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METABOLIC CAPABILITIES OF SULFUR OXIDIZING BACTERIA AND THEIR ROLE IN WATER POLLUTION

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ABSTRACT

This report describes investigations into the physiology of microorganisms that are commonly involved in the oxidation of inorganic sulfur compounds. The metabolic activities of these bacteria play a potent role in several fields of economic importance such as strip mining operations, water pollution, corrosion, metallurgy, petroleum technology and soil fertility processes.

The oxidation of inorganic sulfur compounds was studied in the chemolithotrophs Thiobacillus denitrificans, Thiobacillus A_2, Thiobacillus neapolitanus, and a photolithotroph Rhodopseudomonas palustris. Cell suspensions from all of the thiobacilli catalyzed completely the oxidation of sulfide, thiosulfate, and sulfite to sulfate. The oxidation of thiosulfate in R. palustris was light-dependent and involved tetrathionate and trithionate as the major intermediates in the formation of sulfates.

Almost all of the thiobacilli (e.g. T. neapolitanus and T. denitrificans) were capable of producing approximately 7.5 moles of sulfuric acid aerobically from 3.75 moles of thiosulfate per gram of cellular protein per hr. By far the most prolific producer of sulfuric acid (or sulfates) from the anaerobic thiosulfate oxidation with nitrates was T. denitrificans which was capable of producing 15 moles of sulfates from 7.5 moles of thiosulfate with concomitant reduction of 12 moles of nitrate resulting in the evolution of 6 moles of nitrogen gas/g protein/hr. The oxidation of sulfide was mediated by the flavoprotein system and cytochromes of b, c, o, and a-type. This process was sensitive to flavoprotein inhibitors, antimycin A, and cyanide. Thiosulfate oxidation, on the other hand involved cytochrome c:O_2 oxidoreductase region of the electron transport chain and was sensitive to

cyanide only. The anaerobic oxidation of thiosulfate by T. denitrificans, however, was severely inhibited by the flavoprotein inhibitors because of the splitting of the thiosulfate molecule into the sulfide and sulfite moieties produced by the thiosulfate-reductase. Accumulation of tetrathionate and to a small extent trithionate and pentathionate occurred during anaerobic growth of T. denitrificans. These polythionates were subsequently oxidized to sulfate with the concomitant reduction of nitrate to N_2. Intact cell suspensions catalyzed the complete oxidation of sulfide, thiosulfate, tetrathionate, and sulfite to sulfate with the stiochiometric reduction of nitrate, nitrite, nitric oxide, and nitrous oxide to nitrogen gas thus indicating that NO_2^-, NO, and N_2O are the possible intermediates in the denitrification of nitrate. This process was mediated by the cytochrome electron transport chain and was sensitive to the electron transfer inhibitors. In addition, the APS-reductase pathway was also operative. The latter aspect was absent in T. novellus and Thiobacillus-A_2. In all of the thiobacilli the inner as well as the outer sulfur atoms of thiosulfate were oxidized at approximately the same rate by intact cells. The sulfide oxidation occurred in two stages: (1) a cellular-membrane-associated initial and rapid oxidation reaction which was dependent upon sulfide concentration, and (2) a slower oxidation reaction stage catalyzed by the cell-free extracts, probably involving polysulfides. In T. novellus and T. neapolitanus the oxidation of inorganic sulfur compounds is coupled to energy generation through oxidative phosphorylation, however, the reduction of pyridine nucleotides by sulfur compounds involved an energy-linked reversal of electron transfer.

Descriptors:

Sulfides, Sulfates, Sulfur bacteria*, Microorganisms*, Acid bacteria, Sulfur*.

Identifiers:

Sulfur oxidizing bacteria, Sulfite, Dissimilatory nitrate reduction, Chemosynthetic bacteria, Autotrophic energy metabolism, Purple sulfur bacteria.

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CHAPTER I

INTRODUCTION

The objectives of this project were:

(a) To conduct investigations, under laboratory conditions, into the physiology and metabolism of organisms commonly found in the polluted waters and in the strip-mine runoffs.

(b) To conduct studies on the chemistry of microbial oxidation and reduction of inorganic sulfur and nitrogen compounds and to elucidate the factors involved in the control of such microbial metabolic activities in order to seek a solution to the problems involved in the pollution of waters.

(c) To investigate the feasibility of creating the optimal environmental conditions for the establishment of a "normal" Sulfur and Nitrogen Cycle in waters, under laboratory conditions, in order to eliminate the toxic compounds from the environments and thus making the process of mineralization more useful to the plants and other life systems.

It is now well established that biological as well as non-biological transformations of organic and inorganic materials are the major cause of water pollution. As a result of diversified biochemical activities of a vast abundance of physiologically heterogeneous microflora in aquatic biosphere, it is important that a study of their biochemical potentialities is undertaken before their quantitative role in the pollution of waters and the recovery of the streams can be evaluated. Of particular interest are the microorganisms which play dominant role in the transformations of sulfur and nitrogen compounds. Thus the activities of sulfur-oxidizing and sulfate-reducing bacteria are known to be responsible for many of the characteristics of the acid mine wastes. The important role of sulfur oxidizing organisms in the formation of sulfuric acid, the sulfate reducing bacteria in the production

of sulfide minerals, and the iron oxidizing microorganisms in sulfide ore leaching present problems of great economic importance which is of significant interest to sanitary engineers, geologists, and microbiologists alike.

The sulfides produced by the sulfate reducing bacteria lead to the formation of petroleum in waters since sulfides act as chemical reducing agents for the transformation of organic acids and sugars into hydrocarbons. In addition to their inherent ability to produce sulfuric acid from reduced sulfur compounds, some of the sulfur oxidizing microorganisms are capable of reducing nitrates and other nitrogen-oxides to nitrogen gas at a fairly rapid rate. Thus the toxicity of the metabolic products of sulfur bacteria (eg. sulfides and sulfuric acid) to many life forms coupled with the ability of these bacteria to exhaust the biosphere of the supply of one of the most essential available nutrients i.e. nitrogen, make these organisms of significant ecological importance.

In view of the above explained roles of these organisms investigations were undertaken to elucidate the physiology and metabolism of sulfur bacteria since this approach appeared to be the key to the development of control measures. It was felt that once the basic biochemical mechanisms were elucidated, the mechanism (s) of influences by the environments should become available for explanation and for further course of action involving remedial measures.

CHAPTER II

RESEARCH PROCEDURES

Description of Organisms - The following organisms were used in the current work:

i) Thiobacillus dentrificans, an obligate chemoautotrophic bacterium which can actively oxidize inorganic sulfur compounds either aerobically or anaerobically; in the latter case either nitrate or nitrite can serve as the terminal oxidant. The aerobic growth conditions were similar to those for T. neapolitanus, while nitrate was included in the growth medium when the bacterium was grown anaerobically. In the latter case the nitrate-nitrogen is lost as nitrogen gas.

ii) Thiobacillus-A_2, a facultative chemoautotroph which can rapidly oxidize inorganic sulfur compounds under aerobic conditions only. The organism can grow anaerobically provided the source of energy and cell carbon is furnished by an organic compound such as succinate, and an oxy-anion such as nitrate serves as the oxidant which is finally reduced to nitrogen gas.

iii) Thiobacillus neapolitanus, an obligate chemoautotrophic bacterium which is able to catalyze a rapid oxidation of sulfides, elemental sulfur, and several other reduced sulfur compounds under laboratory conditions and in natural environments. This organism was grown aerobically in an inorganic mineral salt medium with thiosulfate as the sole energy source and carbon dioxide as the sole source of cellular carbon.

iv) Rhodopseudomonas palustris, a facultative photoautotroph capable of oxidizing sulfur compounds and synthesizing cells from carbon dioxide in the presence of light only. Under these conditions the bacterium requires a complete anaerobiosis for growth.

Growth of bacteria and preparation of cell-free extracts. Thiobacillus denitrificans was grown on thiosulphate under anaerobic conditions with nitrate as the final electron acceptor in an inorganic medium described by Sargeant, Buck, Ford & Yeo (1966).

Thiobacillus-A_2 was grown according to the method described by Taylor & Hoare (1969) using thiosulphate, succinate or glutamate as individual substrates for autotrophic and heterotrophic growth. With succinate as the carbon and energy source, the bacteria were grown aerobically as well as anaerobically and in the latter case nitrate served as the terminal oxidant.

The organisms were harvested during the early log phase using a DeLaval continuous flow centrifuge maintained at 6°. In the case of Thiobacillus-A_2 the bacterial paste was washed three times with 0.05M-Tris-HCl buffer pH 8·0. The wet packed organisms were suspended in the same buffer (30%, w/v) containing 0·3 M-sucrose, 5 mM-$MgCl_2$, 0·5 mM-Na_4EDTA and 0·5 mM-glutathione (reduced form). Organisms were broken by sonic disruption for 3 min in a Bronson Sonifier (Heat Systems Ultrasonics, Inc.) operated at full power; the temperature was maintained at 5°. The suspension was centrifuged first at 30,000 g for 30 min and further at 144,000 g for 60 min yielding the appropriate cell-free preparations. The cell-free extracts from T. denitrificans were prepared as described previously (Peeters & Aleem, 1970).

Enzyme assays. All determinations, unless otherwise indicated, were made with the 144,000 g supernatant and the various enzymes were measured using well-established methods. The optical procedure described by Ochoa (1955) was used for the assay of citrate synthetase (EC 4.1.3.7). isocitrate dehydrogenase (EC 1.1.1.42), malate dehydrogenase according to the method described by Sottocasa, Kuylenstierna, Ernster & Bergstrant (1967); succinyl CoA synthetase (EC 6.2.1.5) was determined

as described by Bridger, Ramaley & Boyer (1969). For succinate dehydrogenase (I.3.99.I) the spectrophotometric adaptation of the manometric method with phenazine methosulphate was used (Veeger, DerVartanian & Zeylemaker, 1969). The decline in fumarate concentration was followed at 300 nm as described by Hill & Bradshaw (1969) for the assay of fumarase (EC 4.2.I.2). The α-ketoglutarate dehydrogenase was measured by the method of Kaufman (1955) and also by the method of Amarasingham & Davies (1965) using 3-acetylpyridine-NAD. Other enzymes assayed were: aconitase (EC 4.2.I.3), Afinsen (1955); malate synthetase (EC 4.I.3.2), Dixon & Kornberg (1965); isocitrate lyase (EC 4.I.3.I), Olson (1959) and 3-ketoacyl CoA transferase (EC 2.8.3.5), Pearce, Leach & Carr (1969). As the cell-free preparations from the two organisms showed a powerful NADH oxidase activity, those determinations which involved NADH oxidation or NAD^+ reduction were performed anaerobically or in the presence of I mM-potassium cyanide; the latter completely blocked the NADH oxidase activity.

Specific activities are reported as nanomoles substrate converted/min/mg protein. The following millimolar extinction coefficients were used in the calculations: reduced NADP and NAD (340 nm.), 6·2; cis-aconitic acid (240 nm.), 4·88; thio-ester bond in succinyl CoA and acetyl CoA (230 nm.), 4·5; 2, 6-dichlorophenol indophenol (600 nm.), 20·6; glyoxylic semicarbazone (252 nm.), I2.4. For furmarate the millimolar extinction coefficient at 240 nm. is 2·II. We have observed that the ratio between the extinction of fumarate at 240 and 200 nm was 63; and, since fumarate measurements were made at 300 nm, we used a millimolar extinction coefficient of 0.0335 in our calculations. Finally, a value of II·9 was used for the magnesium complex of acetoacetyl

CoA (Stern, Coon, DelCampillo & Scheider, 1956). Protein was determined by the method of Gornall, Bardawill & David (1949).

Manometry. Oxygen uptake or nitrogen evolution was measured at 30° in a Gilson Differential-Respirometer. Anaerobic measurements were made under Helium atmosphere. The main compartment of the reaction vessel contained cells or cell-free extracts (2-3 mg protein/ml) and 150 μmoles of phosphate, pH 6.9. After thermal equilibration the substrates were added from the side-arm.

Spectrophotometry. Spectrophotometric measurements were made in a Cary, Model 14, recording spectrophotometer and in a dual wavelength double-beam spectrophotometer equipped with a dual-pen recorder. Experimental details are provided in the legends to the figures.

Analytical Methods. Thiosulfate and polythionates were estimated by Sorbo's method (Sorbo, 1957), and protein was determined by the biuret method (Gornall et al., 1949).

Gas Analysis. In the preliminary experiments analysis of the gaseous mixture produced upon anaerobic incubation of thiosulfate with either nitrate, nitrite, nitric oxide or nitrous oxide was carried out by gas chromatography as described by Barbaree and Payne (1967) and Payne and Riley (1969). In each case nitrogen gas was found to be the final product. After nitrogen gas was identified as the end product by gas chromatography, gas evolution was measured at 30°C using standard manometric techniques with helium as the gas phase. The Warburg flask contained resting cell suspensions containing 2-4 mg protein per ml in the main compartment, 0.2 ml of 20% KOH and a fluted filter paper in the center well, and thiosulfate and nitrate or nitrite in the side arm. The final volume was made up to 2.0 ml with 0.05 M

potassium phosphate buffer, pH 7.0. After thermal equilibrium the contents of the side arm were mixed with the cell suspension and the gas evolution was recorded.

When the reduction of nitric oxide gas was measured, the reaction flask was first flushed with helium for 15 min and then a small amount of nitric oxide gas (98.5% purity, Matheson Company, Inc., New Jersey) was added by means of a gas-tight syringe through the side arm sealed with a rubber stopper. The reaction was started by the addition of thiosulfate from the second side arm. In order to ascertain that nitric oxide was the nitrogenous substance during the initial reduction of nitrite, the method described by Walters and Taylor (1964) was employed. When nitric oxide was generated, the procedure employed was similar to the one used by Baalsrud and Baalsrud (1954).

Difference Spectra. The difference absorption spectra were obtained by means of a Cary, Model 14, double beam recording spectrophotometer. The intact cell suspensions, under anaerobic conditions, contained thiosulfate in the treatment cuvette whereas the reference cuvette lacked the substrate. Potassium nitrate, potassium nitrite, nitrous oxide or nitric oxide were used as the terminal electron acceptors.

Protein Estimation. Protein was determined by the biuret method of Gornall et al. (1949).

Chemicals. All chemicals were obtained from commercial sources and were of the highest purity available. The ATP, CoA, acetyl CoA, acetoacetyl CoA, $NADP^+$, NADPH, NAD^+, NADH and 3-acetylpyridin-NAD were purchased from Sigma Chemical Co., St. Louis, Missouri, U.S.A.

Thiobacillus neapolitanus was grown autotrophically as described by Vishniac and Santer (1957). The cultures were continuously neutralized

with 2.0 M potassium carbonate, maintaining the final pH at about 7.0. Cells were collected at 4° in a DeLaval continuous flow centrifuge and washed subsequently with 0.05 M Tris-HCl buffer (pH 7.5). The cells were disrupted by passing through a French press at 17,000 P.S.I. and the extract was centrifuged at 8000 g for 20 min to remove large fragments and unbroken cells.

Phosphorylation Studies with Whole Cells

The intact cells were washed twice with 0.02 M potassium phosphate buffer (pH 7.5) containing 3% NaCl and finally suspended in a small volume of this solution to give a final concentration of 86 mg dry weight of cells per 10 ml suspension. Two milliliters of this suspension were pipetted in a flask and incubated on a shaker at 30° for 1 min, and thereafter appropriate substrate (25 mM thiosulfate or 2-ME, 5 mM sulfide) was added. Samples for ATP measurements were taken at times indicated and deproteinized suspensions (Gibson and Morita, 1967) were allowed to stand at 10° for 10 min to facilitate the extraction of ATP. The amount of ATP formed was measured by the luciferin-luciferase assay procedure described by Strehler (1965).

Phosphorylation Studies with Cell-Free Extracts - The supernatant fractions obtained after centrifugation at 8000 g were diluted to give a final protein concentration of 9.7 mg/ml to minimize endogenous oxygen consumption and phosphorylation. Samples for ATP measurements were taken at various times after substrate addition (6.5 mM) and deproteinized, and the amount of ATP formed was measured as described above.

Assay of Acid-Induced ATP Formation - Cells were washed and suspended in 0.02 M potassium phosphate buffer (pH 7.5) containing 3% NaCl to give a final concentration of 20 mg dry weight of cells per 5.0 ml

suspension. An aliquot of 0.5 ml of this suspension was pipetted in a flask and incubated for 1 min in ice on a shaker; thereafter 0.8 ml of acid buffer (3mM HCl, pH 2.5; or a mixture of 3 mM succinate and 3 mM glutamate, pH 3.8) was quickly added with a syringe. After incubation in acid buffer as indicated, 1.8 ml of 0.1 M Tris-HCl (pH 8.3) containing 5 μmol of inorganic phosphate was added, and phosphorylation was allowed to occur for 15 sec. The reaction was stopped and the amount of ATP formed was measured as described above. The control flasks had the same contents except that acidic and basic buffers were mixed together prior to the addition to cell suspensions.

The oxygen consumption was measured polarographically and thiosulfate disappearance was followed by the method of Sorbo (1957). Sulfate was measured turbidimetrically as $BaSO_4$ (Gleen & Quastel, 1953) after deproteinization with 10 mM cadmium acetate. Sulfide concentration was continually monitored by recording decrease in absorbance at 230 nm. Low enzyme and sulfide concentration was necessary for the maximal sensitivity of the assay. The extinction coefficient of sulfide at 230 nm was found to be 1.41 mM. Sulfide solution was relatively more stable when prepared in 0.05 M potassium phosphate buffer, pH 8.0.

Rhodopseudomonas palustris was grown anaerobically in magnetically stirred 10-liter carboys maintained at 30-35°C in a medium (Table 1) modified after Hutner (1946) and Cohen-Bazire et al. (1957). Illumination was provided by a bank of 150-watt incandescent lamps providing 10,000 lux at the culture surface. The organism was first grown on malate (20 mM) plus $S_2O_3^=$ (20 mM) followed by growth on formate (20 mM) plus

TABLE 1

Medium for photoautotrophic growth with thiosulfate[a]

Component	Conc. (mM)	Component	Conc. (μM)
$Na_2S_2O_3 \cdot 5H_2O$	20	$MnSO_4 \cdot H_2O$	1.0
K-phosphate	20	Nicotinic acid	8.0
NH_4Cl	20	$ZnSO_4 \cdot 7H_2O$	4.0
$NaHCO_3$	15	PABA	1.5
$MgSO_4$	2	Thiamin-HCl	0.75
$N(CH_2COOH)_3$	1	$CuSO_4 \cdot 5H_2O$	0.15
$CaCl_2 \cdot 2H_2O$	0.5	$CoCl_2 \cdot 6H_2O$	0.10
EDTA $\cdot Na_2$	0.12	$Na_2B_4O_7 \cdot 10H_2O$	0.05
$FeSO_4 \cdot 7H_2O$	0.10	$(NH_4)/Mo_7O_{24} \cdot 4H_2O$	0.075
		Biotin	0.040

Yeast extract, 50 mg/l; pH 6.8
[a]Modified after Hutner (1946) and Cohen-Bazire et al. (1957).

$S_2O_3^{=}$ (20 mM). Inocula from the latter culture yielded an active photoautotrophic growth with $S_2O_3^{=}$ as the sole electron donor. Subsequent cultures were then maintained and grown in the media containing $S_2O_3^{=}$ as the oxidizable substrate.

Preparation of Cell-Free Extracts. After 2 days of growth the cells were collected at 4°C with a DeLaval continuous flow centrifuge (DeLaval Separator Co., Poughkeepsie, New York). The cells (about 20 g wet weight) were washed twice with 0.05 M Tris-HCl (pH 8.0) and suspended in a medium containing 0.05 M Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 0.5 mM EDTA · Na_2 and 0.5 mM GSH. The cells were disrupted by passing twice through an Aminco French pressure cell at 18,000 PSI. Unbroken cells and large fragments were removed by centrifugation at 10,000 g for 30 min. The resulting chromatophore-rich supernatant (S-10,000) was used as the crude cell-free extract. The chromatophore-poor fraction was obtained as the supernatant after centrifugation of the S-10,000 at 144,000 g for 1 h. The supernatant fraction (S-144,000) was dialyzed for 12 h at 4°C against 0.05 M Tris-HCl (pH 8.0) containing 1.0 mM $MgCl_2$, 0.1 mM EDTA · Na_2 and 0.1 mM GSH.

Determination of ATP-Dependent Reduction of NAD^+. The reaction mixture in a total volume of 3.0 ml in Thunberg-type cuvettes of 1-cm light path contained: 2 µmoles NAD^+, 5 µmoles ATP and 130 µmoles Tris-HCl (pH 8.0). The side arm of the cuvette contained: 5 µmoles $MgCl_2$, 20 µmoles $S_2O_3^{=}$, 0.165 µmoles cytochrome c (horse-heart, Sigma Chemical Co., type II) and the dialyzed cell-free preparation. The control cuvette contained all of the components except ATP. The cuvettes were carefully evacuted and the contents of the side arm were tipped in to start the reaction. Absorbance changes due to oxidation and reduction of cytochrome c and NAD^+ were measured at 550 and 340 nm respectively

in a dual-wavelength split-beam recording spectrophotometer capable of recording absorbance changes at two separate wavelengths at the same time in the same reaction mixture.

ATP Assay. The amount of ATP utilized during reversed electron flow was determined by measurement of NADPH formation with modification of the procedure of Kornberg (1950). The reduction of NAD^+ by reversal of electrons was stopped by adding perchloric acid, followed by neutralization with alkaline triethanolamine according to the method of Gibson and Morita (1967). After centrifugation, the supernatant was assayed for ATP. The reaction mixture in a total volume of 3.0 ml contained 100 μmoles glucose, 15 μmoles $MgCl_2$, 0.52 μmoles $NADP^+$, 2.5 mg hexokinase (Sigma Type IV), and 0.18 units of glucose-6-phosphate dehydrogenase (Sigma Type X). The reaction was started by the addition of 0.3 ml of the sample to be assayed for ATP. The formation of NADPH was followed at 340 nm using a full-scale absorbance change of 0.10 on a Beckman Kintrac VII spectrophotometer.

Protein Determination. Protein was determined in duplicate by the biuret method of Gornall et al. (1949).

Biochemicals were obtained from Sigma Chemical Co., St. Louis, Missouri.

After growth in a medium containing malate (20 mM) plus thiosulfate (20 mM), cells were transferred to a medium containing formate (20 mM) plus thiosulfate (20 mM) and then finally the cells were transferred to a medium containing thiosulfate as the sole oxidizable substrate. Cultures could then be grown with transfers to fresh media every 3-4 days for several months without return to photoheterotrophic growth conditions. Cells were collected, after 3 to 4 days of photosynthetic growth in 10-liter

carboys maintained at 30-35°, in a DeLaval continuous flow centrifuge (DeLaval Separator Co., Poughkeepsie, N.Y.). After washing twice with 0.05 M Tris (pH 8.0) the cells were suspended in 0.05 M Tris (pH 8.0) containing 0.5 mM GSH. For cell disruption the suspension was treated for 2 min in a Biosonic III sonic disintegrator (Bronwill Scientific Co., Rochester, N.Y.) or was passed twice through a French press at 15,000 PSI. After centrifugation at 10,000 g for 30 min to remove cell debris, the supernatant was centrifuged further at 144,000 g for 1 h to yield a clear supernatant fraction (S-144,000). After dialysis for 12 h at 4° against 0.05 M Tris (pH 8.0) containing 0.1 mM GSH, the supernatant was used as the enzyme source.

Assay of enzymatic activities. Thiosulfate-cytochrome c reductase, succinate-cytochrome c reductase, formate-cytochrome c reductase, and NADH-cytochrome c reductase activities were measured by following cytochrome c reduction at 550 nm spectrophotometrically with a Cary, Model 14, or a Beckman Kintrac VII spectrophotometer. The reaction mixture contained 40-45 μmoles of Tris (pH 8.0), 0.01-0.02 ml of 4% cytochrome c(w/v) and the dialyzed (S-144,000) supernatant in a total volume of 1.0 ml and the reaction was started by addition of the respective substrate. Cytochrome c-O_2 oxidoreductase (EC 1.9.3.1) activity was measured by following the decrease in absorbance at 550 nm or by measuring oxygen uptake polarographically with a modified Clark-type oxygen electrode. The reaction mixture contained 0.02 ml of 4% reduced mammalian cytochrome c, 45 μmoles of Tris (pH 8.0) and enzyme supernatant in a total volume of 1.0 ml. Reduced cytochrome c was prepared by bubbling the solution of cytochrome c containing palladium asbestos with hydrogen gas for 10 min.

Products of thiosulfate oxidation. Cell-free extracts (S-144,000) containing 40-50 mg of protein or whole cells equivalent to 20 mg dry weight were added in test tubes containing 0.60 ml of 0.05 M Tris (pH 8.0), 0.02 ml of 1.0 M $MgCl_2$ and 0.01 ml of 0.10 M EDTA. Nitrogen was bubbled through each reaction mixture at 25°. After incubation for 3-5 min, 10 μmoles of Na_2 ($S.SO_3$) labelled with ^{35}S in the outer (S-) position and containing 250 μCi of ^{35}S were added at zero time. Aliquots of 0.02 ml were removed at various time intervals and placed in a tube containing 0.08 ml of ice-cold chromatography solvent to stop the reaction. Aliquots of 0.005 ml were then spotted on Whatman No. 1 filter paper and the products of thiosulfate oxidation were separated by paper chromatography as described by Trudinger (1959) using pyridine: propanol-1: water (3:5:5) by volume as the solvent system. After drying, the chromatograms were sprayed with a 0.5% $AgNO_3$ (w/v) in aqueous NH_3 solution and heated to 100°C to develop the spots. Radioactive spots were located by autoradiography by exposing the chromatograms to Dupont Chronex X-ray film for 2-5 days. Spots were identified by R_f comparison with standards. To distinguish between ^{35}S-thiosulfate and ^{35}S-sulfate, which have similar R_f values, selected spots were treated with excess iodine to oxidize thiosulfate to tetrathionate. Radioactive spots appearing on the chromatograms were cut out and counted in a Packard liquid scintillation spectrometer.

Labelled thiosulfate was obtained from Amersham/Searle Corp., Arlington, IL. Cytochrome c (horse heart type II) and other biochemicals were purchased from Sigma Chemical Co., St. Louis, Mo.

CHAPTER III

DATA AND RESULTS

A. Oxidation of Sulfur Compounds by Thiobacillus denitrificans. The obligate autotroph Thiobacillus denitrificans is a facultative anaerobe capable of deriving its energy from the oxidation of reduced sulfur compounds either using nitrate or oxygen as terminal electron acceptor. Baalsrud and Baalsrud (1954) studied the growth characteristics of this organism and they also conducted some work concerning the intermediates involved in nitrate-reduction. Milhaud et al. (1959) reported that cytochrome c mediated the electron-transport from thiosulfate to nitrate or oxygen. The present report is concerned with a further study of the electron transport systems concerned with the oxidation of various sulfur compounds.

1. Oxidation of Thiosulfate. Thiosulfate was oxidized completely by whole cells anaerobically as well as aerobically (Fig. 1,2). At the end of the oxidation period no thiosulfate was left in the reaction mixture and the amount of nitrogen evolved or oxygen taken up corresponded to the amount required by the following equations:

$$5\ S_2O_3^{=} + 8\ NO_3^{-} + H_2O \rightarrow 10\ SO_4^{=} + 4\ N_2 + 2\ H^{+}$$

$$S_2O_3^{=} + 2\ O_2 + H_2O \rightarrow 2\ SO_4^{=} + 2\ H^{+}$$

Due to the presence of sulfur there was always some endogenous activity. Sulfur-free suspensions were therefore obtained by preincubating the cells for 2 hours with nitrous oxide which the cells can use as an oxidant (Baalsrud and Baalsrud, 1954). This treatment did not affect the activity of intact cells, whereas the endogenous respiration was reduced to zero (Fig. 1).

Effect of Inhibitors Upon Thiosulfate Oxidation by Whole Cells. Under aerobic or anaerobic conditions, cyanide and azide inhibited thiosulfate oxidation (Table 2). The anaerobic oxidation of thiosulfate by nitrate on the other hand, was completely inhibited by the inhibitors of the flavoprotein system as well as by antimycin A, HQNO, and Dicumarol. These inhibitors did not affect the aerobic oxidation process. Rotenone and TTFA, however, caused no inhibition initially of the aerobic thiosulfate oxidation but the reaction stopped after the consumption of 2 or 4 μmoles of oxygen when 5 or 10 μmoles of thiosulfate were present in the reaction mixture.

2. Oxidation of Sulfite. Sulfite was oxidized both anaerobically and aerobically by whole cells, however, the rate of sulfite oxidation was 43% of the rate of aerobic thiosulfate oxidation. Anaerobic rate of sulfite oxidation corresponded to about 12% of the anaerobic thiosulfate oxidation rate.

The experiments were carried out at pH 8 in 0.05 M phosphate buffer. The sulfite was made up as a 0.2 M solution in the same buffer containing 5 mM EDTA. Under these conditions no chemical oxidation of sulfite was observed. It may be seen (Fig. 3) that the amount of oxygen taken up, or nitrogen evolved yielded a complete stochiometry according to the equations:

$$5\ SO_3^= + 2\ NO_3^- + 2\ H^+ \rightarrow 5\ SO_4^= + N_2 + H_2O$$

$$SO_3^= + 1/2\ O_2 \rightarrow SO_4^=$$

The effect of various inhibitors on sulfite oxidation is shown in Table 3. In general, the anaerobic oxidation of sulfite by nitrate was more sensitive to cyanide, azide and the inhibitors of the flavoproteins compared to the aerobic sulfite oxidation.

TABLE 2

Effect of inhibitors on thiosulfate oxidation by T. denitrificans

Inhibitor	Concentration	%Inhibition	
		anaerobic	aerobic
Cyanide	0.1 mM	78	93
Azide	1.0 mM	100	70
Rotenone	0.05 mM	100	00
Amytal	5.0 mM	100	00
TTFA	0.1 mM	100	00
Antimycin A	3 µg/mg protein	100	13
HQNO	1.5 µg/mg protein	100	7
Dicumarol	0.1 mM	100	00

Experimental conditions were the same as described in Figs. 1 and 2.

TABLE 3

Effect of inhibitors on sulfite oxidation

Inhibitor	Concentration	%Inhibition	
		aerobic	anaerobic
Cyanide	0.1 mM	42	80
Azide	1.0 mM	21	70
Rotenone	0.05 mM	30	70
Amytal	5.0 mM	40	68
TTFA	0.1 mM	08	41

Experimental conditions were the same as described in Fig. 3

3. Oxidation of Sulfide. The rate of sulfide oxidation by whole cells was about 41% of the rate of thiosulfate oxidation under aerobic and 50% of that under anaerobic conditions. All experiments were performed at pH 8.0 in 0.05 M phosphate buffer to avoid the evolution of hydrogen sulfide. Due to the hygroscopic properties of sodium sulfide it is impossible to prepare exact solutions volumetrically; in addition, the presence of hydrogen sulfide interfered with the stochiometry of the sulfide oxidation. However, the results indicate that both aerobic and anaerobic oxidations were sensitive to cyanide and other metabolic inhibitors (Table 4).

TABLE 4

Effect of inhibitors on sulfide oxidation

Inhibitor	Concentration	% Inhibition	
		aerobic	anaerobic
Cyanide	0.1 mM	80	94
Azide	1.0 mM	35	89
Rotenone	0.05 mM	67	53
Amytal	5.0 mM	69	44
TTFA	0.1 mM	39	90

Experimental conditions as explained in the text.

Cytochrome systems. Intact cells or cell-free extracts from T. denitrificans were observed to contain cytochromes of the type reported by Trudinger (1961) in the chemoautotroph T. neapolitanus. Thus upon treatment

of the T. denitrificans preparations with thiosulfate, sulfide or sulfite the following absorption peaks were observed: alpha absorption peaks 560-558, 555, 553, 550 mμ; beta absorption peaks 530-528, 523, 521 mμ; gamma absorption peaks 430, 428, 424, 420-417 mμ. The above absorption maxima represent the presence of cytochrome of b and c types. In addition, the Soret peaks of the cytochrome oxidase components (e.g. cytochrome a + a_3) also appeared in the region of 440-450 mμ. All of the cytochromes were reduced by thiosulfate under anaerobic conditions and were rapidly oxidized upon addition of nitrate (Fig. 4). When the cell-free extracts were incubated with NADH or succinate under anaerobic conditions, the cytochromes were also reduced; in both cases the cytochromes were oxidized upon the addition of nitrate (Fig. 5a and 5b). The dithionite or thiosulfate-reduced plus CO minus reduced absorption spectra of intact cells or cell-free preparations indicated the presence of cytochrome o and exhibited absorption maxima at 570, 540 and 418 mμ representing the alpha, beta and gamma peaks respectively; the ratio gamma/alpha peaks being about 14 (Fig. 6).

Reduction of NAD^+ by Thiosulfate. The reduction of pyridine nucleotides by thiosulfate in T. novellus and T. neapolitanus has been shown to be an energy-linked process (Aleem, 1966; 1969). A similar situation was also observed in the case of T. denitrificans (Fig. 7). It was observed that the cell-free extracts from the chemoautotroph catalyzed an ATP-driven reduction of NAD^+ by thiosulfate and the process involved reversal of electron transfer from cytochrome c (reduced by thiosulfate) to NAD^+. In this reaction approximately 20 nmoles of ferrocytochrome c were oxidized under anaerobic conditions coupled to the reduction of 6 nmoles of NAD^+

5. NADH - Oxidase. The oxidation of NADH by cell-free extracts was studied under aerobic conditions. The optimum pH was found to be 7.5. The effect of various inhibitors of the respiratory chain is shown in Table 5a. Like the oxidation of thiosulfate, NADH oxidation was inhibited by cyanide, azide, antimycin A, and HQNO. In addition amytal, rotenone, TTFA and atabrine were potent inhibitors. The inhibition caused by atabrine or TTFA could not be restored by added flavin.

Although the activity was mostly connected with the membrane fractions, the existence of a soluble NADH-oxidase may also be considered from the data obtained (Table 5b). It was observed that NADH could be oxidized by cell-free extracts under anaerobic conditions in the presence of added nitrate; the rate of oxidation, however, was about one half of that of the aerobic NADH oxidation but the inhibition pattern was similar in both cases.

It is of interest to note that the NADH oxidase activity in the cell-free extracts from anaerobically grown T. denitrificans is 10-20 fold greater compared to that reported in the case of T. neapolitanus (Trudinger and Kelly, 1968) or other heterotrophic bacteria (Smith et al., 1967). We have observed that the NADH oxidase activity in cell-free extracts from aerobically grown T. denitrificans was markedly decreased and was of the same order as in T. thioparus or T. neapolitanus. Further work is needed to explain these differences.

6. Cytochrome Oxidase. Mammalian cytochrome c, reduced with hydrogen in the presence of palladium asbestos, was rapidly oxidized by cell-free extracts at the rate of 45 nmoles per minute per milligram protein, and the cytochrome oxidase was completely inhibited by 1mM cyanide or 0.1 mM azide.

TABLE 5a

Influence of inhibitors on NADH-oxidase

Inhibitor	Concentration	% Inhibition
Cyanide	3.0 mM	74
Azide	10.0 mM	40
Amytal	2.0 mM	36
Atabrine	1.0 mM	99
TTFA	0.1 mM	100
Rotenone	50 µM	93
Antimycin A	1 µg/mg protein	20
HQNO	1 µg/mg protein	30

Reaction mixture in a total volume of 3.0 ml contained: 150 µmoles of Tris-HCl (pH 7.5), and cell-free extract containing 6.0 mg protein. The reaction was started by the addition of 0.3 µmoles of NADH to the test cuvette, and the oxidation of NADH was followed at 340 mµ.

TABLE 5b

Localization of NADH-oxidase

Fraction	NADH Oxidized (nmoles/min/mg protein)
5,000 g supernatant	11.5
30,000 g supernatant	23.4
30,000 g pellet	100.0
144,000 g supernatant	218.0
144,000 g pellet	8.0

Experimental conditions were the same as described in Table 5a.

Discussion of Results. Based upon our experimental observations it appears that in T. dentrificans different electron transport pathways are involved in thiosulfate oxidation by molecular oxygen and by nitrate. Aerobically the thiosulfate molecule, as a whole, is oxidized to sulfate through the mediation of cytochrome c and cytochrome c: O_2 oxidoreductase and this reaction is not blocked by antimycin A or HQNO as well as by several of the flavoprotein inhibitors. The anaerobic pathway of thiosulfate oxidation involves the enzymatic transfer of electrons from thiosulfate to nitrate and this process is strongly inhibited by low concentrations of rotenone or TTFA, antimycin A or HQNO, and dicumarol indicating the probable involvement of the flavoprotein(s), cytochrome b and possibly quinones or a vitamin K analogue. It appears that under anaerobic conditions the thiosulfate molecule is reductively cleaved by thiosulfate reductase into the sulfide and sulfite moieties and thus both sulfide and sulfite may in some manner couple with the electron transport chain at the flavoprotein level and therefore, the oxidation of sulfite and sulfide becomes sensitive to the flavoprotein inhibitors. This hypothesis is in harmony with the observed sensitivity of sulfide and sulfite oxidation to the flavoprotein inhibitors. The coupling of sulfite with the flavoprotein must, however, involve an activation energy since the E_0' of the $SO_4^{=}/SO_3^{=}$ system is +0.43 V. Such an activation of sulfite has been observed in the case of Thiobacillus neapolitanus which requires ATP in order for the sulfite to couple with the flavoproteins (Saxena and Aleem, unpublished data). The latter workers observed that this reaction was also sensitive to rotenone or atabrine. In addition possibility cannot be excluded that the

thiosulfate reductase enzyme may also be sensitive to flavin inhibitors. Further work is in progress to elucidate this mechanism. Since both the aerobic and anaerobic thiosulfate oxidations are markedly sensitive to cyanide and azide, it may be concluded that cytochrome oxidase mediates the oxidation of thiosulfate by molecular oxygen as well as by nitrate.

Aubert et al. (1959) reported the presence of a cytochrome of a-type on the basis of an absorption peak at 418 mμ which appeared in a reduced + CO minus reduced spectrum of cell-free extracts from T. denitrificans. However, Trudinger (1967) interpreted this as an indication of the presence of an o-type cytochrome and our observations show that an o-type cytochrome is indeed present in this organism. Sulfite oxidation by molecular oxygen appears to be catalyzed mainly by cytochrome o since this reaction is not markedly sensitive to cyanide or azide (Table 2).

The cell-free preparations from T. denitrificans also catalyze the aerobic oxidation of NADH involving a complete electron transport chain. Although the oxidation of NADH is markedly sensitive to the flavoprotein inhibitors and to a lesser extent to antimycin A and HQNO, relatively much higher concentrations of cyanide or azide are required to achieve a 50-70% inhibition. It would appear therefore that the terminal oxidase involved in the oxidation of NADH is a cytochrome of o-type rather than the normal cytochrome oxidase which is inhibited by low cyanide or azide concentrations in T. denitrificans. A similar situation has been observed in the case of T. neapolitanus (Aleem, 1969). The cell-free extracts from T. denitrificans also catalyze the oxidation of NADH under anaerobic conditions with nitrate as the terminal electron acceptor and this process has been observed to be

mediated by cytochrome systems. In addition the disappearance of the peak at 475 mμ upon addition of nitrate indicates that cytochrome o is probably involved in this process (Fig. 5a).

The cell-free extracts also contain low succinic dehydrogenase activity due to the fact that the cytochromes are reduced by succinate under anaerobic conditions. Thus far, we have not been able to demonstrate the presence of α-ketoglutarate dehydrogenase in this organism and these observations are in agreement with those of Taylor et al. (1969), and do in fact support the hypothesis advanced by Smith et al. (1967) that in obligate chemoautotrophs the tricarboxylic acid cycle is blocked at the α-ketoglutarate dehydrogenase level.

Being an obligate chemoautotroph T. denitrificans must generate reduced pyridine nucleotides with thiosulfate as the energy yielding substrate. However, the reduction of NAD^+ or $NADP^+$ by thiosulfate in T. novellus or T. neapolitanus has been observed to be an energy-dependent reaction (Aleem, 1966, 1969). We have shown that in T. denitrificans, the reduction of NAD^+ by thiosulfate is also ATP-dependent and involves the reversal of electron transfer from ferro-cytochrome c to NAD^+. Further work is in progress to characterize the partial reactions involved in this process.

In view of our experimental findings the pathways of electron transport in T. denitrificans are presented in Fig. 8.

B. The Tricarboxylic Acid Cycle and Related Enzyme Systems in Thiobacillus denitrificans and Thiobacillus thioparus. The cell-free extracts from Thiobacillus denitrificans contained most of the tricarboxylic acid cycle enzymes and their activities were comparable with those found by Smith et al. (1967) and Johnson & Abraham (1969a) in T. thioparus (Table 6).

TABLE 6

Tricarboxylic acid cycle and some related enzymes in cell-free extracts from *Thiobacillus denitrificans* as compared to *Thiobacillus thioparus*

Activities are expressed as nanomoles/min./mg. protein.

Enzyme	T. denitrificans	T. thioparus*
Citrate synthase	30·I	40·7
Aconitase (isocitrate as substrate)	23·I	I2·0
Isocitrate dehydrogenase (NADP-specific)	45·0	204·4
		74·0
α-Ketoglutarate dehydrogenase	0·0	0·0
Succinyl CoA synthetase	0·0	---
Succinate dehydrogenase	0·5	7·5
Fumarase (fumarate as substrate)	2I5·0	79·6
Malate dehydrogenase (oxaloacetate as substrate)	32·3	223·5
		28·0
Isocitrate lyase	9I·5	---
Malate synthetase	I2·0	---
3-Ketoacyl CoA transferase	I6·3	---
Glutamate dehydrogenase	2·6	---

*Smith et al. (1967) and Johnson & Abraham (1969 a).

Assays for α-ketoglutarate dehydrogenase were repeated several times using both the methods described by Kaufman (1955) and Amarasingham & Davis (1965), but without success. In addition, we failed to demonstrate the presence of succinyl CoA synthetase in Thiobacillus denitrificans. The latter enzyme was also absent in the autotrophically grown blue-green algae Anabaena variabilis and Anacystis nidulans (Pearce et al. 1969). Succinate dehydrogenase activity was quite low, but was increased by a factor of 1·5 when pellet was added back to the cell-free extract. It should be noted that, while the apparently higher succinate dehydrogenase activity in T. thioparus was based on the rate of reduction of cytochrome c, we have expressed the specific activity of this enzyme on the basis of the rate of reduction of phenazine methosulphate. Among the other enzymes assayed, isocitrate lyase was present at a high level, while the activity of glutamate dehydrogenase was quite low.

The same enzymes were measured in extracts of Thiobacillus-A2 grown under various conditions (Table 7). Cell-free extracts from the organism grown autotrophically showed some distinct but important similarities with the T. denitrificans enzyme pattern: citrate synthase, isocitrate dehydrogenase, fumarase and malate synthetase were present at about the same level of activity while α-ketoglutarate dehydrogenase and succinyl CoA synthetase could not be detected. Succinate dehydrogenase and glutamate dehydrogenase, however, were present at about the same level in both autotrophically and heterotrophically grown organisms.

Among the aerobic and anaerobic heterotrophic growth conditions examined significant differences in the levels of various enzymes were observed in the case of organisms grown anaerobically on succinate.

TABLE 7

The tricarboxylic acid cycle and some related enzymes in cell-free extracts from Thiobacillus-A2

Activities are expressed as nanomoles/min./ mg. protein.

Enzyme	Organism grown: Aerobically with thiosulphate	Aerobically with glutamate	Aerobically with succinate	Anaerobically with succinate
Citrate synthase	23·2	I0·2	6·0	NT
Aconitase	35·4	NT	2I·5	5I3·0
Isocitrate dehydrogenase	34·8	I40·8	258·0	270·0
α-Ketoglutarate dehydrogenase	0·0	95·I	I0·6	35·3
Succinyl CoA synthetase	0·0	NT	267·0	300·0
Succinate dehydrogenase	7·5	6·4	8·7	42·5
Fumarase	I95·0	I74·0	2II·0	I065·0
Malate dehydrogenase	2I3·0	85·5	I96·3	I95·9
Isocitrate lyase	I6·2	I7·3	9·7	8·7
Malate synthetase	I0·3	NT	I3·2	I44·9
3-Ketoacyl transferase	50·2	3I·3	37·6	42·I
Glutamate dehydrogenase	22·0	2I·6	20·0	30·0

NT = Not tested.

These organisms contained comparatively greater aconitase, succinate dehydrogenase, fumarase and malate synthetase activities. Under all of the heterotrophic growth conditions both the α-ketoglutarate dehydrogenase and the succinyl CoA synthetase were easily detected.

Discussion of Results. Smith et al. (1967) advanced the hypothesis that obligate autotrophs lack both NADH oxidase and α-ketoglutarate dehydrogenase activities and proposed that the absence of these enzymes could provide a biochemical explanation for the inability of strict autotrophs to use organic compounds as sources of energy.

However, NADH oxidase has been demonstrated in many obligate autotrophs. Its presence in substantially high levels has been reported in Nitrobacter agilis (Aleem, 1968; Smith & Hoare, 1968), in Nitrosomonas europaea (Hooper, 1969), in Thiobacillus neapolitanus (Hempfling & Vishniac, 1965; Aleem, 1969), in Thiobacillus strain c (Trudinger & Kelly, 1968), in T. thioparus and T. thiooxidans (Johnson & Abraham, 1969 a), and in T. denitrificans (Peeters & Aleem, 1970 a). Although the role of NADH oxidase in the obligate autotrophs has not yet been well established, the enzyme has been shown to catalyse the process of oxidative phosphorylation with remarkable efficiency in Nitrobacter wynogradskii (Kiesow, 1964) and in N. agilis (Aleem, 1968).

Based upon direct enzymic analyses, and the study of the incorporation of labelled acetate, the existence of an incomplete tricarboxylic acid cycle, lacking α-ketoglutarate dehydrogenase, has been fairly well established in several of the thiobacilli (Smith et al. 1967; Kelly, 1967; Johnson & Abraham, 1969 b), blue-green algae (Hoare & Moore, 1965; Smith et al. 1967; Pearce et al. 1969), and also in two photosynthetic bacteria (Callely, Rigopoulos & Fuller, 1968; Fuller, Smillie, Sisler & Kornberg, 1961).

Our results further substantiate the hypothesis that in most of the autotrophic organisms the tricarboxylic acid cycle is incomplete. In agreement with Taylor, Hoare & Hoare (1969) we were unable to detect the α-ketoglutarate dehydrogenase in Thiobacillus denitrificans. We also failed to demonstrate this enzyme in cell-free extracts from Thiobacillus-A2 when grown autotrophically. Our results further indicate that the absence of α-ketoglutarate dehydrogenase cannot be considered as a causative feature of obligate autotrophy. Indeed, the fact that this enzyme is present in Thiobacillus-A2 when grown heterotrophically suggests that it is repressed under autotrophic growth conditions. As already suggested by Smith et al. (1967), such a repression might be expected, because in autotrophic organisms the Krebs cycle appears to lose its energetic function and may thus fulfil only biosynthetic needs for which the presence of α-ketoglutarate dehydrogenase is not necessary. The same authors could not find such a repression in the facultative autotroph Hydrogenomonas eutropha, but it is interesting to note that Amarisingham & Davis (1965) reported a repression of α-ketoglutarate dehydrogenase in Escherichia coli when this organism was grown on carbon sources that do not require terminal respiration. Although the mechanism of repression under autotrophic growth conditions is not clearly understood at present, one might still expect to find a low α-ketoglutarate dehydrogenase activity even in obligate autotrophs. Smith & Hoare (1968) already reported low level in Nitrobacter agilis, although Aleem (1970) failed to observe any activity of α-ketoglutarate dehydrogenase in pure cultures of this organism. Quite puzzling is the finding of Butler & Umbreit (1969), who reported that the α-ketoglutarate dehydrogenase activities in T. thiooxidans were twice the highest figures

published for E. coli; while Smith et al. (1967), working with the same organism, could not detect any activity

One might further expect that, if the Krebs cycle serves only the biosynthetic needs during chemosynthesis (or autotrophy), the levels of all the enzymes involved would decrease; this was indeed the case in Thiobacillus denitrificans and for most of the enzymes in Thiobacillus-A2 grown autotrophically. Surprising is the absence of succinyl CoA synthetase in both organisms, under autotrophic growth conditions especially, in view of the important biosynthetic role of this enzyme. Pearce et al. (1969) did not find this enzyme in Anacystis nidulans and Anabaena variabilis, and suggested that its function was taken over by the enzyme 3-ketoacyl CoA transferase. Although the latter enzyme was present under both autotrophic and heterotrophic growth conditions, it is not clear why this should be so. It is apparent, therefore, that the control exerted by autotrophic conditions on the heterotrophic cellular metabolism warrants further investigations.

The glyoxylate pathway is present in these organisms, but no evidence can be deduced from our results that this pathway is more important in autotrophic organisms.

C. Intermediates of Denitrification in Thiobacillus denitrificans. The chemoautotrophic sulfur bacterium Thiobacillus denitrificans can grow anaerobically in the presence of nitrate with either thiosulfate or elemental sulfur as the oxidizable substrates (Baalsrud and Baalsrud, 1954). The intact cells of the organism have been shown to catalyze the oxidation of thiosulfate, sulfide and sulfite with either nitrate or molecular oxygen as the terminal electron acceptor involving participation of the electron transport chain (Peeters and Aleem, 1970). The

earlier observations of Baalsrud and Baalsrud (1954) did indicate that nitrite and nitric oxide were the probable intermediates of nitrate reduction by T. denitrificans. Renner and Becker (1970) have reported nitrous oxide as the end product of nitrate, nitrite, or nitric oxide reduction by Corynebacterium nephridii. In addition, it has been suggested by several investigators that nitrous oxide was not an obligatory intermediate in the denitrification process (Allen and Van Niel, 1952; Sacks and Barker, 1952; Delwiche, 1956; Fewson and Nicholas, 1961). Interestingly enough, Payne et al. (1971) has been able to show that the heterotrophic denitrifyer Pseudomonas perfectomarinus could grow anaerobically with nitrate, nitrite, or nitrous oxide as the terminal acceptors; and the cell-free fractions from this organism could catalyze the reduction of nitrite to nitric oxide, nitric oxide to nitrous oxide and the latter was reduced enzymatically to nitrogen gas. Similar results have been obtained by Matsubara and Iwasaki (1971) and Matsubara and Mori (1968) using Alcaligenes faecalis.

The data presented in this paper show that both nitric oxide and nitrous oxide are the intermediates of nitrate or nitrite reduction with nitrogen gas as the final end product in T. denitrificans grown autotrophically on thiosulfate with nitrate as the terminal electron acceptor under anaerobic conditions.

It was shown previously (Peeters and Aleem, 1970) that whole cells of Thiobacillus denitrificans oxidized thiosulfate completely under anaerobic conditions according to the following equation:

$$5\ S_2O_3 = + 8\ NO_3^- + H_2O \dashrightarrow 10\ SO_4^= + 4\ N_2 + 2\ H^+.$$

The data in Fig. 9 show that intact cells also catalyzed a complete oxidation of thiosulfate anaerobically with nitrite as the oxidant which

was reduced to gaseous nitrogen. Thus, the oxidation of 5, 8 and 10 μmoles of thiosulfate resulted in the evolution of 110, 174 and 219 μl of N_2 respectively; the theoretically expected values being 112, 179 and 224 μl when thiosulfate is oxidized according to the following reaction:

$$S_2O_3^= + 2\ NO_2^- + H_2O \dashrightarrow 2\ SO_4^= + N_2 + 2\ H^+.$$

In the above experiment excess nitrite (25 μmoles) was used.

Fig. 10 illustrates the gas production by whole cells, supplied with 25 μmoles of thiosulfate and various amounts of nitrite. It may be seen that the amount of nitrogen produced i.e., 86, 131, 187 and 220 μl respectively corresponded closely with the stoichiometrically quantitative reduction of 8, 12, 16 and 20 μmoles of added nitrite. It was observed, however, that in the presence of nitrite as the terminal electron acceptor there occurred always an over-production of gas. After the initial rapid production of gas had ceased, a subsequent slow gas uptake was observed upon continued shaking of the suspensions until stationary level was reached which yielded a complete stoichiometry according to the above equation. Since there was always some endogenous activity due to the presence of sulfur, the cell suspensions were preincubated for approximately 2 h with nitrous oxide which can be used as an oxidizing agent by T. denitrificans; such suspensions showed no endogenous activity in any of the reaction vessels which lacked either nitrite or thiosulfate or both.

1. Reduction of Nitric Oxide by Whole Cells. It is well extablished that nitrite is the first intermediate in the denitrification of nitrate (Allen and Van Niel, 1952). In early studies by Baalsrud and Baalsrud

(1954] nitric oxide was considered as the first product of nitrite reduction by T. denitrificans. Since nitric oxide is absorbed by alkaline sulphite solution and the product formed on acidification is converted to sulfate and nitrous oxide, we took advantage of this reaction to establish the first product of nitrite reduction. The results of such an experiment are shown in Fig. 11. Trace 1 represents the final stationary level of gas (130 µl) which would be expected from the complete reduction of added 12 µmoles of nitrite. Trace 2 indicates that during the rapid and initial production of nitric oxide the latter was partly absorbed by the alkaline sulfite solution and partly reduced to nitrogen gas by the cells until a stationary stage was reached. Upon addition of 0.05 ml of 25% H_2SO_4 in the sulfite solution a stormy evolution of gas occurred which soon reached a stationary state in a shaking flask. The final quantity of gas evolved (132 µl) was very close to the amount of nitrogen produced in the control flask. Manometric experiments thus revealed that the first product of nitrite reduction by T. denitrificans was nitric oxide. These results also indicated that the over production of gas during the intial stages of nitrite reduction was due to nitric oxide which was produced at a faster rate than that of its conversion to nitrogen gas.

In preliminary experiments it was observed that when nitric oxide gas was provided in excess into the reaction flasks containing nitrate grown cells, nitric oxide proved to be very toxic and the cells were unable to use it as an oxidizing agent anaerobically in the presence of thiosulfate. However, when a small amount of nitric oxide gas, e.g., 190 and 300 µl was supplied (Fig. 12), reduction of nitric oxide gas occurred anaerobically with the concomitant oxidation of thiosulfate

after exhibiting a lag period of about 10 min. The process continued until all of the added nitric oxide was converted to nitrogen. These observations thus provided a direct evidence that nitric oxide is indeed an intermediate in nitrite reduction and that T. denitrificans can reduce nitric oxide further to nitrogen gas.

Since nitric oxide can be generated stoichiometrically from the known amount of added nitrite according to the following equation:

$$KNO_2 + KI + H_2SO_4 \dashrightarrow NO + I + K_2SO_4 + H_2O$$

generated nitric oxide was employed in all subsequent experiments when nitric oxide served as the terminal electron acceptor for thiosulfate oxidation. The results presented in Table 8 show that the generation of nitric oxide occurred according to the added amounts of nitrite; upon addition of thiosulfate from the side arm of the manometric vessel the nitric oxide was reduced to nitrogen gas stoichiometrically by cell suspensions from T. denitrificans. It may be seen that the observed values were well within the limits of experimental accuracy.

2. Effect of Inhibitors on the Denitrification Process. The data in Table 9 show that the oxidation of thiosulfate by nitrate and by nitric oxide was sensitive to the flavoprotein inhibitors such as rotenone, amytal, atabrine and TTFA. Nitrogen evolution from nitrate during the anaerobic oxidation of thiosulfate was almost completely inhibited by antimycin A or NHQNO as well as by cyanide or azide. Under similar conditions about 75-100% inhibition in the rate of N_2 formation from nitric oxide was observed. The inhibition by dicumarol suggests the possible involvement of a quinone or vitamin of K-type. The reduction of nitrite to nitrogen gas was much less sensitive to the electron transport inhibitors.

TABLE 8

Reduction of nitric oxide generated from various amounts of nitrite

Nitrite (μmoles)	Nitric oxide produced		
	Experimental value (μl)	Theoretical value (μl)	Nitrogen produced (μl)
4	94	90	44
8	190	180	88
12	266	269	130
16	344	358	170
20	440	448	216

Each reaction flask contained cell suspensions containing 8 mg protein in the main compartment and various amounts of potsssium iodide and nitrite in the side arm sealed with a rubber stopper. After equilibration and flushing the reaction flask with helium for 15 min, H_2SO_4 was introduced by means of a syringe into the side arm containing KI and nitrite. When the nitric oxide gas evolution was complete, 25 μmoles of thiosulfate were tipped from the second side arm of the reaction flask into the main compartment and N_2 production was measured at 30 C.

TABLE 9

Effect of inhibitors on the anaerobic reduction of nitrate, nitrite, and nitric oxide by thiosulfate in *Thiobacillus denitrificans*

Inhibitor	Concentration	Percent inhibition of gas evolution Electron acceptor		
		NO_3^-	NO_2^-	NO
Rotenone	0.06 mM	60	5	40
Amytal	5.00 mM	82	2	42
Atabrine	0.50 mM	55	5	41
TTFA	1.00 mM	95	5	44
Dicumarol	0.20 mM	100	8	96
Antimycin A	7 µg/mg prot.	100	6	75
NHQNO	7 µg/mg prot.	100	4	76
Cyanide	0.50 mM	100	0	100
Azide	1.00 mM	95	8	75

Nitric oxide was generated by the same procedure as described in Table 8. When NO_3^- or NO_2^- served as the electron acceptors experimental conditions were similar to those described in "Materials and Methods" except that various inhibitors were added as indicated.

3. Participation of Cytochrome Systems in the Process of Denitrification. It was shown previously (Peeters and Aleem, 1970) that the oxidation of thiosulfate by T. denitrificans was mediated by cytochrome systems. When the nitrous oxide-treated cell suspensions were incubated with thiosulfate under anaerobic conditions all of the cytochromes were reduced (Fig. 13, trace 1) and they were readily oxidized under anaerobic conditions by nitrate, nitrite, nitric oxide or nitrous oxide as shown by the respective traces 2, 3, 4 and 5. In order to confirm that the oxidation of the cytochromes by nitric or nitrous oxide was due to the enzymatic and not a chemical reaction, Thiobacillus novellus a facultative chemoautotroph capable of utilizing thiosulfate as the substrate was used as a control. All of the cytochromes were reduced when the cell suspensions from T. novellus were treated with thiosulfate under anaerobic conditions but remained unchanged when nitric oxide or nitrous oxide was passed through the anaerobic suspensions in the treatment cuvette (Fig. 14). These observations thus clearly show and confirm our manometric experiments that nitrate, nitrite, or nitric oxide can serve as the terminal oxidants. The results also suggest that nitrous oxide might also be a possible intermediate in the process of nitrate or nitrite reduction since nitrous oxide also served as an oxidant of the reduced electron transport chain components.

As the dissimilatory nitrate reductase system was markedly sensitive to HNQNO and cyanide (Peeters and Aleem, 1970) the effect of these inhibitors on electron transfer reactions was investigated. The data in Fig. 15 (trace 1) show that the addition of thiosulfate to cell suspensions under anaerobic conditions resulted in the reduction of cytochromes of b- and c-like components. Addition of NHQNO and NO_3^-

under these conditions caused the oxidation of cytochrome c and reduction of cytochrome b; in addition, there was a marked increase in the trough at the 460 nm region indicating reduction of the flavoprotein system (trace 2). The data also showed that the oxidation of the thiosulfate-reduced cytochrome system by NO_3^- was inhibited by cyanide (trace 3). Similar effects of NHQNO and cyanide on the oxidation of thiosulfate-reduced cytochrome system was observed when N_2O or NO served as the terminal electron acceptors under anaerobic conditions.

Unlike the anaerobic oxidation of thiosulfate by nitrate, nitrous oxide or nitric oxide, the oxidation of $S_2O_3^=$ by NO_2^- was relatively insensitive to NHQNO, cyanide and other inhibitors of the electron transport chain. It appears that the nitrite reductase system accepts electrons directly from the flavin level. This is supported by our observations that in the NHQNO-blocked electron transport chain the flavoprotein system did not remain in the reduced state upon addition of NO_2-(trace 2, Fig. 16) and the trough at 460 nm completely disappeared although absorption peaks of reduced cytochrome b were still apparent. Similar results were obtained when NO_2- was added to the S_2O_3 =-treated cell suspensions in the presence of cyanide (trace 3, Fig. 8) e.g., cyanide did not inhibit the oxidation by NO_2^- of the $S_2O_3^=$ -reduced cytochromes and flavins.

Discussion of Results. Based on manometric experiments, results reported in this paper have shown that the stochiometry between thiosulfate oxidized and the gas produced from the reduction of nitrite is 1:2. The overall reaction may thus be represented by the following equation:

$$S_2O_3^= + H_2O + 2\ NO_2^- \rightarrow 2\ SO_4^= + N_2 + 2\ H^+$$

Nitrogen gas has also been shown to be the end product of nitrate reduction

in Thiobacillus denitrificans with thiosulfate and sulfite as the electron donors which yielded the following stoichiometry (Peeters and Aleem, 1970):

$$5\ S_2O_3^= + 8\ NO_3^- + H_2O \rightarrow 10\ SO_4^= - 4\ N_2 + 2\ H^+$$

$$5\ SO_3^= + 2\ NO_3^- + 2\ H^+ \rightarrow 5\ SO_4^= + N_2 + H_2O.$$

More recently Adams et al. (1971) were unable to detect N_2 during nitrate reduction in cell suspensions from T. denitrificans "Oslo" strain, although the reduction products contained both NO and N_2O. These interesting observations might be attributed to the strain differences since the "Oslo" strain is least effective in nitrate reduction by thiosulfate compared to sulfide or sulfite; our strain, on the other hand, appears to be most effective with thiosulfate. Moreover, we have observed that the thiosulfate-reduced cytochrome system was oxidized enzymatically in the presence of either NO or N_2O. The reduction product of N_2O is naturally nitrogen gas. The latter has also been identified by gas chromatography as the end product of nitrate or nitrite reduction by thiosulfate.

Nitric and nitrous oxides have been demonstrated as intermediates of denitrification in other organisms (Payne et al., 1971; Matsubara and Mori, 1968; Renner and Becker, 1970; Kluyver and Verhoeven, 1954; Delwiche, 1959; Radcliffe and Nicholas, 1968; Walker and Nicholas, 1961) and the presence of both NO and N_2O during the reduction of nitrate in T. denitrificans (Adams et al., 1971) suggests that they might be the intermediates of dissimilatory nitrate reduction in the chemoautotroph. In addition, the earlier work of Baalsrud and Baalsrud (1954) suggests nitric oxide as an intermediate of nitrate reduction. We have shown in this work that the first product of nitrite reduction in T. denitrificans is in fact nitric oxide. This is borne out by the use of

an alkaline-sulfite nitric oxide-trap in a reaction mixture for NO_2^- reduction followed by the release of N_2O formed from the sulfite-absorbed-NO upon acidification. Moreover, it has been possible to show the stoichiometric reduction of nitric oxide to N_2 in our manometric experiments. Nitrogen gas is most probably the product of N_2O reduction in T. denitrificans. This process appears to be mediated by the cytochrome system since N_2O causes the oxidation of reduced cytochromes of b- and c-types. This reaction undoubtedly is enzymatic since the anaerobic oxidation of the cytochrome system by N_2O is inhibited by NHQNO as well as by cyanide, moreover, N_2O fails to oxidize the thiosulfate-reduced cytochromes in T. novellus.

The participation of a c-type cytochrome in thiosulfate oxidation by nitrate in T. denitrificans has been suggested earlier by Aubert et al. (1959) and the participation of the electron transport system in the oxidation of inorganic sulfur compounds with O_2 or NO_3^- as terminal electron acceptors was studied subsequently by Peeters and Aleem (1970). The experimental data given in this report have shown clearly that T. denitrificans has the ability to reduce NO_2^-, NO and N_2O to N_2 with the mediation of electron transport chain components. This conclusions is further supported by the observations that all of the enzymatically reduced cytochromes are oxidized by these compounds.

Based on the effect of respiratory chain inhibitors it appears that the transport of electrons from thiosulfate to either NO_3^- or NO is mediated by the flavoprotein, cytochromes of b- and c-type and cytochrome oxidase components. It is interesting to note, however, that although the electron transport from thiosulfate to NO_3^- or NO is blocked by the flavoprotein inhibitors, dicumarol, and by antimycin A

or NHQNO, these inhibitors have no effect when O_2 serves as the final electron acceptor (Peeters and Aleem, 1970). This would indicate that under anaerobic conditions the thiosulfate molecule is cleaved into the sulfide and sulfite moeities which are able to couple the electron transport chain at the flavin level. Under aerobic conditions the thiosulfate oxidation appears to involve an oxidative condensation of two thiosulfate molecules yielding tetrathionate with the release of 2 electrons which reduce cytochrome c:

$$2\ S_2O_3^{=} \rightarrow S_4O_6^{=} + 2\bar{e}$$

2 cytochromes c. $Fe^{3+} + 2\bar{e} \rightarrow$ 2 cytochrome c. Fe^{2+}.

Tetrathionate is further metabolized to sulfate and the reduced cytochrome c is oxidized by the cytochrome oxidase. The latter process should naturally be unaffected by the flavoprotein antagonists and by those inhibitors which are capable of blocking electrons at the level of quinones or between cytochromes of b- and c-type. The insensitivity to flavoprotein inhibitors and to antimycin A or NHQNO of the aerobic thiosulfate oxidation has been observed in T. novellus (Aleem, 1965, 1966) and in T. neapolitanus (Ross et al., 1968; Saxena and Aleem, 1973).

Allen and Van Niel (1952) reported that NO_2^- could be converted to gaseous products in the presence of cyanide while under similar conditions reduction of NO_3^- or N_2O was completely prevented by cyanide. Likewise, Matsubara and Iwasaki (1971) demonstrated that reduction of NO to N_2O and N_2 was markedly inhibited by cyanide or azide whereas the same concentration of these inhibitors had little effect on NO_2^- reduction. The data in Table 2 also show that the transport of electrons from thiosulfate to nitrite is insensitive to any of the electron transport chain inhibitors. It would also appear that the electron transport

chain is modified in the presence of excess nitrite which could accept electrons directly from the flavoprotein system. If this is so then the flavoproteins mediating electrons between thiosulfate and nitrite must be different from those involved in nitrate or nitric oxide reduction since the latter systems are sensitive to rotenone, amytal, atabrine and TTFA while the nitrite reductase system is unaffected by these inhibitors. The observed oxidation by nitrite of the thiosulfate-reduced cytochrome system could be due to the cytochrome peroxidase system which has been found to be quite active in the cell-free extracts (unpublished data). This explanation would also account for the cyanide and NHQNO insensitivity of the oxidation of cytochrome system by nitrite (Fig. 8). In view of our experimental observations the electron transport pathways involved in the dissimilatory reduction of inorganic nitrogen compounds in T. denitrificans are presented in Fig. 17.

D. Oxidation of Sulfur Compounds and Energy Generation in Thiobacillus neapolitanus. Thiobacillus neapolitanus derives energy for CO_2 assimilation and other biosynthetic reactions from the oxidation of inorganic sulfur compounds. Vogler and Umbreit (1942) suggested initially that energy-rich phosphate compounds are produced by thiobacilli during the oxidation of inorganic sulfur compounds. This conclusion has been supported by the work of Kelly and Syrett (1964; 1966) who were able to demonstrate that the reductive CO_2 assimilation coupled to the oxidation of inorganic sulfur compounds in T. thioparus cells was DNP-sensitive especially when sulfide oxidation was the energy source. Results of these workers indicate that in T. thioparus both electron-transport-linked and the substrate-level phosphorylation take place; however, the latter appeared to be linked more to thiosulfate

oxidation. Furthermore, molar growth yield calculations by Hempfling and Vishniac indicate that T. neapolitanus produces more ATP than is accounted for by the substrate-level phosphorylation alone (Hempfling and Vishniac, 1967). These workers, (1965) also demonstrated a DNP-sensitive phosphate esterification coupled to the oxidation of 2-ME, a nonphysiological electron donor resulting in P/O ratios of 0.4; the cell-free extracts, however, failed to catalyze oxidative phosphorylation with sulfide, thiosulfate, or NADH.. Davis and Johnson (1967) were able to demonstrate a DNP-sensitive phosphorylation coupled to sulfite oxidation in T. thioparus but the P/O ratios were only 0.13. Similar P/O ratios with sulfite were reported by Charles and Suzuki (1966) using T. novellus cell-free extracts. The recent work of Cole and Aleem (1967, 1971), however, demonstrated P/O ratios of 0.9 with thiosulfate or ascorbate, 1.9 with succinate, and 2.3 with NADH as the electron donors in T. novellus. In view of these findings the status of oxidative phosphorylation in T. neapolitanus was reinvestigated.

1. Thiosulfate Oxidation by Intact Cells and Cell-Free Extracts. After a short lag freshly prepared intact cells from T. neapolitanus oxidized thiosulfate completely and at a linear rate. There was no polythionate accumulation, and the stoichiometry of oxygen consumed to thiosulfate oxidized (1.96) suggests that thiosulfate was completely converted to sulfate by the following equation:

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

Cell-free extracts, on the other hand, were unable to oxidize thiosulfate completely and a stoichiometry (1.38) of oxygen consumed to $S_2O_3^{2-}$ oxidized suggested the accumulation of incompletely oxidized products(s). Chromatographic analysis of the reaction mixture during

thiosulfate oxidation by cell-free extracts revealed that the products formed were sulfate and tetrathionate. When thiosulfate was supplied as ($S \cdot {}^{35}SO_3^{2-}$) or (${}^{35}S \cdot SO_3^{2-}$), no significant difference in the rate of sulfate production and thiosulfate disappearance was observed with intact cells or cell-free extracts indicating that both the inner and the outer sulfurs were oxidized at the same rate. The thiosulfate-reduced plus CO minus thiosulfate-reduced difference absorption spectra of cell-free extracts revealed that cytochrome of a-type was involved predominantly in thiosulfate oxidation in addition to cytochrome o (Fig. 18).

2. Phosphorylation Coupled to Thiosulfate Oxidation. Attempts to achieve phosphorylation coupled to the oxidation of thiosulfate or sulfite in extracts of T. neapolitanus have yielded negative results. We have observed that the cell-free extracts contained a potent adenylate kinase activity responsible for the formation of 3.6 nmol ATP/min/5 mg protein; the enzyme was unaffected by 10 mM potassium flouride or polyuridylic acid (2 mg/ml reaction mixture). The latter compound has been reported to be effective in inhibiting adenylate kinase from rabbit muscle (Cassuto and Chargaff, 1969). Under our experimental conditions, no ATP was formed from AMP and P_i or from AMP and pyrophosphate.

The endogenous cellular ATP content in T. neapolitanus was about 2 nmol/mg dry weight of cells and was unaffected to a significant extent by growth conditions, by starvation for prolonged periods, or by incubating for 1/2 h with 20 mM β-hydroxybutrate, a substrate found effective in reducing the ATP level in R. rubrum (Smith and Ramirez, 1965). A decrease in endogenous ATP content to 0.4 nmol/mg dry weight in T.

neapolitanus was observed only when the cells were preincubated at pH 9.0. The data in Fig. 19 show that a rapid increase in the cellular ATP occurred upon the addition of thiosulfate, and that in cells preincubated at pH 9.0 for 10 min a net increase in ATP content was sevenfold. The initial rapid rise in cellular ATP was followed by a slow increase for about 15 s, after which the ATP content was decreased. Treatment of whole cells with EDTA (1 mM) did not impair their phosphorylating ability.

The thiosulfate-associated formation of ATP in intact cells of T. neapolitanus was inhibited by the uncouplers of oxidative phosphorylation such as DNP and CCCP (Table 10) at concentrations at which the oxygen consumption was either unaffected or only slightly decreased. However, the process was not affected by inhibitors of the flavoprotein system such as amytal and rotenone, as well as by antimycin A which blocks electron transfer between cytochrome b and c. Cyanide, on the other hand, inhibited both oxygen consumption and phosphorylation.

3. Oxidation of Sulfide. Sulfide was oxidized by whole cells as well as by crude cell-free extracts (Fig. 20). Whereas the ratio of sulfide disappeared to oxygen consumed by whole cells was approximately 1.3, this ratio rose as high as 3.5 in the case of cell-free extracts. Although intact cells oxidized all of the added sulfide in about 5 min, the oxygen consumption continued afterwards although at a much slower rate. These observations suggested the accumulation of some intermediate during sulfide oxidation which was oxidized slowly even after all of the sulfide disappeared. The reaction appeared to occur in two stages and will henceforth be referred to as stage 1 and stage 2.

TABLE 10

ATP formation linked to the oxidation of sulfur compounds by *T. neapolitanus* cells

Inhibitor	Concentration (nmol/mg dry weight of cells)	ATP formed (nmol)		
		2-ME	Sulfide	Thio-sulfate
No	-	28	32	39
CCCP	1	4	1	5
PCP	40	0	0	0
DNP	20	10	25	2
Amytal	160	0	0	35
Atabrine	40	0	6	40
Antimycin A	1*	1	3	29
Cyanide	200	0	0	0

*Concentration expressed in micrograms per milligram protein.

Note: Experimental conditions have been described in Materials and Methods. Inhibitors were added to the reaction mixture as indicated and preincubated with cells for 3 min before the addition of various substrates. The ATP measurements were carried out 20 s after the addition of the electron donors 2-ME, sulfides or thiosulfate. The endogenous level of ATP in the cells in the absence of any added substrate was 32 nmol after a 20 s period and has been substracted from the ATP values given in the table.

We have observed that not all of the sulfide utilized by the whole cells could be recovered in the form of sulfate and must, therefore, be present in some other form. The molar ratio of oxygen consumed and sulfate formed during sulfide oxidation in stage 1 was higher than the one observed when sulfide oxidation proceeded via stage 2. These results suggest that sulfate in stage 2 was formed completely from the intermediate and that this intermediate compound was more oxidized than sulfide. A higher ratio of sulfide to oxygen with cell-free extracts may be due to the loss of stage 2 activity in the process of cell disruption. Although indirect evidence presented here suggested that sulfide oxidation proceeds via the formation of an intermediate compound, sulfate was the only product identified by the chromatographic analysis of the reaction mixture during sulfide oxidation by whole cells or cell-free extracts (solvent system, pyridine-propanol-water 3:5:5).

The particulate cell-free fraction sedimenting at 144,000 g contained the bulk of the stage 1 oxidase activity and no stage 2 activity (Fig. 21). When the particulate and the supernatant fractions were combined, very rapid sulfide oxidation followed by a slower rate characteristic of stage 2 of sulfide oxidation was observed after all the sulfide was utilized. The rate of sulfide oxidation in stage 1 progressively increased with increase in enzyme concentration whereas stage 2 sulfide oxidation was only slightly affected. An increase in sulfide concentration caused the inhibition of oxygen uptake during stage 1 without exhibiting any effect on stage 2 oxidation; the optimum sulfide concentration for stage 1 oxygen consumption was found to be 0.33 mM.

Sulfide oxidation was markedly sensitive to antimycin A (Table 11). Inhibitors of the flavoprotein system, such as amytal and rotenone, caused only partial inhibition of sulfide oxidation which in T. neapolitanus appears to proceed via flavoprotein because the cell-free preparations catalyzed the reduction of added as well as endogenous flavin, as indicated by the trough at 450 nm (Fig. 22). Partial sensitivity of sulfide oxidation to flavoprotein inhibitors was probably due to the ability of sulfide to chemically reduce cytochrome c which could be oxidized by the cytochrome oxidase system. Sulfide oxidase was not inhibited by uncouplers of oxidative phosphorylation but was very sensitive to metalbinding agents such as cyanide and to sulfhydryl group inhibitors such as p-hydroxymercuribenzoate. The sulfide-reduced plus CO minus sulfide-reduced absorption spectra of cell-free extracts from T. neapolitanus revealed absorption bands in the region of 565, 538, and 415 nm, representative of the α, β, and γ peaks of an o-type cytochrome. No peaks corresponding to the a-type cytochrome were observed when sulfide was the electron donor (Fig. 18). The reduced plus CO minus reduced absorption spectra of the cell-free extracts with dithionite (chemically reducible) was indicative of the presence of both b- and a-type cytochromes (Ross et al., 1968).

4. Phosphorylation Coupled to Sulfide Oxidation. When sulfide was added to fresh cell suspensions of T. neapolitanus, a rapid increase in the cellular ATP content was observed (Fig. 23). The initial increase in sulfide-induced ATP formation was somewhat variable although a two- to three-fold increase in ATP content in 5 s was frequently observed in the presence of added 10 mM sulfide. Upon increase in sulfide concentration, a rapid decrease in ATP levels as well as in oxygen

TABLE 11

Effect of inhibitors on the oxidation and utilization of sulfide by the cell-free extracts

Inhibitor	Concentration (M)	Percentage inhibition	
		Oxygen consumption	S^{2-} utilization
Rotenone	5×10^{-5}	48	55
Amytal	5×10^{-4}	17	54
Antimycin A	4*	70	60
Dicoumarol	1×10^{-4}	48	50
CCCP	1×10^{-5}	24	14
DNP	1×10^{-4}	24	28
CN^-	1×10^{-4}	100	27
Azide	1×10^{-3}	37	20
PCMB	2×10^{-4}	100	100

*Concentration expressed as micrograms per milligram protein.

Note: Experimental conditions were the same as those described in Materials and Methods. Enzyme preparations were preincubated for 3 min with various inhibitors. In the absence of any added inhibitor, 33.2 nmol oxygen were consumed concomitant to the utilization of 106 nmol of sulfide per minute.

consumption occurred. The uncouplers of oxidative phosphorylation such as CCCP, DBP, and PCP completely inhibited the ATP formation by whole cells (Table 10). Dinitrophenol, on the other hand, was only slightly effective in causing the inhibition of the phosphorylation. The synthesis of ATP linked to the oxidation of sulfide was effectively inhibited by amytal, atabrine, and antimycin A.

The sulfide oxidation by the cell-free extracts was coupled to phosphorylation, but P/O ratios were always very low and ranged between 0.1 and 0.3, depending upon the physiological stage of the cells. Lower P/O ratios were obtained with samples taken for ATP and oxygen measurements at 3 or 5 min instead of 1 min. Phosphorylation, coupled to sulfide oxidation, was completely inhibited by DNP and CCCP (Table 12).

Respiratory chain inhibitors, such as antimycin A, rotenone, and amytal, only slightly affected the oxygen consumption, but the coupled phosphorylation was completely inhibited. Sulfide oxidation and associated ATP generation was much more sensitive to cyanide than azide.

Phosphorylation Coupled to the Oxidation of 2-Mercaptoethanol. Intact cells as well as cell-free extracts oxidized 2-ME, although the rates of oxidation with whole cells were slower probably due to the inability of 2-ME to penetrate the cell. Upon the addition of 2-ME to the suspensions of intact cells, a slow increase in the ATP content of the cell was observed (Fig. 19). The 2-ME-induced ATP formation in intact cells was sensitive to CCCP and PCP, relatively less sensitive to DNP, and also strongly inhibited by amytal, atabrine, antimycin A, and cyanide (Table 10). The oxidation of 2-ME catalyzed by the cell-free extracts was coupled to ATP synthesis and yielded P/O

TABLE 12

Effect of inhibitors on phosphorylation coupled to sulfide oxidation catalyzed by cell-free extracts from T. neapolitanus

Inhibitor	Concentration (M)	ATP formed (nmol)	O_2 consumed (ng atoms)	P/O
None	-	33.0	110	0.30
Rotenone	5×10^{-5}	0.0	70	0.00
Amytal	5×10^{-4}	0.0	90	0.00
Antimycin A	2*	0.0	77	0.00
CN^-	1×10^{-4}	0.0	0.0	0.00
Azide	1×10^{-3}	3.5	70	0.05
CCCP	1×10^{-6}	0.0	96	0.00
DNP	1×10^{-4}	0.0	97	0.00

*Concentration expressed in micrograms per milligram protein.

Note: Experimental conditions are described in Materials and Methods. Enzyme preparations were preincubated with the indicated inhibitors for 3 min before the addition of substrate.

ratios which ranged between 0.4 and 0.85. As shown in Table 13, phosphorylation coupled to 2-ME oxidation was inhibited by CCCP as well as oligomycin; dinitrophenol was only slightly inhibitory. Inhibitors of flavoprotein system and antimycin A also completely blocked ATP formation without significantly affecting oxygen uptake. Oxidation of 2-ME by cell-free extracts involved predominantly the participation of cytochrome o although a small peak corresponding to cytochrome a was also observed (Fig. 18).

5. Acid-Induced ATP Synthesis by Whole Cells of T. neapolitanus. Intact cells of T. neapolitanus were able to phosphorylate endogenous ADP to form ATP as a result of proton gradient produced artifically by the addition of acid (Fig. 24). The yield of ATP increased with increasing periods of incubation in acid phase reaching maximum in 30 s. A decrease in the ability of intact cells to make ATP was observed after 30 s due probably to the progressive denaturation of the cellular proteins. Although 2 mM HCl or a mixture of 3 mM succinate and 3 mM glutamate could be used to create the acid phase, the succinate-glutamate system was relatively less effective. The greater effectiveness of HCl is probably due to its ability to penetrate the cell membrane rapidly. Interestingly enough, the phenomenon of acid-induced ATP formation could not be observed when the cells were disrupted. Acid-induced cellular ATP formation by intact cells from T. neapolitanus was effectively inhibited by DNP and CCCP at concentrations similar to those which inhibited the substrate-induced ATP synthesis (Table 14). The respiratory chain inhibitors did not have any effect on the proton-linked ATP synthesis in T. neapolitanus intact cells.

TABLE 13

Phosphorylation coupled to the oxidation of 2-mercaptoethanol catalyzed by cell-free extracts from T. neapolitanus

Inhibitor	Concentration (M)	ATP formed (nmol)	O_2 consumed (ng atoms)	P/O
None	-	115	135	0.85
DNP	2×10^{-4}	88	146	0.60
CCCP	2×10^{-6}	0.0	162	0.00
Oligomycin	2*	0.0	120	0.00
Cyanide	2×10^{-4}	0.0	27	0.00
Azide	2×10^{-4}	25	52	0.48
Antimycin A	2*	0.0	140	0.00
Rotenone	5×10^{-5}	0.0	140	0.00
Amytal	2×10^{-4}	0.0	120	0.00

*Concentration expressed as micrograms per milliliter of protein.

Note: Experimental conditions were the same as described in Materials and Methods. The P/O ratios have been corrected for the endogenous ATP synthesis and O_2 uptake values

TABLE 14

Effect of inhibitors and uncouplers on the acid-induced ATP synthesis by whole cells of *T. neapolitanus*

Uncouplers and inhibitors	Concentration (nmol/mg dry weight of cells)	Net ATP formed (nmol)
None	-	30
DNP	15.0	0
CCCP	0.5	0
Rotenone	10.0	24
Antimycin	2.0*	28
Cyanide	15.0	27

*Concentration expressed as micrograms per milligram protein.

Note: Experimental conditions have been described in Materials and Methods. The acid phase produced by HCl addition was maintained for 30 s before neutralization. The endogenous ATP content of the untreated cells was subtracted from the acid-induced ATP formed. The final pH of the untreated cell suspension was the same as that of the treated cells after neutralization.

Discussion of Results. The observations concerning the stoichiometry of oxygen consumed and thiosulfate oxidized by intact cells and cell-free extracts suggests that a considerable degree of alteration occurs in the pathway of thiosulfate oxidation in T. neapolitanus cells and cell-free extracts. Hempfling et al (1967) were unable to demonstrate APS reductase in extracts of this bacterium. Calculations based on molar growth yields indicate that the organism must generate more ATP than is accounted for by the substrate-level phosphorylation alone. The experiments reported here reveal that the ATP content of intact cells of T. neapolitanus rapidly increased sevenfold upon the addition of thiosulfate. That this was due to oxidative phosphorylation was supported by its inhibition with uncouplers. The insensitivity of thiosulfate-linked cellular ATP generation to flavoprotein inhibitors and antimycin A but not to cyanide suggests the coupling of thiosulfate in the electron transport chain at the cytochrome c level, an observation in harmony with that of Trudinger (1961). The observed ATP formation, therefore, appears to occur in the cytochrome c: O_2 oxidoreductase region. Moreover, these observations suggest that the thiosulfate molecule does not undergo a reductive cleavage yielding the sulfur or sulfide and the sulfite moieties whose oxidation and associated phosphorylation is sensitive to flavoprotein inhibitors and to antimycin A. It needs to be emphasized here that the conclusions drawn above are based on the assumption that the site of action of respiratory chain inhibitors in T. neapolitanus is similar to that in the mitochondrial systems. Recently, Cole and Aleem, (1967) have reported that the 144,000 g supernatant cell-free fraction from Thiobacillus novellus catalyzed phosphorylation coupled to the oxidation of

thiosulfate yielding P/O ratio of 0.96. It should, however, be emphasized that the T. novellus cell-free preparations have repeatedly been described to catalyze complete oxidation of thiosulfate to sulfate (Van Pouck, 1962; Aleem, 1965), a phenomenon observed only with the intact cells of T. neapolitanus.

The enzymatic oxidation of sulfide catalyzed by intact cells or cell-free extracts involves an oxidation rate which is dependent on sulfide concentration, and a concentration-independent slower oxidation rate after all the sulfide is utilized. The stoichiometry of sulfide utilized to O_2 consumed by whole cells and cell-free extracts suggests the accumulation of an intermediate product. The 300-400 nm band attributed to the intermediate polysulfide formed during sulfide oxidation in T. concretivorus (Moriarty and Nicholas, 1969) was not seen when sulfide was oxidized by the cell-free extracts. Baer and Carmack (1949) have shown that as the number of sulfur atoms in aliphatic polysulfide chain increases its absorption bands increase in intensity and shift to longer wavelength. Our inability to see an absorption band in the 300-400 nm region may, therefore, be due to the formation of small chains of polysulfide during sulfide oxidation with smaller coefficients of extinction, and also due to the masking effect of interfering compounds present in the cell-free extracts which absorb in this region. It appears that the intermediate formed during sulfide oxidation by T. neapolitanus is membrane-bound because the chromatographic analysis of the supernatant obtained after centrifugation of deproteinized reaction mixture revealed no spot other than sulfate; thus the membrane-bound polysulfide may have remained in the sedimented pellet. This conclusion is further supported by the fact that sulfide oxidation in stage 1 is catalyzed by the particulate fraction only.

Sulfide oxidation has been shown to be coupled to oxidative phosphorylation in T. concretivorus (Moriarty, 1969) and T. novellus (Cole 1971) only. Kelly and Syrett (1964, 1966) have reported, however, that uncoupling agents at concentrations which have little effect on oxygen uptake inhibited CO_2 fixation coupled to sulfide oxidation in T. thioparus. Our results with intact cells as well as with cell-free extracts show that sulfide oxidation is coupled to phosphorylation which is severely inhibited by uncouplers of the oxidative phosphorylation. The sensitivity of the sulfide-linked energy generation process to amytal, anitimycin A, and cyanide suggests the participation of the flavoprotein, cytochrome b, and cytochrome oxidase components. The repression of sulfide oxidation as well as coupled phosphorylation by high concentration of sulfide might partly be due to the inhibition of cytochrome oxidase.

The electron-transport-linked phosphorylation coupled to the oxidation of mercaptoethanol has been demonstrated in cell-free extracts of T. neapolitanus with P/O ratios of 0.4 (Hempfling, 1965). By using shorter incubation periods we have been able to obtain P/O ratios as high as 0.85. The P/O ratios thus obtained were also dependent upon the physiological stage (e.g. early log phase) of the cells. It is of interest to mention that the oxidation of ascorbate in presence of added GSH, cytochrome c, or TMPD was unable to replace 2-ME in catalyzing the formation of ATP. The inhibition of 2-ME-catalyzed ATP generation and stimulation of O_2 uptake by DNP or CCCP is consistent with the idea that the electron transport process in T. neapolitanus is under the control of some intermediate reaction of oxidative phosphorylation which occurs earlier than the formation of ATP. The observed inhibitory action of respiratory

chain inhibitors indicates that the oxidation of 2-ME and coupled phosphorylation involve the participation of coupling sites II and III.

The inhibition of proton-induced ATP formation in intact cells of T. neapolitanus by DNP and CCCP might be due to a proton leak through the cell membrane caused by the uncouplers. A similar phenomenon has been observed in chloroplasts by Neumann and Jagendorf (1965). Our results indicate further that the acid-induced ATP synthesis is not connected with the respiratory chain since the process was not affected by the inhibitors of the electron transport chain. It might be speculated, however, that the oxidation of reduced inorganic sulfur compounds in thiobacilli results in the release of H^+ which may be visualized as forming a proton gradient across the cell membrane resulting in the ATP synthesis. Whether or not the total ATP synthesized by the thiobacilli would reflect combined action of the H^+ gradient and the transmembrane electrical potential has not yet been determined.

E. Thiosulfate Oxidation and Related Reactions Catalyzed by Rhodopseudomonas palustris. Among members of the Athiorhodaceae, Rhodopseudomonas palustris is unique in its ability to assimilate CO_2 or formate photosynthetically with thiosulfate as the electron donor (van Niel, 1944). Even under photoheterotrophic growth conditions the presence of thiosulfate results in the increased cell yield, indicating that the organic electron donors do not compete with or suppress the photoautotrophic metabolism of the bacterium (Rolls and Lindstrom, 1967a, 1967b). Quite obviously the thiosulfate-linked photoautotrophic metabolism in Rps. palustris must generate ATP as well as reducing power in the form of either reduced pyridine nucleotide or some other reductant such as reduced ferredoxin. Unfortunately little is known at present concerning the nature and the mechanism by which the reducing power is generated in Rps. palustris under photoautotrophic growth conditions.

Experimental observations concerned with the mechanism of pyridine nucleotide reduction in photosynthetic bacteria have led to controversies among various investigators. One group favors the conclusion that the photoreduction of pyridine nucleotides is acheived by the direct electron transfer from an excited chlorophyll molecule with the mediation of some cofactors such as ferredoxin and a flavoprotein involving the so-called non-cyclic electron flow process (Arnon et al., 1961; Arnon, 1963; Amesz, 1963; Nozaki et al., 1963; Yamanaka and Kamen, 1965). According to a second school of thought, however, the function of the bacterial light-catalyzed cyclic electron flow lies mainly in the generation of ATP, while the pyridine nucleotide reduction is considered to involve an energy-linked reversal of electron transfer (Bose and Gest, 1963; Gest, 1966). This concept appears to be supported by the initial

observations of Frenkel (1958) that the photoreduction of NAD+ in Rhodospirillum rubrum chromatophores was linked with the oxidation of exogenous $FMNH_2$ or succinate. Chance and Olson (1960) and Chance and Nishimura (1960) suggested that the light-stimulated NAD+ reduction in intact cells of purple sulfur bacteria would involve an energy-linked reversal of electron transfer similar to the one observed in animal mitochondria with succinate as the electron donor (Chance and Hollunger, 1961). The observations of Bose and Gest (1962) revealed subsequently that photoreduction of NAD+ by succinate and photoevolution of hydrogen in R. rubrum was very likely promoted by the "dark" oxidoreduction reactions which could proceed only at the expense of energy-rich compounds generated in the primary light reaction. As a matter of fact succinate-linked ATP-dependent NAD+ reduction in the dark has been demonstrated in chromatophores from R. rubrum (Low and Alm, 1964; Keister and Yike, 1966, 1967), and Rhodopseudomonas capsulata (Klemme 1969). It has also been shown that the photoreduction of NAD+ in intact cells from R. rubrum (Jackson and Crofts, 1968) and Rhodopseudomonas spheriodes (Jones and Whale, 1970) was driven by an energy-linked reverse electron flow process which was inhibited by uncouplers of the energy-transfer reactions. The latter workers reported, however, that in the obligately photoautotrophic green sulfur bacterium Chlorobium thiosulfatophilum NAD+ reduction did not involve an energy-linked reversal of electron transfer and the pyridine nucleotide was reduced directly by the reduced ferredoxin-NAD reductase. The situation has been complicated further by a recent report (Govindjee and Sybesma, 1970) that while in the early stages of growth, the younger cells from the non-sulfur purple bacterium R. rubrum reduced pyridine nucleotide by an

energy-linked reversal of electron flow driven by the energy generated in the light-induced cyclic electron transport, In the case of older cells the photoreduction of NAD+ occurred by a direct light-induced electron transport from exogenous as well as endogenous electron donors.

In view of the conflicting observations and differing views concerning the mechanism of pyridine nucleotide reduction in photosynthetic bacteria, investigations were undertaken to study this process in Rps. palustris grown with thiosulfate as the electron donor and CO_2 as the main source of carbon. It was found that both the chromatophore-rich and the chromatophore-deficient cell-free preparations catalyzed an ATP-dependent NAD+ reduction by thiosulfate in the dark involving a reversal of electron transfer from the level of cytochrome c with the possible mediation of cytochrome b and also the flavoprotein system.

1. Conditions for Photosynthetic Growth on Thiosulfate. In order to obtain a reasonable photosynthetic growth rate with thiosulfate, the non-sulfur bacterium had to be adapted to $S_2O_3^=$ as the electron donor. An adaptation period of 2-3 months was required in order to obtain the same photosynthetic growth rate that occurs normally with malate or formate. Reasons for the long adaptation period are not known. It was essential, however, to increase the iron content (i.e. iron-EDTA) in the thiosulfate medium by a factor of 15 as compared to that used by other workers for the photoheterotrophic growth (Table 1). An addition of 0.005% yeast extract significantly increased the growth rate.

2. Spectral Characteristics of Whole Cells and Cell-free Extracts. The absorption spectrum of whole cells from Rps. palustris grown photosynthetically with $S_2O_3^=$ is presented in Fig. 25. Similar spectral characteristics are known from other photosynthetic bacteria (Clayton,

1963; French and Young, 1956) and have also been reported for Rps. palustris grown in the light on malate (Vredenberg and Amesz, 1966). A similar absorption spectrum was obtained with the 10,000 g supernatant (fig. 26, solid line) and addition of dithionite did not cause any further change in the absorption peaks (dashed line). The absorption spectrum of the 144,000 g supernatant (Fig. 26 B, solid line) was obtained with a preparation similar in protein content to the 10,000 g supernatant (1.7 mg protein/ml). Although very small absorption peaks due to bacteriochlorophyll were still present cytochrome c became visible and dominated the carotenoid absorptions. This fraction was thus designated as the chromatophore-deficient fraction. The addition of dithionite (dashed curve) to this fraction resulted in an increase in absorption of the α-, β-, and γ-peaks of a c-type cytochrome, and also caused a shift in the γ-peak of a reduced c-type cytochrome. The reduced flavins also became visible. More detailed information is given in Fig. 27 representing the dithionite-reduced minus oxidized difference absorption spectra. Both cytochromes of b and c-type were present in the S-10,000 (trace A). The S-144,000, however, exhibited spectral evidence of cytochrome c but did not reveal cytochrome b (trace B). Nevertheless, the complete absence of cytochrome b in this fraction cannot be assumed due to the possible masking by the cytochrome c component (s) and/or to the possibility of an autooxidizable cytochrome b. The presence of flavin components in the S-144,000 fraction is further indicated by the absorption spectra shown in Fig. 28. The addition of NADH to the extract resulted in the reduction of the endogenous cytochrome c (trace A). Upon the addition of Antimycin A to inhibit electron flow, flavin components became further reduced as indicated by the pronounced trough at 450 nm (trace B).

3. Thiosulfate: Cytochrome c Oxido-reductase. The S-144,000 fraction contained an active enzyme system for $S_2O_3^=$ oxidation. Electrons from $S_2O_3^=$ could couple efficiently with the endogenous cytochrome c (Fig. 29, trace A). Antimycin A and NOQNO did not inhibit this activity, (traces B and C). The $S_2O_3^=$ oxidizing system developed during photoautotrophic growth in Rps. palustris thus appears to be a thiosulfate-activating enzyme (Eley et al., 1971) with properties similar to those observed for the thiosulfate: cytochrome c reductase described in the facultatively autotrophic sulfur bacterium Thiobacillus novellus (Aleem, 1965). It was of particular interest, therefore to compare the system of energy-linked reduction of pyridine nucleotides observed in Thiobacillus novellus (Aleem, 1966a) with the system found to be operative in the facultative photoautotroph, Rps. palustris grown with thiosulfate as the electron donor.

4. Thiosulfate oxidation by cell-free extracts. The (S-144,000) fraction was found to contain an active thiosulfate-cytochrome c reductase. Upon the addition of 20 mM thiosulfate to the reaction mixture, there occurred an increase in absorbance at 550 nm indicating reduction of the endogenous cytochrome c (Fig. 30). The rates of reduction were stimulated by the addition of cyanide which inhibited the rapid reoxidation of reduced cytochrome c by cytochrome oxidase. Cytochrome c reduction also occurred without the addition of cyanide indicating that the reduction was not due to rhodanese activity (see Discussion). Figure 30 also reveals that thiosulfate caused the reduction of added mammalian cytochrome c and that the thiosulfate-dependent reduction of endogenous or exogenous cytochrome c did not occur without the addition of enzyme or with boiled enzyme preparations. The thiosulfate-cytochrome

c reductase activity furthermore, was shown to be proportional to the enzyme concentration. A thiosulfate-reduced minus oxidized absorption spectrum of the cell-free extracts showed absorption peaks at 550, 520, and 420 nm corresponding to the α, β, and γ peaks of cytochrome components of the c-type (Fig. 31). The trough at 440-470 nm indicated probably the reduction of endogenous flavin components.

The reduction of cytochrome c by thiosulfate was studied as a function of thiosulfate concentration. A linear increase in enzyme activity was observed in the presence of 1-100 mM thiosulfate without indication of substrate saturation. Similar data have been observed numerous times which always failed to exhibit substrate optima, even at 200 mM thiosulfate concentration.

The thiosulfate-dependent reduction of cytochrome c was previously shown to be insensitive to inhibitors of cytochrome b oxidation such as Antimycin A and NOQNO (Knobloch et al, 1971), and was also insensitive to the flavoprotein inhibitors rotenone, amytal, and atebrin; in addition, the process was not affected by the uncoupling agents, CCP and DNP.

5. Products of thiosulfate oxidation. Figure 32 shows the products of thiosulfate oxidation by whole cells of Rps. palustris as revealed by autoradiography. Radioactive spots on the chromatograms were cut out and counted to determine the amount of radioactivity in each of the oxidation products. Results, shown in Table 15, show the fraction of the total recovered label appearing in each of the products of thiosulfate oxidation. Ten minutes after the addition of labelled thiosulfate to the cell suspension, 54.4% of the label remained in thiosulfate while 45.6% of the total radioactivity was recovered in the oxidized products. Tetrathionate ($S_4O_6^{2-}$), trithionate ($S_3O_6^{2-}$) and sulfate (SO_4^{2-}) contained

90% of the label with the remainder being found in several higher polythionates (Table 15).

Formate, succinate and NADH oxidation by cell-free extracts. The cell-free extract from thiosulfate grown cells were found to catalyze cytochrome c reduction with a variety of substrates. Formate was an effective electron donor and exhibited zero order kinetics at concentrations greater than 75 mM (Fig. 33). A plot of s/v against s (Dixon and Webb, 1964) revealed a K_m of 30 mM for formate. The formate-cytochrome c reductase activity was completely inhibited by 10^{-4} M cyanide. Succinate was about equally as effective as formate for cytochrome c reduction, showing a K_m of 40 mM (data not shown). The reduction of cytochrome c by succinate was, however, not inhibited by cyanide.

NADH proved to be the most effective substrate for cytochrome c reduction, exhibiting a K_m of 0.025 mM (Fig. 34). In addition to cytochrome c reductase activities, the extract also contained active cytochrome c oxidase activity. Thus, the addition of 0.1 mM NADH to the reaction mixture caused the rapid reduction of cytochrome c which was followed by the reoxidation of cytochrome c by the oxidase (Fig. 35, trace 2). The addition of 5×10^{-6} M cyanide resulted in complete inhibition of the oxidase and a greater reduction of cytochrome c (trace 3). Under these conditions, the NADH-cytochrome c reductase was not inhibited by 10^{-4} M rotenone (trace 4), 10^{-3} M amytal (trace 5), 10^{-3} M atebrin (trace 8), and by Antimycin A or NOQNO at concentrations of 2 µg/mg of protein (traces 6 and 7).

7. Oxidation of cytochrome c by cell-free extracts. The cytochrome c-O_2 oxidoreductase activity in the cell-free extracts was further investigated by following the oxidation of reduced mammalian cytochrome c, as indicated by a decrease in absorption at 550 nm, or, by oxygen uptake polarographically. A ratio of nanomoles of cytochrome c oxidized per

TABLE 15

Products of Thiosulfate Oxidation By

Rps. Palustris[a]

R_f	Compound	% of total counts
0.0	(origin)	0.4
0.37-0.39	$S_2O_3^{=}$	54.4
0.40-0.41	$SO_4^{=}$	13.0
0.53-0.58	$S_3O_6^{=}$	8.5
0.71-0.74	$S_4O_6^{=}$	18.7
0.80-0.82	polythionates	2.9
0.85-0.87	polythionates	1.8
0.96-1.00	polythionates	0.4

[a]Products detected after 20 mg dry weight of whole cells were incubated for 10 min with 10 μmoles of ^{35}S-thiosulfate containing 250 μCi of ^{35}S. Samples taken at zero time showed only labelled thiosulfate.

TABLE 16

Stoichiometry of Cytochrome c Oxidized and Oxygen Consumed in the Cytochrome c-O_2 Oxidoreductase Reaction[a]

Mins		Nanomoles		Ratio
		Cytochrome c oxidized	O_2 consumed	Cyt. c/O_2
Exp. I	1.0	37.3	15.6	2.4
	5.0	94.0	34.8	2.7
Exp. II	1.0	31.2	8.06	3.9
	5.0	79.0	19.80	4.0

[a]Reaction mixture in a total volume of 1.0 ml contained 45 μmoles of Tris (pH 8.0), 144,000 g supernatant equivalent to 4.7 mg of protein and 0.02 ml of a 4% solution of reduced mammalian cytochrome c.

nanomole of oxygen consumed of approximately 2.5-4.0 was observed (Table 16).

The effect of various inhibitors on the cytochrome oxidase activity was examined. The oxidase was markedly sensitive to cyanide; 10^{-7} M and 10^{-6}M cyanide caused 50% and 100% inhibition, respectively (Fig. 36). Azide at a concentration of 10^{-4} M caused 50% inhibition. The flavoprotein inhibitor amytal was not effective, even at concentrations of 10^{-3} M. Rotenone, however, showed 50% inhibition at a concentration of 5×10^{-5} M (data not shown). The oxidase was also sensitive to inhibition by atebrin, but Antimycin A and NOQNO were ineffective at concentrations of 1-10 µg/mg of protein. As shown in Fig. 37, EDTA at 1.3×10^{-4} M caused 83% inhibition of the oxidase, and this inhibition was not relieved by 0.5 mM Mg^{2+} or by 2×10^{-4} M Fe^{3+}. However, 2×10^{-4} M Cu^{2+} significantly relieved the inhibition and a concentration 2.5 fold higher caused a complete reversal of the EDTA inhibition. The cyanide and azide inhibition of cytochrome c oxidase were also overcome by Cu^{2+} but not by the addition of other cations.

The effect of uncouplers on cytochrome c oxidase activity was examined to ascertain the possible involvement of a coupling site. All of the uncouplers tested caused a stimulation in the rate of cytochrome c oxidation, with CCCP being the most effective (Table 17). Both Antimycin A and NOQNO at concentrations of 10 µg/mg of protein caused a stimulation of the oxidase activity, presumably by their acting as uncoupling agents.

To further characterize cytochrome c oxidation, the cell-free extracts were reduced with NADH in the presence of cyanide and the sample cuvette was then bubbled with carbon monoxide for 3 min in the dark. The resulting reduced + CO minus reduced difference absorption spectrum showed peaks at 562, 532, and 416-419 nm corresponding to the α, β and γ peaks of carbon monoxide complexes of cytochrome components of the O-type, and a

TABLE 17

Effect of Uncouplers on Cytochrome c:O_2 Oxidoreductase Activity[a]

Uncoupler	Concentration	% Stimulation
CCCP	3×10^{-6}M	25
	3×10^{-5}M	91
DNP	3×10^{-6}M	22
PCP	1×10^{-5}M	13
DBP	1×10^{-5}M	6
Antimycin A	1 µg/mg protein	0
	10 µg/mg protein	63
NOQNO	10 µg/mg protein	57

[a]Experimental conditions as described in Table 16. Oxidation of cytochrome c was followed spectrophotometrically at 550 nm.

single peak of 430 nm corresponding to the γ peak of the carbon monoxide complex of an a-type cytochrome (Fig. 38, trace A). After a period of 20 min in dark at room temperature, the absorption peaks due to o-type cytochrome became more pronounced with a lessening of the 430 nm peak (Fig. 38, trace B).

8. ATP-driven Reversed Electron Flow. Thiosulfate-reduced cytochrome c and under anaerobic conditions, the electrons from ferrocytochrome c could be reversed against the thermodynamic gradient in the presence of added ATP in the dark resulting in a reduction of the pyridine nucleotides. The ATP-driven reduction of NAD+ by $S_2O_3^=$ in the dark was catalyzed by the crude cell-free extract (S-10,000) as well as by the chromatophore-deficient fraction (S-144,000). A simultaneous oxidation of cytochrome c and reduction of NAD+ was observed when absorbance changes at 550 and 340 nm were recorded in a dual-wavelength split-beam spectrophotometer (Fig. 39). A molar stoichiometry of approximately 2:1 was obtained for the cytochrome c oxidized and the NAD+ reduced (see also Fig. 40 and Tables 18 and 19). That the increase in absorbance at 340 nm was in fact due to the reduction of NAD+ was shown by the rapid decrease in absorbance at 340 nm upon the addition of the NADH trapping systems consisting of pyruvate (6 µmoles) and lactic dehydrogenase (50 µg) or acetaldehyde (about 20 µmoles) and alcohol dehydrogenase (from yeast; about 4 µg), respectively. If, on the other hand, the NADH trapping systems were preincubated with the S-144,000 fraction before reverse electron flow occurred, the oxidation of cytochrome c was not accompanied by the increase in absorbance at 340 nm.

9. Factors Affecting the $S_2O_3^=$ linked ATP-driven NAD Reduction by the Chromatophore-poor Fraction, S-144,000. The optimal reaction (i.e.

TABLE 18

Stoichiometry of ATP utilized and NAD reduced in reversed electron flow coupled to cytochrome c oxidation

Expt.	mins.	nmoles			ratio	ratio
		NAD reduced	cyt c oxidized	ATP utilized	cyt. c / NAD	ATP / NAD
1	1.3	24	50	170	2.1	7.1
2	2.6	36	54	185	1.5	5.1
3	3.5	44	93	203	2.1	4.6
4	9.3	56	100	425	1.8	7.6

Experimental conditions were as described under Materials and Methods. The reaction mixture in a total volume of 3.0 ml contained 0.2 ml of the 144,000xg supernatant representing 19 mg of protein. The initial ATP concentration was 500 nmoles per cuvette. Reactions were stopped after the time indicated and the amount of ATP left in the reaction mixture was determined as described in the text.

TABLE 19

Effect of uncouplers and inhibitors on ATP-driven NAD^+ reduction coupled to cytochrome c oxidation

Expt.	uncoupler or inhibitor	Conc.	nmoles cyt.c oxidized	nmoles NAD^+ reduced	ratio cyt.c / NAD
1	none		23.0	13.0	1.8
	CCCP	5 μM	0	0	-
		1 μM	8.7	4.0	2.2
		0.5 μM	21.2	12.0	1.8
	DNP	10 μM	0	0	-
	DBP	100 μM	13.3	0	-
	PCP	100 μM	6.7	0	-
2	none	-	24.6	11.1	2.2
	AsO_4^{---}	8 mM	4.5	0	-
	AsO_4^{---} + PO_4^{---}	8 mM 16 mM	23.7	12.5	1.9
3	none	-	35.6	14.2	2.5
	Antimycin A	(2 μg/mg protein)	20.0	0	-
		(7 μg/mg protein)	3.7	0	-
	NOQNO	(2 μg/mg protein)	16.2	0	-
		(7 μg/mg protein)	1.8	0	-
	BAL	50 μM	0	0	-
	Rotenone	50 μM	3.3	0	-
	Amytal	100 μM	0	0	-

Experimental conditions were as described in Materials and Methods except that 5 μmoles of ATP were used to drive the reversal of electrons during $S_2O_3^{=}$ oxidation. The reaction mixtures used in experiments 1, 2 and 3 contained 11.2, 17.5 and 8.0 mg protein respectively.

ATP-driven anaerobic cytochrome c oxidation and coupled NAD+ reduction) occurred at pH 8.0 (Fig. 41) and in the presence of added 1.7 mM ATP, 7.0 mM $S_2O_3^=$, 0.7 mM NAD+, 1.7 mM $MgCl_2$, and 55 μM cytochrome c (trace B, Fig. 40). No reaction or any absorbance change at 550 nm or 340 nm took place in the absence of any added enzyme preparation (trace A), and the reversed electron flow catalyzed by the chromatophore-deficient enzyme preparations in the dark occurred only when ATP was added (trace B). Although the enzyme preparations were dialyzed for 12 h, they contained 10-30 nmoles of $S_2O_3^=$/mg protein as determined by the method of Sorbo (1957). The exogenous addition of $S_2O_3^=$ was therefore necessary to obtain the optimal substrate concentration for NAD+ reduction. This is evidenced by the enhanced rate of cytochrome c oxidation and coupled NAD+ reduction upon the addition of 7.0 mM $S_2O_3^=$ to the treatment cuvette (trace C); the reaction mixture in both cuvettes was otherwise identical and contained all the components including ATP. Under these conditions omission or addition of NAD+ did not cause any difference in the absorbance changes (trace D) indicating that the amount of endogenous NAD+ in the cell-free extracts was sufficient to saturate the reaction. Addition of $MgCl_2$ (trace E) and cytochrome c (trace F) caused a stimulation in the reverse electron flow process.

10. ATP-dependent Reversal of Electron Transfer from Ferrocytochrome c to NAD+. It was observed that $S_2O_3^=$ could be replaced by reduced mammalian cytochrome c as the source of electrons for NAD+ reduction. Thus under anaerobic conditions the ATP-driven oxidation of ferrocytochrome c occurred at a rapid rate coupled to a concomitant reduction of the pyridine nucleotide. However, in some experiments the stoichiometry of cytochrome c oxidized/NAD+ reduced was less than 2.0 due to the endogenously present thiosulfate and

limiting concentration of the ferrocytochrome c that served as the exogenous electron donor.

11. Stoichiometry of the Energy-linked Reactions. Representative data of some of the typical experiments concerning the stoichiometry of ferrocytochrome c oxidized, NAD+ reduced, and ATP utilized are given in Table 18. It was observed that approximately 2 molar equivalents of ferrocytochrome c were oxidized per equivalent of NAD+ reduced with the utilization of about 4-7 molar equivalents of ATP.

12. Sensitivity of ATP-dependent NAD+ Reduction to Uncouplers and Inhibitors. Since the electrons donated by $S_2O_3^=$ couple at the level of cytochrome c (Fig. 29) the ATP-driven reverse electron transport may be mediated by other electron transport carriers, such as cytochrome b and the flavoprotein system in order to reach the redox level of the pyridine nucleotides. If such is the path of electron transport, uncouplers of energy transfer reactions and inhibitors of the electron transport chain should be expected to inhibit the reverse electron flow reactions. The data in Table 19 show that the ATP-dependent reversed electron flow was markedly sensitive to various uncouplers of the oxidative phosphorylation. Thus the energy-linked oxidation of the ferrocytochrome c as well as the simultaneously occurring NAD+ reduction was inhibited completely by 5 μM CCCP or 10 μM DNP. Although cytochrome c was oxidized partially in the presence of 100 μM DBP or PCP, the process was not coupled with NAD+ reduction. The inhibitory effect of arsenate could be completely reversed by an excess of added phosphate ions. The thiosulfate-linked ATP-driven NAD+ reduction was completely inhibited by antimycin A or NOQNO at 7 μg/mg protein, presumably by blocking electron transport between cytochrome b and c (Table 19). When used at a concentration of 2 μg/mg protein, however,

these inhibitors allowed cytochrome c oxidation which was not coupled with NAD+ reduction. The potent inhibition caused by a low concentration of BAL indicated the possible involvement of -SH groups and/or quinone and a vitamin K analogue. In addition the energy-linked reactions were markedly sensitive to inhibitors of the flavoprotein system; thus the reversed electron flow was completely inhibited by amytal and by low concentrations of rotenone.

Discussion of Results. Although reduced inorganic sulfur compounds support photosynthetic growth in members of Chlorobacteriacea and Thiorhodaceae (Truper and Schlegel, 1964) at least one member of the Athiorhodaceae, e.g., Rps. palustris is known to utilize thiosulfate under photolithotropic growth conditions (Van Niel, 1944; Rolls and Lindstrom, 1967; Knobloch et al, 1971). Since Rps. palustris when grown in a medium containing both thiosulfate and organic substrates exhibits increased cell yields over cells grown with organic substrates one may reach a conclusion, although indirectly, that the photosynthetic sulfur oxidation might be coupled with the electron transport chain. However, little information is available concerning this aspect except some preliminary communications by the authors (Eley et al, 1971, Knoblock et al, 1971) demonstrating a direct coupling of thiosulfate with cytochrome c in the dark in Rps. palustris. Under anaerobic conditions the reduced cytochrome c is re-oxidized by pyridine nucleotides by an energy-dependent reversal of electron transfer. This important physiological event is involved in the generation of photosynthetic reducing power in the organism and is driven either by the light energy, or by ATP in the dark (Knoblock et al, 1971). Since cytochrome c appears to serve as a link between the light and dark reactions,

this study describes further the cytochrome-linked reactions in the photolithotroph.

The thiosulfate: cytochrome c oxidoreductase in Rps. palustris possesses characteristics similar to those of the enzyme system reported earlier in the faculative chemoautotroph Thiobacillus novellus (Aleem, 1965) e.g., the enzyme system being localized chiefly in 144,000 g supernatant and also being insensitive to flavoprotein inhibitors such as amytal, rotenone and atabrine as well as to Antimycin A or NOQNO. The reduction of cytochrome c by thiosulfate is enzymatic since no reaction occurs in the absence of cell-free extracts or without thiosulfate. Thus the oxidation of thiosulfate in Rps. palustris involves the enzymatic transfer of electrons to cytochrome c without mediation of the flavoprotein and cytochrome b components. If the thiosulfate molecule is split into the sulfonyl and sulfane groups then the oxidation of these moieties should become sensitive to the flavoprotein inhibitors and to Antimycin A or NOQNO (Saxena and Aleem, 1971; Peeters and Aleem, 1970). It would appear, therefore, that in Rsp. palustris thiosulfate oxidation involves the oxidative coupling of two thiosulfate molecules resulting in the formation of tetrathionate according to the following reactions:

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

$$2\ \text{cyt. c}\cdot Fe^{3\pm} + 2e^- \rightarrow 2\ \text{cyt. c}\cdot Fe^{2+}$$

In fact the addition of labelled thiosulfate ($^{35}S\text{-}SO_3^{2-}$) to suspensions of whole cells or cell-free extracts in the light or dark, followed by chromatography and autoradiography, revealed that thiosulfate is oxidized to tetrathionate, trithionate, and sulfate with lesser amount of label in several higher polythionates. These observations suggest that in Rps. palustris thiosulfate is oxidized via the following pathway:

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

According to one school of thought the above so-called polythionate pathway operates in at least some of the sulfur oxidizing orgamisms (Trudinger, 1959; 1958; Rittenberg and London, 1964) as opposed to the enzyme systems which oxidize thiosulfate involving a reductive cleavage into the sulfonyl and sulfane moieties (Peck, 1960; Peck and Fisher, 1962; Truper and Pfennig, 1966).

An enzyme related to the thiosulfate-cytochrome c reductase is rhodanese or thiosulfate-cyanide sulfur transferase first isolated from Chromatium-vinosum, strain D by Smith and Lascelles (Smith and Lascelles, 1966) who suggested that rhodanese may function as a thiosulfate-cleaving enzyme:

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

The presence of rhodanese has since been reported in several of the sultur-metabolizing organisms (Smith and Lascelles, 1966; Charles and Suzuki, 1966; Bowen et al, 1965; Yoch and Lindstrom, 1971). However, Yoch and Lindstrom have recently reported that the occurrence of rhodanese in photosynthetic bacteria is not correlated either with the ability to metabolize thiosulfate or with the route of thiosulfate metabolism since the enzyme is also present in the nonthiosulfate oxidizing members of Athiorhodaceae. The thiosulfate-cytochrome c reductase system in Rps. palustris does not require cyanide and it therefore differs from that described in Thiobacillus novellus and Thiobacillus intermedius (Charles and Suzuki, 1966). Although we have observed increased cytochrome c reduction in the presence of cyanide, we interpret this as being due to the inhibition of an active cytochrome oxidase present in the cell-free extracts.

Qadri and Hoare reported that the ability to photoassimilate formate by a strain of Rps. palustris was dependent upon growth in formate medium (1968). Their data show that formate was assimilated as carbon dioxide and molecular hydrogen by action of an inducible formic hydrogenlyase consisting of formic dehydrogenase, hydrogenase and one or more unidentified electron carriers. Although spectral data showed that their preparation contained cytochrome c, they found no evidence that cytochrome c was the electron acceptor for the formic dehydrogenase. Extracts from our strain of Rps. palustris, grown on thiosulfate without formate, oxidized formate via a formate-cytochrome c reductase. Whether these differences are due to the strain or due to the possibility of more than one mechanism of formate assimilation of Rps. palustris is uncertain and warrants further investigation. The observed sensitivity of formate-cytochrome c reductase to cyanide may be due to the involvement of the formic hydrogenlyase system.

The cell-free extracts also contain an efficient NADH-cytochrome c reductase which is insensitive to the flavoprotein inhibitors rotenone, amytal and atebrin as well as to Antimycin A and NOQNO which inhibit electron flow between b-type and c-type cytochrome components. Such data suggest that the 144,000 g supernatant catalyzes a direct transfer of reducing equivalents from NADH to cytochrome c without the involvement of flavoprotein or of cytochrome b. In contrast, the reversal of elctron flow from cytochrome c to NAD catalyzed by the similar cell-free preparation from the same organism has been shown by Knoblock et al. (1971) to be sensitive to these inhibitors. Electron flow in the forward and reverse directions thus might proceed by different pathways. Similar results were obtained by Saxena (1970) with the obligate chemosynthetic bacterium Thiobacillus neapolitanus in which the NADH-cytochrome c reductase is

unaffected by flavoprotein inhibitors and by Antimycin A. It thus appears that the oxidation of NADH by several chemosynthetic and photosynthetic bacteria may involve a direct oxidation coupled to cytochrome c reduction, and this possibility should be fully investigated.

The 144,000 g supernatant also contains an active cytochrome c oxidase for which reduced mammalian cytochrome c functions as a substrate, and for each mole of oxygen consumed, 3.0-4.0 moles of cytochrome c are oxidized; the stoichiometry is thus similar to that observed for mitochondrial preparations. The oxidase is markedly sensitive to low concentrations of cyanide and is also inhibited by azide, though at considerably higher concentrations. The enzyme resembles the "dark cytochrome c oxidase" observed by Kamen and Vernon in R. rubrum (Kamen and Vernon, 1954) in that it is associated with small particles, it catalyzes cytochrome c oxidation independent of light, and it is cyanide sensitive. The inhibition by cyanide and azide, as well as that caused by EDTA, is overcome by the addition of Cu^{2+} ions, suggesting the involvement of copper in the terminal segment of the electron transport chain. The stimulation by uncoupling agents suggest involvement of the coupling-site III in cytochrome c oxidation. The carbon monoxide absorption spectrum shows the cell-free extracts to contain cytochrome components of the a-and O-type which could function as the terminal oxidase(s).

In view of these observations and of those reported in an earlier paper (Knobloch, et al., 1971) concerning the reversal of electron flow, the electron transport mechanisms concerned with cytochrome c-linked reactions in Rps. palustris grown photosynthetically on thiosulfate may be represented as in Fig. 42.

The mechanism described herein concerning the generation of reducing power in bacterial photosynthesis appears to be highly suitable for the interpretation and/or clarification of numerous observations by several workers which do not seem to fit into a unique picture of the function of light energy in the bacterial photosynthetic apparatus. In view of the fact that reducing power is generated in the dark involving an ATP-driven reverse elctron flow catalyzed by the chromatophore-deficient enzymatic system from Rps. palustris, the findings of Chance and Olson (1960) become more intelligible. After activating photosynthesis by infrared illumination in whole cells of R. rubrum and Chromatium vinosum strain D these investigators observed a rapid oxidation of cytochrome which interestingly enough, occurred just before the reduction of pyridine nucleotide began. Likewise when ATP replaces the light reaction in Chromatium the reductive assimilation of CO_2 in the dark observed by Losada et al. (1960) suggests that ATP not only replaces the process of photophosphorylation but that it also should be involved in the generation of reduced pyridine nucleotide. As a matter of fact in preliminary experiments we have observed a thiosulfate-linked ATP-dependent NAD+ reduction in the dark in chromatophore-free extracts from Chromatium vinosum strain D (unpublished data).

We suggest that the primary and possibly the only role of light in the bacterial photosynthetic apparatus of Rps. palustris is to provide ATP via reduction of an unknown electron acceptor and/or ferredoxin followed by a subsequent cyclic electron flow. Moreover, the reduction of pyridine nucleotides via reduced ferredoxin failed to be observed so far in bacterial photosynthetic reactions (Vernon, 1964, 1968). In support of these negative observations, Trebst et al. (1967) using

phenylenediamine as electron donor also concluded that ferredoxin is not required for the reduction of NAD(P)+ by chromatophores from R. rubrum.

Although a succinate-linked ATP-dependent NAD+ reduction has been demonstrated in purple non-sulfur bacteria, this report provides the first conclusive evidence for the energy-linked reduction of NAD+ in the dark by an inorganic electron donor, such as thiosulfate, in photolithotrophically grown Rps. palustris. The process of ATP-dependent reversal of electron transfer has been observed to be catalyzed in the dark either by cell-free frattions containing chromatophores (i.e. S-10,000) or by chromatophore-deficient but cytochrome-rich cell-free preparations (i.e. S-144,000). The latter preparations usually contain $S_2O_3^=$: cytochrome c and ferrocytochrome c: O_2 oxidoreductase activities. Although the characteristics and the properties of the thiosulfate: cytochrome c reductase and the ATP-dependent reversal of electron transfer from $S_2O_3^=$ to NAD+ in Rps. palustris appear to be very similar to those observed in the case of the facultative chemoautotrophic sulfur bacterium Thiobacillus novellus (Aleem, 1966 b), it has not been possible as yet to link thiosulfate oxidation with O_2 uptake in the dark. While the cell-free preparations can consume O_2 with ferrocytchrome c as the electron donor, oxidation of $S_2O_3^=$ occurs either at the expense of reduction of cytochrome c or of a non-physiological electron acceptor such as ferricyanide. The product of thiosulfate oxidation appears to be tetrathionate under these conditions (Rolls and Lindstrom, 1967a) and hence the apparent lack of O_2 uptake in the process.

Neither the thiosulfate oxidation nor the reduction of cytochrome c by $S_2O_3^=$ occurs when the cell-free extracts are heated at 60° for 5 min. The enzymatic reduction of cytochrome c by $S_2O_3^=$ is unaffected by antimycin A

or NOQNO; thus the electrons from $S_2O_3^=$ appear to be transfered to cytochrome c directly with concomitant formation of tetrathionate. It would appear therefore that under our experimental conditions the thiosulfate molecule is not cleaved to yield the sulfide and sulfite moieties whose oxidation should become sensitive to the flavoprotein inhibitors as well as to antimycin A or NOQNO as observed earlier in the case of Thiobacillus dentrificans (Peeters and Aleem, 1970). The pertinent reactions involved in the transport of electrons from thiosulfate to the pyridine nucleotide may thus be presented as follows:

$$2\ S_2O_3^= \dashrightarrow S_4O_6^= + 2e^-$$

$$2\ \text{cytochrome c} \cdot Fe^{3+} + 2e^- \dashrightarrow 2\ \text{cytochrome c} \cdot Fe^{2+}$$

$$ATP + X \dashrightarrow (X\sim P) + ADP$$

$$2\ \text{cytochrome c} \cdot Fe^{2+} + (X\sim P) + NAD^+ + 2H^+ \dashrightarrow 2\ \text{cytochrome c} \cdot Fe^{3+} + X + Pi + NADH + H^+$$

The energy transfer reactions from ATP have been shown to be highly sensitive to DNP, DBP, PCP and CCCP which are well known uncouplers of oxidative phosphorylation. The possible involvement of (X~P) in the process is indicated by the potent inhibition caused by arsenate to the ATP-linked NAD_ reduction by $S_2O_3^=$:

$$ATP + X + AsO_4^{3-} \text{---} (X\sim AsO_4^{3-}) + ADP + Pi$$

and a complete reversal of the inhibition upon the addition of phosphate ions:

$$(X\sim AsO_4^{3-}) + Pi \dashrightarrow (X\sim Pi) + AsO_4^{3-}$$

Although the reduction of cytochrome c by $S_2O_3^=$ is insentitive to the flavoprotein inhibitors as well as to antimycin A or NOQNO, the ATP-driven transfer of electrons from $S_2O_3^=$ to NAD+ becomes very sensitive to these inhibitors. The chromatophore-deficient but cythchrome-containing enzyme system from Rps. palustris represents, therefore, an

active chemosynthetic system whereby under the influence of ATP a weak inorganic reductant ($S_2O_3^=$) can produce a strong reductant (NADH) in the dark involving a reversal of the electron transport chain (Aleem et al., 1963; Aleem, 1965, 1966a, 1966b; Sewell and Aleem, 1969). It should, however, be realized that the observed pathway of energy-linked reversal of electrons from $S_2O_3^=$ to NAD+ must involve a carrier system which is different from the electron transport chain that is employed in the primary light reaction.

In Rps. palustris the reduction of a pyridine nucleotide requires 4 ATP (Table 2). Moreover, 3 ATP and 2 NADH equivalents are needed for 1 equivalent of CO_2 reduced in the carbon reduction cycle. Assuming that 2 light quanta are needed to produce 1 ATP in cyclic photophosphorylation, then 22 quanta must be employed to generate the required 11 ATP equivalents for the photoassimilation process. The quantum yield measurements in photosynthetic bacteria show, however, the requirements of 10 light quanta to photoassimilate CO_2, a process which requires about 112 kcal to fix one gram atom of carbon. Assuming that one mole quantum at 880 nm has an energy content of 35.6 kcal, the efficiency of the photoassimilation process in Rps. palustris would then be $(\frac{112}{22 \times 35.6}) = 14\%$. This would correspond to about 50% of the efficiency of the actual quantum yield based on 10 quanta/CO_2 reduced $(\frac{112}{10 \times 35.6}) = 31\%$. Thus the process of reverse electron flow in the dark as studied in the chroatophore-deficient cell-free preparations yields about 50% of the quantum efficiency measured during the photoassimilation in whole cells of photosynthetic bacteria. Assuming

that the $\Delta F'$ for ATP hydrolysis is about 7 kcal, the observed ATP/NADH quotient of 4 does exhibit an efficiency of almost 100% with respect to energy utilization for the reversal of electrons from ferrocytochrome c (E_o' = + 0.26 V) to NAD+ (E_o' = -0.32 V), representing about 27 kcal of energy.

The possible mechanism involved in the reduction of pyridine nucleotides by thiosulfate in Rps. palustris is outlined in Fig. 43.

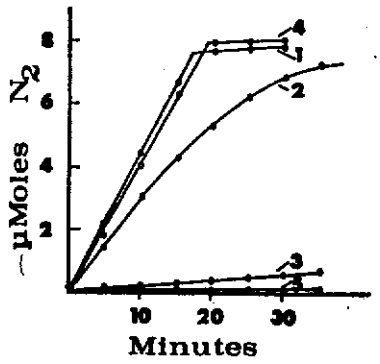


Fig. 1

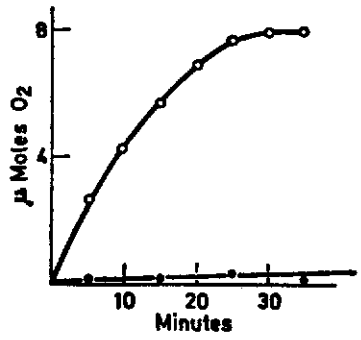


Fig. 2

Fig. 1: Thiosulfate oxidation by intact cells of T. denitrificans under anaerobic conditions. The experimental conditions were the same as described in Material and Methods. Cells before treatment with N_2O: (1) 16 µmoles nitrate and 25 µmoles thiosulfate, (2) 32 µmoles nitrate and 10 µmoles thiosulfate, (3) 16 µmoles nitrate and no thiosulfate tipped in from the side arm. Cells after treatment with N_2O: (4) 16 µmoles nitrate and 25 µmoles thiosulfate, (5) 16 µmoles nitrate and no thiosulfate.

Fig. 2: Thiosulfate oxidation under aerobic conditions by whole cells of T. denitrificans after treatment with N_2O. Experimental conditions were the same as described in Material and Methods: 5 µmoles thiosulfate (°) and no thiosulfate (•).

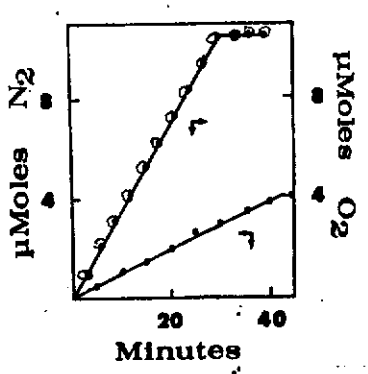


Fig. 3

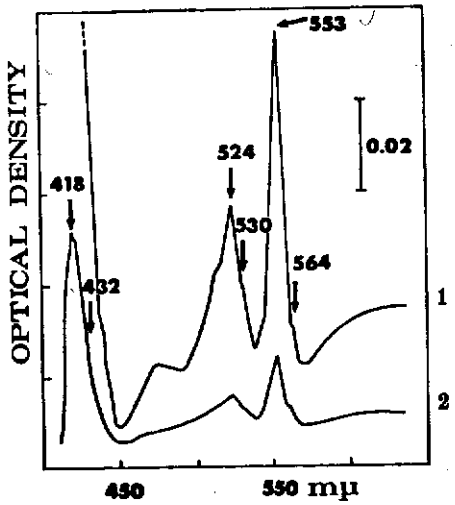


Fig. 4

Fig. 3: Sulfite oxidation by whole cells of T. denitrificans under anaerobic (•) and aerobic (°) conditions. Experimental conditions were the same as described in the text: 20 µmoles of sulfite were added after thermal equilibration together with 16 µmoles of nitrate in the anaerobic experiment.

Fig. 4: Steady-state difference absorption spectra of T. denitrificans cell-free extracts upon treatment with thiosulfate under anaerobic conditions. The reaction mixture contained in a total volume of 2 ml, cell-free extracts containing 4.0 mg protein and 90 µmoles of phosphate buffer (pH 7.0). The treatment cuvette in addition, was supplied with 20 µmoles of thiosulfate (trace 1); trace 2 shows change in absorption after the addition of 100 µmoles of nitrate.

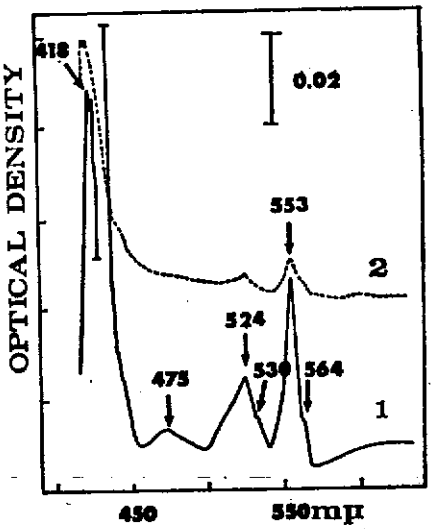


Fig. 5a

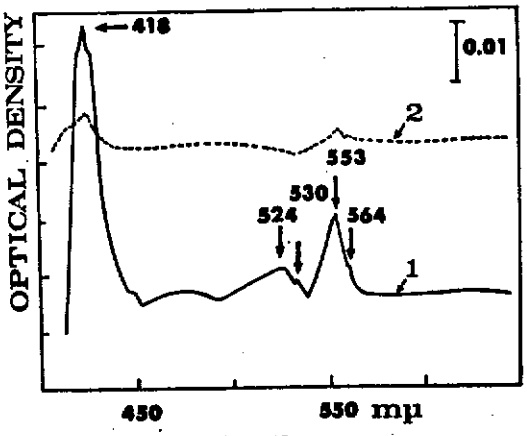


Fig. 5b

Fig. 5a: Steady-state difference absorption spectra of T. denitrificans cell-free extracts upon addition of NADH. The cuvettes contained in a total volume of 2.0 ml, 90 μmoles of Tris-HCl (pH 7.5), cell-free extract containing 2.0 mg protein, and the test cuvette in addition was supplied with 10 μmoles of NADH (trace 1); trace 2 shows change in absorption after the addition of 100 μmoles nitrate.

Fig. 5b: Steady-state difference absorption spectra of T. denitrificans cell-free extracts upon addition of succinate. Conditions were the same as for Fig. 5a except that 10 μmoles of succinate were used instead of NADH.

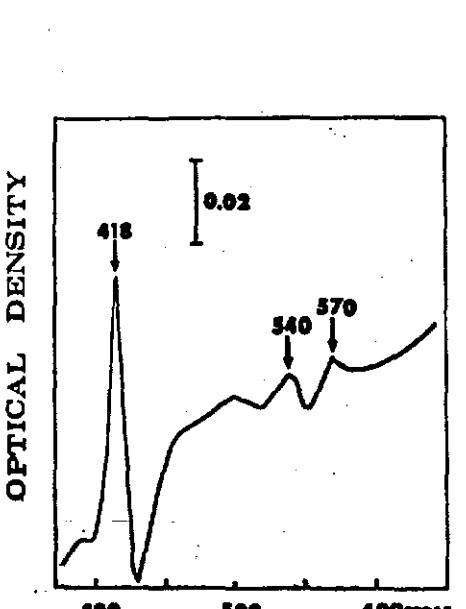


Fig. 6

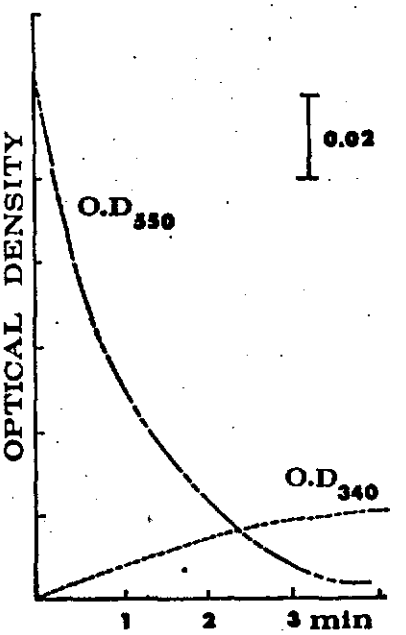


Fig. 7

Fig. 6: Reduced plus CO minus reduced absorption spectrum of T. denitrificans cell-free extracts. Reaction mixture in a total volume of 3.0 ml contained supernatant fraction 30,000 g containing 20 mg of protein in 0.05 M Tris-HCl (pH 7.5) and 0.4 μmoles of dithionite. The treatment cuvette was bubbled with CO for 2 min and the absorption spectra were recorded in a Cary, Model 14, spectrophotometer.

Fig. 7: ATP-dependent reduction of NAD+ by thiosulfate coupled to the oxidation of cytochrome c under anaerobic conditions. The reaction was carried out in Thunberg-type Beckman cuvettes of one cm light path. The reaction mixture in a total volume of 3.0 ml contained: 0.3 ml of enzyme containing 5.0 mg of protein, 55 μM mammalian cytochrome c and 20 μmoles of thiosulfate in the side-arm; 2 μmoles of NAD+, 5 μmoles of $MgCl_2$, 150 μmoles Tris-HCl (pH 7.0), in the main compartment. The treatment cuvette in addition was provided with 3 μmoles of ATP. After evacuation the contents of the side arm were tipped in, and the change in optical density at 340 and 550 mμ was measured simultaneously in a dual wave-length double beam recording spectrophotometer.

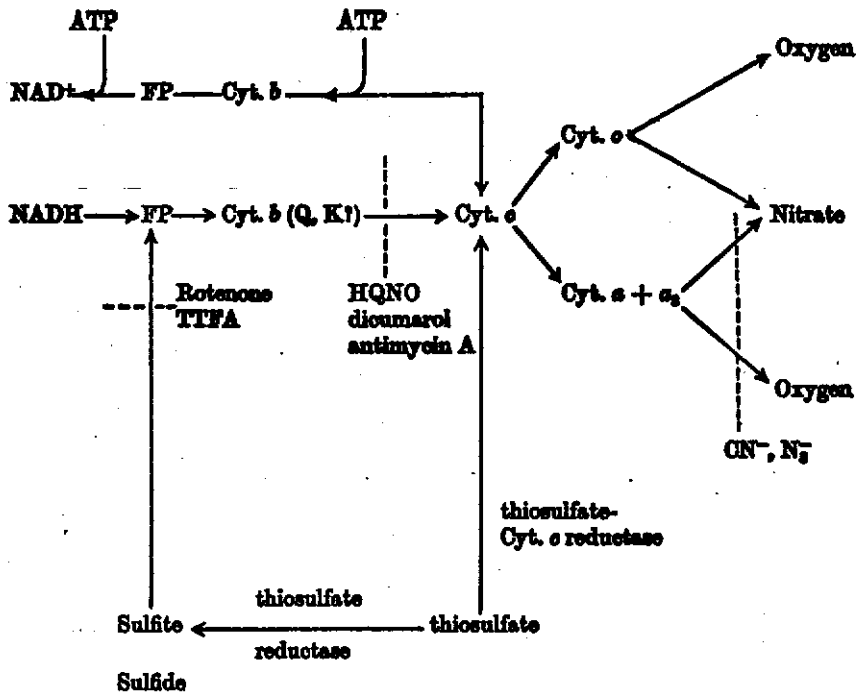


Fig.8. Proposed pathways of electron transport in *T. denitrificans*

Fig. 8: Under anerobic conditions thiosulfate is cleaned by thiosulfate reductase to its sulfide and sulfite moieties which couple with the flavoproteins of the electron transport chain. The transfer of electrons to nitrate is inhibited by rotenone, HQNO, and cyanide. Aerobically thiosulfate couples with cytochrome c and the transfer of electrons to O_2 is not inhibited by rotenone or HQNO; both thiosulfate-cytochrome c reductase and cytochrome oxidase are involved in the aerobic thiosulfate oxidation.

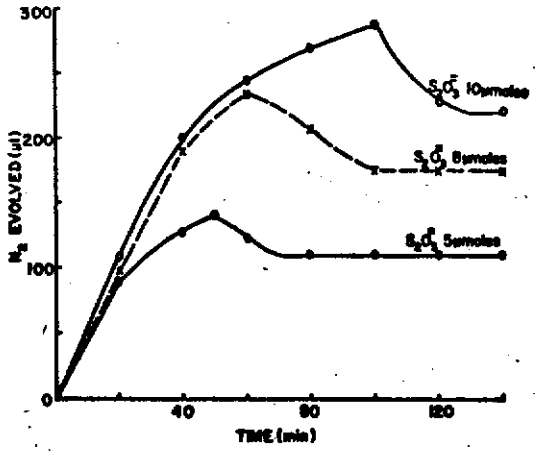


Fig. 9

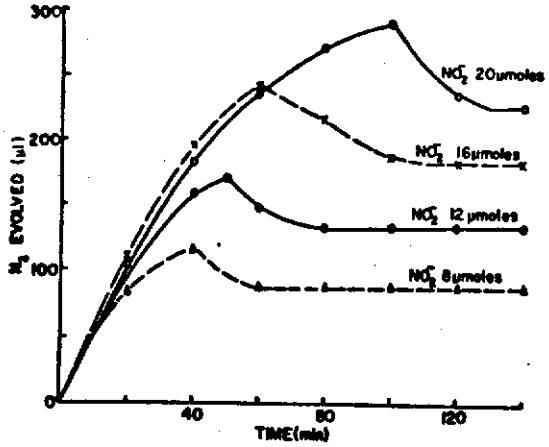


Fig. 10

Fig. 9: Stoichiometry of anaerobic oxidation of thiosulfate by Thiobacillus denitrificans. The experimental conditions are described in Materials and Methods. The gas evolution was measured by Warburg-manometry in the presence of 5, 8 and 10 µmoles of thiosulfate with 25 µmoles of nitrate as the electron acceptor. The reaction mixture contained 5 mg of cellular protein.

Fig. 10: Stoichiometry of anaerobic nitrite reduction and coupled thiosulfate oxidation in Thiobacillus denitrificans. The experimental conditions were similar to those described in Fig. 9 except 8, 12, 15 and 20 µmoles of nitrite were used in the presence of 25 µmoles of thiosulfate.

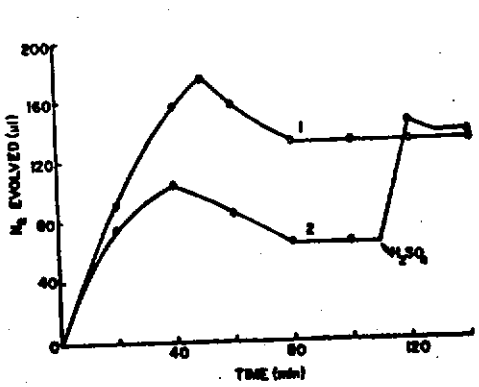


Fig. 11

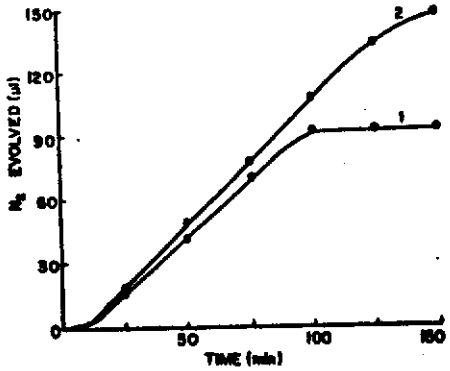


Fig. 12

Fig. 11: Effect of nitric oxide trapping system on nitrite reduction by Thiobacillus denitrificans. The reaction mixture contained 12 µmoles of nitrite, 20 µmoles of thiosulfate and cell suspensions containing 7 mg protein. Trace 1 represents gas production in the control flask. Trace 2 shows gas production in the presence of 0.1 ml 20% alkaline sulfite solution in the side arm; 0.05 ml of 25% H_2SO_4 was added to the sulfite solution after 110 min.

Fig. 12: Reduction of nitric oxide under anaerobic conditions by intact cells of Thiobacillus denitrificans. The experimental conditions are described in "Materials and Methods". The reaction mixture contained 20 µmoles of thiosulfate and cell suspensions containing 5 mg protein. Traces 1 and 2 represent nitrogen formation from 190 and 300 µl of nitric oxide respectively.

Fig. 13

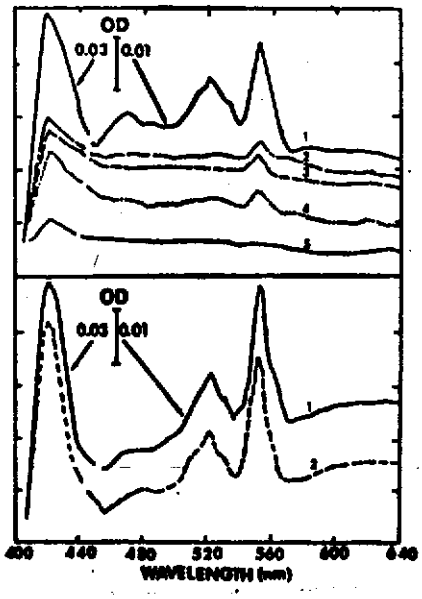


Fig. 14

Fig. 15

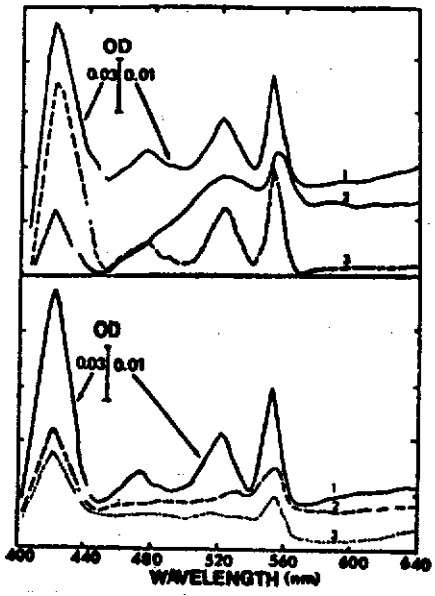


Fig. 16

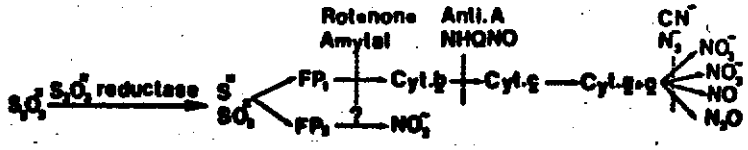


Fig. 17

Fig. 13: The redox state of cytochrome system in Thiobacillus denitrificans intact cells, treated anaerobically with thiosulfate, upon the addition of NO_3^-, NO_2^-, NO and N_2O. The steadystate difference absorption spectra were in a Cary, Model 14, spectrophotometer. The reaction mixture in a total volume of 2.0 ml contained nitrous oxide-treated cell suspensions containing 1 mg protein and 95 µmoles of phosphate buffer, pH 7.0. The treatment cuvette in addition, was supplied with 10 µ moles of thiosulfate (trace 1). Traces 2 and 3 represent change in absorption after the addition of 30 µmoles of nitrate or nitrite, respectively. Traces 4 and 5 respectively show the change in absorption after nitric oxide and nitrous oxide was added in the treatment cuvettes which were fitted with serum caps.

Fig. 14: Effect of nitrous oxide on the thiosulfate-reduced cytochrome system in Thiobacillus novellus cells under anaerobic conditions. The reaction mixture in a total volume of 2.0 ml contained cell suspensions containing 1.2 mg protein and 95 umoles of phopphate buffer, pH 7.0. The treatment cuvette in addition was supplied with 10 umoles of thiosulfate (trace 1). Trace 2 represents change in absorption after nitrous oxide was added in the treatment cuvette fitted with a serum cap.

Fig. 15: Effect of NHQNO and cyanide upon the reduction of cytochromes in whole cells of Thiobacillus denitrificans upon treatment with thiosulfate under anaerobic conditions when nitrate, nitric oxide, or nitrous oxide served as the terminal electron acceptor. Reaction mixture in a final volume of 2.0 ml contained cell suspensions containing 1 mg enzyme protein and 95 μmoles of phosphate buffer, pH 7.0. The treatment cuvette in addition, contained 10 μmoles of thiosulfate. Trace 1 indicates the difference absorption spectrum under anaerobic conditions after the addition of thiosulfate to the treatment cuvette. Traces 2 and 3 represent respectively the difference spectra in the presence of 5 ug NHQNO and 0.1 mM cyanide in both cuvettes upon the addition of nitrate, nitric oxide, or nitrous oxide in the treatment cuvette.

Fig. 16: Effect of NHQNO and cyanide upon the reduction of cytochromes in whole cells of Thiobacillus denitrificans upon treatment with thiosulfate under anaerobic conditions when nitrite served as the terminal electron acceptor. Reaction mixture and experimental conditions were the same as described in Fig. 15. Trace 1 indicates the difference absorption spectrum under anaerobic conditions obtained after the addition of the thiosulfate to the treatment cuvette. Traces 2 and 3 respectively show the difference absorption spectra in the presence of 5 μg of NHQNO and 0.1 mM cyanide in both cuvettes upon the addition of nitrite in the treatment cuvette.

Fig. 17: Probable electron transfer pathways involved in dissimilatory nitrate reduction in Thiobacillus denitrificans. "See Discussion" for the explanation of the branched chain. The oxidation of thiosulfate coupled to the reduction of nitrate, nitric oxide and nitrous oxide is sensitive to the classical inhibitors of the electron transport chain. Since nitrite seldom accumulates in growing cultures, the concentrations of this electron acceptor used in experiments were non-physiological which appear to result in the modification of the respiratory chain thereby making it insensitive to the various inhibitors used.

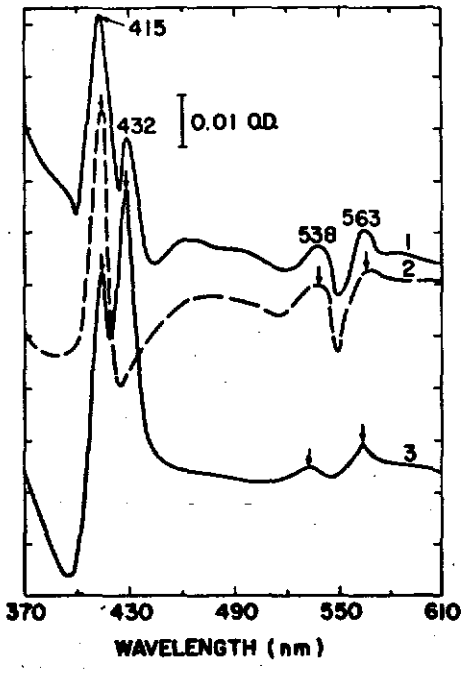


Fig. 18

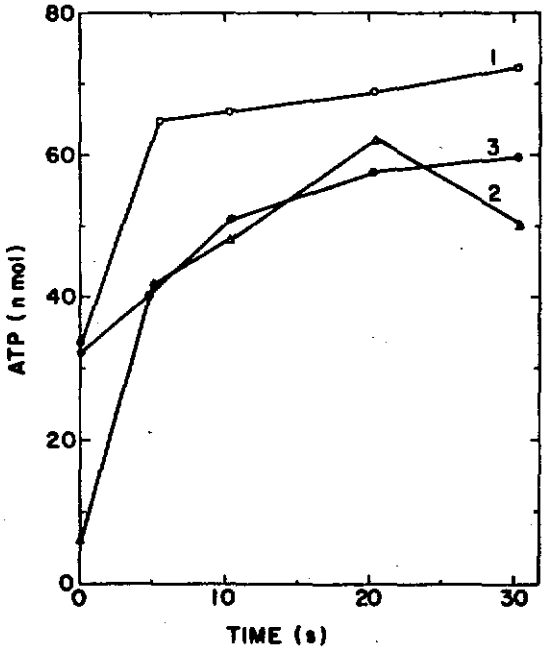


Fig. 19

Fig. 18: Reduced plus CO minus reduced absorption spectra of cell-free extracts from T. neapolitanus. Reaction mixture contained 5.0 mg of enzyme protein, 10 mM substrate as indicated, and 0.05M potassium phosphate buffer (pH 8.0) to make up the volume to 3 ml. CO was bubbled through the experimental cuvette only. 2-ME, trace 1; sulfide, trace 2; thiosulfate, trace 3.

Fig. 19: Phosphorylation with thiosulfate in intact cells of T. neapolitanus. Experimental conditions are described in detail in Materials and Methods. No increase in the ATP content of the cells was observed in the absence of substrate. Thiosulfate at pH 7.5, trace 1; thiosulfate at pH 9.0, trace 2; 2-mercaptoethanol, trace 3.

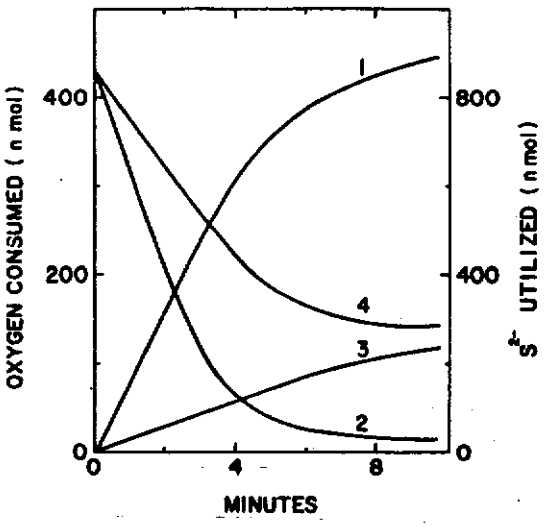


Fig. 20

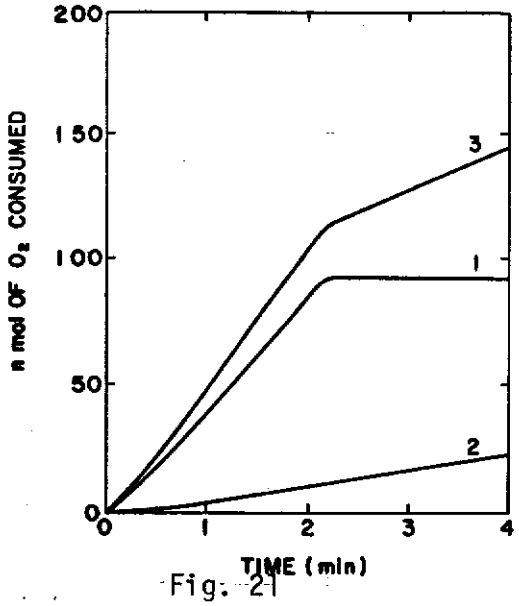


Fig. 21

Fig. 20: Sulfide oxidation by whole cells and cell-free extracts from T. neapolitanus. Reaction mixture in a total volume of 3 ml contained whole cells equivalent to 0.3 mg protein or cell-free extracts containing 0.75 mg of protein as indicated, 850 nmol of sulfide, and 0.05 M potassium phosphate buffer (pH 8.0) to make a final volume of 3 ml. Oxygen consumption was measured as described in Materials and Methods. Sulfide utilization and oxygen consumption by whole cells, trace 2 and trace 1, respectively; sulfide utilization and oxygen consumption by cell-free extracts, trace 4 and trace 3, respectively.

Fig. 21: Localization of stage 1 and stage 2 sulfide oxidase activity. Reaction mixture contained 1 μmol of sulfide, fraction of cell-free extract as indicated, and 0.05 M posassium phosphate buffer (pH 8.0) to make a final volume of 3 ml. Oxygen consumption was measured polarographically. P-144 fraction containing 1.5 mg protein, trace 1; S-144 fraction containing 2.5 mg protein, trace 2; P-144 (1.5 mg protein) + S-144 (2.5 mg protein), trace 3.

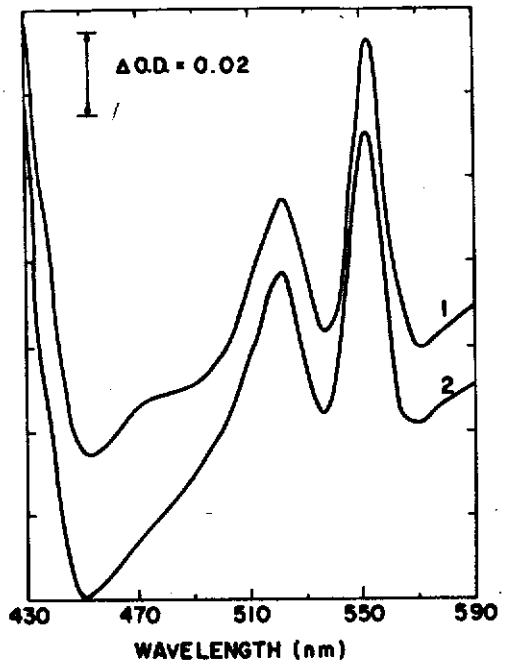


Fig. 22

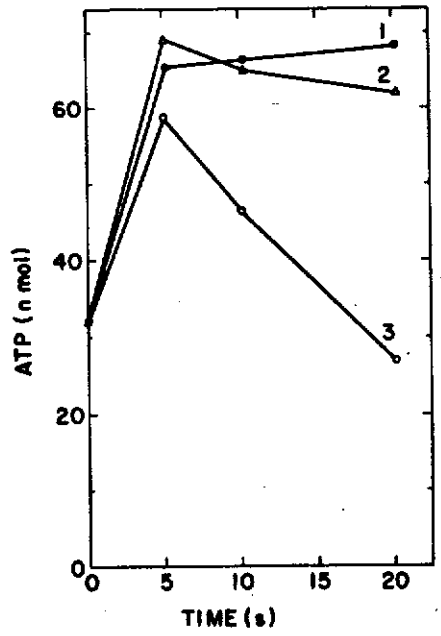


Fig. 23

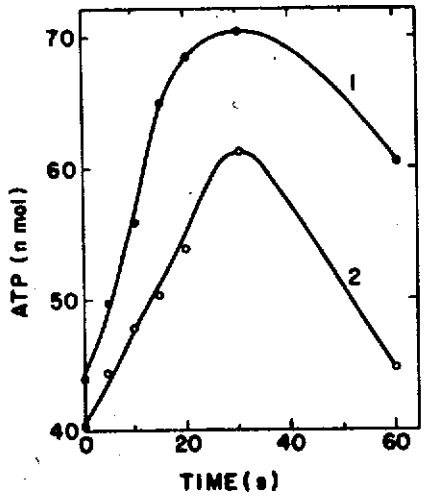


Fig. 24

Fig. 22: Steady-state difference absorption spectra of T. neapolitanus cell-free extracts upon treatment with sulfide. Reaction mixture contained 5 mg of enzyme protein (8000 g supernatant) and 0.05 M potassium phosphate buffer (pH 8.0) to give a final volume of 2.0 ml. In addition, the treatment cuvette was supplied with 10 μmol of sulfide. Endogenous flavin reduction by sulfide, trace 1; reduction of added FMN (50 μM) by sulfide, trace 2.

Fig. 23: Phosphorylation linked to sulfide oxidation catalyzed by intact-cells of T. neapolitanus. Experimental conditions were similar to those described in Materials and Methods except that sulfide was employed as the oxidizable substrate and its concentration was varied as indicated. Sulfide at 5 mM, trace 1; sulfide at 10 mM, trace 2; sulfide at 25 mM, trace 3.

Fig. 24: Acid-induced ATP synthesis by intact cells of T. neapolitanus Experimental conditions are described in detail in Materials and Methods. Acid phase 3 mM HCl (pH 2.5), trace 1; acid phase 3 mM succinate + 3 mM glutamate (pH 3.8), trace 2.

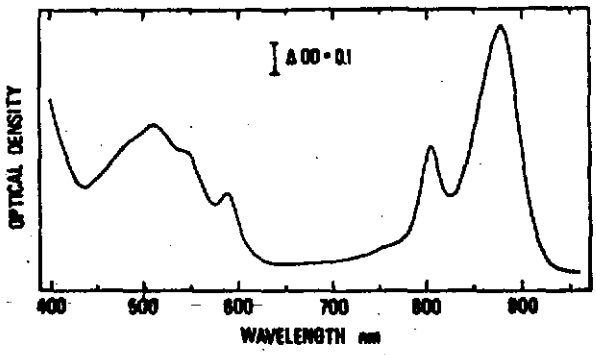


Fig. 25

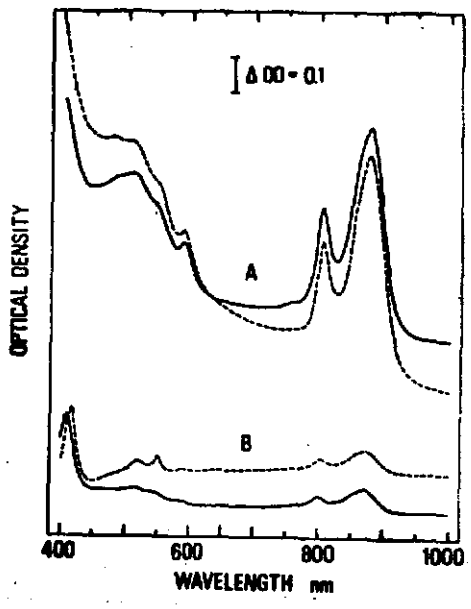


Fig. 26

Fig. 25: Absorption spectrum of whole cell suspension of Rps. palustris grown photosynthetically with thiosulfate. The spectrum was obtained on a Cary, Model 14, spectrophotometer by use of white translucent lucite diffusion plates (Cadillac Plastics No. W-7328) immersed in the cuvettes to minimize scattering by the Shibata technique (Shibata et al., 1954). The protein content was 1.4 mg/ml suspension in a total volume of 3 ml in a cuvette of 1 cm light path. The reference cuvette contained distilled water.

Fig. 26: Absorption spectra of Rps. palustris cell-free preparations on the basis of equal amounts of protein. Trace A indicates the absorption spectrum of the 10,000xg supernatant containing 1.7 mg protein/ml, and trace B shows the spectrum of the 144,000x g supernatnat also containing 1.7 mg protein/ml. The dashed lines represent the steady spectra obtained after the addition of dithionite.

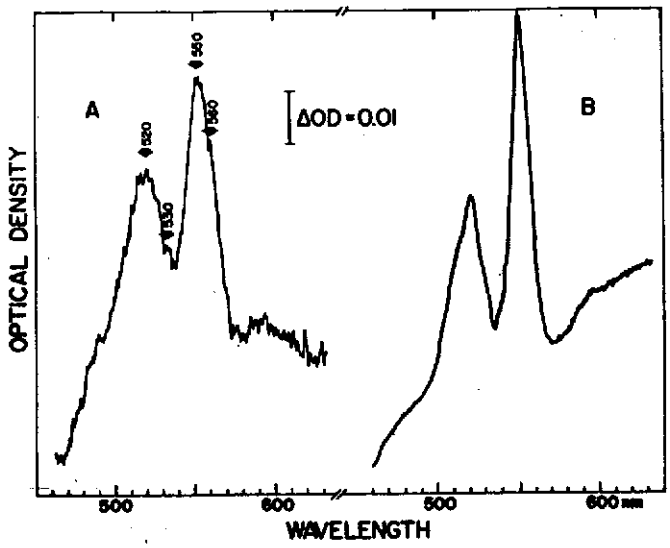


Fig. 27

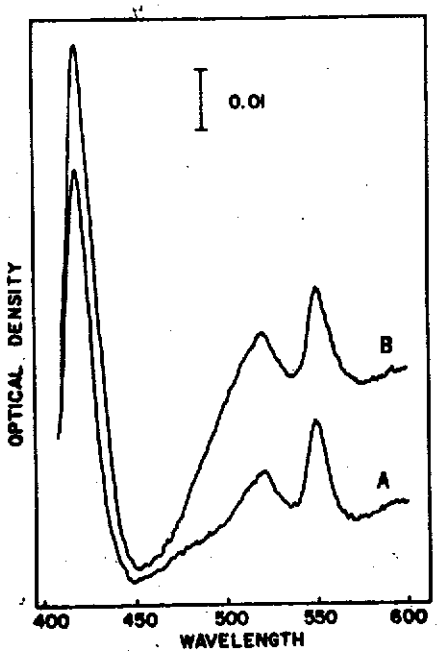


Fig. 28

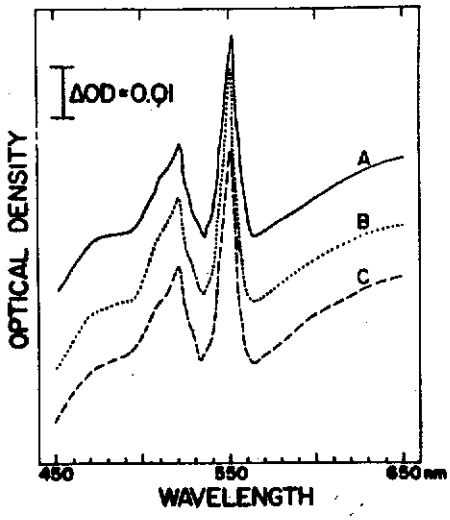


Fig. 29

Fig. 27: Steady-state dithionite-reduced minus oxidized difference absorption spectra of Rps. palustris cell-free extracts. Trace A represents the difference spectrum obtained with the 100,000xg supernatant; protein content was .5 mg/ml. Trace B illustrates the difference spectrum obtained with the 144,000xg supernatant; protein was 2.6 mg/ml. Other conditions were similar to those described in Fig. 26.

Fig. 28: Absorption spectra of the S-144,000 cell-free extract. Reaction micture in a total volume of 1.0 ml contained 50 μmoles Tris (pH 8.0) and cell-free extract containing 11 mg protein. Trace A indicates the steady-state absorption spectrum obtained after the addition of 10 μmoles of NADH to the sample cuvette. Antimycin A (10 μg) was then added to both reference and sample cuvettes and trace B represents the resulting difference absorption spectrum obtained after 5 min incubation.

Fig. 29: Steady state thiosulfate–reduced minus oxidized difference absorption spectra obtained with the 144,000xg supernatant fraction. The cuvettes, in a total volume of 2.0 ml, contained 50 μmoles of Tris (pH 8.0), 1.0 ml of the 144,000xg supernatant fraction containing 23 mg protein and in addition, the test cuvette contained 20 μmoles of thiosulfate. Trace A represents the extent of reduction of the endogenous cytochrome system after 1 min of incubation of the cell-free extract with thiosulfate. Trace B and C indicate the amount of endogenous cytochrome reduced by added thiosulfate, after the cell-free extract had been preincubated for 5 min with 1 μg of antimycin A/mg protein (trace B) and 2 μg of NOQNO/mg protein (trace C), respectively.

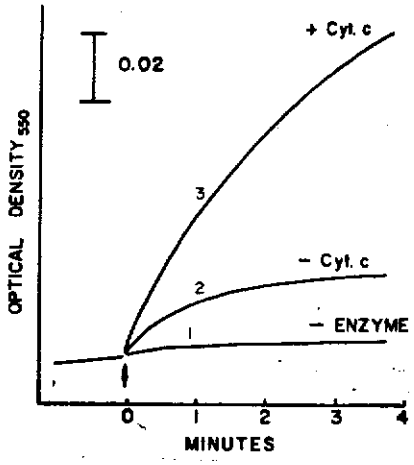


Fig. 30

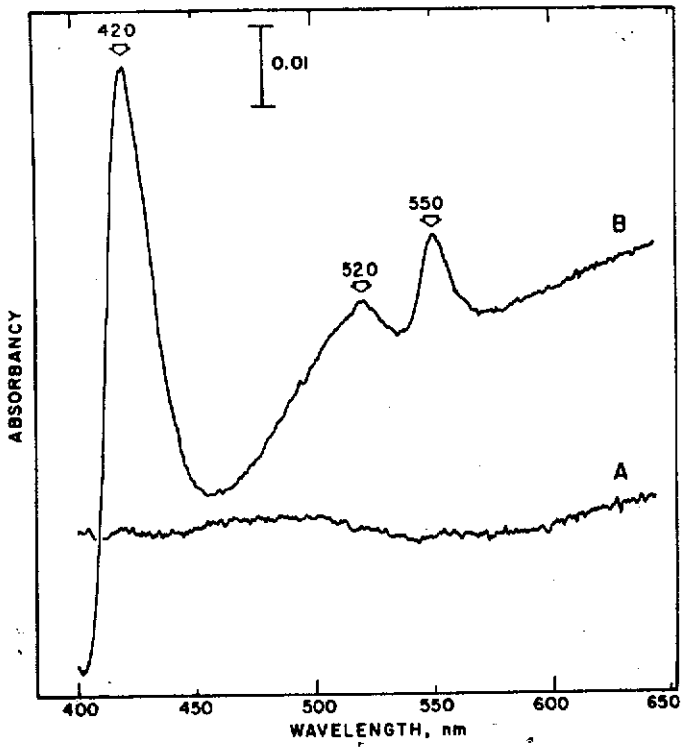


Fig. 31

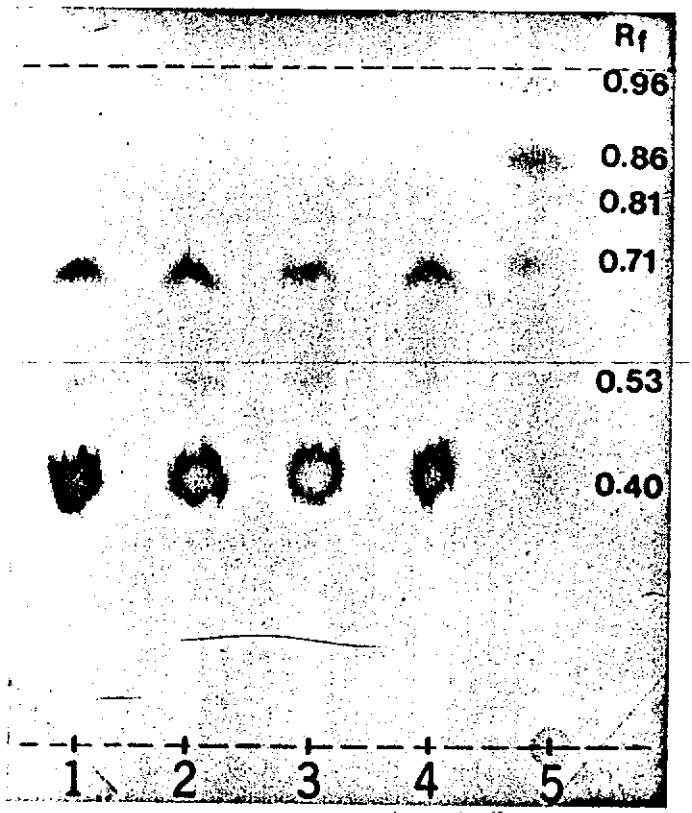


Fig. 32

Fig. 30: Thiosulfate-dependent reduction of endogenous and added cytochrome c by Rhodopseudomonas palustris cell-free extracts. The reaction mixture, in a total volume of 1.0 ml, contained 45 μmoles of Tris-HCl (pH 8.0), 5 X 10^{-6} M cyanide, and where shown, 0.10 ml of enzyme equivalent to 3.8 mg of protein, and 0.02 ml of 4% cytochrome c (horse heart type II). The reaction was started by the addition of 20 μmoles of thiosulfate. Trace 1 shows the kinetics of cytochrome c reduction (endogenous or exogenous) in the absence of enzyme. Traces 2 and 3 show the kinetics of cytochrome c reduction in the presence and absence of added mammalian cytochrome c, respectively.

Fig. 31: Steady-state thiosulfate-reduced minus oxidized difference absorption spectra of the Rps. palustris cell-free extracts. Trace A represents the baseline before the addition of thiosulfate. Trace B represents the difference spectrum obtained 5 min after the addition of 20 μmoles of thiosulfate to the sample cuvette containing 5 mg of protein.

Fig. 32: Autoradiogram showing the products of thiosulfate oxidation by whole cells of Rps. palustris. Whole cells equivalent to 20 mg dry weight were incubated under N_2 in the dark with 10 μmoles of $^{35}S{\cdot}SO_3^{2-}$ containing 250 μc of ^{35}S. Aliquots of 0.02 ml were removed 15 sec (1), 1 min (2), 3 min (3), and 10 min (4 and 5) after the addition of thiosulfate. Oxidation products were separated by chromatography in a pyridine:propanol-1:water (3:5:5) solvent system. Excess iodine was added to one of the 10 min aliquots (5) to oxidize tetrathionate. Radioactive products were located by exposure of the chromatogram to X-ray film. Identification of the products was by R_f comparison to standards as given in Table I.

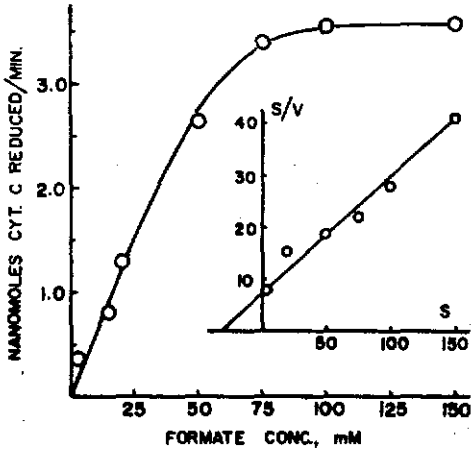


Fig. 33

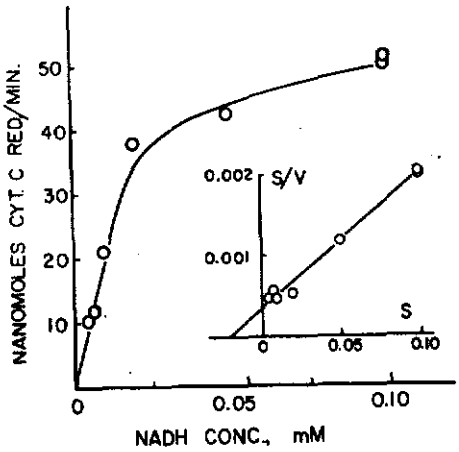


Fig. 34

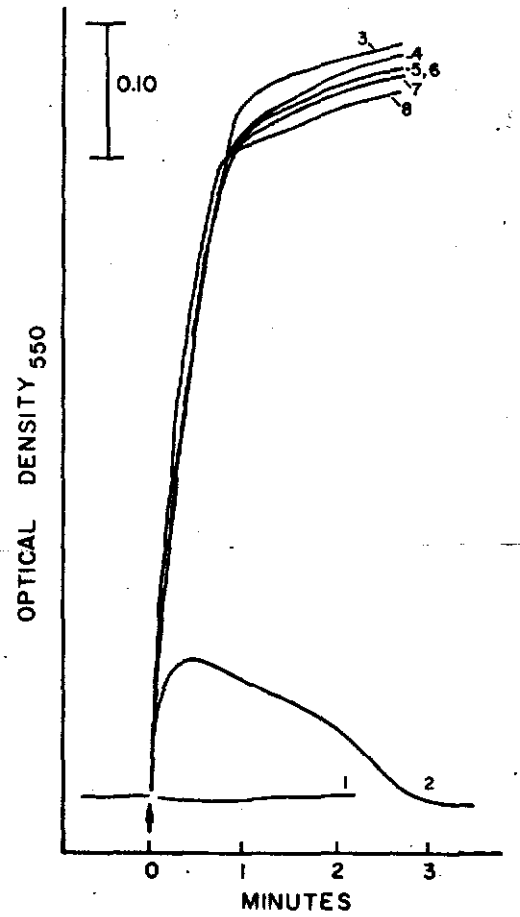


Fig. 35

Fig. 33: Effect of formate concentration on cytochrome c reduction. Reaction mixture in a total volume of 1.0 ml contained 40 μmoles of Tris-HCl (pH 8.0), 0.02 ml of 4% cytochrome c, 0.2 ml of cell-free extracts containing 8.0 mg protein. The reaction was started by the addition of various amounts of formate, as indicated.

Fig. 34: Effect of NADH concentration on cytochrome c reduction. Experimental conditions were similar to those described in Fig. 4 except that NADH was added to start the reaction. Protein content was 7.2 mg.

Fig. 35: The effect of inhibitors of flavoprotein and of cytochrome b oxidation on cytochrome c reduction by NADH. The basic reaction mixture contained in a total volume of 1.0 ml, 45 μmoles of Tris-HCl (pH 8.0), 0.02 ml of 4% cytochrome c, 0.01 ml of the cell-free supernatant containing 0.4 mg of protein, 1.0 μmole of NADH to start the reaction, and other additions as indicated; trace 1, minus enzyme; trace 2, complete; traces 3-8 contained 5 x 10^{-6} M cyanide plus the following inhibitors; trace 3, none; trace 4, 10^{-4} M rotenone; trace 5, 10^{-3} M amytal; trace 4, 10^{-4} M rotenone; trace 5, 10^{-3} M amytal; trace 6, 2 μg/mg of protein of Antimycin A; trace 7, 2 μg/mg of protein of NOQNO; and trace 8, 10^{-3} M atebrin.

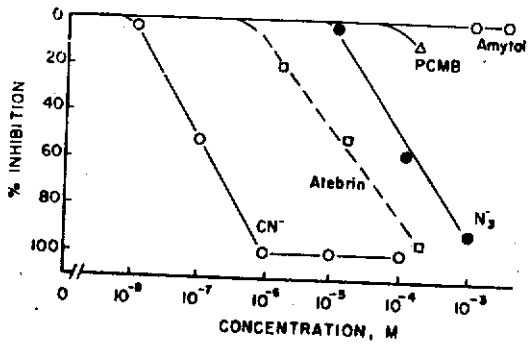


Fig. 36

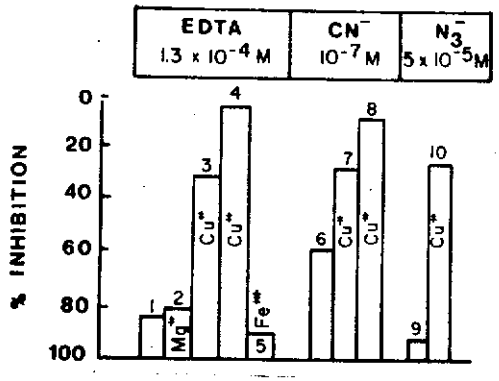


Fig. 37

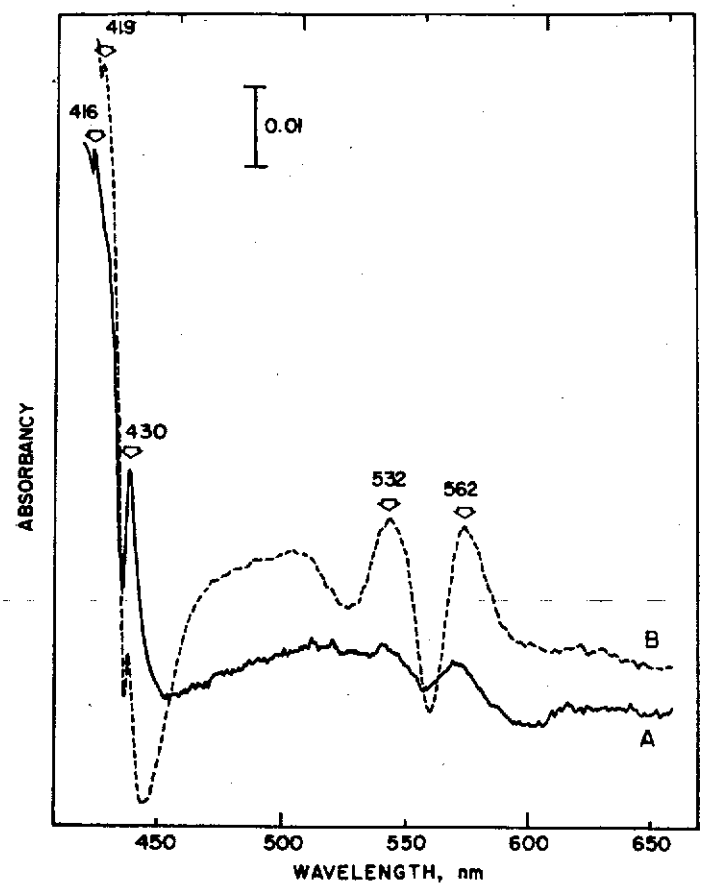


Fig. 38

Fig. 36: Effect of inhibitors on cytochrome c:O_2 oxidoreductase activity. The reaction mixture contained in a total volume of 1.0 ml, 45 µmoles of Tris-HCl (pH 8.0), 0.01 ml of enzyme representing 0.4 mg of protein, 0.02 ml of reduced cytochrome c, and inhibitors as indicated. The oxidation of cytochrome c was followed spectrophotometrically by measuring the decrease in absorbance at 550 nm.

Fig. 37: Effect of EDTA, cyanide and azide on cytochrome c:O_2 oxidoreductase activity. Experimental conditions were the same as described for Fig. 7 with the following additions: 1, EDTA (1.3×10^{-4} M); 2, EDTA + Mg^{2+} (5×10^{-4} M); 3, EDTA + Cu^{2+} (2×10^{-4} M); 4, EDTA + Cu^{2+} (5×10^{-4} M); 5, EDTA + Fe^{3+} (2×10^{-4} M); 6, cyanide (10^{-7} M); 7, cyanide + Cu^{2+} (10^{-6} M); 8, cyanide + Cu^{2+} (10^{-5} M); 9, azide (5×10^{-5} M); 10, azide + Cu^{2+} (5×10^{-4} M).

Fig. 38: Reduced + carbon monoxide minus reduced difference absorption spectra of Rps. palustris cell-free extracts. The difference spectra represents those obtained immediately after bubbling with CO for 3 min (trace A) and after incubation for 20 min in darkness at 25° (trace B). The cuvette contained in a total volume of 1.0 ml, 10^{-5} M cyanide, 6µ moles of NADH and the S-144,000g supernatant equivalent to 35 mg of protein. CO gas phase was 1 atmosphere.

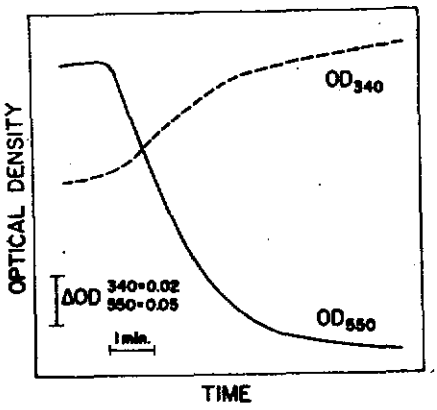


Fig. 39

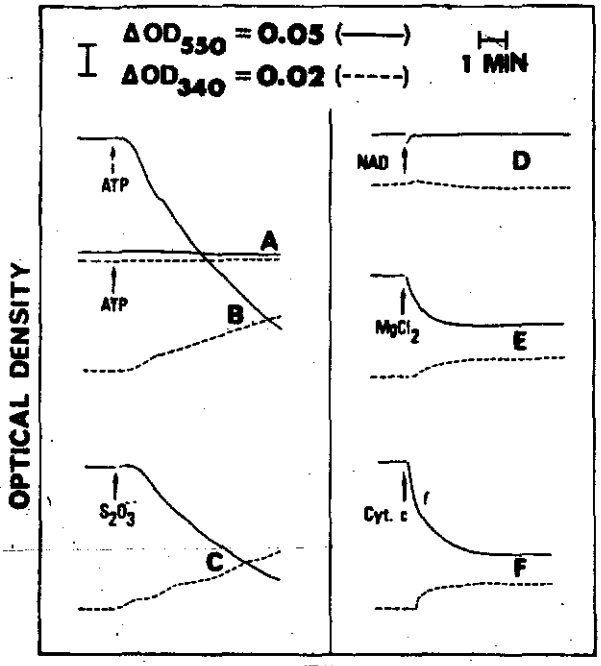


Fig. 40

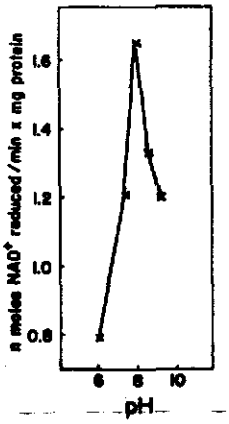


Fig. 41

Fig. 39: ATP-dependent reduction of NAD+ by thiosulfate coupled to the oxidation of cytochrome c, catalyzed by the crude cell-free extract (10,000 xg supernatant). The reaction was carried out in anaerobic Thunberg-type cuvettes of 1 cm light path. Reaction mixture in a total volume of 3.0 ml contained 0.3 ml of cell-free extract containing 6.9 mg protein. In addition the main compartment of the cuvettes contained 125 μmoles Tris (pH 8), 2 μmoles NAD+, and only in the sample cuvette, 5 μmoles ATP. The side arms of the cuvettes contained 20 μmoles $S_2O_3^=$, 5 μmoles $MgCl_2$, and 0.165 μmoles mammalian cytochrome c. After evacuation, the contents of the side arms were tipped in to start the reaction. The changes in optical density at 340 and 550 nm were measured simultaneously in the dual-wavelength split-beam recording spectrophotometer.

Fig. 40: Factors affecting the $S_2O_3^=$ -linked ATP-dependent NAD+ reduction by 144,000xg supernatant fraction from Rps. palustris. The experimental conditions were the same as described in Fig. 39. The complete reaction mixture in a total volume of 3 ml consisted of 0.2 ml of the cell-free extract containing 7.6 mg protein, 1.7 mM ATP, 7 mM $S_2O_3^=$, 0.7 mM NAD+, 1.7 mM $MgCl_2$, and 55 μM mammalian cytochrome c. Arrows indicate the addition of the missing component from the side arm to start the reaction. Thus, the difference absorbance changes were obtained due to ATP (traces A and B; trace A representing the control without cell-free extract added), $S_2O_3^=$ (traces C), NAD (traces D), Mg++ (traces E), and mammalian cytochrome c (traces F). The solid and the dashed lines represent O.D. at 550 and 340 nm respectively.

Fig. 41: Effect of pH on NAD+ reduction by ATP-driven reversed electron flow. Experimental conditions were as described in Fig. 40. Protein content was 0.4 mg/ml.

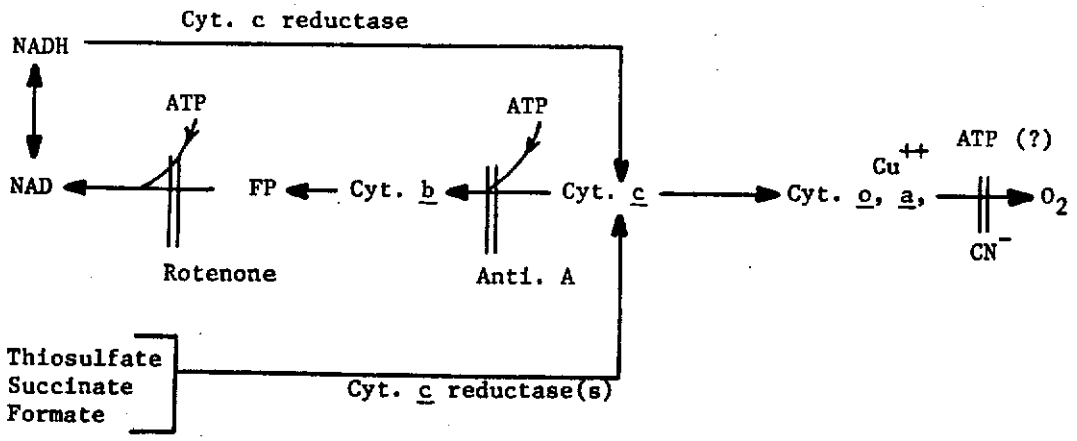


Fig. 42

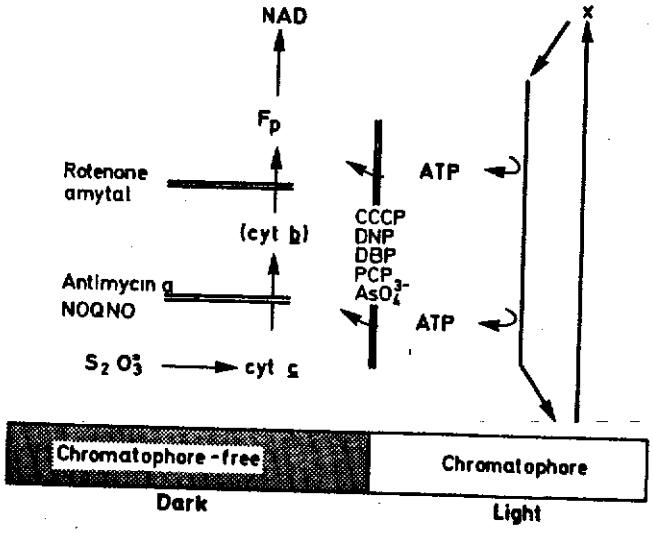


Fig. 43

Fig. 42: Electron Transport Pathways in Rhodopseudomonas palustris.

Fig. 43: Proposed pathways of electron and energy transfer reactions involved in the generation of reducing power in Rps. palustris cell-free extracts (S-10,000 and S-144,000). The light-driven electron transfer and coupled ATP synthesis involve the participation of the photochemical apparatus containing chromatophores and membrane-bound electron transport carriers with X representing a strong reductant produced in the primary photoact. The synthesis of ATP is coupled to the subsequent electron flow along the downhill thermodynamic gradient. It is assumed, however, that the energy generation occurs mainly by cyclic photophosphorylation without the participation of the external electron donor. The function of the latter (i.e. $S_2O_3^=$) is to provide electrons for the "non-cyclic" electron flow mediated by the electron transport carriers (e.g. cyt. c, cyt. b, Fp etc.) with NAD+ as the terminal acceptor. Since the "non-cyclic" electron transfer is driven against the thermodynamic gradient, the process inevitably is energy-dependent. The evidence is further supported by the potent effect of the uncouplers of the energy transfer reactions and inhibitors of the electron transport chain.

CHAPTER IV

CONCLUSIONS

The experimental results that have been obtained and discussed in this report have yielded significant information concerning the metabolism of sulfur compounds by microorganisms that play an important role in the environmental quality of our water resources. Although considerably useful data have been obtained, it was not possible to complete the entire project within the time schedule of the grant support. Hence it is extremely important to continue further investigations in order to meet objectives (b) and (c) as outlined on page 1 of this report. The importance of further work should be quite apparent because of the tremendous applicability of the expected findings in the solution of the water pollution problems. It is recommended, therefore, that the OWRR must consider further continuation of the research as proposed originally in the research proposal that was submitted by the principal investigator. The latter and his staff have invested an enormous amount of time and efforts in the current project which enabled us to develop deep insights into the possible directions of further work with respect to problem - solving research in the area of water pollution.

There has been a great deal of confusion concerning the biochemical reaction mechanisms involving inorganic sulfur compounds because of their chemical interactions. It is our hope that we have been able to make considerable advances in the field of inorganic sulfur metabolism and have provided information that might prove to be useful in the understanding of the sulfur chemistry. It has been possible to demonstrate that the cellular

and /or the subcellular components of sulfur bacteria are the potent biocatalysts for the numerous transformations under both aerobic and anaerobic conditions. In the latter case oxyanions of inorganic nitrogen compounds could serve as oxidants. There is a great deal of interest in the sequence of oxide-reduction reactions and the intermediates that might be involved in sulfur and nitrogen metabolism under physiological conditions and our contributions appear to be significant in elucidating the related pathways and the mechanisms.

In view of our experimental observations and of several other investigators, there can be the following three pathways by which thiosulfate is oxidized by the chemosynthetic and photosynthetic bacteria:

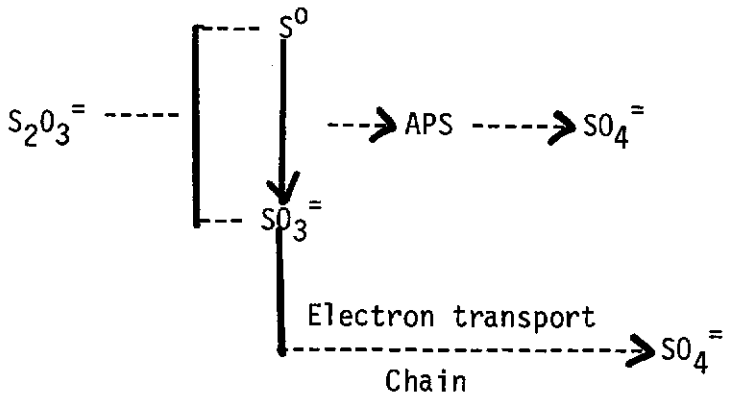
i) The polythionate pathway by which thiosulfate is oxidized to sulfate involving tetrathionate, trithionate, and sulfite as intermediates:

$$S_2O_3^{=} \longrightarrow S_4O_6^{=} \longrightarrow S_3O_6^{=} \longrightarrow SO_3^{=} \longrightarrow SO_4^{=}$$

ii) Reductive cleavage of thiosulfate molecule to the sulfide and sulfite moieties and the subsequent conversion of both to sulfate involving (APS) adenosine phosphosulfate reductase:

$$S_2O_3^{=} \begin{cases} \longrightarrow S^{=} \longrightarrow S^{o} \\ \longrightarrow SO_3^{=} \longrightarrow APS \longrightarrow SO_4^{=} \end{cases}$$

iii) Splitting of the thiosulfate molecule to sulfur and sulfite and the subsequent oxidation of sulfur to sulfite by an oxygenase enzyme; the sulfite could then be oxidized to sulfate with the mediation of either the cytochrome system or the APS reductase.



Our experimental observations suggest that all of the three proposed mechanisms might be involved in the thiobacilli with a few exceptions. In some cases such as in T. denitrificans the aerobic oxidation of thiosulfate occurs with two rate constants:

1. An initial and rapid rate in which both the inner and outer sulfur atoms are oxidized rapidly at almost the same rate; here the thiosulfate molecule appears to undergo the following transformations:

a) $^-S\_SO_3^-$ $\xrightarrow{S_2O_3^= \text{ Reductase}}$ $S^= + SO_3^=$

b) $S^=$ $\xrightarrow{\text{Sulfide-oxidase}}$ $S^o + 2e^-$

c) $S^o + O_2 + H_2O$ $\xrightarrow[\text{(oxygenase?)}]{\text{Sulfur oxidase}}$ $SO_3^= + 2H^+ + 4e^-$

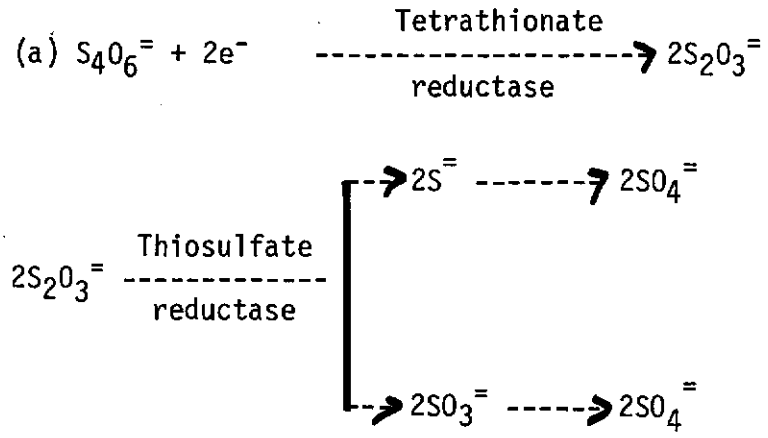
d) $2SO_3^= + 2H_2O$ $\xrightarrow{\text{Sulfite oxidase}}$ $2SO_4^= + 4H^+$

e) $4H^+ + 4e^- + O_2$ $\xrightarrow[\text{Chain}]{\text{Electron transport}}$ $2\ H_2O$

2. The above rapid rate of thiosulfate oxidation is followed by a slower but constant rate in which the product of first oxidation is tetrathionate produced by the condensation of thiosulfate molecules:

$$2S_2O_3^= \longrightarrow S_4O_6^= + 2\ e^-$$

The tetrathionate thus formed can be oxidized by the following reactions:



(b) i) $S_4O_6^{=} \longrightarrow 2SO_3^{=} + 2S^{=}$

ii) $SO_3^{=} + S^0 \longrightarrow S_2O_3^{=}$

iii) $SO_3^{=} + S_2O_3^{=} \longrightarrow S_3O_6^{=}$

In reaction (a) the tetrathionate can be reductively cleaved to produce 2 molecules of thiosulfate, the sulfide and the sulfite moieties of which can be oxidized to sulfate under aerobic or anaerobic conditions. According to reaction (b) the sulfide and sulfite groups which are the cleavage products of tetrathionate would react with thiosulfate molecule to yield trithionate (reaction b iii). Pentathionate may be formed by the interaction of the sulfide moiety with the trithionate molecule:

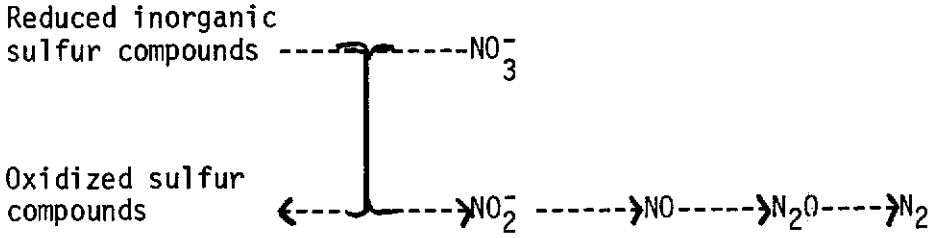
$S_3O_6^{=} + 2S^{=} \longrightarrow S_5O_6^{=}$

Presence of all of the intermediates e.g. tetrathionate, trithionate, and pentathionate has been experimentally verified in cell suspensions oxidizing thiosulfate as well as during the growth of the organisms.

Although trithionate and pentathionate might arise due to non-enzymatic chemical reactions, the oxidation of tetrathionate has been shown to be mediated by the electron transport chain components such as flavoprotein, cytochromes of b and c-type, and cytochrome oxidase components. The initial appearance and accumulation of tetrathionate in growing cultures followed by its oxidation to sulfate, and the complete oxidation of tetrathionate by intact cells under aerobic or anaerobic conditions strongly suggest it to be a true intermediate of oxidative sulfer metabolism. Since the oxidation of tetrathionate involves the participation of respiratory chain it would appear that the compound can be utilized by the thiobacilli as an energy source. Such a possibility is being explored in the subsequent work.

The denitrifying organisms such as Thiobacillus denitrificans pose a serious threat to our environments and the ecosystems. These creatures have been found to produce enormous amounts of sulfuric acid (or sulfates) from the oxidation of inorganic sulfur compounds. The rate of transformations doubles under anaerobic conditions when the sulfur compounds are oxidized at the expense of nitrate reduction (e.g. 15 moles of sulfates produced from thiosulfate oxidation with the concomitant reduction of 12 moles of nitrates/mg cellular protein/hr). Such a high efficiency of these organisms results not only in the accumulation of toxic sulfates and sulfuric acid in the polluted waters but the unprecedented activity of these bacteria can virtually exhaust the biosphere from the available supply of useful form of nitrogen which is an essential elements for all life systems. Our experimental data have shown that almost all of the oxyanious of inorganic nitrogen are reduced by the organism to nitrogen gas which is lost from our water

resources; the sequence of the reduction process appears to be as follows:



The work performed under this project has revealed that the thiobacilli were the most prolific producers of sulfates. If we assume that the rate of sulfur transformation in the natural environments having enrichment of these organisms is approximately 10% of the transformations observed under laboratory conditions, then the sulfur oxidizing bacteria could produce as much as 100-400 moles of sulfates on one hour in the upper one-foot layer of water over an area of one acre. Such a high level of sulfate production undoubtedly poses a serious threat to our environment.

The solution of the problem appears to lie in the chemical and biological treatment of the polluted reservoirs or streams as well as the strip mine areas. The feasibility of employing microbial ecosystems for the removal of sulfates and/or sulfides appears to be highly essential. The potential application of such systems should bring benifits that would far exceed the total cost of this project.

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