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M. I. H. Aleem
University of Kentucky

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METABOLIC ROLE OF SULFATES AND SULFIDES PRODUCING BACTERIA IN POLLUTION OF WATERS

M.I.H. ALEEM
Principal Investigator

University of Kentucky Water Resources Institute
Lexington, Kentucky

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ABSTRACT

Cytochrome transport particles from Nitrobacter catalyzed nitrite, ascorbate as well as NADH oxidation with concomitant phosphate esterification yielding P/O ratios of 1.0, 0.6 and 2.0 respectively. Phosphorylation coupled to nitrite oxidation was not effected by rotenone, amytal or antimycin while 50 and 70% inhibition of the NADH-linked phosphorylation was observed in the presence of HOQNO and rotenone respectively. Cell-free extracts from Nitrobacter also catalyzed an energy-dependent reduction of NAD^+ by nitrite. The reduction of cytochrome c by $N\dot{O}_2$ was energy-dependent which involved the reversal of electrons from cytochrome a_1. The subsequent energy-linked reduction of the flavoproteins and pyridine nucleotides occurred concomitantly with the oxidation of cytochrome c. The process of energy-linked reversal of electron transfer in Nitrobacter was markedly sensitive to all the inhibitors and uncouplers.

The reduction of NAD^+ by thiosulfate in T. neapolitanus was an energy-linked process and involved the reversal of electrons from ferrocytochrome c mediated by flavoproteins. Cell-free extracts also catalyzed the reduction of NAD^+ by sultie at the expense of ATP and involved the participation of the flavoprotein-pyridine nucleotide segment of the respiratory chain. The NADH oxidation by T. neapolitanus was mediated by the flavoprotein and cytochrome systems and this process also appeared to be coupled with energy-generation.

The energy-dependent metabolism of sulfate producing bacteria and nitrite oxidizing bacteria was found to be very markedly sensitive to extremely low quantities of chloro, bromo or nitro-substituted phenols. Investigations

which were conducted to assess the importance of these organisms in natural environments seem to be rather important in designing control measures for the control of biological production of sulfur or nitrogen compounds or acidity in acid-mine streams or spoil bank.

KEY WORDS: *sulfur oxidizing bacteria, * iron oxidizing bacteria, *nitrite oxidizing bacteria, *nitrate reducing bacteria, *sulfates, sulfides, *sulfite, chemoautotrophic bacteria. energy metabolism of autotrophic bacteria.

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CHAPTER I

INTRODUCTION

The aquatic biosphere is subject to chemical diversity as a result of biochemical activities of a vast abundance of physiologically heterogeneous microflora involved in the transformation of sulfur, nitrogen, iron, and phosphorous compounds. Particular attention has been given to microorganisms which play a dominent role in the transformation of sulfur and nitrogen compounds. The potent role of bacteria in sulfur transformations is evident; the toxicity of H_2S and H_2SO_4 to many life forms, the economic importance of sulfur deposits, and the wide variety of oxidation states of sulfur, makes the sulfur cycle of considerable ecological significance. Likewise investigations into the metabolic activities of some of the denitrifying bacteria (i.e. Thiobacillus denitrificans and Micrococcus denitrificans) are also carried out as their role in the nitrogen economy in the aquatic biosphere cannot be underestimated. Moreover, accumulation of nitrites in waters due to the reactions catalyzed by ammonium oxidizers and by nitrate reducing bacteria bears equal importance in that the nitrites so accumulated exert highly toxic effects on the aquatic life. Hence studies were conducted concerning the metabolism of nitrite oxidizing bacteria which remove this toxic compound from the aquatic environments.

It is therefore obvious that a study of the biochemical potentialities of the above mentioned microbial systems was necessary before the effect of strip mining on their presence and their role in the recovery of the streams could be evaluated. With this objective in mind investigations were undertaken to elucidate the mechanisms of metabolic activities of

the microflora involved in the cycling of sulfur and nitrogen compounds so that the experimental findings could be applied to determine the optimal environmental conditions for the establishment of a "normal" sulfur and nitrogen cycle in waters in order that it can be useful to the plants and other biological life systems.

CHAPTER II

Plan for Research-The research plan being followed involves a study, under laboratory conditions, concerning the physiology and metabolism of the organisms commonly found in the polluted waters of the strip-mine areas. The principal microflora constitute the following:

(a) Sulfur oxidizers (Thiobacillus spps)-Investigations are being conducted into the energy metabolism of Thiobacillus neapolitans, Thiobacillus novellus and Thiobacillus denitrificans. The iron oxidizing bacteria have also been included in such studies since these organisms play a dominent role in the oxidation of iron sulfide ores.

(b) Nitrate reducers-Investigations are being carried out on the denitrifying enzymes in Micrococcus denitrificans and Thiobacillus denitrificans. These bacteria reduce nitrates or nitrites to gaseous nitrogen thus causing a severe deficiency of available nitrogen in the biosphere.

(c) Nitrite oxidizers-The toxicity of nitrites can be eliminated by the Nitrobacter spps. in that the latter catalyzes the oxidation of nitrites to nitrates which serve as a readily available nitrogen source for the growth and development of biological life.

(d) Iron oxidizers-Preliminary investigations have been conducted on the metabolism of Ferrobacillus ferrooxidans. The metabolic activities of these organisms also produce acidity in waters and are responsible for rusting of iron materials.

(e) Sulfate reducers-Work has also been initiated on the physiology and metabolic role of Desulfovibrio desulfuricans in the toxic accumulation of sulfides.

Under the proposed program emphasis has been given to the biochemistry of sulfur, iron and nitrogen oxidations and reductions, and, the factors involved in the control of such metabolic activities of the above enumerated flora responsible for water pollution is under investigation. In particular, research is in progress in the following areas:

(i) The electron transport from the donor molecules to oxygen (or an oxyanion such as nitrate or sulfate), and from donor molecule to pyridine nucleotides in the case of chemosynthetic bacteria catalyzing the oxidation and reduction of inorganic sulfur and nitrogen compounds.

(ii) Oxidative phosphorylation occurring concomitantly with electron transport to oxygen or oxyanions.

(iii) A study of inhibitors of the metabolic and electron transport processes.

(iv) Investigations to create optimal environmental conditions for the re-establishment of a "normal" sulfur and nitrogen cycle in waters polluted by strip-mine runoff.

CHAPTER III

EXPERIMENTAL PROCEDURE

The experimental procedures, the equipment used and the methods used for specific analysis in various stages of this study are described in this section.

1. (a) Culture of Nitrobacter agilis: N. agilis (ATCC 9482) was grown in an inorganic liquid medium described previously (1) under forced aeration with 5% CO_2. An active log phase of the culture was obtained with periodic nitrite additions. When the culture was capable of oxidizing 60 mM nitrite within 24 hours for a period of 2 days, the cells were collected in a DeLaval continuous flow centrifuge at 5^{o} and were washed twice with 50 mM Tris-HCl buffer (pH 8.0) by centrifugation at 10,000 xg for 30 minutes in a Sorvall RC-2 refrigerated centrifuge.

   (b) Preparation of cell-free extracts: A 5-g weight of the cell paste was suspended in 15 ml of the sonication medium containing 50 mM Tris-HCl buffer (pH 8.0), 250 mM sucrose, I mM EDTA, and I mM glutathione (reduced). The cell suspension was subjected to sonication at $5^{o}C$ for 20-sec intervals for a total period of 3 min using a Bronson cell disintegrator at maximum output. The broken cell suspension was centrifuged at 10,000 X g for 10 min and the supernatant fraction was further centrifuged at 40,000 X g for 10 min. The resultant supernatant was subjected to centrifugation at 150,000 X g for 60 min and the pellet was suspended in 10 ml of the sonication medium described above. This fraction designated

as P-150,000 was used as the enzyme source in the phosphorylation and electron transport studies.

(c) Measurement of oxidation and coupled phosphorylation: Details of the experimental procedures are given in the legend to the tables and figures. Enzyme fractions containing 2-4 mg protein per ml of reaction mixture, determined according to LOWRY et al. (2), were used in the present experiments. The esterification of ^{32}Pi into ATP was measured in reaction mixtures deproteinized with 5% final concentration of trichloroacetic acid as described previously using a scintillation counter, (3). Oxygen uptake was measured polarographically with a Platinum electrode in identical reaction mixtures used for phosphate esterification, but contained no ^{32}Pi. The difference absorption spectra were obtained using a Cary Model-14 recording spectrophotometer equipped with sensitive slide wire; full deflection 0-0.1 O.D.

(d) ATP-dependent NAD^+ reduction by nitrite in N. agilis: The energy-dependent reduction of pyridine nucleotide by nitrite was measured in a dual split-beam spectrophotometer capable of recording difference absorption changes at two desired wavelengths in the same reaction mixture at the same time. Thus the oxido-reduction changes in cytochrome a1, and c, cytochrome c and flavo-protein, and cytochrome c and NAD^+ could be followed by monitoring the respective wavelength couples, e.g., at 438 and 550 nm, 550 and 450 nm, and 550 and 340 nm.

The reaction was performed in thunberg-type cuvettes of 1 cm light path. The reaction mixture, unless otherwise specified, contained in a total volume of 3.27 ml, cell-free extract containing 7.5 mg

protein, 50 nmoles of cytochrome c (horse heart, Sigma), 5 µmoles of $MgCl_2$, 250 µmoles of Tris-HCL (pH 8.0), 10 µmoles of KNO_2 and 2 µmoles of NAD^+. The treatment cuvette in addition was supplied with 2 µmoles of ATP. The side arm of the cuvettes contained enzyme, Mg^{2+} and cytochrome c. After evacuating and gassing the cuvettes with O_2-free N_2 (repeated 3 times), the contents of the side arm were tipped in, and absorbance changes were recorded at the two desired wavelengths.

2. (a) Culture of Thiobacillus neapolitanus: T. neapolitanus originally isolated by Parker (4) was cultivated autotrophically as described by Vishniac and Santer (5) with slight modifications. The medium contained:

$Na_2S_2O_3 \cdot 5H_2O$	10 gms.
K_2HPO_4	6 gms.
KH_2PO_4	6 gms.
Trace Metal Solution	10.0 ml.
Bromocresol Purple p. 1%	0.4 ml.
Tap water	to 1000 ml.

Composition of Trace Metal Solution:

Ethylenediamine tetra acetic acid	50.0 g.
$ZnSO_4 7H_2O$	22.0 g.
$CaCl_2$	5.54 g.
$MnCl_2\ 4H_2O$	5.06 g.
$FeSO_4\ 7H_2O$	4.99 g.
$(NH_4)_6 Mo_7O_{24}\ 4H_2O$	1.10 g.
$CuSO_4\ 5H_2O$	1.57 g.

$CoCl_2$ $6H_2O$	1.61 g.
$MgSO_4$ $7H_2O$	80.0 g.
NH_4Cl	50.0 g.
Distilled water	to 1000 cc.
Adjusted to pH 6.0 with KOH	

Carboys (5 gallon capacity) contained 15 liters of tap water, trace metal solution and dye. They were fitted with two tubes, one terminating into a gas dispersion fritted glass tube of medium porosity for pushing sterile air and the other acted as an outlet for air. Carboys were sterilized for 50 minutes at 121°C. Solutions of thiosulfate (300 ml. of 50% W/V solution of $Na_2S_2O_3$ $5H_2O$) and phosphate (400 ml. of 20% W/V solution of each of monobasic and dibasic phosphate mixed together) were autoclaved separately for 15 min. at 121°C and aseptically added to the cooled basal medium. The final pH of the medium was 6.6 - 6.8. The medium in the carboy was inoculated with 500 ml of a 24-hour culture of T. neapolitanus grown in a smaller bottle. The phosphate concentration employed for the growth of this organism gave considerably greater buffering ability without affecting the lag period. The cultures were continuously neutralized manually with 2M potassium carbonate, maintaining a pH of about 7.0.

Cultures of T. neapolitanus were maintained by weekly serial transfers into fresh 100 ml. medium in 500 ml. conical flasks. The flasks were incubated on a Gyrotory shaker (New Brunswick Scientific Company) for 24 hours at 30°C and stored in a refrigerator until required.

(b) Collection of cells: The cells were harvested in the log phase at 4°C in a Delaval continuous flow centrifuge (Delaval Separator Company, Poughkeepsie, New York) maintaining the flow rate at

105 ml/min with the aid of a Variable Speed Masterflex pump (Cole Parmer Company, Chicago, Illinois). The harvested cells were washed twice with 0.05 M Tris-HCl buffer (pH 7.5) and suspended in the same buffer to a concentration of 30-40% (wet weight/volume).

(c) Preparation of cell-free extracts: The cells were disrupted by passing through a French pressure cell at 17,000 PSI. The unbroken cells and large fragments were removed by centrifugation at 8000 X g for 20 minutes. For some experiments the cell-free extracts were depleted of endogenous substrate by dialysis for 18 to 20 hours at $4^{o}C$, against 0.05 M Tris-HCl buffer pH 7.5 containing 1.0 mM $MgCl_2$. The cell-free extracts were centrifuged further at 30000 X g for 30 min and the supernatant fraction was designated as 30S. Particulate and soluble fractions were prepared by centrifugation of 30S fraction at 144000 X g for 1 hour. The supernatant fraction thus obtained was designated as S_{144} and the pellet after washing and resuspending in 0.05 M Tris (pH 7.5) was called P_{144}.

(d) Measurement of oxygen uptake: Oxygen uptake was measured polarographically at $30^{o}C$ with a platinum oxygen electrode (Yellow Spring Instrument Company, Inc., Ohio). The reaction mixture in a total volume of 3 ml contained cell-free extracts, 150 µmoles of Tris-HCl (pH 7.5) and thiosulfate as indicated. The reaction was started by adding the substrate.

(e) Assay of energy-dependent reduction of pyridine nucleotides by thiosulfate: The reaction mixture in a total volume of 3.2 ml in a Thunberg-type Beckman cuvette of 1 cm light path, unless otherwise

specified, contained: 0.4 ml enzyme (8000 X g supernatant) containing 15 mg of protein, 125 μmoles of Tris-HCl (pH 7.5), 2 μmoles of NAD^+, 20 μmoles of thiosulfate, 2 μmoles of $MgCl_2$, 3 μmoles of ATP and 165 nmoles of cytochrome c (Sigma Horse Heart Type II). The side arm of the cuvette contained enzyme, Mg^{++}, cyt c and thiosulfate. The control cuvette contained all the components except ATP. The cuvettes were evacuated and the contents of the side arm were tipped in to start the reaction. Absorbance changes due to oxido-reduction of pyridine nucleotides, cytochrome c and flavoprotein were measured at 340, 550, and 450 nm, respectively, with a dual-split-beam spectrophotometer.

(f) Assay of ATP-dependent reduction of pyridine nucleotides by sulfite: The reaction mixture in a total volume of 3.2 ml contained 10 mg of enzyme protein, 125 μmoles of Tris-HCl (pH 7.5), 165 nmoles of cyt c, 2 μmoles of NAD^+, 20 μmoles of AO_3^{2-}, 500 nmoles of EDTA, 2 μmoles of $MgCl_2$ and 3 μmoles of ATP. The side arm of the cuvette contained enzyme, Mg^{++}, cyt. c, sulfite and EDTA. The control cuvette contained all the reagents except ATP. The cuvettes were evacuated and the reaction was started by adding the contents from the side arm. Spectrophotometric measurements were made as described earlier. In these experiments low sulfite concentration was used and EDTA was added to the reaction mixture to minimize the auto-oxidation of sulfite.

(g) Other determinations: The rate of ATP utilized to NAD^+ reduced was obtained by assaying for ADP and AMP in the reaction mixture by the method of Adams (6). Due to the interference caused by

adenylate kinase, present in the cell-free extracts, Pi determination did not prove to be a satisfactory method for ATP measurements.

Thiosulfate cytochrome c reductase was measured spectrophotometrically with mammalian cyt c as the electron acceptor.

3. (a) Bacterial strain: The Ferrobacillus ferroxidans used in this study was isolated from acid strip mine effluents in the Daniel Boone National Forest, McCreary County, Kentucky. The bacterium was obtained in pure culture after isolation of individual colonies.

(b) Isolation of individual colonies: The strain was isolated from individual colonies as follows. A volume of cells from which the precipitated iron had been removed was combined with six volumes of 0.32 mM H_2SO_4 and passed through sterile cellulose acetate filters of 0.47 µm pore size (Millipore Corp., Bedford, Mass.). The filters were then transferred to agar surfaces and incubated for 48 hours at 26°C. The agar contained 0.865 mM $(NH_4)_2SO_4$, 0.495 mM KCI, 2.12 mM K_2HPO_4, 1.52 mM $MgSO_4 \cdot 7H_2O$, and 0.6% (w/v) purified agar (Difco).

(c) Characteristics of the organisms: Organisms isolated from the individual colonies were gram-negative, motile rods measuring 0.6 to 1.0 µm wide and 1.0 to 1.6 µm long in the stained preparation. The organism oxidized ferrous sulfate (130 µmoles of oxygen utilized per min per mg of bacterial protein) but not thiosulfate (less than 1 µmole of oxygen uptake per min per mg of bacterial protein).

(d) Growth of bacteria: Bacteria were grown in 20-liter carboys in a medium containing 34 mM $(NH_4)_2SO_4$, 20 mM KCI, 4.3 mM K_2HPO_4, 3.4 mM

$MgSO_4 \cdot 7H_2O$, 0.092 mM $Ca(NO_3)_2$, and 195 mM $FeSO_4 \cdot 7H_2O$ in 18 liters of demineralized water. The carboys were inoculated with 300 ml of a 48-hr culture and were incubated at 28°C. Cultures were aerated with a mixture of air and CO_2 (3:1, v/v) at a rate of 8 liters per min per 18 liters of medium. Sterile $FeSO_4$ solution was added intermittently as described (7). After 72 hours, the cells were harvested with a De-Laval Gyrotest continuous-flow centrifuge. The cells were then separated from the iron precipitate as described (8).

Bacteria were grown in 600 ml of medium containing 1 mc of $NaH^{14}CO_3$ (New England Nuclear Corp., Boston, Mass.) and 1 mc of $H_3{}^{32}PO_4$ (Tracerlab, Waltham, Mass.). When radioactive bicarbonate was included in the medium, the flask after inoculation was gassed with oxygen for several minutes and then sealed; the $NaH^{14}CO_3$ solution was injected into the medium through a rubber septum. The turnover of the ^{14}C and ^{32}P in the lipids was measured as follows. The bacteria were grown in the presence of the isotopes for 2.3 divisions; the flask was flushed with air, and the $^{14}CO_2$ was trapped in $Ba(OH)_2$. The suspension was centrifuged at 12,000 X g for 20 min at 28°C, and the cells were then resuspended in 1,500 ml of nonradioactive medium. Cell suspensions containing 2.5 g of bacterial protein were inoculated into two flasks; in one flask the medium was pH 1.5 and in the other flask the pH was 3.5. The pH was maintained at these values by the addition of 5N NaOH at 20-min intervals. Samples (200 ml) were withdrawn at 3-hour intervals into an equal volume of ice; the bacteria were harvested

by centrifugation and the lipids were extracted. The lipids were then deacylated and separated chromatographically and the radioactivity was determined.

(e) Lipid extraction: The bacteria were withdrawn into an equal volume of ice, centrifuged, resuspended in 50 mM phosphate buffer (pH 7.6), and extracted by Bligh and Dyer procedure (9, 10). This extraction procedure was reproducible.

(f) Mild alkaline methanolysis: Glycerol phosphate esters were derived from diacyl phospholipids by mild alkaline methanolysis at $0^{o}C$ as described by White (11). The reaction was complete in 2.5 hours. The alkali was neutralized with the weakly acidic cation-exchange resin, Biorex 70 (Bio-Rad Corp., Richmond, Calif.), as described by White (11). The abbreviations used for the glycerol phosphate esters derived from the phospholipids are: glycerolphosphorylethanolamine (GPE) from phosphatidyl ethanolamine (PE), glycerolphosphorylmonomethylethanolamine (GPME) from phosphatidyl monomethylethanolamine (PME), glycerolphosphoryldimethylethanolamine (GPDME) from phosphatidyldimethylethanolamine (PDME), glycerolphosphorylcholine (GPC) from phosphatidyl glycerol (PG) diglycerolphosphoryl glycerol (GPGPG) from cardiolipin (CL), and glycerolphosphorylserine (GPS) from phosphatidyl serine (PS).

(g) Acid hydrolysis: The glycerol phosphate esters derived from the diacyl phospholipids were hydrolyzed in 2 M HCl at $100^{o}C$ for 1 hour (14). This reaction quantitatively splits the nitrogen-containing esters into α-glycerolphosphate (αGP) and ethanolamine (E)-hydrochloride, monomethylethanolamine (ME)-hydrochloride, dimethylethanolamine (DME)-hydrochloride, or choline chloride (CH), as described

by White and Tucker (12). HCl was removed in a stream of nitrogen.

(h) Paper chromatography: Diacyl phospholipids were separated on silica gel-impregnated paper (Whatman SG-81) with solvents of chloroform-methanoly-dissobutyl ketone-acetic acid-water (23:10:45:25:4, v/v) in the first dimension and chloroform-methanol-diisobutyl ketone-pyridine 0.5 M ammonium acetate buffer, pH 10.4 (30:17.5:25:35:6, v/v), in the second dimension, as described by Wuthier (13). The lipids were detected by radioautography and by sprays for amines (14), phosphate, and amino nitrogen (15).

Glycerol phosphate esters derived by deacylation of the phospholipid were separated on aminocellulose paper (Whatman AE-81) with solvents of 3 M formic acid containing 0.4% pyridine in the first dimension and 1.15 M ammonium acetate containing 11.8 mM ethylenediaminetetracetic acid (EDTA) made to pH 5.0 with acetic acid and diluted 3:7 (v/v) with 95% ethanolic 0.26 M ammonium hydroxide in the second dimension (11).

The hydrochlorides of E, ME, DME, and CH were separated on acid-washed paper no. 589 (Schleicher and Schuell, Keene, N. H.) which had been soaked in 1 M KCl and dried, by descending chromatography with a solvent of phenol-n-butyl alcohol-23 N formic acid-water (100 g, 100 ml, 6 ml, 20 ml) which had been saturated with KCl. This system is slightly modified from that reported by Bremer et al. Hydrochlorides were detected with the amine spray (14) or by radioautography.

(i) Thin-layer chromatography: A method for separation of glycerol phosphate esters developed by R. L. Lester involved cellulose thin-layer plates (Eastman Chromagrams 6064, Rochester, N.Y.) and solvents

of 3.8 mM EDTA and 0.7 M ammonium bicarbonate in 90 mM ammonium hydroxide containing 57% (v/v) ethyl alcohol in the first dimension and isobutyric acid-water-concentrated ammonium hydroxide (66:33:1, (v/v) in the second dimension. The lipids were detected by radioautography. The spots corresponding to the radioactivity were cut out, and the esters were deluted in three 2-ml portions of water. The elution was quantitative. The quinone and phospholipids were separated on Silica Gel G thin-layer plates (19). The lipids were detected and eluted from the silica gel as described by White and Frerman (10).

(j) Column chromatography: Neutral lipids and phospholipids were separated by silicic acid chromatography (10). Glycerolphosphate esters derived from the phospholipids were eluted from columns (3 mm by 81 cm) of Dowex 1-8 X (200-400 mesh) in the formate form with the ammonium formate-sodium borate gradient described previously (10) or with 20 mM ammonium formate, pH 9.0.

(k) Analysis: The bacterial protein and the lipid phosphate were determined as described by White and Frerman (10). The bacterial and lipid extracts were saponified and the fatty acids were recovered as described by White (17). The fatty acids were determined colorimetrically (18) with palmitic acid as standard.

(l) Measurement of radioactivity: Samples were assayed for radioactivity in a Packard scintillation spectrophotometer model 2311. The ^{32}P and ^{14}C in the glycerol phosphate esters were determined on paper discs (1.2 to 2.0 cm in diameter) in a scintillation fluid of 9.28 mM 2,5[2 5-terbutyl benzoxazol)]-thiophene in toluene. Where ^{14}C and

^{32}P were determined simultaneously, the overlap of the ^{32}P into the ^{14}C channels was 6%, and the efficiency of counting was 55% for the ^{14}C and 88% for the ^{32}P. Radioautograms were made with Kodak no-screen X-ray film as described previously (11).

CHAPTER IV

RESULTS AND DISCUSSION

During the first year period attention was focused on the energy metabolism of Thiobacillus, Ferrobucillus, Nitrobacter, and Thiobacillus neapolitanus. The results obtained concerning the bioenergetics involved in the chemosynthetic electron transport and coupled generation of energy and reducing power are presented as follows:

I. BIOENERGETICS OF CHEMOSYNTHESES IN NITROBACTER

A. Mechanism of Oxidative Phosphorylation. The process of chemosynthesis in Nitrobacter is driven at the expense of energy and reducing power both of which are tightly coupled with CO_2 reduction and are generated in the process of nitrite oxidation catalyzed by the chemoautotroph. The mechanism of nitrite oxidation by Nitrobacter has been reported by Aleem (19). The data in Table 1 show that the oxidation of nitrite by Nitrobacter particles was coupled to ATP synthesis; oxidation of one nitrite molecule yielded one ATP. The phosphorylation coupled to nitrite oxidation was unaffected by rotenone or amytal and antimycin A. The NADH - trapping system (pyruvate-lactate dehydrogenase) did not inhibit the oxidation of nitrite nor the coupled phosphate esterification. These results thus suggest that the entry of nitrite into the electron transport chain occurs beyond the flavin or cytochrome b region toward the O_2 side. Based on spectrophoto-metric observations and the use of uncouplers and inhibitors of the energy generations and energy transfer reactions within the electron transport chain it was established that the entry of nitrite is effected at the cytochrome a - level and that the electron transport from nitrite to molecular oxygen involves cytochromes

TABLE 1

Phosphorylation Coupled to Nitrite Oxidation by Nitrobacter Electron Transport Particles

Reaction mixture (1.3 ml) contained 4.0 mg of enzyme protein, 35 μmoles of Tris-HCl (pH 8.0), 1 mg of hexokinase from yeast (Sigma Chemical Co.,) 17 μmoles of glucose, 7 μmoles of $MgCl_2$, 7 μmoles of NaF, 0.2 μmoles of ADP, 4.6 μmoles of potassium phosphate (pH 8.0), $2.5 \cdot 10^5$ counts per min ^{32}Pi and additions as indicated. The enzyme was preincubated for 5 min with inhibitors before addition of nitrite where indicated. Reaction was stopped with 5% final concentration of trichloroacetic acid after 2 min.

Additions	Total ^{32}Pi uptake (counts/min)	Total ATP formed (μmole)	Total O_2 uptake (μatom)	P/O
None	520	0.01	0.00	0.00
NO_2^- (1 μmole)	13050	0.24	0.30	0.80
NO_2^- + antimycin A (10 μg)	9783	0.18	0.21	0.86
NO_2^- + pyruvate (2 μmoles) + lactate dehydrogenase (50 μg)	13674	0.27	0.30	0.90
NO_2^- + Rotenone (250 μM)	8696	0.16	0.18	0.90

of *a*, and a_3- type.

The data presented in Table 2 shows that with ascorbate as the electron donor, the *Nitrobacter* electron transport particles could yield a p/o ratio of 0.6 indicating only one energy coupling site between cytochrome *c* and molecular oxygen. It may also be seen (Table 2) that the same electron transport particles which catalyzed nitrite oxidation and coupled phosphorylation involving only the terminal part of the electron transport chain could catalyze the oxidation of NADH and coupled phosphate esterification involving a complete electron transport sequence mediated by the flavoproteins and cytochromes of *b*, *c*, and a_3 -type. The oxidation of NADH yielded p/o ratios of about 2.0 and the phosphorylation was found to be sensitive to amytal, rotenone, antimycin A, and 2-n-heptyl 4-hydroxyquinoline-N-oxide (HOQNO). A cross-over point was observed between the flavoproteins and the cytochrome *c* when the electron transport from NADH was blocked by either rotenone or antimycin A. Moreover, the NADH oxidase was inhibited by cyanide. Thus it appears that all the conventional sites of energy conservation were functional when NADH served as the electron donor.

The experimental data thus indicated that:

a) Nitrite oxidation by *Nitrobacter* is mediated by cytochrome *a*, and cytochrome oxidase components thereby generating ATP only in the terminal region of the electron transport chain.

b) With Nitrite as the electron donor, the coupled ATP synthesis is independent of the participation of the pyridine nucleotides or the flavoproteins.

c) Oxidation of exogenously added NADH and the coupled phosphorylation is mediated by Flavoproteins and cytochrome systems.

TABLE 2

Phosphorylation Coupled to the Oxidation of Ascorbate and NADH by Nitrobacter Electron Transport Particles

Experimental conditions were similar as described in Table 1, except the reaction contained $4.4 \cdot 10^5$ counts/min ^{32}Pi. Additions were made as indicated and the reaction was allowed to proceed for 5 min.

Additions	Total ^{32}Pi uptake (counts/min)	Total ATP formed (µmole)	Total O_2 uptake (µatom)	P/O
None	428	0.004	0.00	0.00
+ Ascorbate (5 µmoles) + TMPD (50µM)	43875	0.500	0.90	0.55
+NADH (1µmole)	54300	0.58	0.32	1.8]
+NADH +Antimycin A (10µg)	26214	0.28	0.24	1.16
+ NADH + NOQNO (10µg)	20597	0.20	0.22	0.90
+ NADH + Rotenone (250µM)	5617	0.06	0.10	0.60
+NADH + Pyruvate (2µmoles) + lactate dehydrogenase (50µg)	514	0.005	0.00	0.00

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+ NADH + Rotenone (250µM)	5617	0.06	0.10	0.60
+NADH + Pyruvate (2µmoles) + lactate dehydrogenase (50µg)	514	0.005	0.00	0.00

B. Energy-linked Reverse Electron Flow: Nitrite oxidation by Nitrobacter should not only yield energy but it should also be coupled to the generation of reducing power. The reducing equivalents from nitrite, therefore, should share a pathway of electron transport to molecular oxygen yielding a coupled synthesis of ATP as well as a pathway of energy dependent electron transfer to pyridine nucleotides since the latter are involved in the reduction and assimilation of CO_2 for the synthesis of cellular components. The pathway of electron transport from nitrite to oxygen mediated by cytochrome c and cytochrome a, originally reported by Aleem and Nason (20) were later modified by Aleem (19) in that nitrite oxidation involved the participation of cytochrome a, and a_3-like components and the reduction of cytochrome c by nitrite was energy-dependent. Similar results were reported by Kiesow (21) who in addition observed that the reduction of cytochrome c by cytochrome a, was ATP-dependent and that this reaction was inhibited by dibromophenol.

Sewell and Aleem (22) observed that cell-free extracts from Nitrobacter catalyzed an energy-dependent reduction of NAD^+ by nitrite and this process could be driven either at the expense of added ATP or by the energy generated from nitrite oxidation. Fig. 1 shows the carriers involved in the energy-linked transfer of electrons from nitrite to NAD^+. It may be seen that concomitant to the ATP-dependent reduction of cytochrome c by nitrite there occurred oxidation of cytochrome a, (as indicated by the decrease in absorption at 438 nm). The energy-linked oxidation of ferrocytochrome c also resulted in the initial reduction of flavoprotein system (decrease in absorption at 450 nm), and its subsequent oxidation caused the coupled reduction of exogenously added NAD^+ (increase in absorption at 340 nm).

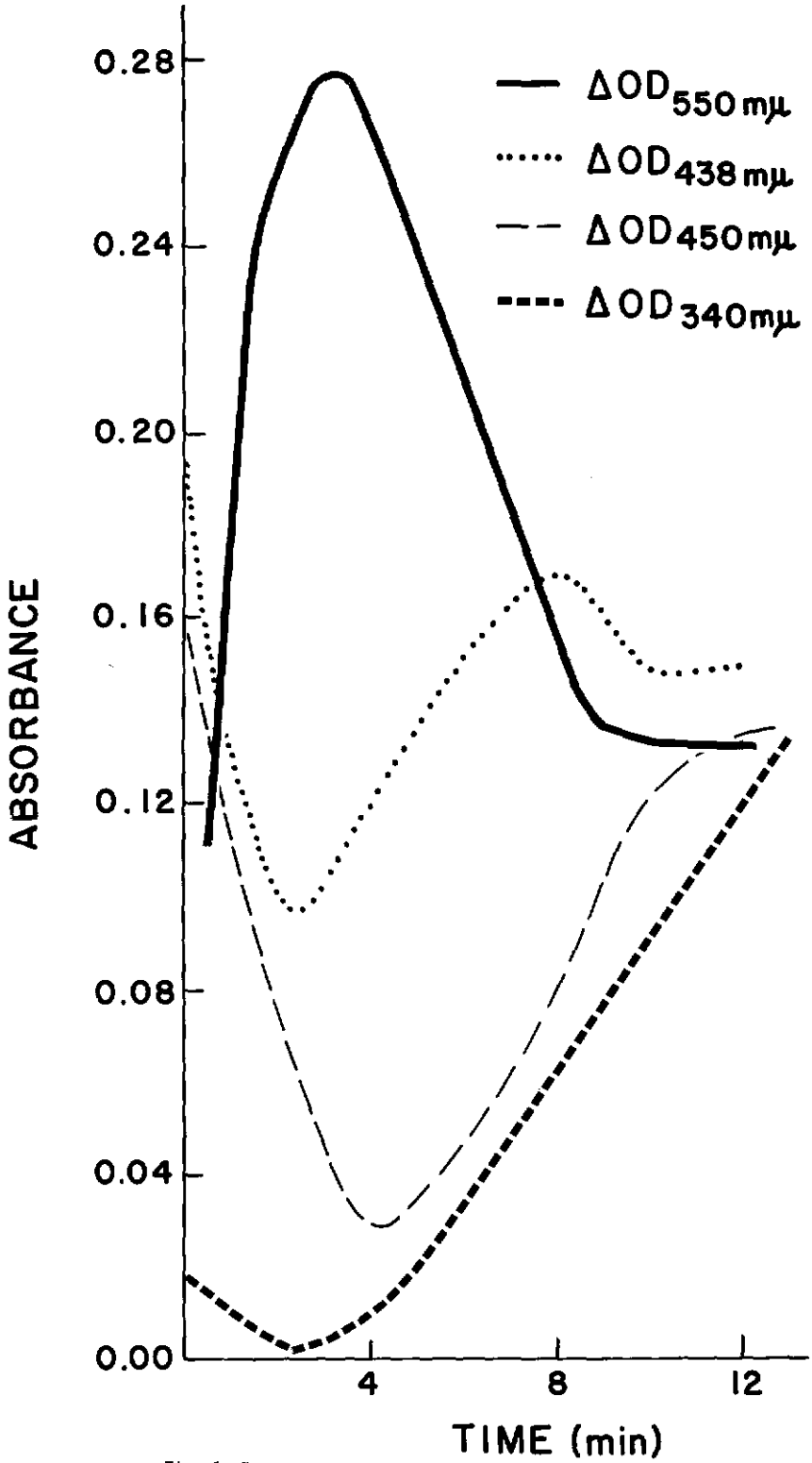

Fig. 1 Pathways of ATP-dependent reversal of electron transfer from NO_2^- to NAD^+. The reaction mixture in a total volume of 3.1 ml contained Nitrobacter cell-free extract containing 7.5 mg of enzyme protein, 250 μ moles of Tris-HCl (pH 8.0), 2 μ moles of NAD^+, 10 μ moles of NO_2^-, 5 μ moles of Mg^{2+}, 2 μ moles of ATP, and 2.0 mg of cytochrome c (Sigma, horse heart, Type II). The reaction was carried out in thunberq-type cuvettes of 1 cm light path. The side arm of the cuvette contained enzyme, ATP, Mg^{2+}, and cytochrome c. The control cuvette contained all the components except ATP. The cuvettes were evacuated and the contents of the side arm were tipped in to start the reaction. Absorption changes corresponding to the redox states of cytochrome a_1, cytochrome c, FMN and NAD^+ were measured at 438, 550, 450 and 340 nm respectively in a dual wavelength double-beam spectrophotometer.

These results thus clearly show that the reduction of cytochrome c by nitrite was an energy dependent reaction which involved reversal of electron transfer from cytochrome a_1. The subsequent energy-dependent reduction of the flavoproteins and pyridine nucleotides occurred concomitantly with the oxidation of cytochrome c.

The data concerning the stoichiometry of ATP-driven NAD^+ reduction by nitrite is presented in Table 3. It may be seen that between 4-5 ATP equivalents are utilized per equivalent of NAD^+ reduced. These observations are in harmony with the calculated energetics of the overall reverse electron flow process which involves a free energy gap of some 35 Kcal. When ATP was limiting, the energy-linked reduction of 1 mole of NAD^+ by nitrite required the concomitant oxidation of 2 molecules of cytochrome c. However, when the process was driven by an optimal ATP concentration (0.7 mM), the rate of NAD^+ reduction was about 1.5 fold compared to the rate of cytochrome c. oxidation.

Presented in Table 4 is the effect of inhibitors on the ATP-dependent reverse electron flow from nitrite to oxygen. The process of energy-linked reversal of electron transfer in Nitrobacter was markedly sensitive to the inhibitors of the flavoprotein systems as well as to antimycin A or HOQNO. In addition, cyanide was observed to be a potent inhibitor. The uncouplers of oxidative phosphorylation caused a strong inhibition of the ATP-linked reverse electron transfer. This process was also inhibited by oligomycin.

Since the reversal of electron transfer from nitrite to NAD^+ is driven by ATP from the level of cytochrome a_1 in Nitrobacter, the process has to be driven against a thermodynamic gradient of some 38 Kcal for which 3-4 nitrite molecules must be oxidized to yield a coupled synthesis of required

TABLE 3

Stoichiometry of ATP-Dependent Reduction of NAD^+

Experimental conditions were the same as in Fig. 1 except that various ATP levels were employed as indicated in the table. 'Net ATP used' corresponds to the P_i released with respect to the presence and absence of added $NAD.^+$

Total ATP employed (µmoles)	Net ATP used (µmoles)	Net NAD^+ reduced (µmoles)	Ratio ATP/NADH
1	0.670	0.169	4.0
2	1.515	0.283	5.3
3	2.370	0.414	5.7

TABLE 4

Effect of inhibitors on the ATP-Dependent Reverse Electron Flow from NO_2^- to NAD^+

The enzyme preparation was preincubated for 3 min. with various inhibitors. The experimental conditions were similar to those described in Fig. 1 In the absence of added inhibitor, 46 nmoles of cytochrome c. were oxidized with the concomitant reduction of 43 nmoles of NAD^+.

Inhibitor	Concn.	Inhibition(%)	
		Cytochrome c oxidized	NAD+ reduced
Atabrine	100μM	77	77
Rotenone	10μM	45	34
Thenoyltrifluoroacetone	1.7mM	64	71
Amytal	2mM	40	41
Antimycin A	1.5μg/mg protein	64	63
HQNO	5.3μg/mg protein	67	68
CN^-	0.3 mM	77	76
	0.7 mM	90	97
CCCP	17μM	55	82
Pentachlorophenol	20μM	100	100
2,4-Dibromophenol	10μM	100	97
2,4-Dinitrophenol	33μM	100	95
Oligomycin	0.8μg/mg protein	43	73
	2μg/mg protein	61	81

ATP molecules to bridge this gap. Further work is in progress to establish the energetics of the reverse electron transfer pathways in Nitrobacter.

2. OXIDATIVE PHOSPHORYLATION AND ENERGY-LINKED REACTIONS IN THIOBACILLUS NEAPOLITANUS.

A. Electron Transport and Coupled Phosphorylation: Adenosine triphosphate (ATP) and reduced pyridine nucleotides (NADH) are the driving forces for the carbon reduction cycle and other cellular biosynthetic reactions in Chemosynthetic bacteria. It was previously shown by Aleem and Huang (23) that the cell-free extracts of the chemoautotroph T. neopolitanus required ATP and NADH for the active reduction of carbon dioxide. Aleem (24) has also reported earlier that the generation of reduced pyridine nucleotides by thiosulfate in this organism is an energy-linked process which is driven by ATP. Hempfling and Vishniac (25) were unable to obtain phosphorylation coupled to the oxidation of thiosulfate in T. neopolitanus. However, investigations by Hempfling (26) concerning the molar growth yields of T. neopolitanus indicate that the organism produces more ATP than is accounted for by the substrate-level phosphorylation alone. The results in Table 5 show that thiosulfate oxidation by the T. neopolitanus cell-free extracts is coupled to the generation of ATP; P/O ratios ranged from 0.4 to 0.8. The phosphate esterification coupled to thiosulfate oxidation was markedly sensitive to 2,4-dinitro-phenol (DNP). It was also observed that the pathway of electron transfer from thiosulfate to molecular oxygen in this organism is mediated by cytochromes of c types and cytochrome oxidase components of a_3 and o-type. Although cytochrome b was reduced in intact cells or cell-free extracts in the presence of $S_2O_3^{=}$, neither its oxidation nor

TABLE 5

Phosphorylation coupled to thiosulfate oxidation catalyzed by *T. neapolitanus* cell-free extracts.

Exp. I, cells broken by Mini-Mill, 3 mg enzyme protein used. Exp. II, cells broken by Omni mixer, 2 mg enzyme protein used. Complete reaction mixture in a total volume of 3.0 ml contained cell-free supernatent fraction 10,000xg, 10 µmoles $MgCl_2$, 6 µmoles K_2HPO_4, 250 BSA, 1 µmole ADP, 40 µmoles glucose, 0.5 mg hexokinase, 20 µmoles KF, 6 µmoles $Na_2S_2O_3$, and 300 µmoles Tris-HCl (pH 7.0). Reaction vessel was shaken at 28° and the experiment was terminated after 30 min. Oxygen uptake was measured polarographically in identical reaction mixtures.

Conditions	ATP formed µmoles	O_2 consumed µatoms	P/O
Exp. I Complete	0.873	2.16	0.40
" minus BSA	0.00	3.30	0.00
" minus $S_2O_3^=$	0.00	0.00	0.00
Exp. II Complete	1.35	1.62	0.83
" + 10^{-4}M DNP	0.24	1.50	0.16
" minus $S_2O_3^=$	0.00	0.00	0.00

the electron transport was inhibited by antimycin A. Moreover, reduction of flavins required ATP. It therefore, appears that thiosulfate enters at the cytochrome c-level and thus the observed phosphorylation occurs in the cytochrome oxidase regions of the electron transport chain of T. neapolitanus.

B. Pyridine Nucleotide Reduction by Thiosulfate in T. neapolitanus. It was observed that cell-free extracts from T. neapolitanus catalyzed the reduction of NAD^+ or $NADP^+$ by thiosulfate in the presence of added ATP. The rate of ATP-dependent $NADP^+$ reduction by thiosulfate was much faster compared to that of NAD^+ reduction.

C. Carriers involved in the energy-linked transfer of electrons from $S_2O_3^=$ to NAD^+. The reduction of NAD^+ by thiosulfate involves the participation of the electron transport chain at the level of cytochrome c. The data in Fig. 2 show the thiosulfate-linked reduction of exogenously added flavins was ATP dependent. Both FMN or FAD could be reduced by thiosulfate in the presence of ATP. Added FMN, however, was a more effective electron acceptor from thiosulfate. The observed ATP-dependent FMN reduction must involve an energy-linked reversal of electron transfer from cytochrome c. It may be seen that addition of ATP caused the initial reduction of FMN by thiosulfate and the subsequent oxidation of FMN was accompanied with a concomitant reduction of NAD^+. The stoichiometry of 1:1 between FMN oxidized and NAD^+ reduced was not observed because FMN is an intermediary electron carrier. These findings suggest that the reduction of NAD^+ by ferrocytochrome c probably involves a reversal of the sequence of reactions of a normal respiratory chain for which ATP served as the driving force.

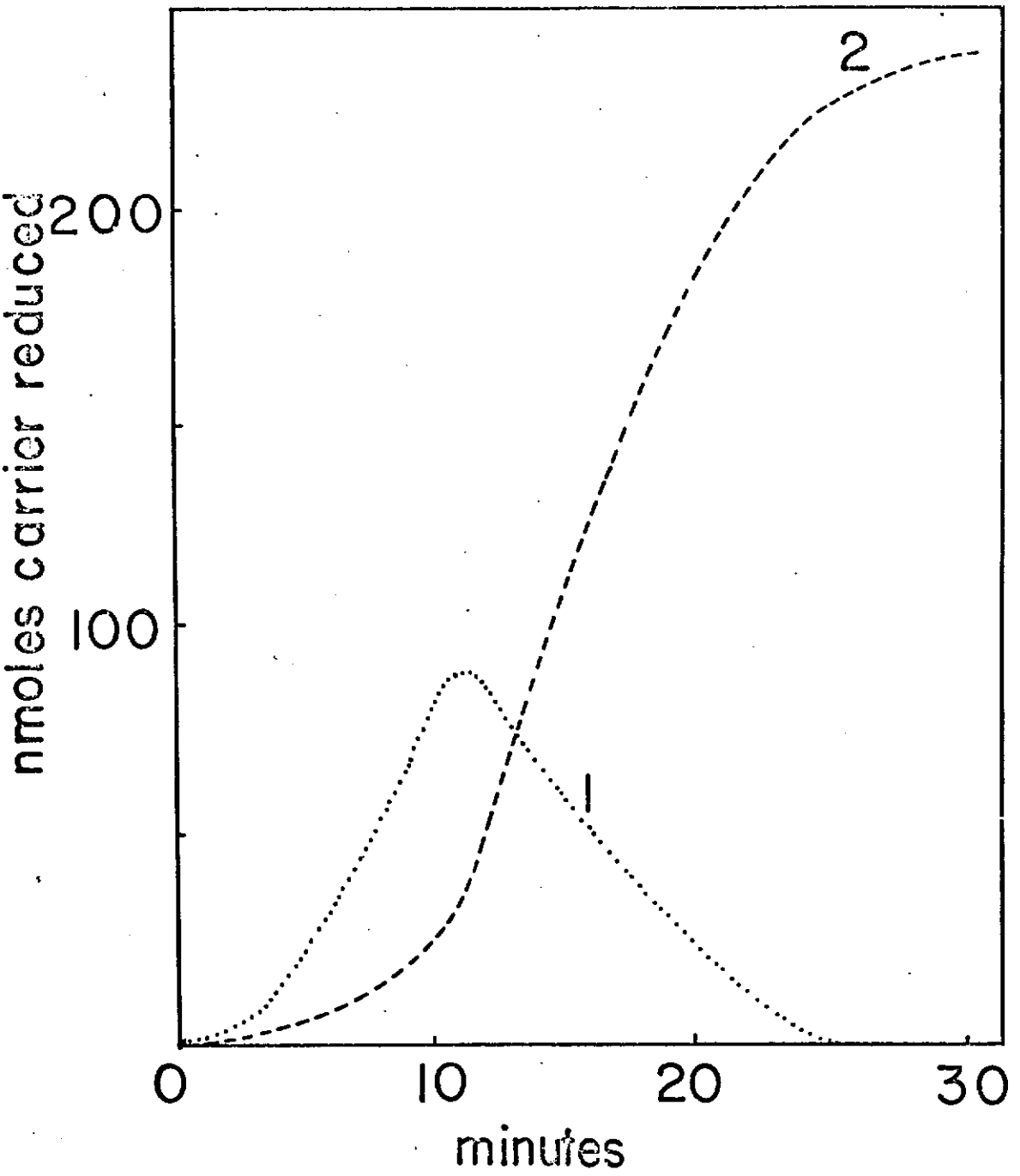

Fig. 2 Carrier involved in the energy-linked transfer of electrons from $S_2O_3^{2-}$ to NAD^+. The experimental conditions were similar to those described in Materials and Methods except that 0.07 mM FMN was included in the reaction mixture. FMN, trace 1; NAD^+, trace 2.

D. ATP/NADH quotient: Our preliminary experiments showed that both ADP and AMP were formed in the reaction mixture as a result of hydrolysis of ATP for NAD^+ reduction. Extracts from T. neapolitanus contained an active adenylate kinase (Keq at 27^o = 1.001 calculated from the free energy valves of Burton (27) capable of catalyzing the formation of 3.6 nmoles of ATP/min/5 mg protein when ADP concentration was 100 umolar. The ADP formed as a result of hydrolysis of ATP may therefore, be converted to ATP and AMP; ATP being recycled for NAD^+ reduction. The total ATP utilized for NAD^+ reduction would thus be equivalent to the sum of AMP and ADP formed. As is shown in Table 6 the ratio of ATP utilized to NAD^+ reduced was found to be 2.5 with thiosulfate as the electron donor. These results thus suggest that each ATP must be utilized by the enzyme system in such a way that it bridges a potential gap of +0.21V involving at least two thermodynamic barriers, e.g.,the reduction of flavin by cytochrome c and the reduction of NAD^+ by flavin.

E. Effect of inhibitors on the energy-linked reversal of electron transfer:

The energy-dependent nature of NAD^+ and FMN reduction was confirmed by the inhibitory action of various uncouplers of oxidative phosphorylation. The energy-linked reversal of electron transfer from thiosulfate or ferrocytochrome c to FMN as well as to NAD^+ was markedly sensitive to DNP, CCCP, and oligomycin (Table 7). Moreover, inhibitors of the flavoprotein system such as rotenone and amytal caused the inhibition of both NAD^+, the reduction of FMN was markedly sensitive to antimycin A and HOQNO. It is also found that the inhibition of NAD^+ reduction by rotenone and amytal is overcome by the addition of menadione which, however, failed to relieve the inhibition caused by antimycin A or HOQNO of the energy-dependent NAD^+ reduction. These observations clearly indicate that

TABLE 6

Stiochiometry of ATP-Dependent reduction of NAD^+

Experimental conditions were the same as described in Materials and Methods except that the ATP levels were varied as indicated in the Table.

ATP additions	Net NAD^+ reduced in 50 min.	Net ATP used in 50 min.	ATP/NADH
(mole)	(mole)	(mole)	
1.0	0.131	0.33	2.56
3.0	0.236	0.61	2.58
5.0	0.30	0.76	2.55

TABLE 7

Effect of inhibitors on the ATP-Dependent reduction of flavin and pyridine nucleotide by thiosulfate

The enzyme preparations were preincubated for three minutes with various inhibitors. The experimental conditions for the reduction of NAD^+ and FMN were similar to those described in Materials and Methods except that in the latter case cyt. c was omi-ted and NAD^+ was replaced by 200 nmoles of FMN. The FMN or NAD^+ were reduced at a rate of 5 nmoles/min in the absence of inhibitors.

Inhibitor	Concn. (mM)	Inhibition (%) FMN reduction	Inhibition (%) NAD reduction
Rotenone	0.1	80	70
Amytal	2.0	100	90
TTFA	0.1	100	100
Atabrine	0.3	70	35
Antimycin A	3 µg/mg prot.	95	40
HQNO	3 µg/mg prot.	95	88
DNP	0.1	88	90
CCCP	0.02	60	80
Oligomycin	2.0	88	95
Cyanide	1.0	9	43

menadione (vit. K_3) constitutes a bypass of electron transfer between cytochrome b and NAD^+.

The results reported thus suggest that the energy-dependent reverse electron flow from thiosulfate to NAD^+ proceeds via the scheme proposed in Fig. 3 and is in harmony with the effect of inhibitors which act specifically at various segments of the respiratory chain. It is of interest to mention that the energy transfer reactions in T. neapolitanus appear to involve the reversal of a sequence of reactions of the oxidative phosphorylation although the chains involved in the electron transport from NADH to O_2 and from ferrocytochrome c to NAD^+ might be different and spatially separated.

F. Pyridine nucleotide reduction by sulfite in T. neapolitanus. Charles and Suzuki (28) and Peck (29) indicated that Thiobacilli oxidize thiosulfate involving sulfite as a probable intermediate. It has been reported that the oxidation of sulfite to sulfate proceeds via the formation of adenosine-5-phosphosulfate (APS) in Thiobacillus thioparus (29). Sulfite oxidation in T. novellus has, however, been shown to proceed through the cytochrome systems and was coupled to ATP generations by oxidative phosphorylation (28,30). The energetics and pathways of the energy-dependent reversal of electron flow from sulfite to NAD^+ was studied in T. neapolitanus.

G. Reduction of NAD^+, FMN and cytochrome c by sulfite in the presence of ATP: Although the cell-free extracts could catalyze the enzymatic reduction of cytochrome c by thiosulfate, added sulfite failed to reduce either endogenous or added mammalian cytochrome c. Similar results were reported by Hampfling et al. (31). The data in Fig. 4 present the pattern of energy-linked reduction of added FMN and cytochrome c by sulfite. Addition of ATP caused the initial reduciton of flavin as well as cytochrome c. The

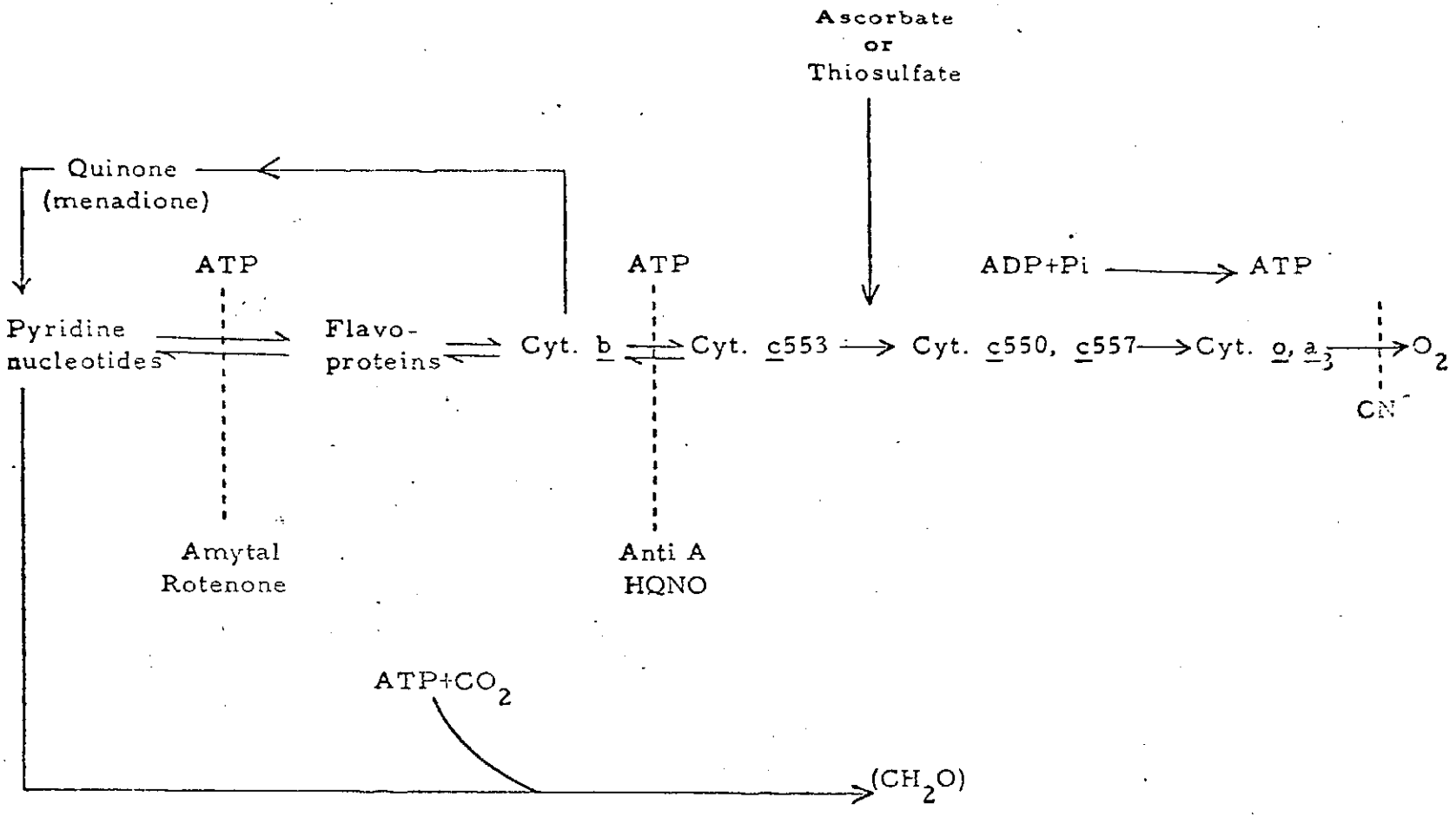

Figure 3. Proposed mechanism of thiosulfate-linked electron and energy transfer pathways in T. neapolitanus.

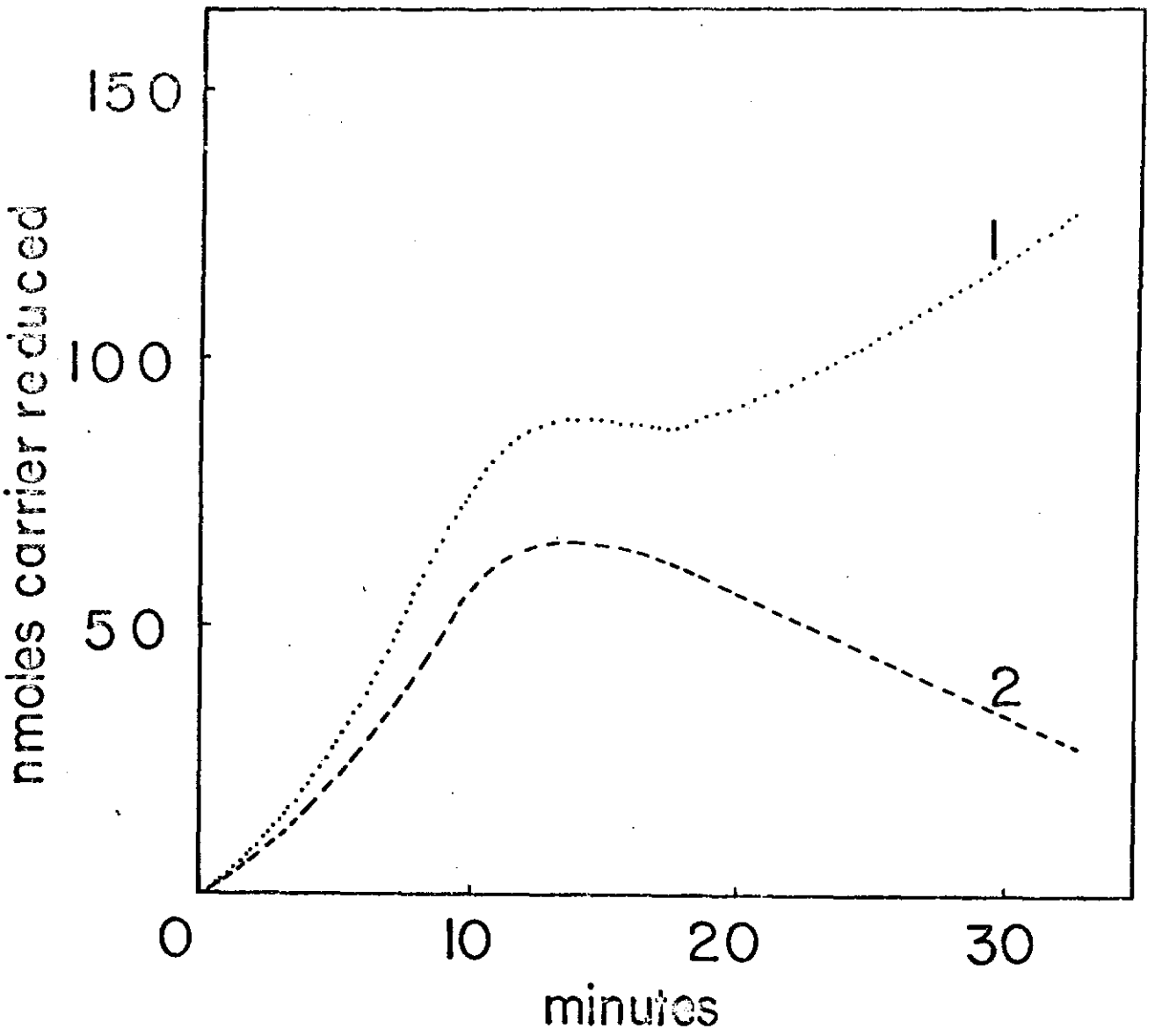

Fig. 4 ATP-dependent reduction of FMN and cytochrome c by sulfite. The experimental conditons were the same as described in Materials and Methods for the ATP-dependent NAD^+ reduction by sulfite except that NAD^+ was omitted and 200 n moles of FMN were incorporated in the reaction mixture. Oxidation-reduction of FMN and cytochrome c was followed by recording absorption changes at 450 and 550 nm, respectively, in a duel-split-beam spectrophotometer: FMN, trace 1; cytochrome c, trace 2.

reaction leveled off after 87 nmoles of FMN and 65 nmoles of cytochrome c were reduced; FMN reduction, however, resumed again and was coupled to the subsequent oxidation of cytochrome c involving an energy-dependent reverse electron flow. The observed initial energy-dependent reduction of flavin could not be due to reversal of electrons from cytochrome c as the reduction of cytochrome c occurred simultaneously along with the reduction of FMN. This indicated that sulfite was capable of reducing flavin directly under the influence of ATP and that the reduction of cytochrome c occurred subsequently due to a spontaneous flow of electrons from reduced FMN. The second phase of energy-dependent FMN reduction was accompanied with the subsequent oxidation of cytochrome c and is apparently linked to the energy-dependent reversal of electrons from cytochrome c.

Upon incorporation of NAD^+ in the reaction mixture, the energy-linked redox behavior of FMN and cytochrome c remained unchanged but no reduction of NAD^+ was observed until the energy-dependent reversal of electrons from cytochrome c to FMN was initiated (Figure 5). The reduction of NAD^+ under these conditions (e.g., in the presence of 2.7 mM ATP) was relatively slow because added FMN competed with NAD^+ for electrons.

H. Effect of inhibitors on the ATP-dependent reduction of cytochrome c, FMN and NAD^+ by sulfite

Experiments were designed to study the effect of some of the inhibitors upon the ATP-dependent reduction of FMN and cytochrome c by sulfite (Table 8). The energy-dependent nature of FMN reduction by sulfite

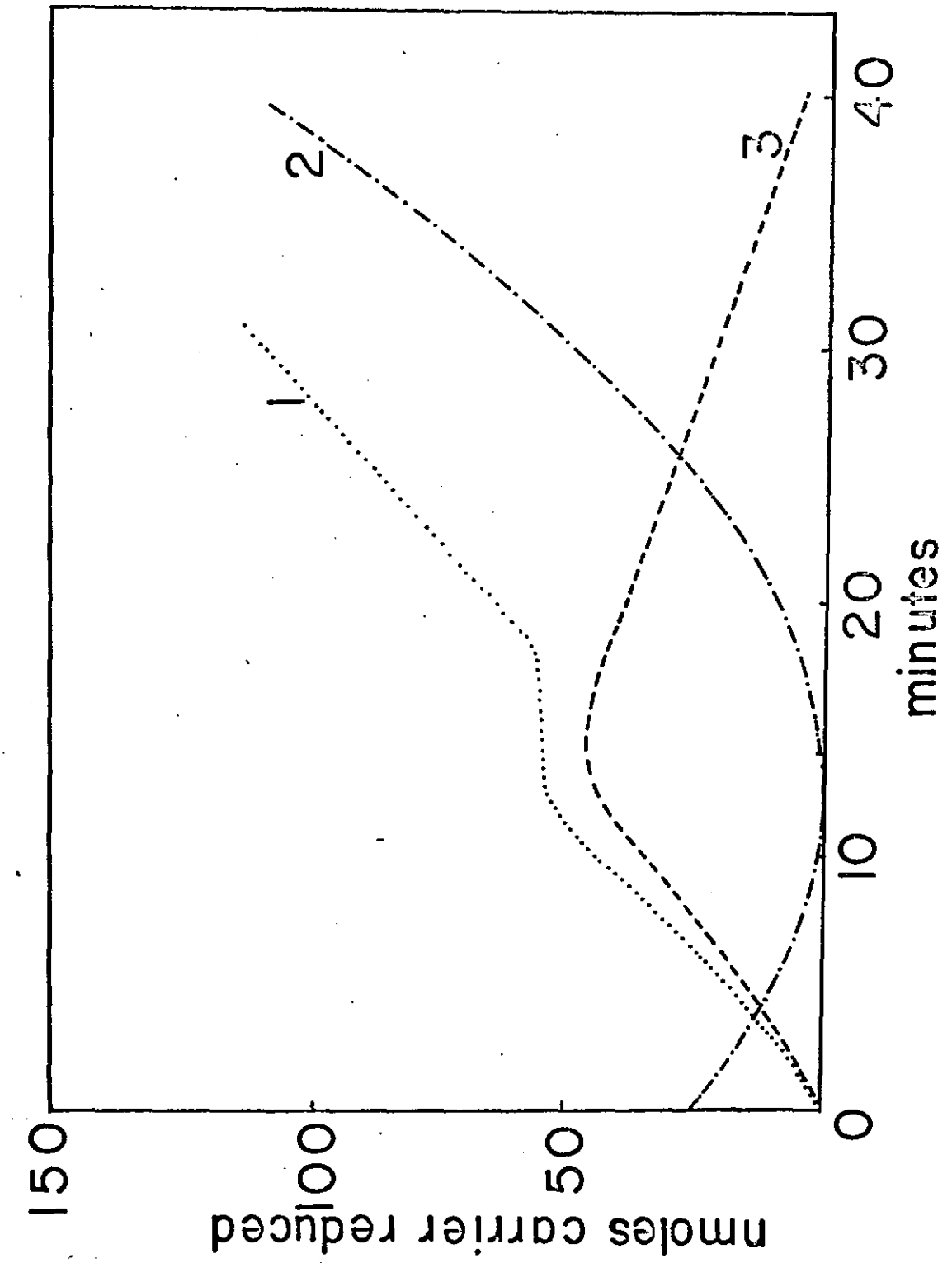

Fig. 5 Reduction of NAD^+, FMN and cytochrome c by sulfite in the presence of ATP. The experimental conditons were the same as described in Materials and Methods for the ATP-dependent NAD^+ reduction by sulfite except that 200 n moles of FMN were added in the reaction mixture and 8 μ moles of ATP were used: FMN, trace 1; cytochrome c, trace 3; NAD, trace 2.

TABLE 8

Effect of inhibitors on the ATP-Dependent reduction of FMN by sulfite and subsequent flow of electrons from flavin to cytochrome c.

The enzyme preparations were preincubated for 3 min. with various inhibitors. The experimental conditions were the same as described in Materials and Methods except that NAD^+ was omitted and 200 nmoles of FMN were incorporated in the reaction mixture. When no inhibitor was present, 32 nmoles of FMN and 29 nmoles of cyt. c were reduced in 5 minutes.

Inhibitor	Conc. (mM)	FMN Reduced	Cyt. c Reduced
Amytal	1.0	96	98
Rotenone	0.1	90	98
Antimycin A	1 µg/mg protein	60	100
HQNO	1 µg/mg protein	48	100
DNP	0.1	90	94
Anit A+DNP	1 µg/mg protein +0.1	100	100
HQNO + DNP	1 µg/mg protein +0.1	100	100
Oligomycib	2.0	100	100
Oligomycin + Antimycin A	2.0 + 1 µg/mg protein	100	100

was confirmed by the inhibitory action of dinitrophenol, an inhibitor and uncoupler of energy transfer reactions. As expected, cytochrome c reduction was also inhibited by DNP. It may be seen that the addition of HQNO and antimycin A resulted only in the partial inhibition of FMN reduction. These compounds, however, caused the complete inhibition of cytochrome c reduction by sulfite. Thus, it would appear that the initial energy-dependent reduction of flavin was not due to the reverse electron flow from cytochrome c but that the reduction of cytochrome c occurred at the expense of reduced flavin; the reduction of flavoprotein in the presence of HQNO and antimycin A, however, was completely inhibited by DNP and oligomycin. The initial ATP-dependent reduction of flavin by sulfite and, subsequently, the reduction of cytochrome c was also effectively inhibited by amytal and rotenone.

The ATP-dependent reduction of cytochrome c and NAD^+ was effectively inhibited by uncouplers of oxidative phosphorylation such as 2, 4 DNP and CCCP (Table 9). Dicoumarol was capable of inhibiting specifically the energy-linked reduction of cytochrome c by sulfite without significantly affecting the NAD^+ reduction. These observations suggest that dicoumarol inhibits in the cytochrome b-coenzyme Q region. Inhibitors of the flavoprotein system such as amytal and rotenone, as well as HQNO and antimycin A which block the electron transfer between cytochrome b and c, strongly inhibited the reduction of cytochrome c. Thus, cytochrome c reduction may be visualized as due to a spontaneous flow of electrons from the flavin, which could be reduced by sulfite only in the presence of ATP.

TABLE 9

Effect of inhibitors on the ATP-Dependent reduction of cytochrome c and NAD^+ by sulfite.

The experimental conditions were the same as described in Materials and Methods except that the inhibitors were added to the reaction mixture as indicated. The extracts were preincubated with each inhibitor for 3 min. In the absence of added inhibitor, 27 nmoles of cyt. c and 22 nmoles of NAD^+ were reduced in 5 minutes.

Inhibitor	Concn. (mM)	% Inhibition	
		Cyt. c Reduced	NAD Reduced
Amytal	2.0	96	59
Rotenone	0.1	92	72
HQNO	2 µg/mg protein	100	36
Dicoumarol	10.0	90	14
DNP	0.1	90	95
CCCP	0.01	100	64
PCP	0.2	86	87
Oligomycin	2.0	100	77
Arsenate	10.0	25	5

Our results indeed show that sulfite is capable of reducing pyridine nucleotide in T. neapolitanus only in the presence of ATP. Previous reports have shown that the reduction of pyridine nucleotides by thiosulfate, sulfite or ascorbate is an energy-driven reaction and involved the reversal of electron transfer from cytochrome c (32). We have observed that while thiosulfate enters the respiratory chain of T. neapolitanus at the level of cytochrome c, sulfite can couple directly with flavoprotein in the presence of ATP.

An interesting point concerning the reduction of flavin by sulfite lies in the fact that the E_0' of the sulfite/sulfate system being +p.41 V when calculated from the free energy values of Gibbs and Schiff (33) is appreciably higher than that of the flavin (E_0' = 0.067 V; Chance and Williams (34). It is possible that T. neapolitanus can modify sulfite in some way in the presence of ATP, so as to lower the redox potential of sulfite. It appears likely that the modified form of sulfite is in the form of "Activated Sulfite".

Based on the effect of inhibitors and uncouplers there seem to be two phases of ATP-linked FMN reduction; one in which FMN is reduced directly by sulfite under the influence of ATP and the other which is due to an energy-dependent reversal of electrons from cytochrome c involving the concomitant reduction of pyridine nucleotide or flavin and oxidation of cytochrome c.

The mechanism of sulfite oxidation in T. neapolitanus is apparently different from that reported for Thiobacillus novellus (30). Sulfite oxidation in the latter case is not stimulated by AMP and is coupled to the reduction of cytochrome c. Hempfling et. al. (31) has recently purified sulfite oxidase from T. neapolitanus which is capable of transferring the

reducing equivalents from sulfite directly to oxygen. He also suggested that oxidative phosphorylation is not linked to sulfite oxidation in extracts of T. neapolitanus. However, we have observed that sulfite can couple directly with the flavoproteins in the presence of ATP and can be oxidized therefore, via the electron transport chain. Hence, sulfite oxidation involves the segment of the respiratory chain from flavin to oxygen mediated by the cytochrome system and is probably coupled to the generation of energy involving oxidative phosphorylation.

3. Characteristics of NADH oxidase in T. neapolitanus cell-free extracts: The oxidation of NADH was observed to be mediated by flavo-proteins and cytochrome systems. The results in Table 10 indicate that the NADH oxidase in T. neapolitanus was inhibited only by 20% either by cyanide or azide when used at 2 mM concn. Relatively high concentrations of antimycin A or 2, N-heptyl-4-hydroxyquinoline N-oxide(5µg/mg/ enzyme protein) were required for a 30% inhibition. These inhibitors intercept electron transfer between cytochrome b and c in mammalian respiratory chain. Amytal, an inhibitor of the flavoprotein system caused a 50% inhibition when supplied at a 2 mM concn; likewise a 25 µM concn of rotenone inhibited the enzyme by about 40%. When various combinations of the inhibitors were used at these saturation concentrations, the inhibitory effects were additive. It was also observed that the addition of 33 µM FMN to the reaction mixture caused a five-fold stimulation of the NADH-oxidase activity but the inhibition pattern by various inhibitors was virtually unchanged even in the presence of added flavin. It therefore appears that in the in vitro experiments NADH oxidase is only partially coupled to the cytochrome oxidase system, and that the bulk of the NADH is oxidized by molecular oxygen through the mediation of autooxidizable components such as flavins, cytochrome b and cytochrome c 553. Nevertheless, a part of the

TABLE 10

Characteristics of NADH oxidase from T. neapolitanus cell-free extracts

Reaction mixture in a total volume of 2.0 ml contained cell-free extract (2.0 mg protein) and 100 µmoles of Tris-HCL (pH 8.0). The reaction was started by the addition of 0.2 µmoles of NADH to the treatment cuvette and decrease in optical density at 340 mµ was recorded in a double beam spectrophotometer. Various inhibitors were added as indicated. The cell-free extracts catalyzed the oxidation of NADH at a rate of 20 mµmoles/min/mg protein.

Additions	Inhibitor concn.	Inhibition %
NADH	-	-
NADH + Amytal	2mM	50
NADH + Amytal + CN-	2mM + 2mM	70
NADH + CN	2mM	20
NADH + NaN	2mM	19
NADH + Rotenone	25 µM	40
NADH + HOQNO	10 µg	30
NADH + Rotenone + HOQNO	25 µM + 10 µg	60
NADH + Antimycin A	10 µg	30

electron transport pathway appears to be tightly coupled as evidenced by its sensitivity to amytal or rotenone, antimycin A or quinoline N-oxide, and cyanide or azide. Such inhibitions at various segments of the electron transport chain do in fact suggest the possible existence of the loci of the energy coupling steps. Although ATP formation coupled to NADH oxidation by O_2 could not be demonstrated, however, under anaerobic conditions rapid reduction of the cytochrome systems occurred. Upon the addition of FMN, the cytochrome c was completely oxidized with concomitant reduction of cytochrome b and the flavin. Thus it would appear that the process of electron transport from NADH to cytochrome c mediated by the flavo-proteins and cytochrome b was coupled to the generation of energy which effected the reverse electron flow even when NADH was feeding electrons. This is further supported by our observations that in the presence of added amytal, or uncouplers of energy transfer reactions, the energy-linked oxidation of cytochrome c did not occur in T. neapolitanus cell-free extracts treated with NADH under anaerobic conditions. Further work is in progress to elucidate whether all the energy coupling sites in the electron transport chain of this chemoautotroph are functional when NADH is used as an electron donor.

4. Energy Metabolism of Ferrobacillus

a. Electron Transport Systems: Intact cells of Ferrobacillus were found to catalyze iron oxidation through the mediation of cytochromes c_1, c, and a_1. During this process cytochrome b was also reduced probably involving an energy-linked reversal of electron transfer. While iron oxidation by the Ferrobacillus cells was markedly sensitive to azide, the latter did not affect the iron oxidation by the cell-free extracts which usually lacked cytochrome a_1 component and therefore, the oxidation of

iron catalyzed by cell-free preparations appear to involve the mediation of some autooxidizable components such as a cytochrome of $\underline{c}_1$ -type. A similar situation was observed when ascorbate was used as an electron donor; cytochromes $\underline{c}$, $\underline{c}_1$, and $\underline{a}_1$, were initially reduced followed by the reduction of cytochrome $\underline{b}$ in intact cells or cell-membrane fractions. Succinate donated electrons to membrane fractions producing absorption maxima characteristic of the cytochromes of $\underline{b}_1$, $\underline{c}$, and $\underline{a}_1$. Upon addition of 1 mM ATP and NAD, the reduced cytochrome components were oxidized with concomitant reduction of the pyridine nucleotide under anaerobic conditions, thus indicating that the energy-dependent reverse electron flow was effected from cytochrome $\underline{a}_1$.

At pH 7.0, NADH served as an electron donor in cell-free extracts of Ferrobacillus yielding absorption maxima characteristic of the presence of cytochromes of $\underline{b}_1$, $\underline{b}_5$, $\underline{c}$, $\underline{c}_1$, and $\underline{a}_1$-type. The reduction of cytochromes by NADH was inhibited in the order of 70% in the presence of 10 µg antimycin A; and, a 10 mM concn. of amytal caused a 50% inhibition in the enzymatic reduction of cytochromes by NADH. The presence of NADH oxidase in Ferrobacillus cell-free extracts was occasionally observed; the enzyme was found to be insensitive to cyanide or azide but the activity was completely abolished after boiling the cell-free extracts.

Ferrobacillus cells were analyzed for the presence of quinones. The benzoquinone extracted and isolated by thin layer chromatography, revealed an absorption maxima in isooctane at 275 mµ in its oxidized form; the reduced form absorbed at 285 mµ. On silicone-impregnated paper, a single red spot sppeared with an Rf value of 0.42. This Rf value corresponded to the Rf value of coenzyme Q_8.

b. Phospohlipids of Ferrobacillus ferroxidans: Ferrobacillus ferrooxidans has the capacity to grow at very acid pH values. However the function of the electron transport system of this organism in cell-free preparations for both respiration and reversed electron transport has a pH optimum near pH 7.0 (Aleem and Short, 1968). This implies that the internal pH is maintained near neutrality either by an active process or that the membrane is impermeable to hydrogen ions. Since phospholipids may possibly play an active role in the transport systems used by the cell to maintain its internal environment, the phospholipid metabolism of F. ferrooxidans was examined. Because of the marked difference in the pH of the medium and that necessary for function of the respiratory system in this organism, possible involvement of the phospholipids in active transport could be exaggerated. In addition, F. ferrooxidans has been reported to contain a unique lipid composition. From this unique lipid composition phosphatidyl serine has been postulated to be involved in iron metabolism. In this report the phospholipids of F. ferrooxidans are identified and shown to be similar to those of the thiobacilli. We have shown that the metabolism of the phospholipids was relatively independent of the pH of the growth medium.

The lipid composition of the chemoautotroph Ferrobacillus ferrooxidans was examined. Fatty acids represented 2% of the dry weight of the cells and 86% of the total were extractible with organic solvents. About 25% of the total fatty acids were associated with diacyl phospholipids. Polar carotenoids, the benzoquinone coenzyme Q-8 and most of the fatty acids were present in the neutral lipids. The phospholipids were identified

as phosphatidyl monomethylethanolamine (42%), phosphatidyl glycerol (23%), phosphatidyl ethanolamine (20%), cardiolipin (13%), phosphatidyl choline (1.5%), and phosphatidyl dimethylethanolamine (1%) by chromatography of the diacyl lipids, by chromatograph in 4 systems of the glycerol phosphate esters derived from the lipids by mild alkaline methanolysis and by chromatographic identification of the products of acid hydrolysis of the esters. The strain of F. ferrooxidans used in this study was isolated from a typical location in nature, had morphological and biochemical properties characteristic for the organism and was isolated from distinct single colonies several times. No trace of phosphatidylserine (PS), glycerol-phosphorylserine or serine could be detected in the lipid extract or in derivatives of that extract. This casts some doubt on the postulated involvement of PS in iron metabolism. After growth in the presence of ^{14}c and ^{32}p, there was essentially no difference in the turnover of either isotope in the glycerolphosphate ester derived from each lipid in cells grown at pH 1.5 or at pH 3.5.

Further investigations are in progress concerning the significance of the benzoquinone and phospholipids in the energy metabolism of the iron oxidizing autotroph.

CHAPTER V

SUMMARY

Since the activities of sulfur bacteria are known to be responsible for many of the characteristics of acid-mine wastes, a comprehensive study of their biochemical potentialities is necessary before the effect of strip-mining on their presence and their role in recovery of the stream can be evaluated. Investigations were therefore aimed at elucidating the physiology and metabolism of these bacteria as this approach appears to be the key to the development of control measures. Once the basic biochemical mechanisms are elucidated, the mechanism of influences by the environments (e.g. pH, substrate concentration, temperature, etc.) will become available to explanation.

The results of our investigations have shown that thiobacilli are the most prolific producers of sulfates from the oxidation of sulfur compounds. Thus we have observed that under laboratory conditions, an average population of thiobacilli approximates 10^8 cells/cc. (or 0.075 mg dry weight) in a growth medium. The rate of sulfate production was observed to be in the order of 10 - 40 umoles/hr/mg dry wt. of cells. Assuming that the rate of sulfur transformation in natural environments having enrichment of these organisms is approximately 10% of the transformations observed under laboratory conditions, then the thiobacilli could produce as much as 100 - 400 moles of sulfates in one hour in the upper one-foot of water in an area of one acre. Such a high level of sulfate production of course poses a serious threat in our environments. Our findings that concentrations as low as 10^{-6} -10^{-7} M of chloro or bromo-substituted phenols are effective in the complete inhibition of the energy metabolism of thiobacilli, might prove to be useful in designing experiments to control sulfate production by the sulfur oxidizing bacteria.

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