MECHANISM OF THIOSULFATE OXIDATION BY A FACULTATIVE AUTOTROPH *THIOBACILLUS NOVELLUS*

by

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TO DOODLES

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ABSTRACT

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The mechanism of carbon dioxide fixation by Thiobacillus novellus was studied with $C^{14}O_2$ using both whole cells and cell-free extracts. Under autotrophic conditions with thiosulfate as substrate the organism fixed CO_2 through the carboxydismutase pathway and the phosphoenolpyruvate pathway in accordance with the mechanisms found in other autotrophs. Under heterotrophic conditions with glucose as substrate, however, the carboxydismutase pathway was repressed and the phosphoenolpyruvate pathway was the only pathway found.

Both whole cells and extracts of T. novellus oxidized thiosulfate, sulfur, sulfite and sulfide, but tetrathionate was oxidized only by whole cells. The sulfur-oxidizing enzyme was found to be similar to the enzyme found in other thiobacilli requiring reduced glutathione as cofactor. Sulfide was oxidized with an intermediary formation of sulfur. Sulfite was oxidized through a cytochrome system involving sulfite:cytochrome c oxidoreductase, cytochrome c and cytochrome oxidase. Rhodanese activity was found in extracts of this organism. A mechanism of thiosulfate oxidation was proposed where thiosulfate is initially cleaved to sulfur and sulfite by a rhodanese-like enzyme, sulfur is then oxidized to sulfite by the sulfur-oxidizing enzyme, and finally sulfite is oxidized to sulfate through a cytochrome system.

Sulfite:cytochrome c oxidoreductase was partially purified and its properties were studied. It was found to be a new enzyme distinct from APS-reductase of Thiobacillus thioparus. The enzyme reduced

ferricyanide or cytochrome c with sulfite stoichiometrically and was specific for sulfite as substrate. The enzyme was inhibited by sulfhydryl inhibitors and various monovalent anions. The inhibition by NaCl was competitive with respect to sulfite concentrations. The K_m value for sulfite was found to be 4×10^{-5}M at pH 8.0 and 2×10^{-6}M at pH 6.5. The activity, however, was much higher at pH 8.0. The significance of these findings is discussed in relation to the mechanism of sulfite and thiosulfate oxidations by T. novellus.

Oxidative phosphorylation was shown in extracts of T. novellus during sulfite oxidation and was concluded to be the mechanism of energy generation in this organism rather than the substrate-level phosphorylation mechanism proposed for T. thioparus.

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ABBREVIATIONS

ADP	-	adenosine-5'-diphosphate
AMP	-	adenosine-5'-phosphate
APS	-	adenosine-5'-phosphosulfate
ATP	-	adenosine-5'-triphosphate
Cyt	-	cytochrome
DEAE	-	diethylaminoethane
DNP	-	2, 4 dinitrophenol
EDTA	-	ethylenediaminetetracetate
FDP	-	fructose-1,6-diphosphate
F6P	-	fructose-6-phosphate
GSH	-	reduced glutathione
G6P	-	glucose-6-phosphate
NAD	-	oxidized nicotinamide adenine dinucleotide
NADP	-	oxidized nicotinamide adenine dinucleotide phosphate
PEP	-	phosphoenolpyruvate
3-PGA	-	3-phosphoglyceric acid
RDP	-	ribulose-1,5-diphosphate
R5P	-	ribose-5-phosphate
Ru5P	-	ribulose-5-phosphate
TCA	-	trichloracetic acid
Tris	-	tris (hydroxymethyl) aminoethane

INTRODUCTION

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The thiobacilli hold a unique position among the non-photosynthetic bacteria since some members are strict autotrophs whereas others are facultative, i.e., they are capable of either autotrophic or heterotrophic growth. Autotrophically grown thiobacilli utilize either thiosulfate or sulfur as energy-source, but there is general disagreement as to the end-products formed, and only the report of Peck (1960) and Peck and Fisher (1962) have substantiated their results by enzymatic evidence.

Since both whole cells and extracts of T. novellus actively oxidized thiosulfate to sulfate without accumulation of polythionates and the extracts had both the sulfur- and sulfite-oxidizing systems, thiosulfate oxidation by this organism was investigated in order to elucidate the enzymatic mechanisms involved. Initial studies of the oxidation of sulfite revealed that AMP was not stimulatory. The enzyme responsible was subsequently purified and found to be sulfite oxidase rather than APS-reductase. During purification of the sulfite oxidizing enzyme, an enzyme which oxidized elemental sulfur to thiosulfate was also isolated and found to be similar to that observed in T. thiooxidans (Suzuki 1965) and T. thioparus (Suzuki and Silver 1966). Since sulfur and sulfite were intermediates of thiosulfate metabolism, it was believed that a scission of thiosulfate was the initial reaction. The enzyme responsible was subsequently found to be rhodanese.

Since T. novellus does not metabolize thiosulfate according to the pathway involving substrate-level phosphorylation reactions, the possibility of oxidative phosphorylation was studied. Oxidative phosphorylation was found to be the mechanism for deriving energy in this organism. The pathway of CO_2 fixation was also investigated in order to compare the mechanism of T. novellus with that of the obligately autotrophic thiobacilli. The results indicated that the synthesis of cellular carbon proceeded by way of the 3-PGA pathway and the PEP carboxylase system in accordance with the mechanism in other thiobacilli.

It is hoped that as a result of the findings of this investigation a better understanding of the metabolism of reduced sulfur compounds and of CO_2 by this organism will be obtained, and that some of the discrepancies of other investigations will be resolved.

HISTORICAL

HISTORICAL

Winogradsky (1887) established the fundamental principles of autotrophy among bacteria, when he concluded from studies of certain sulfur- and iron-oxidizing organisms that all their energy requirements were derived from the oxidation of incompletely oxidized inorganic compounds and all of the carbon by the fixation of CO_2. His experiments were conducted with members of the genus Beggiatoa which recently have been found to be unable to grow on the energy released from sulfur, and are heterotrophic (Starkey, 1962). Thus whereas the concepts of autotrophy were correct, the organisms from which they were drawn did not live autotrophically as they were believed to.

Among the autotrophic bacteria are those which metabolize reduced inorganic sulfur compounds to sulfate and are classified in the genus Thiobacillus (Breed et al., 1957). In this genus are found both strictly autotrophic and facultatively autotrophic species. This discussion will be concerned with the physiology of Thiobacillus novellus, including appropriate information about the metabolism of other species of the genus. Some aspects of the metabolism of carbon compounds will also be presented. For reviews on the thiobacilli, C. B. Van Niel (1954), Lees (1955), and Vishniac and Santer (1957) should be consulted. The mechanisms of autotrophic carbon dioxide fixation, are well documented in the report of Elsden (1962), while those of heterotrophic CO_2 assimilation are found in the report of Wood and Stjernholm (1962). Because of these extensive reviews,

details will be treated very briefly here.

General physiology of Thiobacillus novellus

Starkey (1935) isolated a small, non-motile, gram-negative, non-sporulating rod, which grew on organic as well as inorganic carbon sources. When grown with CO_2 as carbon source, it utilized thiosulfate as a source of energy and was therefore a facultative autotroph. This organism was named Thiobacillus novellus. On an inorganic salt medium containing thiosulfate, the acidity of the medium was increased as growth progressed (Starkey, 1934[a]). Since the organism developed best at pH between 8.0 and 9.0, it somewhat resembled Thiobacillus thioparus which developed best at pH close to neutral except that the latter was motile and accumulated sulfur during thiosulfate oxidation. These two organisms were unlike Thiobacillus thiooxidans (Waksman and Joffe, 1921) which also oxidized thiosulfate or elemental sulfur, but developed best in an acid environment (Starkey, 1934[b]). On the basis of these differences T. novellus and T. thioparus are considered as alkaline thiosulfate oxidizers, and T. thiooxidans as acid sulfur oxidizer (Umbreit, 1962). Among the facultative thiobacilli are T. novellus, the best known of the group, T. coproliticus, isolated from a piece of Triassic coprolite (Lipman and McLees 1940), and T. intermedius, the most recently isolated member of the genus (London, 1963).

Although externally supplied organic compounds do not support growth of the obligately autotrophic thiobacilli in the absence of

sulfur compounds, at least some such compounds penetrate into the cell. Waksman and Starkey (1922) have found that the rate of sulfur oxidation by T. thiooxidans increased slightly in the presence of glucose. Glucose slowly disappeared during sulfur oxidation and its consumption was proportional to the growth of the organism (Starkey, 1925).

Suzuki (1958) incubated T. thiooxidans whole cells and extracts with C^{14}-labelled glucose and found $C^{14}O_2$, as well as labelled amino acids, sugars and polysaccharides. In view of these findings, obligate autotrophy cannot be explained in terms of cell-walls or membranes impermeable to organic matter. The difference between obligate and facultative thiobacilli probably lies in the inability of the former to generate sufficient amounts of energy for growth from the metabolism of organic compounds, and may be quantitative rather than qualitative.

Mechanism of carbon dioxide fixation

The autotrophic mechanism of carbon dioxide fixation in bacteria is the same as that found by Calvin and his associates using C^{14}-labelled CO_2 during investigations of a photosynthetic alga. In this mechanism CO_2 is directly involved in only one reaction, namely, the formation of 3-phosphoglyceric acid (3-PGA) which was labelled in the carboxyl group and was the earliest stable product detected in the experiments (Calvin and Benson, 1948; Bassham et al., 1950;

Benson et al., 1950). The 3-PGA arose from a condensation of $C^{14}O_2$ with ribulose-1,5-diphosphate (RDP) (Calvin and Massini, 1952; and Bassham et al., 1954). The enzyme catalyzing this reaction was termed RDP carboxylase or carboxydismutase. (Weissbach and Horecker, 1956).

The RDP arose from ribulose-5-phosphate (Ru5P) and adenosine-5'-triphosphate (ATP) by a reaction catalyzed by phosphoribulokinase (Hurwitz et al., 1956). Ru5P could be replaced by ribose-5-phosphate (R5P) in the presence of phosphoriboisomerase.

PGA is converted to hexose primarily by a reversal of the Embden-Meyerhof pathway, being first reduced to triose phosphate, which is then converted to fructose-1,6-diphosphate (FDP) by aldolase, fructose-6-phosphate (F6P) arising by phosphate removal of FDP.

Pentose phosphates are regenerated by the action of transketolase and transaldolase, enzymes commonly involved in carbohydrate metabolism. Thus from the evidence available, only two enzymes unique to the autotrophic mechanism of CO_2 fixation are involved, namely, carboxydismutase catalyzing the condensation reaction between CO_2 and RDP, and phosphoribulokinase forming RDP from Ru5P and ATP.

The mechanism of CO_2 fixation in chemosynthetic bacteria is well documented in the literature, and its occurrence by way of the RDP carboxylating mechanism was found in T. thioparus (Santer and Vishniac, 1955), Thiobacillus denitrificans (Trudinger, 1955, 1956; Milhaud et al., 1956) and T. thiooxidans (Suzuki and Werkman, 1958[a]).

Although there is overwhelming support for the 3-PGA pathway as

a means of CO_2 fixation in autotrophs, it should be pointed out that other mechanisms exist for the incorporation of CO_2 into organic carbon. The Wood-Werkman reaction (Wood and Werkman, 1938) is essential for the operation of the tricarboxylic acid cycle and for the biosynthesis of various amino acids. Utter and Kurahashi (1953, 1954[a,b]) isolated oxalacetic carboxylase from liver. The enzyme catalyzed a reversible reaction between phosphoenolpyruvate (PEP), CO_2 and oxalacetate requiring inosine or guanosine diphosphate as phosphate acceptor. Phosphoenolpyruvate carboxylase (PEP carboxylase) isolated from spinach leaves (Bandurski and Greiner, 1953) catalyzed the formation of oxalacetate and orthophosphate from PEP and CO_2 in an irreversible reaction. Both of these enzymes were present in T. thiooxidans (Suzuki and Werkman, 1957, 1958[a]) and were apparently responsible for the rapid labelling of the β-carboxyl group of aspartate and γ-carboxyl group of glutamate in the whole cell experiments (Suzuki and Werkman, 1958[b]). The carboxydismutase activity of T. novellus was dependent upon growth conditions since in cells grown on organic media the enzyme level fell to less than 2% of the autotrophic level (Vishniac and Trudinger, 1962). Similar results were also obtained in an extension of these studies with the same organism (Aleem, 1965).

Oxidation of sulfur compounds

The thiobacilli are a small group of microorganisms capable of obtaining all the energy required for growth from the oxidation of

reduced inorganic sulfur compounds to sulfate. In spite of the wealth of literature available on the oxidation of sulfur compounds by thiobacilli, the mechanism whereby this is accomplished still is a controversial topic.

When grown on thiosulfate, some thiobacilli produced tetrathionate along with sulfur and sulfate (Gleen and Quastel, 1953; Vishniac, 1952; Jones and Happold, 1961; Vishniac and Trudinger, 1962), while others produced only sulfur and sulfate (Starkey, 1935; Parker and Prisk, 1953; Peck, 1960). The variation in end-products obtained by different workers has been interpreted as being due to growth conditions as well as the organisms investigated (Peck, 1962). According to Starkey (1934[a,b]) and Parker and Prisk (1953) T. novellus did not produce tetrathionate during growth on thiosulfate. Thiosulfate was oxidized according to the overall equation:

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

Variations in the results of growth experiments have resulted in widely divergent views on the mechanism of thiosulfate oxidation. Vishniac and Santer (1957), based on the observation (Vishniac, 1952) that tetrathionate was produced during thiosulfate oxidation of resting cells of T. thioparus, have suggested a transformation of the sulfur atoms of thiosulfate by way of the formation of several polythionates. Further support for this hypothesis came from the isolation of a soluble enzyme from Thiobacillus X (Trudinger, 1961) which catalyzed the quantitative conversion of thiosulfate to tetra-

thionate. A similar enzyme was obtained by Santer while investigating T. thioparus and autotrophic T. novellus (Trudinger and Vishniac, 1962). London and Rittenberg (1964) demonstrated the accumulation of polythionates during thiosulfate oxidation by cell-free extracts of T. thioparus and T. thiooxidans and the oxidation of tetrathionate by the same extracts. This pathway involving polythionates as intermediates between thiosulfate and sulfate still suffers from the lack of knowledge of specific enzymes responsible for the overall oxidation of thiosulfate to sulfate.

Skarzyński et al., (1957), on the basis of growth experiments with S^{35}-labelled thiosulfate ($S\text{-}SO_3^{2-}$), have concluded that thiosulfate scission occurs at the cell-membrane and the outer sulfur (S-) is the only part of the molecule that enters the cell and is metabolized. Peck (1960) proposed a similar mechanism based on studies of thiosulfate oxidation by extracts of T. thioparus and suggested that the initial reaction was the reduction of thiosulfate to sulfide and sulfite. Sulfide was converted to sulfur and sulfite eventually to sulfate. The enzyme specific for each reaction was also shown and is outlined in the following equations:

$$2S_2O_3^{2-} + 4H^+ + 4e^- \xrightarrow[\text{reductase}]{\text{thiosulfate}} 2SO_3^{2-} + 2H_2S$$

$$2H_2S + O_2 \xrightarrow[\text{oxidase}]{\text{sulfide}} 2S^\circ + 2H_2O$$

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

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

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

Overall reaction: $2S_2O_3^{2-} + O_2 + AMP + 2P_i + 4H^+ \longrightarrow 2S^\circ + 2SO_4^{2-} + ATP + 2H^+$

A unique feature of this scheme is the production of ATP by a substrate-level phosphorylation reaction. This mechanism was satisfactory in view of the fact that it explained the origin of sulfur and the method by which energy was derived. In the light of more recent investigations, however, this scheme became questionable as a general metabolic pathway of thiosulfate by thiobacilli.

Firstly, during investigations of T. novellus it was found that thiosulfate oxidation by extracts proceeded without the requirement of GSH, and that sulfite oxidation did not require AMP. Subsequently, the enzyme sulfite oxidase (sulfite:cytochrome c oxidoreductase) was isolated and purified (Charles and Suzuki, 1965). This enzyme quantitatively converted sulfite to sulfate stoichiometrically reducing mammalian and native cytochrome c. Secondly, Suzuki (1965) isolated and purified an enzyme from T. thiooxidans which converted elemental sulfur to thiosulfate with GSH as cofactor. Further investigations revealed the presence of this enzyme also in T. thioparus, and the

initial product of enzymatic sulfur oxidation was shown to be sulfite, thiosulfate arising by a non-enzymatic reaction between sulfite and sulfur (Suzuki and Silver, 1966). The requirement for GSH by the enzyme in order to oxidize sulfur has been explained by the nucleophilic attack of GSH on the S_8 molecule to form a linear polysulfide chain, GSS_nH, which was shown to be the actual substrate for oxidation.

On the basis of the above findings an alternate pathway has been proposed for thiosulfate oxidation (Suzuki and Silver, 1966) by assuming that the initial scission of thiosulfate by GSH leads to sulfur and sulfite instead of sulfide and sulfite as in the original scheme proposed by Peck (1960).

The new scheme is as follows:

$$SSO_3^{2-} + GS^- \longrightarrow GSS^- + SO_3^{2-}$$

$$GSS^- + O_2 + H_2O \longrightarrow GS^- + SO_3^{2-} + 2H^+$$

$$2SO_3^{2-} + 2H_2O \longrightarrow 2SO_4^{2-} + 4e^- + 4H^+$$

$$4e^- + O_2 + 4H^+ \longrightarrow 2H_2O$$

Overall reaction: $SSO_3^{2-} + 2O_2 + H_2O \longrightarrow 2SO_4^{2-} + 2H^+$

Metabolism of energy by thiobacilli

Thiobacilli have to convert the energy of oxidation of inorganic sulfur compounds into the form of ATP, which is required for the conversion of CO_2 to cell materials. The mechanism of ATP generation

by thiobacilli is also a controversial subject.

In the mechanism proposed by Peck (1960) ATP is generated by substrate-level phosphorylation reactions during thiosulfate oxidation.

On the other hand, the inhibition of oxidation by carbon monoxide, cyanide and azide, (Volger et al., 1942; Iwatsuka and Mori, 1960) and the presence of cytochromes (Trudinger, 1958; Skarzyński et al., 1956) suggest that these organisms can generate biological energy by means of oxidative phosphorylation. In fact, Milhaud et al., (1957) have shown that T. denitrificans incorporated inorganic phosphate into ATP only when thiosulfate oxidation was proceeding. Finally, Hempfling and Vishniac (1965), demonstrated oxidative phosphorylation in cell-free extracts of Thiobacillus X using mercaptoethanol as substrate. Thus, although the thiobacilli may be regarded as unique with regard to the production of energy by substrate-level phosphorylation, they may also derive energy through oxidative phosphorylation, the mechanism observed in other autotrophic forms, (Lees, 1960).

MATERIALS AND METHODS

MATERIALS AND METHODS

Organism and medium

The organism used in this investigation, Thiobacillus novellus (ATCC 8093), was a gift of Dr. R. L. Starkey and was converted to autotrophy by repeated transfer into a modified Starkey's medium No. 3 (Starkey 1934[a]) containing decreasing glucose concentrations and increasing concentrations of thiosulfate. Autotrophic T. novellus was grown in the same medium with glucose replaced by 1.0% thiosulfate. This medium had the following composition: 10 g $Na_2S_2O_3 \cdot 5H_2O$, 4.0g $K_2H\ PO_4$, 1.5g $KH_2\ PO_4$, 0.02g $CaCl_2 \cdot 2H_2O$, 0.1g $MgSO_4 \cdot 7H_2O$, 0.3g $(NH_4)_2SO_4$, 0.02g $MnSO_4 \cdot 2H_2O$, 0.02g $FeCl_3 \cdot 6H_2O$ and water to a final volume of 1 liter.

Four ml 0.2% phenol red was added to 19 liters of the medium in Pyrex glass carboys to give an observable color. The pH was maintained between 7 and 8 as growth progressed by the addition of aliquots of a heat-sterilized 10% Na_2CO_3 solution.

Small amounts of bacteria were grown in Erlenmeyer flasks with mechanical shaking, whereas large batches of bacteria were grown in carboys containing the heat-sterilized medium, through which was bubbled a mixture of air containing about 5% CO_2. After incubation at 30° for about six days, the bacteria were harvested in a Sharples centrifuge*, washed twice in 0.02M potassium phosphate (pH 7.0). The washed cells were suspended in 20 volumes 0.04M potassium

*52,000 x g

phosphate (pH 7.0) and stirred at 5° overnight. These cells were collected by centrifugation at 12,100 x g washed once more as previously described and either stored at -20° or used immediately for making extracts.

Cells used for experiments based on carbon dioxide fixation were grown in the following medium: K_2HPO_4, 4g; KH_2PO_4, 4g; $KHCO_3$, 0.4g; NH_4Cl, 0.4g; $Na_2S_2O_3 \cdot 5H_2O$, 10g; or glucose, 10g; 20 ml trace element mixture and water to a final volume of 1 liter. The trace element mixture consisted of KH_2PO_4, 3.0g; $CaCl_2 \cdot 2H_2O$, 1.0g; $MgSO_4 \cdot 7H_2O$, 0.5g; $MnCl_2$ 0.1g; $FeCl_3 \cdot 6H_2O$, 0.1g; and water to a final volume of 1 liter. The pH was adjusted to 7.8 by adding KOH, the precipitate was allowed to settle and the supernatant decanted and used as trace-element mixture. The same procedures as described above were used for harvesting, washing and storing.

Preparation of cell-free extracts

Extracts were prepared by sonication of a 25% suspension of wet cells in 0.04M potassium phosphate (pH 7.0) under an atmosphere of nitrogen, for 15 min in a water-cooled (5°) 10 kc Raytheon sonic disintegrator. Cell-debris and unlysed cells were removed by centrifugation at 12,100 x g for 20 min. The manipulation of all extracts and purified enzymes was conducted at 0 -5°.

Dialysis of extracts was carried out in a cellulose bag against

500 ml of 0.002M Tris-HCl (pH 8.0) for 12 hr with four changes of the dialyzing medium.

Determination of protein

Protein was determined by the method of Lowry et al., (1951) with powdered bovine albumin as standard.

Preparation of cytochrome oxidase

Either of two methods was used in the preparation of cytochrome oxidase. The first of these involved centrifugation of crude extracts at 144,000 x g for one hour in a Spinco Model L. The precipitate was resuspended in a volume of 0.002M potassium phosphate buffer (pH 7.0) equal to the original volume of crude extract and dialysed against 0.002M Tris-HCl (pH 8.0), and used as cytochrome oxidase. As an alternative, crude extracts were diluted with an equal volume of 0.2M Tris-HCl (pH 8.0) and treated with crystalline $(NH_4)_2SO_4$. The fraction which precipitated between 0-40% $(NH_4)_2SO_4$ was resuspended and dialysed as outlined above.

Chemicals and reagents

Sodium tetrathionate was prepared from sodium thiosulfate according to the method of Trudinger (1964). APS was synthesized and isolated according to the method of Reichard and Ringertz (1959). All other reagents and chemicals used were obtained from commercial sources. $Na_2C^{14}O_3$ was obtained from Radiochemical Centre, Amersham,

England; horse-heart cytochrome c Type II, alumina c gel (13% solids), GSH, AMP, ATP (Tris salt, from muscle), NADP, NAD, N,N' dicyclohexylcarbodiimide, catalase (liver, 2 times crystallized), hexokinase, p-hydroxymercuri benzoate, barium salts of 3-PGA and F6P, sodium salts of G6P and FDP, glutamic acid, serine, valine and glycylglycine from Sigma Chemical Co., St. Louis, Missouri, U.S.A.; G6P dehydrogenase and ADP (trisodium salt) from C. F. Boehringer & Soehne, Mannheim, Germany; benzyl viologen and glycine from British Drug Houses, Toronto, Ontario; DEAE-cellulose from Brown Co., Berlin, New Hampshire, U.S.A.; 2-mercaptoethanol and succinic acid from Eastman Organic Chemicals; polyacrylamide gel with reagents from Canalco, Bethesda, Maryland, U.S.A.; Atebrin (quinacrine hydrochloride), Mann Research Laboratories Inc.; Powdered bovine albumin, Armour Laboratories, Chicago, U.S.A.; alanine, leucine, malic acid and fumaric acid from Nutritional Biochemical Corporation, Cleveland, Ohio, U.S.A.; precipitated powdered sulfur from Baker Chemical Co., N.J., U.S.A.; Norit-A (technical), sodium thiosulfate (technical), sodium sulfite, sodium sulfide and citric acid from Fisher Chemical Co., N.J., U.S.A.

Barium salts of phosphate esters were converted to the corresponding sodium salts by treatment with the Na-form of Dowex 50 resin.

Paper Chromatography

One - and two - dimensional descending techniques were used with

Whatman No. 1 filter paper throughout the investigation. The papers were all washed in 2N acetic acid for 15 min with frequent agitation, rinsed in several changes of distilled water, and dried at room temperature before use.

Solvents

The following solvents were used in the separation of the various compounds chromatographed. With the exception of phenol solvent, the others were prepared fresh before use.

Phenol solvent (80 ml melted distilled phenol and 20 ml water was used as the first solvent followed by propanol-propionic acid - H_2O (2:2:1). One-dimensional chromatography was carried out in ethyl acetate - formic acid - H_2O (3:3:1).

Sprays

The different compounds on the chromatograms were located by spraying the paper with various specific sprays.

Amino acids were revealed with a ninhydrin reagent (Kornberg, 1958). After spraying, the paper was heated at 100° for 10 min.

Organic acids separated by an acid solvent were detected with a mixed acid-base indicator (Aronoff, 1956).

Sugars were identified with the aniline phthalate spray (Partridge, 1949). Hexoses appeared as brown spots after drying whereas pentoses were pink.

Phosphorylated sugars were sprayed according to the procedure of Bandurski and Axelrod (1951) using an ammonium molybdate reagent.

$C^{14}O_2$ fixation by whole cells

For these experiments, 900 mg (wet weight) cells were incubated at 30° for 4 hr in 250 ml growth medium with thiosulfate and with shaking. The cells were collected and divided into three portions. Each portion was incubated with shaking for 30 min in a 50 ml rubber-stoppered Erlenmeyer flask with 80 µmoles potassium phosphate (pH 8.0); either 20 µmoles glucose, 40 µmoles sodium thiosulfate or no substrate; and water in a final volume of 4 ml. At the end of the 30 min incubation period, the contents of each flask were sucked into a 5 ml glass syringe followed immediately by 1.0 ml of a $Na_2C^{14}O_3$ mixture, 10 µmoles Na_2CO_3 and 67 µmoles potassium phosphate (pH 8.0) containing a total of 2.5×10^7 c.p.m. At various time intervals after addition of the $C^{14}O_2$, 1.0 ml aliquots of the mixture were squirted into boiling ethanol in conical glass centrifuge tubes, which were further boiled for 5 min, cooled and acidified in a fume-hood with a drop of glacial acetic acid in order to evolve unfixed $C^{14}O_2$ from the reaction mixtures. The tubes were then centrifuged to remove cell-debris and the supernatants were used for determination of radioactivity, and for chromatography and radioautography.

Cell-free fixation of radioactive CO_2

The complete reaction mixture contained 200 µmoles Tris-HCl (pH 7.5), 10 µmoles R5P, 10 µmoles ATP, 10 µmoles $MgCl_2$, 10 µmoles $NaHCO_3$, 10 µmoles $Na_2C^{14}O_3$ (2.5×10^7 c.p.m.), 8.0 mg protein and H_2O to a final volume of 3.0 ml. The reaction mixtures were incubated in Warburg flasks under nitrogen atmosphere at 30° for 30 min. The reaction was stopped by the addition of 0.5 ml TCA (50%) to each flask in a fume-hood. The precipitate formed was removed by centrifugation and 25λ of the supernatants were used for determination of radioactivity incorporated. The remaining supernatant was evaporated to 0.5 ml and 0.1 ml was spotted on a chromatogram.

Radioactivity counting

Radioactivity was measured with a lead-shielded "micromil" end-window, continuous gas-flow Geiger-Müller tube (Nuclear Chicago Model D-47) with a Nuclear Chicago Model 181A scaler. All counts were corrected for background and the counting time was selected so as to make the standard deviation within 5% of the total count.

Preparation of radioactive samples for counting

The radioactivity in the ethanol soluble fraction from $C^{14}O_2$ fixation experiments was counted by plating 0.1 ml onto aluminum planchets by means of a micropipette fitted with a propipette attachment. The sample was then evenly distributed by spreading with 0.5 ml

acetone and dried under an infra-red lamp. No correction for self-absorption was necessary since evaporation of the sample resulted in an extremely thin film on the planchet surface.

Radioautography

The position of radioactive compounds on the paper chromatograms was located by radioautography. Kodak no-screen medical X-ray film was fastened to the chromatogram by paper clips and the initial alignment marked by removal of small wedges from 2 adjacent sides. The whole was wrapped in black paper and stored in a cassette under a heavy object thus ensuring uniform contact between the chromatogram and the film. From the total counts per min found in each sample and from the expected number of spots per sample an exposure time of 2 days to 2 weeks was sufficient. The film was developed according to the manufacturer's specification.

Elution and transfer of spots

The compounds on the chromatograms, located through radioautography were transferred to the origins of new chromatograms for further separation and identification. The spot on the chromatogram was removed by making a wedge-shaped cut into the paper to include the spot concerned. The paper-wedge was stapled to another strip of paper which was immersed into water in a trough in a chromatography tank so that the wedge was hanging down from the trough and its apex just

fit into a tube which collected about 5 ml eluate. The material thus collected was evaporated to 0.1 ml by blowing a continuous stream of air over its surface.

Identification of radioactive compounds

Radioactive compounds in the ethanol soluble fraction were separated by two-dimensional paper chromatography. The chromatograms were thoroughly air-dried in a fume-hood after each solvent treatment and radioautograms prepared as outlined earlier. The radioactive spots were eluted with water, evaporated and rechromatographed one-dimensionally at 5° together with authentic compounds. The known compounds were sprayed with suitable spraying reagents for their detection while unknowns were located from radioautograms.

Determination of sulfur compounds

Thiosulfate and polythionates. Thiosulfate and tetrathionate were determined according to the procedure of Sörbo (1957), after the removal of proteins by Cd^{2+} ions. Thiosulfate was converted to thiocyanate in the presence of cyanide and cupric ions, then thiocyanate was determined as the iron complex by adding a ferric nitrate reagent. The optical density was read in a Klett-Summerson photoelectric colorimeter with a No 42 filter. Polythionates were converted to thiocyanate without the addition of cupric ions.

Sulfate. Sulfate was determined according to the benzidene method

of Letonoff and Reinhold (1936). Since this method was not always reliable and was affected by the presence of thiosulfate, the methods were always confirmed qualitatively by the formation of $BaSO_4$ precipitate upon treatment of the uranyl acetate supernatant with a solution of $BaCl_2$ in HCl. In some experiments $BaSO_4$ was isolated and determined gravimetrically.

Sulfite. Sulfite was determined by iodometric titration after hydrolysis of a formaldehyde-bisulfite complex with alkali as described by Suzuki and Silver (1966).

Sulfide. Sulfide was determined according to the methylene blue method of Fogo and Popowsky (1949), the resulting blue solution was measured in a Klett-Summerson photoelectric colorimeter with a No 66 filter.

Oxidation of sulfur compounds

Thiosulfate and tetrathionate. Oxidation of thiosulfate and tetrathionate was measured manometrically at 30° in a Warburg apparatus. The standard reaction mixture for the determination of thiosulfate - and tetrathionate - oxidizing activity contained, unless otherwise stated, 60 µmoles Tris-HCl (pH 8.0), 5.0 µmoles potassium phosphate (pH 8.0), 5.0 µmoles sodium thiosulfate or 7.5 µmoles sodium tetrathionate, enzyme and H_2O to make a final volume of 3.0 ml. The reaction was started by tipping the substrate from the side-arm of the Warburg vessel.

Sulfite. Sulfite oxidation was measured manometrically at 30° in a Warburg apparatus, or spectrophotometrically following the reduction of either ferricyanide or cytochrome c. Reaction mixtures for the manometric method consisted of 25 µmoles Tris-HCl (pH 8.0), 10 µmoles Na_2SO_3 in 5mM EDTA, enzyme and H_2O to a final volume of 3.0 ml. The reaction was started by the addition of substrate from the side-arm of the Warburg flask.

The spectrophotometric assay procedure for enzyme was a modification of the method of Peck (1965). The reaction mixture contained 5.0 µmoles Tris-HCl (pH 8.0), 1.5 µmoles $K_3Fe(CN)_6$ or 0.2 µmole cytochrome c, 5.0 µmoles Na_2SO_3 in 5mM EDTA, enzyme and water to a final volume of 3.0 ml. The reduction of ferricyanide or cytochrome c was followed in a Unicam SP-700 recording spectrophotometer (0.5 cm or 1.0 cm cell) at 400 mµ and 550 mµ respectively. The reduction of ferricyanide at low sulfite concentrations was followed in an Aminco Dual Wavelength spectrophotometer with λ_1 at 429 mµ and λ_2 at 440 mµ. The cytochrome c procedure was much more sensitive and required much less enzyme than the ferricyanide method. The activity of the enzyme was expressed as the number of µmoles of ferricyanide or cytochrome c reduced per hour calculated from the initial linear rate of reduction.

Sulfur. Sulfur oxidation was measured manometrically. Reaction mixtures consisted of 60 µmoles Tris-HCl (pH 8.0), 10 µmoles potassium phosphate (pH 8.0), sulfur 32 mg, 5.0 µmoles GSH where necessary,

enzyme and water to a final volume of 3.0 ml. The reaction was started by the addition of enzyme and gluthathione in separate side-arms of double side-arm flasks to the main compartment of the flask.

Sulfide. Sulfide oxidation was also measured manometrically. Reaction mixtures consisted of 10 µmoles Na_2S, 10 µmoles potassium phosphate (pH 8.0), 200 µmoles Tris-HCl (pH 8.0), enzyme and water to a final volume of 3.0 ml. The reaction was started by the addition of enzyme and substrate.

Oxidative phosphorylation in cell-free extracts

Oxidative phosphorylation during sulfite oxidation was studied by coupling ATP formation to the reduction of NADP with hexokinase and glucose-6-phosphate dehydrogenase (Pinchot, 1953). The standard reaction mixture contained 60 µmoles Tris-HCl (pH 7.8), 10 µmoles $MgCl_2$, 10 µmoles potassium phosphate (pH 7.8), 0.5 mg hexokinase, 10 µmoles glucose, 20 µmoles Na_2SO_3 in 5mM EDTA, 10 µmoles ADP, 20 µmoles NaF, extract (6.0 mg protein) and water to a final volume of 2.0 ml. The reaction was stopped by boiling the reaction mixtures from manometric experiments for 3 min. The precipitated protein was then removed by centrifugation. The supernatant was analyzed for glucose-6-phosphate by measuring the amount of NADP reduced in the presence of glucose-6-phosphate dehydrogenase at 340 mµ in a Unicam SP-700 recording spectrophotometer. The standard reaction mixture contained 100 µmoles

glycylglycine buffer pH 7.8, 1.0 μmole NADP, 0.2 mg glucose-6-phosphate dehydrogenase, 0.2 ml supernatant and water to a final volume of 2.0 ml.

RESULTS

RESULTS

I. Fixation of carbon dioxide

The mechanism of carbon dioxide fixation by T. novellus was investigated using both whole cells and extracts.

$C^{14}O_2$ fixation by whole cells

Washed cells of T. novellus rapidly incorporated radioactive carbonate into organic compounds. The fact that there was a greater uptake of $C^{14}O_2$ in the presence of thiosulfate than in its absence suggested the release of energy required for the CO_2 fixation process during thiosulfate oxidation. Typical results showing incorporation of radioactivity are found in Table I.

Identification of radioactive compounds

Radioactive compounds in the various samples were separated by two-dimensional paper chromatography and their locations revealed by radioautography. The labelling pattern for samples from endogenous fixation and fixation in the presence of glucose (based on the Rf's of the various spots) were similar, and differed from fixation in the presence of thiosulfate. Spots from this latter sample were eluted and rechromatographed one-dimensionally along with known compounds. From a comparison of the Rf's and the intensity of the spots on the radioautogram, it was found that the majority of the activity after 2 sec exposure was located in 3-PGA; aspartic acid and a phos-

TABLE I

$C^{14}O_2$ uptake in whole cells of *T. novellus*

Time	Endogenous fixation c.p.m.	plus glucose c.p.m.	plus $Na_2S_2O_3$ c.p.m.
2 sec	1.5×10^3	4.1×10^3	9.0×10^3
10 sec	4.3×10^3	1.2×10^4	2.3×10^4
30 sec	1.6×10^4	2.1×10^4	4.5×10^4
2.0 min	5.1×10^4	1.4×10^5	8.1×10^4

The reaction was carried out under the conditions described in Materials and Methods.

phorylated sugar (glucose-6-phosphate or fructose-1,6-diphosphate) being the other compounds labelled.

After 10 sec exposure 4 spots were detected, 3-PGA which still had most of the radioactivity incorporated, a phosphorylated sugar which seemed to have the next highest amount of radioactivity and aspartic and glutamic acids.

Since 3-PGA was one of the earliest compounds labelled, the mechanism appeared to be similar to that in plants (Calvin and Benson, 1948) and in other species of thiobacilli (Santer and Vishniac, 1955; Trudinger, 1955, 1956; Milhaud et al., 1956; and Suzuki and Werkman, 1958[a]). For these reasons no further investigation was undertaken. The presence of aspartate and glutamate indicated the presence of PEP carboxylase which is believed to be responsible for the labelling of the β-carboxyl group of aspartate and γ-carboxyl group of glutamate, respectively (Suzuki and Werkman, 1958[b]).

$C^{14}O_2$ fixation by extracts

Results of $C^{14}O_2$ incorporation experiments by extracts of autotrophically grown cells and cells grown on glucose mineral salts medium are shown in Table II. From these results it can be seen that maximal incorporation occurred in autotrophic extracts only in the presence of R5P and ATP. There was also considerable uptake in the absence of ATP. This can be explained as being the result of endogenous metabolism. The incorporation of CO_2 in the absence of

TABLE II

$C^{14}O_2$ incorporation by cell-free extracts of T. novellus

Reaction mixture	Extracts from glucose grown cells. Total c.p.m.	Extracts from autotrophic cells. Total c.p.m.
Complete	$< 2 \times 10^2$	1.5×10^5
Minus R5P	$< 2 \times 10^2$	3.7×10^3
Minus ATP	$< 2 \times 10^2$	1.1×10^4

The reaction was carried out under the conditions described in Materials and Methods.

R5P can be interpreted as showing that this intermediate was either present in extracts, or was formed during endogenous metabolism. There was very little fixation by extracts grown on glucose.

The only compound detected by chromatography and radioautography as a result of $C^{14}O_2$ fixation by cell-free extracts was 3-PGA.

These results clearly demonstrated the presence of carboxydismutase in autotrophically grown cells, but not in heterotrophically grown cells. Thus the autotrophic growth was essential for the formation of this enzyme.

II. Oxidation of inorganic sulfur compounds

Oxidation of thiosulfate and related inorganic sulfur compounds by both whole cells and cell-free extracts was studied in order to elucidate the mechanism of thiosulfate oxidation.

Oxidation of thiosulfate

Both whole cells and cell-free extracts oxidized thiosulfate completely and at a linear rate as shown in Figs. 1 and 2, even though different batches of cells and extracts showed different activities. There was no polythionate accumulation during the thiosulfate oxidation by T. novellus cells or extracts. At the end of the oxidation period, there was no thiosulfate left in the reaction mixtures. The amount of oxygen consumed and the amount of sulfate determined both by colorimetric and gravimetric methods corresponded to the amounts expected from Eqn. I in whole cell experiments.

Fig. 1. Thiosulfate oxidation by whole cells of T. novellus.

The reaction mixture was the same as that described in Materials and Methods with 10 mg (wet weight) whole cells.

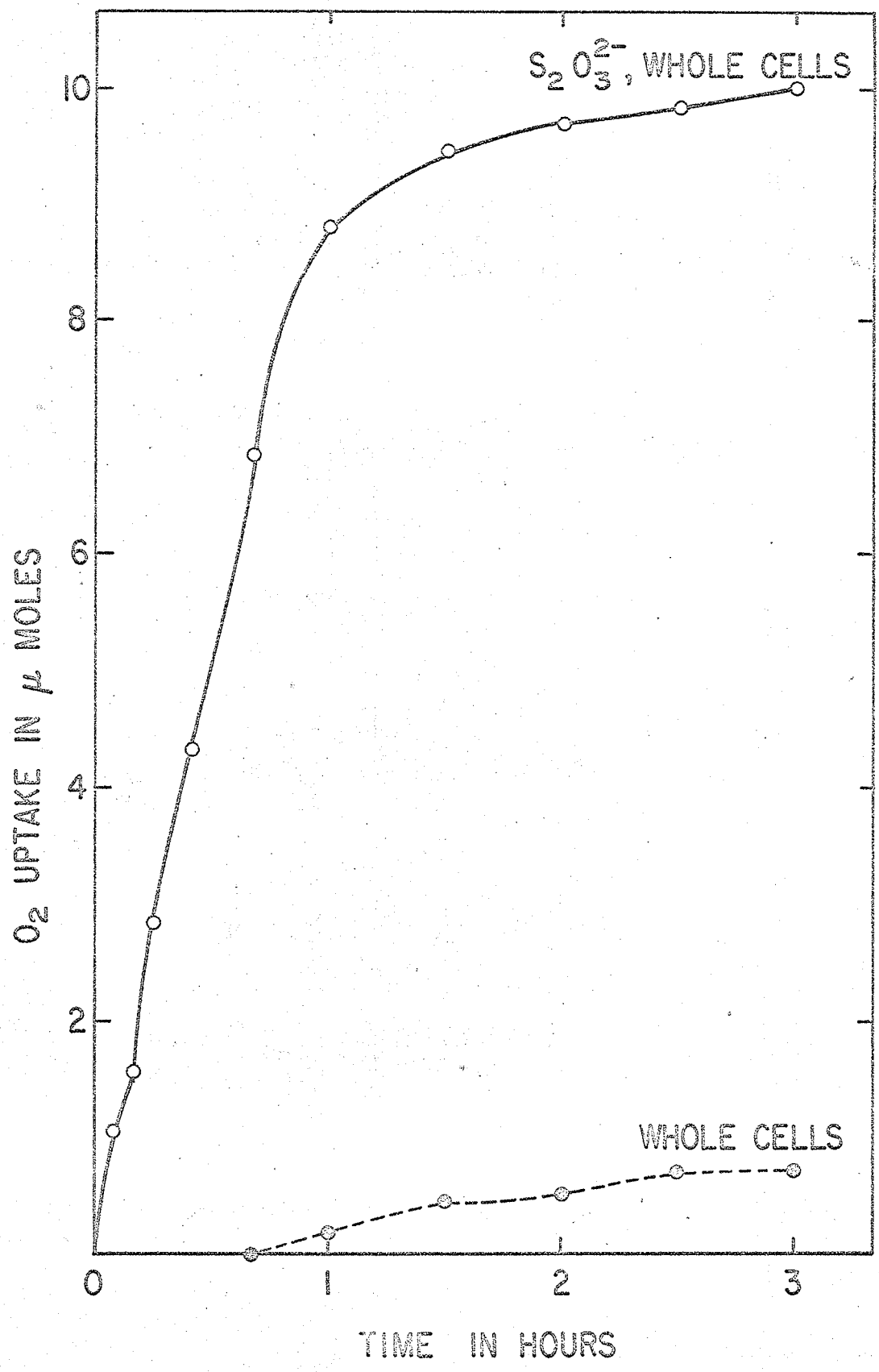
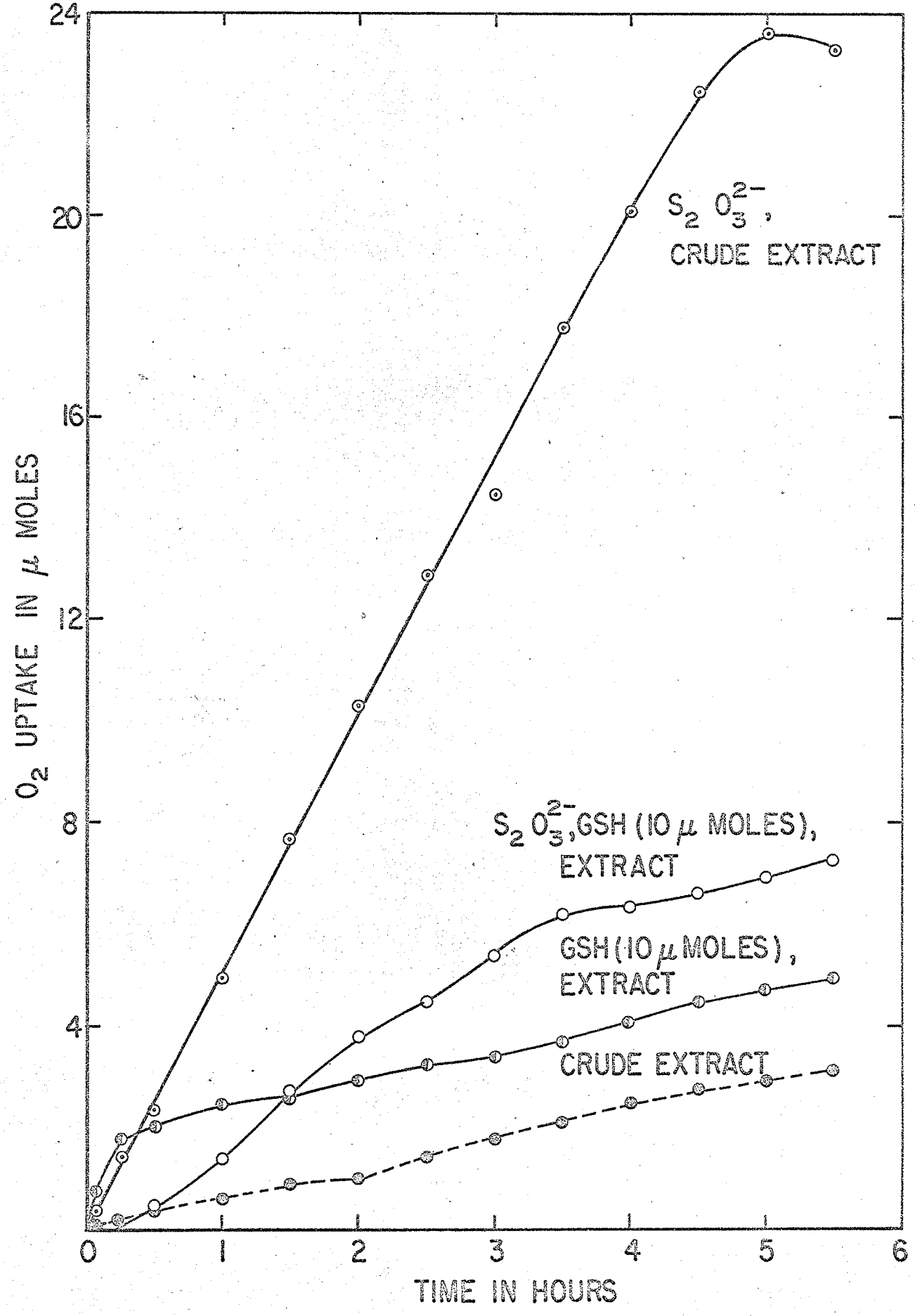

$S_2O_3^{2-}$, WHOLE CELLS
WHOLE CELLS
O_2 UPTAKE IN μ MOLES
TIME IN HOURS
0
1
2
3
2
4
6
8
10

Fig. 2. Thiosulfate oxidation by crude extracts of *T. novellus*.

The reaction mixture contained Tris-HCl (pH 8.0), 200 μmoles; potassium phosphate (pH 8.0), 10 μmoles; $Na_2S_2O_3$, 10 μmoles; GSH (where required), 10 μmoles; extract, 6.0 mg.


24
20
16
12
8
4
0
1
2
3
4
5
6
O_2 UPTAKE IN μ MOLES
TIME IN HOURS
$S_2O_3^{2-}$, CRUDE EXTRACT
$S_2O_3^{2-}$, GSH (10 μ MOLES), EXTRACT
GSH (10 μ MOLES), EXTRACT
CRUDE EXTRACT

$$S_2O_3^{2-} + H_2O + 2O_2 \longrightarrow 2SO_4^{2-} + 2H^+ \qquad (1)$$

With cell-free extracts, however, the amount of sulfate determined varied among different batches of extracts from 50 to 75% of the theoretical amount in agreement with the results reported by London and Rittenberg (1964). The uniform rate of thiosulfate oxidation shown in Fig. I is unlike the oxidative pattern observed by Vishniac (1952) with T. thioparus cells where there was an initial rapid oxygen uptake which corresponded to the formation of tetrathionate, followed by a slower oxidation period which coincided with the disappearance of tetrathionate.

The uniform rate of thiosulfate oxidation was observed also by London and Rittenberg (1964) with extracts of T. thioparus and T. thiooxidans. They detected the accumulation of polythionates with the former organism but not with the latter. Thiosulfate was oxidized with a consumption of 2 moles of oxygen for every mole of thiosulfate by both whole cells and cell-free extracts as expected from Eqn. 1 (Figs. 1 and 2). GSH was inhibitory for the oxidation by cell-free extracts in contrast with the results of Peck (1960) and Peck and Fisher (1962) with T. thioparus.

Oxidation of tetrathionate

Since tetrathionate is the key intermediate in the pathway involving polythionates, the oxidation of tetrathionate by T. novellus was investigated.

Although polythionates did not accumulate during thiosulfate oxidation, tetrathionate was rapidly oxidized to sulfate by whole cells. The cell-free extract, however, did not oxidize tetrathionate. Since the extract could oxidize thiosulfate to sulfate, it is not likely that tetrathionate was an intermediate.

Oxidation of sulfite

Sulfite was rapidly oxidized to sulfate either by whole cells or cell-free extracts, 0.5 mole oxygen being consumed for every mole of sulfite oxidized (Table III). Cell-free extracts contained an active enzyme which reduced either ferricyanide or cytochrome c with sulfite (Charles and Suzuki, 1965).

No requirement was found for AMP and other constituents of the APS-reductase assay system. In order to establish the different nature of the sulfite-oxidizing system of T. novellus from that of the APS-reductase of T. thioparus, sulfite oxidase was purified from T. novellus extracts while following the sulfite: ferricyanide oxidoreductase activity as described later.

As shown in Table III sulfite oxidase alone did not consume oxygen with sulfite, but in the presence of mammalian cytochrome c and cytochrome oxidase a rapid oxidation took place. The crude cytochrome oxidase preparation and cytochrome c alone did not oxidize sulfite. As shown in Fig. 3 sulfite oxidase reduced cytochrome c with sulfite as an electron donor. The addition of cytochrome

Fig. 3. Reduction of mammalian cytochrome c with sulfite and sulfite oxidase and its oxidation with cytochrome oxidase and air.

The reaction mixture contained in a final volume of 3.0 ml:Tris-HCl (pH 7.8), 25 μmoles; Na_2SO_3 in 5 mM EDTA, 5 μmoles; sulfite oxidase, 0.35 μg protein; mammalian cytochrome c, 0.15 μmole; and T. novellus cytochrome oxidase, 0.4 mg protein. The spectrum was followed in a Unicam SP-700 recording spectrophotometer with a 1 cm anaerobic cell. The reference cell contained all the reagents except Na_2SO_3. The reaction was started by the addition of Na_2SO_3 to the sample cell and the cell made anaerobic by evacuation. A: One min after the addition of Na_2SO_3. B: 6 min after the addition of Na_2SO_3. Cytochrome oxidase was then added from the side arm. C: 5 min after the addition. Air was then introduced and the cell and contents were shaken for 1 min. D: 5 min after the introduction of air.

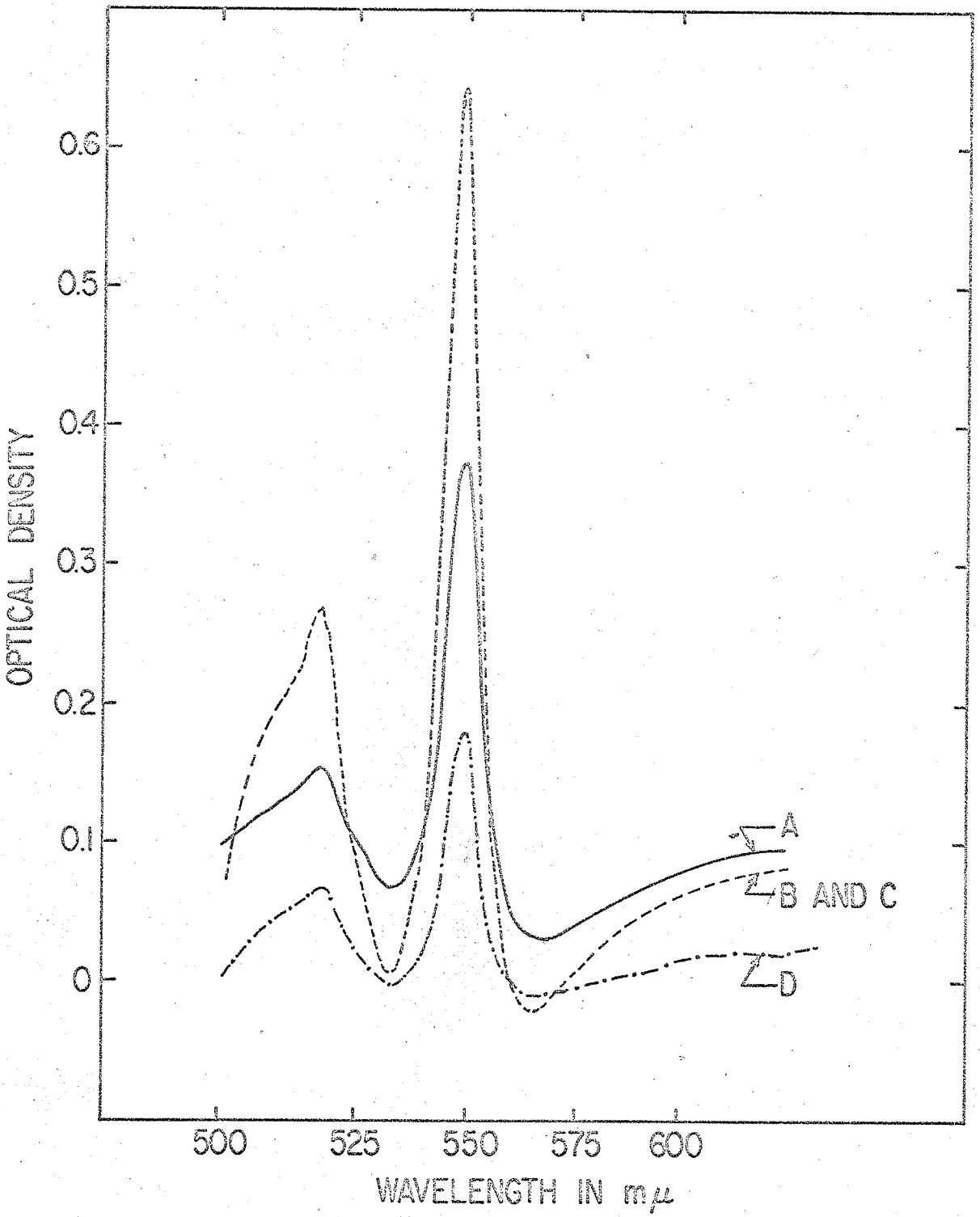

OPTICAL DENSITY
0.6
0.5
0.4
0.3
0.2
0.1
0
500
525
550
575
600
WAVELENGTH IN mμ
A
B AND C
D

TABLE III

Sulfite oxidation by whole cells, extracts and sulfite oxidase

Enzyme source	Na_2SO_3 (μmoles)	Time for completion of oxidation (min)	O_2 consumed in 30 min (μmoles)
Whole cells	10.0	25	4.5
Crude extract	10.0	25	4.7
Sulfite oxidase	5.0	-	0.0
Plus cyt c	5.0	-	0.0
Plus cyt oxidase	5.0	-	0.0
Plus cyt c plus cyt oxidase	5.0	15	2.1

Reaction mixtures contained (in μmoles): Tris-HCl (pH 7.8), 80; Na_2SO_3 in 5mM EDTA as indicated; whole cells 10 mg (wet weight) or crude extract 6 mg protein; and H_2O to a final volume of 3.0 ml. For purified sulfite oxidase experiments: Tris-HCl (pH 8.0), 30; Na_2SO_3 in 5mM EDTA as indicated; mammalian cytochrome c, 0.15; and cytochrome oxidase; 0.4 mg when indicated; sulfite oxidase, 90 μg protein and H_2O to a final volume of 3.0 ml.

oxidase and introduction of air resulted in the reoxidation of reduced cytochrome c. A similar spectrum change was observed in the manometric experiments of Table III. In the absence of cytochrome oxidase, cytochrome c was reduced by sulfite and remained reduced. Table IV shows the inhibition of sulfite oxidation by crude extracts with known cytochrome c oxidase inhibitors. Azide was a potent inhibitor of the reaction. The inhibition by cyanide required a higher concentration for the same degree of inhibition.

From these results, the mechanism of sulfite oxidation by T. novellus was formulated as follows:

$$SO_3^{2-} + 2\text{cyt } c\ Fe^{3+} + H_2O \xrightarrow[\text{oxidase}]{\text{sulfite}} SO_4^{2-} + 2\text{cyt } c Fe^{2+} + 2H^+ \quad (2)$$

$$2\text{cyt } c\ Fe^{2+} + 1/2O_2 + 2H^+ \xrightarrow[\text{oxidase}]{\text{cyt}} 2\text{cyt } c\ Fe^{3+} + H_2O \quad (3)$$

Since the natural electron acceptor for sulfite oxidase is a native cytochrome c of T. novellus as shown later, the enzyme is referred to as sulfite: cytochrome c oxidoreductase.

Sulfur oxidation by whole cells and extracts

Whole cells of T. novellus oxidized elemental sulfur in the absence of GSH, although GSH stimulated the oxidation in early incubation periods (Fig. 4). Cell-free extracts, on the other hand, oxidized sulfur only in the presence of GSH (Fig. 5).

An enzyme which oxidizes elemental sulfur with GSH as cofactor

Fig. 4. Sulfur oxidation by whole cells of T. novellus.

The reaction mixture was the same as that described in Materials and Methods with 10 mg (wet weight) whole cells.

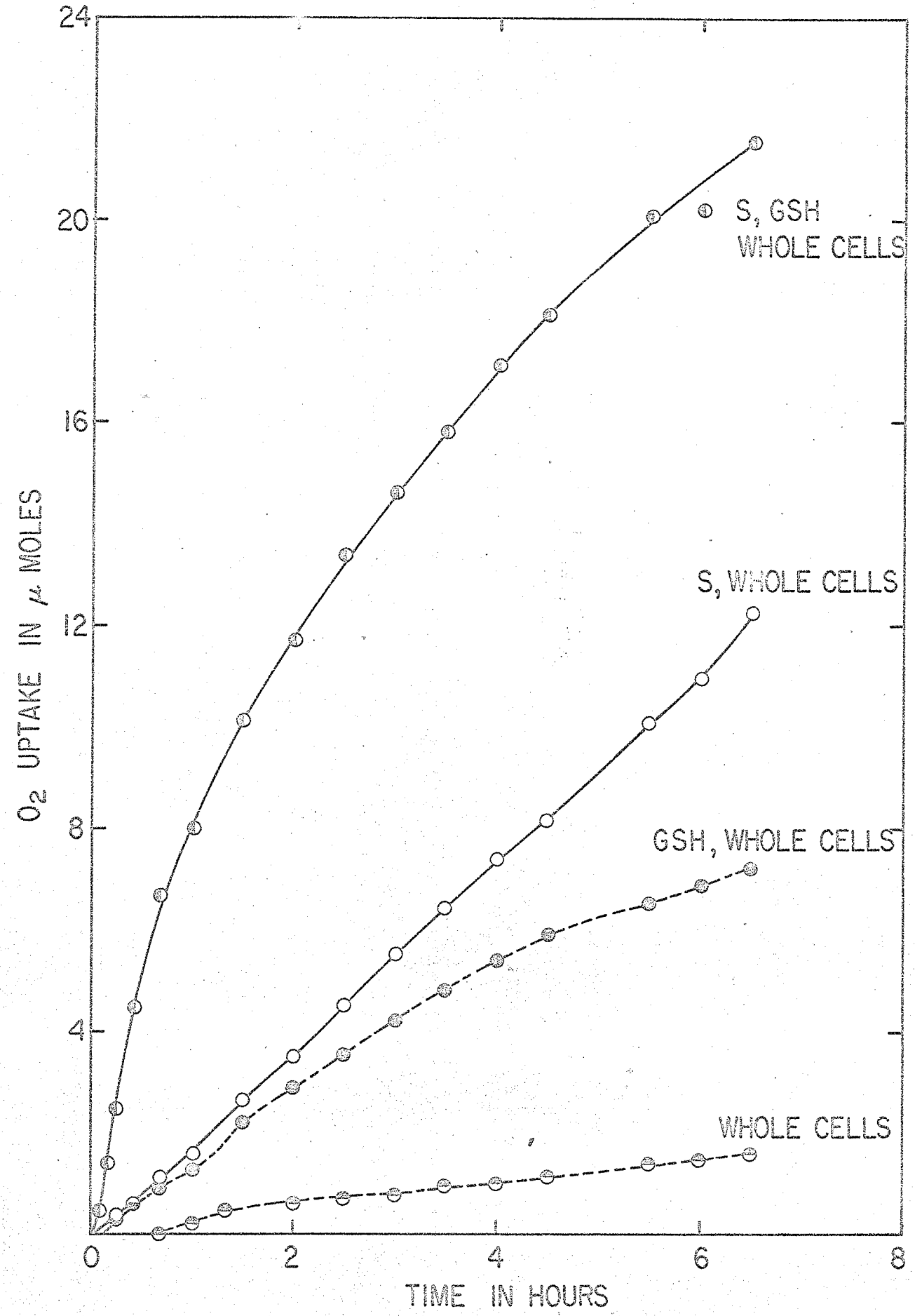

24
20
16
12
8
4
0
2
4
6
8
O_2 UPTAKE IN μ MOLES
TIME IN HOURS
S, GSH
WHOLE CELLS
S, WHOLE CELLS
GSH, WHOLE CELLS
WHOLE CELLS

TABLE IV

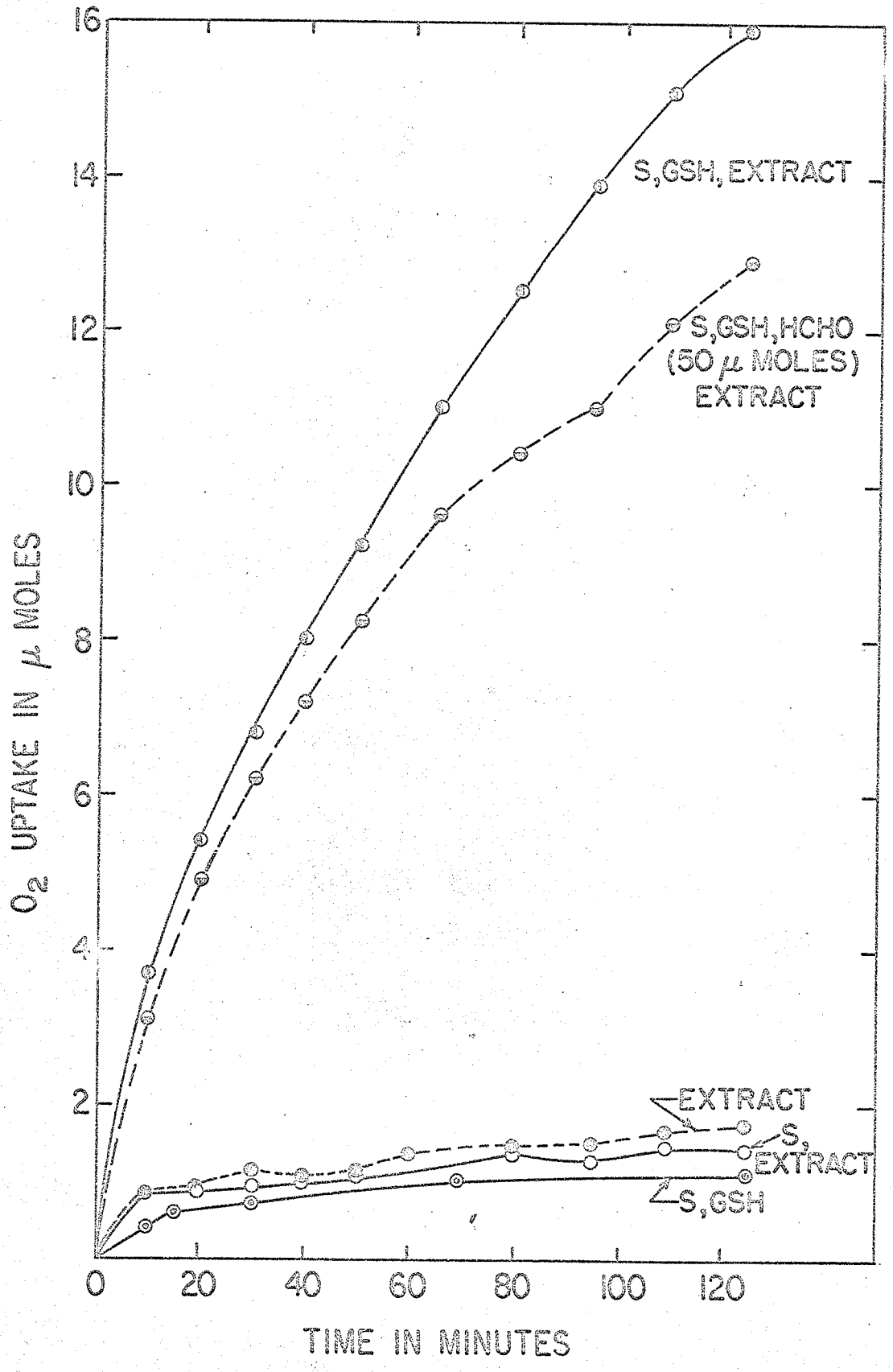
Inhibition of sulfite oxidation by cyanide and azide

Inhibitor	Concentration	O_2 Uptake (μmoles)	% Inhibition
None	-	7.1	0.0
KCN	4×10^{-3} M	6.0	15.5
	8×10^{-2} M	0	100.0
NaN_3	4×10^{-3} M	0.6	91.5

Reaction mixtures contained: Tris-HCl (pH 8.0), 200 μmoles, Na_2SO_3 in 5mM EDTA, 10 μmoles; potassium phosphate (pH 8.0) 10 μmoles; extract, 4.4 mg protein; inhibitor and water to a final volume of 2.5 ml.

Fig. 5. Sulfur oxidation by crude extracts.

The reaction mixture was the same as that described in Materials and Methods with 6.0 mg extract.


16
14
12
10
8
6
4
2
0
20
40
60
80
100
120
O_2 UPTAKE IN μ MOLES
TIME IN MINUTES
S, GSH, EXTRACT
S, GSH, HCHO (50 μ MOLES) EXTRACT
EXTRACT
S, EXTRACT
S, GSH

has been isolated and partially purified by Suzuki (1965) from T. thiooxidans and Suzuki and Silver (1966) from T. thioparus. The reaction catalyzed by the enzyme is the initial oxidation of sulfur to sulfite followed by a non-enzymatic formation of thiosulfate from sulfur and sulfite:

$$S + O_2 + H_2O \xrightarrow{\text{enzyme}} SO_3^{2-} + 2H^+ \qquad (4)$$

$$S + SO_3^{2-} \longrightarrow S_2O_3^{2-} \qquad (5)$$

The only product isolated from the oxidation of sulfur by whole cells or extracts of T. novellus was sulfate, since the sulfite-oxidizing system was very active. Even in the presence of formaldehyde, which effectively trapped sulfite during sulfur oxidation by purified enzyme preparations of T. thiooxidans and T. thioparus, the only product was sulfate. This result is apparently due to the very high affinity of T. novellus sulfite oxidase for sulfite as will be shown later. The sulfur-oxidizing enzyme of T. novellus was partially purified during the purification of sulfite oxidase. The enzyme was eluted from DEAE-cellulose with 0.2M potassium phosphate and was free of sulfite oxidase activity. The enzyme was often associated with a c-type cytochrome and attempts to purify it further with ethanol or acid precipitation resulted in complete loss of enzyme activity. Although the activity of partially purified enzyme was quite low, it was possible to show the accumulation of thiosulfate during sulfur oxidation and the specificity for

GSH as cofactor. Mercaptoethanol and cysteine did not replace GSH. The ratio of oxygen consumed to thiosulfate formed was 1 to 1 in accordance with Eqns. 4 and 5. Thus the enzyme is very similar to the sulfur-oxidizing enzyme of T. thiooxidans (Suzuki, 1965) and T. thioparus (Suzuki and Silver, 1966).

Thiosulfate-cleaving system

Since cell-free extracts of T. novellus oxidized thiosulfate in the absence of GSH and without the accumulation of polythionates, it was postulated that thiosulfate (SSO_3^{2-}) was initially cleaved to sulfur (S) and sulfite (SO_3^{2-}) which were then oxidized by the sulfur-oxidizing system and sulfite-oxidizing system, respectively. Cell-free extracts did not reduce cytochrome c with thiosulfate. Since the same extracts reduced cytochrome c with sulfite very rapidly, the failure of cytochrome c reduction was taken as the absence of thiosulfate-cleavage reaction. Upon addition of cyanide, however, the extracts reduced cytochrome c with thiosulfate as shown in Table V. The reduction was faster at a thiosulfate concentration of 3.3 mM than 33mM, apparently due to the salt inhibition of sulfite oxidase as will be shown later.

These results suggested the presence of rhodanese in extracts. Rhodanese was demonstrated recently in extracts of Thiobacillus denitrificans by Bowen et al., (1965). In the presence of rhodanese the cytochrome reduction by thiosulfate may be explained by a com-

TABLE V

Requirements for the assay of thiosulfate-cleaving enzyme

System	µmoles cyt c reduced /mg protein /hr.
Complete	52
Complete plus 90 µmoles $S_2O_3^{2-}$	25
Minus cyt c	0
Minus $S_2O_3^{2-}$	0
Minus enzyme	0
Minus KCN	0

The complete system: Tris HCl (pH 7.8), 25 µmoles; cyt c, 0.15 µmole; $Na_2S_2O_3$, 10 µmoles; KCN, 0.3 µmole; extract, 2.2 mg protein in a total volume of 3.0 ml.

bination of Eqn. 6:

$$SSO_3^{2-} + CN^- \xrightarrow{\text{rhodanese}} SCN^- + SO_3^{2-} \qquad (6)$$

and Eqn. 2. In this scheme sulfite formed by the action of rhodanese on thiosulfate is oxidized by sulfite oxidase to sulfate with the reduction of cytochrome c.

The rhodanese activity in cell-free extracts of T. novellus was directly demonstrated by incubating the extracts (2.2mg protein) with 10 μmoles of thiosulfate, 10 μmoles of KCN and 25 μmoles Tris-HCl (pH 7.8) in a total volume of 3.0 ml at 30° C. After 60 min 5 μmoles of thiocyanate were formed. Under these conditions the omission of KCN, thiosulfate or extracts resulted in complete lack of thiocyanate formation.

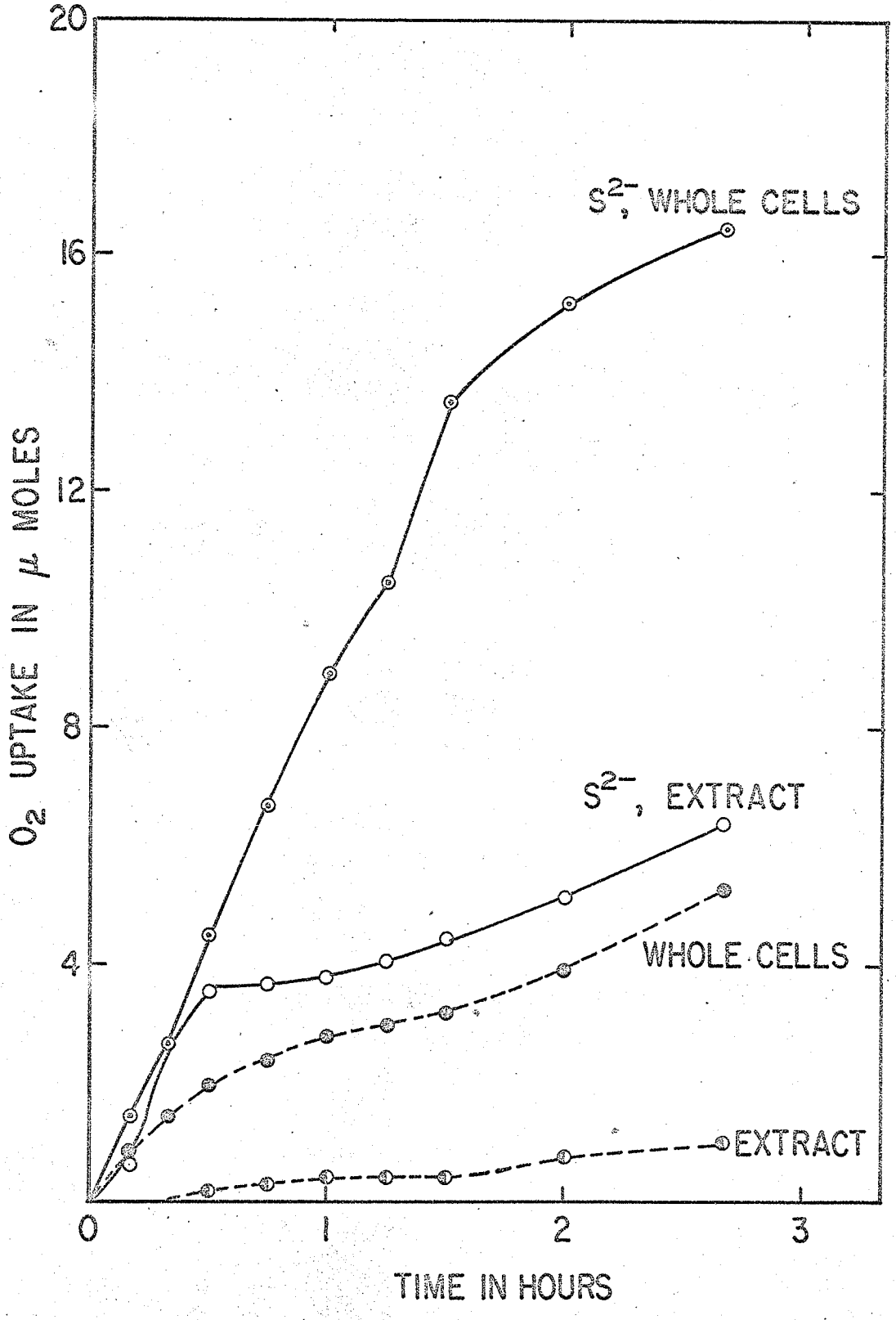
Sulfide oxidation

Sulfide was oxidized by both whole cells and crude extracts (Fig. 6). Whole cells oxidized sulfide rapidly and at a linear rate until all the substrate was utilized. The only product formed was sulfate. Crude extracts, however, initially oxidized sulfide rapidly resulting in colloidal sulfur formation visible in the reaction mixture which had become cream-colored and turbid after 20 min incubation. Subsequent to this rapid oxidation there was a slower oxidation period during which the sulfur disappeared.

The products of cell-free sulfide oxidation were sulfate and

Fig. 6. Sulfide oxidation by T. novellus.

The reaction mixture consisted of Tris-HCl (pH 8.0), 100 µmoles; potassium phosphate (pH 8.0), 10 µmoles; Na_2S, 5.0 µmoles; and either whole cells, 32 mg (wet weight), or extract, 6.0 mg.


20
16
12
8
4
0
1
2
3
O_2 UPTAKE IN μ MOLES
TIME IN HOURS
S^{2-}, WHOLE CELLS
S^{2-}, EXTRACT
WHOLE CELLS
EXTRACT

thiosulfate. From these experiments it is evident that sulfide was first oxidized to sulfur and thiosulfate as was reported for T. thiooxidans (Suzuki and Werkman, 1959). Sulfur and thiosulfate were then slowly oxidized to sulfate. Although the extracts oxidized thiosulfate, the oxidation of elemental sulfur required GSH as shown in previous sections. The disappearance of colloidal sulfur during long incubation periods may be due, therefore, to its colloidal nature. The rapid and linear rate of sulfide oxidation by whole cells may be attributed to the ability of the cells to oxidize elemental sulfur.

III. Purification and properties of sulfite:cytochrome c oxidoreductase

Since sulfite oxidase (sulfite:cytochrome c oxidoreductase) seemed to be a key enzyme in the oxidation of thiosulfate by T. novellus and also since this enzyme seemed to be a new enzyme distinct from APS-reductase of T. thioparus (Peck, 1961), the enzyme was purified from cell-free extracts of T. novellus and its properties studied.

Purification and stability of sulfite:cytochrome c oxidoreductase

Crude cell-free extracts of T. novellus were diluted with an equal volume of 0.2M Tris-HCl (pH 8.0) and solid ammonium sulfate was added to a concentration of 40%. After 20 min the suspension was centrifuged at 17,300 x g for 20 min. The supernatant was then dialysed against several changes of 0.002M Tris-HCl (pH 8.0). To a DEAE-cellulose column, 1.0 cm x 15 cm, was added 15 ml dialysed

supernatant. A red band of cytochrome adhered at the top of the column. The column was eluted with successive additions of 20 ml of 0.002M, 40 ml each of 0.01M and 0.02M and 20 ml each of 0.04M, 0.2M and 0.5M potassium phosphate buffer (pH 7.0). Cytochrome gave two major elution peaks. Approximately half the amount of cytochrome was eluted with 0.002M buffer and the other half with 0.2M. The enzyme was always located in the fraction eluted in 0.02M phosphate. Tubes containing the enzyme were pooled and the enzyme was concentrated in a dialysis bag placed in crystalline sucrose at 5°. The concentrated enzyme was dialysed against several changes of 0.002M Tris-HCl (pH 8.0) and made up to the original volume with the same buffer. To 5.0 ml extract from DEAE-cellulose step, containing 250 µg protein per ml, was added 1.2 ml alumina cγ gel (40 mg/ml). Most of the enzyme activity was located in the supernatant and the protein concentration was reduced to 106 µg protein per ml. As shown in the typical purification results of Table VI, these purification procedures resulted in a 77-fold purification of the enzyme with 25% recovery of the total activity.

The purified enzyme preparation retained high activity for more than six months when stored at -20° even with repeated freezing and thawing. However, treatment of enzyme with 50% ethanol resulted in significant loss of activity. The activity was also completely destroyed by heating at 60° for 1 min or 55° for 5 min. The

TABLE VI

Purification of sulfite oxidase

Fraction	Total Protein mg/ml	Total activity $Fe(CN)_6^{3-}$ in umoles reduced/hr	Specific activity $Fe(CN)_6^{3-}$ in umoles reduced/mg protein/hr	Per cent recovery
Crude extracts	15.2	1722	113	100
Supernatant after 40% $(NH_4)_2SO_4$	7.0	1870	267	109
DEAE-cellulose Supernatant	0.32	945	2950	55
Alumina C_γ gel	0.106	920	8670	53

addition of sulfite afforded some protection since the enzyme retained at least 75% of the activity in the presence of 0.05M sodium sulfite when heated at 55° for 5 min.

Effect of enzyme concentration

The relationship between activity and protein concentration was determined by the ferricyanide assay procedure. A linear relationship was obtained as shown in Fig. 7.

Comparison with APS-reductase

The reduction of ferricyanide by sulfite with purified sulfite oxidase was inhibited by AMP and other constituents of the APS-reductase assay system (Peck, 1961) as shown in Table VII, AMP is one of the substrates of APS-reductase and the lack of requirement by a purified enzyme preparation is a clear proof that the enzyme is not APS-reductase.

Further proof of dissimilarity was obtained by measuring the rate of APS reduction to AMP and sulfite with reduced benzyl viologen. Reaction mixtures contained (in μmoles): Tris-HCl (pH 8.0), 60; benzyl viologen, 0.5; $Na_2S_2O_4$, 0.5; APS, 10; enzyme and water in a final volume of 2.0 ml. Hydrosulfite was added to the benzyl viologen in a side-arm under an atmosphere of helium gas, and the reaction was started by tipping the contents of the side-arm into the Warburg flask. Neither crude extracts nor sulfite

Fig. 7. Effect of enzyme concentration.

The reaction mixture consisted of Tris-HCl (pH 8.0), 10 μmoles; $K_3Fe(CN)_6$, 1.5 μmoles; Na_2SO_3 in 5 mM EDTA, 5.0 μmoles; purified enzyme and H_2O to a final volume of 1.5 ml.

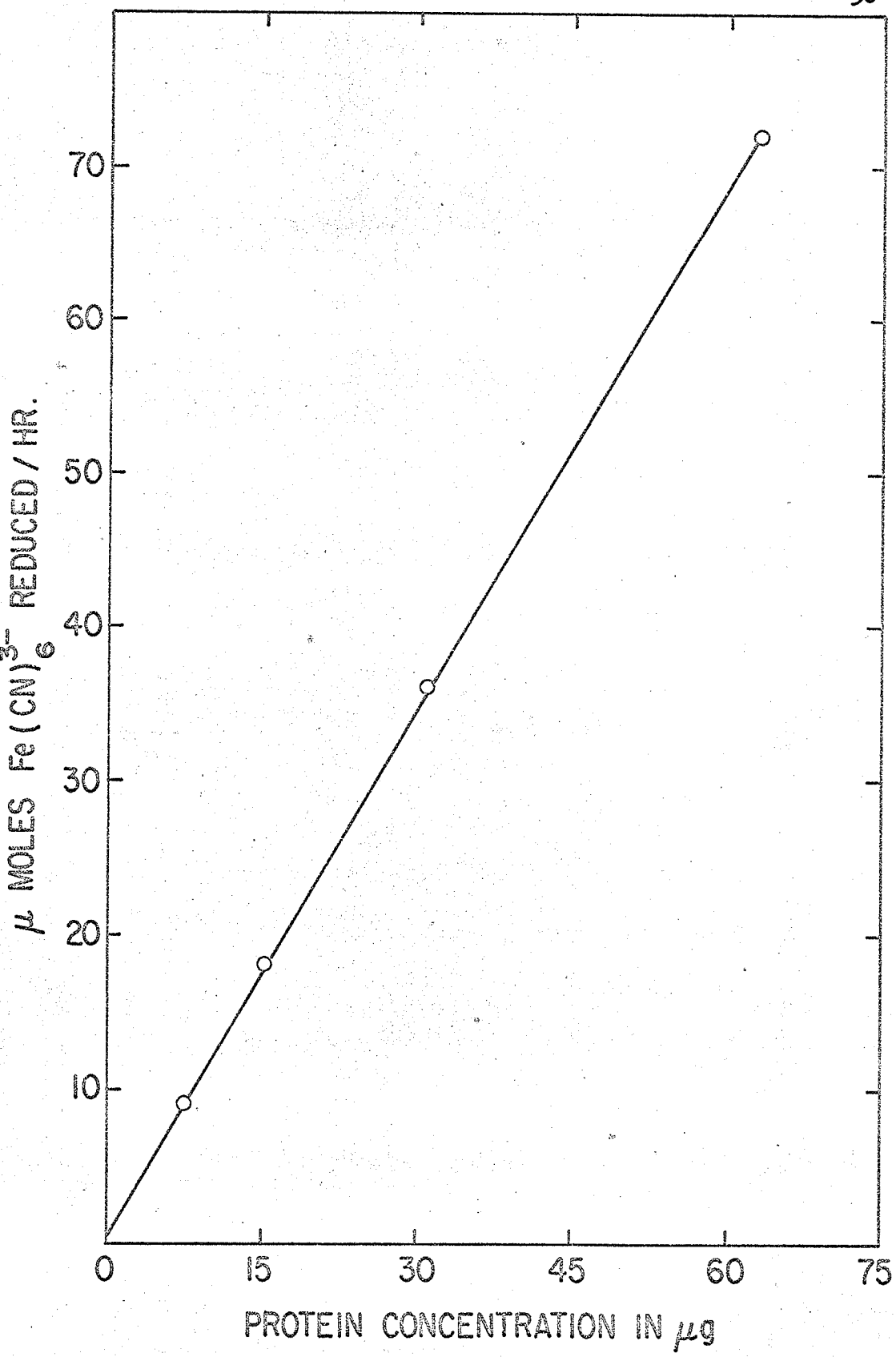

70
60
50
40
30
20
10
μ MOLES Fe(CN)$_6^{3-}$ REDUCED / HR.
0
15
30
45
60
75
PROTEIN CONCENTRATION IN μg

TABLE VII

Inhibition of sulfite:cytochrome c oxidoreductase by constituents of APS-reductase assay

Additions	Activity in µmoles $K_3Fe(CN)_6$ reduced/mg protein/hr	% inhibition
A. None	2,707	0
B. 10 µmoles AMP	2,030	25
C. 10 µmoles NaF	2,233	17.5
D. 5.0 µmoles EDTA	2,165	20
E. Combination of B,C,D	1,353	50

Reaction mixtures contained, Tris-HCl buffer (pH 8.0), 50 µmoles; $K_3Fe(CN)_6$, 1.5 µmoles; Na_2SO_3 in 5mM EDTA, 5.0 µmoles; enzyme protein, 19 µg; water and additions to make a final volume of 1.5 ml. Activity without additions taken as 100%.

oxidase of T. novellus decolorized the dye after 4 hr at 30°. Crude extracts of T. thioparus (ATCC 8158) oxidized the dye completely in the presence of APS within 45 min.

Stoichiometry of ferricyanide and cytochrome c reduction

Stoichiometry of the sulfite oxidase reaction was investigated and it was found that 2.0 moles of $Fe(CN)_6^{3-}$ were reduced for each mole of sulfite added according to the following equation:

$$SO_3^{2-} + H_2O + 2Fe(CN)_6^{3-} \longrightarrow SO_4^{2-} + 2H^+ + 2Fe(CN)_6^{4-} \quad (7)$$

Similarly 2.0 moles of mammalian cytochrome c were reduced for every mole of sulfite oxidized according to the equation:

$$SO_3^{2-} + 2\text{cyt } c\ Fe^{3+} + H_2O \longrightarrow SO_4^{2-} + 2\text{cyt } c\ Fe^{2+} + 2H^+ \quad (8)$$

This calculation was based on the assumption that the mM extinction coefficient of cytochrome c was 27.6 (Margoliash, 1954).*

Effect of pH on enzyme activity

The optimal pH of enzyme reaction was around pH 8.0 with potassium phosphate buffers, while with Tris-HCl, a plateau occurred from pH 7.0 to 7.8 after which there was a sharp decrease in activity. The results are shown in Fig. 8.

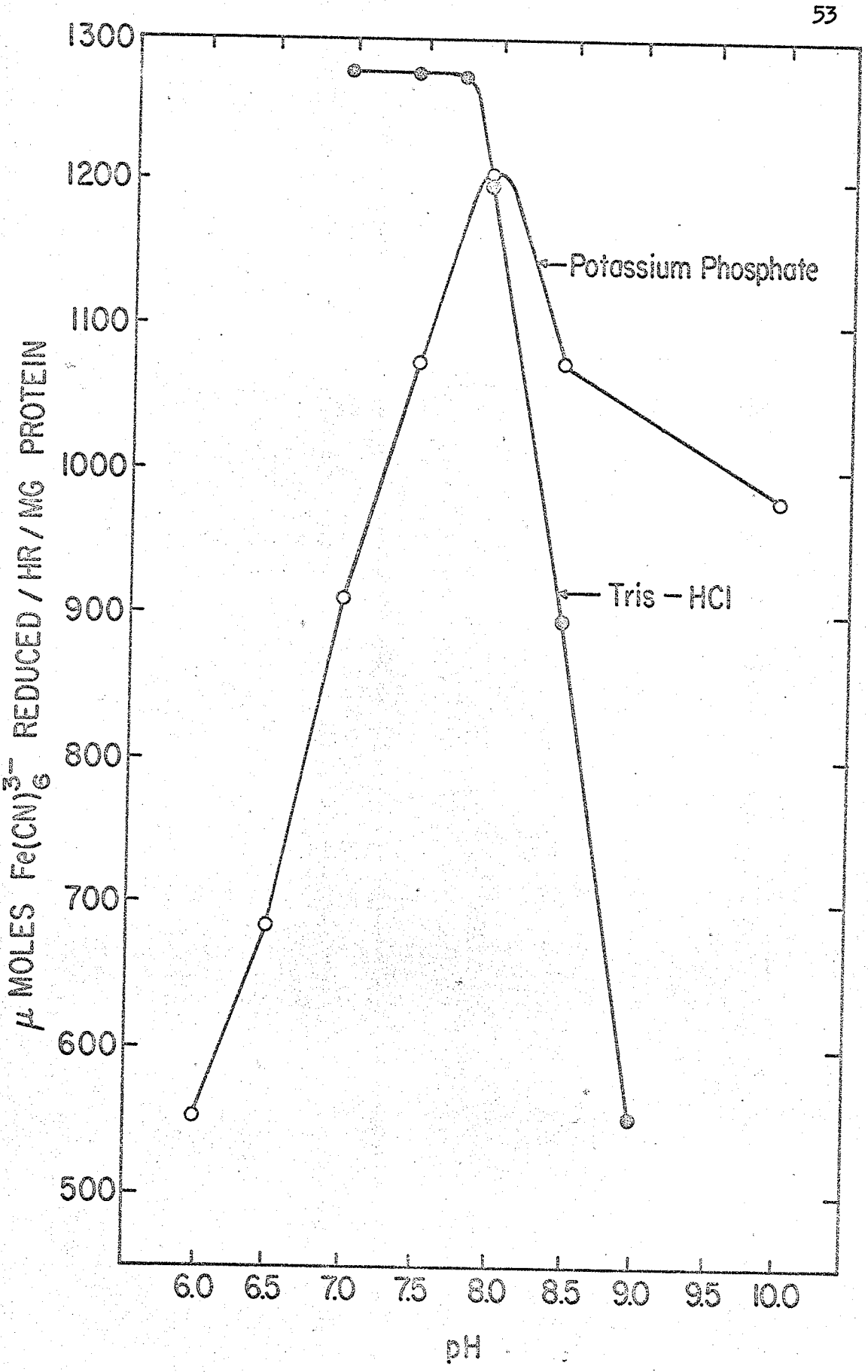
Cofactor requirements

No cofactor requirement has been found for the enzyme. Dialysis of extracts or purified enzyme against several changes of 0.002M

*at 550 mμ

Fig. 8. Effect of pH.

The reaction mixture contained Tris-HCl or potassium phosphate, 5.0 μmoles, $K_3Fe(CN)_6$, 1.5 μmoles; Na_2SO_3 in 5 mM EDTA, 5.0 μmoles; enzyme protein 30 μg and water to a final volume of 1.5 ml.


1300
1200
1100
1000
900
800
700
600
500
μ MOLES $Fe(CN)_6^{3-}$ REDUCED / HR / MG PROTEIN
Potassium Phosphate
Tris – HCl
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
10.0
pH

Tris-HCl (pH 8.0) for 8 hr at 3-5° did not result in significant loss of activity. The enzyme was not inhibited by atebrin at 3×10^{-4} M and the purified enzyme showed only one absorption peak at 280 mμ in the ultraviolet range and occasionally a small γ-peak of contaminating native cytochrome c in the visible range. Although at least 25% of the total enzyme activity was retained during purification procedures, the cytochrome content decreased to less than 0.1% of the amount present in crude extracts.

Substrate specificity

Using both the ferricyanide and the cytochrome c assay procedures, it was found that the enzyme was specific for sulfite, since if replaced by thiosulfate, cysteine or GSH, no reduction of the electron acceptors occurred. It was observed also, that NO_2^- and NH_2OH which function as substrates for a sulfite reductase (EC 1.8.1.2) from E. coli (Kemp et al., 1963) did not replace sulfite in the cytochrome c assay procedure. Table VIII shows the activity of sulfite oxidase in the presence of various substrates.

Effect of substrate concentration

The effect of substrate concentration on enzyme activity was investigated by both the ferricyanide and cytochrome c assay methods. Since the K_m of enzyme for sulfite was very low an Aminco Dual Wavelength Spectrophotometer was used to follow the reduction of ferri-

TABLE VIII

Effect of various substrates on sulfite oxidase activity

System	% activity
Complete	100
No enzyme	0
No $Fe(CN)_6^{3-}$ or cyt *c*	0
No SO_3^{2-}	0
No SO_3^{2-} plus $S_2O_3^{2-}$	0
No SO_3^{2-} plus cysteine	0
No SO_3^{2-} plus GSH	0
No SO_3^{2-} plus $NaNO_2$	0
No SO_3^{2-} plus $NH_2OH.HCl$	0

The complete reaction mixture contained: Tris-HCl (pH 7.8) 5.0 µmoles; $K_3Fe(CN)_6$, 1.5 µmoles; or cyt *c*, 0.1 µmole; substrate, 5.0 µmole; enzyme protein 30 µg for the ferricyanide procedure or 0.35 µg for the cytochrome *c* procedure and water to a final volume of 1.5 ml. The complete system contained sulfite as substrate.

cyanide. The reduction of cytochrome c was followed in a Unicam Sp-700 recording spectrophotometer. The experiments were carried out at both pH 8.0 and 6.5 in potassium phosphate buffers. The double reciprocal plots of Lineweaver and Burk (1934) gave linear lines as shown in Fig. 9 and the K_m values were calculated from these plots. At pH 8.0 the K_m for sulfite was 4×10^{-5}M with the cytochrome c method and 2×10^{-5}M with the ferricyanide method. The K_m value for sulfite at pH 6.5 was found to be 2×10^{-6}M using the cytochrome c procedure. No determination was possible for the ferricyanide method due to low activity of the enzyme at pH 6.5 and the low concentration of sulfite required for determination. Thus sulfite has over 10-fold higher affinity for the enzyme at pH 6.5 than at pH 8.0 although the rate of reaction is much slower at pH 6.5 as indicated by a large decrease in the maximal velocity (Fig. 9).

Effect of buffer and salt concentrations

As shown in Table IX high concentrations of buffer inhibited the oxidation of sulfite by the enzyme. The type of inhibition was studied using NaCl and found to be competitive as shown in Fig. 10. From the slope of the double reciprocal plots of Fig. 10 the K_i for NaCl was calculated as 4.5×10^{-3}M. Since KCl showed the same degree of inhibition as NaCl the inhibition may be due to the concentration of anions.

Fig. 9. Effect of substrate concentration.

The reaction mixture contained potassium phosphate (pH 8.0 or 6.5), 25 μmoles; cyt c, 0.2 μmoles; Na_2SO_3 in 5 mM EDTA as required; enzyme protein, 0.3 μg and water to a final volume of 3.0 ml.

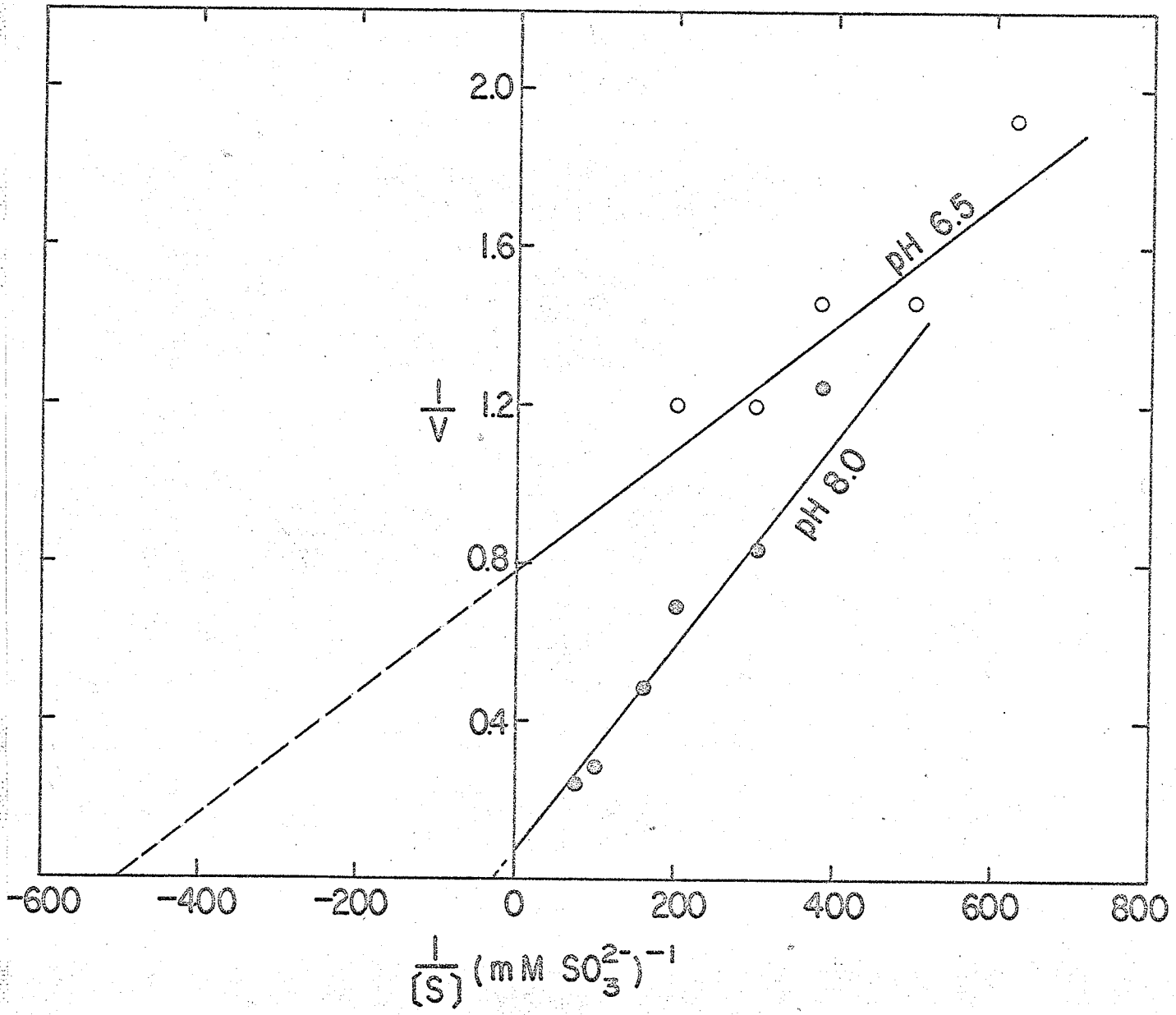

2.0
1.6
1.2
0.8
0.4
$\frac{1}{V}$
pH 6.5
pH 8.0
-600
-400
-200
0
200
400
600
800
$\frac{1}{[S]}$ (mM SO_3^{2-})$^{-1}$

TABLE IX

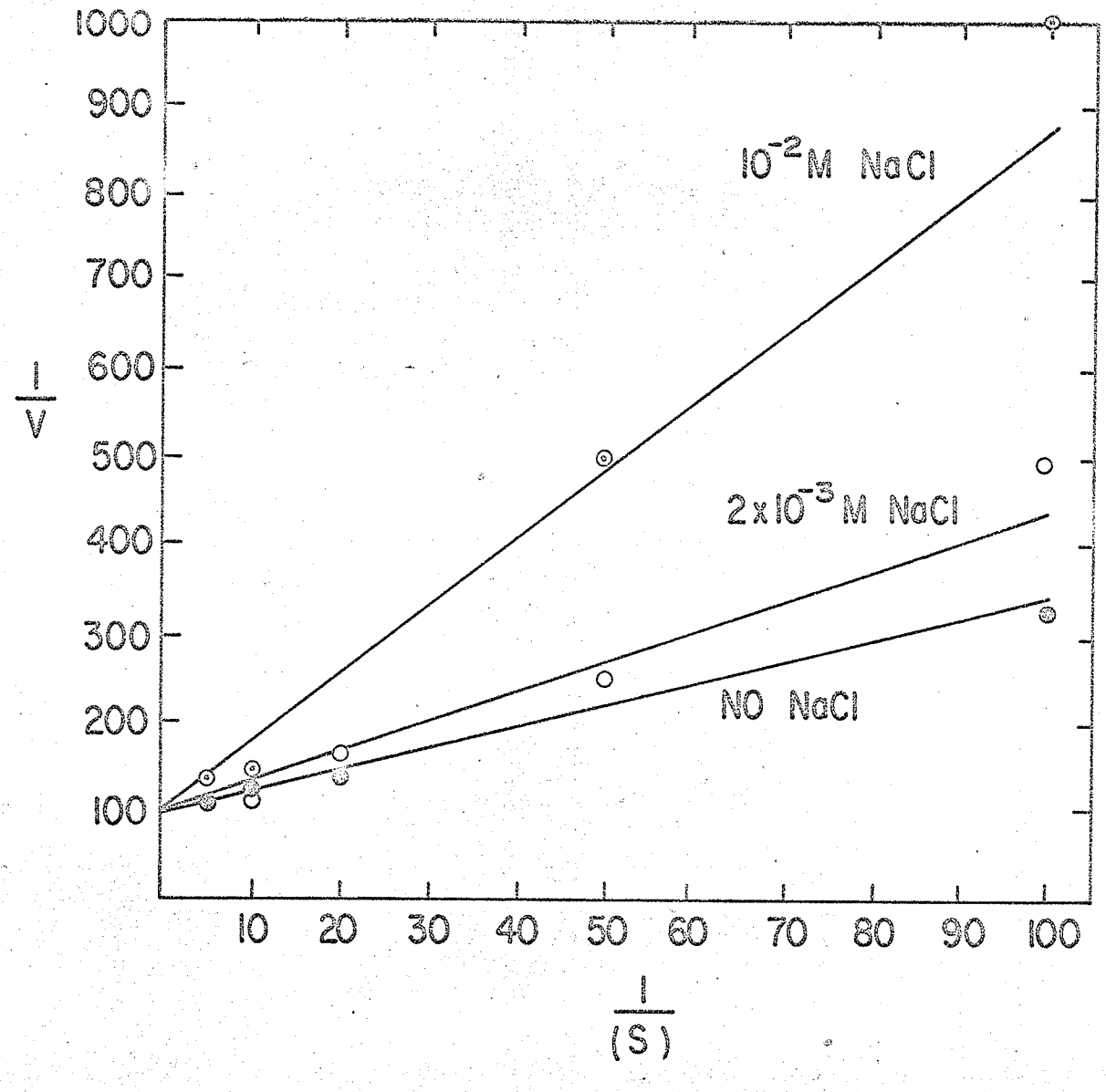
Effect of various buffers on sulfite oxidase

Buffer	Molar Concentration	Activity % of control
Control (no buffer)	-	100
potassium phosphate	1.67×10^{-3}	91.2
" "	3.3×10^{-3}	91.2
" "	1.65×10^{-2}	50.0
Tris-HCl	1.67×10^{-3}	113
	3.3×10^{-3}	95.6
	1.65×10^{-2}	57.7
Tris-acetate	1.67×10^{-3}	113
	3.3×10^{-3}	86.5
	1.65×10^{-2}	72

The reaction was carried out under standard conditions using the ferricyanide assay method at pH 8.0, except that the concentration of buffers varied as indicated.

Fig. 10. Inhibition of sulfite oxidase by NaCl.

S: μmoles of Na_2SO_3: initial velocity expressed as O.D. change per min in an Aminco Dual Wavelength spectrophotometer. The reaction mixture contained in a total volume of 3.0 ml (1 cm cell) potassium phosphate, 5.0 μmoles; $K_3Fe(CN)_6$, 3.6 μmoles; NaCl, 6 or 30 μmoles; enzyme protein 7.6 μg and H_2O.


1000
900
800
700
600
500
400
300
200
100
1/V
10⁻² M NaCl
2x10⁻³ M NaCl
NO NaCl
10
20
30
40
50
60
70
80
90
100
1/(S)

It is interesting to note that ammonium sulfate used during the purification of enzyme had no inhibitory effect on enzyme activity. $MgSO_4$ at 2.67×10^{-2}M did not inhibit the enzyme. From these observations it seems that only monovalent anions inhibit the enzyme activity. This problem was not investigated any further.

Various metals tested at 10^{-4}M had very little effect on the enzyme activity. These included $CaCl_2$, $MgSO_4$, $ZnSO_4$, $CuSO_4$, $FeCl_3$, $NiCl_2$ and Na_2MoO_4. $MnSO_4$ inhibited the reaction by 70% at 2.67×10^{-4}M but this was apparently due to the effect on the non-enzymatic oxidation of sulfite with air (Tager and Rautanen, 1955). A control experiment showed a rapid disappearance of sulfite in the presence of $MnSO_4$ as determined by iodometric titration.

Effect of various sulfhydryl inhibitors

Table X shows that Hg^{++}, AsO_2^-, N-ethylmaleimide and p-hydroxymercuribenzoate were inhibitory and that GSH added to the reaction mixture reversed this inhibitory effect. Inhibition by p-hydroxymercuribenzoate was rapid and was almost complete within ten minutes. Inhibition by N-ethylmaleimide, on the other hand, was progressive with time as shown in Fig. 11.

Electrophoresis pattern of enzyme

Polyacrylamide gel electrophoresis of the enzyme was undertaken to examine the purity. Gel was stained with buffalo-black to locate proteins. After destaining, the preparation was found to possess

TABLE X

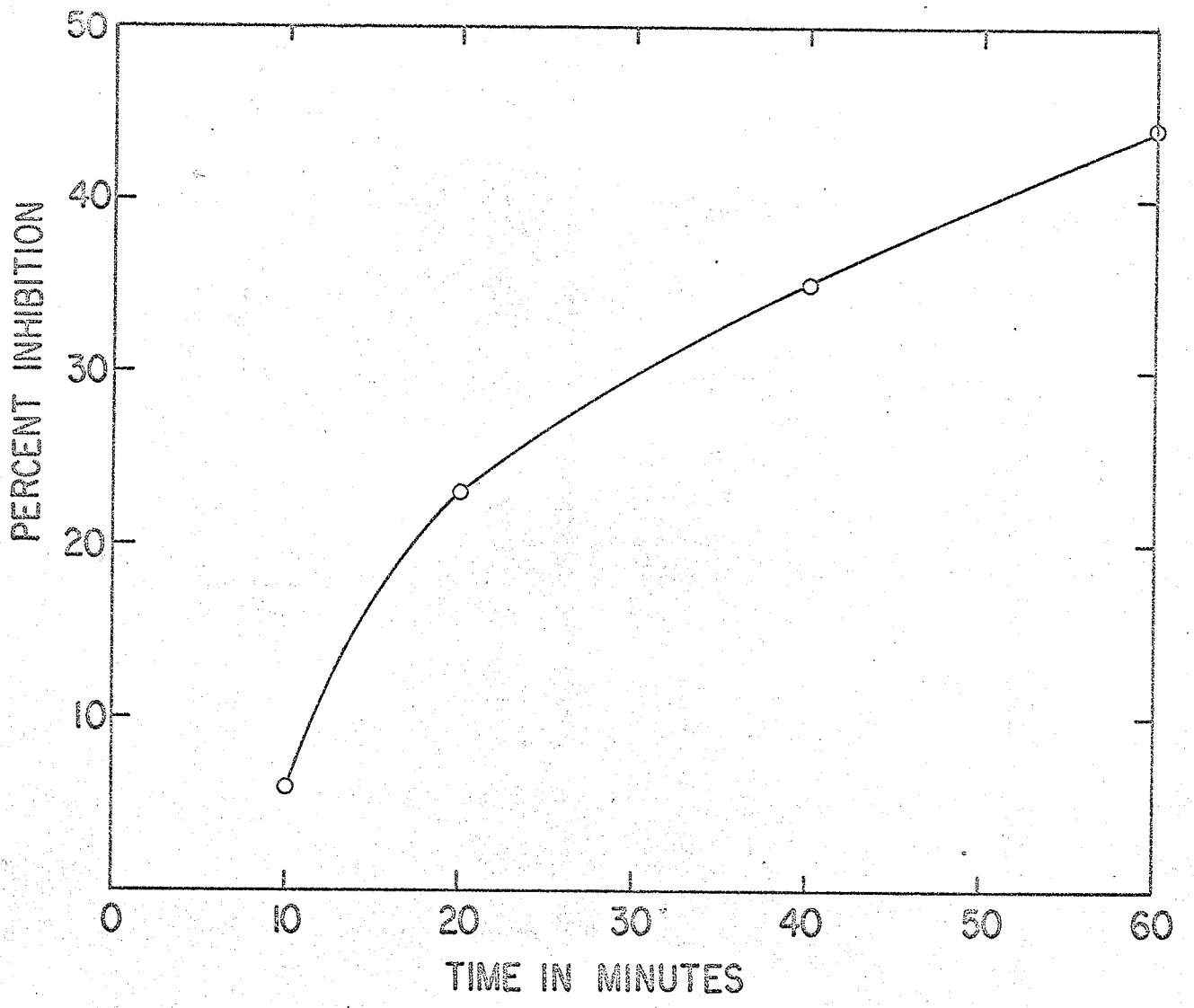
Effect of various sulfhydryl inhibitors on enzyme activity

Additions	Conc'n M	Activity % of control
N-ethylmaleimide	6.67×10^{-4}	70
p-hydroxymercuribenzoate	1.3×10^{-3}	8
" " "	6.67×10^{-4}	14
$HgCl_2$	6.67×10^{-4}	44
$NaAsO_2$	2.0×10^{-3}	69
p-hydroxymercuribenzoate	1.3×10^{-3}	103
plus GSH	3.3×10^{-3}	

The reaction was carried out under standard conditions using the ferricyanide assay method at pH 8.0, except that the concentration of inhibitors varied as shown. Enzyme 30 µg.

Fig. 11. Inhibition by N-ethylmaleimide.

The reaction mixture contained in a total volume of 1.5 ml, Tris-HCl (pH 8.0), 5.0 μmoles; $K_3Fe(CN)_6$, 1.5 μmoles; N-ethylmaleimide, 1.0 μmoles; Na_2SO_3 in 5 mM EDTA, 5.0 μmoles; enzyme protein 19 μg and water.


50
40
30
20
10
PERCENT INHIBITION
0
10
20
30
40
50
60
TIME IN MINUTES

two intensely stained and four very faint bands indicating the presence of traces of contaminating protein. Attempts were made to locate the enzyme directly in the gel through the formation of Prussian blue (Peck, 1965). A gel was incubated in the assay medium for about 40 min, rinsed with distilled water and placed in a ferric chloride solution. An intense band of Prussian blue was located in an area occupied by the two intensely stained bands of the buffalo-black procedure. The precise position of enzyme activity was not clearly established by this procedure and was not investigated further.

Electron acceptors

Several natural and artificial electron acceptors were tested as coupling agents during sulfite oxidation by sulfite oxidase. Sulfite alone reduced phenazine methosulfate, 2,6 dichlorophenol-indophenol and neotetrazolium chloride rapidly and without the mediation of enzyme thus making these acceptors unsuitable for assays. In the presence of enzyme and sulfite, methylene blue was not reduced anaerobically and NADP, NAD and atmospheric O_2 did not act as electron acceptors. However, enzyme in the presence of sulfite readily reduced ferricyanide and mammalian and native cytochrome c spectrophotometrically. The reduction of cytochrome c was much more rapid than that of ferricyanide with even one tenth the amount of enzyme. Thus cytochrome c is a much better electron acceptor for the enzyme. In manometric studies it was further observed that the addition of crude

cytochrome oxidase to a reaction mixture consisting of buffer, enzyme, sulfite and mammalian cytochrome c resulted in rapid oxygen uptake and reoxidation of the cytochrome.

These results were confirmed using T. novellus cytochrome c which was eluted from the DEAE-cellulose with 0.002M potassium phosphate during the purification of sulfite oxidase. The spectrum of this cytochrome is shown in Fig. 12. This cytochrome was not autooxidizable.

The native cytochrome c was reduced in the presence of sulfite and sulfite oxidase (Fig. 13A). The reaction was time-dependent and the 550 mμ peak of cytochrome increased with time. Upon addition of a crude cytochrome oxidase, cytochrome c was temporarily partially oxidized apparently due to the transfer of electrons to the oxidase (Fig. 13B). This was accompanied by an increase in absorption around 600mμ indicating the reduction of cytochrome oxidase. Upon further incubation the α and β peaks of reduced cytochrome c became much more marked indicating the continuous reduction of cytochrome c by sulfite and sulfite oxidase (Fig. 13C). With the introduction of air, however, these peaks of cytochrome c virtually disappeared (Fig. 13D).

Effect of catalase

The stoichiometric relationship of oxygen uptake to sulfite oxidized always proceeded according to the equation:

Fig. 12. Spectrum of T. novellus cytochrome c.

Cytochrome c purified as outlined in the text was scanned in a Unicam SP-700 recording spectrophotometer. Reduced cytochrome (-----) was obtained by mixing a few crystals of $Na_2S_2O_4$ with the oxidized cytochrome (———) in the cuvette.

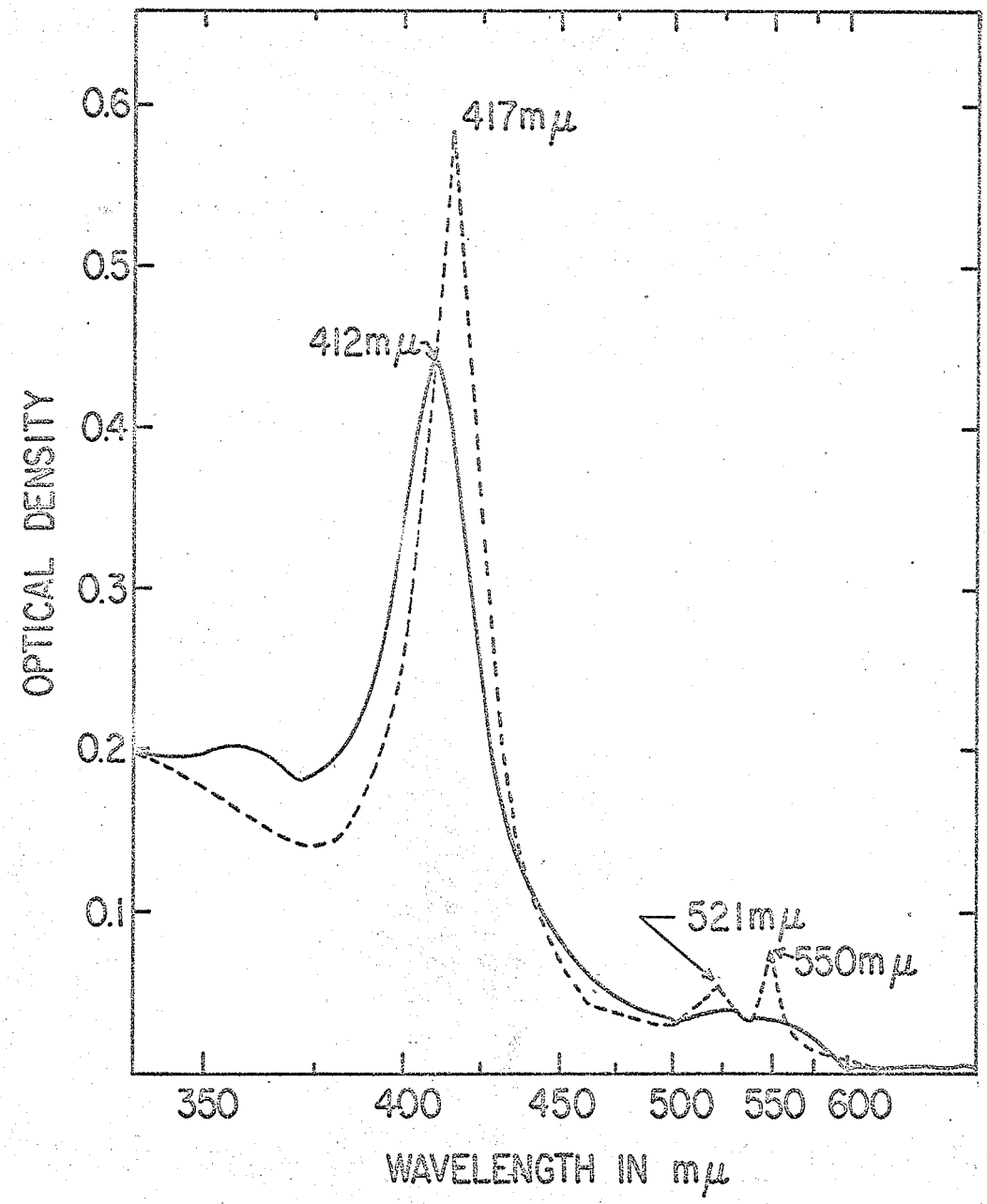
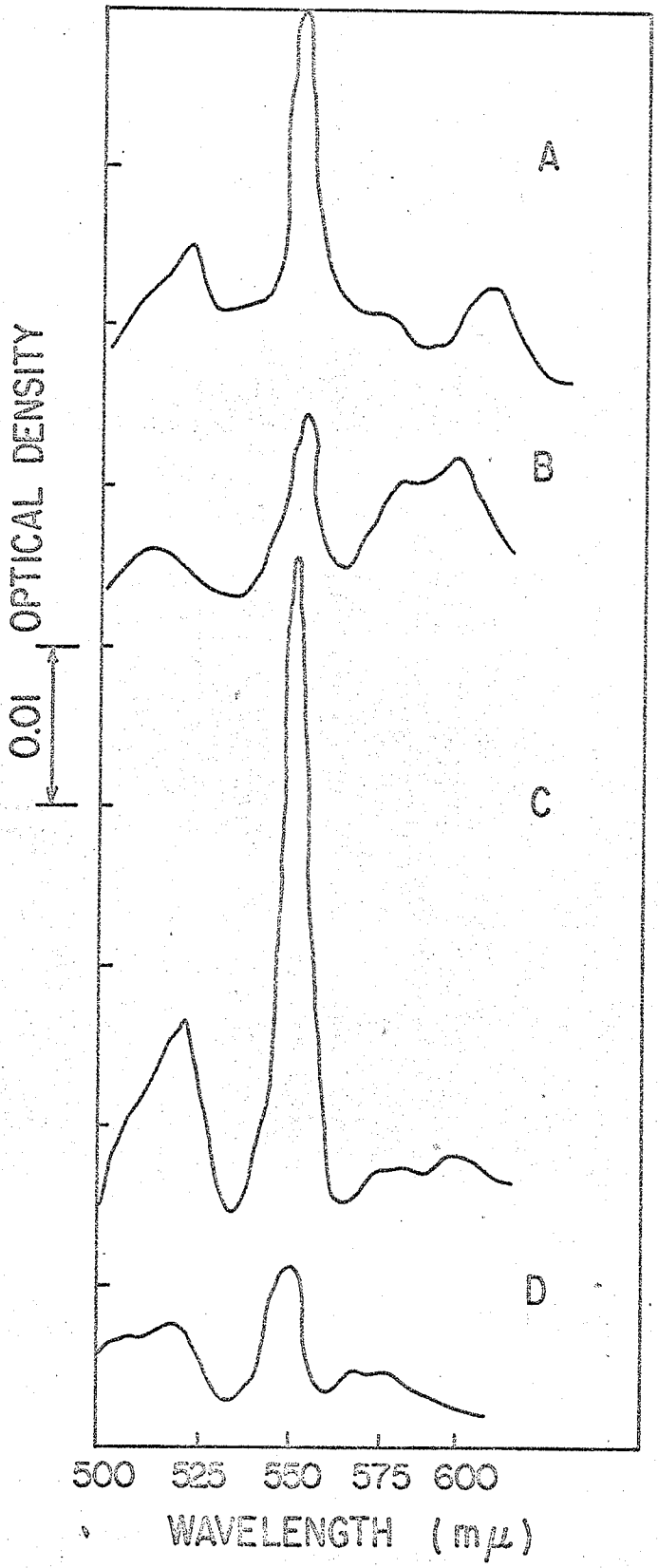

0.6
0.5
0.4
0.3
0.2
0.1
OPTICAL DENSITY
417mμ
412mμ
521mμ
550mμ
350
400
450
500
550
600
WAVELENGTH IN mμ

Fig. 13. Reduction of T. novellus cytochrome c with sulfite and sulfite oxidase and its oxidation with cytochrome oxidase and air.

The reaction mixture contained in a final volume of 3.0 ml: Tris-HCl (pH 7.8), 25 μmoles; Na_2SO_3 in 5 mM EDTA, 5 μmoles; sulfite oxidase, 0.35 μg; T. novellus cytochrome c 0.5 mg and T. novellus cytochrome oxidase 0.4 mg. The spectrum was followed in a Unicam SP-700 recording spectrophotometer with a 1 cm anaerobic cell. The reference cell contained all the reagents except Na_2SO_3. The reaction was started by the addition of Na_2SO_3 to the sample cell and the cell was evacuated. A: 5 min after the addition of Na_2SO_3. Cytochrome oxidase was then added from the side-arm. B: 2 min after the addition of cytochrome oxidase. C: 8 min after the addition. Air was then introduced and the cell was shaken for 1 min. D: 1 min after the introduction of air.


A
B
C
D
0.01 OPTICAL DENSITY
500 525 550 575 600
WAVELENGTH (mμ)

$$SO_3^{2-} + 1/2\ O_2 \longrightarrow SO_4^{2-} \qquad (9)$$

Peroxide generation during the oxidation of sulfite by enzyme could result in a similar stoichiometry even though some of the oxygen was the result of catalase activity (MacLeod et al., 1961). However, since the enzyme did not couple directly to oxygen even with added cytochrome c it was unlikely that peroxide was involved in this reaction. Conclusive evidence for this was obtained when it was found that the total amount of oxygen consumed for sulfite oxidation was not affected in the presence of catalase and 1.0% ethanol. As shown in Fig. 14 the oxidation of 10 µmoles of sulfite consumed approximately 5 µmoles of oxygen even in the presence of catalase and ethanol.

IV. Oxidative phosphorylation

Since sulfite oxidase of T. novellus did not seem to involve a substrate-level phosphorylation system through APS-reductase and ADP-sulfurylase a possibility of oxidative phosphorylation during sulfite oxidation was investigated.

Evidence for oxidative phosphorylation during sulfite oxidation by cell-free extracts of T. novellus is shown in Table XI.

From the results listed in Experiment I of Table XI, 0.9 µmole ATP was formed with a P/O ratio of 0.08 in the complete system. In the absence of sulfite or under anaerobic conditions, no phosphorylation took place.

Fig. 14. Effect of catalase during sulfite oxidation by sulfite: cytochrome c oxidoreductase.

Reaction mixtures consisted of the following. Tris-HCl (pH 8.0), 80 μmoles; Na_2SO_3 in 5 mM EDTA, 10 μmoles; catalase, 0.2 mg; ethanol, 1.0%; crude extract, 6.0 mg protein and water to a final volume of 2.0 ml.

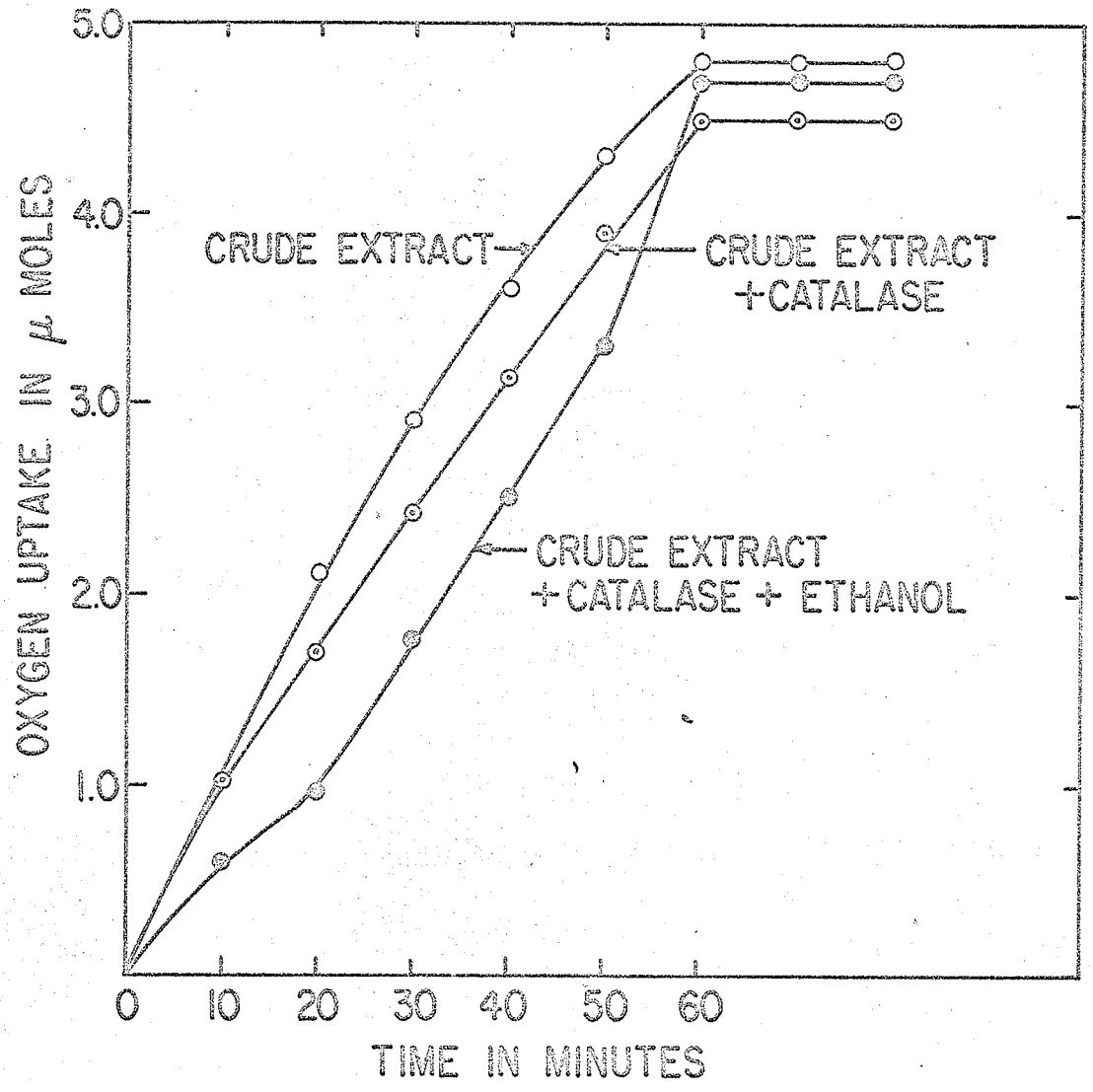

5.0
4.0
3.0
2.0
1.0
OXYGEN UPTAKE IN μ MOLES
CRUDE EXTRACT
CRUDE EXTRACT
+CATALASE
CRUDE EXTRACT
+CATALASE + ETHANOL
0
10
20
30
40
50
60
TIME IN MINUTES

TABLE XI

Phosphorylation coupled to sulfite oxidation by T. novellus extracts

Experiment	System	Total NADPH formed (μmoles)	Total O_2 uptake (μmoles)	*P/O
I	Complete	0.9	5.7	0.08
	Minus SO_3^{2-}	0.0	1.3	0.0
	Anaerobic complete	0.0	-	-
II	Complete	2.0	4.2	0.23
	Minus NaF	2.5	4.1	0.32
	Minus ADP plus AMP	0.0	4.3	0.0
	Minus ADP and NaF plus AMP	0.0	4.3	0.0
	Minus SO_3^{2-}	1.5	1.0	0.75

Reaction carried out at 30° for 1 hr under standard conditions described in Materials and Methods. * P/O ratios: not corrected for endogenous metabolism or adenylate kinase.

Some cell-free preparations showed a very pronounced adenylate kinase activity which was not inhibited even in the presence of 2×10^{-2} M NaF. Thus the results of Experiment II in Table XI illustrate this very clearly since there was considerable ATP formation in the absence of sulfite. Further evidence for the presence of very active adenylate kinase was observed by the omission of NaF from the reaction mixture which resulted in an approximately 20% increase in phosphorylation over a similar reaction mixture which contained fluoride. It was also observed that AMP did not replace ADP for the esterification of phosphate.

The effect of an uncoupler for oxidative phosphorylation, 2,4-dinitrophenol (DNP), was investigated. From the results of Table XII it can be seen that DNP inhibited the phosphorylation coupled to sulfite oxidation. Since adenylate kinase activity of most cell-free extracts was quite strong, the oxidative phosphorylation by T. novellus was not investigated any further.

TABLE XII

Inhibition of oxidative phosphorylation by dinitrophenol

Inhibitor	Conc'n (M)	Total NADPH formed (μmoles)	Total O_2 uptake (μmoles)	P/O
None	-	1.30	2.7	0.24
DNP	5×10^{-4}	1.07	3.1	0.17

Reaction carried out at 30° for 1 hr under standard conditions described in Materials and Methods.

DISCUSSION

DISCUSSION

I. Fixation of carbon dioxide

Two classes of compounds have generally been obtained as the earliest products labelled during $C^{14}O_2$ fixation by chemoautotrophic bacteria (Aubert et al., 1956, Suzuki and Werkman, 1958[b] and Bergman et al., 1958). These compounds are sugars, sugar phosphates and 3-PGA on the one hand, and aspartic, glutamic and malic acids and some other amino acids on the other. On the basis of these findings it is believed that there are at least two systems involved in the fixation of carbon dioxide by chemoautotrophic bacteria, (Suzuki and Werkman, 1958[a]) the carboxydismutase pathway, elucidated by Calvin and his associates in plants, and the so-called Wood-Werkman reaction (Wood and Werkman, 1938) catalyzed by PEP carboxylase (Bandurski and Greiner, 1953).

In $C^{14}O_2$ fixation experiments with whole cells of T. novellus preincubated with thiosulfate, 3-PGA was found among the earliest compounds labelled suggesting the presence of carboxydismutase. The rapid labelling of aspartic and glutamic acids suggested the presence of PEP carboxylase. The labelled phosphorylated hexose was probably formed from labelled 3-PGA. It would thus appear that the carboxydismutase system and the PEP carboxylase reaction play a major role in the fixation of carbon dioxide by this organism, as has been observed for other thiobacilli.

Cells incubated either in buffer or in glucose showed the same pattern of labelling in which amino acids were the chief radioactive compounds detected, but no 3-PGA. These results indicated the requirement of thiosulfate for operation of the carboxydismutase pathway. Since these thiosulfate-grown cells could oxidize glucose rapidly and could grow on glucose without an extended lag period, the failure of glucose to replace thiosulfate could be due to the repression of carboxydismutase rather than the lack of energy and reducing power required for the operation of the 3-PGA pathway. This problem, however, requires further investigation.

Cell-free experiments clearly demonstrated the presence of carboxydismutase in the thiosulfate-grown cells of T. novellus, but not in the cells grown on glucose. These findings further support the report of Vishniac and Trudinger (1962) who found that growth of the organism on organic media resulted in a level of carboxydismutase activity that was only 2% of that found in autotrophic cells.

II. Oxidation of inorganic sulfur compounds

The ability of whole cells and cell-free extracts of T. novellus to oxidize thiosulfate to sulfate without intermediary accumulation of polythionates suggested the initial scission of thiosulfate between the two sulfur atoms rather than the condensation to tetrathionate. The inability of cell-free extracts to oxidize

tetrathionate is a further support to this theory. The oxidation of thiosulfate by extracts, however, did not require the presence of GSH in contrast with the results of the ATCC strain of T. thioparus (Peck, 1962 and Peck and Fisher, 1962) but in agreement with those of the strains of T. thioparus and T. thiooxidans isolated from natural sources (London and Rittenberg, 1964). Thus the cleavage of thiosulfate is not due to thiosulfate reductase and the products are probably sulfur and sulfite rather than sulfide and sulfite.

Although sulfide was oxidized by both whole cells and extracts, sulfide is not likely to be an intermediate of thiosulfate oxidation. Colloidal sulfur which accumulated during sulfide oxidation by extracts was never observed during thiosulfate oxidation by the same extracts.

The presence of a sulfur-oxidizing enzyme and rhodanese in extracts of T. novellus is very interesting. The sulfur-oxidizing enzyme, an iron-containing oxygenase (Suzuki, 1965) catalyzes the reaction of Equation 4.* When purified from T. thioparus the enzyme showed a rhodanese activity. Rhodanese, first crystallized from beef liver by Sörbo (1953) and kidney by Westley and Green (1959) has been shown to catalyze the scission of thiosulfate by a double displacement with an enzyme sulfur complex as an intermediate (Green and Westley, 1961; Westley and Nakamoto, 1962). Since the sulfur-oxidizing enzyme also contains labile sulfide (Suzuki and

$$* \quad S + O_2 + H_2O \xrightarrow{\text{Enzyme}} SO_3^{2-} + 2H^+$$

Silver, 1966), it is tempting to postulate that the two activities reside in the same protein. Further work is required to clarify the situation. It should be noted, however, that the addition of 32 μg crystalline beef liver rhodanese (Sigma, Type III) to the extracts of T. novellus did not stimulate the rate of oxidation of thiosulfate.

Failure of the extracts of T. novellus to reduce cytochrome c in the absence of cyanide may be explained by the requirement of the sulfur-oxidizing enzyme for oxygen. Without aeration sulfur may not be cleaved from thiosulfate and no sulfite may be formed to reduce cytochrome c. This theory is supported by the oxidation of both sulfur atoms of thiosulfate to sulfate by the extracts in manometric experiments where the reaction mixtures are constantly aerated. In this context the role of cyanide in the spectrophotometric experiments may simply be to replace oxygen and to dispose of the sulfur atom cleaved from thiosulfate. These reactions may be written in the following equations:

$$SSO_3^{2-} + \text{Enzyme} \longrightarrow \text{Enzyme-S} + SO_3^{2-} \quad (10)$$

$$\text{Enzyme-S} + O_2 + H_2O \longrightarrow \text{Enzyme} + SO_3^{2-} + 2H^+ \quad (11)$$

$$\text{Enzyme-S} + CN^- \longrightarrow \text{Enzyme} + SCN^- \quad (12)$$

where enzyme is either the sulfur-oxidizing enzyme or rhodanese. The role of GSH in thiosulfate oxidation by T. thioparus (Peck, 1960 and Peck and Fisher, 1962) may be as an acceptor of the sulfur atom as postulated by Suzuki and Silver (1966). In this connection it should

be mentioned that some extracts of T. novellus, when stored for one year with repeated freezing and thawing, showed a long lag before thiosulfate oxidation. The addition of GSH removed such a lag period. Thus it seems that the sulfur-oxidizing enzyme, under certain conditions, required the mediation by a thiol group for the transfer of the sulfur atom of thiosulfate. The involvement of thiol groups for thiosulfate oxidation was first suggested by Lees (1960) and was recently supported by the experiments of Trudinger (1965) who showed the inhibition of thiosulfate oxidation by Thiobacillus neapolitanus in the presence of thiol inhibitors. Rhodanese of T. denitrificans was also inhibited by thiol inhibitors (Bowen, Butler and Happold, 1965). In view of a recent finding by Davidson and Westley (1965) that a tryptophan rather than a sulfhydryl group is involved in the binding of sulfur to rhodanese, the role of thiol groups in the oxidation of thiosulfate may be an indirect one, i.e., that of mediation.

While this investigation was being conducted, Aleem (1965) reported the oxidation of thiosulfate by extracts of T. novellus and isolation of an enzyme thiosulfate:cytochrome c oxidoreductase. Although some of his results agreed with ours, it is difficult to make a direct comparison since he did not analyze the products of oxidation. His thiosulfate:cytochrome c oxidoreductase may be either the thiosulfate-oxidizing enzyme isolated by Trudinger (1961) from

Thiobacillus X which forms tetrathionate, or a mixture of sulfite oxidase and rhodanese since it required cyanide for the reduction of cytochrome c at a low thiosulfate concentration. In the absence of cyanide the affinity for thiosulfate was very low and the K_m calculated from his data fell in the range of 10^{-1}M. In the presence of cyanide, however, the K_m was around 10^{-4}M. Since neither extracts nor purified sulfite oxidase of T. novellus catalyzed the reduction of cytochrome c with thiosulfate even at 2×10^{-1}M the difference may be due to the growth conditions. He grew the cells in the medium of Vishniac and Santer (1957) which contains EDTA and a large number of trace metals.

III. Sulfite:cytochrome c oxidoreductase

From the results reported, the oxidation of sulfite by T. novellus is definitely catalyzed by sulfite oxidase and cytochrome oxidase according to Eqns. 2 and 3.

Sulfite is a key intermediate in the oxidation of thiosulfate and accumulates under certain conditions during thiosulfate oxidation by T. novellus (De Ley and Van Poucke, 1961). Peck (1960) has shown that the oxidation of sulfite in T. thioparus was catalyzed by APS-reductase forming APS from AMP and sulfite. In this mechanism APS-reductase together with ADP-sulfurylase and adenylate kinase are believed to produce high-energy phosphate bonds of ATP through substrate-level phosphorylation reactions. A sulfite oxidase

activity of oat mitochondria is also stimulated by AMP (Tager and Rautanen, 1955), but it is not certain whether this enzyme activity is due to APS-reductase. From the evidence presented it is clear that T. novellus does not oxidize sulfite by the APS pathway.

Further, the results clearly indicate that sulfite oxidase of T. novellus, in contrast with APS-reductase of T. thioparus, does not require AMP for ferricyanide reduction with sulfite, does not oxidize a reduced viologen dye with APS and finally does reduce cytochrome c with sulfite. The similarities are that both enzymes reduce ferricyanide with sulfite and are inhibited by sulfhydryl inhibitors.

The sulfite oxidase of T. novellus is similar to an enzyme isolated from liver (MacLeod et al., 1961) in several respects. Both enzymes were inhibited by sulfhydryl inhibitors, showed a very high affinity for sulfite and reduced either cytochrome c or ferricyanide. Oxygen and methylene blue, on the other hand, served as electron acceptor only for the liver enzyme which was associated with cytochrome b_{-5}. H_2O_2 was produced during the aerobic oxidation of sulfite by the liver enzyme, but not during the oxidation by T. novellus extracts.

The low K_m value of the enzyme for sulfite has an advantage for the oxidation of thiosulfate by the organism. If thiosulfate is initially cleaved to sulfur and sulfite as proposed previously,

a rapid removal of sulfur and sulfite through oxidation is essential since the equilibrium is favoured for the synthesis of thiosulfate.

A ten-fold decrease in the K_m of enzyme for sulfite when the pH is changed from 8.0 to 6.5 may implicate HSO_3^- ions as the actual ionic form of substrate. With APS-reductase (Peck, 1965) the K_m values were identical at pH 6.4 and 8.6. A competitive inhibition of the sulfite oxidase by NaCl and the inhibition by other monovalent anions, but not by divalent anions, also support the idea that the actual substrate is probably HSO_3^- and not SO_3^{2-}. Further investigation is necessary in order to establish the actual ionic form of substrate. It should be noted that the enzyme reaction was much faster at pH 8.0 than at pH 6.5 at higher concentrations of sulfite (Fig. 8).

A competitive inhibition of the enzyme by increasing salt concentrations has been reported for carbamyl phosphate synthetase (Kennedy and Grisolia, 1965) and ribonuclease (Irie, 1965). The K_i values, however, for these inhibitions were much higher than that found for the sulfite oxidase of T. novellus which was 4.5×10^{-3}M.

IV. Oxidative phosphorylation

Whereas energy generation is accomplished by substrate-level phosphorylation reactions in the mechanism of sulfite oxidation proposed by Peck, in T. novellus oxidative phosphorylation is

apparently the means by which energy is derived during sulfite oxidation. This was shown by the reduction of a native cytochrome c with sulfite by sulfite oxidase, the reoxidation with air by cytochrome oxidase, and finally the demonstration of oxidative phosphorylation during sulfite oxidation by cell-free extracts. The results listed in Table XII reveal that DNP at a concentration of 5×10^{-4}M inhibited phosphorylation by 30%. It is known that bacterial systems are relatively insensitive to this inhibitor which uncouples phosphorylation from the oxidative reactions in mammalian mitochondrial systems (Smith, 1962). In view of the fact that adenylate kinase present in many cell-free preparations interfered with the study of oxidative phosphorylation using the spectrophotometric method, the use of P^{32} may be more advisable for a further study of this problem.

CONCLUSIONS

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From the results obtained during the course of these investigations, it is concluded that the mechanism proposed for thiosulfate oxidation in T. thioparus (Peck, 1960) is not the mechanism followed in T. novellus. An alternate mechanism is proposed according to the following equations, where thiosulfate is oxidized to sulfate by a combination of four enzyme systems and energy is derived during sulfite oxidation by oxidative phosphorylation.

$$S_2O_3^{2-} \xrightarrow{\text{thiosulfate-cleaving enzyme}} S + SO_3^{2-}$$

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

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

$$4\text{cyt } \underline{c}\ Fe^{2+} + O_2 + 4H^+ \xrightarrow{\text{cyt oxidase}} 4\text{cyt } \underline{c}\ Fe^{3+} + 2H_2O$$

$$2ADP + 2P_i \xrightarrow{\text{oxidative phosphorylation}} 2ATP$$

Overall reaction: $S_2O_3^{2-} + 2O_2 + H_2O + 2ADP + 2P_i \longrightarrow 2SO_4^{2-} + 2H^+ + 2ATP$

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