THE MECHANISM OF THIOSULFATE OXIDATION BY *THIOBACILLUS THIOOXIDANS* 8085

BY

ROSEMARIE JEFFERY Y. MASAU

A Thesis
submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the degree of

MASTER OF SCIENCE

Department of Microbiology
University of Manitoba
Winnipeg, Manitoba

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The Mechanism of Thiosulfate Oxidation by *Thiobacillus thiooxidans* 8085

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Rosemarie Jeffery Y. Masau

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree

of

Master of Science

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ABSTRACT

Abstract

Growth of *Thiobacillus thiooxidans* 8085 on 9K medium containing thiosulfate instead of sulfur, as the sole energy source, was successfully attained. The optimum growth pH was maintained at 5.0 during cultivation. The cultivation period for *T. thiooxidans* on thiosulfate was 4 - 5 days at 28°C. The final cell yield with 1 % thiosulfate in a 4 liter reactor was 425 mg wet cells/L. The culture was continuously maintained with fresh medium. The harvested cells were washed and stored in 0.1M sodium citrate pH 6.0 buffer at 4°C where full activity of the cells remained after 3 weeks.

The mechanism of thiosulfate oxidation in *T. thiooxidans* 8085 was studied at pH 5.0 as well as pH 2.3 and pH 7.0. The oxidation of other reduced inorganic sulfur substrates tetrathionate, sulfur dissolved in dimethyl sulfoxide (DMSO), sulfur suspended in Tween 80 and sulfite were also studied. The oxygen consumption rate and the total oxygen consumed were determined for all substrates in the presence of various metal chelators, inhibitors and uncouplers. Results showed that metal chelations had an effect on the substrate oxidation depending on specific substrate. Most substrate oxidation showed stimulation with the addition of metal chelators except sulfite oxidation which was inhibited.

Stoichiometric studies in the presence of inhibitors 2-n-heptyl-4-hydroxyquinoline N-oxide (HQNO) and N-ethylmaleimide (NEM) in the oxidation of thiosulfate showed that in 0.1M sodium citrate at pH 5.0, thiosulfate was initially cleaved to sulfur and sulfite by rhodanese before further oxidation. In 9K medium and 0.1M

sodium sulfate at pH 2.3, thiosulfate was oxidized to tetrathionate by thiosulfate-oxidizing enzyme. Sulfite was established as an intermediate in sulfur oxidation.

The cytochrome system of *T. thiooxidans* 8085 in both whole cells and cell-free crude extracts was also studied with various substrates, inhibitors and uncouplers. Full reduction of the whole-cell cytochromes with thiosulfate, tetrathionate, DMSO sulfur and sulfite was achieved in 0.1M sodium citrate at pH 5.0. However, full cytochrome reduction of cell-free crude extracts could only be achieved with sulfite in 0.1M Tris-HCl pH 7.5, while thiosulfate, tetrathionate and DMSO sulfur could reduce only cytochrome *c* and only in 0.1M sodium citrate at pH 5.0.

The cytochrome system of *T. thiooxidans* 8085 was tentatively identified by respective absorption peaks : c_γ-420, b_γ-433, $a_\gamma(d_\gamma)$-442, c_β-525, b_β-535, c_α-555, b_α-566, a_α-602 and d_α-635. In the presence of inhibitors, HQNO, cyanide and azide, results suggested that a branched chain electron transport system similar to that of *Azotobacter vinelandii* existed. The cytochrome *d* is the main terminal oxidase of the branch chain system. The second branch of the system is believed to carry a much lower flux of electrons to cytochrome *a* terminal oxidase.

Acknowledgment

I would like to extend my deepest gratitude and sincerest thanks to my advisor, Dr. Isamu Suzuki, for his guidance, teaching and support in both inspirational and financial throughout the research and the preparation of this thesis.

Also many thanks to the members of the department of Microbiology and in particular Dr. P. Y. Maeba, Dr. R. M. Lyric and Dr. L. Van Caeseele for serving as my committee members.

For the utmost, I would like to thank God, my mother, my father and my two brothers for their never ending love and support for the past 7 years of my life here in Canada.

I would like to dedicate my thesis to my mother and father, Mr. and Mrs. Dora and Jeffery Masau, to whom I am forever grateful.

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List of abbreviations

CCCP	Carbonyl cyanide-m-chlorophenylhydrazone
2,4-DNP	2,2-dinitrophenol
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediamine tetraacetate
HCl	Hydrochloric acid
HQNO	2-heptyl-4-hydroxyquinoline-*N*-oxide
milli-Q water	Milli-Q UF PLUS water
NEM	N-ethylmaleimide
o-phenanthroline	orthophenanthroline
p-rosaniline	pararosaniline
ppm	parts per million
S°	Elemental sulfur
Tiron	4,5-Dihydroxy-1,3-benzenedisulfonic acid disodium salt

INTRODUCTION

Introduction

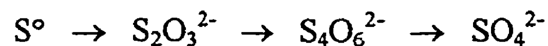
The existence of microorganisms can be shown virtually anywhere and everywhere; from the gut of human beings to deep-sea vents; from a very ideal environment to the most extreme, forbidding environment that would be disastrous to humans. These forbidding environments that are home to some microbes have either very near boiling temperatures or near freezing temperatures and conditions from very acidic to very alkaline. Microorganisms that are happily living in these punishing habitats, are called 'extremophiles'. The extreme environments once thought to be sterile abound with microbes that may be useful in many aspects of application in a variety of industries (Michael T. Madigan et al., 1997).

Thiobacillus species are one of many extremophiles that are categorized into acidophiles. Acidophiles thrive best in environment with pH below 5. These acidic environments are naturally found as a result of geochemical activities such as the production of sulfurous gases in hydrothermal vents and some hot springs; and from the metabolic activities of certain acidophiles themselves (Madigan et al.,1997).

Thiobacillus species which have been isolated from industrial mineral leaching operations or from sites of natural leaching are capable of attacking mineral sulfides. The species are further subdivided according to their preferred temperatures for growth. The most important mesophiles are the chemolithotrophic bacteria, *Thiobacillus ferrooxidans*, *Thiobacillus thiooxidans*, and *Leptospirillum ferrooxidans*. These three species are highly acidophilic with optimal growth pH of 1.5 to 2.0. They are also obligate autotrophs and have optimum growth temperature of 25 - 35°C. *T. thiooxidans*

obtains energy from the oxidation of reduced sulfur compounds. On the contrary, *L. ferrooxidans* obtains energy from the oxidation of ferrous ion. As for *T. ferrooxidans*, it can obtain energy for growth from either ferrous iron or reduced sulfur compounds. These species can share a common habitat where they attack and rapidly degrade mineral sulfides such as pyrite (FeS_2) (Rawlings et al., 1995).

With their ability, thiobacilli are also important in soil systems. They play an essential role in the sulfur cycle processes which is important in agriculture. Sulfur is an essential nutrient for crop production where it is found naturally or artificially added in the form of fertilizer. Elemental sulfur is the most concentrated form of sulfur used as a fertilizer. However, it is not available for the uptake by plants until it is oxidized to SO_4^{2-} ions. (Germida and Janzen, 1993) and this is where the bacteria come into play. The biological oxidation of elemental sulfur in soils is shown in the following sequence, although some products may result from abiotic side reactions.



$$S^\circ \rightarrow S_2O_3^{2-} \rightarrow S_4O_6^{2-} \rightarrow SO_4^{2-}$$

These sulfur compounds can be oxidized to SO_4^{2-} by thiobacilli and the SO_4^{2-} is then made available for uptake by plants. Thiobacilli were long thought to be the most important bacteria for the oxidation of S° in soil (Germida and Janzen, 1993).

Aside from being important in the agricultural aspect, they can also bring great benefits to the mining industry. The insoluble metal deposits (usually metal sulfides or oxides) can be converted to soluble metal sulfates by these microorganisms. The solubilization process as the results of microbial metabolism is termed 'bioleaching' or so called 'biohydrometallurgy' (Rawlings et al., 1995).

Bioleaching was studied extensively, but most of the work was directed toward the mechanism of ferrous iron oxidation and indirect leaching of ferric iron. The oxidation of inorganic sulfur compounds by these acidophilic thiobacilli is more complex, especially in *T. ferrooxidans* which can oxidize either ferrous iron or sulfur. *T. thiooxidans* was selected as the organism to be studied because it can only oxidize sulfur compounds and not ferrous iron, therefore the oxidation is expected to be easier to study. Thiosulfate was selected as the substrate since almost all work on *T. thiooxidans* was carried out with elemental sulfur as growth substrate and yet thiosulfate is an important inorganic sulfur compound in nature.

HISTORY

History

Microbial leaching

Biohydrometallurgy is a study of the interrelationships between the biosphere and the lithosphere. Its roots are in geomicrobiology, microbial ecology and microbial biochemistry which are all affiliations of microbiology and hydrometallurgy (Giovanni Rossi, 1990).

The existence of the interaction between microbes and minerals was first established by geologists and microbiologists which can be dated back to 1838 where C. S. Ehrenberg discovered the association of *Gallionella ferruginea* with ochreous deposits of bog iron. So the beginning of the fundamentals of geomicrobiology were established. The second contributor after C. S. Enrenberg was the renowned Russian scientist, S. N. Winogradsky, who can be rightly considered as the founder of soil microbiology and the discoverer of chemolithoautotrophy, the precursor of biohydrometallurgy (Giovanni Rosssi, 1990).

In 1902, Nathansohn isolated the first strain of *Thiobacillus* species oxidizing inorganic sulfur compounds. Rudolfs and Helbronner (1922) reported the oxidation of pyrite and zinc sulfide by microorganisms in soil, although they did not characterize the organism. These reports further led to the use of these microorganisms for the production of soluble phosphate from mixtures of pyrite, elemental sulfur and phosphate rock in agriculture soils and for economical utilization of low grade zinc sulfide ores (Giovanni Rossi, 1990).

However, there was a silent period for about two decades when the topic of interactions between microbes and minerals was neglected. Finally, in the 1940s, the neglected topic was resuscitated but not in regards to microbiological or geological sciences but with the concern of environmental issues. The concern of environmental pollution was generated after the discovery of Ohio river pollution by several subbituminous coal-mines operating in Pennsylvania. The pollution was caused by some millions of tons of sulfuric acid annually being discharged into the river. This led to an alarming level of acid pollution such that authorities had to take action in finding measures to eliminate it. Therefore, a series of research projects have been conducted on the issues and the results of these investigations revealed that (1) sulfuric acid was generated by the oxidation of the iron sulfides associated with coal and that the rate of this oxidation was much higher than that expected by inorganic chemistry experience (indicated a role for biological oxidation); (2) the occurrence of sulfur-oxidizing bacteria in acid mine drainage waters; (3) that ferrous iron released by pyrite oxidation into the acid mine drainage waters was being oxidized to ferric iron at a much higher rate than indicated by inorganic chemistry data; as soon as these effluents were mixed with water, a characteristic yellow-red colloid (called 'yellow boy' by the mining people) formed and settled out (Giovanni Rossi, 1990).

Clearly, the enhancement of oxidation indicated that reactions were due to biological catalysts that originate from microbial activities. With further investigation, an organism was isolated that had close resemblance to *Thiobacillus thiooxidans*. In 1950 and 1951, a chemolithoautotrophic bacterium *Thiobacillus ferrooxidans*, which derives its energy from the oxidation of inorganic compounds and carbon for biosynthesis from

CO_2 was isolated. This bacterium was also found to be responsible for the oxidation of ferrous iron to ferric iron. With the ability of *T. ferroxidans* to oxidize both sulfur and the iron moieties, it therefore can oxidize not only pyrites (FeS_2) but also other metal sulfides, such as chalcopyrite ($CuFeS_2$), chalcocite (Cu_2S), covellite (CuS), bornite (Cu_5FeS_4), tetrahedrite ($Cu_8Sb_2S_7$) and molybdenite (MoS_2) (Giovanni Rossi, 1990).

From then on, intensive research began and many scientists from around the world joined the search for bioleaching organisms. In the past 40 years of on going research, it was found that *T. ferrooxidans* was not the sole player in the oxidation of metal sulfide minerals but other microorganisms also contributed to the oxidation processes.

Through all this research, the mystery of the processes behind this leaching phenomenon was finally unveiled and these microorganisms were shown to be true biological catalysts in the chemical leaching processes, forming the foundation of bioleaching.

The conventional way of the mining industry has been developed over many centuries: crude ores are dug from the earth, crushed, minerals concentrated and then the metals are extracted with extreme high heat of smelting with toxic gas production. With this conventional way of mining, it poses a great problem to the environment. As we are in an era of more conscience towards the well-being of our planet; we look for other more friendly, kinder, gentler methods. With the method of bioleaching, minerals can be extracted from ores with fewer of the harsh effects than conventional methods (Moffat, 1994).

Bioleaching has always been associated with copper mining but other more recent associations are uranium and gold-bearing arsenopyrite. This technology can also be applied to other mineral extraction containing sulfides of zinc, lead, cobalt, nickel, bismuth, and antimony (Goldstein et al., 1993).

The first miners to make use of bacterial action were the romans in the Rio Tinto Copper mine in Spain. They noticed blue tailings which indicated the presence of copper but had no idea how the metal got into the solution (Moffat, 1994).

The reason why copper mining is more in association with biomining methods is because copper is often found in the lower grade ores bound up in a sulfide matrix. If one was to use the conventional way of smelting, it would come up to a great cost. Lower grade ores can be simply dumped outside a copper mine and be treated with sulfuric acid to encourage the growth of *T. ferrooxidans*. The bacteria can degrade and further solubilize the copper where it is further extracted out from the solution. And the sulfuric acid can be recycled through the ore again (Moffat, 1994).

Currently, 25% of all copper production, worth more than $1 billion annually, comes from the application of bioprocessing. Therefore, it has placed as one of the most important industrial applications of biotechnology in any area of the world today (Moffat, 1994).

A fair amount of studies and research have been conducted and are still on-going extensively on these 'tiny miners'. In order to fully utilize these 'tiny miners' in the optimum capacity, its physiology, biochemistry, and the genomic point of view have to be fully grasped. Mechanism of ferrous iron oxidation to ferric iron ($Fe^{2+} \rightarrow Fe^{3+}$) by *T.*

ferrooxidans is simple, but the oxidation of inorganic sulfur compounds by thiobacilli is complex.

A number of different species of thiobacilli have different oxidative capabilities, but most can oxidize sulfide, sulfur and thiosulfate in general. The mechanism of inorganic sulfur oxidation by thiobacilli has been studied for a number of years now. Although inorganic sulfur compounds are oxidized with various reactions according to their reduction degrees, in all cases, the oxidation process is completed by the oxidation of sulfite to sulfate (Kazuo Nakamura et al., 1995).

Therefore, sulfur and sulfite oxidation are still the key reactions in the oxidation of these inorganic sulfur compounds (Suzuki et al, 1994).

Oxidation of inorganic sulfur compounds pathway :

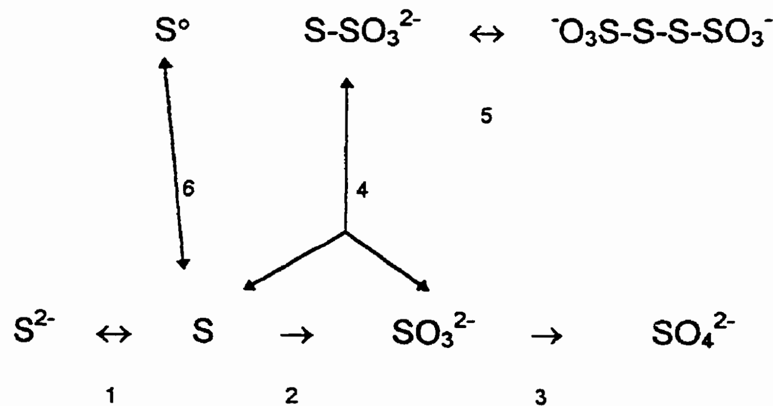


Figure 1. Oxidation of inorganic sulfur compounds.
The enzymes responsible for the reactions were identified as follows : reaction 1, sulfide oxidase; 2, sulfur-oxidizing enzyme; 3, sulfite oxidase or APS reductase; 4, rhodanese (thiosulfate-cleaving enzymes, sulfur transferase); and 5, thiosulfate-oxidizing enzyme (Suzuki et al., 1994).

The sulfur oxidation scheme illustrated in Figure 1 was formulated in 1974 by I. Suzuki and showed the general mechanism of oxidation of these sulfur compounds. Till today, this scheme still stands and satisfies most experimental results (Suzuki et al., 1994).

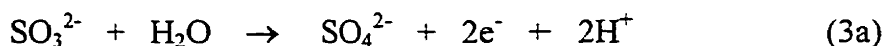
Reaction 1 involved the membrane-bound sulfide oxidase in the oxidation of sulfide to sulfur (Suzuki et al., 1994).

$$S^{2-} \rightarrow S + 2e^- \quad (1)$$

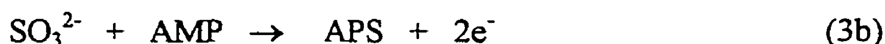
Reaction 2 was based on a series of evidence and findings that the sulfur grown *T. thiooxidans* and *T. ferrooxidans* and thiosulfate-grown *Thiobacillus thioparus* (accumulating sulfur as intermediate) and *Thiobacillus novellus* had sulfur-oxidizing enzyme (reaction 2 in the presence of reduced glutathione, GSH) (Suzuki et al., 1994).

$$S^\circ + O_2 + H_2O \rightarrow H_2SO_3 \quad (2)$$

Reaction 3a involved sulfite oxidase which was found in *T. thioparus*, and *T. ferrooxidans*. As for reaction 3b, adenosine phosphosulfate (APS) reductase is found in *Thiobacillus denitrificans* and *T. thioparus* (Suzuki et al., 1994).



$$SO_3^{2-} + H_2O \rightarrow SO_4^{2-} + 2e^- + 2H^+ \quad (3a)$$

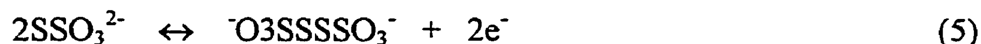


$$SO_3^{2-} + AMP \rightarrow APS + 2e^- \quad (3b)$$

In reaction 4, the enzyme rhodanese, also considered as sulfur transferase present in *T. denitrificans* and *T. novellus*. Sulfite can readily react with sulfur chemically to form thiosulfate (Suzuki et al., 1994).

$$SSO_3^{2-} \leftrightarrow S + SO_3^{2-} \quad (4)$$

The thiosulfate-oxidizing enzyme catalyzed reaction 5 in *Thiobacillus neopolitanus, T. thioparus* and *T. ferrooxidans* (Suzuki et al., 1994).



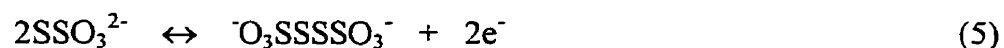
$$2SSO_3^{2-} \leftrightarrow {}^-O3SSSSO_3^- + 2e^- \qquad (5)$$

Reaction 6 involved the interconversion of a single sulfur atom (S) to elemental sulfur octet (S_8 or S°).

$$S \leftrightarrow S^{\circ} \qquad (6)$$

Based on Figure 1, reactions 4 and 5 are the area where my studies were focused.

$$SSO_3^{2-} \leftrightarrow S + SO_3^{2-} \qquad (4)$$

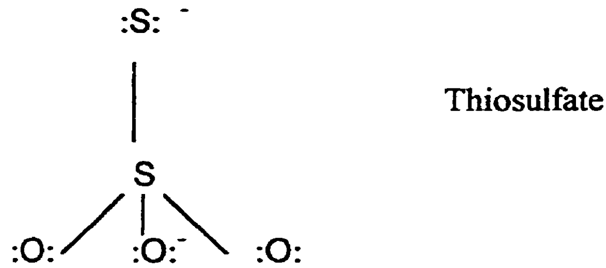


$$2SSO_3^{2-} \leftrightarrow {}^-O_3SSSSO_3^- + 2e^- \qquad (5)$$

The main goal and purpose of my study was to identify the pathway of oxidation of thiosulfate by *T. thiooxidans* ATCC 8085 and its relationship with cytochromes.

General chemistry of thiosulfate

In nature, thiosulfate can be found in most soils and also in significant quantities in certain marine environments. It is easily produced from the sulfur added as fertilizer by means of oxidation with oxygen. It is also found as the by-product of mammalian cysteine catabolism and is present in urine. In humans, it is produced by metabolism and contributes to the sulfane pool although its concentration in body fluids is always low. Thiosulfate is an asymmetric molecule which posses a sulfane (outer) sulfur and a sulfonyl (inner) sulfur atom (Roy and Trudinger, 1970).



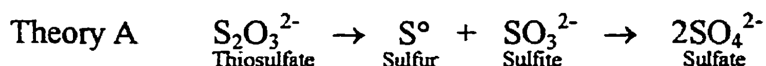
Thiosulfate may be viewed as being derived from sulfate by substitution of an oxygen atom by sulfur. Thiosulfate is relatively stable around pH 7. In alkaline solutions thiosulfate decomposes to sulfide and sulfate or sulfite depending on the condition. Below pH 4-5(acid) thiosulfate decomposes rapidly in a complex manner, the end product depending upon the acidity of the solutions. In strongly acid media, sulfur dioxide, sulfur and polythionates are produced; at medium acidity high molecular weight sulphanes are found while in weakly acid media thiosulfate is converted almost entirely to sulfur and bisulfite.

$$8\ HS_2O_3^- \rightarrow S_8 + 8HSO_3^-$$

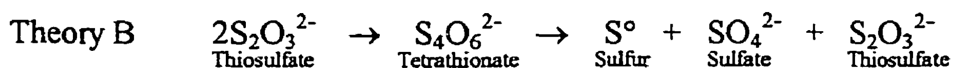
Since thiosulfate is found transiently in many environments, it is therefore commonly used as an energy source for photoautotrophic or chemolithotrophic microorganisms, but their assimilation has been seldom studied and their importance in bacterial physiology is not well understood. However, the utilization of thiosulfate as an electron donor for aerobic growth is quite well documented particular with *Thiobacillus* species. In all the bacteria studied so far, thiosulfate cleavage is performed by one of these enzymes; thiosulfate reductase, polythionate reductase, thiosulfate sulfur transferase or S-sulfocysteine synthase. The growth of *T. thiooxidans* on thiosulfate is normally achieved only at higher pH of 4.5 to 6 with an optimum at pH 5.0 (Barton and

Shively, 1968; Nakamura et al., 1990). However, the oxidation of thiosulfate by sulfur-grown *T. thiooxidans* achieved a optimum at pH 2.3 instead (Suzuki et al, 1992). These results suggested that *T. thiooxidans* may have a different enzyme system operating for thiosulfate oxidation in the sulfur-grown cells and those grown on thiosulfate (Nakamura et al., 1990). The previous study of Chan and Suzuki (1994) failed to grow *T. thiooxidans* on thiosulfate at pH 2.3. In this study, a successful growth on thiosulfate at pH 5.0 was able to be achieved. A better view of understanding on the mechanism of thiosulfate oxidation might be attained if the cells are grown on thiosulfate.

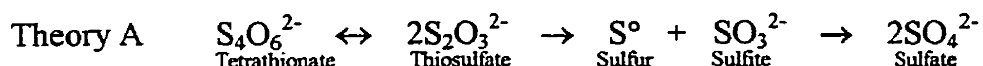
Here, the study is based on two main theories on thiosulfate oxidation. Namely theory A and theory B (to be discussed in more detail in the next heading). Theory A involved the enzyme rhodanese (thiosulfate-cleaving enzyme, sulfur transferase).



Theory B involved the thiosulfate-oxidizing enzyme converting two moles of thiosulfate to tetrathionate. Further, tetrathionate hydrolase catalyzes the hydrolysis of tetrathionate to thiosulfate, sulfur and sulfate.

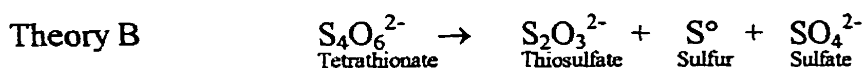


The mechanism of tetrathionate oxidation is also based on two main theories. Theory A involving tetrathionate reductase catalyzes the splitting up of one mole of tetrathionate to two moles of thiosulfate. Then, thiosulfate is further cleaved by the enzyme rhodanese.



Theory A $S_4O_6^{2-}$ (Tetrathionate) $\leftrightarrow$ $2S_2O_3^{2-}$ (Thiosulfate) $\rightarrow$ $S°$ (Sulfur) + SO_3^{2-} (Sulfite) $\rightarrow$ $2SO_4^{2-}$ (Sulfate)

Theory B involves the enzyme tetrathionate hydrolase which catalyzes the hydrolysis of tetrathionate to thiosulfate, sulfur, and sulfate.



Theory B $S_4O_6^{2-}$ (Tetrathionate) $\rightarrow$ $S_2O_3^{2-}$ (Thiosulfate) + $S°$ (Sulfur) + SO_4^{2-} (Sulfate)

These theories can be tested by inhibition studies using N-ethylmaleimide (NEM), and 2,*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO). The following are the theories showing quantitative equations involving the inhibition experiment studies.

Theory

Based upon the sulfur oxidation scheme in Figure 1, thiosulfate can be oxidized in two ways, namely A and B (Suzuki, 1999).

Thiosulfate oxidation

A. Rhodanese (Thiosulfate-cleaving enzyme, Sulfur transferase)

(1) $S\text{-}SO_3^{2-} + 2H_2O \leftrightarrow S + H_2SO_3 + 2OH^-$

Rhodanese catalyzes the cleavage of thiosulfate into sulfur and sulfite.

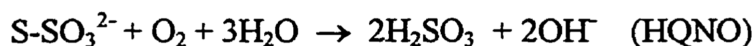
Sulfur-oxidizing enzyme

(2) $S + O_2 + H_2O \rightarrow H_2SO_3$

Sulfur-oxidizing enzyme catalyzes the oxidation of sulfur to sulfite.

In the presence of HQNO which inhibits sulfite oxidation :

(3): (1) + (2)



$$S\text{-}SO_3^{2-} + O_2 + 3H_2O \rightarrow 2H_2SO_3 + 2OH^- \quad \text{(HQNO)}$$

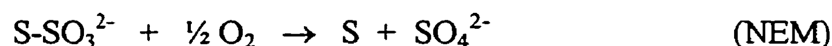
Sulfite oxidase + Electron transfer system

$$(4)\ H_2SO_3 + \frac{1}{2}O_2 \rightarrow SO_4^{2-} + 2H^+$$

Final step, involving the oxidation of sulfite to sulfate as the end product.

In the presence of NEM inhibitor which inhibits sulfur oxidation :

(5): (1) + (4)



$$S\text{-}SO_3^{2-} + \frac{1}{2}O_2 \rightarrow S + SO_4^{2-} \qquad \text{(NEM)}$$

In the absence of inhibitor :

(6): (1) + (2) + 2x(4)

$$S\text{-}SO_3^{2-} + 2O_2 + H_2O \rightarrow 2SO_4^{2-} + H^+$$

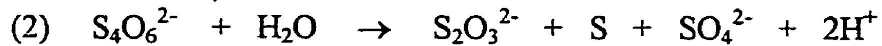
Therefore, the complete oxidation of one mole of thiosulfate will consume two moles of O_2 producing two moles of sulfate (sulfuric acid) as the end product.

B. Thiosulfate-oxidizing enzyme + Electron transfer system

$$(1)\ 2S_2O_3^{2-} + \frac{1}{2}O_2 + 2H^+ \rightarrow S_4O_6^{2-} + H_2O$$

Thiosulfate-oxidizing enzyme catalyzes the formation of tetrathionate from two moles of thiosulfate.

Tetrathionate hydrolase



(2) $S_4O_6^{2-} + H_2O \rightarrow S_2O_3^{2-} + S + SO_4^{2-} + 2H^+$

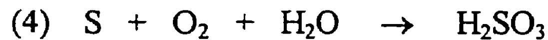
The hydrolysis of tetrathionate is catalyzed by tetrathionate hydrolase; giving one mole each of thiosulfate, sulfur, and sulfate.

In the presence of NEM which inhibits sulfur oxidation :

(3): (1) + (2)

$S_2O_3^{2-} + \frac{1}{2}O_2 \rightarrow S + SO_4^{2-}$ (NEM)

Sulfur-oxidizing enzyme

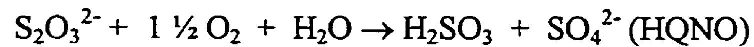


(4) $S + O_2 + H_2O \rightarrow H_2SO_3$

Sulfur-oxidizing enzyme catalyzes the oxidation of sulfur to sulfite.

In the presence of inhibitor HQNO which inhibits sulfite oxidation :

(5): (1) + (2) + (4)



$S_2O_3^{2-} + 1\frac{1}{2}O_2 + H_2O \rightarrow H_2SO_3 + SO_4^{2-}$ (HQNO)

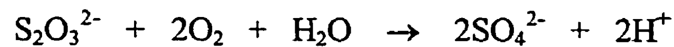
Sulfite oxidase + Electron transfer system

(6) $H_2SO_3 + \frac{1}{2}O_2 \rightarrow SO_4^{2-} + 2H^+$

Final step, sulfite is further oxidized to sulfate by sulfite oxidase.

In the absence of inhibitors :

(7) : (1) + (2) + (4) + (6)



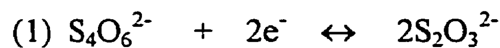
$S_2O_3^{2-} + 2O_2 + H_2O \rightarrow 2SO_4^{2-} + 2H^+$

Therefore, the complete oxidation of one mole of thiosulfate through the tetrathionate hydrolase pathway will consume two moles of O_2 producing two moles of sulfate (sulfuric acid).

Tetrathionate oxidation

The oxidation of tetrathionate can also be directed in two different ways, namely A and B (Suzuki, 1999).

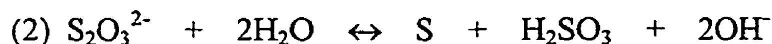
A. Tetrathionate reductase (Thiosulfate-oxidizing enzyme ?)



(1) $S_4O_6^{2-} + 2e^- \leftrightarrow 2S_2O_3^{2-}$

Tetrathionate reductase catalyzes the splitting up of one mole of tetrathionate into two moles of thiosulfate.

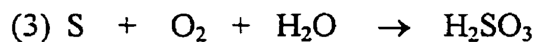
Rhodanese



(2) $S_2O_3^{2-} + 2H_2O \leftrightarrow S + H_2SO_3 + 2OH^-$

Rhodanese further catalyzes the cleavage of thiosulfate into sulfur and sulfite.

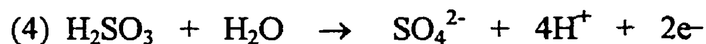
Sulfur-oxidizing enzyme



(3) $S + O_2 + H_2O \rightarrow H_2SO_3$

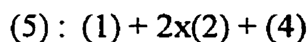
Sulfur-oxidizing enzyme catalyzes the oxidation of sulfur to sulfite.

Sulfite oxidase

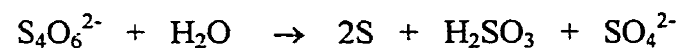


(4) $H_2SO_3 + H_2O \rightarrow SO_4^{2-} + 4H^+ + 2e^-$

Sulfite is further oxidized to sulfate.



(5) : (1) + 2x(2) + (4)



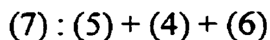
$$S_4O_6^{2-} + H_2O \rightarrow 2S + H_2SO_3 + SO_4^{2-}$$

(No O_2)

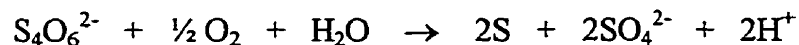
Electron transfer system

(6) $2e^- + 2H^+ + \frac{1}{2} O_2 \rightarrow H_2O$

In the presence of inhibitor NEM which inhibits sulfur oxidation :



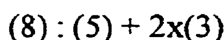
(7) : (5) + (4) + (6)



$$S_4O_6^{2-} + \frac{1}{2} O_2 + H_2O \rightarrow 2S + 2SO_4^{2-} + 2H^+$$

(NEM)

In the presence of inhibitor HQNO which inhibits sulfite oxidation :

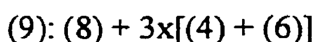


(8) : (5) + 2x(3)

$$S_4O_6^{2-} + 2O_2 + 3H_2O \rightarrow 3H_2SO_3 + SO_4^{2-}$$

(HQNO)

In the absence of inhibitor :

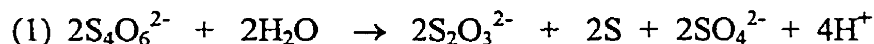


(9): (8) + 3x[(4) + (6)]

$$S_4O_6^{2-} + 3\frac{1}{2} O_2 + 3H_2O \rightarrow 4SO_4^{2-} + 6H^+$$

Therefore, with the oxidation of one mole of tetrathionate, three and one half molesof oxygen are consumed to produced four moles of sulfate.

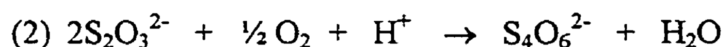
B. Tetrathionate hydrolase



(1) $2S_4O_6^{2-} + 2H_2O \rightarrow 2S_2O_3^{2-} + 2S + 2SO_4^{2-} + 4H^+$

Tetrathionate hydrolase catalyzes the hydrolysis of two moles of tetrathionate producing two moles of thiosulfate, sulfur, and sulfate.

Thiosulfate-oxidizing enzyme + Electron transfer system



(2) $2S_2O_3^{2-} + \frac{1}{2} O_2 + H^+ \rightarrow S_4O_6^{2-} + H_2O$

Two moles of thiosulfate are oxidized to one mole of tetrathionate.

In the presence of inhibitor NEM :

(3) : (1) + (2)

$$S_4O_6^{2-} + \frac{1}{2} O_2 + H_2O \rightarrow 2S + 2SO_4^{2-} + 2H^+ \quad \text{(NEM)}$$

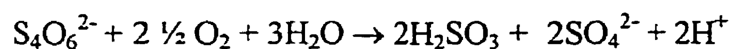
Sulfur-oxidizing enzyme

$$(4) \quad 2S + 2O_2 + 2H_2O \rightarrow 2H_2SO_3$$

Two moles of sulfur oxidized to two moles of sulfite by sulfur-oxidizing enzyme.

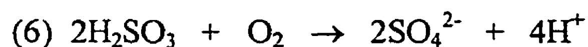
In the presence of inhibitor HQNO :

(5) : (1) + (2) + (4)



$$S_4O_6^{2-} + 2\frac{1}{2} O_2 + 3H_2O \rightarrow 2H_2SO_3 + 2SO_4^{2-} + 2H^+ \quad \text{(HQNO)}$$

Sulfite oxidase + Electron transfer system

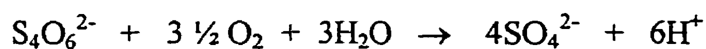


$$(6) \quad 2H_2SO_3 + O_2 \rightarrow 2SO_4^{2-} + 4H^+$$

Sulfite further oxidized to sulfate.

In the absence of inhibitor :

(7) : (1) + (2) + (4) + (6)



$$S_4O_6^{2-} + 3\frac{1}{2} O_2 + 3H_2O \rightarrow 4SO_4^{2-} + 6H^+$$

Therefore, with the involvement of tetrathionate hydrolase, three moles of oxygen are consumed for the oxidation of one tetrathionate producing four moles of sulfate.

MATERIALS AND METHODS

Materials

Chemical

Chemicals were of the highest grade commercially available.

General chemicals were obtained from Fisher Scientific (Fairlawn, New Jersey, U.S.A) or Mallinkrodt Canada Inc. (Pointe Claire, Quebec, Canada). Sodium thiosulfate pentahydrate and precipitated sulfur powder were obtained from British Drugs Houses (BDH) Limited (Toronto, Canada). Sodium tetrathionate was obtained from Aldrich chemical company Inc. (Milwaukee, U.S.A). Potassium sulfite was obtained from Matheson, Coleman and Bell (Norwood, Ohio and East Rutherford, New Jersey, U.S.A) All inhibitors : 2-n-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO), N-ethylmaleimide (NEM), carbonyl-m-chlorophenylhydrazone (CCCP), and 2,4-dinitrophenol (2,4-DNP) were obtained from Sigma Chemical company (U.S.A).

Culture medium

The basal medium was the 9K medium of Silverman and Lundgren (1959) which contained per liter of glass-distilled water: 3.0 g $(NH_4)_2SO_4$, 0.1 g KCl, 0.5 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 0.014 g $Ca(NO_3)_2.4H_2O$, and 0.18 mg $FeSO_4.7H_2O$ (i.e. 10 mL of Millipore filtered 18 mg/L $FeSO_4.7H_2O$ solution added to the sterilized culture medium). The pH of the culture medium was adjusted to 5.0 with concentrated sulfuric acid. The media were sterilized by autoclaving.

Starkey No.1 medium contained the following (per liter) : 0.3 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4.7H_2O$, 0.018 g $FeSO_4.7H_2O$, 3.5 g K_2HPO_4 and 0.25 g $CaCl_2$.

Starkey No.2 medium contained the following (per liter) : 4.0 g KH_2PO_4, 4.0 g K_2HPO_4, 0.3 g $(NH_4)_2SO_4$, 0.019 g $CaCl_2$, 0.05 g $MgSO_4.7H_2O$, 0.014 g $MnSO_4.H_2O$ and $FeCl_3.\ 6H_2O$.

Substrate

Sodium thiosulfate (10 g/100 mL) was the source of substrate. It was sterilized by Millipore filtration. Source of the water used was Milli-Q UF PLUS water.

pH Indicator

A 0.5 % solution of bromophenol blue (tetrabromophenolsulfonephthalein) was used as pH indicator. The pH range was 3.0 - 4.6, with the color change from yellow $\rightarrow$ blue violet. The indicator solution was autoclaved before using.

Methods

The adaptation of sulfur grown *T.thiooxidans* (ATCC 8085) to thiosulfate

T. thiooxidans (ATCC 8085) grown on elemental sulfur for 4 days at 28°C were used as the seed culture for the adaptation to thiosulfate.

The growth medium for sulfur grown *T. thiooxidans* (ATCC 8085) was Starky No.1 medium which consisted of 0.3 g $(NH_4)_2SO_4$, 3.5 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.25 g $CaCl_2$, and 18 mg $FeSO_4.7H_2O$ per liter and adjusted to pH 2.3 with sulfuric acid. The cells were grown on powdered sulfur (BDH, precipitated sulfur, 10 g per liter) which was spread evenly on the surface.

After 4 days of growth the sulfur-grown cells were used as inoculum (10 %) for the thiosulfate medium. The culture liquid was transferred carefully without disturbing the remaining sulfur to microcentrifuge tubes (Eppendorf tube) and cells were collected and washed three times with autoclaved 9K growth medium at pH 5 in a microcentrifuge (IEC, Micro-MB centrifuge 3615, 14000 rpm) for 3-5 mins. The washing was necessary to remove excess sulfuric acid. All procedure steps were done aseptically.

The adaptation growth of thiosulfate grown cells were done in 250 mL Erlenmeyer flasks. The washed cells (10 %) were inoculated into 80 mL of autoclaved 9K media and 10 mL of sterilized 10 g/100 mL $Na_2S_2O_3$ (1 %) was added as substrate bringing a total volume of 100 mL. Two drops of the 0.5% Bromophenol blue pH indicator were also added.

The flasks were kept stationary for 2 to 4 days before placing on the shaker (150 rpm). The disappearance of thiosulfate was followed by the determination of thiosulfate using Sörbo's method (1957). Sulfite and tetrathionate determinations as described later were also performed.

Transfer of the flask culture into a reactor

The flask culture that had the optimum pH, i.e. pH 5, was used as an inoculum (10 %) for the growth of *T. thiooxidans* in a 400 mL reactor. The reactor was equipped with an automated titrator (Radiometer Copenhagen, pH meter 28) to maintain the pH at 5 with 5% potassium carbonate (K_2CO_3). It was also continuously aerated through a glass sparger and a magnetic stirrer. Sodium thiosulfate (1 %) was added as substrate.

Another transfer to a 4 liter reactor was made using the 400 mL of culture from the 400 mL reactor as inoculum. The purpose of growing in a reactor was to obtain large quantities of cells for experimental studies.

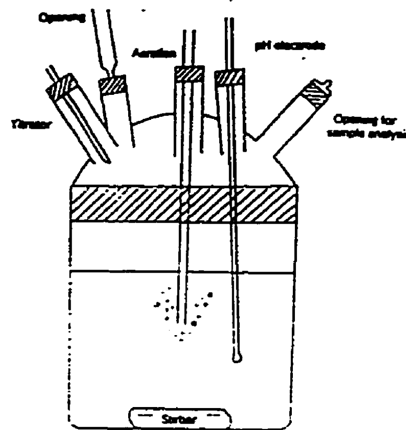


Fig. 2 Culture fermentor (reactor).

Cultivation

The cultivation was carried out on a continuous basis where 400 mL of the 4 liter culture was left as the 10 % inoculum for the next batch of culture and the remainder of culture was harvested for cells. The reactor was refilled with fresh media and substrate. The cultivation was carried for 4 to 5 days at 28°C.

Cell harvesting

After the completion of growth at 4 to 5 days (i.e. the utilization of thiosulfate was completed), the 4 liter culture was harvested by centrifugation at 8,000 x g for 10 min. The cell pellet was collected and was divided into two portions. One portion was

washed twice with milli-Q water and the other washed twice with 9K pH 5 medium with centrifugation in between (10,000 x g, 10 min). Both kinds of washed cells were suspended in 0.1 M sodium citrate (pH 6) at a concentration of 50 mg wet cells per mL. When cells contained high endogenous activity, the cell suspensions were stirred for 20-30 min to get rid of most of the internal stored substrate. When the oxidation activity of the cells was high, the cell suspension could be stored at 4°C for 2 weeks without losing much of the activity. However, activity of the cells decreased when 9K medium was used for cell storage. The use of Silverman 9K medium for storing the cells adversely affected the cell activity. The activity of cells gradually decreased with time. The storage of cells in citrate buffer (pH 6.0) retained cell activity better (Nakamura et al., 1990). For this reason, 0.1M sodium citrate pH 6.0 was used for cell suspension and storage.

Preparation of cell-free extracts

The *T. thiooxidans* (ATCC 8085) cells suspended in 0.1 M sodium citrate were washed once with 0.1 M Tris-HCl (pH 7.5) buffer and suspended in the same buffer at a wet cell concentration of 200 mg/mL.

There were two kinds of treatment; trypsin treated and trypsin untreated. The trypsin treated cells were prepared as follows: 3 mg of trypsin per mL was added to the Tris-HCl suspended cells and after stirring for 20min, 3 mg trypsin inhibitor was added to stop the action of trypsin. After 5 min stirring the cell suspension was passed three times through a French pressure cell at 110 Mpa, followed by centrifugation at 10,000 x g for 10 min to remove the bulk of cell debris. The supernatant was termed as crude cell-free

extracts. The supernatant was translucent with a reddish brown color. The cell debris pellets were also kept for analysis.

For the trypsin untreated cells, the crude cell-free extracts were prepared as above without going through the trypsin treatment.

Oxidation of thiosulfate and other substrates by whole cells and cell free extract

Thiosulfate oxidation was assayed by measurement of oxygen consumption at 25°C in a thermostated vessel equipped with a Teflon membrane-covered Clark oxygen electrode (Gilson Medical Electronic Oxygraph machine).

The reaction mixture for thiosulfate (0.01M) or other substrate (0.1M sulfite with EDTA, 0.01M tetrathionate, 32 g/100 mL Tween 80 sulfur, and 5 mg/10 mL DMSO sulfur) oxidation contained in a total volume of 1.2 mL, 2.5 mg wet cells (50 µL of 50 mg/mL) or cell-free extracts and a specific amount of substrate in a buffer.

Oxidation of the above mentioned substrate was studied in different buffers at different pH's. Buffers used were 0.1 M sodium citrate, 9K medium, 0.1 M sodium sulfate, 0.1 M potassium phosphate, 0.1 M potassium nitrate and 0.1 M sodium chloride at pH 2.3, 5 and 7.

Metal chelation effects

Addition of metal chelators to the substrate oxidation experiments was to see if the effect of 0.1 M sodium citrate buffer in providing the highest oxidation activity for all substrates was due to the suspected metal chelation. Chelators tested were

ethylenediamine tetraacetate (disodium) EDTA, orthophenanthroline, 2,2' dipyridyl, and 4,5-dihydroxy-1,3-benzenedisulfuric acid disodium salt (Tiron).

Inhibition studies

The purpose of inhibition studies was to inhibit a specific reaction steps in thiosulfate oxidation leading to predictable accumulation of intermediates with expected stoichiometries. Inhibitors used were N-ethylmaleimide (NEM) , 2-n-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO), cyanide (KCN), azide (NaN_3), 2,4-dinitrophenol (2,4-DNP) and carbonyl cyanide-m-chlorophenylhydrazone (CCCP). Inhibition studies were done in both experimental analyses; oxidation of substrate studies and cytochrome oxidation/reduction studies.

Determination of thiosulfate (growth experiments)

Thiosulfate was determined by the method of Sörbo (1957). To 3.2mL sample containing 0.2 - 2.0 μmole thiosulfate was added 2.0 mL 0.2 N NH_4OH, 0.5 mL 0.1 M KCN, 0.3 mL 0.1 M $CuCl_2$ and 0.5 mL ferric nitrate reagent (10 % $Fe(NO_3)_2.9H_2O$ in 13% HNO_3) with mixing after each addition. After 15 min of incubation, the optical density was determined using a Diode Array spectrophotometer (Hewlett Packard 8452A) at 460 nm.

Determination of tetrathionate (growth experiments)

Tetrathionate was determined by the same procedure as thiosulfate except the sample was incubated with NH_4OH and KCN for 30 min prior to the addition of ferric

nitrate reagent. After 15 min of incubation, $CuCl_2$ was added and the optical density was read at 460 nm.

Modified tetrathionate determination (oxidation experiments)

Modification was done in three aspects : (1) sample and reagent volumes were scaled down to 1/3 (1/3.2 to be exact) of the original volume. (2) a higher concentration of NH_4OH (1.0 M) was used for sodium citrate buffer assays, (3) mixtures were centrifuged after incubation with KCN to remove cells. Due to the high buffering capacity of sodium citrate, more NH_4OH was needed to bring the pH up. Further, 0.1 mL of concentrated HNO_3 was added after the addition of ferric nitrate reagent to counteract the high NH_4OH concentration used.

Cold cyanolysis (oxidation experiments)

Tetrathionate was determined colorimetrically after cyanolysis by the method of Sörbo (1957). To 1.0 mL reaction mixture, 0.625 mL of 0.2 N NH_4OH (1.0 M NH_4OH for sodium citrate assays) and 0.156 mL of 0.1 M KCN were added. After 30 min of incubation for cold cyanolysis at room temperature, the mixture was centrifuged to remove cells. To the supernatant, 0.156 mL of ferric nitrate reagent [10% $Fe(NO_3)_2.9H_2O$ in 13% HNO_3] was added to the final reaction mixture. After an equilibrium time of 15 min, the intensity of color was determined at 460 nm against a control cuvette containing all the reagents and water and then compared to a standard curve prepared with different known amounts of thiocyanate.

Determination of sulfite

Pararosaniline method (West and Gaeke, 1956) was used.

A 1.0 ml sample of the reaction mixture was withdrawn in a 5 mL of 0.1 M sodium tetrachloromercurate II which was then centrifuged to get rid of cells (10,000 x g, 15min). To the supernatant, 0.5mL of 0.2% formaldehyde and 0.5 mL of HCl-bleached pararosaniline (0.04 %) were added with mixing in between. After 30 min of incubation, the intensity of color was determined at 554 nm against the reagent blank and compared to the standard curve (0.02 - 0.2 μmole).

Modified sulfite determination

This modified sulfite determination was applied specifically to sodium citrate buffer assay. The problem with sodium citrate buffer was that an appropriate color of sulfite determination could not be obtained. The natural color of determination for sulfite is light pink to pink-purple but as for sodium citrate buffer assays, the color appeared to be blue-purple to very dark blue-purple with some precipitation occurring. This color normally appeared 10 - 20 min after all the reagents were added. Since sodium citrate has a very strong buffering capacity, pH might have an effect. As for the precipitation, this only occurred when cells are present. The dark matter precipitation could be composed of mercuric sulfide (HgS) which is black in color in cubic crystal (β-form). The transition temperature is from (red to black) 386°. Black form can exist indefinitely in metastable state at room temperature (The Merck Index; 10th addition. P.841, 1983).

Polysulfide may accumulate on the cell surface during the growth and may react with mercury ions found in the sodium tetrachloromercurate II reagent at a certain pH.

The modification of sulfite determination for sodium citrate buffer assays was simply the addition of 20 μL of concentrated HCl after the mercury and formaldehyde steps. This was followed by the addition of *p*-rosaniline. Color intensity was measured at 554 nm after 20 min incubation. With the addition of 20 μL of concentrated HCl, an appropriate color formation was achieved and no precipitation occurred.

Determination of elemental sulfur

The procedure for determination of elemental sulfur was done according to Barlett and Skoog (1954).

The reagents to be prepared were acetone solvent which was prepared by diluting 50 mL of water to one liter with technical grade acetone, sodium cyanide solution (0.1 g NaCN in 100 mL acetone solvent) and ferric chloride solution (0.4 g $FeCl_3.6H_2O$ in 100 mL acetone solvent). A standard sulfur solution of 50 ppm sulfur on a weight per volume basis was prepared by dissolving 50 mg of elemental sulfur (BDH, precipitated sulfur) in 10 mL of petroleum ether (petroleum spirit, Analar, boiling point range 80-100°C, BDH chemicals) and diluting it further by 100 times with petroleum ether.

The procedure of Barlett and Skoog (1954) was scaled down to 1/10 (0.5 mL) except for the volume of ferric chloride solution which was only reduced to 1/5 (1 mL). A standard curve obtained from 25, 50, 75 and 100 nmoles elemental sulfur in 0.5 mL petroleum ether gave a linear line.

Elemental sulfur in the reaction mixture (1.2 mL) was extracted with 2 mL petroleum ether by 15 - 20 s of vigorous vortex mixing (Scientific Products S8220 deluxe mixer, maximum speed 60 Hz) in a 25 mL corex centrifuge tube with screw cap (Corning 8446).

Then the mixture was centrifuged at 6,000 x g for 10 min; and 0.5 mL of the top layer was carefully removed for sulfur determination.

The top layer of the 0.5 mL sample was mixed with 1.5 mL of sodium cyanide solution and 0.5 mL of acetone solvent to bring up to a total of 2.5 mL. After 2 min of the cyanolysis reaction, 0.5 ml of the mixture was withdrawn and mixed with 1 mL of ferric chloride solution. After 10 min of incubation, the absorbance was taken at 464 nm with Hewlewtt Packard 8452A diode array spectrophotometer in a 1-cm light path quartz cuvette against a blank prepared from 1.2 mL water (or whatever buffer was used) instead of the reaction mixture treated by the same procedures as the experimental sample.

In the standard curve was obtained with the extraction procedure; the value of absorbance (*A*) at 464 nm increased linearly with the amount of sulfur and approximately followed the relationship $A = (0.2 \pm 0.01)$ X amount of sulfur with fresh reagents. Basically, the absorbance values were identical to the values in the standard curve for sulfur obtained without extraction after considering the dilution factor of 4. Therefore, the petroleum ether extraction of elemental sulfur from an aqueous phase was considered to be nearly 100 %.

Cytochrome studies using Shimadzu Multipurpose recording Spectrophotometer (MPS-50L)

All absorbance spectrum of cytochrome oxidation/reduction states studies were done using a Shimadzu Multipurpose Recording spectrophotometer model MPS-50L at room temperature with 1-cm light path quartz cuvette.

Both intact cells and cell-free crude extracts were analyzed. Difference spectra were taken between samples which contained cells or extracts with and without substrates. A complete reduction of *T. thiooxidans's* cyctochrome system of both intact cells and cell-free extracts was obtained by the addition of the reducing agent dithionite. A very small amount of crystalline crystal dithionite was added directly to the quartz cuvette containing intact cells or cell-free extract in a specific buffer and pH.

Reduction of the cytochrome system of *T. thiooxidans* was carried out with substrates : thiosulfate, tetrathionate, sulfite and DMSO sulfur. The amounts of substrate used was in excess of O_2 present for the complete oxidation. The scanning speed was 3 min from 400 - 800 nm and the scanning was repeated many times to follow the spectral change with time.

After the complete reduction of cytochromes, the cuvette which contained cells or extracts and the reference cuvette was shaken a few times to introduce O_2. Scanning of the re-oxidized cytochromes was carried out immediately.

In the substrate reduction studies of the cytochromes, the effect of inhibitors were also analyzed. Inhibitors used were 2-n-heptyl-4-hydroxyquinoline *N*-oxide (HQNO), N-ethylmaleimide (NEM), cyanide, azide, CCCP and 2,4-DNP. Inhibitors HQNO, NEM, cyanide and azide were added to the sample cuvette only but CCCP and 2,4-DNP were added to both sample and reference cuvette. This is because CCCP and 2,4-DNP are yellow coloured compounds and therefore will interfere with the absorptions. Both CCCP and 2,4-DNP were added to sample and reference cuvettes to cancel out interfering absorptions.

RESULTS

Results

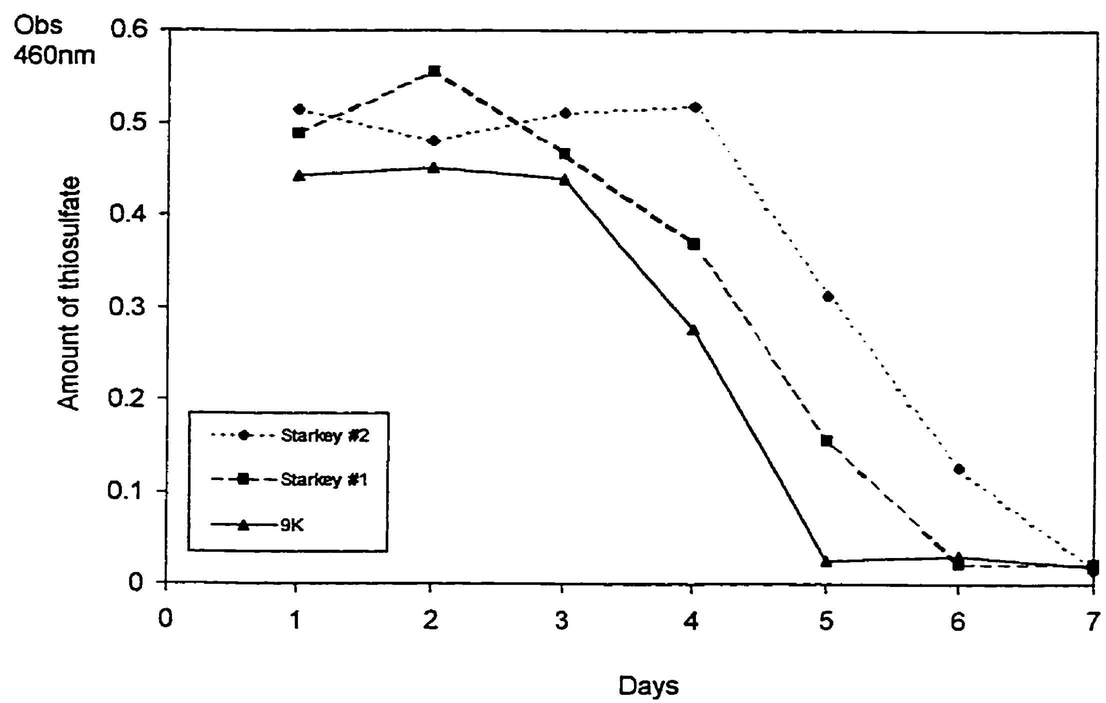
Cultivation of T. thiooxidans on thiosulfate

There is no uniformity among *T. thiooxidans* strains in their ability to oxidize thiosulfate and grow on thiosulfate. Barton and Shively (1968) and London and Rittenberg (1964) grew some strains successfully in thiosulfate media (Nakamura et al., 1990). Among these strains of *T. thiooxidans* that are able to grow in thiosulfate media is *T. thiooxidans* S3 studied by Nakamura et al. (1990). This strain readily oxidizes thiosulfate in the medium and grows rapidly at pH 5.0, the optimum (Nakamura et al., 1990). *T. thiooxidans* ATCC 8085 did not grow on thiosulfate at pH 2.3, but sulfur grown cells were able to oxidize thiosulfate at the pH optimum of pH 2.3 (Chan and Suzuki, 1994). Nakamura (1990) suggested that *T. thiooxidans* may have different enzyme systems operating for thiosulfate oxidation in sulfur-grown cells and those grown on thiosulfate. In order to obtain a clear perspective on the mechanism of thiosulfate oxidation, the cells must be cultivated in a thiosulfate medium.

In this study, the same strain of *T. thiooxidans* ATCC 8085 was used to try to grow in a thiosulfate medium again. Through many trials and errors in finding the perfect growth conditions mainly the growth medium and the amount of aeration, the cultivation of *T. thiooxidans* ATCC 8085 in thiosulfate medium has finally been attained. Initial studies were carried out in shake flask experiments with 100 mL mediium shaking at 150 rpm. As seen in Figure 3, the best growth medium was 9K medium by Silverman and Lungren (1959), adjusted to pH 5.0 showing the complete utilization of thiosulfate

in 5 days as compared to 6 and 7 days in Starkey No. 1 and Starkey No. 2 medium adjusted to the same pH respectively. This could be due to the amount of phosphate present in each medium since the 9K medium has the least amount of phosphate. This medium was selected for a large scale growth experiment in reactors. The amount of aeration and stirring in the 4 liter fermentor (reactor) had to be moderate, requiring adjustment based on the growth stage of the culture. A large amount of colloidal/elemental sulfur accumulated with some production of sulfide gas if the aeration was too low making the culture anaerobic. If this occurred, one could increase the aeration and the colloidal/elemental sulfur and sulfide gas would disappear within a day.

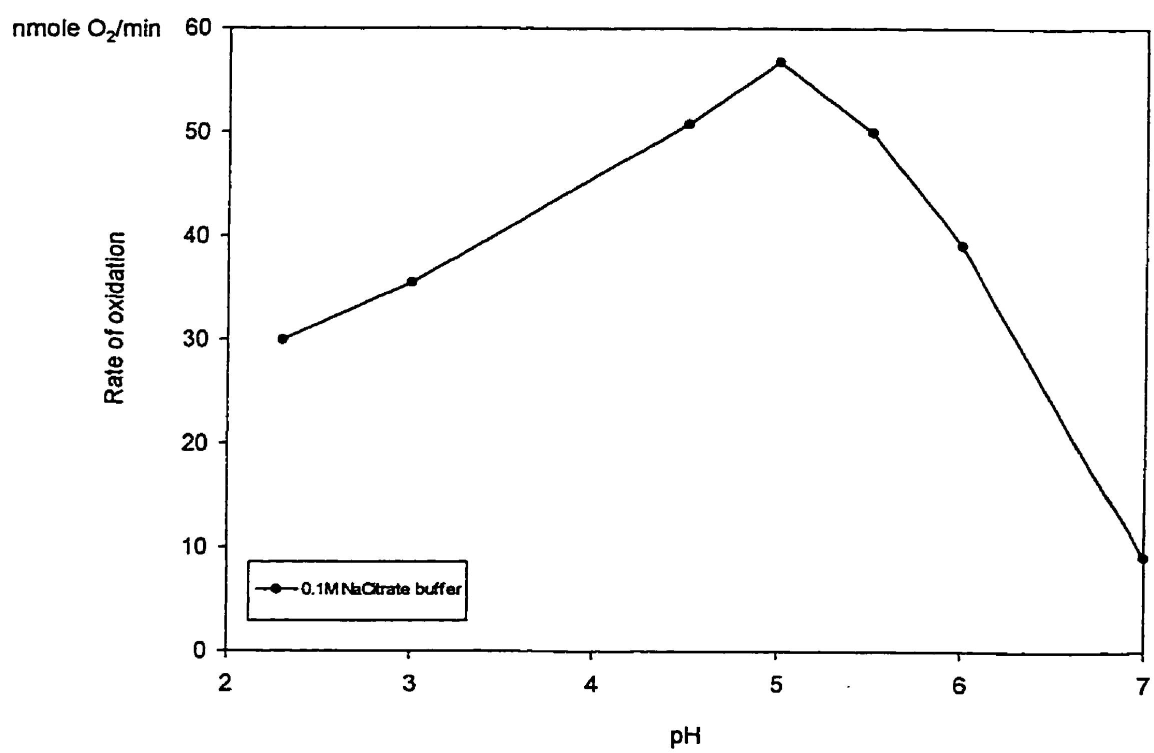
Figure 3. Growth medium experiment.
The preferred growth medium for *T. thiooxidans* was determined by following the disappearance of substrate thiosulfate. Starkey No. 2, Starkey No. 1, and 9K medium took 5, 6 and 7 days respectively for the complete utilization of thiosulfate by *T. thiooxidans*. The preferred growth medium was 9K medium.


Obs
460nm
Amount of thiosulfate
0.6
0.5
0.4
0.3
0.2
0.1
0
0
1
2
3
4
5
6
7
Days
Starkey #2
Starkey #1
9K

An adaptation period of 4-5 days in cultivating cells on thiosulfate was needed when the seed culture from sulfur grown cells was used. However, no adaptation period was needed when the seed culture was grown in a thiosulfate medium. This factor was also observed by Nakamura et al. (1990) with their strain. The final cell yield on 1 % thiosulfate media was 0.425 g wet cells/L.

Figure 4 shows the optimum pH, pH 5.0, in the oxidation of thiosulfate by these thiosulfate grown cells in 0.1M sodium citrate buffer. A reasonable range of pH for thiosulfate oxidation can be seen i.e. from 4.5 - 6.0 which was also observed by Nakamura et al. (1990).

Figure 4. Optimum pH for the oxidation of substrate thiosulfate in sodium citrate buffer by *T. thiooxidans*. The highest rate of oxidation was achieved at pH 5.0.



nmole O2/min
Rate of oxidation
60
50
40
30
20
10
0
2
3
4
5
6
7
pH
0.1M NaCitrate buffer

Oxidation study

<u>*The oxidation of thiosulfate and other substrates*</u>

Various buffers aside from sodium citrate were also analyzed for the oxidation of thiosulfate as shown in Figures 5, 6, and 7 with three increasing concentrations of 0.1 μmole, 1.0 μmole, and 10.0 μmoles sodium thiosulfate. Among the three concentrations of thiosulfate, 1.0 μmole was the best substrate concentration for the pH 5.0 sodium citrate giving the highest oxidation rate. For the complete oxidation of thiosulfate 0.1 μmole was the amount used since the oxygraph reaction vessel contained only 0.3 μmole O_2 in 1.2 mL. For the oxidation rate determinations, 1.0 μmole thiosulfate was used.

Among the various buffers analyzed, 0.1 M sodium citrate, 9K medium, 0.1 M sodium sulfate, 0.1 M potassium phosphate, 50 mM potassium nitrate and 0.1 M sodium chloride at pH 2.3, 5.0, and 7.0, 0.1 M sodium citrate buffer at pH 5.0 provided the best condition for thiosulfate oxidation having an oxidation rate of 80 nmol O_2/min compared to 20 - 30 nmol O_2/min by the other buffers. At pH 2.3, however, the 9K medium was the best for the oxidation of thiosulfate, particularly at low concentrations (70 - 80 nmole O_2/min with 0.1 μmole thiosulfate).

Figure 5. 0.1 μmole of thiosulfate oxidation study.
The oxidation of 0.1 μmole thiosulfate was studied in various buffers and pH's.
The cells analyzed was washed in water and suspended in 0.1M sodium citrate pH 6.0 buffer.

Figure 6. 1.0 μmole of thiosulfate oxidation study.
The oxidation of 1.0 μmole thiosulfate was studied in various buffers and pH's.
The cells analyzed was washed in water and suspended in 0.1M sodium citrate pH 6.0 buffer.

Figure 7. 10.0 μmoles of thiosulfate oxidation study.
The oxidation of 10.0 μmole thiosulfate was studied in various buffers and pH's.
The cells analyzed here was washed in water and suspended 0.1M sodium citrate pH 6.0 buffer.

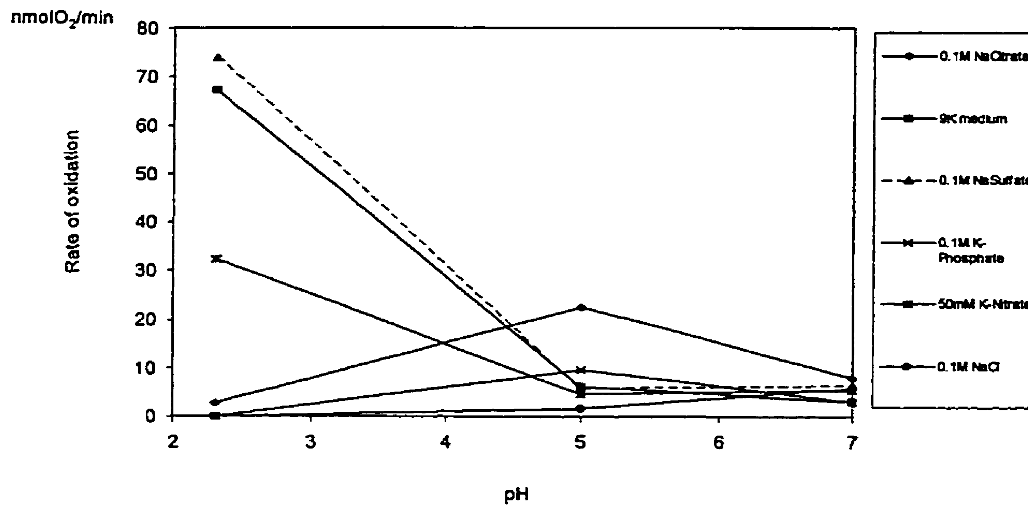


Fig. 5

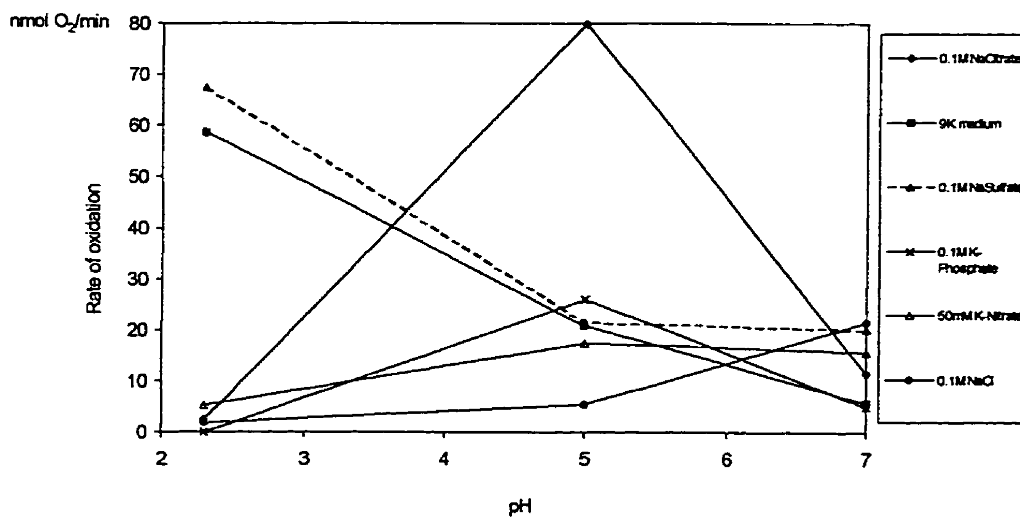


Fig. 6

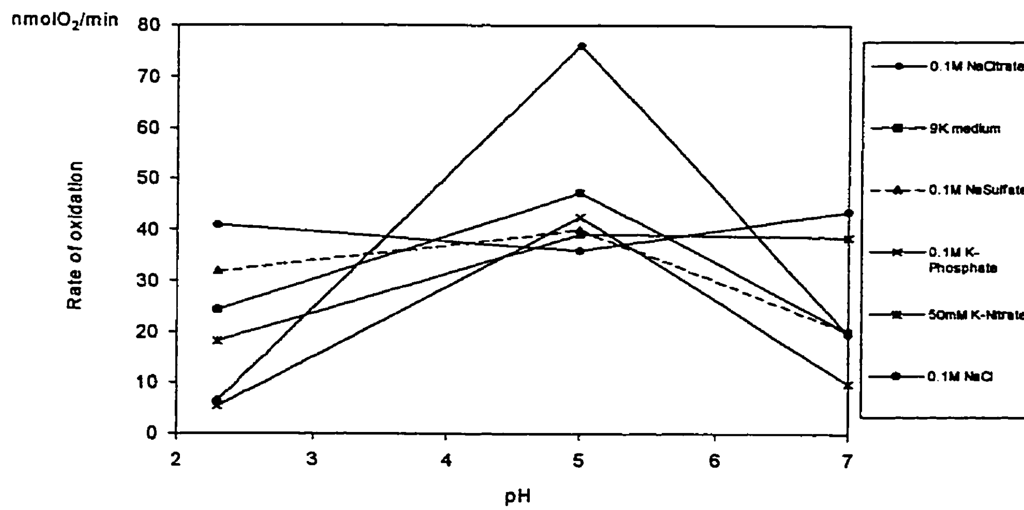


Fig. 7

Tetrathionate, DMSO sulfur, Tween 80 sulfur, and sulfite were also studied in various buffers and conditions as substrates for oxidation. The oxidation rates for tetrathionate, DMSO sulfur, Tween 80 sulfur, and sulfite in 0.1 M sodium citrate at pH 5.0 were 60, 65, 35 and 8 nmol O_2/min respectively, as shown in Figures 8, 9, 10 and 11. Clearly 0.1 M sodium citrate again provided the best condition for all substrate oxidations at pH 5.0. However, the thiosulfate oxidation rate remained the highest among the rates of oxidation of all the substrates tested.

Note that two kinds of cell treatment were analyzed throughout the experiment; (1) twice water washed, 0.1 M sodium citrate (pH 6.0) suspended cells and (2) twice 9K medium washed, 0.1 M sodium citrate (pH 6.0) suspended cells. The results with the water washed cells are shown unless otherwise specified as the results were similar and the water washed cells normally showed higher activities.

Figure 8. Tetrathionate oxidation study.
The oxidation of 60.0 nmole of tetrathionate was studied in 3 different buffers; 0.1M sodium citrate, 9K medium and 0.1M sodium sulfate buffers at different pH's. Two different kind of cells were analyzed; water washed and 9K media washed cells. Both cells were suspended in 0.1M sodium citrate pH 6.0 buffer.

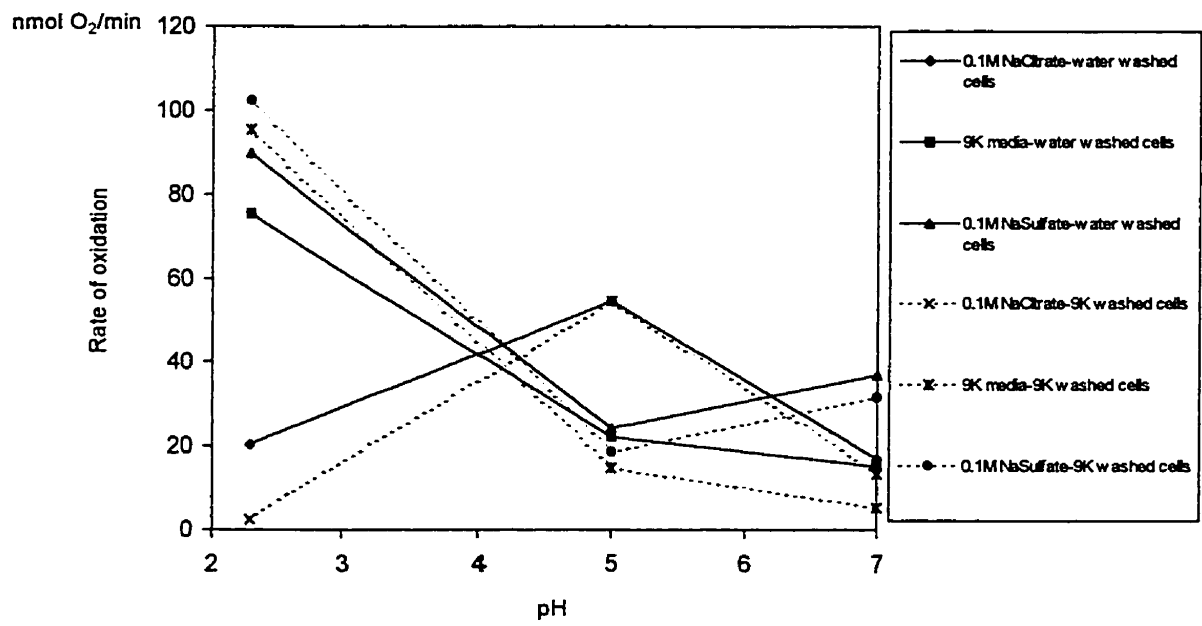

nmol O2/min
Rate of oxidation
120
100
80
60
40
20
0
2
3
4
5
6
7
pH
0.1M NaCitrate-water washed cells
9K media-water washed cells
0.1M NaSulfate-water washed cells
0.1M NaCitrate-9K washed cells
9K media-9K washed cells
0.1M NaSulfate-9K washed cells

Figure 9. DMSO S° oxidation study.
The oxidation of 5 µg DMSO S° was studied in various buffers and pH's.
The cells analyzed was washed in water and suspended in 0.1M sodium citrate pH 6.0 buffer.

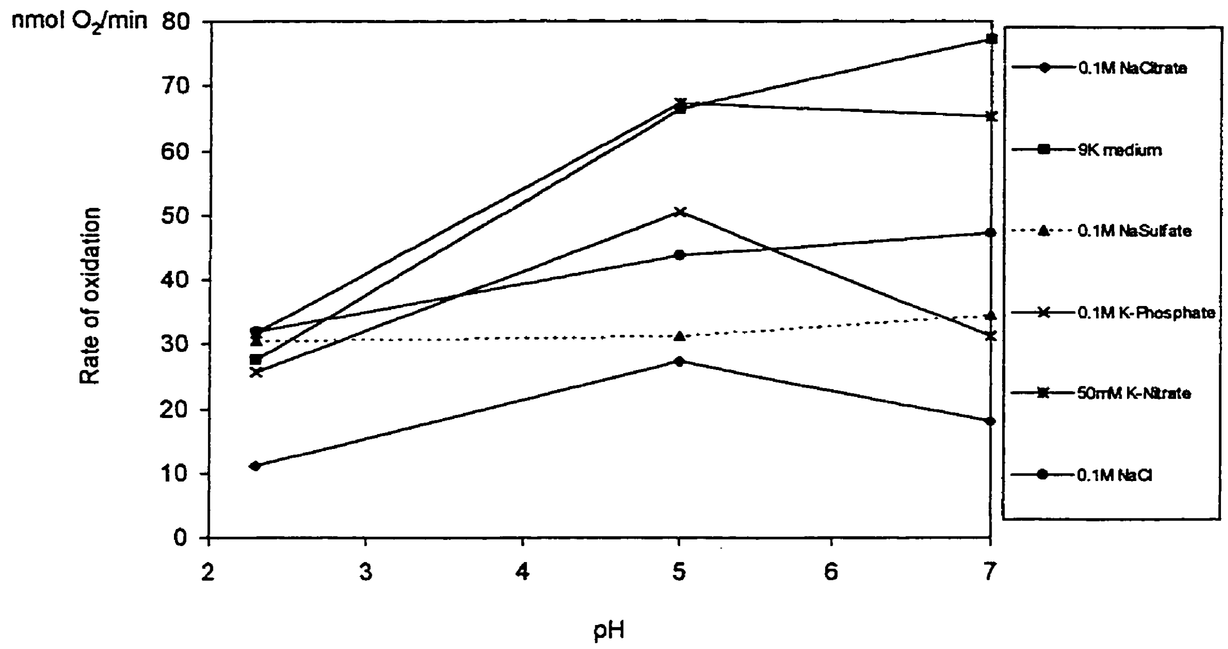

nmol O_2/min
Rate of oxidation
80
70
60
50
40
30
20
10
0
2
3
4
5
6
7
pH
0.1M NaCitrate
9K medium
0.1M NaSulfate
0.1M K-Phosphate
50mM K-Nitrate
0.1M NaCl

Figure 10. Tween 80 sulfur oxidation study.
The oxidation of 0.032 g Tween 80 sulfur was studied in various buffers and pH's.
Kind of cells analyzed : water washed, 0.1M sodium citrate pH 6.0 suspended.

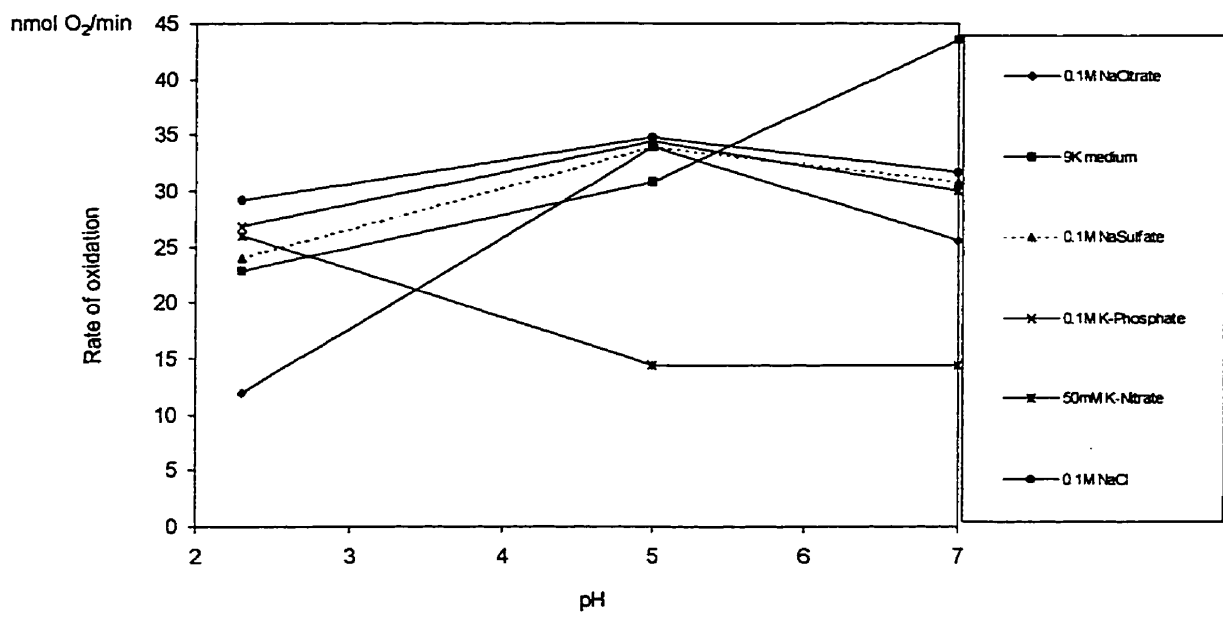
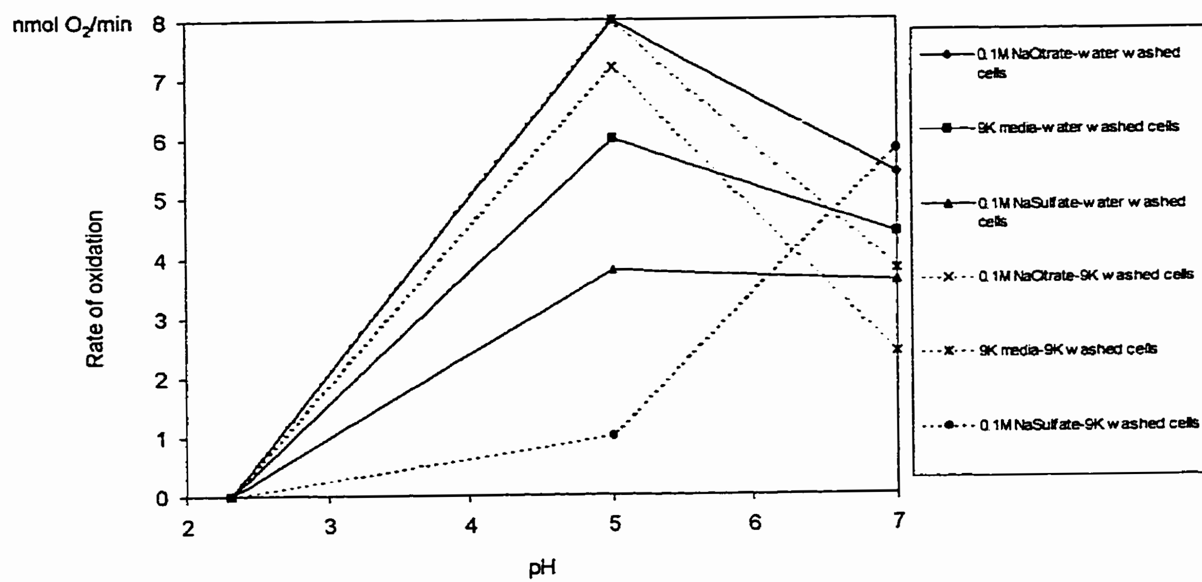

nmol O2/min
Rate of oxidation
45
40
35
30
25
20
15
10
5
0
2
3
4
5
6
7
pH
0.1M NaCitrate
9K medium
0.1M NaSulfate
0.1M K-Phosphate
50mM K-Nitrate
0.1M NaCl

Figure 11. Sulfite oxidation study.
The oxidation of 0.2 μmole of sulfite in EDTA solution was studied in 3 different buffers; 0.1M sodium citrate, 9K medium, and 0.1M sodium sulfate buffer at different pH's. Two kinds of cells were analyzed; water washed and 9K medium washed cells. Both suspended in 0.1M citrate pH 6.0 buffer.


nmol O_2/min
Rate of oxidation
pH
0.1M NaCitrate-water washed cells
9K media-water washed cells
0.1M NaSulfate-water washed cells
0.1M NaCitrate-9K washed cells
9K media-9K washed cells
0.1M NaSulfate-9K washed cells

Effect of metal chelators on substrate oxidation

Surprisingly, the complete oxidation of thiosulfate in 9K growth media at pH 5.0 could not be demonstrated with 0.1 μmole thiosulfate. One would assume that it should provide the best condition for thiosulfate oxidation for it is the growth medium for the cells. Results in Figures 5 - 7 suggest that a much higher concentration of thiosulfate is required for faster oxygen consumption in 9K at pH 5.0, but is not suitable for stoichiometric studies. Sodium citrate at pH 5.0 (0.1M) on the other hand provided the fast and complete oxidation of thiosulfate. This led into an investigation of why the 9K medium at pH 5.0 could not provide the right condition for thiosulfate oxidation. As sodium citrate is known to be a metal chelator, various metal chelators; EDTA, o-phenanthroline, 2,2'-dipyridyl and tiron were tested in the 9K pH 5.0 medium. As shown in Figure 12, EDTA stimulated the rate of thiosulfate oxidation at pH 5.0 in the 9K medium and 0.1M sodium sulfate. The complete oxidation of thiosulfate in the 9K pH 5.0 medium can thus be established with the addition of EDTA. Note that the cell treatment used here was washing in the 9K pH 5.0 and suspending in the 9K pH 5.0, maintaining its original state. Cells that were washed in the 9K pH 5.0 but suspended in 0.1 M sodium citrate showed no effect with EDTA in the 9K pH 5.0 as they were already suspended in the citrate buffer (results not shown).

Figure 12. The effect of EDTA in thiosulfate oxidation.
The oxidation of 0.1 μmole thiosulfate was studied with the addition of 5 μmole of EDTA in 3 different buffers; 0.1M sodium citrate, 9K medium, and 0.1M sodium sulfate at different pH's. Kind of cells analyzed : 9k medium washed, 9K medium suspended. The cell treatment was to maintain them in their original state.
The complete oxidation of thiosulfate in 9K medium and sodium sulfate buffer was achieved.

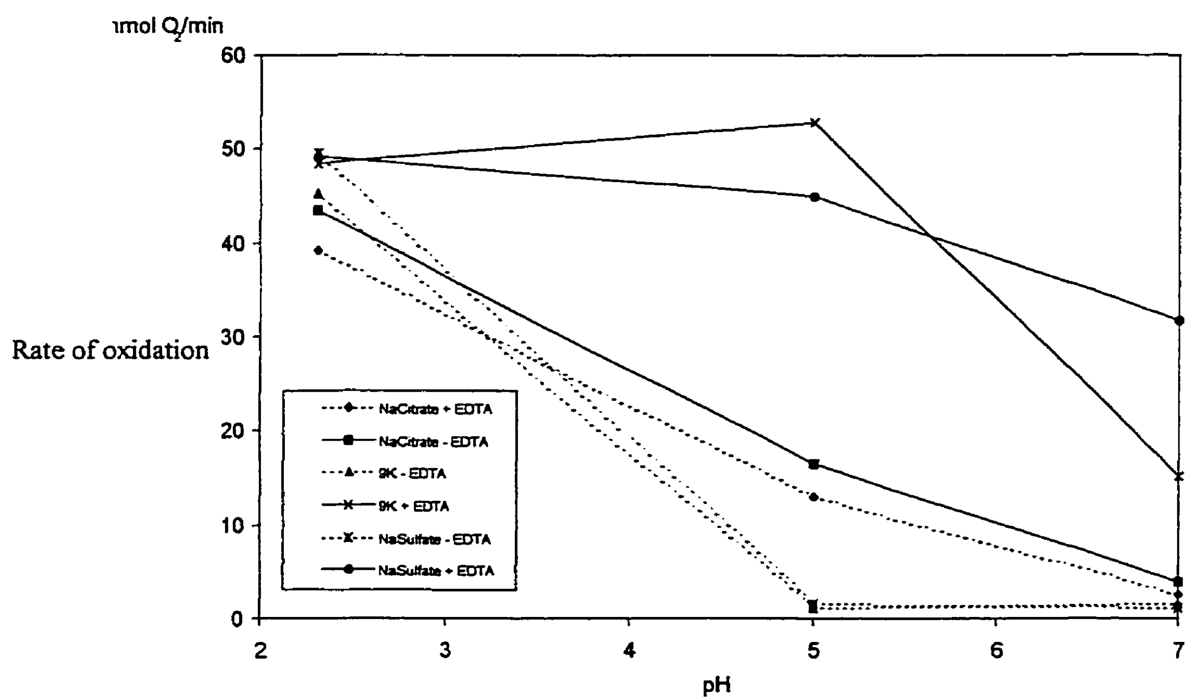

ımol Q2/min
Rate of oxidation
60
50
40
30
20
10
0
2
3
4
5
6
7
pH
NaCitrate + EDTA
NaCitrate - EDTA
9K - EDTA
9K + EDTA
NaSulfate - EDTA
NaSulfate + EDTA

Figure 13 shows the effect of various metal chelators on the oxidation of thiosulfate by water washed, citrate suspended cells in sodium citrate buffer. In general, metal chelators EDTA, o-phenanthroline and 2,2'-dipyridyl seemed to stimulate thiosulfate oxidation. In 9K medium the stimulatory effect was similar but less pronounced (Figure 14). The same stimulatory effect was also shown for tetrathionate oxidation in Figure 15 and 16 although here all four chelators including tiron stimulated activity in the citrate buffer (Figure 15). The effect of metal chelators on sulfur oxidation, however, showed some stimulation and some inhibition (Figures 17 and 18). At pH 5.0, EDTA inhibited the sulfur oxidation in the citrate buffer (Figure 17), but stimulated the oxidation in the 9K medium (Figure 18). The inhibition could be related to the strong inhibition of sulfite oxidation by EDTA (Figure 19). As for sulfite oxidation, all metal chelators had inhibitory effects in both sodium citrate and 9K medium assays as shown in Figures 19 and 20.

Figure 13. The effect of metal chelators on thiosulfate oxidation in citrate buffer.
The oxidation of 0.1 μmole of thiosulfate was studied with the addition of various metal chelators in 0.1M sodium citrate buffer.
Kind of cells analyzed : water washed, 0.1M sodium citrate pH 6.0 suspended.

Figure 14. The effect of metal chelators on thiosulfate oxidation in 9K medium.
The oxidation of 0.1 μmole thiosulfate was studied with the addition of various metal chelators in 9K medium.
Kind of cells : water washed, 0.1M sodium citrate pH 6.0 suspended.

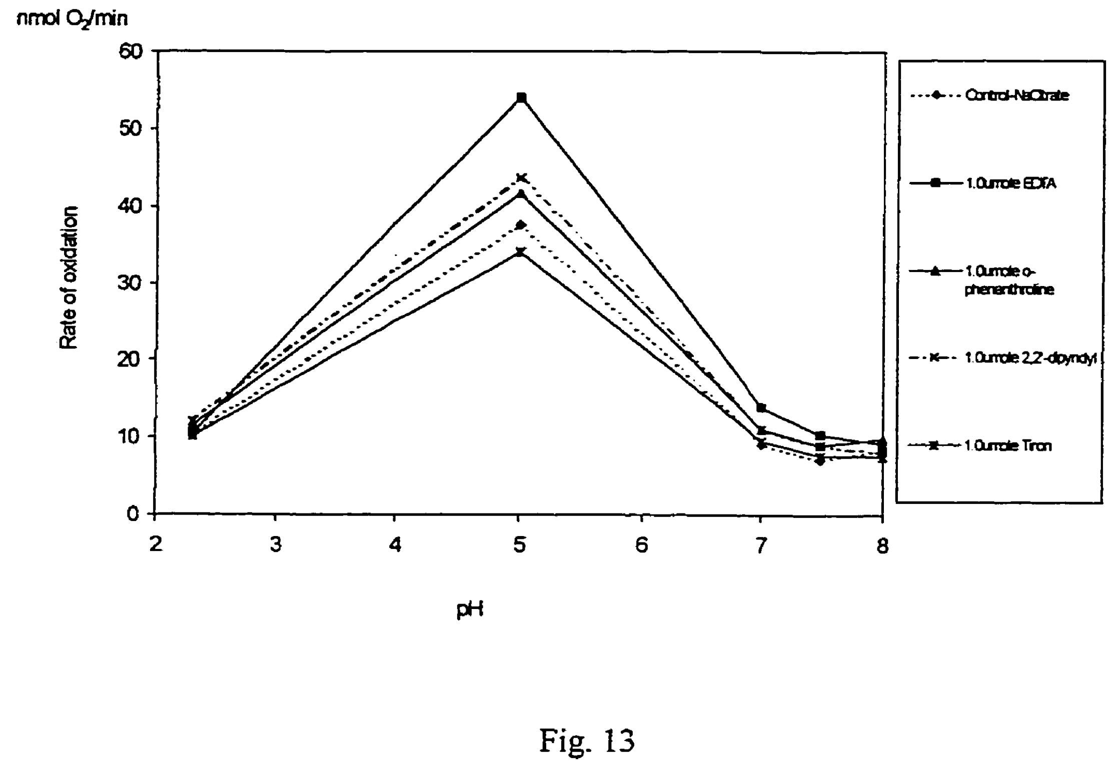


Fig. 13

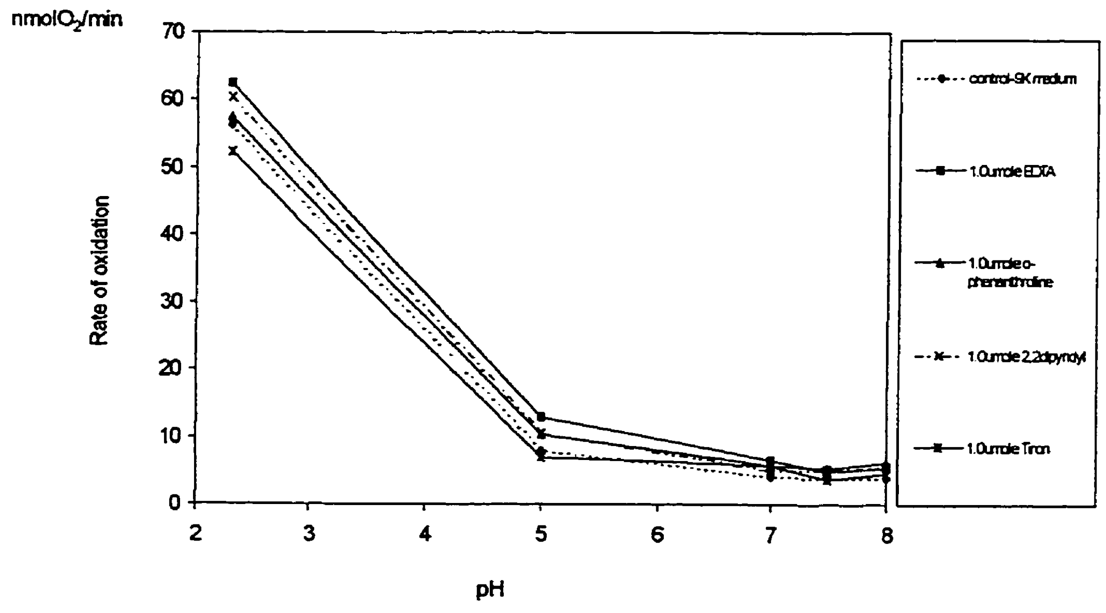


Fig. 14

Figure 15. The effect of metal chelators on tetrathionate oxidation in citrate buffer. The oxidation of 60.0 nmole tetrathionate was studied with the addition of various metal chelators in 0.1M sodium citrate buffer.
Kind of cells : water washed, 0.1M sodium citrate pH 6.0 suspended.

Figure 16. The effect of metal chelators on tetrathionate oxidation in 9K medium. The oxidation of 60.0 nmole tetrathionate was studied with the addition of various metal chelators in 9K medium.
Kind of cells : water washed, 0.1M sodium citrate pH 6.0 suspended.

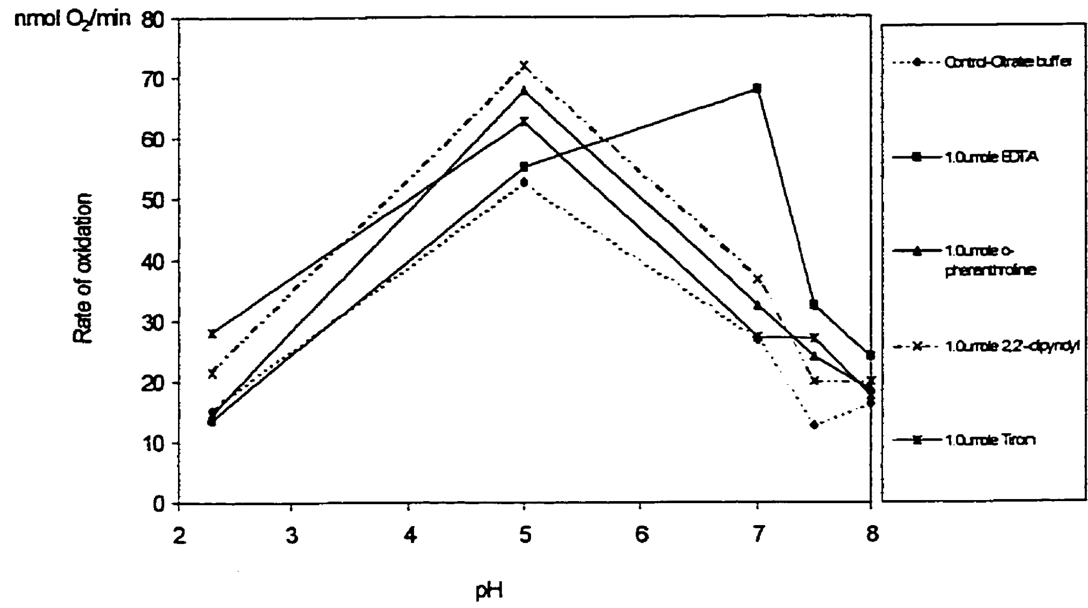


Fig. 15

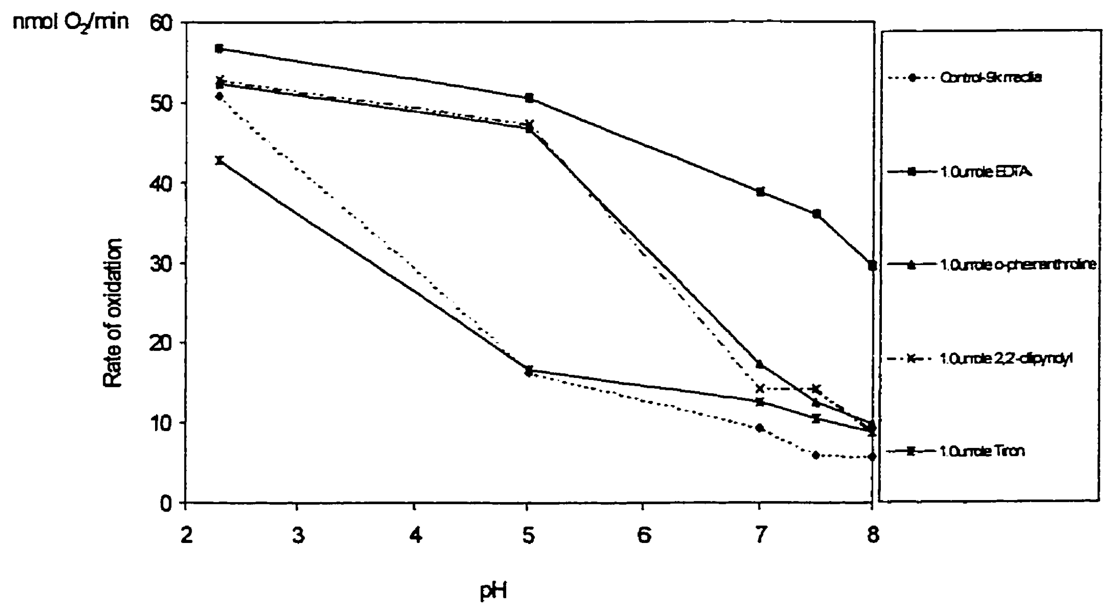


Fig. 16

Figure 17. The effect of metal chelators on DMSO S° oxidation in citrate buffer. The oxidation of 5.0 μg DMSO S° was studied with the addition of various metal chelators in 0.1M sodium citrate buffer.
Kind of cells : water washed, 0.1M sodium citrate pH 6.0 suspended.

Figure 18. The effect of metal chelators on DMSO S° oxidation in 9K medium. The oxidation of 5.0 μg DMSO S° was studied with the addition of various metal chelators in 9K medium.
Kind of cells : water washed, 0.1M sodium citrate pH 6.0 suspended.

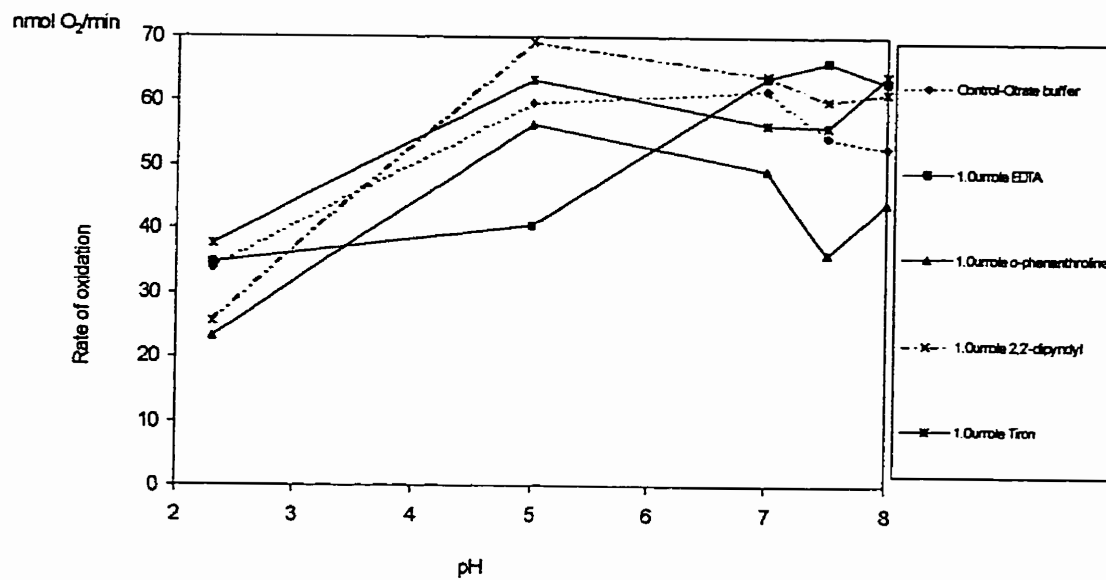


Fig. 17

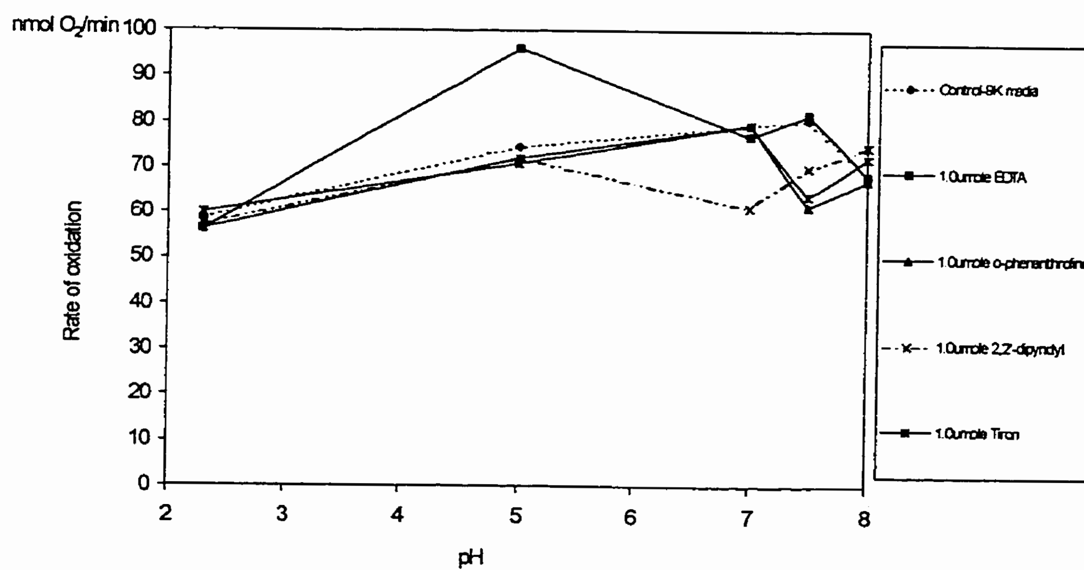


Fig. 18

Figure 19. The effect of metal chelators on sulfite oxidation in citrate buffer.
The oxidation of 0.2 μmole sulfite was studied with the addition of various metal chelators in 0.1M sodium citrate buffer.
Kind of cells : 9K washed, 0.1M sodium citrate pH 6.0 suspended.

Figure 20. The effect of metal chelators on sulfite oxidation in 9K medium.
The oxidation of 0.2 μmole sulfite was studied with the addition of various metal chelators in 9K medium.
Kind of cells : 9K washed, 0.1M sodium citrate pH 6.0 suspended.

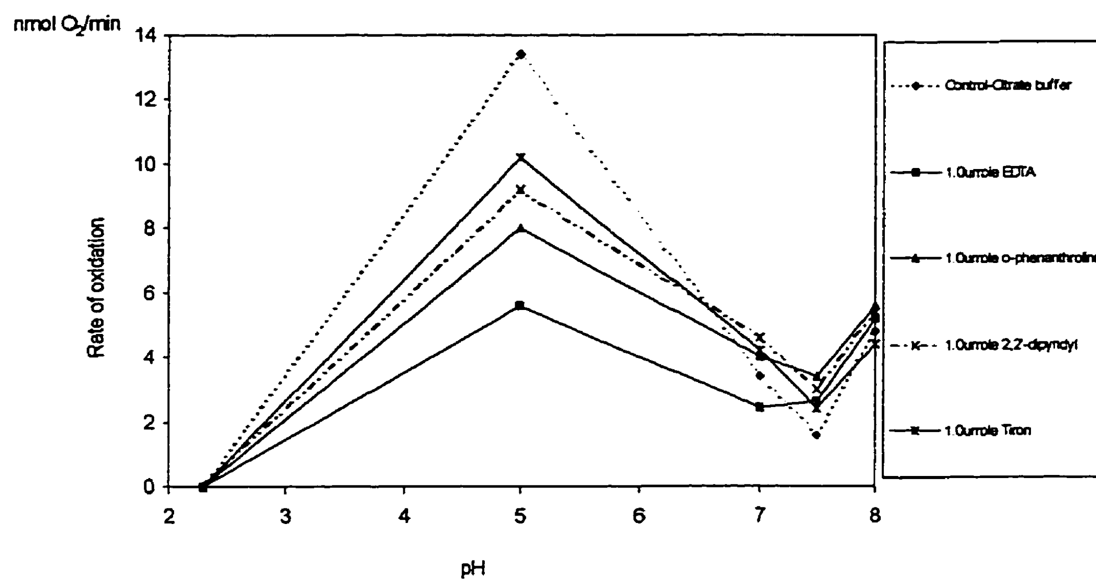


Fig. 19

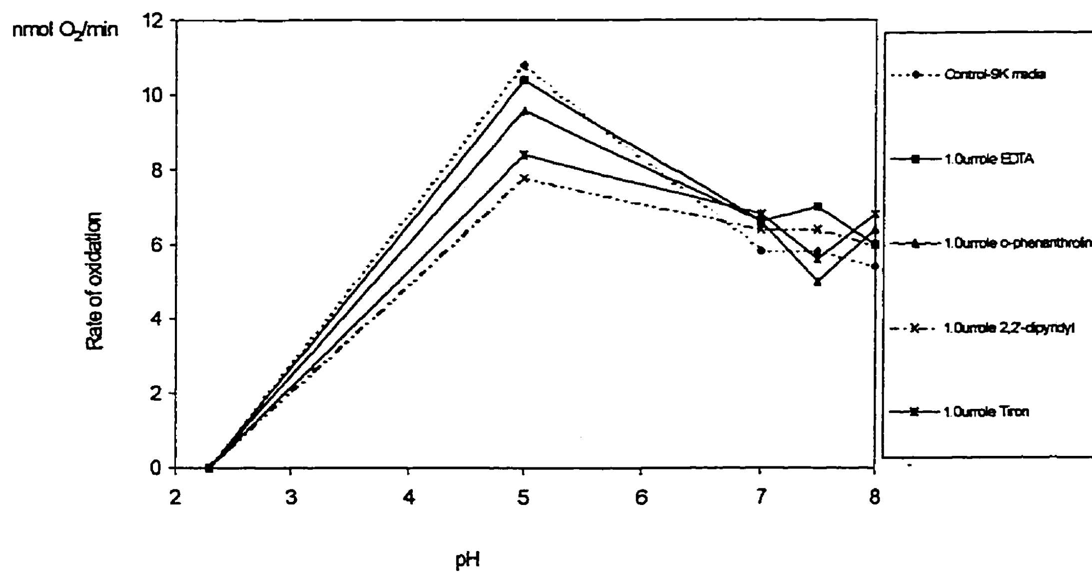


Fig. 20

Effect of azide and cyanide on substrate oxidation

The effects of azide and cyanide on thiosulfate oxidation are shown in Figures 21 and 22. In both citrate and 9K buffers, azide and cyanide inhibited thiosulfate oxidation with azide showing a much stronger effect.

In tetrathionate oxidation (Figures 23 and 24), azide showed a clear inhibition in both buffers, but cyanide had a little effect in the citrate buffer and stimulated the oxidation rate in the 9K buffer at pH 5.0.

DMSO sulfur oxidation in the present of cyanide showed some stimulation at pH 5.0 in both the citrate buffer and the 9K medium, but azide again inhibited the oxidation strongly (Figures 25 and 26).

As for sulfite oxidation, azide strongly inhibited it in the citrate buffer but not at all in the 9K medium (Figures 27 and 28). Cyanide was not included in the figures because the chemical oxidation of sulfite was stimulated by cyanide.

Figure 21. The effect of azide and cyanide on thiosulfate oxidation in citrate buffer.
The oxidation of 0.1 μmole thiosulfate was studied in the presence of inhibitor azide and cyanide in citrate buffer.
Kind of cells : water washed, 0.1M sodium citrate pH 6.0 suspended.

Figure 22. The effect of azide and cyanide on thiosulfate oxidation in 9K medium.
The oxidation of 0.1 μmole thiosulfate oxidation was studied in the presence of azide and cyanide in 9K medium.
Kind of cells : water washed, 0.1M sodium citrate pH 6.0 suspended.

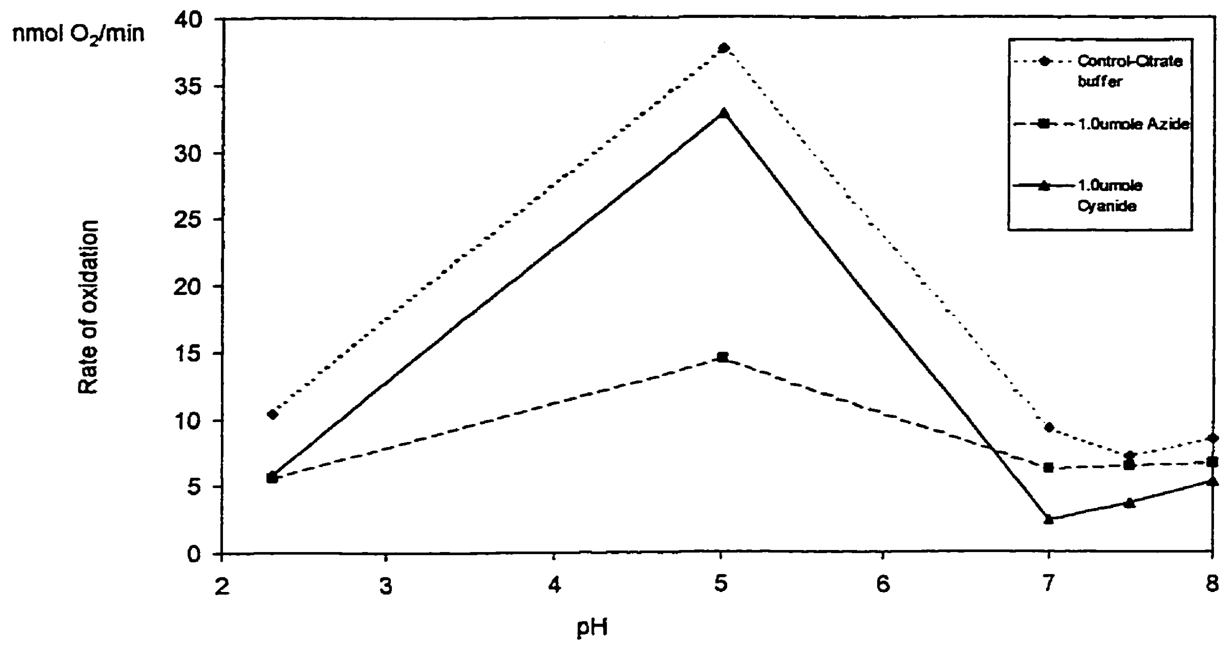


Fig. 21

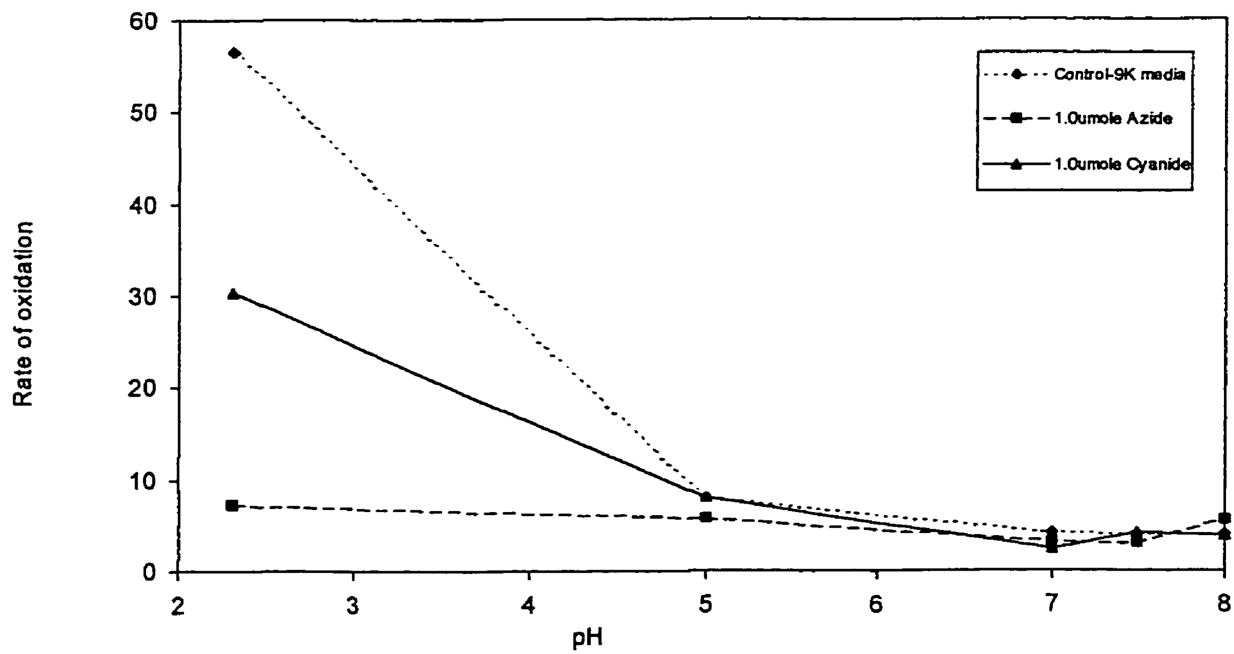


Fig. 22

Figure 23. The effect of azide and cyanide on tetrathionate oxidation in citrate buffer. The oxidation of 60.0 nmole tetrathionate was studied in the presence of inhibitor azide and cyanide in 0.1M sodium citrate buffer.
Kind of cells : water washed, 0.1M sodium citrate pH 6.0 suspended.

Figure 24. The effect of azide and cyanide on tetrathionate oxidation in 9K medium. The oxidation of 60.0 nmole tetrathionate was studied in the presence of inhibitors azide and cyanide in 9K medium.
Kind of cells : water washed, 0.1M sodium citrate pH 6.0 suspended.

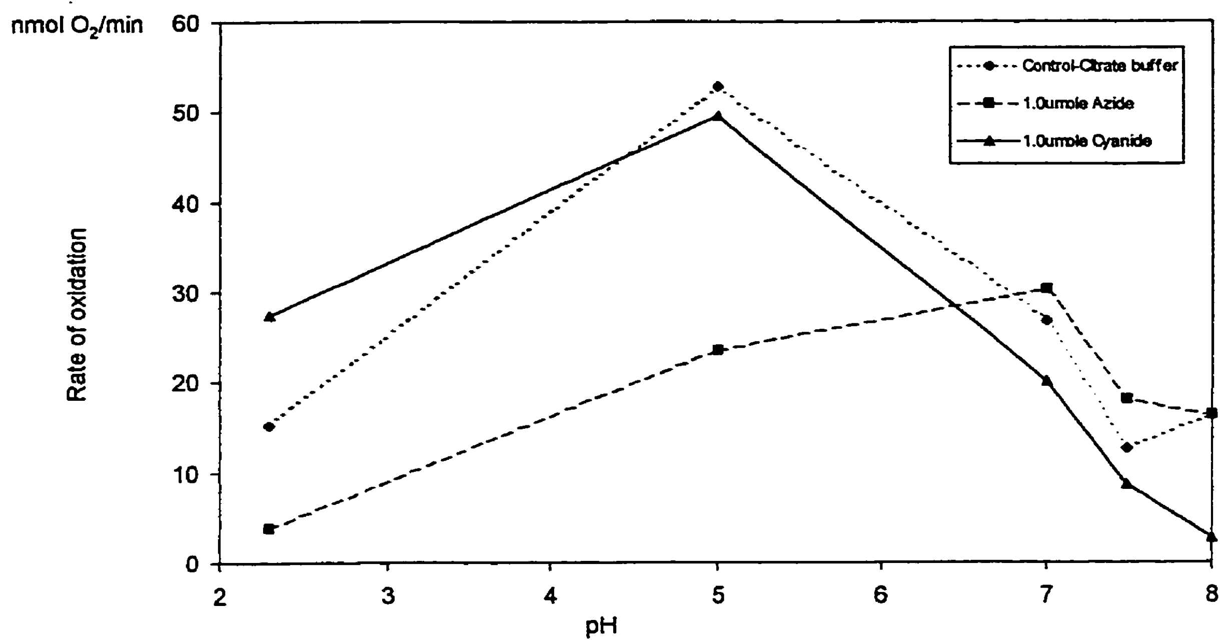


Fig. 23

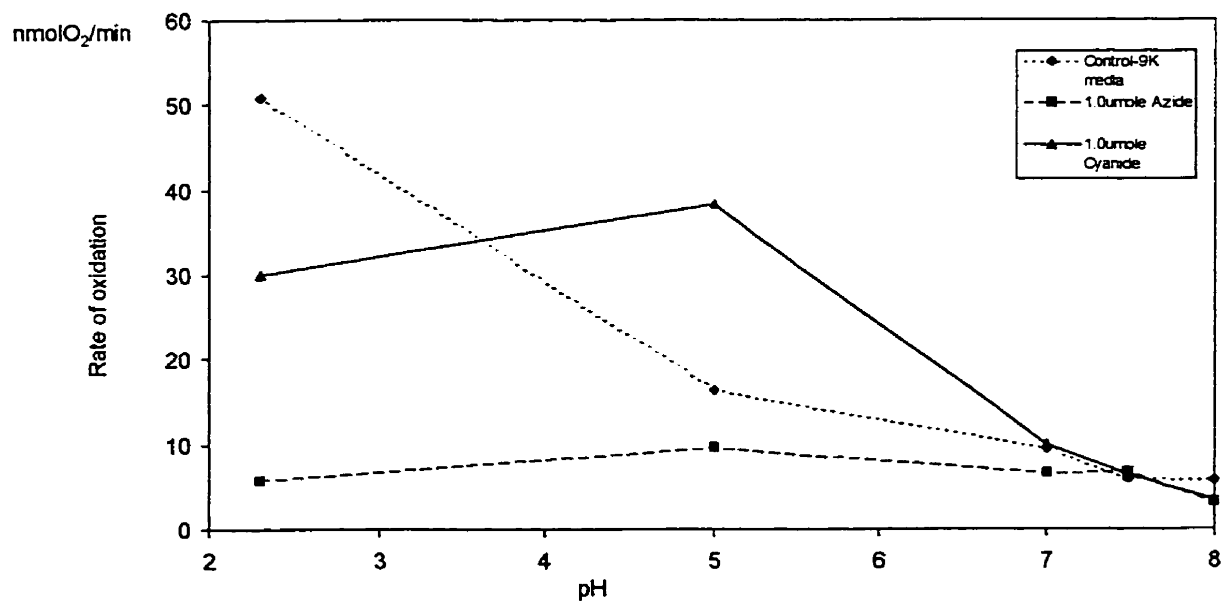


Fig. 24

Figure 25. The effect of azide and cyanide on DMSO S° oxidation in citrate buffer. The oxidation of 5 μg DMSO S° was studied in the presence of inhibitors azide and cyanide in 0.1M sodium citrate buffers.
Kind of cells : water washed, 0.1M sodium citrate pH 6.0 suspended.

Figure 26. The effect of azide and cyanide on DMSO S° oxidation in 9K medium. The oxidation of 5 μg DMSO S° was studied in the presence of inhibitors azide and cyanide in 9K medium.
Kind of cells : water washed, 0.1M sodium citrate pH 6.0 suspended.

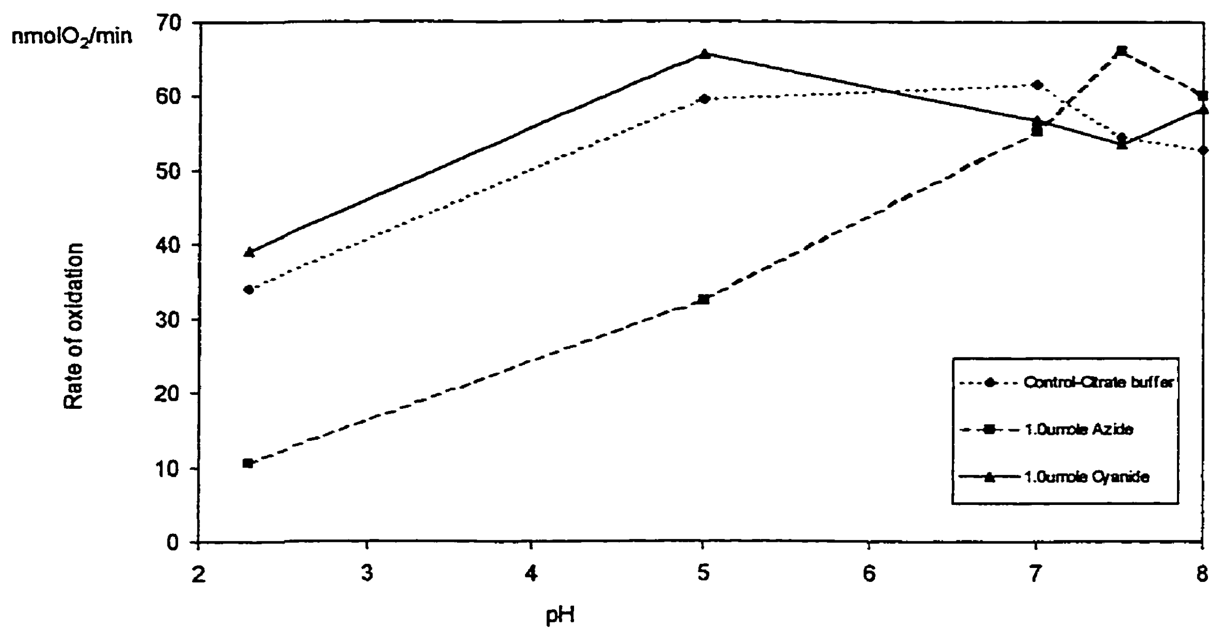


Fig. 25

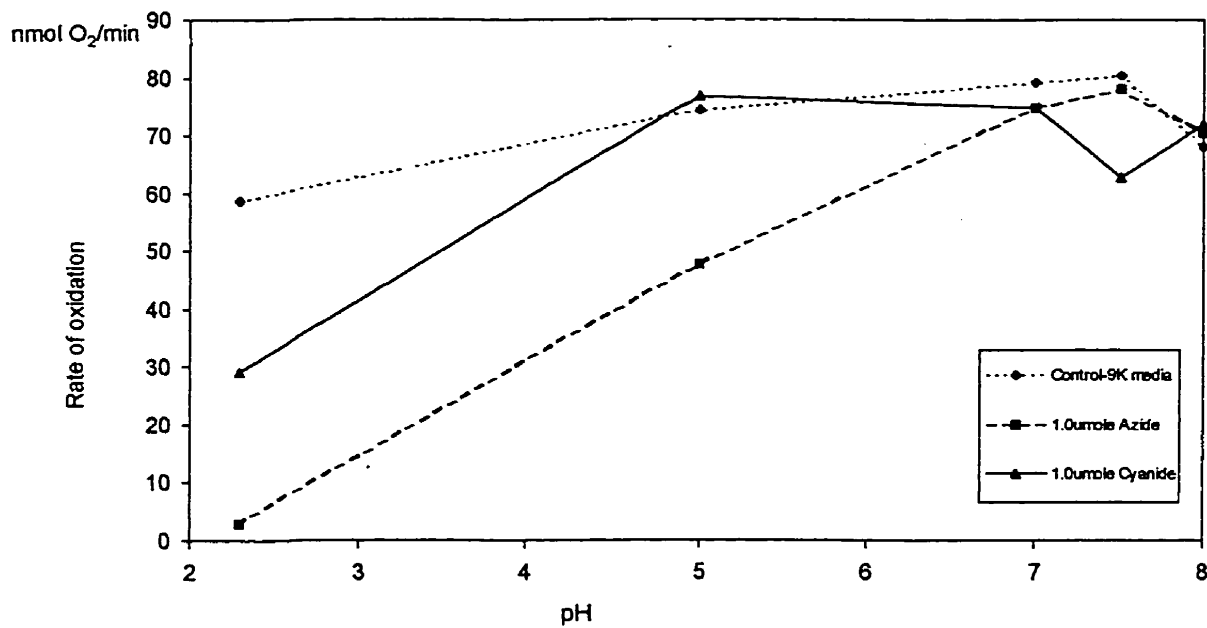


Fig. 26

Figure 27. The effect of azide and cyanide on sulfite oxidation in citrate buffer. The oxidation of 0.2 μmole sulfite in EDTA solution was studied in the presence of inhibitors azide and cyanide in 0.1M sodium citrate buffer.
Kind of cells : water washed, 0.1M sodium citrate pH 6.0 suspended.

Figure 28. The effect of azide and cyanide on sulfite oxidation in 9K medium. The oxidation 0.2 μmole sulfite in EDTA solution was studied in the presence of inhibitors azide and cyanide in 9K medium.
Kind of cells : water washed, 0.1M sodium citrate pH 6.0 suspended.

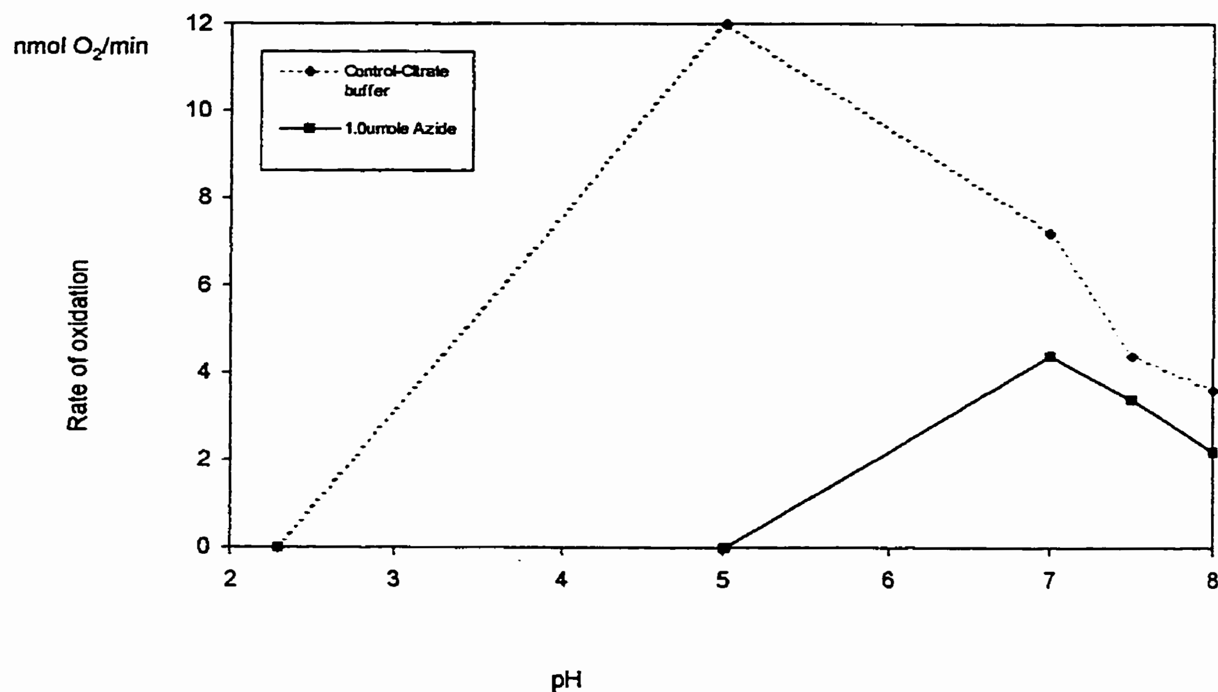


Fig. 27

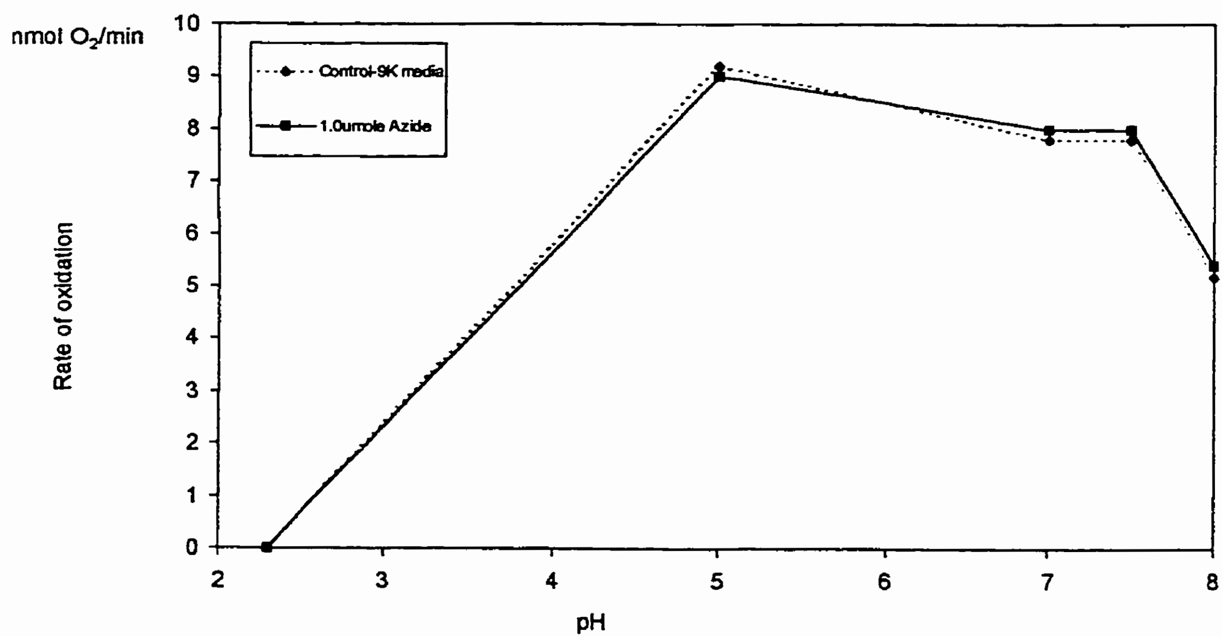


Fig. 28

Effect of other inhibitors and uncouplers on thiosulfate and tetrathionate oxidations

Thiosulfate oxidation

Figures 29, 30 and 31 show the effect of NEM, 2,4-DNP, HQNO and CCCP on thiosulfate oxidation in three different buffers : citrate, 9K medium, and sodium sulfate. Generally, the degree of inhibition with each inhibitor depended on different assay buffer conditions. In citrate pH 5.0, HQNO and NEM showed the greatest inhibition and 2,4-DNP the least, followed by CCCP. At pH 2.3 in 9K and sodium sulfate buffers, they all inhibited by various degrees. These three assay systems, i.e. citrate pH 5.0, 9K pH 2.3 and sodium sulfate pH 2.3 were selected as the conditions to be focused on and the stoichiometric studies were mainly conducted on these three conditions where the complete oxidation of thiosulfate can be established.

Figure 29. The effect of inhibitors; NEM, 2,4-DNP, HQNO and CCCP on thiosulfate oxidation in citrate buffer.
The oxidation of 0.1 μmole thiosulfate was studied in the presence of various inhibitors in 0.1M sodium citrate buffer.
Kind of cells : water washed, 0.1M sodium citrate pH 6.0 suspended.

Figure 30. The effect of inhibitors NEM, 2,4-DNP, HQNO and CCCP on thiosulfate oxidation in 9K medium.
The oxidation of 0.1 μmole thiosulfate was studied in the presence of various inhibitors in 9K medium.
Kind of cells : water washed, 0.1M sodium citrate pH 6.0 suspended.

Figure 31. The effect of inhibitors NEM, 2,4-DNP, HQNO, and CCCP on thiosulfate oxidation in sulfate buffer.
The oxidation of 0.1 μmole thiosulfate was studied in the presence of various inhibitors in 0.1M sodium sulfate buffer.
Kind of cells : water washed, 0.1M sodium citrate pH 6.0 suspended.

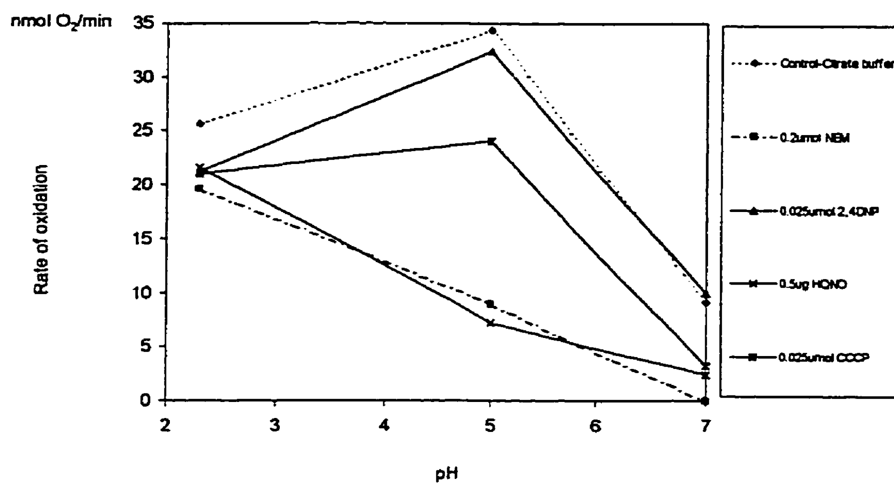


Fig. 29

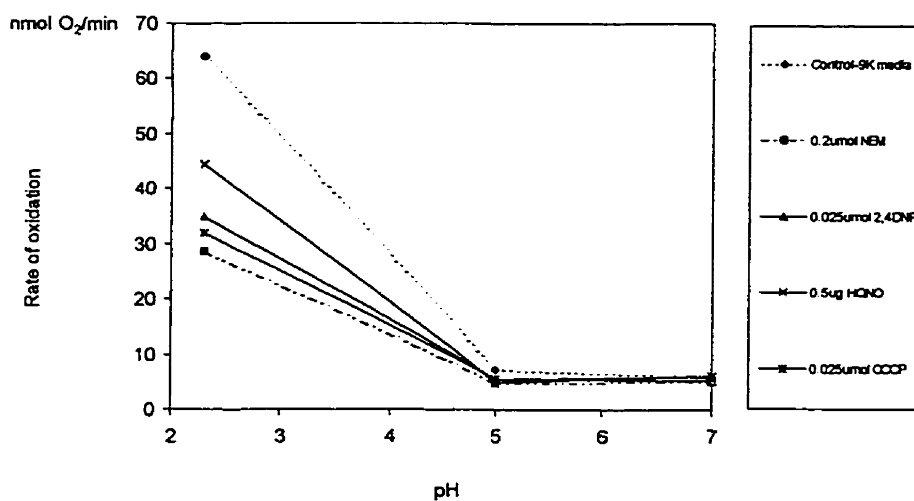


Fig. 30

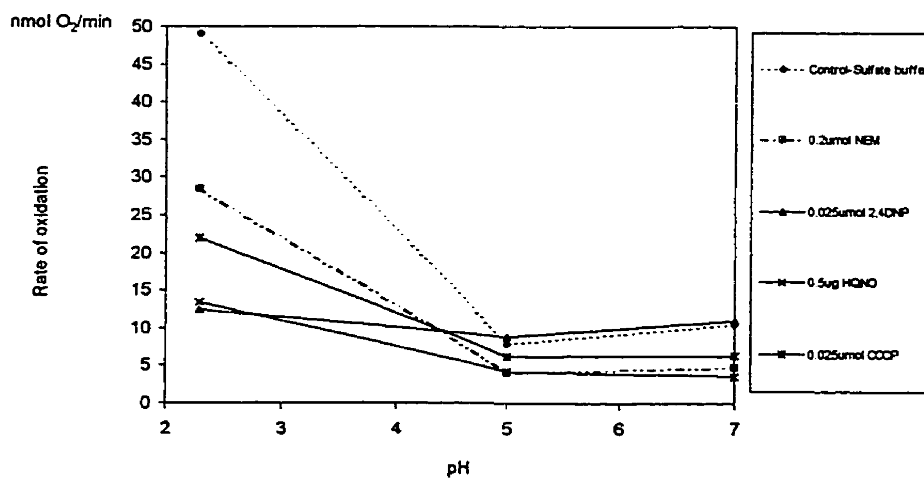


Fig. 31

Tetrathionate oxidation

Tetrathionate oxidation was inhibited by all these inhibitors and uncouplers, but was most sensitive to NEM inhibition and least sensitive to 2,4-DNP in citrate at pH 5.0 as shown in Figure 32. In 9K medium and sulfate at pH 2.3, they were all inhibitory, but 2,4-DNP and CCCP were strongest inhibitors (Figures 33 and 34). NEM was also inhibitory, but HQNO was the least inhibitory in 9K and actually stimulatory in the sulfate buffer. In these two buffers at pH 5.0, however, NEM was the strongest inhibitor and HQNO was also inhibitory in sulfate. CCCP and 2,4-DNP were not inhibitory at all. Hallberg et al.(1996) reported that *Thiobacillus caldus* KU, a thermophilic acidophile was inhibited by CCCP in tetrathionate oxidation at pH 3.0. The strong inhibitory effect of uncouplers at pH 2.3 agrees with their role as "protonophore" or proton translocating compound. The penetration of protons from the acid environment to the cell must be harmful for tetrathionate oxidation. Even in the citrate buffer their inhibitory effect is pronounced at pH 2.3.

Figure 32. The effect of inhibitors NEM, 2,4-DNP, HQNO, and CCCP on tetrathionate oxidation in citrate buffer.
The oxidation of 60.0 nmole tetrathionate was studied in the presence of various inhibitors in 0.1M sodium citrate buffer.
Kind of cells : water washed, 0.1M sodium citrate pH 6.0 suspended.

Figure 33. The effect of inhibitors NEM, 2,4-DNP, HQNO, and CCCP on tetrathionate oxidation in 9K medium.
The oxidation of 60.0 nmole tetrathionate was studied in the presence of various inhibitors in 9K medium.
Kind of cells : water washed, 0.1M sodium citrate pH 6.0 suspended.

Figure 34. The effect of inhibitors NEM, 2,4-DNP, HQNO, and CCCP on tetrathionate oxidation in sulfate buffer.
The oxidation of 60.0 nmole tetrathionate was studied in the presence of various inhibitors in 0.1M sodium sulfate buffer.
Kind of cells : water washed, 0.1M sodium citrate pH 6.0 suspended.

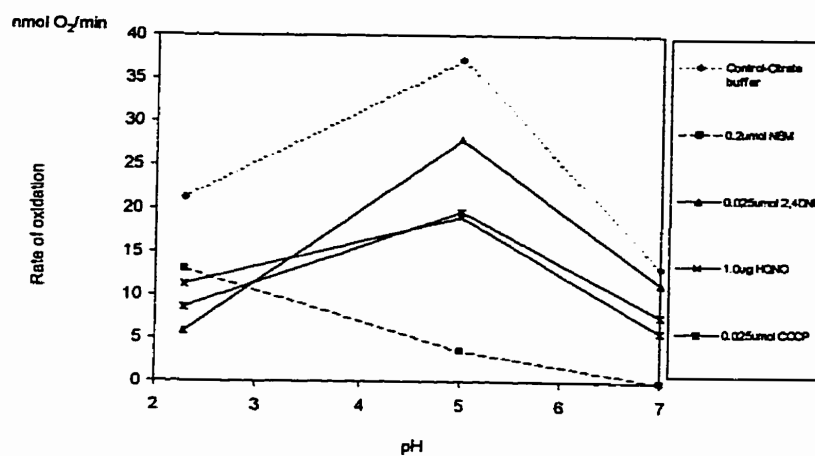


Fig. 32

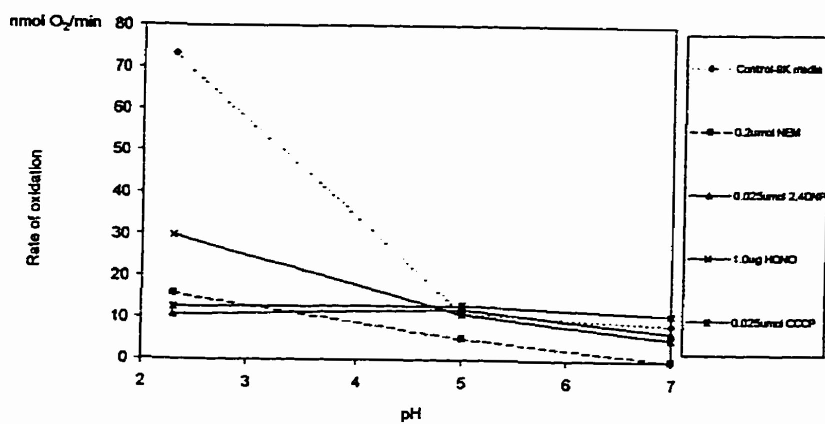


Fig. 33

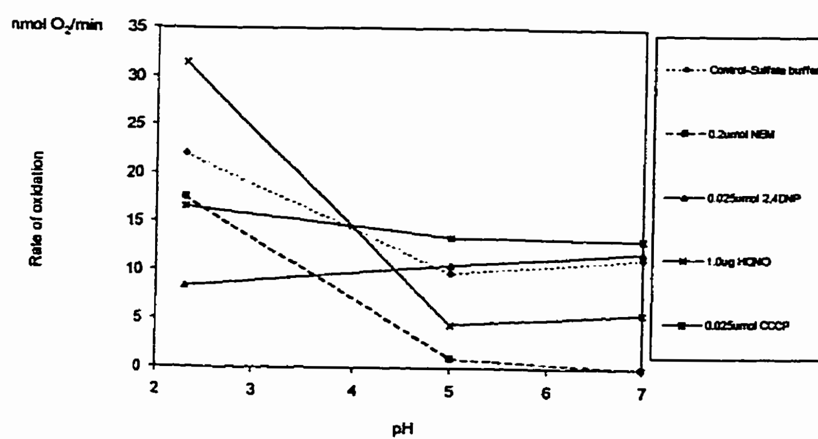


Fig. 34

Stoichiometric studies

Stoichiometric studies on thiosulfate, tetrathionate and sulfur oxidation were based on (a) sulfite determination, b) sulfur determination, and c) tetrathionate determination in addition to measurement of oxygen consumption.

Tables 1, 2 and 3 show the results of sulfite determination with inhibitor HQNO in thiosulfate, tetrathionate and DMSO sulfur oxidations. HQNO is supposed to inhibit sulfite oxidation.

I. Thiosulfate oxidation - HQNO

In the presence of HQNO, thiosulfate oxidation in 0.1 M sodium citrate pH 5.0 assay showed that theory A was applied.

Theory A $S\text{-}SO_3^{2-} + O_2 + 3H_2O \rightarrow 2H_2SO_3 + 2OH^-$ (HQNO)

Based on Table 1, in the presence of HQNO, 160 nmoles of sulfite accumulated from 100 nmoles of thiosulfate, which was close to the value expected in theory A where two sulfites are formed from one thiosulfate. Sulfite determination in this case was very difficult because of the instability of sulfite and long reaction periods required for the complete oxidation due to the slow rate of oxidation in the presence of HQNO.

In 9K at pH 2.3 in the presence of HQNO, theory B of thiosulfate oxidation seemed to be followed where one sulfite is formed from one thiosulfate.

Theory B $S\text{-}SO_3^{2-} + 1\frac{1}{2}O_2 + H_2O \rightarrow H_2SO_3 + SO_4^{2-}$ (HQNO)

Results showed 84 nmoles of sulfite formed from 100 nmoles thiosulfate with consumption of 150 nmoles of oxygen.

However, in 0.1 M sodium sulfate at pH 2.3, only half the amount of sulfite as expected from theory B was found. This could be due to the less inhibitory effect by

HQNO of sulfite oxidation (only 34.2 % inhibition of O_2 consumption rate of thiosulfate oxidation).

Also in 9K at pH 5.0 in the presence of EDTA, the amount of sulfite formed was much less than in the citrate buffer and was closer to the value expected from theory B rather than A.

Table 1. Sulfite accumulation during thiosulfate oxidation in the presence of an inhibitor HQNO

Substrate + 1.0 μg HQNO (in specific pH and buffer)	Sulfite formed (nmol)	O_2 consumed (nmol)	Oxidation rate	
			nmol O_2/min	% Inhibition by HQNO
0.1 M sodium citrate pH 5				
100 nmol thiosulfate Control	19	180	34.4	
100 nmol thiosulfate + HQNO	160	150	6.8	80.2
9K pH 5 + 5 μmol EDTA				
100 nmol thiosulfate Control	0	160	49.2	
100 nmol thiosulfate + HQNO	73	130	22.4	54.5
9 K pH 2.3				
100 nmol thiosulfate Control	1.2	200	49	
100 nmol thiosulfate + HQNO	84	150	24	51.0
0.1 M sodium sulfate pH 2.3				
100 nmol thiosulfate Control	0	195	76	
100 nmol thiosulfate + HQNO	44.4	180	50	34.2

II. Tetrathionate oxidation - HQNO

Table 2 shows that in tetrathionate oxidation, the amount of sulfite formed was insufficient for either theory (two for A, three for B). In the citrate buffer at pH 5.0 the value of 96 nmoles may be considered to be close to 120 nmoles expected for theory B. The difficulty with tetrathionate is that it can react with sulfite as well as polysulfide found in the cells and therefore an appropriate determination could not be achieved.

Table 2. Sulfite accumulation during tetrathionate oxidation in the presence of an inhibitor HQNO

Substrate + 1.0 µg HQNO (in specific pH and buffer)	Sulfite formed (nmol)	O_2 consumed (nmol)	Oxidation rate	
			nmol O_2/min	% Inhibition by HQNO
0.1 M sodium citrate pH 5				
60 nmol tetrathionate Control	39	170	60.8	
60 nmol tetrathionate + HQNO	96	150	22.4	63.2
9K pH 5 + 5 µmole EDTA				
60 nmol tetrathionate Control	7	180	50.8	
60 nmol tetrathionate + HQNO	18	140	27.8	45.3
9K pH 2.3				
60 nmol tetrathionate Control	0	160	66	
60 nmol tetrathionate + HQNO	57	130	37.2	43.6
0.1 M sodium sulfate pH 2.3				
60 nmol tetrathionate Control	0	160	74	
60 nmol tetrathionate + HQNO	55	125	31	58.1

III. Sulfur oxidation - HQNO

In the presence of HQNO (Table 3), sulfur oxidation in citrate buffer, 9K medium, sodium sulfate buffer and phosphate at pH 5.0 showed that the amount of sulfite accumulated was approximately (73 - 88 %) 1:1 to sulfur which is consistent with the equation.

$$S + O_2 + H_2O \rightarrow H_2SO_3 \qquad (HQNO)$$

Table 3. Sulfite accumulation during DMSO S° oxidation in the presence of an inhibition HQNO

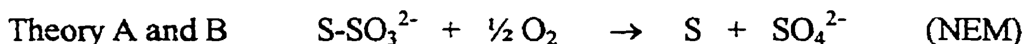
Substrate + 1.0 µg HQNO (in specific pH and buffer)	Sulfite formed (nmol)	O_2 consumed (nmol)	Oxidation rate	
			nmol O_2/min	% Inhibition by HQNO
0.1 M sodium citrate pH 2.3				
5 µg DMSO S° Control	3	180	20.8	
5 µg DMSO S° + HQNO	33	100	9.8	52.9
0.1 M sodium citrate pH 5				
5 µg DMSO S° Control	21	195	56.4	
5 µg DMSO S° + HQNO	123	145	35.6	36.9
0.1 M sodium citrate pH 7				
5 µg DMSO S° Control	86	205	65.6	
5 µg DMSO S° + HQNO	49	130	37.6	42.7
9K pH 2.3				
5 µg DMSO S° Control	16	185	54.4	
5 µg DMSO S° + HQNO	55	140	14	74.3
9K pH 5				
5 µg DMSO S° Control	3	180	54.8	
5 µg DMSO S° + HQNO	137	125	29.2	46.7

Substrate + 1.0 µg HQNO (in specific pH and buffer)	Sulfite formed (nmol)	O_2 consumed (nmol)	Oxidation rate	
			nmol O_2/min	% Inhibition by HQNO
9K pH 7				
5 µg DMSO S° Control	11	180	58.4	
5 µg DMSO S° + HQNO	116	115	34.4	41.1
0.1 *M sodium sulfate pH 2.3*				
5 µg DMSO S° Control	0	190	54.4	
5 µg DMSO S° + HQNO	69	150	16.4	69.9
0.1 M sodium sulfate pH 5				
5 µg DMSO S° Control	3	195	56.8	
5 µg DMSO S° + HQNO	122	130	34.4	39.4
0.1 M sodium sulfate pH 7				
5 µg DMSO S° Control	4	180	62	
5 µg DMSO S° + HQNO	120	140	44.4	28.4
0.1 M potassium phosphate pH 2.3				
5 µg DMSO S° Control	6	180	51.6	
5 µg DMSO S° + HQNO	62	135	16.4	68.2
0.1 M potassium phosphate pH 5				
5 µg DMSO S° Control	6	175	59.6	
5 µg DMSO S° + HQNO	114	125	36.4	38.9
0.1 M potassium phosphate pH 7				
5 µg DMSO S° Control	10	180	64.8	
5 µg DMSO S° + HQNO	110	115	38.8	40.1

The sulfite recovery was lower at pH 2.3 due to SO_2 volatility, but at pH 7.0 the recovery was similar to that of pH 5.0 (with the exception of citrate).

IV. Thiosulfate oxidation - NEM

In theory (A and B), one sulfur will be formed from one thiosulfate in the presence of NEM.



Theory A and B $S\text{-}SO_3^{2-} + \frac{1}{2} O_2 \rightarrow S + SO_4^{2-}$ (NEM)

Table 4 was based on the effect of NEM in the accumulation of elemental sulfur during the oxidation of thiosulfate and tetrathionate oxidation. NEM is supposed to inhibit the oxidation of sulfur to sulfite. Results showed that one mole of sulfur was formed from every mole of thiosulfate with ½ mole of oxygen being consumed in 0.1 M sodium citrate at pH 5.0, 9K at pH 2.3, and 0.1M sodium sulfate at pH 2.3 in agreement with the above equation.

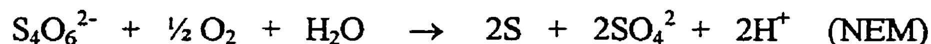
Table 4. Sulfur accumulation during thiosulfate and tetrathionate oxidations in the presence of an inhibitor NEM

Substrate + 1.0 μmol NEM (in specific pH and buffer)	Sulfur formed (nmol)	O_2 consumed (nmol)	Oxidation rate	
			nmol O_2/min	% Inhibition
0.1M sodium citrate pH 5				
100 nmol thiosulfate Control	0	200	43.2	
100 nmol thiosulfate + NEM	96	50	12.8	70.4
200 nmol thiosulfate + NEM	188	30	5	88.4
9K pH 5 + 5 μmol EDTA				
100 nmol thiosulfate Control	44	180	51.2	
100 nmol thiosulfate + NEM	16	40	7.2	85.9
200 nmol thiosulfate + NEM	24	50	9.6	81.3
0.1M sodium sulfate pH 5				
100nmol thiosulfate Control	0	170	24	
100 nmol thiosulfate + NEM	64	40	12.8	46.7
200 nmol thiosulfate + NEM	80	50	11.6	51.7
9K pH2.3				
100 nmol thiosulfate Control	56	190	25.6	
100 nmol thiosulfate + NEM	108	50	16.4	35.9
200 nmol thiosulfate + NEM	220	160	10.2	60.2
0.1M sodium sulfate pH 2.3				
100 nmol thiosulfate Control	12	190	25.6	
100 nmol thiosulfate + NEM	116	50	30	0
200 nmol thiosulfate + NEM	200	100	30	0

Substrate + 1.0 μmol NEM (in specific pH and buffer)	Sulfur formed (nmol)	O_2 consumed (nmol)	Oxidation rate	
			nmol O_2/min	% Inhibition
0.1 M sodium citrate pH 5				
60 nmol tetrathionate				
Control	0	180	20	
60 nmol tetrathionate				
+ NEM	68	20	2.8	86
120 nmol tetrathionate				
+ NEM	200	100	30	88
9K pH 5 + 5 μmol EDTA				
60 nmol tetrathionate				
Control	0	170	45.2	
60 nmol tetrathionate				
+ NEM	16	20	4.4	90.3
120 nmol tetrathionate	88	15	2.4	94.7
0.1 M sodium sulfate pH 5				
60 nmol tetrathionate				
Control	0	150	23.2	
60 nmol tetrathionate				
+ NEM	72	30	9.6	58.6
120 nmol tetrathionate	60	20	4.8	79.31
9K pH 2.3				
60 nmol tetrathionate				
Control	0	140	50	
60 nmol tetrathionate				
+ NEM	132	40	14.4	71.2
120 nmol tetrathionate				
+ NEM	196	60	14.8	70.4
0.1 *M sodium sulfate pH 2.3*				
60 nmol tetrathionate				
Control	44	170	50.8	
60 nmol tetrathionate	124	60	21.6	57.5
120 nmol tetrathionate	120	40	18	64.6

V. Tetrathionate oxidation - NEM

In theory (A and B), two sulfur will be formed from one tetrathionate in the presence of NEM.



$$S_4O_6^{2-} + \frac{1}{2}O_2 + H_2O \rightarrow 2S + 2SO_4^{2} + 2H^+ \quad \text{(NEM)}$$

Most of the results showed less than two sulfur accumulation except for 9K pH 2.3 assay and citrate pH 5.0 (120 nmoles tetrathionate) where it accumulated approximately two sulfur from one tetrathionate. The concentration of tetrathionate seems to be critical depending on the buffer and pH on the stoichiometry of sulfur accumulation. This was probably due to the chemical interaction among polythionates, endogenous polysulfides, sulfur and sulfite.

VII. Thiosulfate oxidation - NEM - HQNO

Table 5 shows the tetrathionate accumulation studies when tetrathionate determination was carried out in the presence of both inhibitors HQNO and NEM during thiosulfate oxidation. In theory A rhodanese cannot cleave thiosulfate since both the oxidation of sulfur and that of sulfite will be inhibited. In theory B the oxidation of thiosulfate to tetrathionate should not be inhibited, but the amount of tetrathionate accumulated could depend on the rate of tetrathionate hydrolase reaction. Thus, thiosulfate oxidation should be totally inhibited in theory A without any tetrathionate formation, while in theory B two moles of thiosulfate should be oxidized to one mole of tetrathionate : $2S\text{-}SO_3^{2-} + \frac{1}{2}O_2 + 2H^+ \rightarrow S_4O_6^{2-} + H_2O$ (HQNO + NEM)

Possibly followed by tetrathionate hydrolase

Results showed that in the presence of both HQNO and NEM, in 0.1 M sodium citrate at pH 5.0 assays, no tetrathionate was formed with almost complete inhibition of O_2 consumption. As for 9K at pH 2.3 and 0.1 M sodium sulfate at pH 2.3 assays, results showed that one tetrathionate was formed from two thiosulfates.

Table 5. Tetrathionate accumulation during thiosulfate oxidation in the presence of inhibitors HQNO and NEM

Substrate + 1 μg HQNO + 1 μmol NEM (in specific pH and buffer)	Tetrathionate formed (nmol)	O_2 consumed (nmol)	Oxidation rate	
			nmol O_2/min	% Inhibition
0.1 M sodium citrate pH 5				
100 nmol thiosulfate Control	0	200	61.6	
100 nmol thiosulfate + HQNO + NEM	0	30	4	93.5
200 nmol thiosulfate + HQNO + NEM	0	25	4	93.5
9K pH 2.3				
100 nmol thiosulfate Control	0	200	39.6	
100 nmol thiosulfate + HQNO + NEM	57	50	12	69.7
200 nmol thiosulfate + HQNO + NEM	102.5	50	13.8	65.2
0.1 M sodium sulfate pH 2.3				
100 nmol thiosulfate Control	0	190	63.2	
100 nmol thiosulfate + HQNO + NEM	73	100	22	65.2
200 nmol thiosulfate + HQNO + NEM	98.4	100	23.6	62.7

These results suggest that in 0.1 M sodium citrate at pH 5.0, the oxidation of thiosulfate followed theory A. In acidic conditions, 9K at pH 2.3 and 0.1 M sodium sulfate at pH 2.3, theory B was followed without tetrathionate hydrolysis.

The study of cytochromes

The reduction and oxidation of cytochromes were studied with various substrates. Unless otherwise indicated 0.1M sodium citrate at pH 5.0 was used as the buffer. The effects of inhibitors and metal chelator (EDTA) were also studied.

Reduction of cytochrome with dithionite

Table 6 shows the fully reduced peaks of cytochromes observed in intact cells/cell free crude extracts difference spectra with reducing agent dithionite. Reduced peaks were 420, 433, 442, 525, 535, 555, 566, 602, and 635 nm and tentatively indicated as cytochrome c_γ, b_γ, $a_\gamma(d_\gamma)$, c_β, b_β, c_α, b_α, a_α, and d_α respectively. These reduced cytochrome peaks are illustrated in Figures 35 and 36. These peaks were used as standards for the analysis of difference spectra of substrate-reduced cells or extracts. There was sometimes a peak at 650 - 660 nm in the difference spectra of cells when cytochrome *d* was more oxidized in the sample cuvette compared to the reference cuvette.

Table 6. Reduction of whole cell/cell-free crude extract with reducing agent dithionite

Addition of a few cystals of dithionite	Reduced peaks (nm)								
	420 c_{γ}	433 b_{γ}	442 a_{γ}/d_{γ}	525 c_{β}	535 b_{β}	555 c_{α}	566 b_{α}	602 a_{α}	635 d_{α}

The same reduced peaks were observed in whole cells and in crude extracts (with or without the cell debris added after centrifugation).

Figure 35. Full cytochrome reduction of whole cells by reducing agent dithionite. Cytochrome *c*, *b*, *a*, and *d* were all present.

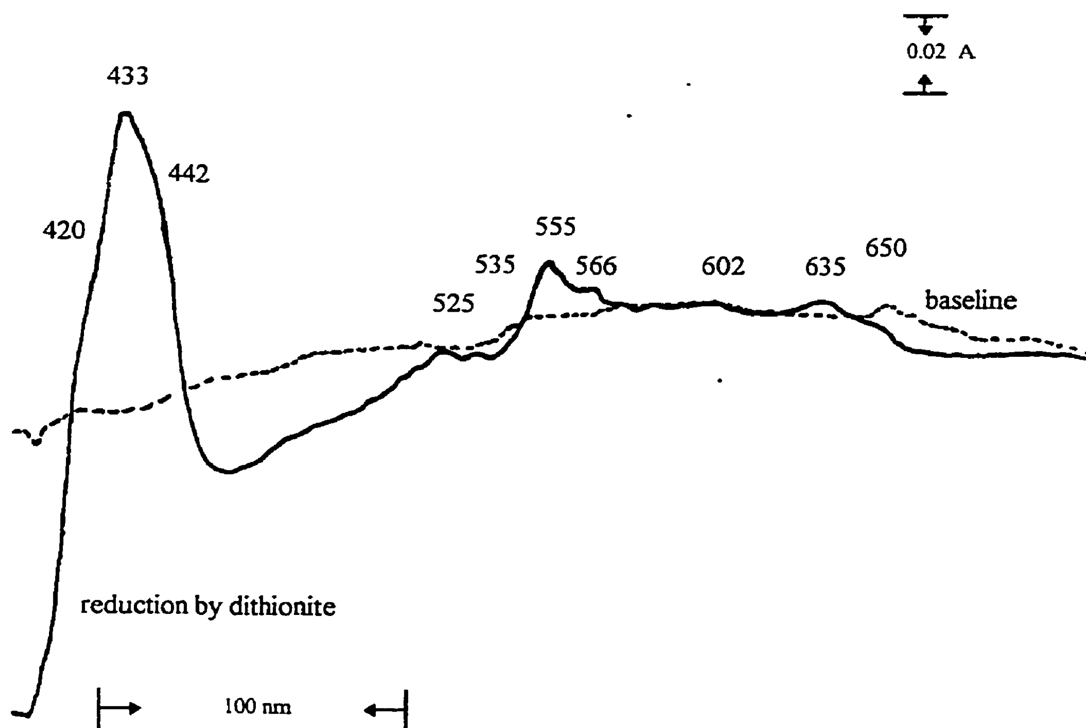
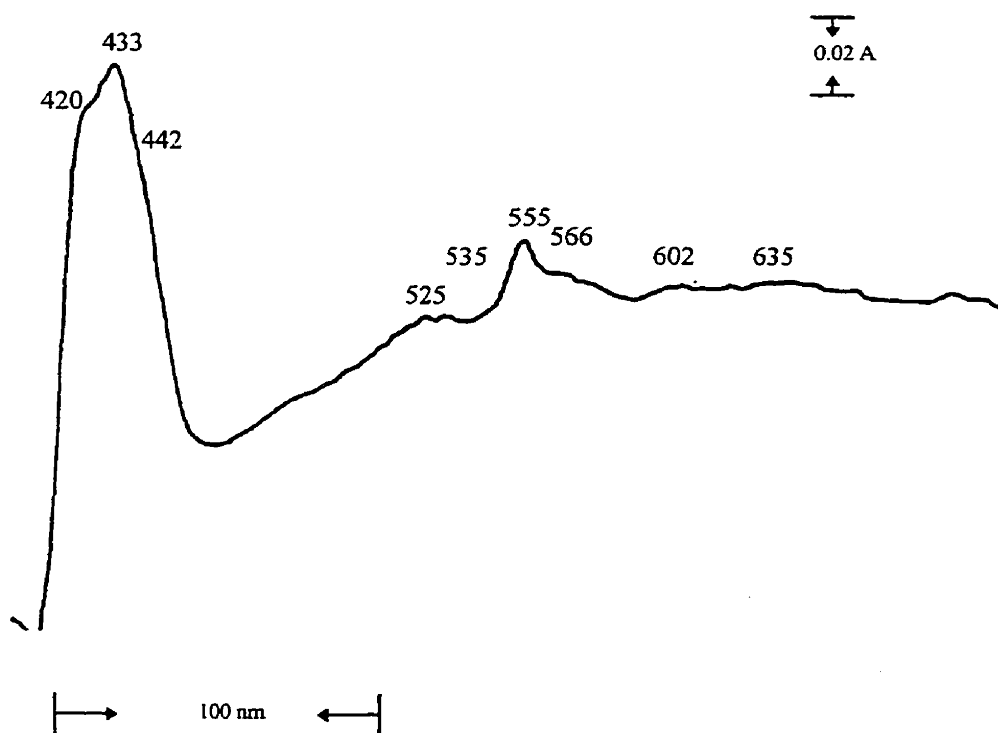

0.02 A
433
442
420
555
535
566
602
635
650
525
baseline
reduction by dithionite
100 nm

Figure 36. Full cytochrome reduction of cell-free crude extract by reducing agent dithionite. Cytochrome *c*, *b*, *a* and *d* were all present.


433
420
442
555
566
535
602
635
525
0.02 A
100 nm

Reduction of cytochromes with substrate

A time course study of intact cells following changes in the reduction of cytochromes with time was carried out with various substrates as shown in Table 7, 8, 9, 10 and the spectrums shown in Figures 37, 38, 39 and 40.

Table 7. Reduction of cytochrome with substrate 1.0 μmole thiosulfate against time

Time (min)	Reduced peak (nm)							
Cells/Cells (before addition of substrate)	Baseline							
0 time (Initial reduction upon addition of substrate)	420	555						
5	420	527	555	565	640			
10	420	433	440	525	535	555	565	602	635
17 (aeration; introduction of O_2 by shaking the cuvette)	420	440	525	555	635				
20	420	433	440	525	555	565	635		
25	420	433	440	525	555	565	640		

Figure 37. Cytochrome reduction of whole cells by substrate thiosulfate (1.0 μmole). Cytochrome *c*, *b*, *a* and *d* were present.

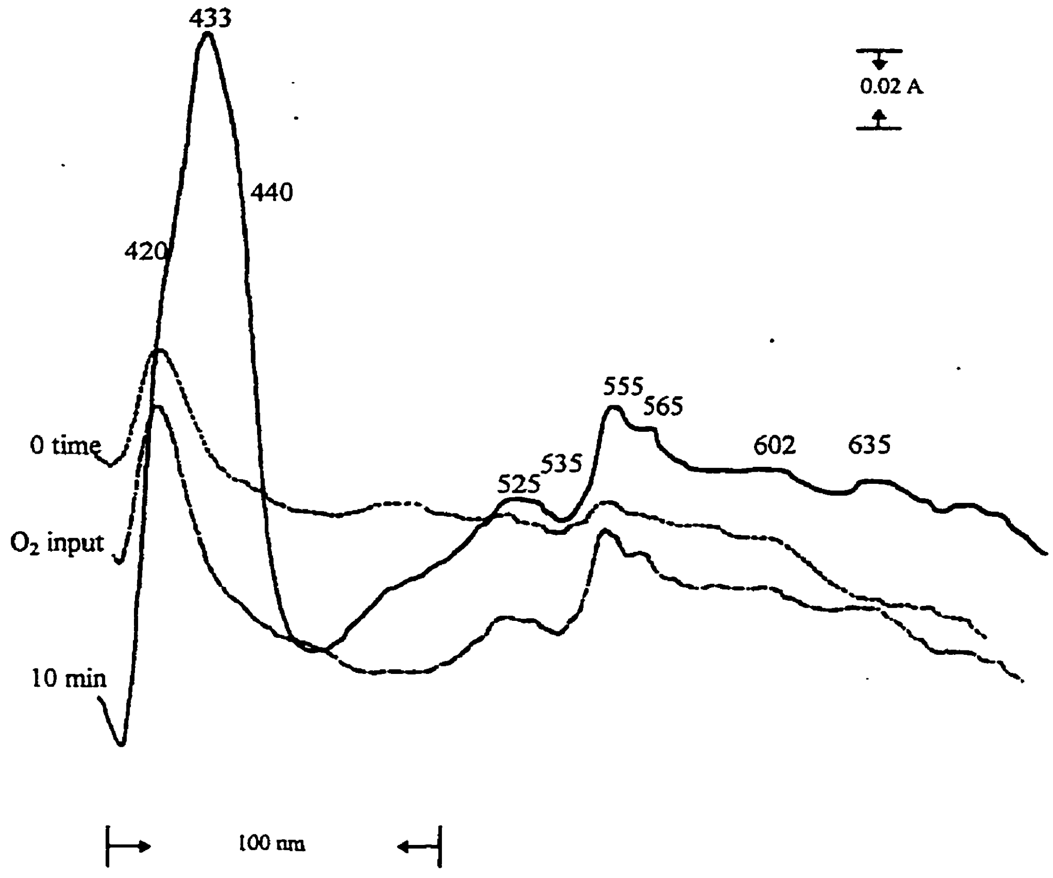
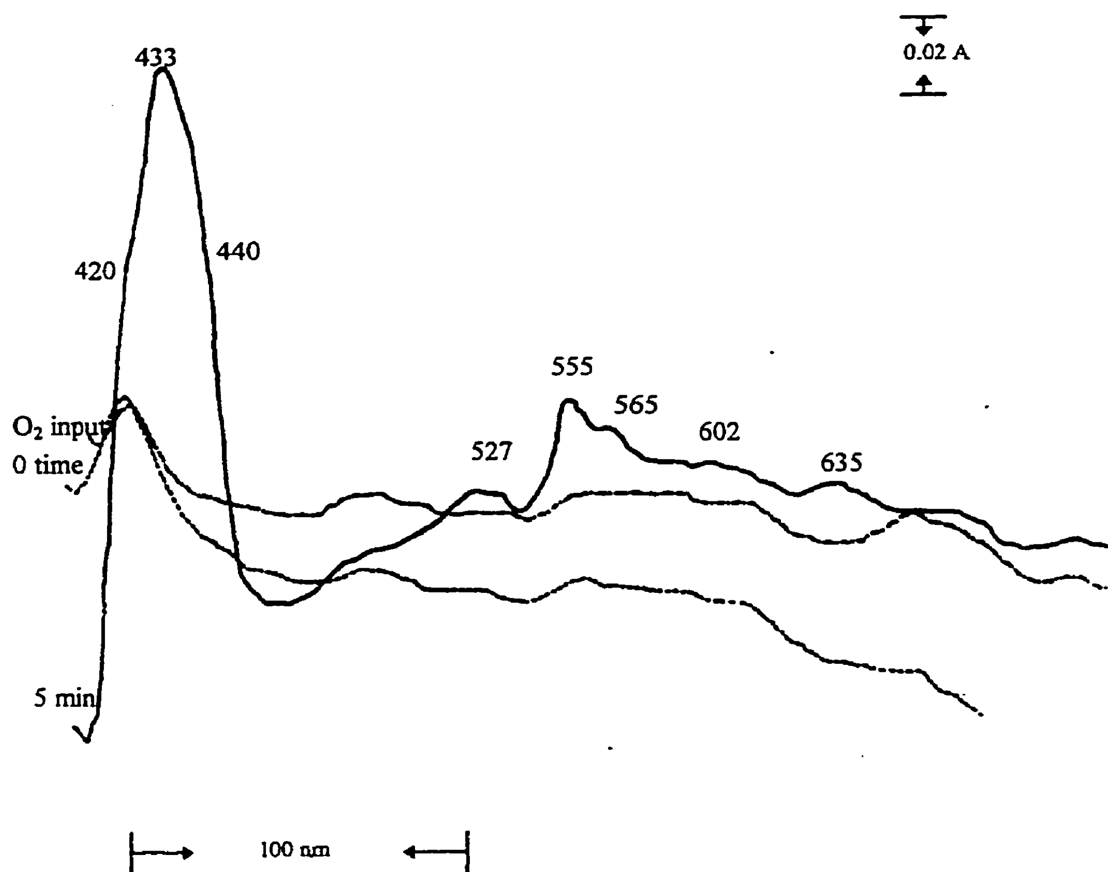

433
0.02 A
440
420
555
565
0 time
535
602
635
525
O_2 input
10 min
100 nm

Table 8. Reduction of cytochrome with 1.0 µmole substrate tetrathionate against time

Time (min)	Reduced peak (nm)							
Cells/Cells	Baseline							
0 time (Reduction)	420	560						
5	420	433	440	527	555	565	602	635
10	420	433	440	527	555	565	602	635
15 (aeration)	420							
20	420	433	440	555	565	635		
25	420	433	440	555	565	635		

Figure 38. Cytochrome reduction of whole cells by substrate tetrathionate (1.0 μmole). Cytochrome *c*, *b*, *a*, and *d* were present.



0.02 A
433
420
440
555
565
602
527
635
O_2 input
0 time
5 min
100 nm

Table 9. Reduction of cytochromes with substrate 0.5 μg DMSO S° against time

Time (min)	Reduced peak (nm)					
Cells/Cells	Baseline					
0 time (Reduced)	420	525				
5	Baseline					
10	420	433	440	530	555	565
14	420	433	440	525	555	565	602
20 (aeration)	Baseline					
25	No re-reduction because no substrate left					
30	"					

Conclusion : Cytochrome *d* was not reduced but cytochrome *a* instead.

Figure 39. Cytochrome reduction of whole cells by substrate DMSO sulfur (0.5 μg). Cytochrome *c*, *b* and *a* present only.

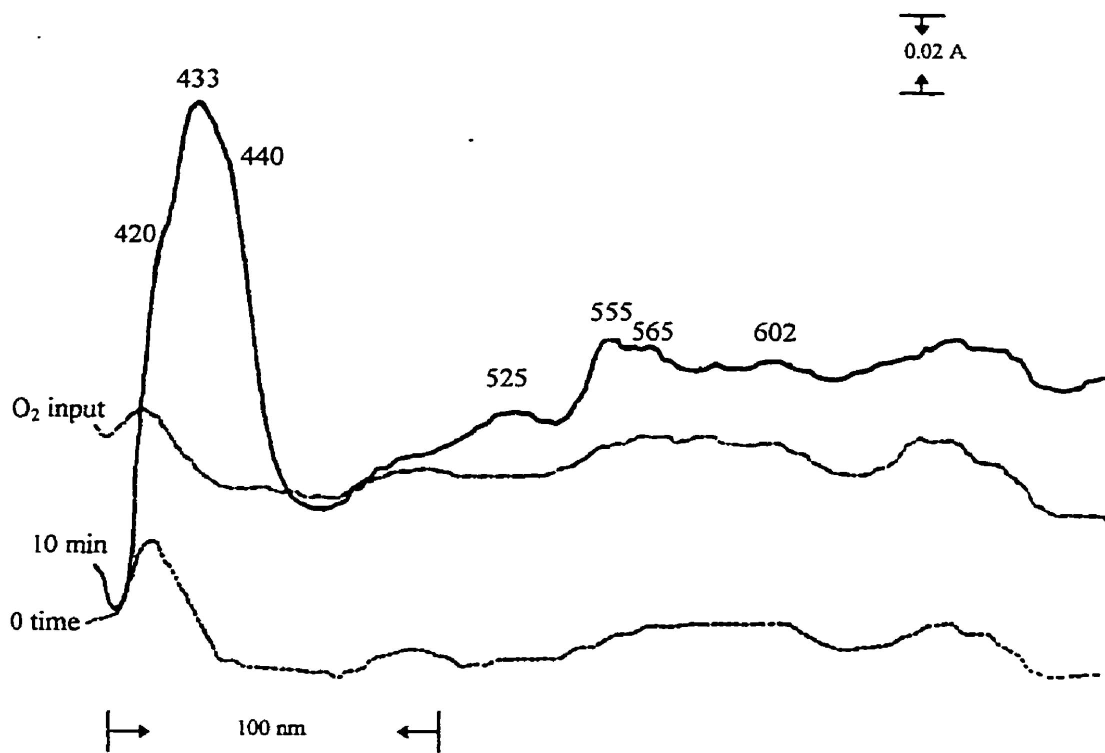
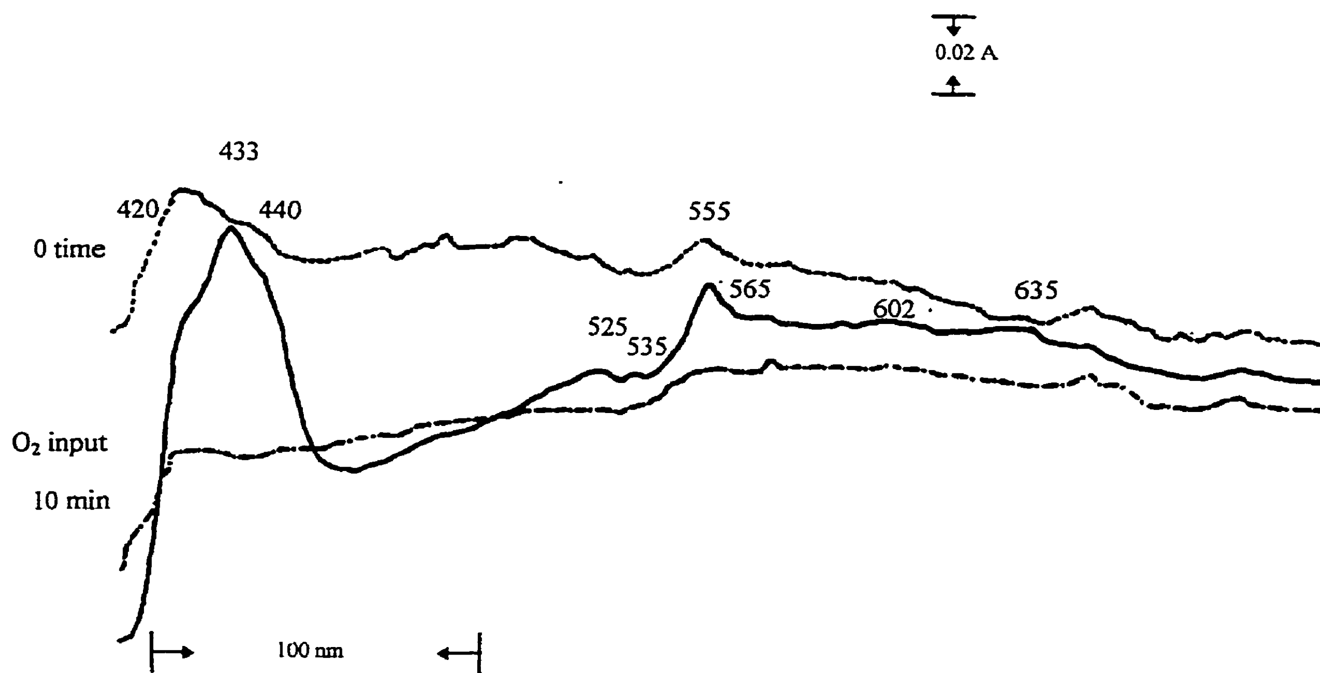

0.02 A
433
440
420
555
565
602
525
O_2 input
10 min
0 time
100 nm

Table 10. Reduction of cytochromes with 0.2 μmole sulfite + 0.1 μmole EDTA against time

Time (min)	Reduced peak (nm)								
Cells/cells	Baseline								
0 time	420	525	550						
5	420	433	440	525	555	565	602	635	
10	420	433	440	525	535	555	565	602	635
15 (aeration)	Baseline								
20	Baseline (substrate completely utilized)								
25	Baseline								
30	Baseline								

Figure 40. cytochrome reduction of whole cells by substrate sulfite (1.0 μmole in EDTA solution). Cytochrome *c*, *b*, *a* and *d* were present.


0.02 A
433
420
440
555
0 time
565
602
635
525
535
O2 input
10 min
100 nm

In general, results showed that cytochrome *c* was first reduced, followed by cytochrome *b* and finally cytochrome *d* or *a*. The cytochrome *d* peak at 635 nm was normally more pronounced than the cytochrome *a* peak at 602 nm. At time 0, i.e. immediately upon the addition of substrate, peaks at 420, 555, and 565 nm first appeared always. At 5 - 10 minutes later, peaks of 433, 440 (shoulder), 565, and 635 nm became pronounced. A time course spectrum can be seen in Figures 37, 38, 39 and 40. Cytochromes were almost fully reduced with excess substrates (thiosulfate, tetrathionate). Reduction of cytochromes by sulfite was slow and incomplete in whole cells since sulfite oxidation was slow, but was faster and more complete in cell-free extracts as shown later. The reduction of cytochrome by DMSO sulfur however, was unusual in that the initially reduced cytochrome *c* was once oxidized before the reduction of cytochromes *c*, *b*, and *a*. No cytochrome *d* reduction was observed.

Effect of EDTA on the reduction/oxidation of cytochromes

The assays were done in 9K at pH 5.0 with whole cell system in the presence of EDTA. There was no effect by EDTA on the reduction of cytochrome with thiosulfate as substrate (Table 11). However, in the substrate oxidation study, EDTA greatly stimulated the oxidation of thiosulfate (Figure 12). This indicated that EDTA effect might not involve the cytochrome system and that the EDTA-catalyzed thiosulfate oxidation might be chemical or non-biological.

A slight effect of EDTA was observed in the reduction of cytochromes with tetrathionate and DMSO sulfur in that the reduction of cytochromes was achieved 5 minutes earlier compared to the controls.

As for the reduction of cytochromes with sulfite in the presence of EDTA, a 15 minute delay in the reduction of cytochrome indicated the inhibition effect agreed with the inhibition of sulfite oxidation by EDTA shown in Figures 19 and 20.

Table 11. Effect of 5 μmole EDTA on the reduction/oxidation of cytochromes (based on whole-cells experiment; cells washed in 9K pH 5 and suspended in 9K pH 5)

Substrate + 5 μmole EDTA in 9K at pH5.0	Time required for full reduction of cytochrome (min)	
	- EDTA	+ EDTA
No substrate, EDTA only	Baseline	Baseline
1.0 μmole Thiosulfate + EDTA	10	10
1.0 μmole Tetrathionate + EDTA	10	5
5 μg DMSO S° + EDTA	10	5
1.0 μmole Sulfite in EDTA	15	30

Effect of inhibitor HQNO on the reduction of cytochrome

In the presence of HQNO, the reduction of both cytochromes *b* and *d* were inhibited with thiosulfate, tetrathionate, DMSO sulfur and sulfite (Tables 12 -15). A delayed reduction of cytochromes *b* and *d* with thiosulfate was observed (after 30 min in Table 12), but the reduction of cytochrome *b* was completely inhibited with the rest of the substrates. In the presence of HQNO, cytochrome *d* reduction was also inhibited in all substrates but a reduced peak of *a* 605 (610) was found instead. Cytochrome *c* was reduced by all substrates. The results suggested that HQNO inhibits the reduction of

cytochrome *b* and cytochrome *d* by these substrates and the electrons are channeled through cytochrome *c* and cytochrome *a*.

Table 12. Effect of 1.0 µg HQNO on the reduction of cytochromes with 1.0 µmole thiosulfate (based on whole cell experiment)

Time (min)	Reduced peaks (nm)							
Cells/Cells	baseline							
Addition of 1.0 µg HQNO with cells only	"							
0 time (reduction)	420	525	555					
5	420	525	555	605				
10	420	525	555	605				
15 (aeration)	420	525	555	605				
30	420	433	440	525	555	565	605	640

Conclusion : 1. HQNO inhibits the reduction of cytochrome *b* and cytochrome *d*. The reduction is achieved only 30 min later.

2. Cytochrome *c* and cytochrome *a* (605) are reduced quickly.

Table 13. Effect of 1.0 μg HQNO on the reduction of cytochromes with 1.0 μmole tetrathionate (based on whole cells experiment)

Time (min)	Reduced peaks (nm)			
Cells/Cells	Baseline			
Addition of 1.0 μg HQNO and cells only	‘’			
0 time (Reduced)	420	525	555	602
5			‘’	
15			‘’	
20			‘’	
24 (aeration)	420	525	555	

Conclusion :
1. HQNO inhibits cytochrome *b* reduction completely.
2. Cytochrome *d* reduction was also completely inhibited. Only cytochrome *a* (602) appeared.

Table 14. Effect of 1.0 μg HQNO on the reduction of cytochromes with 0.5 μg DMSO S°
(based on whole cells experiment)

Time (min)	Reduced peaks (nm)			
Cells/Cells	Baseline			
Addition of 1.0 μg HQNO and cells only	Baseline			
0 time (reduced)	420	440	555	610
5	420	525	555	610
10			‘’	
15 (aeration)	420	525	555	

Conclusion :
1. HQNO inhibits cytochrome *b* reduction completely.
2. Only cytochrome *a* and cytochrome *c* were found.

Table 15. Effect of 1.0 μg HQNO on the reduction of cytochromes with 1.0 μmole sulfite + 0.1 μmole EDTA (based on whole cells experiment)

Time (min)	Reduced peaks (nm)		
Cells/Cells	Baseline		
Addition of 1.0 μg HQNO with cells only	Baseline		
0 time (reduced)	423	555	610
5	423	555	
10	423	555	
15	420		
20 (aeration)	Baseline		

Conclusion :

1. HQNO inhibits cytochrome *b* reduction completely.
2. HQNO inhibits cytochrome *d* reduction completely. Only cytochrome *a* and cytochrome *c* were found.

Effect of inhibitor NEM on the reduction of cytochrome

Reduction of the cytochrome system was completely inhibited with all substrates in the presence of NEM (Table 16). This result is compared to the oxidation experiments (Table 4) where the oxygen consumption rate was inhibited by NEM in the thiosulfate and tetrathionate oxidations (70 % - 90 %). Perhaps the rate of cytochrome reduction was more strongly inhibited than the rate of oxidation (cytochrome oxidase).

Table 16. Effect of 1.0 μmole NEM on the reduction of cytochromes with various substrates (thiosulfate, tetrathionate, DMSO sulfur, and sulfite) (based on whole cells experiment)

Time (min)	Reduced peaks (nm)
0 - 30	Reduction of cytochrome system completely inhibited/shut down

Effect of inhibitors azide and cyanide on the reduction of cytochrome (Table 17 and 18)

The presence of 1.0 μmole azide or cyanide did not affect the reduction of cytochromes by thiosulfate, tetrathionate, and DMSO sulfur. As for sulfite, azide inhibited the reduction of cytochromes nearly completely, but cyanide had little effect. Azide also inhibited the oxidation of sulfite completely seen in Figures 27 and 28.

Table 17. Effect of 1.0 μmole Azide on the reduction of cytochromes with substrates, thiosulfate, tetrathionate, DMSO sulfur, and sulfite.(based on whole cells experiment)

Azide + substrate	Effects
Azide + thiosulfate	No effect or inhibition on cytochrome reduction
Azide + tetrathionate	Cyrochrome *a* and *d* remained reduced when O_2 introduced. No oxidation of cytochrome *a* and *d*.
Azide + DMSO sulfur	
Azide + sulfite	No reduction of cytochromes

Table 18. Effect of 1.0 μmole KCN on the reduction of cytochromes with various substrates; thiosulfate, tetrathionate, DMSO sulfur , and sulfite. (based on whole cells experiment)

Time (min)	Reduced peaks (nm)
0 - 30	No effect on the reduction of cytochromes except for cytochrome *a*. Cytochrome *a* is not reduced.

Effect of CCCP and 2,4 DNP on the reduction of cytochrome (Table 19)

Effect of CCCP

Cytochrome *c* but not cytochrome *b* was reduced by thiosulfate and tetrathionate in the presence of CCCP, but neither cytochrome was reduced by DMSO sulfur and sulfite in the presence of this uncoupler.

Effect of 2,4-DNP

The reduction of cytochromes with either thiosulfate or tetrathionate was not affected by 2,4-DNP, but the reduction of cytochrome *b* was inhibited with DMSO sulfur and the reduction of both cytochrome *b* and *c* was inhibited with sulfite in the presence of 2,4-DNP. Thus, CCCP was a stronger inhibitor than 2,4-DNP and the reduction of cytochromes with sulfite was the most sensitive to the inhibition, followed by that with DMSO sulfur.

Table 19. Effect of 0.025 μmole CCCP and 0.025 μmole 2,4-DNP on the reduction of cytochromes with various substrates; thiosulfate, tetrathionate, DMSO sulfur, and sulfite (based on whole cells experiment)

CCCP + substrate	Effects (based on the reduction of cytochromes *b* and *c*)
CCCP + thiosulfate	Cytochrome *c* is reduced, but not cytochrome *b*
CCCP + tetrathionate	Cytochrome *c* is reduced, but not cytochrome *b*
CCCP + DMSO sulfur	No reduction of cytochromes
CCCP + Sulfite	No reduction of cytochromes
2,4-DNP + thiosulfate	No inhibition of cytochrome reduction
2,4-DNP + tetrathionate	No inhibition of cytochrome reduction
2,4-DNP + DMSO sulfur	Cytochrome *c* is reduced, but not cytochrome *b*
2,4-DNP + sulfite	No reduction of cytochromes

Reduction of cell-free crude extract cytochromes with various substrates

Cytochrome *c* was the only cytochrome reduced with thiosulfate, tetrathionate, and DMSO sulfur in the crude extract in 0.1 M sodium citrate at pH 5.0 (Table 20 and Figure 41). These substrates did not reduce any cytochromes in 0.1 M Tris-HCl at pH 7.5.

The only substrate that gave a full reduction of the cytochrome system of crude extracts was sulfite in 0.1 M Tris-HCl at pH 7.5 (Table 20 and Figure 41). The time course study indicated that cytochrome *c* was first reduced, followed by cytochrome *b* and finally cytochrome *d* and perhaps *a*.

The addition of pellet composed of cell debris from the crude extract preparation to the thiosulfate, tetrathionate, DMSO sulfur assays had no effect at all. However, with the addition of pellet to the sulfite assays, shortening by 5 minutes in the reduction of cytochrome was observed.

Table 20. Reduction of cell-free crude extract with various substrates
(crude extract prepared without the treatment of trypsin)

Substrate/Time (min)	Reduced peaks (nm)							
1.0 μmole thiosulfate (20 min)	420	555						
1.0 μmole tetrathionate (20 min)	420							
0.1 mg DMSO sulfur (20 min)	420	555						
1.0 μmole sulfite + 0.1 μmole EDTA								
Cells/Cells	Baseline							
0 time (reduced)	420							
10	420	430	440	525	555	563	602	635
20	420	430	440	525	555	563	602	635
35 (aeration)	420	555						
With the addition of cell pellet	Reduction of the cytochromes achieved 5 min faster.							

Note : thiosulfate, tetrathionate, DMSO sulfur assays are done in 0.1M sodium citrate pH 5.0 buffer.
sulfite assays done in 0.1 M Tris-HCl pH 7.5.

Conclusion :

1. Thiosulfate, tetrathionate, DMSO sulfur substrate assays are able to reduce cytochrome c only.
2. Substrate sulfite only can fully reduce all cytochromes.

Figure 41. Cytochrome reduction of cell-free crude extract by substrates thiosulfate (1.0 μmole), tetrathionate (1.0 μmole) and DMSO sulfur (0.5 μg). All three substrates only be able to reduced cytochrome *c*.

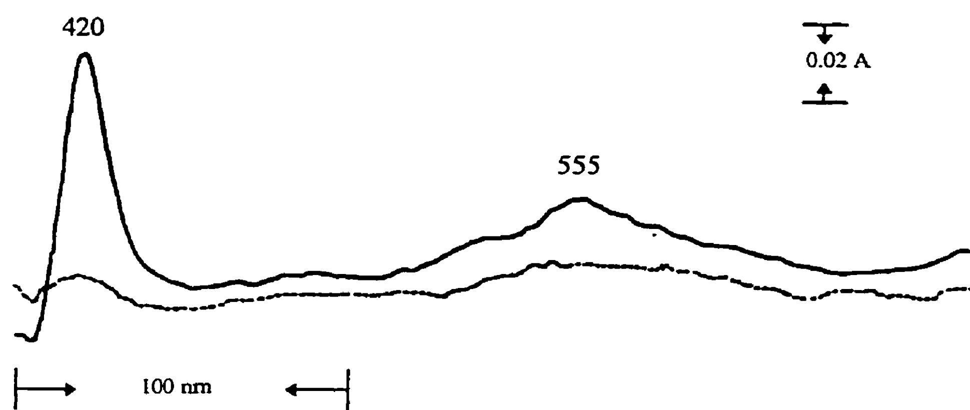
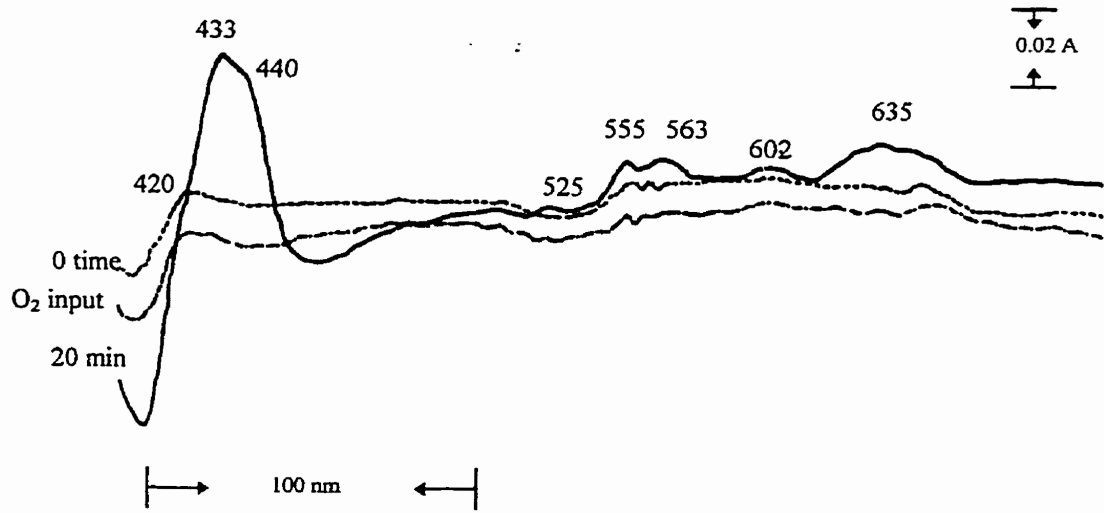

420
0.02 A
555
100 nm

Figure 42. Cytochrome reduction of cell-free crude extract by substrate sulfite (1.0 μmole in EDTA 0.5 μmole solution). Cytochrome *c*, *b*, *a* and *d* were all reduced by sulfite.


433
440
420
525
555
563
602
635
0.02 A
0 time
O2 input
20 min
100 nm

Effect of various inhibitors on the reduction of cell-free crude extract with sulfite (Table 21 and 22)

In the presence of HQNO or NEM, the reduction of cytochromes was inhibited except for some reduction of cytochrome *c* (Table 21). The HQNO results agreed with those with cells, but NEM inhibited the cytochrome reduction completely in cells (Table 16).

Both azide and cyanide gave partial inhibition, i.e., a delay and a reduced peak height in the reduction of cytochromes were observed. Crude extracts seemed to show less sensitivity to azide compared to the whole cell system (Table 17).

There was no effect on the reduction of cytochrome in the presence of 2,4-DNP with sulfite. Partial inhibition was observed in the presence of CCCP with sulfite, i.e., a delay and a reduced peak height in the reduction of cytochrome.

The degree of inhibition of cytochrome reduction in the crude extracts by these inhibitors can be related only partially to the degree of inhibition in the oxidation rate of sulfite by crude extract by these inhibitors as shown in Table 22. HQNO inhibits both the reduction of cytochromes and oxygen consumption, but NEM inhibits only the reduction of cytochromes although the initial fast O_2 consumption rate shown slows down and levels off after several minutes. Azide and CCCP inhibits the reduction of cytochromes partially, but the oxygen consumption rate is little affected. They may affect only the rate of reduction of cytochromes and not the oxidation rate. If the latter rate is the limiting factor in oxygen consumption with sulfite, then the inhibition of the reduction rate may not be affected by the oxidation rate.

Table 21. Effect of various inhibitors on the reduction of cell-free crude extract cytochromes with 1.0 μmole sulfite + 0.5 μmol EDTA in 0.1 M Tris-HCl pH 7.5

Inhibitors	Reduced peaks (nm)
Control	At 15 min : 420, 433, 440, 555, 563, 635
1.0 μg HQNO	420, 555 (only cytochrome *c* reduced)
1.0 μmole NEM	420, 555 (only cytochrome *c* reduced; crude extract was less sensitive to NEM than whole cells)
1.0 μmole Azide	Partial inhibition. (reduction peak height is lower than control particularly cyt.*b*; crude extract showed less sensitivity to azide than whole cells) At 20 min : 420, 433, 440, 555
1.0 μmole KCN	Little inhibition At 17 min : 420, 430, 440, 555
0.025 μmole 2,4 DNP	No effect At 15 min : 420, 430, 440, 555, 565
0.025 μmole CCCP	Partial inhibition (reduction peak height is lower than control particularly cyt.b; crude extract less sensitive to CCCP than whole cells) At 20 min : 420, 430, 440, 550

Table 22. Oxidation rate of cell-free crude extract with 1 μmole sulfite + 0.5 μmole EDTA in the present of inhibitors (This table is in relation with table 17)

Inhibitors	Rate of oxidation (nmole O_2/min)
Control	32.6
1.0 μg HQNO	5.6
1.0 μmole NEM	25.8 ↓
1.0 μmole Azide	28.6 ↓
1.0 μmole KCN	30.8
0.025 μmole CCCP	30.8
0.025 μmole 2,4 DNP	29.4

↓ = the rate of oxidation leveled off after 3 minutes.

DISCUSSION

Discussion

Cultivation

Thiosulfate is found transiently in many environments and is therefore commonly used as an energy source for photoautotrophic or chemolithotrophic microorganisms. The assimilation aspect, however, has been seldom studied and its importance in bacterial physiology is not well understood, although the utilization of thiosulfate as an electron donor for aerobic growth is quite well documented in *Thiobacillus* species. Since thiosulfate is one of the reduced inorganic sulfur compounds found widely in nature, it is important to understand the general mechanism of thiosulfate oxidation by these bacteria. It will further lead to the full understanding of the general pathway of sulfur oxidation and therefore the efficiency of biotechnology and biohydrometallurgy can be fully grasped.

Not all *Thiobacillus thiooxidans* strains have the ability to oxidize thiosulfate but the cultivation of *T. thiooxidans* (ATCC 8085) on thiosulfate medium from sulfur has been attained in this study. An adaptation period of 4 - 5 days in cultivating cells on thiosulfate was needed when the seed culture from sulfur grown cells was used but no adaptation period was needed when the seed culture was grown on thiosulfate.

The preferred growth medium was Silverman Lundgren 9K medium as seen in Figure 3 with the least amount of phosphate compared to Starkey No.1 and Starkey No.2. This can be also seen in the rate of oxidation of thiosulfate in phosphate buffer in Figure 5, 6, and 7 where its rate was much lower than citrate buffer. The optimum pH for

growth and oxidation of thiosulfate was 5.0 (Figure 4) which was also demonstrated by Nakamura et al. (1990).

Substrate oxidation

Based on the substrate oxidation studies, the rate of oxygen consumption with thiosulfate, tetrathionate, and sulfite was highest in 0.1M sodium citrate buffer at pH 5.0 (Figures 5, 6, 7, 8 and 11) compared to the other buffers used. Figure 5, 6 and 7 showed the best substrate concentration for thiosulfate oxidation was 1.0 μmole; having 80 nmoles O_2/min. A concentration of 10.0 μmoles thiosulfate showed some substrate inhibition having a lower rate of oxidation of 70.7 nmoles O_2/min. Thiosulfate oxidation at pH 2.3 exhibited a biphasic pattern in all buffers, indicating that tetrathionate was produced from thiosulfate oxidation as seen with other thiobacilli. (Silver and Lundgren 1968, Lyric and Suzuki 1970, Kelly et al. 1988, Meulenberg et al. 1992, and Linström et al. 1996). Thiosulfate is easily oxidized to tetrathionate by the thiosulfate-oxidizing system which was first established by Trudinger in *Thiobacillus neopolitanus* (Trundiger, 1961). Only in acidic conditions was thiosulfate found to be stoichiometrically oxidized to tetrathionate, which was also observed by Chan and Suzuki (1994). The stoichiometeric oxidation studies will be discussed later.

Complete oxidation of thiosulfate at pH higher than 5.0 could not be achieved. Throughout the various substrate oxidations, pH 5.0 was the optimum pH for substrate oxidation. Among all substrates; thiosulfate, tetrathionate, DMSO sulfur, Tween 80 sulfur and sulfite, thiosulfate was the substrate that gave the highest rate of oxidation, which is theoretically as it should be as the organism was grown on thiosulfate. On the contrary, sulfite had the lowest oxidation rate of 8 nmoles O_2/min compared

to 80 nmoles O_2/min seen for thiosulfate (Figures 5, 6 and 7). This is due to the difficulty in the uptake of sulfite by the cells. Sulfite enters the cell in the form of SO_2 gas or H_2SO_3, sulfurous acid, passing the cell membrane into the cytoplasm (Takeuchi and Suzuki, 1994).

Metal chelation effect

The best condition for most substrate oxidation was attained in 0.1M sodium citrate buffer at pH 5.0. Somehow, metal chelation by citrate was suspected to have a great effect on the mechanism of oxidation. The kind of cells used for most of the experimental analysis shown were washed in water and suspended in 0.1M sodium citrate pH 6.0. This treatment produced cells with a greater activity in the substrate oxidation as compared to cells washed in 9K medium pH 5.0 and suspended in 0.1M sodium citrate pH 6.0. The washing of cells with milli Q water might in the process get rid of most metals contained inside and outside of the cells, which explained a little why sodium citrate buffer gave the best condition for substrate oxidation. The complete oxidation of 0.1 μmole thiosulfate could only be achieved in 0.1M sodium citrate at pH 5.0 but not in 9K medium pH 5.0. Theoretically, the latter should provide the best condition for thiosulfate oxidation for it was the growth medium for the cells. This further lead to an investigation of why the 9K medium at pH 5.0 could not provide the right condition for thiosulfate oxidation.

As metal chelation might be involved in the mechanism of oxidation, various metal chelators; EDTA, o-phenanthroline, 2,2'-dipyridyl and tiron were tested in various buffers. In Figure 12, EDTA was tested in 9K medium and results showed that the rate of thiosulfate oxidation was stimulated by EDTA. The complete oxidation of thiosulfate in

9K growth medium at pH 5.0 can thus be established with the addition of EDTA. The cells analyzed for this experiment were washed in 9K medium pH 5.0 and suspended in 9K medium pH 5.0, maintaining its original state. Cells that were washed in 9K pH 5.0 but suspended in citrate buffer showed no effect with EDTA in 9K medium pH 5.0 as they were already suspended in the citrate buffer, with a chelation effect (results not shown).

A second reason why the rate of oxidation of thiosulfate was found to be low and incomplete in 9K medium pH 5.0 was the concentration of thiosulfate used was too low. In the cultivation, 10 g/L of thiosulfate (40 mM) was used (50 μmole per 1.2 mL) compared to 0.1 μmole thiosulfate used in the studies. Results in Figure 5, 6 and 7 suggested that a much higher concentration of thiosulfate was required for faster oxygen consumption in 9K medium pH 5.0. However, with such a high concentration of thiosulfate, stoichiometric studies on the mechanism of oxidation could not be undertaken. A lower concentration of 0.1 μmole of thiosulfate was needed to quantitate the amount of oxygen consumed in the stoichiometric studies and this only can be achieved in 0.1M sodium citrate at pH 5.0, 9K medium at pH 2.3 and 0.1M sodium sulfate at pH 2.3. Therefore, for most part of the stoichiometric studies, only assays in 0.1M sodium citrate pH 5.0, 9K pH 2.3 and 0.1M sodium sulfate pH 2.3 were analyzed.

In general, metal chelators EDTA, o-phenanthroline and 2,2'-dipyridyl seems to stimulate thiosulfate oxidation (Figures 13 and 14). The same effect was also shown for tetrathionate oxidation in Figures 15 and 16, although here all four chelators including tiron stimulated in citrate buffer (Figure 16). The effect of metal chelators on sulfur oxidation, however, showed some stimulation and some inhibition (Figures 17 and 18) at

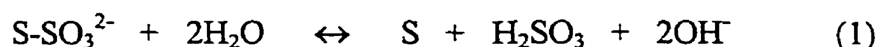
pH 5.0. The inhibition could be related to the strong inhibition of sulfite oxidation by EDTA (Figure 19). For sulfite oxidation, all metal chelators had strong inhibitory effects in both citrate and 9K medium assays as shown in Figures 19 and 20. Strong inhibition by metal chelators 2,2'-dipyridyl, o-phenanthroline, and EDTA was of sulfite oxidase of *Thiobacillus thioparus* (Lyric and Suzuki, 1970) and was considered to be related to the non-heme iron present in the enzyme. The inhibition could not be reversed by the addition of ferrous or ferric iron to the inhibited enzyme (Lyric and Suzuki, 1970). There was some stimulation in the oxidation of thiosulfate and sulfite when ferrous iron (Fe^{3+}) was added in the absence of chelators (Results not shown). Chemical sulfite oxidation is known to be stimulated in the presence of metals, particularly iron.

The effect of azide and cyanide on the oxidation of thiosulfate could be seen in Figures 21 and 22. In both citrate and 9K buffers, cyanide showed less inhibition compared to azide. The results were similar in tetrathionate oxidation (Figures 23 and 24). In DMSO sulfur oxidation, cyanide showed inhibition but also some stimulation in citrate and 9K medium at pH 5.0. Azide again showed strong inhibition in the oxidation of DMSO sulfur (Figures 25 and 26). For sufite oxidation, azide strongly inhibited in citrate buffer but not 9K medium (Figures 27 and 28). Cyanide was not included in the Figures because the chemical oxidation of sulfite was stimulated by cyanide. These results observed with the presence of azide and cyanide will be related and compared to the cytochromes studies.

Stoichiometric studies

Based on the stoichiometic studies of thiosulfate in sulfite determination, sulfur determination and tetrathionate determination, both theory A and B were applied

depending on the specific condition of the substrate oxidation. Assay condition in 0.1M sodium citrate pH 5.0, the oxidation of thiosulfate followed theory A; i.e. the oxidation of thiosulfate first catalyzed by rhodanese (thiosulfate-cleaving enzyme, sulfur transferase) giving sulfur and sulfite as intermediates.

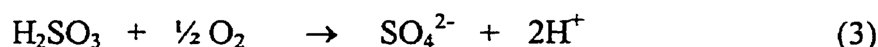


$$S\text{-}SO_3^{2-} + 2H_2O \leftrightarrow S + H_2SO_3 + 2OH^- \qquad (1)$$

The sulfur will be further oxidized by sulfur-oxidizing enzyme to sulfite.

$$S + O_2 + H_2O \rightarrow H_2SO_3 \qquad (2)$$

Finally, sulfite is oxidized to sulfate by sulfite oxidase involving the electron transfer system.



$$H_2SO_3 + \tfrac{1}{2}O_2 \rightarrow SO_4^{2-} + 2H^+ \qquad (3)$$

Final equation : $S\text{-}SO_3^{2-} + 2O_2 + H_2O \rightarrow SO_4^{2-} + H^+$

However, in acidic conditions particularly in 9K and 0.1M sodium sulfate at pH 2.3, the oxidation of thiosulfate followed theory B. Theory B involved the thiosulfate-oxidizing enzyme and electron transfer system which catalyzes the oxidation of thiosulfate to tetrathionate.

$$2S\text{-}SO_3^{2-} + \tfrac{1}{2}O_2 + 2H^+ \rightarrow S_4O_6^{2-} + H_2O \qquad (1)$$

Tetrathionate hydrolase then catalyzes the hydrolysis of tetrathionate to thiosulfate, sulfur and sulfate.

$$S_4O_6^{2-} + H_2O \rightarrow S\text{-}SO_3^{2-} + S + SO_4^{2-} + 2H^+ \qquad (2)$$

Thiosulfate and sulfur will be further oxidized to sulfate by sulfur-oxidizing enzyme and sulfite oxidase giving sulfate as the end product.

For the stoichiometric studies of terathionate oxidation, the results obtained were inconclusive. There was difficulty in the determination of sulfite and sulfur in

tetrathionate oxidation with inhibitors HQNO and NEM. In the presence of individual inhibitors, the sulfite and sulfur formed were less than what theory A and B proposed. The problem with tetrathionate is that it can react actively with sulfite and polysulfide found on the cells and therefore an appropriate stoichiometry could not be determined.

Stoichiometric studies for sulfur oxidation was clear cut. In the presence of HQNO, one mole of sulfite accumulated from one mole of sulfur in assays done in citrate buffer, 9K medium, sodium sulfate buffer at both pH 5.0 and 7.0.

$$S + O_2 + H_2O \rightarrow H_2SO_3 \qquad \text{(HQNO)}$$

Therefore, without inhibitor, sulfite will be oxidized to sulfate by sulfite oxidase and electron transfer system.

$$S + 1\,½\,O_2 + H_2O \rightarrow SO_4^{2-} + 2H^+$$

In conclusion, theory A is followed for thiosulfate oxidation in 0.1M sodium citrate at pH 5.0, and theory B is followed for 9K medium and 0.1M sodium sulfate at pH 2.3. The enzyme which catalyzes the oxidation of thiosulfate to tetrathionate has been shown to be located in the periplasm of some species of thiobacilli (Hazeu et al.1988, Kelly et al.1988 and Meuleuberg et al.1992).

Cytochrome studies

Four cytochromes were found in *T. thiooxidans* 8085 based on the full reduction of the cytochromes by reducing agent dithionite. Therefore, c_γ-420, b_γ-433, a_γ/d_γ-442, c_β-525, b_β-535, c_α-555, b_α-566, a_α-602, and d_α-635 illustrated in the spectrum Figure 35. Both whole cells and cell-free crude extracts gave the same reduced peaks by dithionite.

Reduction of cytochrome by substrates

The reduction of whole cell cytochromes with substrates thiosulfate, tetrathionate and sulfite based on a time course study consistently reduced cytochrome *c* first (at 0 time), followed by cytochrome *b* (5 - 10 min later) then finally cytochrome *d* (0 - 15 min later). However, substrate DMSO sulfur followed a different route where cytochrome *c* was reduced first at 0 time, the reduced cytochrome *c* peak then disappeared at 5 minutes, the reduction of cytochrome *b* was then achieved at 10 minutes and finally cytochrome *a* was reduced instead of *d* at 14 minutes.

Effect of EDTA in the reduction of cytochrome

Addition of EDTA had no effect on the cytochrome reduction by thiosulfate. Some stimulation by EDTA in the cytochrome reduction with tetrathionate and sulfur was observed, i.e. cytochrome reduction was achieved 5 minutes earlier compared to control. In contrast, EDTA exhibited inhibition in cytochrome reduction with sulfite, i.e. cytochrome reduction was achieved 15 minutes later compared to the control. This inhibition effect by EDTA is also consistent with the oxidation of sulfite. (Figures 19 and 20).

Effect of HQNO in the reduction of cytochrome

In the presence of HQNO, the whole cell cytochrome reduction pathway with substrate thiosulfate, tetrathionate and sulfite changes. HQNO inhibited the reduction of cytochrome *b* and *d*. The electrons now channeled from cytochrome *c* to *a* instead. With DMSO sulfur, cytochrome *b* reduction was inhibited, where as the reduction of cytochromes *c* and *a* was achieved, the same results with other substrates. These results suggest that a branched chain electron transport system exists in *T. thiooxidans* 8085.

Effect of azide and cyanide in the reduction of cytochrome

Partial inhibition of the whole cell and cell-free crude extract cytochrome reduction was found in the presence of azide with sulfite but not with cyanide. The same affect were also found by Charles and Suzuki (1966) with *T. novellus* where azide was inhibitory but not cyanide. The whole cell cytochrome reduction by thiosulfate, tetrathionate and DMSO sulfur was not affected much either with azide or cyanide. In the oxidation study with whole cells, azide inhibited the oxidation of thiosulfate, tetrathionate, DMSO sulfur and sulfite somewhat, but not cyanide. The insensivity to cyanide clearly indicated that cytochrome *d* was present because cytochrome *d* is not effected by cyanide. There have been a number of observations with whole cells and particulate preparations which indicated that cytochrome oxidase activity of cytochrome *d* is less sensitive to inhibition with cyanide than that of other oxidases (Lemberg and Barrrett, 1973). Jones and Redfearn (1967a) also noted the relative insensitivity of the cytochrome $d + b_1$ electron pathway of *Azotobactor vinelandii* to both cyanide and azide.

Cell-free crude extract cytochrome reduction by substrate

The full reduction of cytochromes in the cell-free crude extracts only can be achieved with substrate sulfite in assay system with 0.1M Tris-HCl pH 7.5; seen in Figure 42. The reduced peaks include cytochromes *c, b, a,* and *d.* Thiosulfate, tetrathionate, DMSO sulfur could not fully reduce the cytochromes. Only cytochrome *c* was reduced in 0.1M sodium citrate pH 5.0; seen in spectrum Figure 41. No reduction of cytochrome could be seen in assay system 0.1M Tris-HCl pH 7.5. The addition of pellet (cells debris, from the separation of supernatant crude extract) had no effect on the reduction of cytochromes.

Effect of inhibitors and uncouplers in the reduction of cytochrome of cell-free crude extract

In the presence of inhibitors HQNO and NEM, only cytochrome *c* was reduced by sulfite. NEM strongly inhibited the reduction of cytochromes in whole cells with all substrates. Uncouplers 2,4-DNP and CCCP showed less inhibition in the reduction of cytochrome in cell-free crude extracts but more with whole cells. This is true because these proton translocating compounds should not have any effect in the cell-free crude extract but will disrupt the of proton gradient in the whole cell system.

In conclusion of the cytochrome studies, *T. thiooxidans* 8085 seems to have a branched chain electron transport system operating which shares some similarity with *A. vinelandii*.

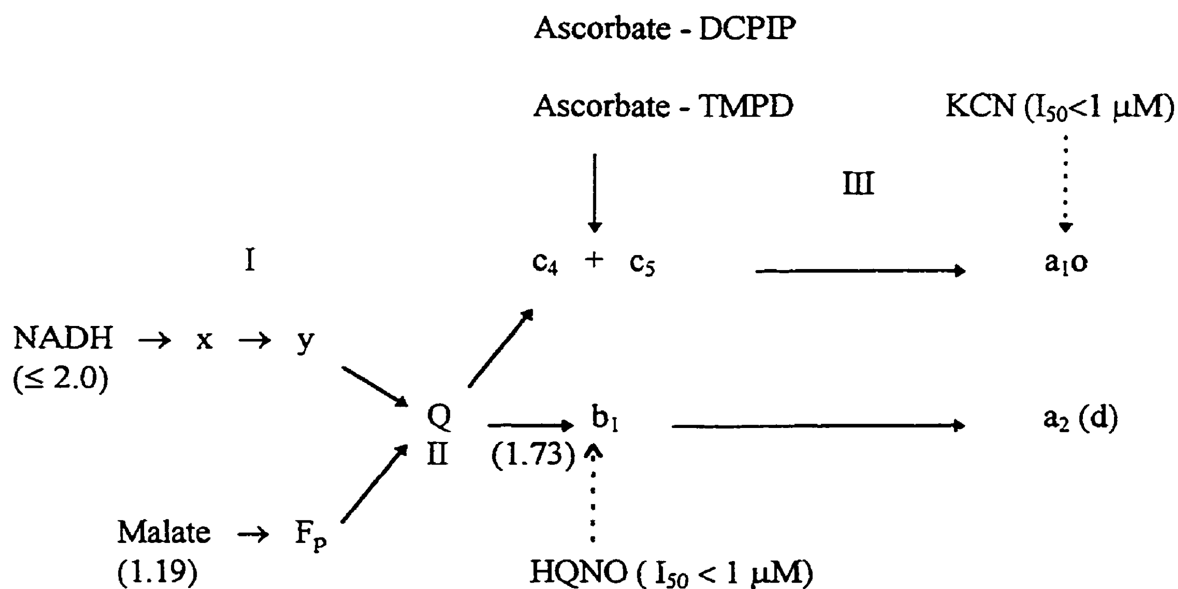


Figure 43. The respiratory system of *A. vinelandii*. I, II and III represent the three energy coupling sites. Numbers in parenthese refer to electron transfer rates (μmoles substrate oxidized/min mg protein) in absence of ADP. Broken arrows indicate inhibitor action. Solid arrows indicate artificial electron transfer (Jones et al., 1971).

Cytochrome *d* is the main terminal oxidase of the branched chain system. The second branch of the system is believed to carry a much lower flux of electrons and to terminate with the *o* and a_1 terminal oxidase (Jones and Redfearn, 1967). The minor path is insensitive to HQNO. Parallelism of the c type cytochrome with cytochrome *b* is shown by the inability of HQNO to affect the steady state reduction of cytochrome *c*. Therefore, electrons flowing through the minor branch are transferred to oxygen by :

Hydroquinone → cytochrome c → a → O_2

This minor path cannot support more than 30 % of the total flux of the respiratory chain (Sagi-Eisenberg and Gutman, 1979). One observation is that when air was introduced to oxidize the reduced cytochrome peaks, cytochrome *c* always remained reduced. This was also found by Jones and Redfearn (1967), and indicated that cytochrome c_4 and c_5 to be on a different path than cytochrome b_1, since the c-type cytochrome showed a greater reduction in the aerobic steady state than the b type cytochrome.

Kodama et al. (1970) in the study of their *T. thiooxidans* found cytochrome *a, b* and *c* types in the particulate fraction. The same findings were also reported by Nakamura et al. (1991) with *T. thiooxidans* JCM 7814, cytochromes *a, b* and *c* found in the membrane fraction. Results by Sugio et al. (1982) agreed with my findings that *T. thiooxidans* ON 106 had cytochromes *c, b, a* and *d,* where c-type cytochrome was present in the soluble fraction and, b-, d- and a-type cytochromes in the membrane. In conclusion for the cytochrome studies, a branched chain electron transport system might exist in *T. thiooxidans* similar to that *in A. vinelandii* consisting of cytochromes *c, b, a* and *d.*

The general conclusion of my thesis work on *T. thiooxidans* 8085 is that metal chelations have a great effect on the mechanism of thiosulfate oxidation as well as other substrates. The mechanism of thiosulfate oxidation at 0.1M sodium citrate at pH 5.0 followed theory A, involving the direct splitting of thiosulfate to sulfur and sulfite as intermediates. In acidic condition, 9K and 0.1M sodium sulfate at pH 2.3, theory B applied where thiosulfate was first oxidized to tetrathionate then it was further hydrolyzed to thiosulfate, sulfur and sulfate. The respiratory system of *T. thiooxidans* 8085 may consist of a branched chain electron transport system that poses some similarity to *A. vinelandii* consisting of cytochrome *c*, *b*, *a*, and *d*.

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