

Theonyltrifluoroacetone as either an inhibitor of the electron transport chain between non-heme iron protein and coenzyme Q or an uncoupler of oxidative phosphorylation, inhibited thiosulfate oxidation only at a high concentration(10^{-3}M) in all preparations.

The data in Table 15 show the effects of inhibitors and uncouplers of oxidative phosphorylation and artificial electron acceptors on the thiosulfate-oxidizing system in T. novellus.

The rate of thiosulfate oxidation was inhibited markedly if the thiosulfate-oxidizing systems were preincubated with oligomycin, an inhibitor of oxidative phosphorylation. The inhibition by oligomycin was dependent upon the concentration of the inhibitor(Fig. 21). The energy transfer from the electron transport chain to ATP formation at all three sites in the mitochondrial system was also reported to be inhibited by oligomycin (Lardy et al., 1958; Slater, 1968; Slater and Ter Welle, 1969).

In contrast to oligomycin, only a slight inhibitory effect of the uncouplers of oxidative phosphorylation such as 2,4-dinitrophenol(DNP) and carbonyl cyanide-m-chlorophentlhydrazone(CCCP) on the thiosulfate-oxidizing system was observed after preincubation of the system with the inhibitors. These uncouplers are known to be inhibitors of oxidative phosphorylation, ATP-dependent NADP reduction,

Table 15. Effect of inhibitors and uncouplers of oxidative phosphorylation, and artificial electron acceptors on the thiosulfate-oxidizing system of T. novellus.

Thiosulfate-oxidizing system	Compound	Concentration (M)	Activity (%)
1. Intact cells	None	-	100
	Oligomycin[b]	50 μg	0
		5 μg	88
	Valinomycin[b]	50 μg	100
	Valiomycin plus potassium chloride	50 μg	100
		5×10^{-4}	100
	2,4-Dinitrophenol (DNP)	10^{-3}	86
		5×10^{-4}	94
		10^{-4}	100
	Carbonyl cyanide-m-chlorophenyl hydrazone (CCCP)[b]	10^{-3}	69
		5×10^{-4}	84
		10^{-4}	96
	Sodium arsenate	10^{-3}	100
	2,6-Dichlorophenolindophenol(DCIP)	10^{-4}	100
	Phenazine methosulfate (PMS)	10^{-4}	100
	Methylene blue(MB)	10^{-4}	100
II. Crude cell-free extract	None	-	100
	Oligomycin[b]	50 μg	0
		5 μg	86
	Valinomycin[b]	50 μg	100
	Valinomycin plus potassium chloride	50 μg	
		5×10^{-4}	100
		10^{-3}	80

Table 15. continued

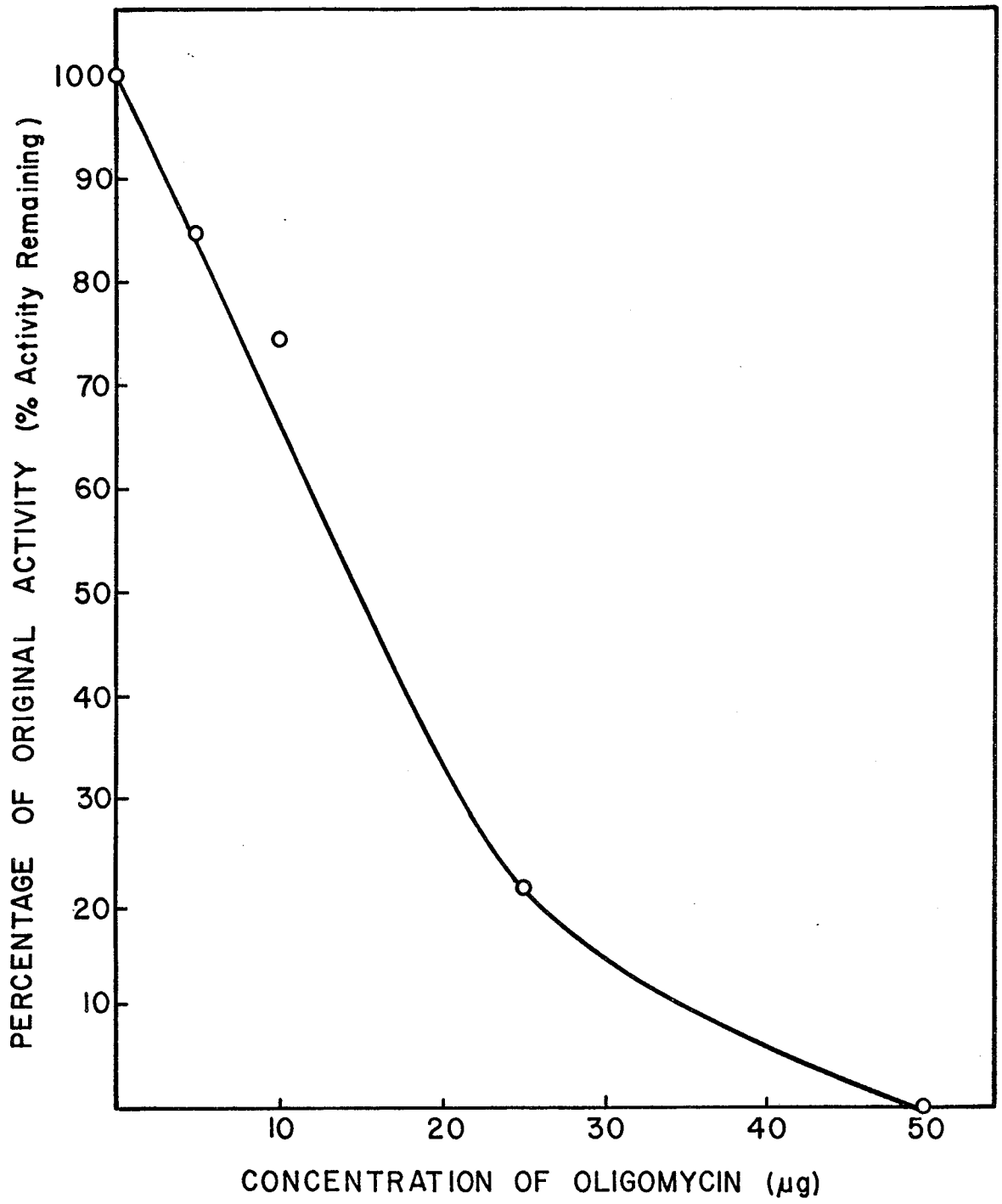
	DNP	$5x10^{-4}$	94
		10^{-4}	110[a]
	CCCP[b]	10^{-3}	60
		$5x10^{-4}$	74
		10^{-4}	90
	Dicoumarol[b]	10^{-4}	100
	Sodium arsenate	10^{-4}	100
	2,6 DCIP	10^{-4}	100
	PMS	10^{-4}	100
	MB	10^{-4}	100
III. Membrane vesicles	None	-	100
	Oligomycin[b]	50 µg	0
		5 µg	85
	DNP	10^{-3}	113[a]
		$5x10^{-4}$	105[a]
		10^{-4}	102[a]
	CCCP[b]	$5x10^{-4}$	63
		10^{-4}	80
		$5x10^{-5}$	94
	Dicoumarol[b]	10^{-4}	97
	Sodium arsenate	10^{-4}	100
	2,6 DCIP	10^{-4}	100
	PMS	10^{-4}	100
	MB	10^{-4}	100

The experimental conditions were the same as those described in Table 12.

a: Activation.

b: Dissolved in DMSO.

Fig. 21. Effect of concentration of oligomycin on the inhibition of thiosulfate-oxidizing activity in isolated membrane vesicles.
The experimental procedure was the same as described in Table 15.


PERCENTAGE OF ORIGINAL ACTIVITY (% Activity Remaining)
100
90
80
70
60
50
40
30
20
10
10
20
30
40
50
CONCENTRATION OF OLIGOMYCIN (µg)

proton pumping, and ion transport in bacteria(Harold, 1970) including T. novellus(Cole and Aleem, 1970; Cole and Aleem, 1973). 2,4-Dinitrophenol was also reported to inhibit thiosulfate oxidase in whole cells of T. novellus(Aleem, 1965). It should be, however, noted that 2,4-dinitrophenol in the present study inhibited the thiosulfate-oxidizing activity in crude cell-free extracts only at high concentrations($5x10^{-4}$ and 10^{-3}M) and activated the activity at a low concentration(10^{-4}M), whereas the inhibitor stimulated the thiosulfate-oxidizing activity in membrane vesicles at all concentrations tested(10^{-4} to 10^{-3}M).

Dicoumarol and sodium arsenate as uncouplers of oxidative or substrate-level phosphorylations had little or no effect on the activity. There was also no detectable change on the thiosulfate-oxidizing activity in either intact cell or crude cell-free extract when valinomycin or a combination of valinomycin and K^+ was added.

Specific inhibitory effects of ammonium oxidation by 2,6,-dichlorophenolindo phenol(DCIP), phenazine methosulfate(PMS) and methylene blue(MB) have been reported in the intact cells of Nitrosomonas europaea (Hooper and Terry, 1973). They suggested that these artificial electron acceptors inhibited ammonia oxidation by oxidizing an intermediate compound or a reduced enzyme or by bypassing reactions coupled to hydroxylamine oxidation. However, the thiosulfate oxidation in

Fig. 22. Effect of trypsin treatment on thiosulfate-oxidizing activity of various preparations of T. novellus.

The experimental procedures were identical with those of Materials and Methods. Aliquots were withdrawn at regular time intervals as indicated and immediately assayed for thiosulfate-oxidizing activity. The results are presented as a percentage of the activity at time zero. Whole cells plus 200 μg of trypsin, ○—○ ; crude extract, △—△ ; crude extract plus 1 mg of trypsin, ▲—▲ ; crude extract plus 2 mg of trypsin, ▲---▲ ; crude extract plus 2 mg of trypsin and 3 mg of trypsin inhibitor, ●—● ; membrane vesicles, □—□ ; membrane vesicles plus 1 mg of trypsin, ■—■ ; membrane vesicles plus 2 mg of trypsin, ■---■ and membrane vesicles plus 2 mg of trypsin and 3 mg of trypsin inhibitor, ◑—◑ .

T. novellus was not inhibited by these dyes at a final concentration of 10^{-4}M(Table 15).
It should be worthy of note that if the various inhibitors were added after thiosulfate oxidation had been initiated, there appeared to be either a partial inhibition or no inhibition on the rate of thiosulfate oxidation.

Effect of Proteolytic Enzymes Treatments on Thiosulfate-oxidizing Activity

The action of proteolytic enzymes on the thiosulfate-oxidizing activity was tested with the whole cells, the cell-free extracts and isolated membrane vesicles.
The oxidation of thiosulfate by cells was not affected by treatment with proteolytic enzymes when the fresh, intact cells of T. novellus(2 mg wet weight cells) were incubated with either 100-200 µg of trypsin or with 100-200 µg of pronase for 20 minutes(Fig. 22 and 23). At these conditions almost no protein was lost due to digestions with both enzymes(Table 16).

In contrast, proteolysis of the cell-free extract and the isolated membrane vesicles by either trypsin or pronase resulted in an inactivation of the membrane-bound thiosulfate-oxidizing activity. As shown in Fig. 22, a progressive decline of the activity to a plateau was observed by trypsin treatment. Incubation of the enzyme

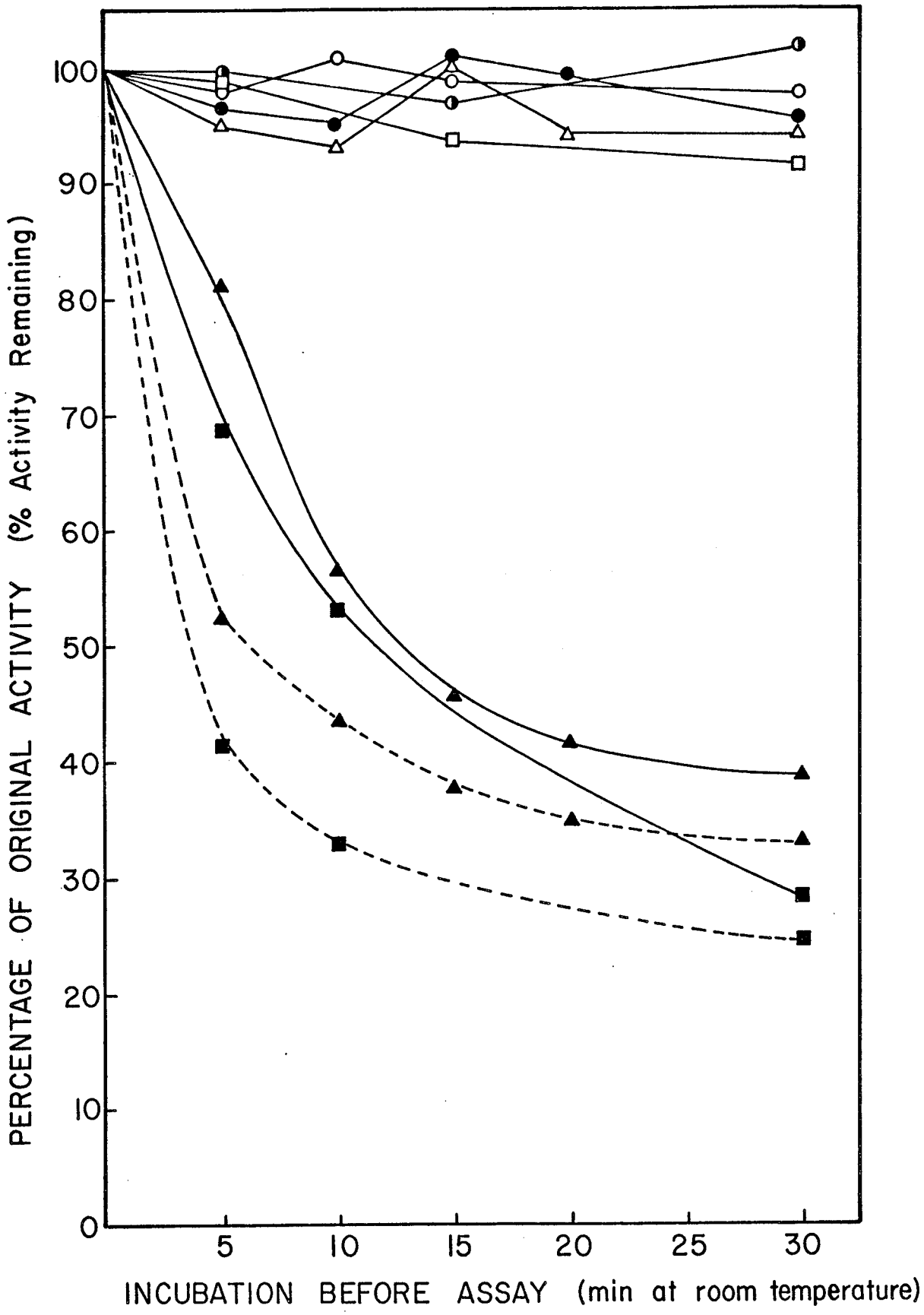

PERCENTAGE OF ORIGINAL ACTIVITY (% Activity Remaining)
100
90
80
70
60
50
40
30
20
10
0
5
10
15
20
25
30
INCUBATION BEFORE ASSAY (min at room temperature)

Fig. 23. Effect of pronase digestion on thiosulfate-oxidizing activity of various preparations of T. novellus.

The intact cells, cell-free extracts and membrane vesicles were prepared as described in Materials and Methods.

The experimental conditions were as described in Materials and Methods. Intact cells, ⊙——⊙; intact cells plus pronase(200 µg), ●——●; crude extracts, △——△; crude extracts plus pronase(100 µg), △——△; crude extracts plus pronase(200 µg), ◑——◑; membrane vesicles, ■——■ and membrane vesicles plus pronase(200 µg), □---□.

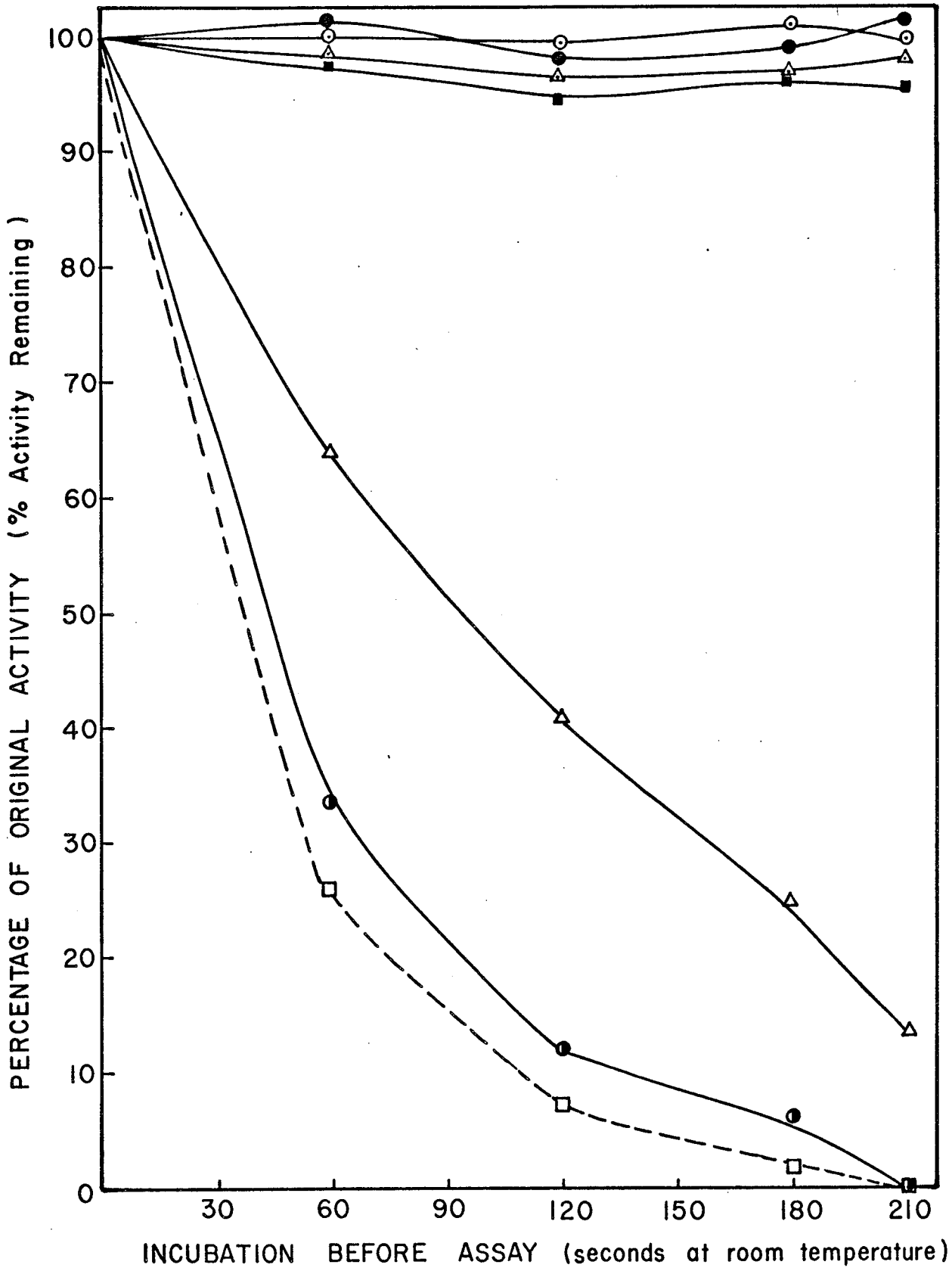

PERCENTAGE OF ORIGINAL ACTIVITY (% Activity Remaining)
100
90
80
70
60
50
40
30
20
10
0
30
60
90
120
150
180
210
INCUBATION BEFORE ASSAY (seconds at room temperature)

Table 16. Effect of preincubations with trypsin and pronase on release of protein and inhibition of thiosulfate-oxidizing activity of the intact cells and isolated membrane vesicles.

Reaction	Total protein (mg)	Treatment				Inhibition of thiosulfate-oxidizing activity (%)
		Trypsin supernatant fraction (mg of protein)	Trypsin pellet suspension (mg of protein)	Pronase supernatant fraction (mg of protein)	Pronase pellet suspension (mg of protein)	
1. Intact cells	0.193	0	0.186	-	-	0
2. Intact cells	0.203	-	-	0	0.191	0
3. Membrane vesicles	1.5	0.72	0.74	-	-	70
4. Membrane vesicles	1.5	-	-	1.12	0.36	100

The enzyme preparations were prepared from fresh cells, either intact cells(10 mg wet weight cells) or the membrane vesicles(7.5 mg of protein) were preincubated at room temperature(25°C) with 1 mg of trypsin for 30 minutes or with 1 mg of pronase for 5 minutes, and aliquots(2 mg of intact wet cells and 1.5 mg of membrane vesicle proteins) were removed and immediately assayed for the thiosulfate-oxidizing activity in a Gilson Oxygraph as described in Materials and Methods.

After sedimentation by centrifugation, each fraction was determed for protein as described in Materials and Methods.

Table 17. Effect of pronase concentration on proteolytic action of thiosulfate-oxidizing system.

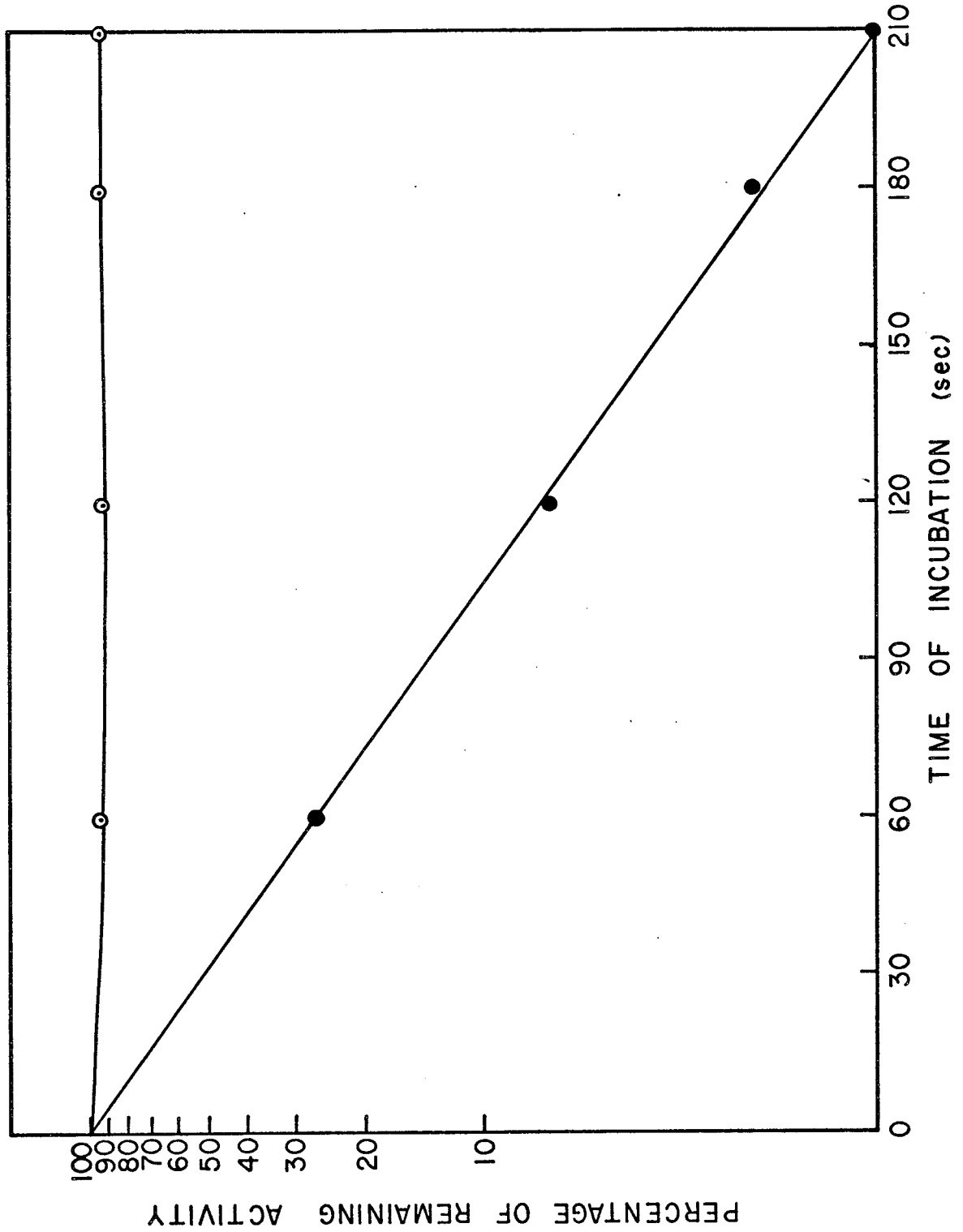
Concentration used for pronase treatment	Thiosulfate-oxidizing activity (nmole O_2 consumption per min)	Inhibition (%)
None	54	0
1 µg/ml(0.45 PUK units)	43	20
10 µg/ml(4.5 PUK units)	27	50
100 µg/ml(45 PUK units)	0	100
200 µg/ml(90 PUK units)	0	100

The cell-free extracts were prepared from active fresh cells, 1.5 mg of enzymes were preincubated with various amounts of pronases at room temperature(25°C) for 5 minutes as described in Materials and Methods. The thiosulfate-oxidizing activity in the reaction mixture in a total volume of 1.5 ml was assayed by using a Gilson Oxygraph as described in Materials and Methods.

preparations with trypsin in the presence of an excess of trypsin inhibitors resulted in no appreciable loss of activity within the experimental period, indicating that trypsin exerts its effect through proteolytic action. Data for the stability of thiosulfate-oxidizing activity under the experimental conditions in the absence of trypsin is included in the figure. The trypsin treatment of fresh cells of T. novellus did not release protein in soluble fraction after centrifugation, but about 50% of the total protein was solubilized and 70% of activity were lost from membrane vesicles in 30 minutes by this treatment(Table 16). However, the treatment with pronase caused a rapid decrease of the thiosulfate-oxidizing activity within the first 2-3 minutes, indicating extensive damage to the membrane(Fig. 22). The inactivation of activity by pronase as a function of time as shown as a semilogarithmic plot in Fig. 24. The straight-line relationship indicated that the proteolytic inactivation reaction was pseudo-first order. These results are understandable because it is well known that the two proteolytic enzymes have very different proteolytic specificities(Smith, 1967).

The effect of various amounts of pronase on the cell-free extract was shown in Table 17. Treatment of the cell-free extract with low levels of pronase(1 μg per ml containing 0.45 PUK units) had slight inhibitory effect on the activity. When the pronase

Fig. 24. The inactivation of thiosulfate-oxidizing activity of membrane vesicles by pronase. The conditions were as described in the legend to Fig. 23. The membrane vesicles(1.5 mg of protein) were preincubated at room temperature with and without 200 µg of pronase in 1.0 ml of 0.1 M Tris acetate buffer-0.02 M potassium phosphate(pH 7.5). Aliquots were removed at various times as indicated and immediately assayed for thiosulfate-oxidizing activity. The membrane vesicles used were prepared by Sepharose 4B column chromatography. Control(no pronase), ⊙——⊙ and with 200 µg pronase, ●——● .


PERCENTAGE OF REMAINING ACTIVITY
100
90
80
70
60
50
40
30
20
10
0
30
60
90
120
150
180
210
TIME OF INCUBATION (sec)

concentration was raised to 10 µg (4.5 PUK units) per ml, a more extensive inhibition of thiosulfate-oxidizing activity began to appear, while digestion with higher levels of pronase (100 or 200 µg per ml) reduced the activity very drastically. These observations indicated that the protein nature of the thiosulfate-oxidizing system in membrane complex had an important role for the system.

The thiosulfate-oxidizing activity was neither labilized nor stabilized to proteolytic inactivation in the presence of thiosulfate. A possible nonspecific protection by anions or cations against inactivation by trypsin and pronase was not examined. Reduced glutathione did not activate the thiosulfate-oxidizing activity in inactivated preparations by proteolytic digestions.

Treatments with trypsin and pronase had no effect on both NADH oxidase and sulfite-oxidizing system in the crude cell-free extracts of T. novellus.

Effect of Lipase, Phospholipase A, C or D and Lysozyme on the Thiosulfate-oxidizing Activity

When intact cells of T. novellus were preincubated at room temperature(25°C) with lipase, phospholipase A, C, or D, and lysozyme, no inhibition of thiosulfate-oxidizing activity occured (Table 18) and the cells were not lysed.

Table 18. Effect of lipase, phospholipase A, C, or D, and lysozyme on the thiosulfate-oxidizing activity of intact cells and isolated membrane vesicles.

Reaction system	Treatment	Activity (%)
Experiment I.		
Intact cells	None	100
Intact cells	Lipase: 50 μg(1,000 units)	100
Membrane vesicles	None	100
Membrane vesicles	Lipase: 10 μg(200 units)	95
	50 μg(1,000 units)	95
Experiment II.		
Intact cells	None	100
Intact cells	Phospholipase A: 100 μg(100 I.U.)	100
Membrane vesicles	None	100
Membrane vesicles	Phospholipase A: 10 μg(10 I.U.)	93
	100 μg(100 I.U.)	60
	100 μg(100 I.U.) minus Ca^{2+}	61
	100 μg(100 I.U.) plus EDTA	99
Experiment III.		
Intact cells	None	100
Intact cells	Phospholipase C: 100 μg(100 I.U.)	99
Membrane vesicles	None	100
Membrane vesicles	Phospholipase C: 10 μg(10 I.U.)	97
	100 μg(100 I.U.)	95

Table 18. continued

Experiment IV.		
Intact cells	None	100
Intact cells	Phospholipase D: 200 μg(0.1125 E.U.)	100
Membrane vesicles	None	100
Membrane vesicles	Phospholipase D: 50 μg(0.00225 E.U.)	99
	200 μg(0.1125 E.U.)	96
Experiment V.		
Intact cells	None	100
Intact cells	Lysozyme: 10 μg	99
Membrane vesicles	None	100
Membrane vesicles	Lysozyme: 2 μg	100
	10 μg	99

The treatment of either intact cells(2 mg wet weight cells) or isolated membrane vesicles was carried out as described in Materials and Methods.
The preparation of the membrane vesicles from fresh crude cell-free extracts and the assay of thiosulfate-oxidizing activity were the same as those described in Materials and Methods.

The results tabulated in Table 18, showed a relatively high resistance of the thiosulfate-oxidizing activity of the membrane vesicles to lipase, phospholipase C, phospholipase D or lysozyme. The activity of the membrane vesicles was somewhat more sensitive to phospholipase A. The thiosulfate-oxidizing activity was reduced by nearly 40% when the membrane vesicles were treated with 100 µg(100 I.U.) of phospholipase A in the presence of 2mM $CaCl_2$, but the turbidity of the membrane vesicle suspensions was not affected by treatment.

Phospholipase A is known to be inactivated by EDTA(Zakim, 1970) because of its chelating action on divalent cations. The inactivation of thiosulfate-oxidizing activity by phospholipase was prevented by EDTA, while the omission of $CaCl_2$ had no effect.
Inactivating effects of phospholipase A were also observed with the oxidation of succinate and NADH in submitochondrial particles(Burstein et al., 1971) and with the reversed electron transfer and energy-dependent transhydrogenase of beef heart submitochondrial particles (Luzikov et al., 1973).

The above observations showed that the thiosulfate-oxidizing system as a membrane-bound enzyme complex was a phospholipoprotein in nature implying the involvement of the phospholipid structure of the membrane as an important determinant for the activity.

Unfortunately, the attempts to regenerate the original thiosulfate-oxidizing activity from the phospholipase A treated membrane vesicles by incubating with lecithin micelles was unsuccessful. However, this approach using either phospholipase A or C has been proved to be successful in several membrane-bound enzymes(McConnell et al., 1966; Duttera et al., 1968; Martonosi, 1968; Martonosi, et al., 1968; Fleischer et al., 1972; Zakim, et al., 1973).

By contrast, the results on lysozyme action indicated that the hydrolysis of the murein layer of the membrane vesicles did not appear to play a role for the oxidation of thiosulfate. It was also interesting to note that the sulfite-oxidizing system and sulfur-oxidizing enzyme activity in the cell-free extracts were not affected significantly by treatment with phospholipase A.

Effect of Detergents on Thiosulfate-oxidizing Activity of Crude Cell-free Extract and Isolated Membrane Vesicles.

The effects of ionic and non-ionic detergents on thiosulfate-oxidizing activity of the cell-free systems were tested before attempting to resolve the membrane-bound complex of the thiosulfate-oxidizing system with any of the detergents.

As shown in Table 19, the thiosulfate-oxidizing

Table 19. Effect of detergents on thiosulfate-oxidizing activity of crude cell-free extract and isolated membrane vesicles.

Reaction system	Treatment	% final concentration	Activity(%)	
			Before dialysis	After dialysis *
Cell-free extract	None	-	100	100
	Sodium deoxycholate	0.1	54	58
		0.2	36	43
		0.4	12	19
		0.5	6	18
		1.0	1	7
Membrane vesicles	None	-	100	100
	Sodium deoxycholate	0.1	69	NT
		0.2	64	NT
		0.4	44	NT
		0.5	38	36
		1.0	32	41
	Triton X-100	0.5	100	100
		1.0	94	93
	Tween 80	0.5	100	100
		1.0	94	93

Experimental procedures were the same as described in Materials and Methods.

* After dialysis at $4^{o}C$ for 30 minutes, there were considerable losses of the thiosulfate-oxidizing activity in the control samples of crude cell-free extracts and membrane vesicles. Therefore, all results were recorded as percentage of inhibition of the control samples.

NT: not tested.

activity of the crude cell-free extract and isolated membrane vesicles were strongly inhibited when the preparations were preincubated at 4°C for 30 minutes with an ionic detergent, sodium deoxycholate at concentrations ranging from 0.5 to 1.0%(v/v). As an indication of lysis, incubation of the samples with sodium deoxycholate caused a clearing of the suspensions. Under these conditions, parallel with these changes about 60 to 100% of the thiosulfate-oxidizing activity was lost. However, the amount of protein which would be liberated from the detergent-treated membrane vesicles in a form not sedimentable on centrifugation at high speed was not determined.

When the isolated membrane vesicles were incubated at 4°C for 30 minutes with a non-ionic detergent, Triton X-100, at final concentrations of 0.5 or 1.0%(v/v), the treated preparations showed only small loss of thiosulfate-oxidizing activity. At Triton X-100 concentration of 1% (v/v) only 6% of the activity was lost(Table 19).

Similar results by another non-ionic detergent, Tween 80, were obtained with the thiosulfate oxidation in the isolated membrane vesicles. Since the treatments by the non-ionic detergents could solubilize some proteins from the membrane vesicles, removal of the proteins, i.e. non-enzymatic proteins, by such treatments would be expected to result in an increased specific activity of the enzyme. The possibility was, however, not examined.

Amphiphiles like sodium deoxycholate are known to be suitable reagents for disruption of lipid-protein interactions by completing for lipid binding sites on the proteins(Helenius and Simons, 1975).
Sodium deoxycholate is also supposed to solubilize most extensively proteins and phospholipids in parallel and to induce conformation changes in certain membrane proteins. On the other hand, Triton X-100 at low concentrations solubilize less extensively proteins and lipids in parallel, but at increasing concentrations a net protein solubilization cease while residual lipids are completely solubilized (Kirkpatrick and Sandberg, 1973; Kirkpatrick et al., 1974).

The inhibitory effects of detergents implied again that both protein and phospholipid were involved in the oxidation of thiosulfate.

It was, however, observed that most of thiosulfate-oxidizing activity inactivated by detergents could not be regained by dialysis.

Attempts to remove the detergents and recover the activity from the detergent-enzyme mixtures by the use of a gel filtration through a Sephadex G-25 column also met with failure, less than 10% of the activity being recovered after elution with the standard buffer.

The above observations indicated that the detergent treatments resulted in irreversible loss of thiosulfate-oxidizing activity by causing an extensive damage in the

system.

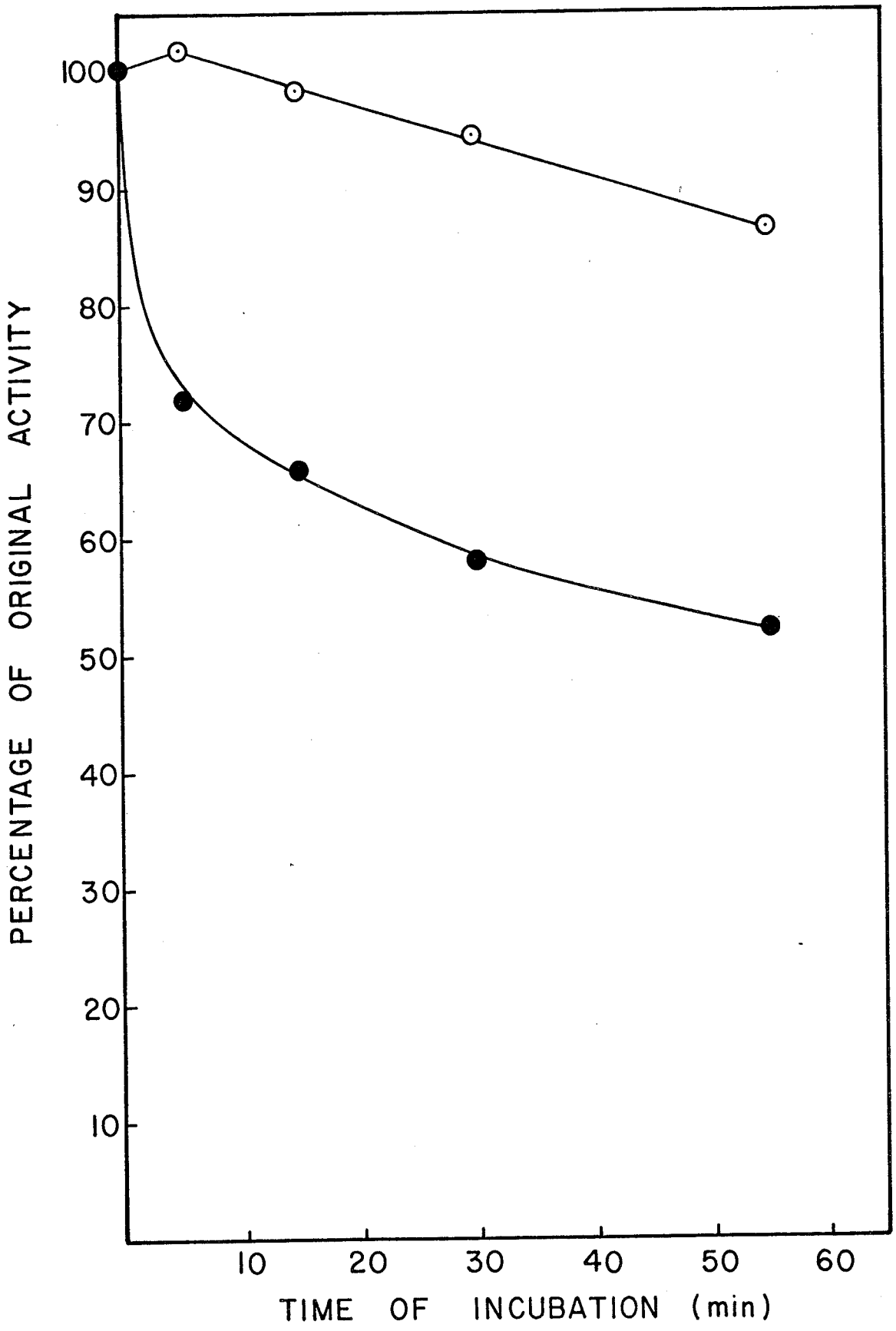
Effect of Lysolecithin on Thiosulfate-oxidizing Activity

Fig. 25 demonstrates that the thiosulfate-oxidizing activity of cell-free extracts is sensitive to treatment with lysolecithin. Although the degree of inhibition was time-dependent, the rate of activity dropped only to 30% of the initial activity in the presence of lysolecithin within 5 minutes incubation and decreased very slowly afterwards from this level. Such treatment did not result in a change in the turbidity of the reaction mixtures.

Lysolecithin as a disruptive agent is known to inhibit respiration and uncouple phosphorylation in the mitochondrial electron transport particles(Witter et al., 1957; Honjo and Ozawa, 1968).

On the other hand, lysolecithin have been reported to be effective solubilizing agents for membrane bound proteins in the mitochondrial inner membrane and small non-vesicular fragments of membrane were obtained by the treatment of electron transport particles(Komai, et al., 1973; Capaldi, et al., 1973; Sadler, et al., 1974). Lysolecithin has also been used as the most active naturally occuring phospholipid in stimulation of hydroxylation reaction in microsomes(Fisher and Kaufman, 1972). Therefore,

Fig. 25. Effect of lysolecithin on thiosulfate-oxidizing activity in the cell-free extracts. Experimental procedure was the same as described in Materials and Methods. Control(lysolecithin untreated), ⊙—⊙ and lysolecithin treated, ●—● .


PERCENTAGE OF ORIGINAL ACTIVITY
100
90
80
70
60
50
40
30
20
10
10
20
30
40
50
60
TIME OF INCUBATION (min)

it is interesting to see whether this agent can be used in isolating the intermediate enzymes such as sulfur-oxidizing enzyme and sulfite-cytochrome c oxidoreductase etc. However, it has not been further investigated.

Effect of Sucrose and Glycerol on Thiosulfate-oxidizing Activity

When crude cell-free extracts were preincubated with sucrose for 15 minutes at 4°C and tested for the thiosulfate-oxidizing activity, sucrose showed 34% inhibition at 10%(w/v) and 49% inhibition at 20%(w/v) concentration. Glycerol was also inhibitory, exhibiting 50% inhibition at 20%(w/v) concentration after pre-incubation at 25°C for 10 minutes. Although not shown, the inhibitory effect was proportional to the concentration of glycerol up to 20%(w/v).

Effects of Various Compounds on Thiosulfate-oxidizing System

The effects of various compounds on thiosulfate-oxidizing activity in the isolated membrane vesicles were tested. ATP, ADP, AMP, cyclic AMP, FAD, FMN, $NaBH_4$, NaF, hydrazine sulfate, bovine serum albumin(fraction V), spermine HCl, spermidine and ferricyanide, each present

at a final concentration of 10^{-4}M to 10^{-3}M were without effect. However, fractionation of the membrane vesicles between 40 and 90%(w/v) of saturation with ammonium sulfate at 4°C resulted in a complete loss of the thiosulfate-oxidizing activity. The complete removal of ammonium sulfates by using either prolonged dialysis or chromatography on Sephadex G-25 did not regenerate the activity, indicating that the inactivation by fractionation of ammonium sulfate was apparently irreversible.

The thiosulfate-oxidizing activity in the isolated membrane vesicles was strongly inhibited by the chaotrophic anions and dissociating agents such as potassium thiocyanate, potassium trichloroacetate, urea, guanidine HCl and sodium dodecyl sulfate. When the membrane vesicles were treated with these agents for 10 minutes at 25°C, a 98% loss of activity resulted from treatment with 0.4 M potassium thiocyanate; 100% loss with 0.4 M potassium trichloroacetate, and 100% loss with 2 M urea, 2 M guanidine HCl and 5 mM sodium dodecyl sulfate.

When the effects of short-chain primary alcohols on thiosulfate oxidation in the isolated membrane vesicles were examined, n-propanol, n-butanol and ter-butanol completely inhibited the activity at 0.5 M. By contrast, the shorter carbon-chain primary alcohols such as methanol and ethanol were considerably less inhibitory, showing only 25 to 33% inhibition at the final concentrations of 0.5 M.

Spectrophotometric Study of the Thiosulfate-oxdizing System

When the active crude cell-free extracts and isolated membrane vesicles were reduced with hydrosulfite, the absorption spectra taken in a Shimadzu Multipurpose Recording Spectrophotometer indicated the presence of the following types of cytochromes: cytochrome c (550 nm and 551 nm), cytochrome b(560 nm) and cytochrome a(600 nm). A trough at 450 nm and small absorption peaks at 475 nm and 640 nm indicated also the presence of flavin and cytochrome d. The active preparations, when reduced with thiosulfate instead of hydrosulfite, showed identical absorption peaks. However, the absorption peaks were much lower than these with hydrosulfite. These aspects were not investigated further.

Dissociation and Reconstitution of the Thiosulfate-oxidizing System

As mentioned earlier, the isolated membrane fraction, the thiosulfate-oxidizing enzyme complex, oxidized thiosulfate by itself and the additions of soluble or 5 times concentrated soluble fraction obtained by either differential centrifugation or column chromatographic methods had no stimulative effect on the overall

thiosulfate-oxidizing activity. The soluble fractions contained high rhodanese and sulfite: cytochrome c oxidoreductase activity and almost all of the soluble cytochromes.

Furthermore, when the membrane fraction obtained by a Sepharose 4B column chromatography was combined with purified intermediate enzymes such as rhodanese, sulfur-oxidizing enzyme or sulfite: cytochrome c oxidoreductase, no activation of thiosulfate-oxidizing activity was obtained. From these observations, it was believed that differential centrifugation and column chromatographic techniques were not suitable to resolve the thiosulfate-oxidizing enzyme complex from the crude cell-free extracts of T. novellus. Therefore, as a first step towards reconstitution of the thiosulfate-oxidizing system a systematic study of the various possible means for dissociation of the intermediate enzymes involving in thiosulfate oxidation from all tightly bound multienzyme complex particles was undertaken.

As shown in Table 20, the isolated membrane fraction had high activities of cytochrome c oxidase and NADH oxidase(electron-transport system) in which could be accounted for all activity presented in the crude cell-free extract and a membrane-bound rhodanese, but not activities of sulfur-oxidizing enzyme and sulfite-oxidizing enzyme system(sulfite: cytochrome c oxidoreductase).

Table 20. Activities of membrane-bound rhodanese and soluble rhodanese in the isolated membrane vesicles and soluble fraction of T. novellus.

Preparation	Protein		Thiosulfate-oxidizing activity		Rhodanese activity	
	Total protein (mg)	% of cell-free extract	Total activity (μmole O_2 consumed /min)	% of cell-free extract	Total activity (μ equivalent of SCN- /min)	% of cell-free extract
Crude extract	110	100	7.8	100	16.8	100
Membrane fraction	56.1	51.1	7.3	93.6	7.1	42.2
Soluble fraction	50.9	46.3	0	0	9.5	56.5

The various fractions were prepared by differential centrifugation using 5 ml of crude extracts as described in Materials and Methods.

The measurements of protein, thiosulfate-oxidizing activity and rhodanese activity were the same as described in Materials and Methods.

Treatments of the isolated membrane fractions with trypsin and sodium deoxycholate resulted in releases of sulfur-oxidizing activity and sulfite-oxidizing enzyme system from the membrane-bound enzyme complex system. However, these two enzymes were not released by extraction with salt solution, osmotic shock, extensive sonication with a Insonator(biosonic probe) at high pH(9.5) or treatments with phospholipase A, C and D.

Treatment of the membrane fraction by either chaotrophic agents(KSCN, guanidine HCl and urea) or organic solvents(15%, v/v, ethanol, 15%, v/v, methanol, 30%, v/v, n-propanol, 15%, v/v, n-butanol and 10%, v/v, acetone) did not release the sulfur-oxidizing enzyme and sulfite-oxidizing system. A thermal solubilization of membrane vesicles using heat treatment was also unsuccessful.

In addition, any attempts to reconstitute the thiosulfate-oxidizing system with either dissociated enzyme components or partially purified intermediate enzymes involved in thiosulfate oxidation resulted into complete inactivation of the system.

(1) Release of Sulfur-oxidizing Enzyme Activity from Active Membrane Vesicles

The isolated membrane vesicles did not oxidize colloidal sulfur in the presence of reduced glutathione. However, although the difficulties in obtaining reproducible results with various preparations were experienced, the sulfur-oxidizing enzyme activity could be obtained by treatment of the membrane-bound enzyme complex, but not by digestion with pronase. The result from a typical experiment is shown in Fig. 26.

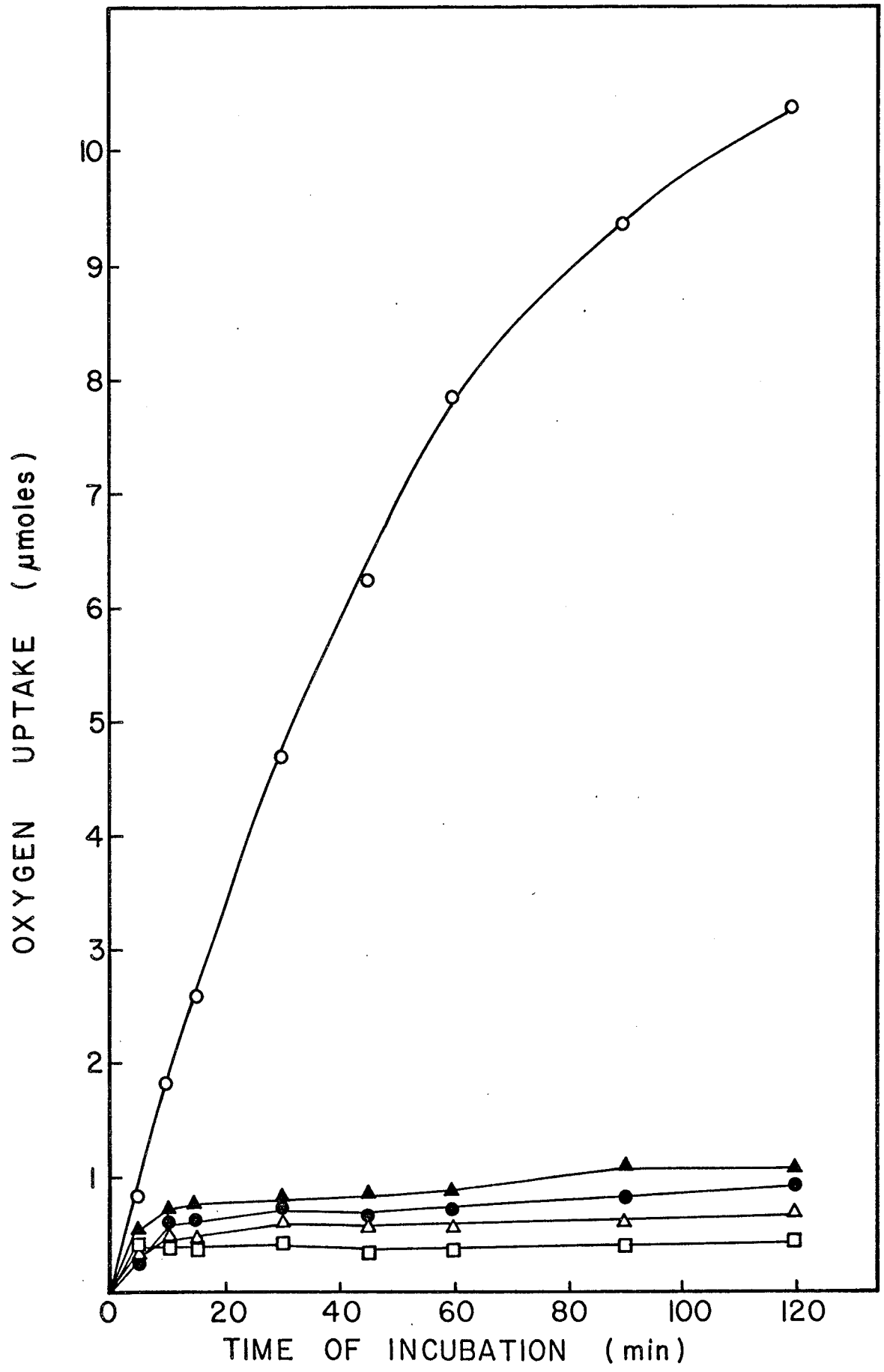
The thiosulfate-oxidizing activity remaining in the active membrane vesicles after treatment of trypsin was approximately 30% of the original activity. The sulfur-oxidizing enzyme which oxidized elemental sulfur with GSH as cofactor was isolated and partially purified by Suzuki (1965) from T. thiooxidans and by Suzuki and Silver(1966) from T. thioparus. They proposed that the enzyme was oxidized initially sulfur to sulfite in the presence of a catalytic amount of GSH followed by a non-enzymatic formation of thiosulfate from sulfur and sulfite:

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

$$S + SO_3^{2-} \xrightarrow{\text{non-enzymatic}} S_2O_3^{2-}$$

The same enzyme also reported from whole cells and cell-free extracts of T. novellus with the exception that the only product was sulfate, because the sulfite-oxidizing

Fig. 26. Release of sulfur-oxidizing enzyme activity from membrane vesicles by treatment of trypsin. The active membrane vesicles from crude extracts were prepared by differential centrifugation and treated by trypsin as described in Materials and Methods.
Measurements of sulfur-oxidizing enzyme activity and thiosulfate-oxidizing actvity were the same as described in Materials and Methods.
Treated membrane vesicles + S + GSH, O—— O; untreated membrane vesicles + S + GSH, ▲——▲ ; treated membrane vesicles + GSH, ●—— ● ; treated membrane vesicles + S, △——△ and S + GSH, □—— □ .


OXYGEN UPTAKE (μmoles)
10
9
8
7
6
5
4
3
2
1
0
20
40
60
80
100
120
TIME OF INCUBATION (min)

systems of these preparations were very active. The sulfur-oxidizing enzyme in the trypsin treated vesicles oxidized colloidal sulfur to thiosulfate in the presence of GSH and the ratio of oxygen consumed to thiosulfate formed was 1:1. Thus the situation was reminiscent of the sulfur-oxidizing enzyme of T. thiooxidans and T. thioparus.

(2) Release of Sulfite-oxidizing System by Sodium Deoxycholate Treatment

When the membrane-bound multienzyme complex was incubated at room temperature(26°C) for 30 minutes with sodium deoxycholate and dialyzed against 0.1 M Tris acetate-0.02 M potassium phosphate buffer(pH 7.5) for 4 hours to remove residual detergents, the treated preparation oxidized sulfite to sulfate in the presence of cytochrome c-550(horse heart, type III) as shown in Table 21. Thus the treated mixture was subjected to a high speed centrifugation at 105,000 x g for 120 minutes to isolate a sulfite: cytochrome c oxidoreductase. As shown in Table 22, it was found that the soluble fraction exhibited the sulfite: cytochrome c oxidoreductase activity, while the suspended pellet showed cytochrome c oxidase activity. Although the activity of the solubilized sulfite-oxidizing activity was much lower than that of crude extracts, it

Table 21. Release of Sulfite-oxidizing activity from isolated membrane vesicles with sodium deoxycholate.

Reaction system	Thiosulfate-oxidizing activity (μmole O_2 consumed/min)	Sulfite-oxidizing activity (μmole O_2 consumed/min)
1. Crude extract	7.8	7.86
2. Isolated membrane vesicles	7.6	0
3. Sodium deoxycholate-treated membrane vesicles	0	0.05
4. 3 plus cytochrome c-550(horse heart, type III)	0	0.98

The crude cell-free extracts were prepared and membrane vesicles were isolated by differential centrifugation using 5 ml of crude extracts as described in Materials and Methods. The detergent-treated membrane vesicles were prepared by incubating the active membrane vesicles at room temperature(26°C) for 30 minutes with 0.2%(w/v) of sodium deoxycholate and dialyzed against 0.1 M Tris-acetate-0.02 M potassium phosphate buffer(pH 7.5) for 4 hours at 4°C with two changes of the buffer.
The thiosulfate-oxidizing activity and sulfite-oxidizing activity were assayed as described in Materials and Methods.

Table 22. Isolation of sulfite: cytochrome c oxidoreductase and cytochrome c oxidase from sodium deoxycholate-treated membrane vesicles.

Reaction system	Sulfite-oxidizing activity (μmole O_2 consumed /min)	Sulfite: cytochrome c oxido reductase (μmole cytochrome c reduced/min)	Cytochrome c oxidase (μmole cytochrome c oxidized/min)
1. Isolated membrane vesicles	0	0	31.4
2. Sodium deoxycholate treated membrane vesicles	0.05	NT	30.9
3. Pellet fraction from 2	0	0	31.1
4. Soluble fraction from 2	0	4.08	0
5. 3 plus 4	0	NT	NT
6. 3 plus 4 plus cytochrome c-550 (horse heart, type III)	0.98	NT	NT

The experimental procedures were the same as described in Table 21. Fractions 3 and 4 were prepared by centrifugating fraction 2 at 105,000 x g for 120 minutes. All enzyme activities were measured as described in Materials and Methods.

NT: Not tested.

was possible to demonstrate that the sulfite: cytochrome c oxidoreductase could be nondestructively dissociated by treatment of sodium deoxycholate to a soluble form.

Electron-microscopic Study on the Thiosulfate-oxidizing System

The electron micrographs of active crude cell-free extracts and isolated membrane vesicles shown in Fig. 27 and Fig. 28 were obtained with negative staining. Both micrographs revealed unique spherical structures not penetrated by the stain, of relatively homogeneous size(100 to 400 nm in diameter). Interestingly enough, the membrane vesicles isolated by Sepharose 4B column chromatography appeared larger than those from crude extracts. In addition to these larger structures smaller particles(20 to 50 nm in diameter) were seen in both micrographs.

The electron micrograph of ultra-thin section of isolated membrane vesicles is shown in Fig. 29. This micrograph indicated that the larger structures observed in the negatively stained preparations actually consisted of smaller vesicles of 20 to 50 nm in diameter. The ultra-thin sectioning procedure seemed to have disrupted the organization of the larger spherical structure, but this micrograph indicated that there probably was no

outer membrane layer for the structures. These aggregated structures, however, must have been stable during the isolation of membrane vesicles by either Sepharose 4B column chromatography or ultracentrifugation(105,000 x g for 90 minutes). In fact the membrane vesicles were aggregated into larger structures(Fig. 28) during the isolation from the crude extracts.

Fig. 27. Electron micrograph of T. novellus cell-free extracts negatively stained with potassium phosphotungstate.
Approximately x 20,000.
The active cell-free extracts were prepared by the standard method as described in Materials and Methods. The experimental procedures were the same as described in Materials and Methods.

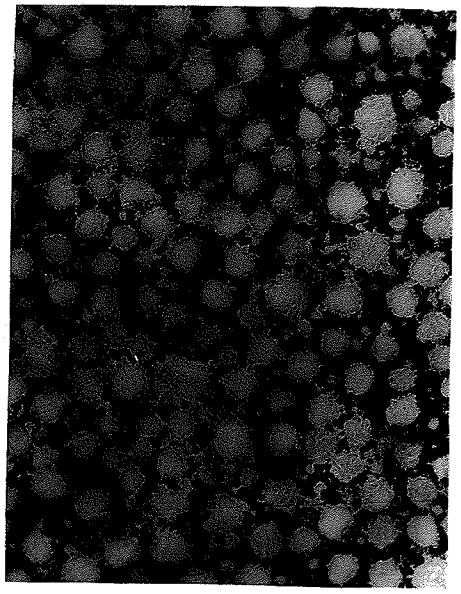


Fig. 28. Electron micrograph of T. novellus membrane vesicles negatively stained with potassium phosphotungstate.
Approximately x 20,000.
The active membrane vesicles were prepared by Sepharose 4B column chromatography as described in Materials and Methods.
The experimental procedures were the same as described in Materials and Methods.

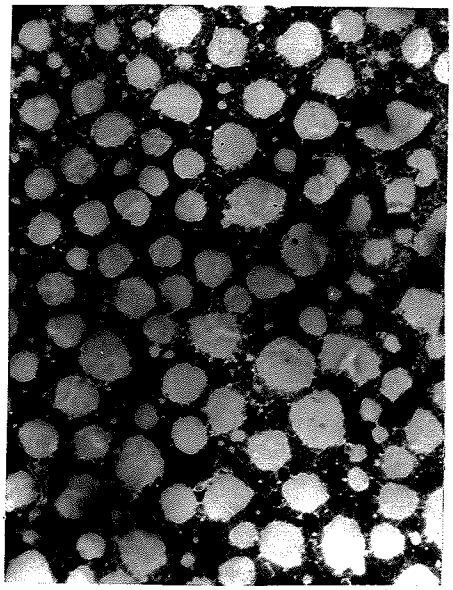
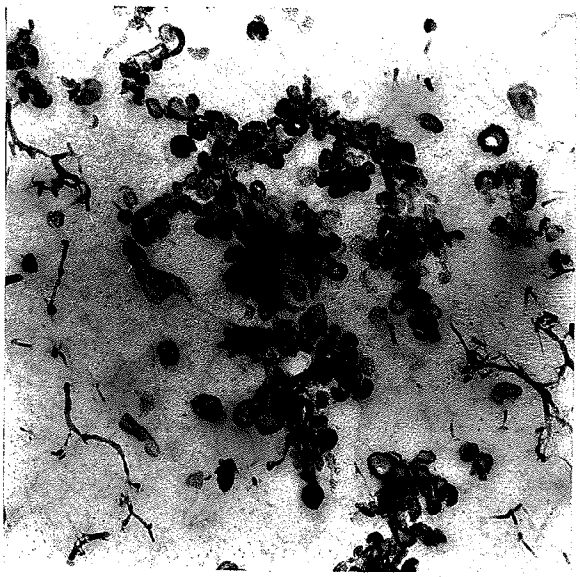


Fig. 29. Electron micrograph of thin-section of *T. novellus* membrane vesicles.
Approximately x 50,000.
The active membrane vesicles were prepared by Sepharose 4B column chromatography as described in Materials and Methods.
The experimental procedures were as described in Materials and Methods.



DISCUSSION

DISCUSSION

In the present study it is unequivocally shown that thiosulfate can be oxidized to sulfate by whole cells, crude cell-free extracts and isolated membrane-bound preparations of T. novellus.

The use of a Clark Oxygen Electrode (a Gilson Oxygraph) proved to be a suitable tool for stud[illegible] thiosulfate-oxidizing system. This assay method was reliable, rapid and reproducible and the assay was possible with small amounts of preparations at the low concentration of thiosulfate.

Before attempting to isolate the active cell-free thiosulfate-oxidizing system from intact T. novellus cells, it was imperative to develop a growth condition for the organism that would result in active intact cells. The results in the foregoing sections showed that the composition of the medium, the control of pH, the degree of aeration, the temperature and duration of cultivation were factors affecting both the growth of bacterial cells and the thiosulfate-oxidizing and sulfite-oxidizing specific activities. With the development of a cultivation method with these factors controlled as described in Materials and Methods, little difficulty was encounte[illegible] in obtaining the active intact cells for the oxidation of thiosulfate with good cell yields. As shown in Fig. 3,

the specific activities of both thiosulfate-oxidizing activity and sulfite-oxidizing activity increased during log phase and peaked during early stationary phase of the culture, indicating a close relationship in the synthesis of these two enzyme activities. The active cells thus obtained always produced cell-free extracts capable of oxidizing thiosulfate when the intact cells were disrupted by sonic oscillation under a nitrogen atmosphere.

In order to prepare the most active cell-free thiosulfate-oxidizing system from the active cells, the effect of various methods was assessed by centrifuging the treated systems at 2,000 x g for 20 minutes, and subsequently measuring the activity of crude cell-free extracts that remained in the supernatant fractions. Among the techniques for disruption of the cells tested, breakage of cells by sonic oscillation with a nitrogen gas for 15 minutes yielded the most active cell-free extracts in which about 10 to 14% of the activity of intact cells remained in supernatant fractions (Table 3). These cell-free extracts did not contain any unbroken intact cells when the extracts were examined under either a phase contrast microscope or an electron microscope. Therefore, this method was accordingly adopted for the preparation of active crude cell-free extracts of T. novellus.

By sonic treatment of intact cells under air, instead of nitrogen the thiosulfate-oxidizing activity

of the cell-free extract was markedly reduced, while the sulfite-oxidizing activity was not. These observations indicated the impairment of the thiosulfate-oxidizing system during sonication in the presence of oxygen. Reduced glutathione (10^{-3}M) as a free radical scavenger had no protective effect.

Kodama and Mori(1968) also reported that the sulfur-oxidizing cell-free extracts could only be obtained by sonic disruption of T. thiooxidans cells under nitrogen atmosphere, while sulfite-oxidizing system was stable for sonic treatment in the presence of air. The cell-free extracts of T. thiooxidans and T. thioparus which had the sulfur-oxidizing enzyme were also prepared by sonication under nitrogen atmosphere (Suzuki, 1965; Suzuki and Silver, 1966). Moreover, in the present work, it was observed that the crude extracts prepared from aged active cells (at 4°C for 5 days), frozen cells or less active intact cells oxidized thiosulfate slowly, but still oxidized sulfite rapidly. In view of these observations, it seems that the initial stage of thiosulfate oxidation (thiosulfate cleavage step) and sulfur-oxidizing enzyme system may be more sensitive to oxygen and may require more intact structural integrity of the membrane than that of sulfite-oxidizing system. It should be mentioned, however, that there was no increased recovery of thiosulfate-oxidizing activity when intact cells were broken in the

presence of bovine serum albumin as a stabilizer or protector of the system during sonication (result not shown). The use of spheroplast for obtaining active crude extracts has also been unsuccessful with this organism (Table 3).

Of particular interest is the fact that the thiosulfate-oxidizing of T. novellus resided exclusively in particular(membrane) fraction of the cell-free preparation, whereas the thiosulfate-oxidizing system reported by Aleem(1965) was a soluble enzyme. The results presented in Table 4 where almost all of the thiosulfate-oxidizing activity was sedimentable by differential centrifugation of the crude cell-free extract at 105,000 x g for 90 minutes indicated that the system was preferentially located on the membrane-bound insoluble fraction. This result was further supported by either Sepharose 4B column chromatography of crude extracts(Fig. 6) or electron-microscopic study of crude extracts and isolated membrane vesicles (Fig. 27, 28 and 29). From these results it is concluded that the thiosulfate-oxidizing system of T. novellus is closely associated with the membrane fraction.

The results of the sucrose gradient sedimentation study(Fig. 7) also support this view.

On the other hand, Aleem(1965) reported that crude cell-free extract (20,000 x g for 60 minutes

supernatant fraction) of T. novellus oxidized thiosulfate and a soluble fraction (144,000 x g for 60 minutes supernatant fraction) had the thiosulfate: cytochrome c reductase activity. In his thiosulfate oxidase system, the exogenous addition of cytochrome c (type III, from horse heart) actually stimulated the rate of thiosulfate oxidation and the Km value for thiosulfate was much higher(2×10^{-1}M) than that of present study(1.2×10^{-4}M). Since full stoichiometry was not established for Aleem's thiosulfate-oxidizing system, it is not even possible to say whether the thiosulfate oxidase activity in his crude extracts was due to the thiosulfate-oxidizing enzyme isolated by Trudinger(1961a and b) from Thiobacillus X which forms tetrathionate, or a mixture of rhodanese and sulfite-oxidizing system as pointed out by Charles and Suzuki(1966a).

In the present study, however, all cell-free preparations (crude extract, isolated membrane vesicles and soluble fraction) had strong rhodanese activity (Table 20), but not thiosulfate-oxidizing enzyme activity, suggesting that the thiosulfate-oxidizing enzyme was not involved in the oxidation of thiosulfate.

Charles and Suzuki(1966a) reported that both whole cells and crude cell-free extracts oxidized thiosulfate to sulfate without polythionate accumulation, consuming 2 moles of O_2 for every mole of thiosulfate

oxidized. The product of the thiosulfate-oxidizing system in whole cell experiment was identified as sulfate using colorimetric and gravimetric methods. The present results obtained in Table 7 and 8 support the above stoichiometry even in the cell-free extract and isolated membrane vesicle experiments. Furthermore, direct evidence for such a stoichiometric relationship was obtained by employing thiosulfate labelled in either the inner or outer positions to show that sulfate ($S^{35}O_4^{2-}$) was formed from both sulfur atoms of thiosulfate in accordance with stoichiometric expectations. It seems, thus, quite clear that the thiosulfate-oxidizing system in T. novellus catalyzes a complete oxidation of thiosulfate to sulfate as follows:

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

It should be pointed out, however, that any attempts to show the formation of sulfate(S-35) from S-35 labelled thiosulfate with isolated membrane vesicles were unsuccessful. The reason for this is not clear at the present time. A strong possibility is that sulfate may have been trapped inside the membrane vesicles. However, it is obvious that a further work is needed to explain the exact reason for this anomaly.

In the thiosulfate oxidation catalyzed by isolated

membrane vesicles, the Km value for thiosulfate was somewhat higher than the one obtained with the whole cells (1.2×10^{-4}M versus 2×10^{-5}M). Although no clear-cut explanation appears to be available for this phenomenon, it may be due to a conformational change of protein during isolation of the thiosulfate-oxidizing system. Similar observation was reported in the cell envelope fragments of T. ferrooxidans which oxidized ferrous ion to ferric ion (Bodo and Lundgren, 1974).

The optimum pH of cell-free thiosulfate-oxidizing system was found to be 7.5. This finding is not suprising, since T. novellus grows best at pH 7.5. The optimum temperature was around 25°C for the crude cell-free extracts, the value very close the optimum temperature 26°C for growth of this organism.

The study on the stability of thiosulfate-oxidizing system showed a marked instability during storage at 4°C and 25°C and upon freezing and thawing (Figs. 8, 9 and 10). The system was more sensitive to slow freezing at -20°C than that of rapid freezing at -74°C. Interestingly, storage under nitrogen atmosphere and at high protein concentrations (above 6 mg per ml) improved the stability of the thiosulfate-oxidizing system. However, attempts to preserve the full activity of the system during the storage by using

various known stabilizers were unsuccessful. The instability of the thiosulfate-oxidizing system is not easily explained, but may be a function of any one or all of the following factors: oxygen sensitivity; alterations in the structural organization at the level of cellular-membrane; change in reactive sulfhydryl groups at the active centers of thiosulfate-oxidizing system and change in endogenous respiration. Nevertheless, it has not been further investigated and the exact cause of the instability of the thiosulfate-oxidizing system is not known at present time.

Activation of less active thiosulfate-oxidizing system could be achieved by several activators such as GSH, NADH and sulfite (Table 8), but not by bovine serum albumin, Mg^{2+}, Cu^{2+}, spermine, spermidine, DTT or mercaptoethanol. The results obtained in Table 9 showed that the rates of thiosulfate oxidation by activation of the system were comparable to those of active cell-free systems. The nature of the activation effect of GSH appears to be different from those of NADH and sulfite. The less active crude extracts prepared in the presence of air or from frozen cells were activated only by GSH. By contrast, the cell-free extracts obtained from less active intact cells or aged cells (stored at $4^{o}C$ for 5 days) and cell-free extracts inactivated by storage at $4^{o}C$ for 12 hours were activated

by GSH, NADH and sulfite. In order to activate less active thiosulfate-oxidizing systems (less damaged preparations) it was absolutely necessary that thiosulfate was added to the reaction mixture before complete oxidation of NADH or sulfite oxidation (result not shown). From these results it can be deduced that the thiosulfate-oxidizing system may require an initial reducing power which can be supplied by NADH, sulfite, GSH or endogenous electron flow. Similar activation effects of NADH and hydroxylamine on the ammonia oxidation was suggested by Kwok(1968) and Suzuki and Kwok(1969).

The effect of GSH on thiosulfate-oxidizing system is of considerable interest. GSH is the predominant low-molecular weight thiol present in the most living cells and one function ascribed to GSH is the maintenance of protein thiol groups in the reduced state to regulate cellular metabolic activity. In the severely impaired thiosulfate-oxidizing system GSH acted as a specific activator. GSH had no effect on the thiosulfate-oxidizing activity of active cell-free system at low concentrations (below 5×10^{-4}M), but inhibited the activity at high concentrations (above 10^{-3}M) in the active or less active preparations (Tables 10 and 11 and Fig. 19).
The activation effect was possibly due to the reduction of disulfide groups to active sulfhydryl groups by GSH in these partially inactivated preparations.

The inhibition effect at higher concentrations of GSH may be more complicated. It was observed that the thiosulfate-oxidizing activity was completely inhibited by preincubation for a longer period (20 minutes) with a high concentration of GSH(10 mM). These inhibitions at high GSH concentrations may be explained by a possible attachment of glutathione to the active sulfhydryl groups of enzymes as mixed disulfides leading to catalytically inactive species. Furthermore, if GSH was added after the initiation of thiosulfate oxidation, it had little inhibitory effect on the rate of thiosulfate oxidation (result not shown). This observation suggests that thiosulfate possibly binds to the same site as glutathione and can effectively prevent the formation of irreversibly inactive mixed disulfides in the presence of high concentrations of GSH.
The activation effect of GSH on the less active preparations obtained from frozen cells indicated that the effect may be related to damaging alterations in the structural organization of the membrane complex system since freezing and thawing are known to bring about structural changes in membranes and membrane-bound enzymes in bacteria (Camerino and King, 1965; Aithal and Ramasarmer, 1969 and 1971; Aithal et al., 1971). Possibly these preparations required GSH to regenerate active sulfhydryl groups, which in undamaged

preparations were probably regenerated by endogenous electron donors.

The effect of GSH as discussed above imply that the thiosulfate-oxidizing system contains essential active sulfhydryl groups or disulfide bridges, that are important for the oxidation of thiosulfate by this membrane-bound complex system. The precise mechanism of these activation or inactivation process, however, remains unclear and it must await for further critical experimentations. The results obtained in this thesis suggest that the reactive sulfhydryl groups, the level of oxygen tension (oxidation-reduction level of the system) and the structural integrity of membranes are inter-related and play some important roles in the thiosulfate-oxidizing system.

The importance of the sulfhydryl groups is further supported by the fact that the thiosulfate-oxidizing activity in all preparations was markedly inhibited by various sulfhydryl inhibitors (Table 13). Inhibition of the activity by the addition of either mercaptide-forming agents or alkylating agents may be taken as an index of essential sulfhydryl groups. It should be noted, however, that the action of the sulfhydryl inhibitors may be indirect, altering the native conformation or masking the reactive sites via steric effect, with a consequent loss of activity.

Since all the activity for thiosulfate oxidation was lost upon treatment with mercaptide-forming agent (10^{-3}M of p-HMB), it is more likely that the reactive sulfhydryl residue modified by the reagent does participate directly in the catalytic mechanism. It is probable that all sulfhydryl inhibitors were reacting with the critical sulfhydryl groups at or near the active center of the thiosulfate-oxidizing system. No attempt was made, however, to reverse these inhibitions with reduced sulfhydryl groups such as GSH, DTT or cysteine.

The potent and specific inhibitory effect of KCN (Table 12) on the thiosulfate-oxidizing system might suggest an involvement of a metal ion or a metal component in the oxidation of thiosulfate. The apparently irreversible inactivation of thiosulfate-oxidizing system by incubation with cyanide was observed and any attempt to reactivate the system with various metal ions after cyanide treatment has so far been without success. Since other metal binding agents did not inhibit the activity the effect of cyanide was probably not due to its metal chelating ability. This assumption is supported further by the fact that no metal was required for the thiosulfate oxidation. Since KCN inhibited activity of the thiosulfate-oxidizing system, its inhibition of thiosulfate oxidation may be explained by its action on cytochrome c oxidase. It is also conceivable that

cyanide inactivation may be due to cyanide forming a complex with iron-sulfur proteins involved in thiosulfate oxidation in analogy with the reported inhibition of xanthine oxidase (Massey and Edmondson, 1970). The purified sulfur-oxidizing enzyme and sulfite: cytochrome c oxidoreductase of T. thioparus contained non-heme iron and labile sulfide (Suzuki and Silver, 1966; Lyric and Suzuki, 1970b).

There is another possibility to explain the action of cyanide, viz., the inactivation may be due to cyanolysis of an active group (persulfide linkage) essential for catalysis: protein $-S-S^- + CN^- \longrightarrow$ protein $- S^- + SCN^-$. If it is true, then it would seem more likely that the cyanide action is associated with an active site persulfide of the thiosulfate-oxidizing system. This area clearly needs more investigation.

Although the effectiveness of electron-transport chain inhibitors on the thiosulfate-oxidizing system varied from inhibitor to inhibitor, the results obtained in Table 14 indicated electron-transport system was definitely involved in the oxidation of thiosulfate. The lack of sensitivity of thiosulfate-oxidizing system to some of the generally used inhibitors of mitochondrial electron-transport chain (i.e., Antimycin A) may be due to differences between mitochondrial and bacterial systems as pointed out by Raff and Mahler(1972).

The absorption spectra and NADH oxidase activity of the isolated membrane complex supported further this notion of electron transport involvement. It is well documented that the oxidative reactions of inorganic sulfur compounds in thiobacilli couple to electron-transport system and the process of oxidative or substrate-level phosphorylation (Roy and Trudinger, 1970; Peck, Jr., 1968; Suzuki, 1974).

It has been reported that neither flavin nor cytochrome b mediated electron transfer between thiosulfate and oxygen in T. novellus (Aleem, 1965). From his results, Aleem concluded that electron flow from thiosulfate to molecular oxygen went through cytochromes of the c and a types. In the present work, however, the isolated membrane-associated complex contained flavin and cytochromes b and d in addition to cytochromes c and a and the thiosulfate-oxidizing system of all preparations was inhibited by inhibitors blocking flavin and cytochrome b sites. Moreover, cytochrome c-550 (horse heart, type III) acted neither as an electron acceptor nor as activator for the thiosulfate-oxidizing system in T. novellus, suggesting that cytochrome c-550 may not be the site for entry of thiosulfate. It is obvious that a further critical study is needed in this area.

As shown in Table 15, the strong inhibitory

effect of oligomycin on the thiosulfate-oxidizing complex system suggests that the energy transfer from the electron-transport chain to ATP formation couples to the oxidation of thiosulfate in the membrane-associated thiosulfate-oxidizing system of T. novellus.

In the recent work, Bramlett and Peck(1975) reported an involvement of superoxide radical (O_2^-), a form of oxygen anion, in the cytochrome c coupled assay of APS reductase of Desulfovibrio vulgaris in the presence of air. They observed that anaerobiosis caused a complete inhibition of enzyme activity and superoxide dismutase exhibited strong inhibitory effect (up to 85% inhibition) on the reduction of cytochrome c. In contrast, the reduction of ferricyanide was not inhibited by anaerobiosis or superoxide dismutase, implying that superoxide anions were not involved in the ferricyanide coupled assay of APS reductase. Cassel and Fridovich(1975) reported that the autoxidation of ferrocytochrome c, under a variety of conditions, generated the superoxide radical which could then dismute to H_2O_2 and O_2 or could reduce ferricytochrome c back to ferrocytochrome c. Superoxide dismutase, by accelerating the dismutation of O_2^-, prevented the back reaction and thus exposed the true rate of reaction of ferrocytochrome c with molecular oxygen. As a theory it is possible that

the superoxide radical may participate in the oxidation of thiosulfate. However, when the effect of the superoxide dismutase on the thiosulfate-oxidizing system was tested, the thiosulfate-oxidizing activity in the crude extracts of T. novellus was shown to be totally unaffected by either superoxide anions generated from xanthine and xanthine oxidase system or superoxide dismutase (0.5 µg per 4 mg of protein). The addition of catalase had no effect on the activity in the crude cell-free extracts (results not shown). These results suggest that either the mechanism of thiosulfate oxidation does not involve superoxide radical, or if it does, the superoxide anion is not freely accessible to the dismutase added.

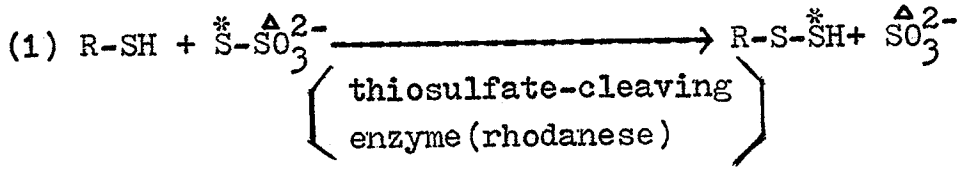
The inactivation of the membrane-associated thiosulfate-oxidizing system by trypsin or phospholipase A clearly demonstrates that it is phospholipo-proteinaceous in nature. The modification of the thiosulfate-oxidizing complex with trypsin seems to be a very specific proteolysis. This rather specific cleavage of the isolated membrane vesicles is very interesting and the enzymatic digestion appears to be a promising method for dissociation of the membrane-associated thiosulfate-oxidizing system since such treatment released the sulfur-oxidizing enzyme activity from the membrane-associated multienzyme complex (Fig. 26).

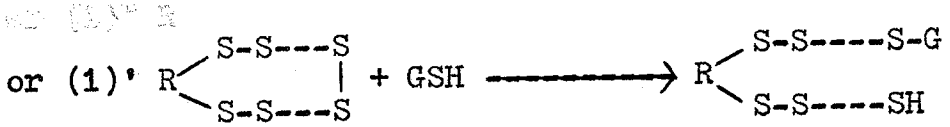
Trumpower et al.(1974) reported that rhodanese activity could be enhanced by controlled digestion with trypsin. They showed that the trypsin treatment of the native bovine rhodanese resulted in the cleavage of the 32,600 molecular weight enzyme into a polypeptide with molecular weight 28,800 which had higher sulfur transferase activity than the native enzyme. It was also reported that a partially purified sulfur-oxidizing enzyme of T. thioparus showed a rhodanese activity (Charles and Suzuki, 1966a). Therefore, it is tempting to assume that rhodanese as thiosulfate-cleaving enzyme and sulfur-oxidizing enzyme may reside closely in the membrane-associated thiosulfate-oxidizing system. If sulfite-oxidizing system is also present, thiosulfate can be oxidized to sulfate by a series of reactions initiated by rhodanese. This point has been fully discussed by Charles and Suzuki(1966a). As can be seen in Table 20, the isolated membrane vesicles contained nearly half the amount of total activity of rhodanese present in the crude cell-free extracts.

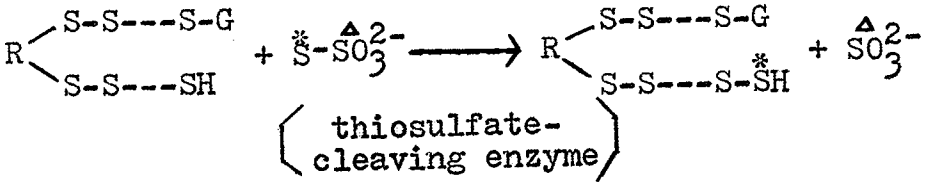
Recently, a membrane-associated sulfite oxidase of T. denitrificans was isolated and characterized by Aminuddin and Nicholas(1974a and b). They observed that the system could be coupled to oxygen, nitrate or ferricyanide as an electron acceptor. However, when the enzyme was purified after solubilization with

deoxycholate, the purified enzyme coupled only with ferricyanide, indicating that the sulfite oxidase complex was disrupted by deoxycholate treatment. In this study, it was possible to dissociate the sulfite: cytochrome c oxidoreductase and cytochrome c oxidase from the isolated thiosulfate-oxidizing complex by means of sodium deoxycholate treatment (Tables 21 and 22). Therefore, it is clear that the membrane-associated multienzyme complex system of thiosulfate oxidation in T. novellus consists of all known intermediary enzyme components which are tightly bound to each other or linked to membrane structure.

Correlating previous observations made by Charles and Suzuki(1966a) with the results obtained in the present study, a plausible scheme for thiosulfate oxidation by T. novellus is outlined in the following equations:







(2) $R\text{-}S\text{-}\overset{*}{S}H + O_2 + H_2O \longrightarrow R\text{-}SH + \overset{*}{S}O_3^{2-} + 2H^+$

(sulfur-oxidizing enzyme)

or

(2)' $R\begin{cases} S\text{-}S\text{---}S\text{-}G \\ S\text{-}S\text{---}S\text{-}\overset{*}{S}H \end{cases} + O_2 + H_2O \longrightarrow R\begin{cases} S\text{-}S\text{---}S\text{-}G \\ S\text{-}S\text{---}SH \end{cases} + \overset{*}{S}O_3^{2-} + 2H^+$

(sulfur-oxidizing enzyme)

(3) $2\overset{*}{S}\overset{\Delta}{S}O_3^{2-} + 4$ cytochrome c $(Fe^{3+}) + 2H_2O$
$\longrightarrow 2\overset{*}{S}\overset{\Delta}{S}O_4^{2-} + 4$ cytochrome c $(Fe^{2+}) + 4H^+$

(sulfite: cytochrome c oxidoreductase)

(4) 4 cytochrome c $(Fe^{2+}) + O_2 + 4H^+ \longrightarrow$
4 cytochrome c $(Fe^{3+}) + 2H_2O$

(cytochrome c oxidase)

Sum : $\overset{*}{S}\text{-}SO_3^{2-} + 2O_2 + H_2O \longrightarrow 2\overset{*}{S}\overset{\Delta}{S}O_4^{2-} + 2H^+$

Where R-SH is thiosulfate-cleaving enzyme in the active thiosulfate-oxidizing system, $R\begin{cases} S\text{-}S\text{---}S \\ S\text{-}S\text{---}S \end{cases}$ is thiosulfate-cleaving enzyme in the impaired thiosulfate-oxidizing system and $R\begin{cases} S\text{-}S\text{---}S\text{-}G \\ S\text{-}S\text{---}SH \end{cases}$ is thiosulfate-cleaving enzyme in the impaired thiosulfate-oxidizing system activated by GSH.

As can be seen in this scheme, the initial step of thiosulfate oxidation might play a very important role for the overall reaction. When the initial step is damaged, a certain amount of reduced glutathione must

be added into the reaction system in order to activate the thiosulfate-oxidizing system.

Although substantial progress has been made in the past decade in illuminating the mechanism for the oxidation of intermediates involved in the thiosulfate oxidation by thiobacilli, the detailed mechanism of thiosulfate oxidation in thiobacilli is far from complete and merits further investigation. It is hoped that these studies will lead ultimately to a deeper understanding of the mechanism of thiosulfate oxidation in thiobacilli.

Future studies on the role of membranes, the nature of electron transport components, the reconstitution and the energetic aspect of the thiosulfate-oxidizing system will certainly further our understanding of the mechanism of thiosulfate oxidation in thiobacilli.

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