THE OXIDATION OF INORGANIC SULPHUR COMPOUNDS IN RELATION TO DENITRIFICATION IN *THIOBACILLUS DENITRIFICANS*

by

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A thesis submitted in fulfilment of
the requirements for the degree of
Doctor of Philosophy

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March 1974.

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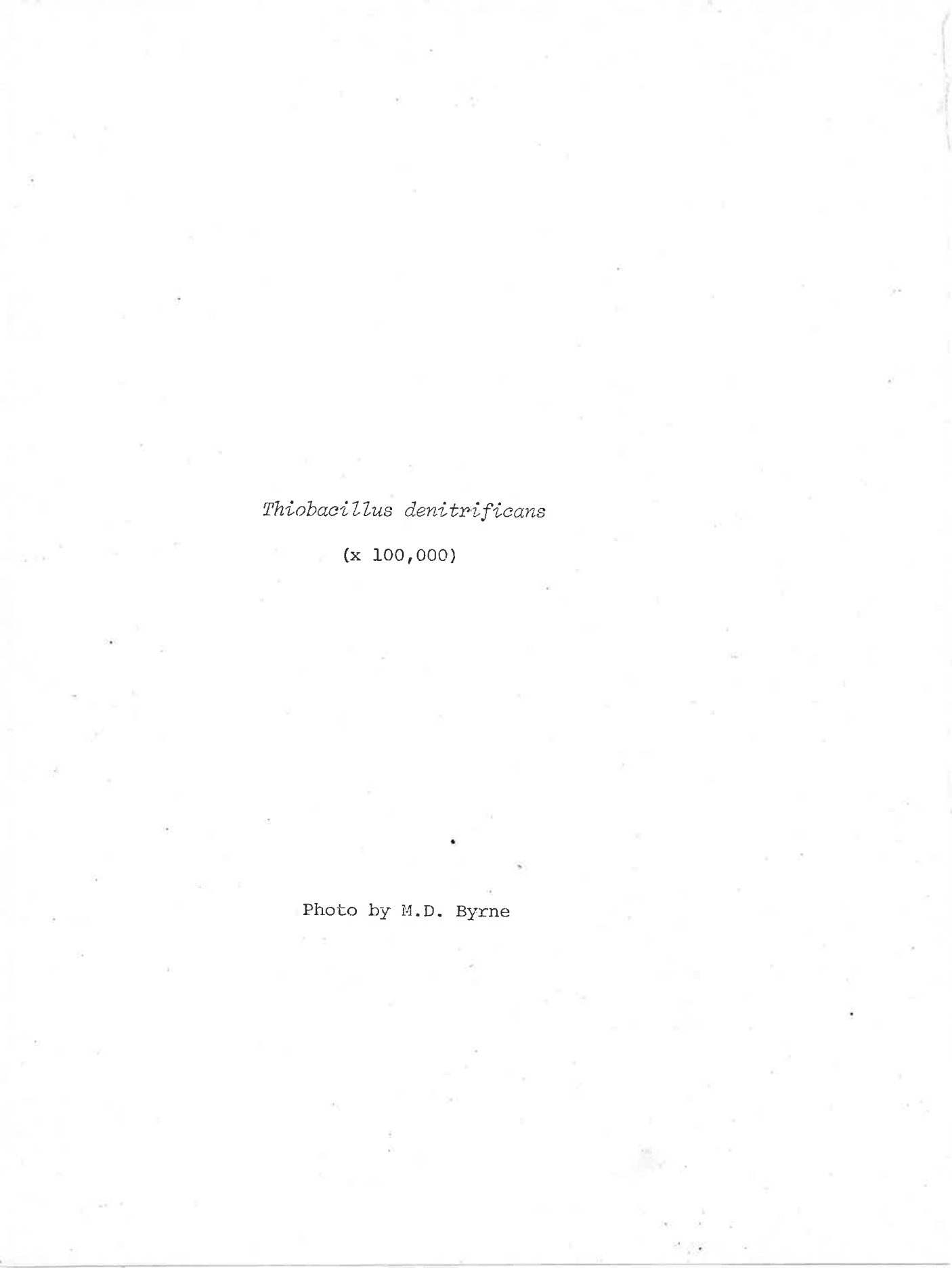

Thiobacillus denitrificans

(x 100,000)

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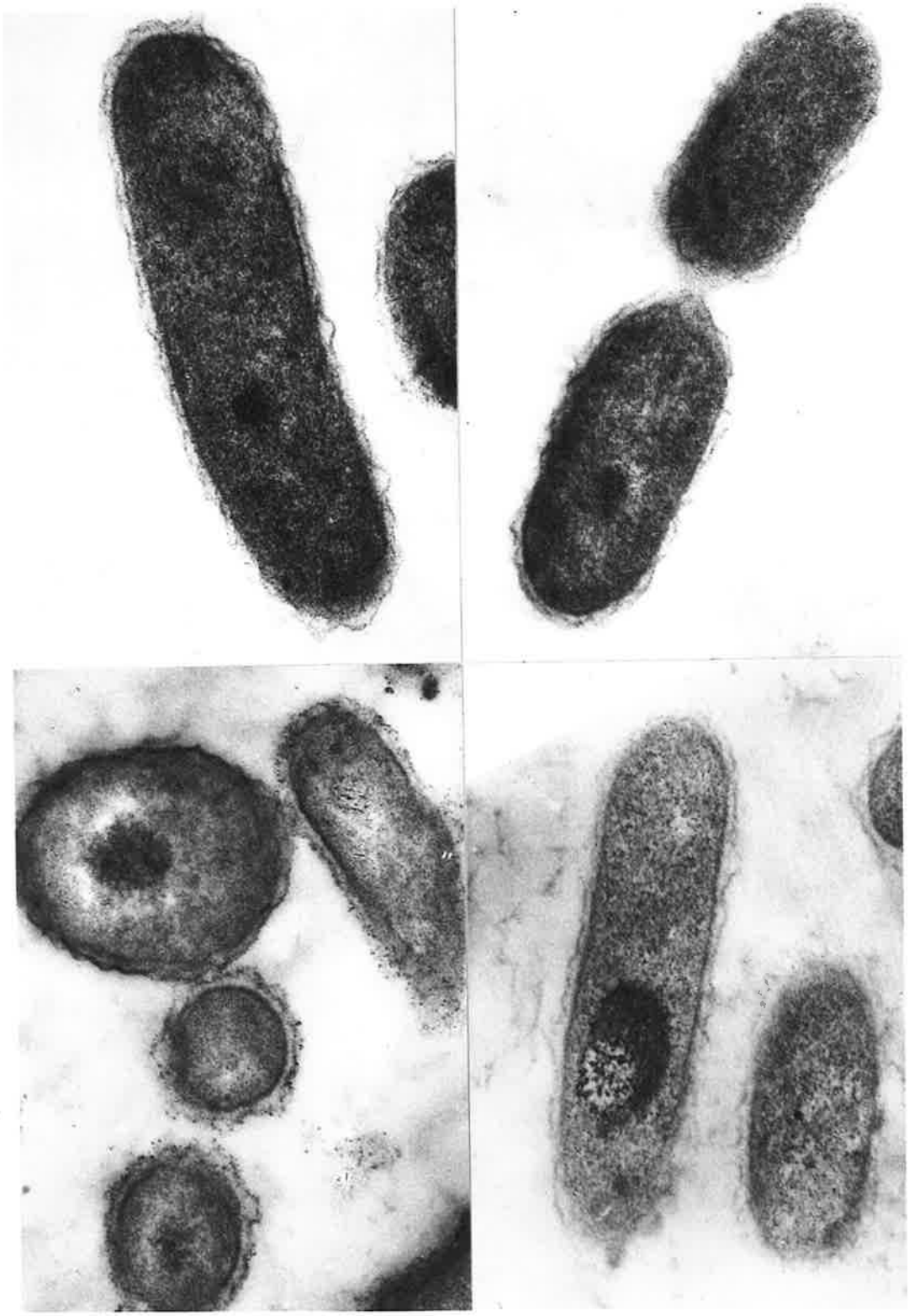

PREFACE

Part of the work described in this thesis has been presented to the Australian Biochemical Society Annual Conferences (Sydney, 1972; Canberra, 1973) and at the 9th International Congress of Biochemistry (Stockholm, 1973). Some of the results have been published or submitted for publication in the journals listed:

1. Sulphide oxidation and nitrate reduction in *Thiobacillus denitrificans*.
   M. Aminuddin & D.J.D. Nicholas,
   Proc. Aust. Biochem. Soc. 5, 26 (1972).

2. Sulphide oxidation linked to nitrate and nitrite reduction in *Thiobacillus denitrificans*.
   D.J.D. Nicholas & M. Aminuddin,
   9th Intern. Congress Biochem. Abstract Book, p. 241, 4i 14 (1973).

3. Sulphide oxidation linked to the reduction of nitrate and nitrite in *Thiobacillus denitrificans*.
   M. Aminuddin & D.J.D. Nicholas,
   Biochim. Biophys. Acta 325, 81-93 (1973).

4. The oxidation of sulphide and sulphite in *Thiobacillus denitrificans*.
   M. Aminuddin & D.J.D. Nicholas,
   Proc. Aust. Biochem. Soc. 6, 28 (1973).

5. An AMP-independent sulphite oxidase from *Thiobacillus denitrificans*. - Purification and properties.
   M. Aminuddin & D.J.D. Nicholas,
   J. gen. Microbiol. - In press.

6. Electron transfer during sulphide and sulphite oxidation in *Thiobacillus denitrificans*.
   M. Aminuddin & D.J.D. Nicholas,
   J. gen Microbiol. - In press.

7. Adenosine 5'-triphosphate-generating systems in *Thiobacillus denitrificans*.
   M. Aminuddin, A.M. Rofe, C.A. Adams & D.J.D. Nicholas,
   J. gen. Microbiol. - Submitted for publication.

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Finally, I wish to express my sincere gratefulness to my wife, Arfah, and to my parents, Mr. and Mrs. Rouse.

DECLARATION

I hereby declare that the work presented in this thesis has been performed by myself between January 1971 and October 1973. To the best of my knowledge no material described herein has been submitted in any previous application for a degree in any university, or reported by any other person, except where due reference is made in the text.

NOMENCLATURE AND ABBREVIATIONS

The enzymes mentioned in this thesis are listed below with their numbers and systematic names as recommended by the Enzyme Commission (Florkin & Stortz, 1965).

Trivial Name	E.C. Name and Number
Adenylate kinase	ATP : AMP phosphotransferase E.C. 2.7.4.3.
ADP-sulphurylase	ADP : sulphate adenylyltransferase E.C. 2.7.7.5.
Alcohol dehydrogenase	Alcohol : NAD oxidoreductase E.C. 1.1.1.1.
Adenosine triphosphatase	ATP phosphohydrolase E.C. 3.6.1.3.
ATP-sulphurylase	ATP : sulphate adenylyltransferase E.C. 2.7.7.4.
Catalase	Hydrogen peroxide : hydrogen peroxide oxidoreductase E.C. 1.11.1.6.
Inorganic pyrophosphatase	Pyrophosphate phosphohydrolase E.C. 3.6.1.1.
APS-kinase	ATP : adenylylsulphate-3'-phospho-transferase E.C. 2.7.1.25.
Cytochrome oxidase	Ferrocytochrome *c* : oxygen oxidoreductase E.C. 1.9.3.1.
Glutathione reductase	Reduced NAD(P) : oxidized glutathione oxidoreductase E.C. 1.6.4.2.

The standard abbreviations for chemicals and symbols in general follow either the tentative rules of the IUPAC-IUB Commision on Biochemical Nomenclature (*Biochem. J.* (1966) 101, 1-7) or the Instructions to Authors for the *Biochemical Journal* (*Biochem. J.* (1973) 131, 1-20).

Chemicals

ATP	adenosine 5'-triphosphate
ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-phosphate
APS	adenosine 5'-phosphosulphate
PAPS	adenosine 3'-phosphate 5'-phosphosulphate
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
DNP	2,4-dinitrophenol
EDTA	ethylenediamine tetraetic acid (Na salt)
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
GSH, GSSG	glutathione and its oxidized form
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine phosphodinucleotide
P_i	inorganic phosphate
PP_i	inorganic pyrophosphate
Tris	*tris*(hydroxymethyl)aminoethane

DIECA	diethyldithiocarbamate (Na salt)
NEM	N-ethylmaleimide
pCMB	para- chloromercuribenzoic acid
POPOP	1,4-bis-(2-(4-methyl-5'-phenyloxazolyl))-benzene
PPO	2,5-diphenyloxazole
PMS	phenazine methosulphate
HOQNO	2-heptyl-4-hydroxyquinoline-N-oxide
CO	carbon monoxide
-SH	sulphydryl
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
TCA	trichloroacetic acid
S_8	elemental sulphur
DD-H_2O	double glass-distilled water
BAL	British Anti Lewisite (2,3-dimercapto-1-propanol)

Symbols and Units

kcal	kilocalories
c.p.m.	counts per minute
d.p.m.	disintegrations per minute
E'_o	standard electrode potential at a given pH
E	absorbance (extinction)
g	gram

g	unit of gravitational field
hr	hour(s)
Ki	inhibitor constant
Km	Michaelis constant
l	litre
µg	microgram
µmole	micromole
µl	microlitre
mg	milligram
ml	millilitre
mmole	millimole
µM	micromolar
mM	millimolar
pmole	picomole
nmole	nanomole
min	minute
M.W.	molecular weight
%	per cent
lb	pound
sec	second
u.v.	ultra violet
λ	wavelength
µCi	microcurie

mCi	millicurie
S10	supernatant fraction obtained after centrifuging disrupted cells of *Thiobacillus denitrificans* at 10,000 x *g* for 30 min
S144 and P144	supernatant and pellet fractions, respectively, obtained after centrifuging S10 at 144,000 x *g* for 90 min

Temperatures are expressed as degrees Centigrade (^{o}C)

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SUMMARY

1. The work described in this thesis is mainly concerned with the metabolism of inorganic sulphur and nitrogen compounds and their inter-relationships in the chemoautotrophic bacterium, *Thiobacillus denitrificans*.

2. Cells and crude extracts (S10) catalysed the enzymic oxidation of sulphide, which may be coupled via the respiratory chain to either oxygen, nitrate or nitrite as terminal electron acceptors. Enzyme activity was associated mainly with membrane fraction (P144).

Cell suspensions reduced nitrate and nitrite to NO, N_2O and N_2 gases when sulphide was the electron donor. Sulphide-linked nitrate reductase was detected in crude extracts (S10) but the P144 and S144 fractions catalysed the reaction only when they were recombined. Sulphide linked nitrite reductase activity, located mainly in the P144 fraction, had a pH optimum of 7.5 with one mole of sulphide oxidized per mole of nitrite reduced.

The initial product of sulphide oxidation was a membrane-bound polysulphide. In the absence of either nitrate or nitrite, sulphide was oxidized to polysulphide and sulphite. When nitrate was present sulphide was oxidized to sulphate with

a concomitant reduction of nitrate to nitrite. Under anaerobic conditions and in the presence of nitrite, sulphide was oxidized to polysulphide only. The formation of this membrane-bound polysulphide was inhibited by CO.

3. The oxidation of elemental sulphur was catalysed by an enzyme present in the S144 fraction; GSH and a low molecular weight, heat-stable factor were required for the reaction. Sulphur oxidation may be linked to oxygen uptake in the S144 fraction and to nitrate reduction in the crude extracts (S10). The initial product of sulphur oxidation was sulphite.

4. There are two sulphite oxidizing enzyme systems, namely a soluble APS-reductase and a particulate, AMP-independent sulphite oxidase. The latter enzyme may be linked to either oxygen uptake, the reduction of ferricyanide or nitrate reduction while the former was linked to ferricyanide only.

Oxygen uptake coupled to sulphite oxidation in the P144 fraction was not affected by AMP but was inhibited non-competitively by nitrate.

Sulphite oxidase and APS-reductase were purified and their properties compared.

5. Thiosulphate oxidation located in the membrane fraction may be linked to either oxygen uptake or nitrate reduction. Thiosulphate utilised oxygen only after adding GSH whereas nitrate reduction occurred with thiosulphate alone.

$^{35}S\text{-}SO_3^=$ was oxidized to ^{35}S-sulphide, ^{35}S-polysulphide, ^{35}S-tetrathionate and ^{35}S-sulphate. In the presence of GSH $S\text{-}^{35}SO_3^=$ was oxidized mainly to ^{35}S-sulphite but when nitrate was substituted for GSH, the main products were ^{35}S-tetrathionate and ^{35}S-sulphate.

6. Nitrate was oxidized to nitrite by cell suspensions and crude extracts (S10) when thiosulphate, sulphide, sulphite and NADH were the electron donors. These compounds also reduced nitrite in intact cells but in crude extracts nitrite was reduced by sulphide and NADH only.

Cytochromes of the *c*, *a* and *d* types were detected in crude extracts. In the membrane fraction (P144) cytochromes of the *c* and *d* types reduced by sulphide under anaerobic conditions were reoxidized by either oxygen or nitrite. Sulphite, however, reduced only cytochrome *c* which was reoxidized by nitrate and oxygen but not by nitrite. In the S144 fraction cytochromes of the *c* and *a* types reduced by sulphite were reoxidized by oxygen but nitrate and nitrite were ineffective. Cytochromes of the *a*

and *d* types combined with CO and these effects were reversed by light.

7. Particulate fractions (P144) catalysed the phosphorylation of ADP to ATP during the oxidation of either various inorganic sulphur compounds or NADH. The production of ATP was verified by the firefly luciferin-luciferase enzyme as well as by following the incorporation of ^{32}Pi into ATP. During the oxidation of either sulphide, sulphite or NADH, ATP production was inhibited by 2,4-dinitrophenol and oligomycin as well as by compounds that restrict electron transfer. Under anaerobic conditions, intact cells produced ATP during either sulphide oxidation linked to nitrite reduction or the oxidation of sulphite coupled to the reduction of nitrate.

In the S144 fraction, ATP was formed from APS and Pi. The S144 fraction contained high activities of ATP-sulphurylase, inorganic pyrophosphatase and adenylate kinase but ADP-sulphurylase activity was relatively low. The contribution of these enzymes to substrate level phosphorylation was investigated.

8. ATP-sulphurylase was purified about 250-fold and some of the properties of the enzyme were studied.

1. LITERATURE REVIEW

1.1 The Metabolism of Inorganic Compounds of Sulphur and Nitrogen by Bacteria

The study of the metabolism of inorganic compounds of nitrogen and sulphur stems from the pioneering work of Sergei Winogradsky who introduced and firmly established his revolutionary concept of chemosynthesis. He discovered that sulphide was oxidized by species of Beggiatoa (Winogradsky, 1888) and that nitrification occurred in the nitrifying bacteria (Winogradsky, 1890). In 1894 he further demonstrated the fixation of free nitrogen by *Clostridium pasteurianum* (Winogradsky, 1894). The sulphate reducing bacteria were isolated by Beierinck (1895) and Dehérain (1897) discovered bacterial denitrification. At the turn of the century Nathansohn (1902) isolated the thiobacilli species and a few years later Kaserer (1906) discovered the hydrogen bacteria. These major discoveries still form the basis of present concepts on the role of these bacteria in the nitrogen and sulphur cycles in nature.

1.1.1 Some common features of sulphur and nitrogen in biological systems

Sulphur and nitrogen, although different chemically,

have many common features in biological systems (Bandurski, 1965).

(i) Both undergo oxidation-reduction reactions involving an 8-electron change; NO_3^- (valency, +5) to NH_3 (-3), and $SO_4^=$ (+6) to $S^=$ (-2).

(ii) Both elements are usually incorporated into organic forms at their lowest oxidation states.

(iii) Sulphate and nitrate (the highest oxidation states) function as electron acceptors for photosynthetic reactions or for the oxidation of either organic or inorganic metabolites.

(iv) Sulphide and ammonia (the lowest oxidation states) may be reoxidized and the resultant energy incorporated into energy-rich compounds.

1.1.2 The Nitrogen Cycle

The ultimate source of nitrogen for all forms of life is inorganic nitrogen. The nitrogen atom undergoes a series of oxidation-reduction changes mediated by chemical or biochemical reactions, the overall system constituting the well-known Nitrogen Cycle in nature.

Some 80% of the earth's atmosphere contains N_2 ($N \equiv N$) which is either converted into its oxides by electrical discharges in the

atmosphere or is "fixed" by certain microorganisms, e.g. *Azotobacter* (Nicholas & Fisher, 1960), *Clostridium* (Carnahan *et al.*, 1960), *Rhodospirillum* (Schneider *et al.*, 1960), blue-green algae (Schneider *et al.*, 1960), *Chromatium* (Arnon *et al.*, 1960), *Rhizobium* (Bergersen, 1960) and some strains of *Thiobacillus ferrooxidans* (Mackintosh, 1971).

The early product of biological nitrogen fixation is NH_3 and this is incorporated into the organic compound, glutamic acid (Carnahan *et al.*, 1960).

The oxidation of ammonia to nitrate, which is termed 'nitrification' is carried out by *Nitrosomonas* and *Nitrobacter* (Hoffman & Lees, 1953; Lees & Simpson, 1957; Aleem & Alexander, 1958; Wallace & Nicholas, 1969).

Nitrate is reduced by many microorganisms and plants to the level of NH_3 and is incorporated into amino acids, proteins and other cellular constituents. This process is termed 'nitrate assimilation'. Certain microorganisms are also able to use nitrate as a terminal electron acceptor in place of oxygen. This microbial process is termed nitrate respiration or dissimilation (Nason, 1962; Nicholas, 1963).

1.1.3 Transformation of sulphur compounds

Organic and inorganic sulphur compounds are constantly being released and utilized in nature and these transformations have

been incorporated into a Sulphur Cycle that is comparable in many respects to the Nitrogen Cycle.

The complete oxidation of reduced sulphur compounds by chemolithotrophic, photolithotrophic and some heterotrophic organisms gives rise to sulphate (Vishniac & Santer, 1957). This oxidation is balanced in nature by the biological reduction of sulphate to sulphide. Sulphate serves as a sulphur source for plants and for most microorganisms. The most active sulphate reducing system is associated with the bacteria, *Desulphovibrio* and *Desulphotomaculum* which utilize sulphate as a terminal electron acceptor. The production of H_2S by these bacteria is of sufficient magnitude to give rise to geological deposits of sulphide and sulphur (Subba Rao, Iya and Sreenivasaya, 1949; Butlin & Postgate, 1953; Jones *et al.*, 1956).

For purposes of comparison the Nitrogen and Sulphur Cycles are presented in Figure 1.

1.2 The Oxidation of Inorganic Sulphur Compounds by *Thiobacillus*

The genus *Thiobacillus* comprises a small number of closely related species of bacteria which are gram-negative, non-sporulating rods measuring 0.5 by 1-3 μ and, except for the non-motile *Thiobacillus novellus*, are polarly flagellated. The energy

Figure 1. The Nitrogen and Sulphur Cycles.

Adapted from Bandurski (1965).

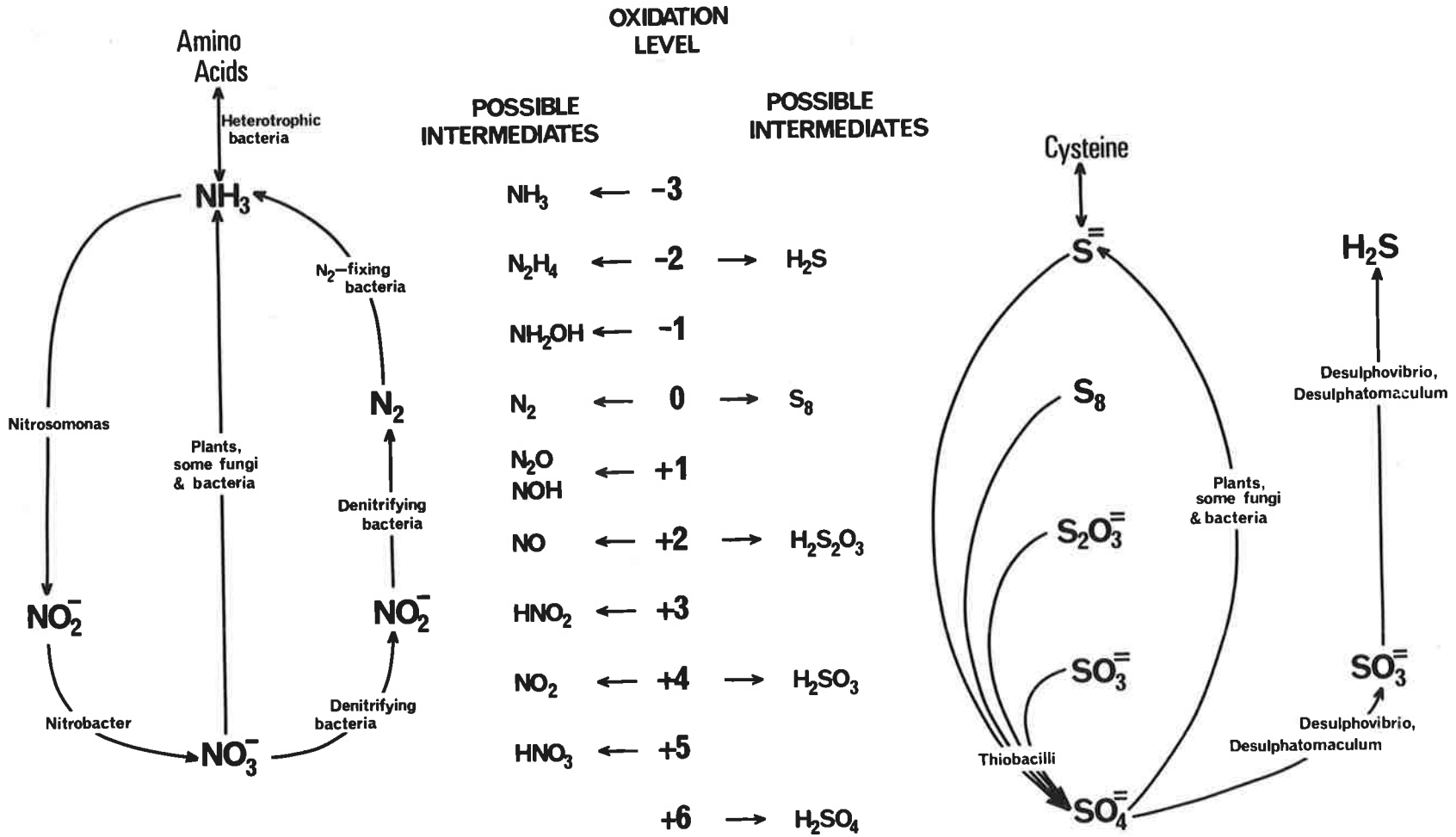

Amino Acids
Heterotrophic bacteria
NH_3
N_2–fixing bacteria
N_2
Nitrosomonas
Plants, some fungi & bacteria
Denitrifying bacteria
NO_2^-
NO_2^-
Nitrobacter
Denitrifying bacteria
NO_3^-
OXIDATION LEVEL
POSSIBLE INTERMEDIATES
POSSIBLE INTERMEDIATES
NH_3 ← -3
N_2H_4 ← -2 → H_2S
NH_2OH ← -1
N_2 ← 0 → S_8
N_2O NOH ← +1
NO ← +2 → $H_2S_2O_3$
HNO_2 ← +3
NO_2 ← +4 → H_2SO_3
HNO_3 ← +5
+6 → H_2SO_4
Cysteine
$S^=$
H_2S
Desulphovibrio, Desulphatomaculum
S_8
Plants, some fungi & bacteria
$S_2O_3^=$
$SO_3^=$
$SO_3^=$
Desulphovibrio, Desulphatomaculum
Thiobacilli
$SO_4^=$

Figure 1

metabolism of the thiobacilli is uniquely adapted to produce all the energy required from the oxidation of inorganic sulphur compounds to sulphate. All members of the genus may utilize carbon dioxide as the sole source of carbon for the synthesis of cellular material (Santer & Vishniac, 1957; Trudinger, 1955, 1956; Milhaud, Aubert & Millet, 1956; Aubert, Milhaud & Millet, 1957; Suzuki & Werkman, 1958; Iwatsuka, Kuno & Maruyama, 1962; Gale & Beck, 1967).

Since these organisms grow in relatively simple inorganic salt media containing an oxidizable sulphur compound and dissolved CO_2, they are considered to be autotrophs (Kelly, 1971). However, recent evidence suggests that some thiobacilli, previously regarded as obligate chemolithotrophs, will grow on organic substrates and are able to assimilate a range of organic compounds. These thiobacilli include *T. novellus* (Vishniac & Santer, 1957), *T. perometabolis* (London & Rittenberg, 1967), *T. neapolitanus* (Kelly, 1967; Saxena & Vishniac, 1970), *T. denitrificans* (Taylor, Hoare & Hoare, 1971; Taylor & Hoare, 1971) and *T. ferrooxidans* (Tabita & Lundgren, 1971; Shafia *et al.*, 1972). Indeed, Rittenberg (1972) has questioned the whole concept of obligate chemoautotrophy.

The classification of the thiobacilli has not been satisfactorily resolved. Johnstone, Townshend & White (1961) claimed that various strains of thiobacilli can be derived from a single species of these

organisms. A recent multivariate analysis by Hutchinson, Johnstone & White (1969) has indicated that several distinct species exist and has grouped many of the 'intermediate' forms reported previously. A better way of examining the inter-relationships between groups of microorganisms is to compare the overall base composition of their DNA complement. This would compare the thiobacilli on the basis of the whole genome, which is a distinct advantage over the multivariate analysis. Jackson, Moriarty & Nicholas (1968) have extracted DNA from various species of thiobacilli and their studies confirmed the work of Hutchinson, Johnstone & White (1965, 1966, 1967) that distinct groups of thiobacilli exist.

The ability of *T. denitrificans* and *T. thiocyanooxidans* to metabolise nitrate and thiocyanate respectively, is not considered to be a unique feature of these thiobacilli (Wooley, Jones & Happold, 1962). Moreover, Baalsrud & Baalsrud (1954) reported that repeated sub-culturing of *T. denitrificans* in air led to a loss in its denitrifying ability and under these conditions these bacteria are indistinguishable from *T. thioparus* (Vishniac & Santer, 1957).

The thiobacilli oxidize sulphide, elemental sulphur, thiosulphate, tetrathionate and sulphite to sulphate (Roy & Trudinger, 1970). The usual substrate for growth, however, is either elemental sulphur or thiosulphate, the latter being preferred

for technical reasons. Much of the research on the thiobacilli has been concerned with the mechanisms of oxidation of inorganic sulphur compounds. Despite considerable experimentation the intermediate compounds formed in these processes have not been firmly established and the relation of inorganic sulphur compound oxidation to energy metabolism is still largely unclear. The general physiology and biochemistry of the thiobacilli has been reviewed by Vishniac & Santer (1957), Lees (1960, 1962), Peck (1962), and Kelly (1968, 1971) while the current status of the pathways of reduced inorganic sulphur compounds has been reviewed in detail by Peck (1968), Trudinger (1967, 1969) and Roy & Trudinger (1970).

1.2.1 Sulphide oxidation

The oxidation of sulphide to sulphate involves the release of eight electrons and is a highly exergonic process (Table 1). These electrons may be coupled to the respiratory chain thereby deriving ATP by oxidative phosphorylation.

The ability of the thiobacilli to oxidize sulphide has long been recognized but the mechanism for this oxidation has not been satisfactorily elucidated. Parker & Prisk (1953) demonstrated the uptake of oxygen during sulphide oxidation and proposed that elemental sulphur was the intermediate. However, this was not observed by Vishniac & Santer (1957) with washed cell preparations

Table 1. Free energy of reactions of some sulphur compounds.

The data presented below were taken or calculated from Latimer (1952), Gibbs & Schiff (1959) and Robbins & Lipmann (1958*B*).

Reaction	ΔF (kcal/mole)
$H_2S + \frac{1}{2}O_2 \longrightarrow S + H_2O$	-50.2
$S + O_2 + H_2O \longrightarrow SO_3^{=} + 2H^+$	-59.7
$SO_3^{=} + H_2O \longrightarrow SO_4^{=} + 2H^+ + 2e$	-33.4
$SO_3^{=} + AMP \longrightarrow APS + 2e$	-18
$APS + PP_i \longrightarrow ATP + SO_4^{=}$	-6 to -11
$S_2O_3^{=} + 2O_2 + H_2O \longrightarrow 2SO_4^{=} + 2H^+$	-100
$2S_2O_3^{=} + \frac{1}{2}O_2 + 2H^+ \longrightarrow S_4O_6^{=} + H_2O$	-5.2
$2S + O_2 + H_2O \longrightarrow S_2O_3^{=} + 2H^+$	-17

of *T. thioparus*, by Charles (1969) with *T. perometabolis* or by London & Rittenberg (1964) who used extracts of *T. thioparus* and *T. thiooxidans*. In these cases the major products, apart from sulphate, which accumulated, were thiosulphate and polythionates. These were also formed in the experiments of Suzuki & Werkman (1959). Postgate (1963) has pointed out that reactions catalysed by enzymes should be distinguished from those occurring chemically, e.g. at physiological pH, in the presence of light and oxygen, thiosulphate is chemically produced from sulphide. These chemical reactions occur more readily when traces of heavy metals, especially copper, are present (Postgate, 1963). In recent reviews, Trudinger (1967, 1969) has proposed that sulphide is linked initially to a disulphide forming a polysulphide.

Kelly & Syrett (1966) showed that DNP strongly inhibited ATP formation coupled to sulphide oxidation in *Thiobacillus* sp. Strain C indicating that oxidative phosphorylation involves the oxidation of sulphide via an electron transfer chain. This was also demonstrated in *T. neapolitanus* by Saxena & Aleem (1973). Cell-free preparations of *T. novellus* (van Poucke, 1962) and *T. thioparus* (London, 1963) oxidize sulphide with a concomitant reduction of cytochrome *c*.

Moriarty & Nicholas (1969) have followed the oxidation of

sulphide in whole cells and extracts of *T. concretivorus* by means of a sulphide electrode. The extracts of *T. thiooxidans* and *T. thioparus* were found to oxidize sulphide at a rate comparable to that in *T. concretivorus*. The reaction occurs in two stages. First, there is a rapid oxidation which is dependent on the amount of both sulphide and crude extract added (Stage I) followed by a second slower reaction (Stage II). These authors found that free sulphide ($S^{=}$) is rapidly utilized by membrane fragments with a concomitant electron transfer, probably via a cytochrome chain to oxygen as a terminal acceptor. The kinetic data for sulphide oxidation, together with the inactivity of boiled fractions, show clearly that the oxidative reactions are enzymic, in contrast with the suggestion that in *T. thiooxidans* the oxidation of sulphide is a heat-stable and non- enzymic reaction (Adair, 1966).

Subsequently Saxena & Aleem (1973) also found that sulphide oxidation in *T. neapolitanus* proceeds via two stages and that the first stage is catalysed by a membrane fraction with the formation of a membrane-bound polysulphide. The second stage, however, is catalysed by a soluble cell-free fraction.

It is also interesting to note that in animal systems sulphite and a protein-bound intermediate are formed during the oxidation of sulphide (Baxter & van Reen, 1958 *A*, *B*).

1.2.2 Oxidation of elemental sulphur

The ability of thiobacilli to oxidize elemental sulphur is induced by growth on sulphur. This was shown by Margalith, Silver & Lundgren (1966) with *T. ferrooxidans* and later confirmed by Silver & Lundgren (1968).

Suzuki & Werkman (1959) and Silver & Lundgren (1968) showed that the oxidation of sulphur by extracts of *T. thiooxidans* necessitates the addition of substrate concentrations of glutathione and they suggested that sulphur is reduced to sulphide prior to oxidation:

$$S + 2GSH \longrightarrow H_2S + GSSG$$

$$NADPH + H^+ + GSSG \xrightarrow[\text{reductase}]{GSH} NADP^+ + 2GSH$$

Reduced glutathione is regenerated by a glutathione reductase (Suzuki & Werkman, 1960).

Suzuki (1965*A*) partially purified an enzyme from *T. thiooxidans* which oxidizes sulphur to thiosulphate in the presence of trace amounts of GSH. Polysulphide, generated by the reaction of GSH on the sulphur (S_8) ring, was presumed to be the actual substrate for the enzyme.

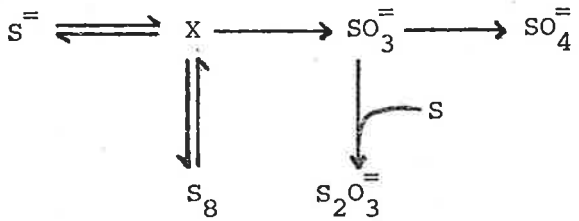
A low but significant incorporation of ^{18}O from $^{18}O_2$ into $S_2O_3^=$ was observed in the presence of this enzyme, suggesting an oxygenase-type activity (Suzuki, 1965*B*). Further studies on the sulphur oxidizing enzyme in thiobacilli showed that sulphite was in fact the initial product of the enzyme reaction (Suzuki & Silver, 1966; Silver & Lundgren, 1968), and that thiosulphate arises from a non-enzymic condensation of sulphur and sulphite.

Cell-free systems from *T. neapolitanus* (Taylor, 1968) and *T. thiooxidans* (Tano & Imai, 1968) which oxidize sulphur to sulphate have been described. These systems differ from that described by Suzuki (1965 *A*, *B*) in not requiring GSH. In fact under certain conditions, thiols actually inhibit the oxidation of sulphur. Adair (1966) has reported a particulate sulphur oxidizing enzyme in *T. thiooxidans* in contrast to Suzuki's system which was soluble. A particulate sulphur oxidizing fraction which requires the addition of soluble components for activity has been briefly reported by Mori, Kodama & Marunouchi (1967).

The sulphur-oxidizing system in a strain of *T. thiooxidans* has recently been characterized (Kodama & Mori, 1968; Kodama, 1969). The system, resolved into three fractions, was reconstituted by recombining the sub-units. Both soluble and particulate fractions

are required for the oxidation of elemental sulphur. The soluble fraction is further separated into two fractions by passing through a collodion membrane resulting in a permeable component and an impermeable one. The former contains a low molecular weight and relatively heat-stable compound which is indispensable for the reconstitution of the sulphur oxidizing system and was found to be a pyridine nucleotide. Oxidation of NADH and NADPH is catalysed by the particulate fraction, the latter being more readily oxidized than the former. Oxidation of NADH and sulphur was inhibited by cyanide, pCMB and CO (photo-reversible). However, inhibitors of sulphur oxidation such as 8-hydroxyquinoline and salicyladoxime had no effect on the oxidation of NADH indicating that the site blocked by such reagents is involved only in the sulphur-oxidizing system.

Since sulphur may, under some circumstances, arise from sulphide (and vice versa) a common early intermediate may be involved in the oxidation of both these compounds to sulphate. Roy and Trudinger (1970) have tentatively schemed the oxidation of sulphide and sulphur as follows:-



where X may be a derivative of glutathione or perhaps a membrane-bound thiol.

1.2.3 Sulphite oxidation

There are two main pathways of sulphite oxidation in the thiobacilli. An AMP-dependent sulphite oxidation has been reported in *T. thioparus* (Peck, 1961; Peck, Deacon & Davidson, 1965; Lyric & Suzuki, 1970*B*), *T. denitrificans* (Bowen, Happold & Taylor, 1966) and *T. neapolitanus* (Hempfling, Trudinger & Vishniac, 1967). Peck (1960, 1962) proposed the following sequence of reactions for sulphite oxidation:-

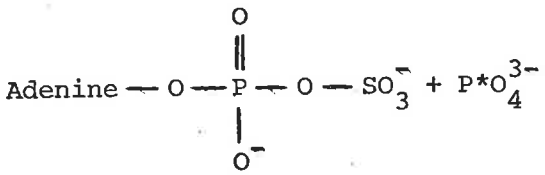
$$SO_3^{=} + AMP \xrightarrow{\text{APS-reductase}} APS + 2e$$

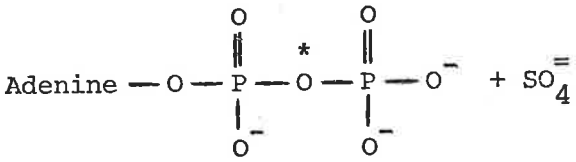
$$APS + Pi \xrightarrow{\text{ADP-sulphurylase}} ADP + SO_4^{=}$$

$$2ADP \xrightarrow[\text{kinase}]{\text{Adenylate}} AMP + ATP$$

That the above pathways are functional is supported by the fact that the oxidation of sulphite by extracts of *T. thioparus* is stimulated by AMP (Peck, 1961) and is accompanied by the formation of APS (Peck, Deacon & Davidson, 1965). Furthermore, the presence of the above enzymeshave been demonstrated in *T. thioparus* and other

thiobacilli (Peck, 1961). Peck & Fisher (1961, 1962) showed that the esterification of inorganic phosphate occurs concomitantly with AMP-dependent sulphite oxidation. Santer (1959) observed that O_2 from phosphate is incorporated into $SO_4^{=}$ during the oxidation of thiosulphate by *T. thioparus*. This may be explained from the mechanism of ADP-sulphurylase (Peck & Stulberg, 1962) where O_2 is transferred from ^{18}O-labelled inorganic phosphate to the bridge oxygen of ADP.





The enzyme APS-reductase has been isolated from *Desulphoribrio desulphuricans*, where it plays a central role in sulphate reduction (Peck, 1959) and is apparently very similar to the enzymes isolated from the thiobacilli. The purified enzyme from *T. denitrificans* contains FAD and ferric iron (Bowen, Happold & Taylor, 1966). Although N-ethylmaleimide inhibits APS-reductase from *T. denitrificans*

the results with other thiol-binding reagents indicate that enzyme activity does not depend on thiol groups. Lyric & Suzuki (1970*B*) have purified APS-reductase from *T. thioparus*. They claim that the enzyme contains FAD, non-haem iron and labile sulphide. The presence of both acid-labile and non-haem iron has been considered to be characteristic of a system in which iron undergoes cyclic reduction and reoxidation (Handler, Rajagopalan & Aleman, 1964). Thiosulphate, nitrite or hydroxylamine does not replace sulphite as a substrate for this enzyme (Kemp *et al.*, 1963).

Previous workers have been unsuccessful in linking APS-reductase to electron acceptors other than ferricyanide. Lyric & Suzuki (1970*B*), however, have reported a coupling to yeast cytochrome *c* in *T. thioparus*. They suggested that failure to detect coupling to cytochromes in the past may have been due to lower activity with the cytochrome (about 25% of the activity with ferricyanide) and to a higher pH optimum (9.5 compared to 7.4 for ferricyanide). Coupling to horse heart cytochrome *c* was also reported but this had only 29% of the activity with yeast cytochrome *c*.

Bowen, Happold & Taylor (1966) and Peck, Deacon & Davidson (1965) have demonstrated APS formation by purified APS-reductase from *T. denitrificans* and *D. desulphuricans* respectively, in the absence of

any added electron acceptor, presumably because of the reactivity of the enzyme with oxygen. Peck (1968) suggested that the electrons released during APS formation are transferred to molecular O_2 by way of an electron-transfer system. The demonstration of AMP-dependent sulphite oxidation coupled to yeast cytochrome *c* in *T. thioparus* supports this idea (Lyric & Suzuki, 1970*B*).

The second enzymic sulphite oxidation does not require AMP and may involve a different mechanism. Cook (quoted by Adair, 1966) did not detect APS-reductase in *T. thiooxidans*. Moreover, there is no evidence that APS is an intermediate in sulphite oxidation in *T. concretivorus* (Moriarty, 1969). Rapid sulphite oxidation in the absence of added AMP is also catalysed by extracts of *T. denitrificans* (Milhaud, Aubert & Millet, 1958), and of *T. thioparus* after treatment with activated charcoal (London & Rittenberg, 1964) and by washed 'cell fragments' of *T. thiooxidans* (Adair, 1966).

A sulphite oxidizing enzyme ($SO_3^=$: cytochrome *c* oxidoreductase) which couples sulphite oxidation to either cytochrome *c* or ferricyanide has been purified from *T. novellus* (Charles & Suzuki, 1966*B*) and mammalian liver (Howell & Fridovich, 1968). This enzyme is soluble, relatively stable and once purified is unable to couple to oxygen as the terminal acceptor. Since the enzyme from *T. novellus* is strongly inhibited by thiol-binding reagents it is likely that

-SH groups may be involved. The enzyme is specific for sulphite and shows no activity with thiosulphate, cysteine or glutathione. In crude extracts of *T. novellus*, the oxidation of sulphite is inhibited by azide and cyanide and it would appear that the oxidation results from the combined action of the sulphite oxidizing enzyme and a cytochrome oxidase (Charles & Suzuki, 1966*B*).

$$SO_3^{=} + 2\ \text{Cyt. } c - Fe^{3+} \longrightarrow SO_4^{=} + 2\ \text{Cyt. } c - Fe^{2+}$$

$$2\ \text{Cyt. } c - Fe^{2+} + 2H + \tfrac{1}{2}O_2 \longrightarrow 2\ \text{Cyt. } c - Fe^{3+} + H_2O$$

Lyric & Suzuki (1970*A*) have also isolated a sulphite oxidizing enzyme from *T. thioparus* which is similar to that purified from *T. novellus* (Charles & Suzuki, 1966*B*). The enzyme, which coupled more readily to cytochrome *c* than to ferricyanide, did not function with O_2 as an electron acceptor. The enzyme contains non-haem iron but labile sulphide was not detected. The marked inhibition by phosphate is thought to result from pseudo-product inhibition because of the structural similarity to sulphate.

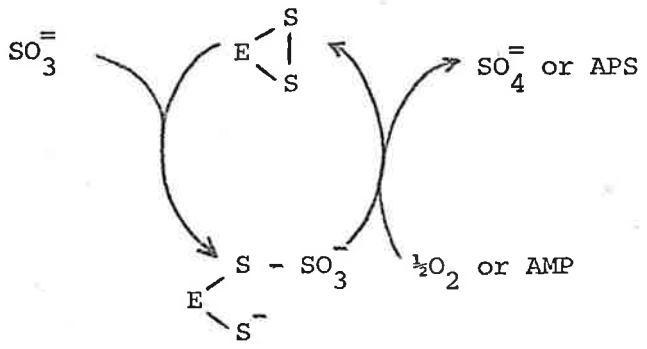
Other workers have reported a particulate sulphite oxidizing enzyme which is coupled to electron carriers of the respiratory chain. In *T. concretivorus* sulphite oxidation is mediated by an enzyme which transfers electrons through the cytochrome system to oxygen or to

ferricyanide (Moriarty, 1969). The involvement of the electron transfer chain is confirmed by the reduction of cytochromes on adding sulphite and by the effects of various inhibitors. Inhibition of sulphite oxidation by azide, assayed by oxygen uptake or by ferricyanide reduction, suggests that a metal may be present at the actual site (Moriarty, 1969). The non-competitive nature of the inhibition indicates that it does not bind sulphite initially. Sulphydryl groups are probably involved at the substrate-binding site because pCMB and NEM inhibited sulphite oxidation. Thus, the active site may contain thiols and a metal. A sulphite oxidizing enzyme in *T. thiooxidans* was also reported to be membrane-bound (Adair, 1966).

Peeters & Aleem (1970) found that sulphite oxidation in *T. denitrificans* is coupled to either O_2 or to nitrate via flavoprotein and cytochromes. Adams, Warnes & Nicholas (1971*A*), found that the sulphite oxidizing system was coupled to a nitrate reductase in particulate fractions of the same bacterium. Sulphite oxidation by these fractions was not affected by AMP and inhibitor and spectral studies indicate that electron transport occurs by way of various electron carriers of the respiratory chain.

A sulphite oxidizing enzyme from *T. neapolitanus* has been partially purified (Hempfling, Trudinger & Vishniac, 1967). In

contrast to the enzyme from *T. novellus* (Charles & Suzuki, 1966*B*), that from *T. neapolitanus* reacts with both ferricyanide and O_2 and the reaction is stimulated 1.5 to 2-fold by AMP. The fact that the relative activities in the presence and absence of AMP remained fairly constant throughout the course of purification suggests that a single enzyme may catalyse both the AMP-dependent and AMP-independent sulphite oxidation. Hempfling (quoted by Trudinger, 1967) suggested that sulphite oxidation by the *T. neapolitanus* enzyme may involve the formation of an enzyme-sulphite intermediate which reacts with AMP to form APS or more slowly with water to form sulphate. Since reduced glutathione and, under some conditions, thiol-binding reagents inhibit the *T. neapolitanus* enzyme (Hempfling, Trudinger & Vishniac, 1967), a disulphide group was suggested as a possible binding site for sulphite on the enzyme molecule:-



Trudinger (1969) suggests that the sulphite oxidizing enzyme of

Charles & Suzuki (1966*B*) is a modified APS-reductase which has lost the ability to react with AMP.

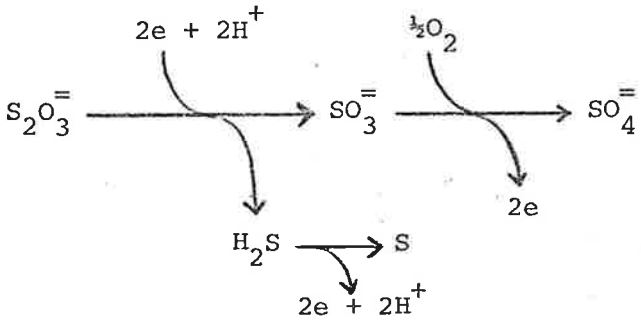
1.2.4 Thiosulphate oxidation

Two pathways have been proposed for the oxidation of thiosulphate to sulphate. One is a polythionate pathway whereby thiosulphate is oxidized to tetrathionate and then to sulphate with the probable formation of other polythionate intermediates (Nathansohn, 1902; Vishniac, 1952; London & Rittenberg, 1964; Pankhurst, 1964). The other pathway involves an initial cleavage of thiosulphate to yield two one-sulphur moieties as proposed by Peck and his associates (Peck, 1962, 1968).

The cleavage of thiosulphate yields sulphite and either sulphide or elemental sulphur. The latter is a common product of thiosulphate metabolism and arises from the sulphane sulphur group of thiosulphate (Skarzynsky & Szczepkowski, 1959; Skarzynski, Szczepkowski & Weber, 1960; Peck & Fisher, 1962; Trudinger, 1964).

Peck (1960) and Peck & Fisher (1962) reported that AMP and substrate concentrations of GSH, homocysteine or cysteine stimulated thiosulphate oxidation in extracts of *T. thioparus* and that sulphur and sulphate, instead of polythionates, are formed. The presence of a glutathione-dependent thiosulphate reductase led Peck to propose that thiosulphate is initially cleaved to sulphide and

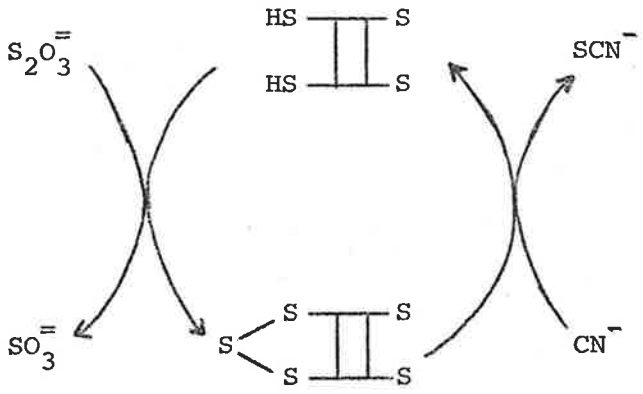
sulphite, which are subsequently oxidized to sulphur and sulphate respectively.



This hypothesis was later modified by Suzuki & Silver (1966) who proposed that the initial cleavage resulted in sulphur and sulphite; GSH is only required in catalytic amounts. They have also presented evidence for sulphur and sulphite oxidizing enzymes in organisms grown on thiosulphate and suggested a pathway for complete oxidation of thiosulphate to sulphate where enzymes have been demonstrated for each stage. Enzymes have not been found, however, for each step in the polythionate pathway.

Charles & Suzuki (1966*A*) suggested that the enzyme rhodanese, which is present in some thiobacilli, may function in the cleavage of thiosulphate to 'S' and sulphite. The enzyme catalyses the cyanolysis of $S_2O_3^=$ to SCN^- and $SO_3^=$. Rhodanese may also be responsible for the reduction of thiosulphate by extracts of *T. thioparus*, since rhodanese enzymes from mammalian tissue and from *Bacillus subtilis* have been shown to catalyse both the cyanolysis and the reduction

of thiosulphate (Sorbo, 1953; Westley & Green, 1959; Villarejo & Westley, 1963, 1966). Bowen, Butler & Happold (1965) suggested a mechanism for rhodanese action in *T. denitrificans* involving the formation of an intermediate trisulphide from thiosulphate and thiol groups on the enzyme:



It is interesting to note that the first step of this reaction mechanism resembles the reductive cleavage of thiosulphate as proposed by Peck and his colleagues (Peck, 1962, 1968).

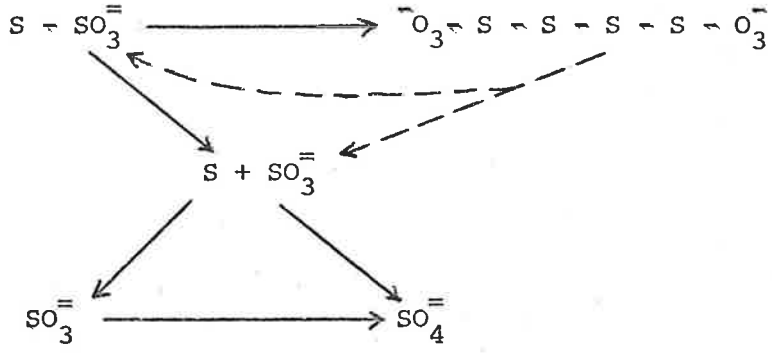
Many workers have reported the formation of polythionates during thiosulphate oxidation. In reviewing earlier work, Vishniac & Santer (1957) proposed a mechanism involving the polythionates as intermediates. Tano, Asano & Imai (1968) have shown that extracts of *T. thiooxidans* oxidize thiosulphate to tetrathionate and trithionate. Moriarty (1969) reported that thiosulphate is oxidized

to tetrathionate by extracts of *T. concretivorus*. Nevertheless, the extent of polythionate formation are variable depending on experimental conditions (see Roy & Trudinger, 1970, for details).

A number of possible mechanisms for thiosulphate oxidation has been advanced to accommodate the various hypotheses (Lees, 1960; Vishniac & Trudinger, 1962). Moriarty (1969) found that the inner (oxidized labelled S atom of thiosulphate ($S^{35}SO_3^=$) is oxidized to sulphate by *T. concretivorus* without the formation of any detectable intermediates. The products of oxidation with the outer (reduced) labelled S atom of thiosulphate ($^{35}SSO_3^=$) were sulphate, thiosulphate and elemental sulphur or a protein-bound form of sulphur which remained at the origin during electrophoresis.

Lyric & Suzuki (1970*C*) proposed that in *T. thioparus*, thiosulphate may either be converted to tetrathionate by the thiosulphate oxidizing enzyme or cleaved to form sulphur and sulphite. The thiosulphate oxidizing enzyme appears to initiate a secondary pathway which operates under conditions of high initial thiosulphate concentration. By oxidizing thiosulphate to tetrathionate the organism is then able to utilize this source of energy. An important property of the enzyme is its inhibition by sulphite. Unless the formation of tetrathionate from thiosulphate is inhibited a 'short circuit' will occur in the metabolic scheme resulting in continual recycling of

sulphur compounds (Lyric & Suzuki, 1970*C*). Once the reaction is stopped thiosulphate and tetrathionate can then be further metabolised to sulphate.



1.2.5 Electron transport

The oxidation of inorganic sulphur compounds in the thiobacilli is closely associated with a respiratory chain consisting of cytochromes and other components of electron transport systems. Thus, Cook & Umbreit (1963) reported that *T. thiooxidans* contains benzoquinone and the importance of quinones in the oxidation of sulphur is suggested by a parallel decrease in both sulphur oxidation and cellular ubiquinones when irradiated by ultraviolet light (Adair, 1968). Moriarty & Nicholas (1969) identified ubiquinone as a component of the respiratory chain involved in sulphide oxidation in *T. concretivorus*.

Cytochrome pigments have been detected in a number of species of thiobacilli. A soluble cytochrome *b* (α-band 557 nm) has been purified from *Thiobacillus X* (Trudinger, 1961 *A*, *B*). This cytochrome is reduced by thiosulphate in the presence of thiosulphate oxidizing enzyme. The cytochrome *b* (α-band 563 nm) from *T. concretivorus* mediates electron flow in both the sulphite and sulphide oxidation pathways (Moriarty & Nicholas, 1970*B*). Aleem (1965) found that cytochrome *b* in *T. novellus*, grown autotrophically with thiosulphate, does not mediate electron flow between that substrate and oxygen. Cytochrome *b* has also been detected in *T. denitrificans* (Peeters & Aleem, 1970).

Several cytochromes of the *c* type have been purified and characterized. A comparison of the properties of cytochrome *c* from various species of thiobacilli have been presented by Roy and Trudinger (1970). Milhaud, Aubert & Millet (1958) isolated a cytochrome *c*-552 (E_o' at pH 7.0 of + 0.27V) which mediates electron flow between either thiosulphate or sulphite and nitrate. Three cytochrome *c* types were isolated from *Thiobacillus X* - cytochrome *c*-550, *c*-553.5 and *c*-557 (Trudinger, 1961 *A*, *B*). All three were autoxidizable at pH 7.0 but none combined with CO. Cytochrome *c*-553.5 was shown to participate in the oxidation of thiosulphate to tetrathionate by the thiosulphate oxidizing enzyme in

Thiobacillus X (Trudinger, 1961*B*).

Aleem (1969) has demonstrated a cytochrome *c* complex (absorption maxima at 550, 553, 555 and 557 nm) in *T. neapolitanus*. Electrons from thiosulphate and sulphite enter the respiratory chain of *T. neapolitanus* at the level of cytochrome *c* and terminate with oxygen via cytochrome aa_3. NADH oxidation is also coupled to cytochrome *c* in *T. neapolitanus*.

A cytochrome *c*-550 which has been purified from *T. concretivorus* (Moriarty & Nicholas, 1969) is involved in the oxidation of sulphide to polysulphide. Carbon monoxide can bind to this cytochrome, thus inhibiting the production of polysulphide. A cytochrome *c*-550 was also found in *T. thioparus* (Cook & Umbreit, 1963). The properties of a purified sulphite oxidizing enzyme which uses cytochrome *c* as an electron acceptor from this bacterium has been studied by Lyric & Suzuki (1970*A*). Cytochrome *c*-550 from *T. novellus* was partly purified and shown to be the electron acceptor for a purified sulphite oxidizing enzyme in *T. novellus* (Charles & Suzuki, 1966*B*). This cytochrome was subsequently purified and studied by Yamanaka *et al.* (1971). More recently, Kusai & Yamanaka (1973) separated a cytochrome *c*-553 which is involved in sulphide oxidation in a *Chlorobium* species.

Cyanide inhibited the oxidation of sulphur by *T. thiooxidans* (Vogler, Le Page & Umbreit, 1942; Iwatsuka & Mori, 1960; Adair, 1966; Tano & Imai, 1968*A*) and the reoxidation of cytochromes by a particulate fraction of *T. neapolitanus* (Trudinger, 1961*B*). Sulphite oxidation by crude extracts of *T. novellus* is also inhibited by cyanide.

Iwatsuka & Mori (1960) have reported the inhibition of sulphur oxidation by CO in *T. thiooxidans*. The inhibition was not reversed by light which suggests that the terminal oxidase in this organism differs from the 'classical' cytochrome oxidase. Conversely, CO inhibits thiosulphate oxidation by extracts of *T. novellus* and this effect was reversed by light (Aleem, 1965). The appearance of an absorption band at 600-610 nm in the reduced extracts strongly suggests the presence of cytochrome a-a_3 in *T. novellus*. Moriarty & Nicholas (1969) showed that CO inhibits oxygen uptake during sulphite oxidation and the second (slow) stage of sulphide oxidation. The reversal by light of this inhibition suggests the involvement of a terminal oxidase. These are likely to be cytochromes of the a_1 and d types since a shoulder at 590 nm (cytochrome a_1) and a maximum at 612 nm (cytochrome d) was recorded in the low temperature reduced spectra. Milhaud, Aubert & Millet (1958) reported that difference spectra of CO-reduced extract of *T. denitrificans* showed an

absorption band with a maximum at 418 nm which they interpreted to be a cytochrome of the a-a_3 type. However, Ikuma and Hempfling (quoted by Roy & Trudinger, 1970) have pointed out that this is more likely to be due to cytochrome *o* since they found that the absorption bands for the reduced CO-binding pigment in *T. neapolitanus* was at 570, 540 and 415-417 nm. Moreover, the oxygen affinity of the respiratory system of cells of *T. neapolitanus* is considerably lower than that of cytochrome a-a_3. Trudinger (1961*A*) was unable to detect an *a*-type cytochrome in *T. neapolitanus*. However, Saxena & Aleem (1973) have recently shown that in extracts of this bacterium CO combines with cytochromes of *a* and *o* types during sulphide and thiosulphate oxidation.

Moriarty & Nicholas (1970*B*) have indicated that at least two distinct electron transfer pathways are present in *T. concretivorus*. The one operating during sulphite oxidation is inhibited by CO, azide, HOQNO or piericidin A whereas the other, which is associated with sulphide oxidation, is not.

Peeters & Aleem (1970) reported that thiosulphate oxidation in *T. denitrificans* is linked to nitrate reduction via cytochrome *b*, *c*, *a* and *o* since under anaerobic conditions these cytochromes, reduced by thiosulphate, were reoxidized by nitrate. Similar results were obtained with sulphide, sulphite, NADH and succinate as electron

donors. These workers suggest that electrons enter the respiratory chain at different sites during the aerobic and anaerobic oxidation of inorganic sulphur compounds. Studies with inhibitors indicate that flavoproteins, quinones and cytochrome *b* mediate electron flow to cytochrome *c* during the oxidation of thiosulphate under anaerobic conditions. Under aerobic conditions, however, it appears that electrons proceed directly to cytochrome *c* without involving flavoproteins or cytochrome *b*.

The thiobacilli generate reducing power (NADH) for biosynthetic purposes by an ATP-dependent reversal of electron transport coupled to the oxidation of inorganic sulphur compounds. Energy-dependent reduction of NAD^+ involving the respiratory chain has been demonstrated in *T. novellus* (Aleem, 1965, 1966 *A*, *B*; Aleem & Huang, 1965), *T. neapolitanus* (Aleem, 1969; Saxena & Aleem, 1972) and *T. denitrificans* (Peeters & Aleem, 1970).

1.2.6 Phosphorylation

The occurrence of ATP in the thiobacilli was shown by Barker & Kornberg (1954). In earlier experiments Baalsrud & Baalsrud (1952) have reported that inorganic phosphate is taken up by the thiobacilli during the oxidation of inorganic sulphur compounds. Subsequently, the incorporation of $^{32}P_i$ into ATP was

demonstrated by Milhaud, Aubert & Millet (1957) for *T. denitrificans* and by Vishniac & Santer (1957) for *T. thioparus*. Numerous workers have shown that CO_2 fixation, which may be taken as an index of ATP formation, is coupled to the oxidation of inorganic sulphur compounds (Trudinger, 1955, 1956; Suzuki & Werkman, 1958; Iwatsuka, Kuno & Maruyama, 1962; Johnson & Peck, 1965; Aleem & Huang, 1965).

Evidence for phosphorylation linked to electron transfer has been obtained for cells, and more recently, for cell-free preparations of thiobacilli. Iwatsuka, Kuno & Maruyama (1962) and Beck & Shafia (1964) reported that sulphur oxidation by thiobacilli was strongly inhibited by 2,4-dinitrophenol. Subsequently, Kelly & Syrett (1963, 1964 *A*, *B*, 1966) have demonstrated that DNP inhibits both the formation of ATP and CO_2 fixation in intact cells of *T. thioparus* and *Thiobacillus* sp. strain C.

Phosphorylation coupled to electron transport during the oxidation of mercaptoethanol has been demonstrated in extracts of *T. neapolitanus* (Hempfling & Vishniac, 1965). The system which requires both soluble and particulate components is relatively unstable. The phosphorylation was dependent on electron transfer and a P/O ratio of about 0.4 was recorded. Charles & Suzuki (1966*B*) also reported phosphorylation coupled to sulphite oxidation in extracts of

T. novellus. Similar results were reported by Davis & Johnson (1967) in *T. thioparus*. Ross, Schoenhoff & Aleem (1968) demonstrated a DNP-sensitive phosphorylation during thiosulphate oxidation in extracts of *T. neapolitanus*. Their results were later confirmed by Saxena & Aleem (1973). Moriarty & Nicholas (1970) have shown that ATP is produced during sulphide oxidation in particulate fractions of *T. concretivorus*.

Cole & Aleem (1970) reported that oxidative phosphorylation occurred in the soluble (S144) fraction of *T. novellus*. The system was uncoupled by DNP. A P/O ratio with succinate as substrate of 1.9 was obtained. This is the only reported observation of a 'soluble' oxidative phosphorylating system in any organism. Most of the evidence, however, support the view that oxidative phosphorylation is associated with the membrane-bound respiratory chain.

Unlike other chemolithotrophs, some of the thiobacilli are able to conserve energy by means of substrate phosphorylation during the oxidation of inorganic substrates. Hempfling & Vishniac (1967) have estimated that substrate phosphorylation may contribute as much as 45% of the high-energy phosphate formed during thiosulphate oxidation in continuous culture. Peck (1960, 1962) has postulated a sequence of reactions for ATP production coupled to sulphite oxidation (see Section 1.2.3). Peck & Fisher (1962) further showed

that ATP formation during sulphite oxidation in *T. thioparus* is not inhibited by DNP indicating that phosphorylation linked to electron transfer is not involved. However, the most cogent evidence for substrate level phosphorylation is provided by Kelly & Syrett (1966). These authors observed that phosphorylation coupled to the oxidation of thiosulphate was unaffected by DNP, whereas this uncoupling agent inhibited phosphorylation during sulphide oxidation. They concluded that the DNP-insensitive phosphorylation represents substrate level phosphorylation.

1.3 Activation of Sulphate

Two sulphur-containing nucleotides are usually involved in the metabolism of sulphur compounds in most biological systems. These are adenosine 5'-phosphosulphate (APS) and adenosine 3'-phosphate 5'-phosphosulphate (PAPS). The latter compound has often been referred to as 'active sulphate'.

The two enzymes required for the synthesis of PAPS were first separated from yeast extracts by Wilson & Bandurski (1956), and later characterized by Bandurski, Wilson & Squires (1956), Wilson & Bandurski (1958 *A*, *B*) and Robbins & Lipmann (1956 *A*, *B*, 1957, 1958 *A*, *B*). These enzymes catalyse the following reactions:

ATP-sulphurylase

(1) $ATP + SO_4^{=} \rightleftharpoons APS + PP_i$

APS-kinase

(2) $APS + ATP \rightleftharpoons PAPS + ADP$

In this review only ATP-sulphurylase will be considered.

1.3.1 ATP-sulphurylase

ATP-sulphurylase has been detected in bacteria (Akagi & Campbell, 1962; Varma & Nicholas, 1970, 1971), fungi (Segel & Johnson, 1963), algae (Abraham & Bachhawat, 1963; Hodson *et al.*, 1968), higher plants (Adams & Johnson, 1968; Ellis, 1969; Balharry & Nicholas, 1970; Shaw & Anderson, 1971, 1972) and animals (Pannikar & Bachhawat, 1968; Levi & Wolf, 1969; Shoyab, Su & Marx, 1972).

The equilibrium for the reaction catalysed by ATP-sulphurylase (equation 1 above) is in favour of ATP formation. The apparent equilibrium constant was found to be of the order of 10^{-8} (Robbins & Lipmann, 1958*B*; Wilson & Bandurski, 1958*B*; Akagi & Campbell, 1962) which corresponds to a standard free energy change of + 11 kcal.

Since the pioneering work of Bandurski & Lipmann, ATP-sulphurylase has been purified and characterized from a variety of

plants, animals and microorganisms. The degree of purification achieved is variable, e.g. it ranges from 2 (DeVito & Dreyfuss, 1964) to 1,200-fold (Robbins & Lipmann, 1958*B*) obtained from baker's yeast.

A variety of assay procedures for ATP-sulphurylase has been employed by various workers including radio-assay techniques, molybdolysis and utilization of PP_i (see Roy and Trudinger, 1970, for details). In the present investigations the back reaction, i.e. the formation of ATP, as assayed by a bioluminescence procedure, was employed.

The enzyme preparations from microorganisms and plants maintained their activity for several months when kept at 0^{o} (Tweedie & Segel, 1971*B*; Shaw and Anderson, 1972). The pH optimum for the enzyme lies between 7 to 9. Mg^{++} is essential for enzyme activity in all the systems reported. ATP-sulphurylase is inhibited by high levels of APS, PP_i and sulphate (Levi & Wolf, 1969; Tweedie & Segel, 1971*B*; Hawes & Nicholas, 1973). The apparent Km values range from 0.47 µM (Balharry & Nicholas, 1970) to 2 mM (Pannikar & Bachhawat, 1968) for APS and from 3 µM (Balharry & Nicholas, 1970) to 1.7 mM (Pannikar & Bachhawat, 1968) for PP_i.

The enzymes prepared from various sources also vary in their molecular weights. Thus, ATP-sulphurylase from animal tissues is

about 1 million (Levi & Wolf, 1969), whereas the yeast enzyme varies between 100,000 and 200,000 (Robbins, 1962; Hawes, 1973). It was found that the enzyme from animal tissues was excluded by Sephadex G-200 (Shoyab, Su & Marx, 1972), whereas that from spinach leaves was not (Balharry & Nicholas, 1970; Shaw & Anderson, 1972).

1.4 Dissimilation of Nitrate by Microorganisms

Some bacteria utilize nitrate as an alternative hydrogen acceptor to oxygen. This process is known as nitrate respiration or dissimilatory nitrate reduction (Kluyver, 1953; Verhoeven, 1956; Nicholas, 1963) since little, if any, of the nitrate is incorporated into cell nitrogen. The dissimilation of nitrate proceeds at a much faster rate than does its assimilation to cell nitrogen. In denitrification the process involves the reduction of nitrate to nitrite, nitric oxide, nitrous oxide and molecular nitrogen.

1.4.1 Nitrate and nitrite reductases

Pollock (1946) observed that nitrate reductase is induced by its substrate in bacteria dissimilating nitrate under anaerobic conditions. This was later confirmed by others (Nason & Takahashi, 1958; Delwiche, 1956; Lam & Nicholas, 1968*A*). Verhoeven & Takeda (1956) and Delwiche (1956) were among the first to observe that the rate of nitrate reduction in dissimilaroty nitrate bacteria was less

when they were grown in air. Oxygen affects nitrate dissimilation either by repressing the formation of nitrate reducing enzyme, or by inhibiting the one already present. The synthesis of nitrate reductase is repressed in *Pseudomonas denitrificans* (Sacks & Barker, 1949), *Ps. stutzeri* (Allen & Van Niel, 1952), *Micrococcus denitrificans* (Kluyver & Verhoeven, 1954) and *Ps. aeruginosa* (Collins, 1955) when grown in air.

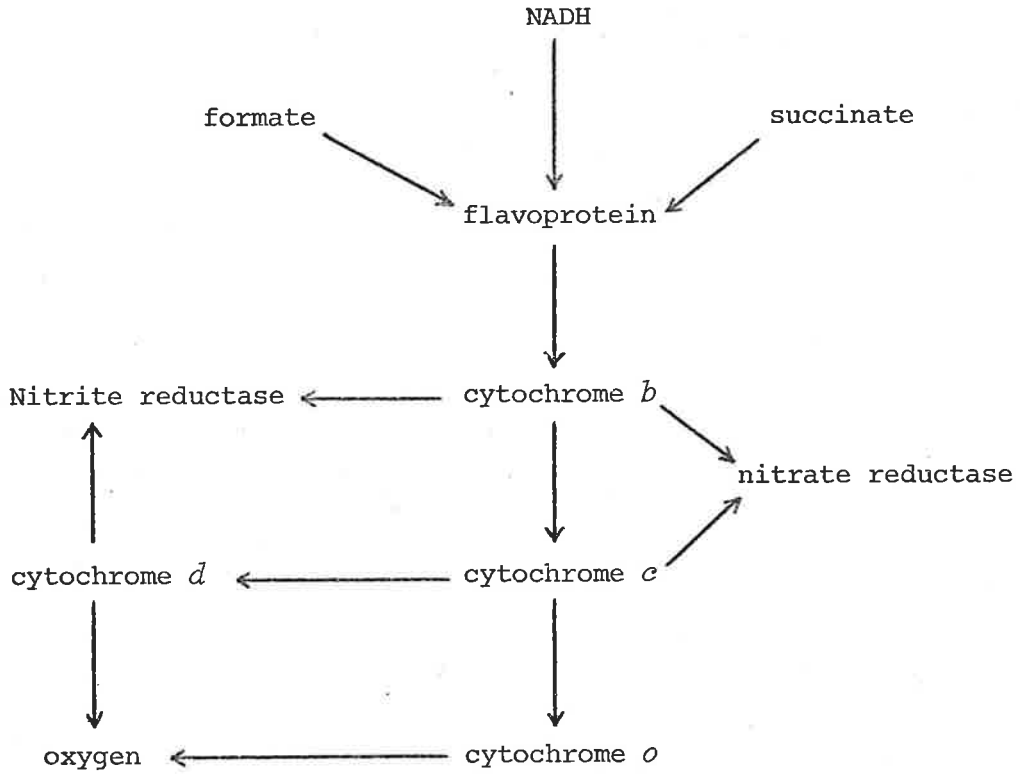
Dissimilatory nitrate reductase has been purified and characterized. The enzyme from *Escherichia coli* was reported to contain cytochrome *b* (Itagaki, Fujita & Sato, 1963). However, in *Ps. aeruginosa* (Fewson & Nicholas, 1961) and *Achromobacter fischeri* (Sadana & McElroy, 1957), nitrate reductase is associated with a *c* type cytochrome. A survey of the nitrate reductase enzymes from various nitrate dissimilatory bacteria suggests that the enzyme differs in its donor specificity as well as in the composition of the electron carriers.

Nitrite reductase in the denitrifying bacteria was first identified by Yamagata (1939) and later purified from *Ps. aeruginosa* by Walker & Nicholas (1961). The latter workers found that the enzyme contained cytochrome *c*. This cytochrome was also associated with a nitrite reductase in *Ps. denitrificans* (Iwasaki & Mori, 1958). Nitrite was found to reoxidize a cytochrome *c* associated with a

nitrite reductase in *Achromobacter* (Cole & Wimpenny, 1966). Lam & Nicholas (1969*C*) and Newton (1969) reported that the enzyme from *M. denitrificans* contains a cytochrome *cd* complex, which can also function as a cytochrome oxidase linking to O_2.

Sato & Niwa (1952) were the first to show that cytochromes were involved as electron carriers in nitrate dissimilation. Thus, they found that cytochrome *b* from *E. coli* was reoxidized by nitrate under anaerobic conditions. Egami, Ishimoto & Taniguchi (1961) observed that nitrate and oxygen respiration required different electron donors, i.e. formate and NADH, respectively, and suggested that more than one respiratory chain may be involved. Nevertheless, based on numerous studies by others, some generalizations may be made about the respiratory chain of nitrate dissimilatory bacteria. Thus, the nitrate and nitrite respiratory pathways which are associated with cell membranes usually utilize the same electron carriers as in systems respiring to oxygen. An increase in the concentrations of electron carriers often accompanies growth under anaerobic conditions with nitrate as the terminal electron acceptor.

The electron transfer pathways that have been described for a variety of denitrifying bacteria may be summarized as follows:



1.4.2 Oxidative phosphorylation

Denitrifying bacteria generate ATP by a phosphorylation coupled to nitrate reduction when they grow under anaerobic conditions with nitrate as the terminal acceptor. Takahashi, Taniguchi & Egami (1957) found that anaerobic reduction of nitrate was accompanied by the incorporation of $^{32}P_i$ into cells of a denitrifying bacterium

isolated from the soil. Similar observations were also made by Ohnishi & Mori (1960) with *Ps. denitrificans*. Subsequently these workers showed that the particulate fractions are involved in the oxidative phosphorylation which was uncoupled by DNP and inhibited by KCN, amytal and antimycin A (Ohnishi & Mori, 1962; Ohnishi, 1963). Oxidative phosphorylation coupled to nitrate reduction was also reported in particulate preparations of *Ps. aeruginosa* (Yamanaka, Ota & Okunuki, 1962). In *M. denitrificans* nitrite was also found to be an effective electron acceptor for oxidative phosphorylation (Naik & Nicholas, 1966).

2. AIM OF THESIS

This thesis is mainly concerned with certain aspects of the biochemistry of inorganic sulphur and nitrogen compounds in the chemoautotrophic bacterium, *Thiobacillus denitrificans*. The following lines of work were undertaken:

(1) To elucidate the mechanisms whereby sulphide, elemental sulphur, thiosulphate and sulphite are oxidized and to purify some of the enzymes involved.

(2) To establish the links between the oxidation of inorganic sulphur compounds and the reduction of nitrate and nitrite.

(3) To study the electron transport systems involved.

(4) To investigate the systems by which ATP is generated in the bacterium and to characterize some of the enzymes involved.

3. EXPERIMENTAL MATERIALS

3.1 Preparation of Standard Solutions

Aqueous solutions were dispensed in double glass-distilled water. Solutions of sulphide were prepared by dissolving washed crystals of $Na_2S.9H_2O$ in oxygen-free distilled water prepared as described by King & Morris (1967). Sulphite solutions were prepared by dissolving $Na_2SO_3.9H_2O$ in double-distilled water containing 0.2 mM Na-EDTA to inhibit its autoxidation to sulphate and also minimise the non-enzymic reduction of ferricyanide by sulphite in the assay system for the sulphite oxidizing enzyme. Both the sulphide and sulphite solutions were prepared fresh prior to use.

3.2 Buffers

All buffers were made up in double-glass distilled water at room temperature. Stock solutions of 0.25 M (K_2HPO_4 : KH_2PO_4), Tris-HCl and citrate buffers were prepared by the method of Gomori (1955) and stored at 2^{o}. The pH of Tris-HCl buffers were adjusted according to temperature (Sigma Technical Bulletin, 106B, 1967), while the pH of other buffers were determined at $20-25^{o}$. A Beckman H5 pH meter was used, standardized with buffer (pH 7.0) provided by

Beckman Instruments Inc. (Fullerton, California, U.S.A.).

3.3 Biological Materials

3.3.1 Bacterium

Thiobacillus denitrificans ('Oslo' strain, NCIB 8377) was purchased from the National Collection of Industrial Bacteria, Torrey Research Station, Aberdeen, Scotland.

3.3.2 Enzymes

Lipase II (hog pancreas), alcohol dehydrogenase, yeast lactate dehydrogenase and catalase (beef liver) were purchased from the Sigma Chemical Co., St. Louis, Missouri, U.S.A., while Calbiochem (Los Angeles, California, U.S.A.) supplied inorganic pyrophosphatase (isolated from *Aspergillus oryzae*, Lot No. 900621).

3.3.3 Others

Marker proteins for gel filtration were purchased from Mann Research Laboratories, New York, U.S.A. Cytochrome *c* (horse heart, types III and VI), bovine serum albumin and firefly lanterns were obtained from Sigma Chemical Co., Missouri, U.S.A.

3.4 Radioisotopes

The following radioisotopes (^{35}S), supplied by The Radiochemical

Centre, Amersham, Bucks, U.K., were used.

^{35}S-labelled Compund	Specific activity (mCi/mmole)	Batch No.
Na_2S	1.2	366401
Na_2SO_4 (aqueous solution, pH 6-8, carrier free)	2.4	414849
Na_2SO_3	22.2	515429
$Na_2S_2O_3$ (inner sulphur atom labelled, $S-^{35}SO_3^=$)	15	537630
$Na_2S_2O_3$ (outer sulphur atom labelled, $^{35}S-SO_3^=$)	14.6	515427

Radioactive sulphide and sulphite were kept sealed under O_2-free N_2 to prevent oxidation.

^{32}Pi in dilute HCl was supplied by the Australian Atomic Energy Commission (Lucas Heights, Sydney, Australia).

3.5 Other Materials and Chemicals

Nucleotides. ATP, ADP, AMP, NADH, NADPH, APS, FMN and FAD were purchased from the Sigma Chemical Co., St. Louis, Missouri, U.S.A.

Chromatographic materials. Whatman DEAE-cellulose (types 11 and 32) and Whatman 3 MM chromatographic paper were supplied by H. Reeve Angel and Co. Ltd, London, U.K.; Pharmacia (Uppsala, Sweden) provided Sephadex G-200 (40-120 μ) and Biorad Laboratories (Richmond, California, U.S.A.) supplied Sepharose-6B and Biogel P-200; starch was from Connaught Medical Research Laboratories (Toronto, Canada).

Non-standard chemicals. Rotenone, Amytal, HOQNO, pCMB, Tris-HCl, 2,4-dinitrophenol, Antimycin A, Glutathione (reduced), phenazine methosulphate (PMS), dithiothreitol, D-luciferin, digitonin were purchased from the Sigma Chemical Co.; 2-5-diphenyloxazole (PPO), 1,4-bis-(4-methyl-5-phenyloxazol-2-yl) benzene (POPOP) were obtained from Packard Instrument Co. (Chicago, Illinois, U.S.A.); blue dextran was from Pharmacia (Uppsala, Sweden); 5,5'-dithiobis-(2-nitrobenzoic acid) from Calbiochem (Carlingford, N.S.W., Australia); mepacrine from Imperial Chemical Co., England; sodium diethyldithiocarbamate (DIECA) from Merck, Darmstadt, Germany; bathocuproin from Fluka AG, Buchs, Swtizerland; Tween 80 from Gurrs Ltd, London, U.K.

Elemental sulphur, prepared by the method of Roy & Trudinger (1970), was washed twice with 10 mM phosphate buffer (pH 7.0) before use. Potassium tetrathionate was prepared as described by Trudinger (1961*B*).

All other chemicals were of the best purity available, obtained

from one or more of the following sources: Ajax Chemical Co. (Auburn, N.S.W., Australia), May and Baker (Dagenham, England), BDH Chemicals Ltd (Poole, Dorset, U.K.).

4. EXPERIMENTAL METHODS

4.1 Culture Techniques

4.1.1 Culture media

Thiobacillus denitrificans 'Oslo' strain was grown in a culture medium based on that of Baalsrud & Baalsrud (1954) with minor modifications. The composition of the "anaerobic" medium is set out in Table 2. For the "aerobic" medium, KNO_3 was omitted and the concentration of NH_4Cl doubled. About 500 ml of fresh inoculum containing approximately 0.5 g cells (wet weight) were added per litre of medium. The pH of the medium was between 7.2 and 7.4 after inoculation. During growth it fell to 6.5.

4.1.2 Maintenance of cultures

The bacterium, maintained in liquid culture, was sub-cultured twice a week. A 10-litre culture, grown under strictly aseptic conditions, was used to inoculate the anaerobic medium and for preparation of fresh inoculum. Microscopic examinations and plating experiments were conducted periodically to check the purity of the cultures.

4.1.3 Anaerobic cultures

Anaerobic cultures were grown batchwise in 40-litre

Table 2. Composition of "anaerobic" culture medium.

The KH_2PO_4 (200 g) was brought to pH 6.5 by adding KOH (31 g). This was made up to one litre, autoclaved (Smith Industries, South Australia) and added at the rate of 10 ml/l of medium. $Na_2S_2O_3.5H_2O$, KNO_3, NH_4Cl and $MgSO_4$ were dissolved and sterilized together. $FeSO_4$, $KHCO_3$ and $CaCl_2$ were autoclaved separately, the three fractions were combined after cooling and made up to volume prior to inoculation.

The composition of the "aerobic" culture solution was the same except that KNO_3 was omitted and the concentration of NH_4Cl doubled.

500 ml of fresh cell suspension (0.1%, w/v) was used for inoculating each litre of medium.

Nutrient	Concentration (g/l)
$Na_2S_2O_3.5H_2O$	5.0
$KHCO_3$	1.0
KNO_3	2.0
NH_4Cl	0.5
KOH	0.3
$MgSO_4.7H_2O$	0.5
KH_2PO_4	2.0
$FeSO_4$	0.01
$CaCl_2$	0.1

containers at 30°. Gas production was observed after about 12 hr and the culture was fully grown within 3 days. During growth the pH was maintained at 7.0 by titrating sterile 25% (w/v) K_2CO_3 into the culture by means of an automatic pH stat unit (Radiometer, Titrator 11 pH meter 8, Copenhagen, Denmark).

4.1.4 Aerobic cultures

Aerobic cultures were grown in 20-litre vessels. Sterile air was sparged through the medium via sintered glass aerators attached to a compressed airline fitted with sterile filters. The pH of the cultures was maintained at 7.0. Approximately 1 g of cells (wet weight), washed twice with 50 mM phosphate buffer (pH 7.0) and resuspended in the same buffer, was used as inoculum. Cultures were fully grown within 3 days.

4.1.5 Harvesting the bacteria

Cells were collected at 2° in a Sorvall RC-2 refrigerated centrifuge fitted with a continuous flow rotor (SS-34). They were washed twice with either 50 mM phosphate buffer (pH 7.0) or 50 mM Tris-HCl buffer (pH 7.5) and, unless used immediately, were stored as a paste at -15°.

4.2 Enzyme Techniques

4.2.1 Preparation of cell-free extracts

Unless otherwise stated, all the work described in this thesis was conducted with extracts prepared from anaerobically-grown cells.

4.2.1.1 Disruption of bacteria

Washed cells suspended (20%, w/v) in either 50 mM phosphate buffer (pH 7.0) or 50 mM Tris-HCl buffer (pH 7.5) both containing 0.2 mM Na-EDTA were disrupted by the following methods. All procedures were carried out at 2^{o}.

Glass homogenizer. The suspended cells were crushed in a glass homogenizer (Kontas Glass Co., Vineland, New Jersey, U.S.A.) by hand. This procedure was repeated between five and ten times. This method was not satisfactory since only about 20% of the cells were broken. Microscopic examination of the homogenate showed that most of the cells were undamaged. However, this technique provided an effective means of preparing a homogenous suspension of whole cells prior to breakage by the other two methods described below.

Ultrasonication. The cell suspension was placed in a double-walled glass vessel. A circulation of ice-cold water

through the outer chamber maintained the cell suspension at 4°. The cells were subjected to ultrasonication at 20 kilocycles/sec, for three 3-minute periods with an M.S.E. Mullard titanium probe. This technique resulted in about 80% cell breakage.

French pressure cell. Cell suspensions were crushed twice in a chilled Aminco French Pressure Cell (American Instrument Co., Maryland, U.S.A.) at 20,000 lb/in^2 (Hughes, Wimpenny & Lloyd, 1971). This resulted in 80-90% disruption of the cells. Because of its effectiveness, simplicity and speed this technique was adopted as the standard procedure for preparing cell-free extracts.

4.2.1.2 Preparation of crude extracts (S10)

The homogenate obtained after disruption of cells by the French Pressure Cell technique was centrifuged at 10,000 X *g* for 30 min in a Sorvall-SS3 automatic superspeed centrifuge (SS-34 rotor) at 2° to sediment unbroken cells and cell debris. The resultant supernatant fraction (S10) was used and is also referred to as the crude extract.

4.2.1.3 Preparation of particulate (P144) and soluble (S144) fractions

The crude extract (S10) was centrifuged at 144,000 X *g* for 90 min (Spinco, Model L, rotor type 50 or Ti50)

resulting in a supernatant (the soluble or S144) and a pellet (the membrane or P144) fraction. The pellet fraction was washed by resuspending it in either 50 mM phosphate or Tris-HCl buffer and re-centrifuging at the same speed for 60 min. A homogenous suspension of the washed pellet was then obtained with the aid of a glass homogenizer. Both the S144 and P144 fractions were dialysed extensively against three changes of 200 volumes of the appropriate buffer and were either frozen or stored at 2^{o}.

4.2.2 Preparation of luciferin-luciferase enzyme

The luciferin-luciferase extract was prepared by the method of Stanley & Williams (1969) with some modifications as follows:- ten firefly lanterns were mashed in a glass homogenizer (Kontas Glass Co., New Jersey, U.S.A.) with 2 ml of cold 50 mM arsenate buffer (pH 7.5). The homogenate, diluted with buffer to yield a final concentration of 0.5 ml buffer per firefly lantern, was centrifuged at 12,000 X *g* for 30 min at 2^{o} in a Sorvall SS3-automatic superspeed centrifuge. To the supernatant fraction were added a few crystals of D-luciferin. The extract was kept at 20^{o} for 24 hr before storing at 0^{o}.

4.2.3 Preparation of heat-stable extract from S144 fraction

The supernatant fraction (S144) obtained on centrifuging

the undialysed crude extract (S10) at 144,000 X g for 60 min, was diluted with 50 mM phosphate buffer (pH 7.0) until its protein concentration was 10 mg/ml. This was then heated by immersing it in a boiling water bath for 5 min. The denatured protein was removed by centrifuging at 5,000 X g for 10 min and the resulting supernatant fraction is referred to as the heat-stable extract.

4.2.4 Partial purification of adenylate kinase

The S144 fraction, prepared as described in Section 4.2.1.3 was precipitated with ammonium sulphate and the pellet formed between 65-85% saturation was resuspended in 50 mM Tris-HCl buffer (pH 7.5) and dialysed for 9 hr against three changes of the same buffer. The dialysed fraction was then loaded on to a DEAE-cellulose column (DE-11; 3.5 X 25 cm) and eluted with 50 mM Tris-HCl buffer (pH 7.5). The active eluates were pooled and concentrated by ultrafiltration under N_2 using a PM-10 membrane and ultra-filtration cell model 52 (Amicon, Mass., U.S.A.) (Section 4.3.8). The concentrated fraction had a specific activity of 93 nmoles ATP produced/min/mg protein and was free of ATP-sulphurylase and inorganic pyrophosphatase activities.

4.2.5 Determination of enzyme activities

4.2.5.1 Nitrate reductase

Nitrate reductase activity was measured by

following the production of nitrite.

Inorganic sulphur compounds as electron donors. Enzyme activity was determined with one of the following compounds as an electron donor:- sulphite, sulphate, thiosulphate and elemental sulphur.

With sulphite the reaction mixture contained Tris-HCl buffer (pH 8.5), 85 µmoles; $NaNO_3$, 5 µmoles; Na-EDTA, 1.5 µmoles; Na_2SO_3, 5 µmoles and 0.1 ml enzyme in a total volume of 2 ml. The reaction initiated by adding the electron donor and incubating for 10 min in open test tubes (10 cm X 1.3 cm) at 30^{o} was terminated with 1 ml 10% (w/v) zinc acetate.

When sulphide was the electron donor the reaction mixture contained Tris-HCl (pH 7.5) or phosphate buffer (pH 7.5), 85 µmoles; Na-EDTA, 1.5 µmoles; Na_2S, 4 µmoles; $NaNO_3$, 5 µmoles and 0.1 ml enzyme in a final volume of 2 ml. The reaction, conducted in glass tubes (7.5 cm X 1.0 cm) stoppered with rubber septa (Suba-seal; Wm. Freeman, Barnsley, Yorks., U.K.), was initiated and incubated as described above. The residual sulphide which strongly inhibited the diazotization reaction of nitrite was removed by precipitating it with zinc acetate which also stopped the enzyme reaction. Thus, after a 10 min incubation 1 ml of 10% (w/v) zinc acetate was added to the reaction mixture.

With thiosulphate as the electron donor the reaction mixture contained in a final volume of 2 ml, citrate buffer (pH 5.0), 85 µmoles; $NaNO_3$, 5 µmoles; $Na_2S_2O_3$, 5 µmoles and 0.1 ml enzyme. The reaction conducted in open glass tubes (10 cm X 1.3 cm) was initiated and incubated as in the previous assays but it was terminated with 1 ml of a mixture of 10% (w/v) zinc acetate and 10% (w/v) mercuric chloride which precipitated proteins and residual thiosulphate.

When the electron donor was elemental sulphur the reaction mixture in a total volume of 2 ml contained phosphate buffer (pH 7.5), 85 µmoles; $NaNO_3$, 5 µmoles; GSH, 5 µmoles; elemental sulphur, 40 mg (dry weight) and 0.1 ml enzyme. The reaction was initiated, incubated and terminated as described for the sulphide-linked nitrate reductase.

The mixtures obtained at the end of the reactions in the above assays were centrifuged at 3,000 X *g* (M.S.E. bench centrifuge) for 5 min and aliquots of the supernatant taken for the chemical determination of nitrite as described in Section 4.4.2. Boiled enzyme preparations were included in each series of reactions as controls. The specific activity of nitrate reductase is expressed in nmoles NO_2^- produced/10min/mg protein.

NADH as an electron donor. The reaction mixture contained phosphate buffer (pH 7.0) 80 µmoles; NADH, 2 µmoles; $NaNO_3$, 2 µmoles and 0.1 ml enzyme in a total volume of 1.8 ml. The reaction was initiated with the electron donor and incubated for 10 min in open tubes (10 cm X 1.3 cm) at 30^{o}. The termination step was modified in order to remove residual NADH which interfered with the diazotization reaction (Medina & Nicholas, 1957; Stulen, 1970). Thus, after incubation, 0.1 ml (N) acetaldehyde was added followed by 0.1 ml of alcohol dehydrogenase (30 µg/0.1 ml phosphate buffer). Nitrite was then determined in an aliquot of the final mixture as described in Section 4.4.2. Enzyme activity is expressed as above.

4.2.5.2 Nitrite reductase

Nitrite reductase activity was determined by following the disappearance of nitrite.

Sulphide as an electron donor. The reaction mixture contained phosphate buffer (pH 7.5), 35 µmoles; Na_2S, 2 µmoles, $NaNO_2$, 1 µmole and 0.1 ml enzyme in a total volume of 1 ml. The reaction, conducted in stoppered tubes, was initiated, incubated and terminated as described for the sulphide-linked nitrate reductase. The mixture was then centrifuged at 3,000 X *g* for 5 min to remove precipitated proteins. Nitrite was then determined

in a suitable aliquot of the supernatant fraction as described in Section 4.4.2. Specific activity of nitrite reductase is expressed in nmoles nitrite reduced/10 min/mg protein.

NADH as an electron donor. Nitrite reductase activity with NADH as an electron donor was measured under anaerobic conditions in Warburg flasks (10 ml capacity) fitted with ground-glass stopcocks. The side arm ports were fitted with rubber septa (Suba-seal). The reaction mixture contained, in the main compartment:- 35 µmoles phosphate buffer (pH 7.5), 1 µmole $NaNO_2$, 1 µmole phenazine methosulphate (PMS) and 0.1 ml enzyme in a total volume of 0.9 ml; in the side arm:- 2 µmoles NADH. The flasks were evacuated and flushed several times with O_2-free N_2 using the apparatus described by Elleway, Sabine & Nicholas (1971). After a pre-incubation period of 5 min at 30^{o} NADH was tipped into the main compartment and the reaction allowed to proceed for 10 min in a shaking water bath. The reaction was stopped by exposing the mixture to air and adding 0.1 ml (N) acetaldehyde followed by 0.1 ml of alcohol dehydrogenase (0.3 mg/ml phosphate buffer, pH 7.0), and 0.8 ml distilled water. After 2 min nitrite was determined in a suitable aliquot of the final mixture (Section 4.4.2). Enzyme activity is expressed as above.

4.2.5.3 Sulphide oxidizing enzyme

Enzymic sulphide oxidation was followed by the following methods:

Sulphide electrode. A Beckman sulphide ion electrode (Model 39610) connected to a Beckman H5 pH meter and recorder (Model 93502) was used to determine the concentration of sulphide in reaction mixtures as described in Section 4.3.1. Specific activity is expressed in µM sulphide oxidized/min/mg protein.

O_2 uptake. This was measured polarographically with a Beckman oxygen electrode (Model 39610) set up and standardized as described in Section 4.3.2. The reaction mixture contained in a final volume of 3 ml, Tris-HCl buffer (pH 7.5), 225 µmoles; Na-EDTA, 1 µmole; $Na_2S.9H_2O$, 1 µmole and 0.1 ml enzyme, the last two components being added with the aid of micro-syringes (Scientific Glass Engineering Co., Melbourne, Australia). Specific activity is expressed in nmoles O_2 utilized/min/mg protein.

Nitrate and nitrite reduction. This was determined as described for the nitrate and nitrite reductases with sulphide as an electron donor (Section 4.2.5.1). Enzyme activities are expressed in nmoles NO_2^- produced/10 min/mg protein and nmoles NO_2^- reduced/10 min/mg protein respectively.

Production of sulphite. The reaction mixture contained in a total volume of 1 ml, 4 µmoles, $Na_2S.9H_2O$; Tris-HCl buffer, 35 µmoles; Na-EDTA, 0.1 µmole and 0.1 ml enzyme. The reaction conducted in stoppered tubes (7.5 cm X 1 cm) was initiated, incubated and terminated as described for the sulphide-linked nitrate reductase (Section 4.2.5.1). The sulphite formed was determined by the basic Fuchsin method of Grant (1947) as described in Section 4.4.3. Enzyme activity is expressed in nmoles SO_3^{2-} formed/10 min/mg protein.

4.2.5.4 Sulphite oxidase (AMP-independent)

Enzyme activity was determined by the following methods:

O_2 uptake. This was measured with an oxygen electrode as described in Section 4.3.2. The reaction was conducted as follows: to 2.8 ml of 50 mM Tris-HCl buffer (pH 8.3) containing 0.2 mM Na-EDTA in the reacting vessel equilibrated at 30^{o} was added 0.1 ml each of 50 mM Na_2SO_3 and enzyme with the aid of micro-syringes. Initial rate is calculated from a tangent to the curve at the point of addition of enzyme. Activity is expressed in nmoles O_2 utilized/min/mg protein.

Nitrate reduction. This was determined as described in Section 4.2.5.1. Specific activity is expressed in nmoles NO_2^- produced/10 min/mg protein.

Ferricyanide reduction. A modification of the APS-reductase assay described by Peck, Deacon and Davidson (1965) was employed. The reaction mixture contained Tris-HCl buffer (pH 8.5), 225 µmoles; Na-EDTA, 1 µmole; Na_2SO_3, 5 µmoles; $K_3Fe(CN)_6$, 5 µmoles and enzyme in a final volume of 3 ml. The reaction, carried out in quartz cuvettes (1 cm), was initiated by adding sulphite to the sample cuvette. The reference cuvette contained all components except sulphite. Changes in absorbance at 420 nm were followed in a Unicam SP-800 recording spectrophotometer equilibrated at 30^o fitted with an expansion scale accessory. Specific activity is expressed in µmoles ferricyanide reduced/hr/mg protein.

Cytochrome c reduction. The reaction mixture in glass cuvettes (1 cm path) contained mammalian or yeast cytochrome *c*, 2 µmoles; Na_2SO_3, 5 µmoles and 0.1 ml enzyme. The final volume was made up to 3 ml with 50 mM Tris-HCl (pH 8.3) containing 0.2 mM Na-EDTA. The reference cuvette contained all components except sulphite which was used to initiate the reaction. Absorbance at 550 nm was measured in a Unicam SP-800 recording spectrophotometer.

Specific activity is expressed in µmoles cytochrome *c* reduced/min/mg protein.

4.2.5.5 APS-reductase

A modification of the method of Peck (1961) was employed. The reaction mixture contained Tris-HCl buffer (pH 7.5) 150 µmoles; Na_2SO_3, 10 µmoles; AMP, 5 µmoles; $K_3(FeCN)_6$, 5 µmoles and enzyme in a final volume of 3 ml. The reaction was carried out in 1 cm cuvettes and was initiated by adding sulphite to the sample cuvette. The reference cuvette contained all components except sulphite. The absorbance at 420 nm was followed in a Unicam SP-800 recording spectrophotometer equilibrated at 30^{o} and fitted with an expansion recorder (Servoscribe type RE 511). Specific activity is expressed in µmoles ferricyanide reduced/hr/mg protein.

4.2.5.6 Sulphur oxidizing enzyme

Enzyme activity was determined either by O_2 uptake or nitrate reduction.

Oxygen uptake. The apparatus used is described in Section 4.3.2. The reaction mixture in a total volume of 2.5 ml contained phosphate buffer (pH 7.5), 120 µmoles; Na-EDTA, 0.4 µmoles; elemental sulphur, 40 mg; GSH, 5 µmoles and 0.1 ml cell extract.

The reaction conducted at 30° was started by adding either GSH or elemental sulphur. Specific activity, calculated as described in Section 4.3.2 is expressed in nmoles O_2 utilized/min/mg protein.

Nitrate reduction. This is described in Section 4.2.5.1. Specific activity is expressed in nmoles NO_2^- produced/10min/mg protein.

4.2.5.7 Thiosulphate oxidizing enzyme

This was determined by the following methods:

(a) Nitrate reduction

This is described in Section 4.2.5.1. Specific activity is expressed in nmoles NO_2^- produced/10 min/mg protein.

(b) Production of sulphite

The reaction mixture contained citrate buffer (pH 5.0) 85 µmoles; Na_2SO_3, 5 µmoles; GSH, 2 µmoles and 0.1 ml extract in a total volume of 2 ml. The reaction was conducted in air for 10 min at 30°. It was terminated with 1 ml of an equal mixture of 10% (w/v) zinc acetate and 10% (w/v) mercuric chloride. After centrifuging at 3,000 X *g* for 5 min an aliquot of the supernatant fraction was taken for sulphite determination (Section 4.4.3).

Enzyme activity is expressed in nmoles SO_3^{2-} produced/10 min/mg protein.

4.2.5.8 Rhodanese

Rhodanese activity was assayed by the method of Bowen, Butler & Happold (1965). The reaction mixture contained phosphate buffer (pH 7.5), 75 µmoles; $Na_2S_2O_3$, 50 µmoles and enzyme in a final volume of 2.5 ml. The mixture was pre-incubated at 30° for 10 min after which 0.5 ml of 0.1M KCN was added. After a further 10 min incubation the reaction was stopped with 0.25 ml 40% (v/v) formaldehyde followed by 2.75 ml of 10% (w/v) $Fe(NO_3)_3$ in 65% (v/v) HNO_3. The extinction at 470 nm was determined and the KCNS formed read from a calibration curve. Enzyme activity is expressed in µmoles KCNS formed/10 min/mg protein.

4.2.5.9 ATP-sulphurylase

Enzyme activity was determined by a firefly bioluminescence procedure either by a continuous method or a sampling technique.

(a) Continuous method

The firefly assay of Balharry & Nicholas (1971) was employed. The reaction vial contained phosphate buffer (pH 7.4), 10 µmoles (1 ml); $MgCl_2$, 0.5 µmole; arsenate buffer (pH 7.5)

neutralized with HCl, 50 µmoles (1 ml); sodium pyrophosphate, 5 nmoles (25 µl); APS, 10 nmoles (50 µl) in a final volume of 3 ml made up with double glass-distilled water. The reaction mixture was then incubated at 20° in a Packard Tri-Carb liquid scintillation spectrometer (Model 3375), which was set up as follows:- The circuit was switched out-of-coincidence so that the photo-multiplier detected individual light flashes (photons). A single channel was used and was set with a gain at 100% and the discriminator settings between 70 to 300. Counts were recorded for 0.1 min at 20°.

The enzyme reaction was initiated by adding 0.1 ml of firefly extract, prepared as described in Section 4.2.2, the vial was then swirled and placed in the well of the spectrometer. Six counts each of 0.1 min were recorded at 0.3 min intervals. A cell-free extract or purified ATP-sulphurylase (20 µl) was added to the vial 2 min after adding the firefly extract and a further 6 counts recorded. After a further 2 min an internal standard of ATP (10 pmoles in 0.1 ml) was added to the vial and the same counting sequence repeated.

A computer program, developed by Balharry & Nicholas (1971), was used to analyse the results of the assay. Specific activity of the enzyme is expressed in nmoles ATP produced/min/mg protein.

(b) Sampling technique

The reaction mixture in a test tube contained Tris-HCl buffer, 35 µmoles; APS, 0.25 µmoles; sodium pyrophosphate, 0.2 µmoles; $MgCl_2$, 0.5 µmole and 0.1 ml enzyme in a total volume of 1 ml. The reaction, carried out at 30^{o}, was started by adding the enzyme and incubating for 1 min. It was terminated with 1.0 ml (v/v) perchloric acid. After centrifuging at 3,000 X *g* for 5 min 0.1 ml aliquot of the supernatant fraction was mixed with 1.9 ml ice-cold distilled water. A suitable aliquot of the diluted sample was then taken for determination of ATP as described in Section 4.4.7. Enzyme activity is expressed in nmoles ATP produced/min/mg protein.

4.2.5.10 ADP-sulphurylase

The method of Adams & Nicholas (1972) was used, where the incorporation of ^{32}P into ADP with APS as the substrate was determined. The reaction mixture contained Tris-HCl buffer (pH 7.5), 50 µmoles; APS, 5 µmoles; inorganic phosphate, 2 µmoles (with 0.2 µCi $^{32}P_i$) and enzyme in a final volume of 0.5 ml. The reaction was initiated by adding the enzyme and after incubating at 30^{o} for 10 min was terminated by heating it in boiling water for a further 5 min. After centrifuging for 10 min in a bench

centrifuge the supernatant fraction was first subjected to column chromatography. DEAE-cellulose (formate form) at pH 6.0 was packed into columns (2 cm X 0.8 cm) and washed with 10 ml water. The supernatant fraction was then loaded on to the column which was eluted with 15 ml of 0.05M NH_4HCO_3. This removes residual $^{32}P_i$ but not the nucleotides. The nucleotides were then eluted with 15 ml of 0.25M NH_4HCO_3. The latter eluates were then evaporated to dryness in a rotary evaporator at 50-60°C, dissolved in 20 ml of water and evaporated to dryness again. This was repeated once more to decompose the NH_4HCO_3. Finally, the dry samples were dissolved in 0.3 ml water. An aliquot of the sample was spotted on to Whatman 3 MM paper and subjected to high voltage electrophoresis for 1.75 hr as described in Section 4.3.4. The electrophoretogram was passed through a Packard radiochromatogram scanner and the radioactive area which corresponded to authentic unlabelled ADP was cut into small portions (1.5 X 2 cm), placed in a scintillation vial and counted by Cerenkov emission in a Packard 3375 Tri-carb liquid scintillation spectrometer (Section 4.3.6.3). Enzyme activity is expressed in nmoles P_i incorporated into ADP/hr/mg protein.

4.2.5.11 Adenylate kinase

The reaction mixture contained in a total volume of 1 ml Tris-HCl buffer (pH 7.5) 35 µmoles; $MgCl_2$, 0.5 µmoles;

ADP, 0.4 µmoles and 0.1 ml enzyme. The reaction was started by adding ADP and incubating at 30° for 1 min. It was terminated with 1.0 ml 5% (v/v) perchloric acid and after centrifuging for 5 min at 3,000 X *g* a 0.1 ml sample of the supernatant fraction was mixed with 1.9 ml of ice-cold water. ATP was then determined in a suitable aliquot of the diluted sample as described in Section 4.4.7. Enzyme activity is expressed in nmoles ATP produced/min/mg protein.

4.2.5.12 Lactate dehydrogenase

The method of Dixon (1955) was used where the reduction of ferricyanide with lactate as the electron donor was determined. Measurements were made at 420 nm in a Unicam SP-800 recording spectrophotometer.

4.2.5.13 Inorganic pyrophosphatase

Pyrophosphatase activity was measured by determining the amount of inorganic phosphate liberated from sodium pyrophosphate. A modification of the method of Akagi & Campbell (1963) was employed. The reaction mixture contained Tris-HCl buffer (pH 7.5), 35 µmoles; $MgCl_2$, 5 µmoles; sodium pyrophosphate, 5 µmoles and enzyme in a final volume of 1 ml. The reaction was started by adding enzyme. After a 10 min incubation at 30° the

reaction was terminated with 1 ml 10% (w/v) trichloroacetic acid. Protein was removed by centrifuging at 3,000 X *g* for 10 min in a bench centrifuge (Measuring and Scientific Equipment) and an aliquot of the supernatant fraction was used for determining inorganic phosphate by the colorimetric method of Fiske & Subba Row (1925) as described in Section 4.4.6. Activity is expressed in nmoles P_i liberated/min/mg protein.

4.2.5.14 ATP-generating systems

(a) Substrate level phosphorylation

APS and P_i as substrates. The reaction mixture contained Tris- HCl buffer (pH 8.0), 50 µmoles; APS, 0.5 µmole; $MgCl_2$, 2 µmoles; sodium orthophosphate, 2 µmoles and 0.1 ml extract in a total volume of 1 ml. The reaction was started by adding the enzyme. After 2 min incubation at 30° it was stopped with 1 ml 5% (v/v) perchloric acid. After centrifuging at 3,000 X *g* for 5 min 0.1 ml aliquot of the supernatant fraction was diluted to 2 ml with cold distilled water. ATP was assayed in a suitable aliquot of the diluted mixture using the firefly method described in Section 4.4.7. Activity is expressed in nmoles ATP produced/min/mg protein.

AMP, sulphite, ferricyanide and P_i

as substrates. The reaction mixture in a scintillation vial contained 1 ml of 10 mM phosphate buffer (pH 7.5), 10 µl of 0.2 mM AMP, 0.1 ml 5 mM $MgCl_2$ and 1 ml 50 mM arsenate-HCl buffer (pH 7.5) made up to a final volume of 3 ml with twice glass-distilled water. The vial was placed in the well of a Packard liquid scintillation spectrometer at 20°. 50 µl of firefly extract, prepared as described in Section 4.2.2 was added to the mixture and six counts each of 0.1 min at 0.3 min intervals were recorded. ATP internal standard (10 pmoles) was then added, followed by 1 µmole each of sulphite and ferricyanide, recording six counts as above after each addition. The results were plotted out and analysed. Activity is expressed in nmoles ATP produced/min/mg protein.

(b) Oxidative phosphorylation

Continuous assay. The reaction mixture in a scintillation vial contained 1 ml of 10 mM phosphate buffer (pH 7.5), 0.1 ml of 5 mM $MgCl_2$, 1 ml of 50 mM arsenate-HCl buffer (pH 7.5) and 0.4 µmoles ADP in a total volume of 3 ml made up with distilled water. The following were added to the vial in order, 50 µl firefly extract, 10 pmoles of internal ATP standard and 1 µmole of electron donor. After each addition the vial was shaken and six counts of 0.1 min each at intervals of 0.3 min were recorded in a Packard liquid

scintillation spectrometer at 20°. The results were plotted and analysed. Activity is expressed in nmoles ATP produced/min/mg protein.

Sampling method. The reaction mixture in a test tube contained Tris-HCl buffer (pH 7.5), 50 µmoles; Na-EDTA, 0.4 µmoles; $MgCl_2$, 5 µmoles; sodium phosphate, 10 µmoles; ADP, 1 µmoles and electron donor 1 µmole in a total volume of 2 ml. The mixture was pre-incubated in air at 30° for 5 min before starting the reaction by adding ADP followed immediately by the electron donor. After a further 2 min incubation 1 ml of cold 5% (v/v) perchloric acid was added to stop the reaction. After centrifuging at 3,000 X *g* for 5 min, 0.1 ml aliquot of the supernatant fraction was diluted to 2 ml with cold distilled water and from this a sample was taken for determining ATP as described in Section 4.4.7.

4.2.6 Determination of Michaelis constant (Km)

This was determined by measuring the initial rate of reaction as a function of the concentration of one substrate while the other(s) were held at a constant saturation level. The Km values were estimated from double reciprocal plots of reaction rate against initial substrate concentration as described by Lineweaver & Burk (1934).

4.2.7 Determination of molecular weight by Sephadex gel-filtration

Estimation of the molecular weights of sulphite oxidase, APS-reductase and ATP-sulphurylase were made by following the method of Andrews (1964) using Sephadex G-200. The column (2.5 X 45 cm) prepared according to Reiland (1971) as described in Section 4.3.7.3 was equilibrated with 50 mM phosphate buffer (pH 7.5) containing 0.2 mM Na-EDTA. The marker proteins used included: horse heart cytochrome *c*, M.W. 12,700 (Sigma grade VI); bovine serum albumin, M.W. 67,000 (Sigma); lactate dehydrogenase, M.W. 140,000 (Sigma); human γ-globulin, M.W. 160,000 and Apoferritin, M.W. 450,000 (Mann Research Laboratories).

Blue dextran (M.W. 2,000,000) and ^{35}S-sulphate were used to determine the void and inclusion volumes after the elution pattern of each protein had been obtained. Distribution coefficient (Kd, Flodin, 1962) for the known globular proteins were determined either by extinction at 280 and 260 nm for inactive proteins or by enzyme activity (Section 4.2.5). Cytochrome *c* and blue dextran were measured by absorbance at 410 nm and 625 nm respectively. ^{35}S-sulphate was determined as in Section 4.3.6.2. 5 ml fractions were collected in the cold using an automatic fraction collector.

The distribution coefficient (Kd) were calculated according to the formula

$$Kd = \frac{Ve - Vo}{Vi}$$

where Ve = elution volume of the protein, Vo = void volume and Vi = inclusion volume.

4.3 General Techniques

4.3.1 Sulphide electrode

A Beckman sulphide ion electrode (Model 37610) was used in conjunction with a calomel reference electrode to determine the concentration of sulphide in reaction mixtures. A Beckman H5 pH meter with an expanded scale connected to a Beckman recorder (Model 93502) was used with the electrode to monitor sulphide concentration. Because the sulphide ion electrode senses sulphide ions as electrical potentials, which are proportional to ionic activities but not to concentration, the millivolt readings were converted to concentration values by means of a calibration curve of electrode potential *versus* concentration of standard sulphide solutions.

Measurement of sulphide were determined in a 25 ml beaker made anaerobic by sealing with a tightly fitting rubber stopper, through

which the electrodes were passed, and flushing with O_2-free N_2. Microsyringes (Scientific Glass Engineering, Melbourne, Australia) were used for gas inlet and escape outlet and for adding reactants. To 5 ml of 50 mM phosphate buffer (pH 7.5) containing 0.2 mM Na-EDTA, 10 mM sodium sulphide solution was added until the required concentration was reached as indicated by the electrode. With the first addition of sulphide a slow utilization occurred until the residual O_2 was depleted. The reaction was started by rapidly injecting the enzyme and re-admitting air into the reaction mixture which was stirred continuously with a magnetic stirrer. All measurements were conducted at 30^{o}. In most experiments 50 µl of 10 mM sulphide was added to give a concentration of 0.1 mM.

The progress curve of the enzyme reaction is exponential and the tangent to this curve at the point of adding the enzyme is an estimate of the initial rate of sulphide oxidation in terms of millivolt/unit time. The molar rate was calculated from a calibration curve of standard sulphide solutions.

Precise sulphide concentration was determined by titration with a standard lead perchlorate solution using the sulphide electrode as the end-point detector as described by Applications Bulletin No. 12, distributed by the Orion Research Incorporated, Cambridge, Massachusetts, U.S.A.

4.3.2 Oxygen electrode

Oxygen uptake was measured polarographically with a Beckman O_2 electrode (Model 39065) fitted with an adaptor box (Model 93260) and connected to a Beckman O_2 analyzer (Model 772) and a Beckman recorder (Model 93502).

The electrode was standardized with either 50 mM Tris-HCl buffer (pH 7.5) or 50 mM phosphate buffer (7.5) both containing 0.2 mM Na-EDTA by the method of Dixon and Kleppe (1966). Thus, at equilibrium, the displacement of the recorder pen corresponded to 0.25 μmoles O_2/ml of buffer, permitting calibration of the electrode. A further correction was unnecessary when enzymes were added to the reaction mixture since enzyme-catalyzed reactions, like other chemical reactions, are determined by their activities rather than the concentrations of the reactants.

The reaction vessel, enclosed in a perspex jacket was maintained at 30^{o} by circulating water from a temperature-controlled bath through the outer chamber. Oxygen uptake during the oxidation of sulphide, sulphite, thiosulphate, elemental sulphur and NADH was determined as follows: the reaction mixture contained enzyme and reductant in a final volume of 3 ml made up with either 50 mM Tris-HCl buffer (pH 7.5 or 8.3) or phosphate buffer (pH 7.0), each

containing 0.2 mM Na-EDTA. The reaction was started by injecting the appropriate reductant through an inlet via a micro-syringe and the decrease in oxygen concentration in the reaction mixture recorded. Enzyme activity was calculated from a tangent to the curve at the point of adding the reductant. The specific activity is expressed in nmoles O_2 utilized/min/mg protein.

4.3.3 Starch-gel electrophoresis

Electrophoresis was carried out on a bed of starch (12% w/v) prepared by the method of Brewer (1970) using the apparatus of Graham (1963). Aliquots of cell extracts or purified enzymes (10 µl each) were applied to Whatman 3 MM chromatography paper section (0.2 X 0.4 cm) which was inserted 1 cm apart into the set gel. An ice pack was placed over the gel and a voltage gradient of 30 volts/cm applied for 1.5 hr. The buffer used for the preparation of the gel was 0.1M tris-citrate (pH 8.1).

To locate the protein bands, the gel was halved horizontally and the slices stained with nigrosine-amido black (1%, w/v nigrosine, 1% w/v amido black in methanol-acetic acid-water, 5 : 1 : 5, by volume).

4.3.4 High voltage paper electrophoresis

4.3.4.1 Separation of inorganic sulphur compounds

This was carried out using the apparatus of Tate (1968). Standard solutions or aliquots of reaction mixtures were spotted 2.5 cm apart at the origin on Whatman 3 MM chromatographic paper (15 cm X 55 cm) near the cathode. The paper was wetted with 0.1M sodium citrate buffer (pH 5.0) and then lightly blotted to remove surface moisture. It was then laid out on a polythene frame and placed into a ceramic tank filled with CCl_4 in such a way that the ends of the paper were connected by wicks to buffer chambers containing 0.1M sodium citrate buffer (pH 5.0). The CCl_4 was cooled by passing cold tap water through the copper coil placed in the centre of the tank. The current from a stabilised power pack (Paton Industries Ltd, South Australia) was applied to the buffer chambers, maintaining a voltage gradient of 30 volts/cm along the paper. The electrophoresis was carried out for 1 hr with either $^{35}SO_4^{2-}$ or thiosulphate or both as marker compounds in each run.

After electrophoresis the paper was dried by hot air and the lanes (30-35 cm X 2.5 cm) separated. Polythionates, thiosulphate and sulphide were detected by dipping the electrophoretogram in $AgNO_3$ solution (8 g in 10 ml water and 90 ml acetone). Sulphite was detected in basic Fuchsin (1% w/v) in 10 ml water and 90 ml

acetone and thiocyanate by $Fe(NO_3)_3$ (10 g) in 2M HNO_3 (10 ml) and acetone (90 ml). Radioactive sulphur compounds (^{35}S) were detected on the electrophoretogram as described in Section 4.3.6.1.

4.3.4.2 Separation of nucleotides

The same procedure as described above was adopted for the separation of nucleotides except that electrophoresis was conducted for 1.5 hr with AMP, ADP, ATP as standards. The nucleotides were detected on the dried paper by u.v. absorption using a hand monitor. Radioactive nucleotides were detected as described in Section 4.3.6.1.

4.3.5 Mass spectrometry

The reaction mixture in a Rittenberg tube contained 2 µmoles $NaNO_2$ or $NaNO_3$ and 5 µmoles of electron donor (Na_2S, Na_2SO_3, $Na_2S_2O_3$ or NADH) in 0.2 ml in one of the arms. The other limb of the tube contained 1.7 ml of 50 mM Tris-HCl buffer (pH 8.5 or 7.5) containing 0.2 mM Na-EDTA and 0.1 ml of 25% (w/v) cell suspensions. A roll of filter paper soaked in 10M KOH was placed inside the cap of the tube to absorb any CO_2 produced. The tube was then rigorously evacuated to 1 X 10^{-5} mm Hg and the contents were then mixed and incubated for 3 hr at 30^{o}. The gases formed were then transferred under high vacuum from the Rittenberg tube

into an MS-2 mass spectrometer (Associated Electrical Industries, Manchester, U.K.) for analysis.

4.3.6 Radioisotope techniques

Radioisotopes in reaction mixtures were separated by either high voltage electrophoresis or DEAE-cellulose chromatography as described in Sections 4.3.4 and 4.3.7.2 respectively. Radioactivity was determined by one of the following methods:

4.3.6.1 Radiochromatogram scanner

Electrophoretogram containing radioisotopes were thoroughly dried and separated into lanes which were then joined end to end. This was then run through a Packard 7201 radiochromatogram scanner. Samples of $^{35}SO_4^{2-}$ were spotted behind the origin to align the chromatogram with the plotted data.

4.3.6.2 Liquid scintillation spectrometry

When quantitative determinations were required, the radioactive areas on electrophoretograms were cut into small portions (1.5 X 2 cm) so that the pieces lay flat when placed in glass vials (Packard Instruments Co., Chicago, Illinois, U.S.A.). Scintillation fluid (1.0 ml), consisting of PPO (0.5%, w/v) and POPOP (0.3%, w/v) in toluene was pipetted into the vials which were

then placed in a Packard Tri-Carb Liquid Scintillation Spectrometer (Model 3375) and assayed.

Radioactivity in solutions (^{14}C or ^{35}S) was measured either by spotting aliquots of samples on to squares (1.5 X 1.5 cm) of Whatman 3 MM chromatographic paper drying and counting as above or by counting aliquots in a scintillation mixture consisting of 2.0 ml of 95% (v/v) ethanol and 5 ml of toluene solution containing PPO and POPOP at the concentrations stated above.

Channel ratios were used to determine the counting efficiency (usually about 80%) by comparison with $^{35}SO_4^{2-}$. This standard has been previously calibrated against a standard (^{14}C) toluene sample (5.73×10^5 d.p.m./g) assuming equal efficiency of ^{14}C and ^{35}S.

4.3.6.3 Cerenkov radiation (for ADP-sulphurylase assay)

Aqueous ($^{32}P_i$) samples were radioassayed by Cerenkov radiation. Samples were made up to constant volume (10 ml) with 50 mM bicarbonate and assayed using a pre-set tritium channel of the Packard Liquid Scintillation Spectrometer. Quenching was always similar within each set of samples so corrections were not made.

4.3.7 Preparation of column support

4.3.7.1 DEAE-cellulose for the separation of proteins

Whatman DE-11 or DE-22 was prepared according to the Whatman Technical Bulletin IE2, first washing with 0.5M HCl, then 0.5M NaOH and finally with water until neutral pH was attained. 0.02M phosphate buffer (pH 7.5) containing 0.2 mM Na-EDTA was used to equilibrate the ion-exchanger in a batchwise fashion and the column was stored at 2^{o} in the presence of 0.1% (w/v) sodium azide. Columns were prepared at room temperature by pouring the homogeneous cellulose slurry onto the column. It was then equilibrated at 2^{o} with buffer until the effluent pH was identical with that of the equilibrating buffer. The enzyme preparation was then loaded on to the column and developed with the appropriate buffer. Fractions were collected in the cold with an automatic fraction collector (Paton Industries, South Australia).

4.3.7.2 DEAE-cellulose for the separation of nucleotides

DEAE-cellulose was also used for the separation of nucleotides. Neutral pre-cycled DE-11 as described above was equilibrated with M formate buffer (pH 6.0). A column

(8 X 1.7 cm) with a flow rate of 150 ml/hr was prepared and washed extensively with water prior to use. The nucleotide mixture was loaded on to the column and eluted with a gradient of equal volumes of 0.2M ammonium bicarbonate and distilled water. Fractions were collected at room temperature using an automatic fraction collector.

4.3.7.3 Sephadex and Sepharose gel-filtration

The recommendations of the manufacturer (Pharmacia Chemicals, Uppsala, Sweden) were generally followed in the preparation of Sephadex G-200 and Sepharose-6B. The beads were stirred in 50 mM phosphate buffer (pH 7.0) containing 0.2 mM Na-EDTA and after settling for 10-20 min the fine particles were removed by decantation. This was repeated several times and after a final decantation was allowed to further equilibrate for 3 days in the same buffer. Sephadex and Sepharose prepared this way were stored at 2^{o} in the presence of 0.1% w/v sodium azide. Columns were prepared at room temperature after the slurry had been degassed under reduced pressure. A homogeneous slurry was usually poured in one operation until the desired height was obtained. The column was equilibrated in the cold with the same buffer.

The void and inclusion volumes of the column were determined by elution patterns of blue dextran (M.W. 2,000,000) and ^{35}S-sulphate respectively. The enzyme preparation was allowed to

adsorb on to the gel and fractions were collected in the cold with an automatic LKB-fraction collector.

4.3.8 Membrane ultrafiltration

Enzyme preparations were concentrated by means of a membrane filter (Diaflo, PM-10, Amicon Corp., U.S.A.). The Diaflo apparatus (capacity, 50 ml) kept in ice during filtration was operated under N_2 at 25-30 lb/in^2. The PM-10 filter retains proteins of molecular weight greater than 10,000.

4.4 Chemical Determinations

4.4.1 Proteins

Proteins were determined with reference to a standard solution of bovine serum albumin by the Folin-Ciocalteau method as modified by Lowry *et al.* (1951). The optical extinction was measured at 750 nm. For a more rapid determination the optical extinction at 260 nm and 280 nm was measured (Kalckar, 1947) and protein determined from the equation:-

$$\text{protein (mg/ml)} = 1.45\ (E_{280}) - 0.74\ (E_{260})$$

4.4.2 Nitrite

Nitrite was determined by the method of Hewitt & Nicholas (1968) as follows:- an aliquot containing 20-100 nmoles

of nitrite was diluted to 2 ml with distilled water. 1 ml each of 1% (w/v) sulphanilamide in 1.0N HCl and 0.1% (w/v) N-1 (naphthylenediamine hydrochloride) were added. After 15 min the optical density of the solution was measured at 540 nm and the nitrite calculated from a standard curve of nitrite standard solutions plotted against optical density.

4.4.3 Sulphite

Sulphite was determined by a modification of the method of Grant (1947). Basic fuchsin colour reagent was prepared fresh before use as follows:- to approximately 200 ml of distilled water was added 11 ml of concentrated H_2SO_4, 4 ml of 3% (w/v) fuchsin in 95% (v/v) ethanol and 1 ml of 40% (v/v) formaldehyde. Then 4 ml of this reagent was added to a sample solution containing sulphite and the absorbance read at 570 nm after exactly 10 min. Sulphite concentration was calculated from a standard curve. The response was linear over the range 0 to 250 nmoles sulphite and was sensitive to 2 nmole changes in the linear range.

4.4.4 Sulphide

Sulphide was determined chemically by modifying the methods of Acree, Sonoff & Splittstoesser (1971) and Gilboa-Garber (1970) as follows:- cadmium chloride (0.7%, w/v) and NaOH (6%, w/v) in the ratio of 5 : 1 were mixed thoroughly. 0.5 ml of this

mixture was added to 1 ml of a sample solution containing between 20 and 200 nmoles sulphide in a stoppered, calibrated centrifuge tube and the mixture shaken thoroughly. After centrifuging at 3,000 x *g* for 10 min the pellet was washed successively with 5 ml 1.5M NaCl (pH adjusted to 8.0 with NaOH) and 5 ml water (pH 8.0). The pellet was then resuspended in water and made up to 1 ml. Then 0.25 ml of 0.1% (w/v) DPD (N,N-dimethyl-p-phenylenediamine sulphate) in 5.5N HCl was added and the mixture shaken immediately until dissolved. After 0.1 ml of 0.01M $FeCl_3$ (in 0.575N HCl) was added the solution was again shaken. After 30 min the abosorbance at 670 nm was measured.

4.4.5 Thiosulphate

This was determined by the method of Sorbo (1957) as follows:- a sample containing between 50 to 1,500 nmoles of thiosulphate was diluted to 4.7 ml with distilled water. To this was added 0.5 ml 0.1M KCN, 0.3 ml 0.1M $CuCl_2$ and 0.5 ml 10% (w/v) ferric nitrate (dissolved in 65%, v/v, HNO_3), mixing well after each addition. The absorbance at 420 nm was then determined and thiosulphate concentration calculated from a standard curve.

4.4.6 Inorganic phosphate

Inorganic phosphate was determined by the colorimetric

method of Fiske & Subba Row (1925), as described by Leloir & Cardini (1957). An aliquot containing inorganic phosphate was diluted to 2 ml with distilled water. 0.5 ml of acid molybdate (2.5%, w/v, ammonium molybdate in 2.5M H_2SO_4) was then added followed by 0.1 ml of colour reagent, mixing well after each addition. The colour reagent was prepared by grinding 1 g 1-amino-2-naphthol 4-sulphonic acid, 3 g Na_2SO_3 and 6 g $Na_2S_2O_5$ in a mortar. This stock mixture was kept at 2^{o} in the dark and the reagent prepared fresh before use by dissolving 0.75 g in 10 ml distilled water. After adding the colour reagent the tubes were left standing at room temperature for 20 min and the absorbance then determined at 750 nm. A series of standard P_i (0-0.4 µmoles) was determined each time.

4.4.7 ATP

The amount of ATP present in reaction mixtures or solutions was determined by the luciferin-luciferase enzyme system of the firefly, *Photinus pyralis*. The light emitted, measured in a Packard Tri-Carb Liquid Scintillation Spectrometer, is directly proportional to the amounts of ATP present.

The settings for the spectrometer were the same as those described for the ATP sulphurylase continuous assay (Section 4.2.5.9(a)). The reaction vial contained 1 ml 10 mM phosphate

buffer (pH 7.4), 0.1 ml 5 mM $MgCl_2$, 1 ml 50 mM arsenate-HCl buffer (pH 7.5) and 0.9 ml double-distilled water. The following additions were made at 2 min intervals.

A : 0.1 ml firefly extract

B : 0.1 ml containing 10 pmoles ATP (internal standard)

C : 0.1 ml aliquot of sample containing between 0 and 100 pmoles ATP.

The counts per min were determined after each addition and ATP calculated from the expression

$$\text{ATP (pmoles)} = \frac{C_{c.p.m.} - B_{c.p.m.}}{B_{c.p.m.} - A_{c.p.m.}} \times 10$$

4.4.8 Cytochromes and flavoproteins

The amounts of cytochromes and flavoproteins in the particulate (P_{144}) and soluble (S_{144}) fractions were determined spectrophotometrically from dithionite-reduced *versus* oxidized difference spectra using the wavelength pairs as extinction coefficients as given by Asano & Brodie (1964). The values are as follows:

	Wavelength Pair		E_{mM}
Cytochrome *a*	598	623	16
" *b*	562	574	20
" *c*	551	540	19
flavoprotein	455	510	11

Cytochrome *d* was determined as the pyridine haemochromogen at 620 nm as described by Newton (1969).

4.5 Other Determination

4.5.1 Absorption spectra

Absorption measurements were made in a Shimadzu Multipurpose Recording Spectrophotometer, Model MPS-50L (Shimadzu Seisakusho Ltd, Tokyo, Japan). Spectra at room temperature were recorded using a 1 cm path cuvette (either 0.4 ml or 3.5 ml capacity) with a photomultiplier voltage of 400 volts, automatic slit control and absorbance ranges of either 0-1 or -0.1 to +0.1.

For routine analysis by colorimetry a Shimadzu QV-50 spectrophotometer was used.

5. EXPERIMENTAL RESULTS

5.1 Sulphide Oxidation

Sulphide was rapidly utilized by whole cells and crude extracts (S10), as shown in Figure 2. Since all sulphide was oxidized within 3 min, little of it was lost to the atmosphere. The membrane fraction (P144) also oxidized sulphide at a rate equivalent to that of the crude extract. Neither the S144 fraction nor the boiled P144 preparation utilized sulphide. When incubated under an atmosphere of nitrogen, neither the cell suspension nor the cell extracts oxidized sulphide. Furthermore, the rate of sulphide oxidation by the membrane fraction was not affected by the addition of the S144 fraction to the reaction mixture, indicating that soluble enzymes are not involved in sulphide oxidation.

Sulphide oxidation in the P144 fraction was directly related to the concentrations of enzyme and substrate. The Km for sulphide was found to be 0.4 mM, as determined by the sulphide electrode (Figure 3A).

5.1.1 Sulphide oxidation linked to various terminal acceptors

5.1.1.1 Oxygen

When sulphide was added to either the crude

Figure 2. Sulphide utilization by cell suspensions and cell-free extracts.

Sulphide utilization was determined polarographically with the sulphide electrode in a reaction mixture described in Section 4.3.1, except that in (A), 0.2 ml cell suspensions (25%, w/v) equivalent to 3 mg bacterial dry weight was used; in (B), the S10 or P144 fraction (4 mg protein) and in (C), the S144 fraction (6 mg protein). At zero time 50 µl 10 mM Na_2S was added.

Figure 2

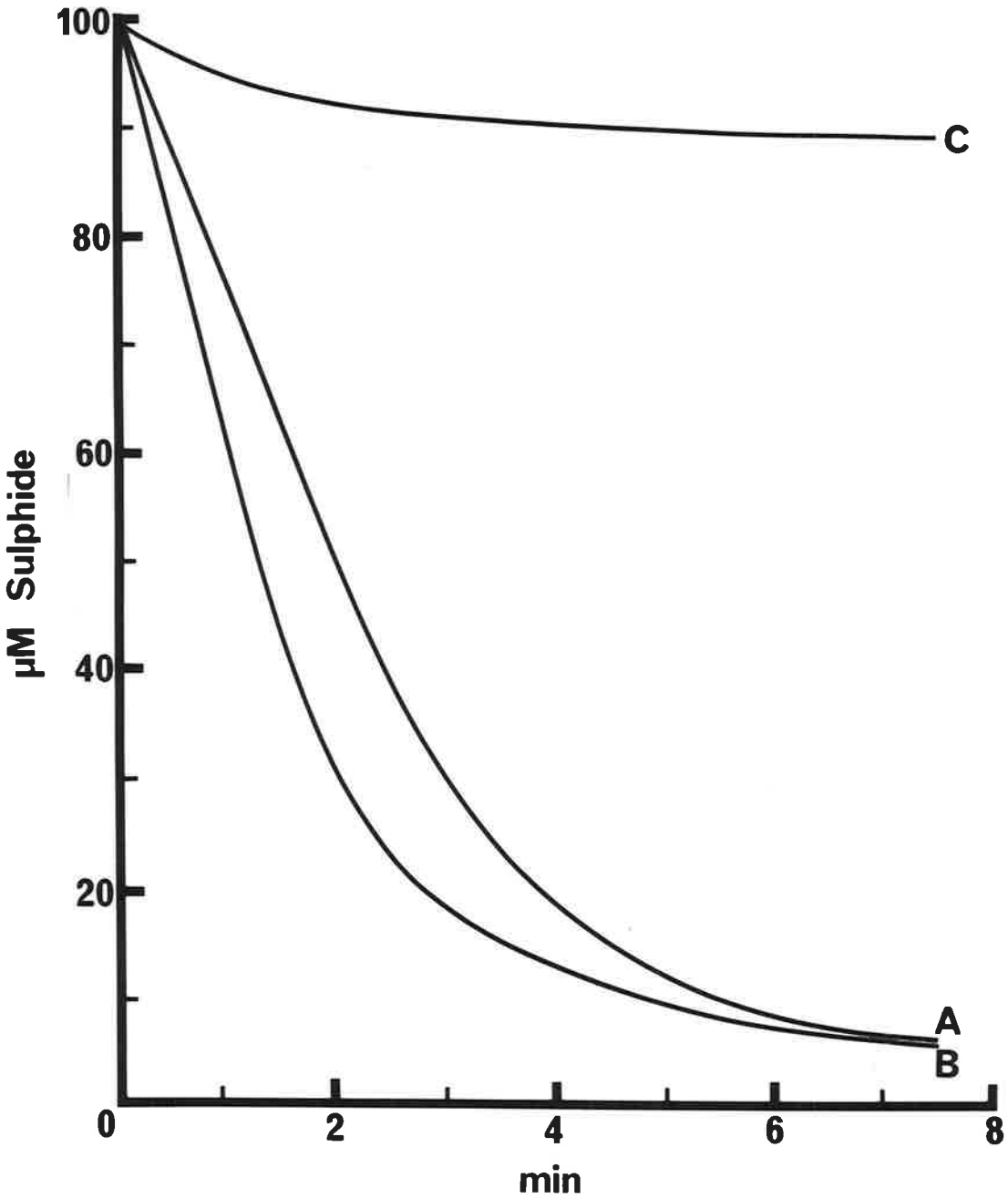


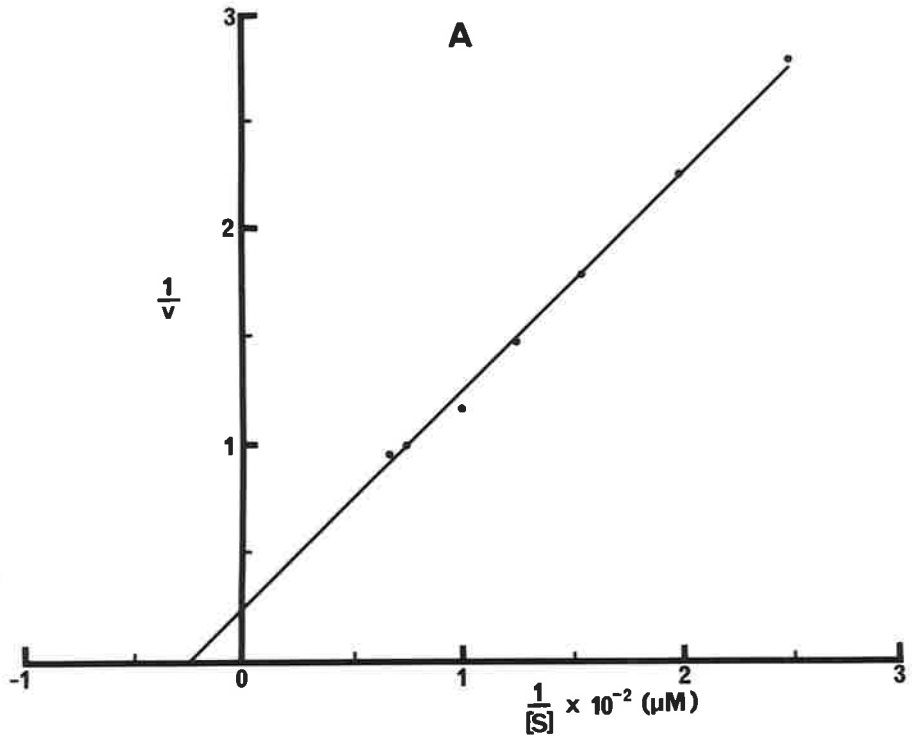
Figure 3. Effect of sulphide concentration on sulphide oxidation and on oxygen uptake.

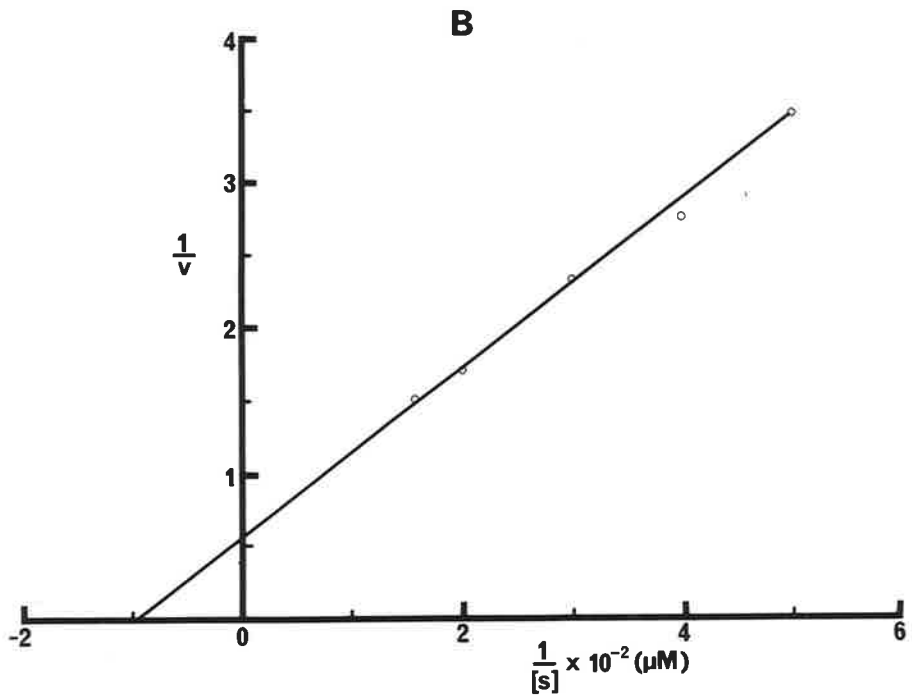
P144 fraction was prepared in 50 mM Tris-HCl buffer (pH 7.5) containing 0.2 mM Na-EDTA (Section 4.2.1,3). Sulphide utilization and oxygen uptake were measured polarographically by the sulphide (Section 4.3.1) and oxygen electrodes (Section 4.3.2) respectively. Sulphide concentration was fixed at levels shown in the figure. The P144 fraction (4 mg protein) was used.

Graph A : Lineweaver-Burk plot of sulphide oxidation *versus* sulphide concentration. The apparent Km was 0.4 mM.

Graph B : Lineweaver-Burk plot of oxygen uptake *versus* sulphide concentration. The apparent Km was 0.1 mM.

Figure 3


A
3
2
1
$\frac{1}{v}$
-1
0
1
2
3
$\frac{1}{[S]} \times 10^{-2}$ (μM)


B
4
3
2
1
$\frac{1}{v}$
-2
0
2
4
6
$\frac{1}{[s]} \times 10^{-2}$ (μM)

extract (S10) or the P144 fraction there was a gradual uptake of O_2 as measured by the electrode method (Figure 4). Although sulphide disappeared after 3 min, oxygen was still being utilized and this reached a maximum in 8 min. The initial rate of oxygen uptake, however, was dependent on the relative amounts of enzyme and sulphide added. The Km for sulphide obtained by this method was 0.1 mM (Figure 3B). The rate of oxygen uptake in the S144 fraction was only 5% of that catalysed by either the S10 or the P144 fractions (Figure 4). The rate of oxygen uptake by the P144 fraction, however, increased when the S144 fraction was added (Figure 5).

Sulphide was effectively oxidized by the membrane fraction (P144) over the pH range 6 to 9, with an optimum of 7.5 as determined by the uptake of oxygen (Figure 6). The stoichiometry between sulphide oxidized and oxygen utilized was 2:1 for the first three min of reaction time (Table 3).

The effects of inhibitors on oxygen uptake during sulphide oxidation are considered in Section 5.5.3.1

5.1.1.2 Nitrate

Adams, Warnes & Nicholas (1971*A*) have shown that on adding sulphide, cells of *T. denitrificans* reduce nitrate to nitrogenous gases. When the crude extract (S10) was incubated

Figure 4. Oxygen uptake in cell fractions during sulphide oxidation.

Cell-free extracts (S10, S144 and P144 fractions) were prepared in Tris-HCl buffer (pH 7.5) containing 0.2 mM Na-EDTA as described in Section 4.2.1. The apparatus for oxygen uptake was set up as described in Section 4.3.2. The reaction mixture in a total volume of 3 ml contained, Tris-HCl buffer (pH 7.5), 150 µmoles; Na-EDTA, 0.5 µmoles; Na_2S, 0.75 µmoles and cell-free extract. The reaction, conducted at 25^{o}, was started by adding sulphide and allowed to continue until equilibrium was reached (about 8 min).

A : S144 fraction (4 mg protein)

B : P144 fraction (3 mg protein)

C : P144 fraction (8 mg protein)

Figure 4

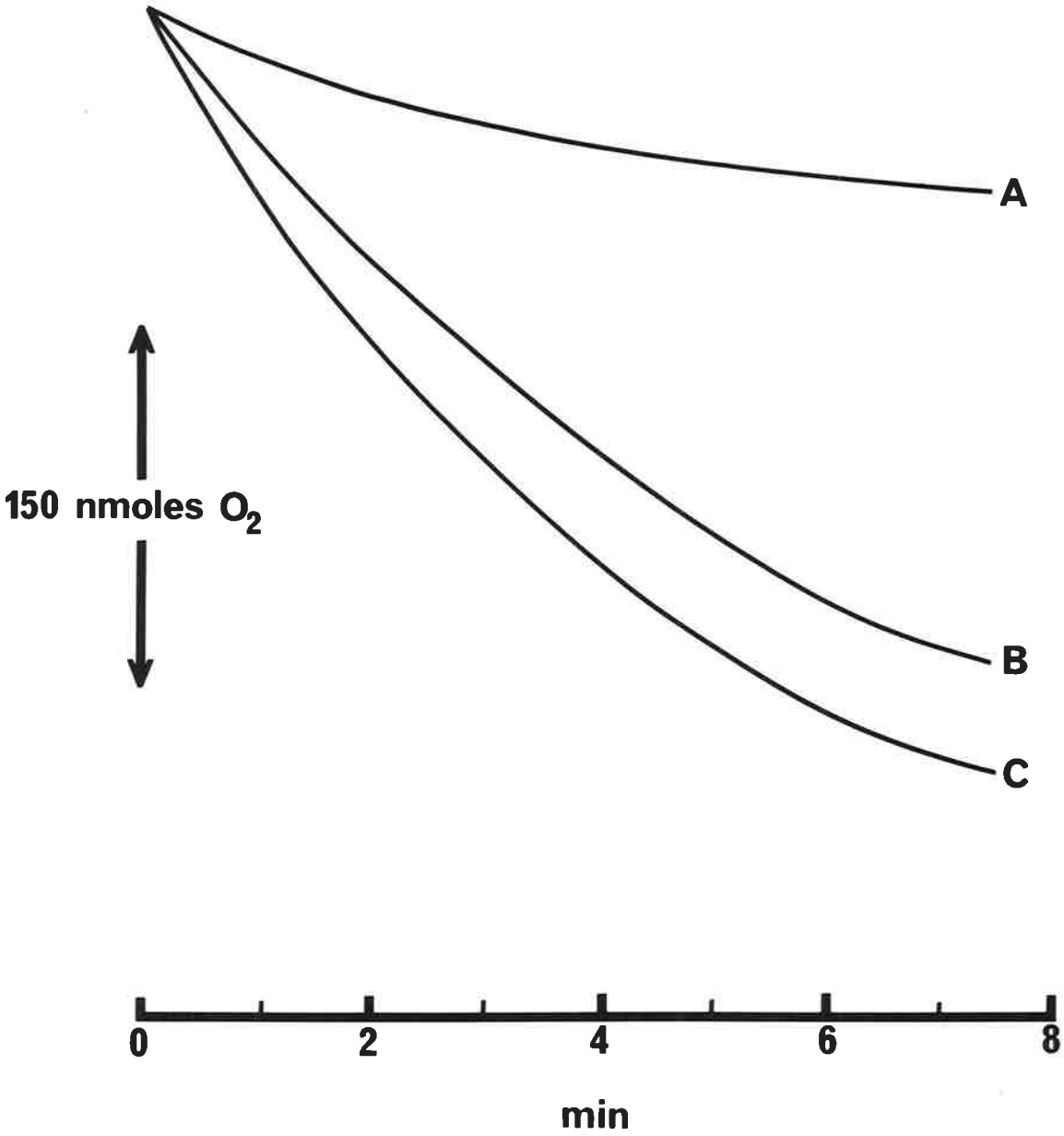


Figure 5. Effect of S144 fraction on oxygen uptake during sulphide oxidation by the P144 fraction.

Oxygen uptake was determined polarographically as described in Section 4.3.2.

A : At zero time the reaction mixture contained 500 µM Na_2S in 3 ml 50 mM Tris-HCl buffer (pH 7.5) with 0.2 mM Na-EDTA. The S144 fraction (4 mg protein) were added as indicated -

(1) after 30 sec

(2) after 4 min

B : At zero time the reaction mixture was as in (A). Additions were -

(3) P144 fraction (4 mg protein) added after 1 min

(4) S144 fraction (4 mg protein) added after 4.5 min

Figure 5

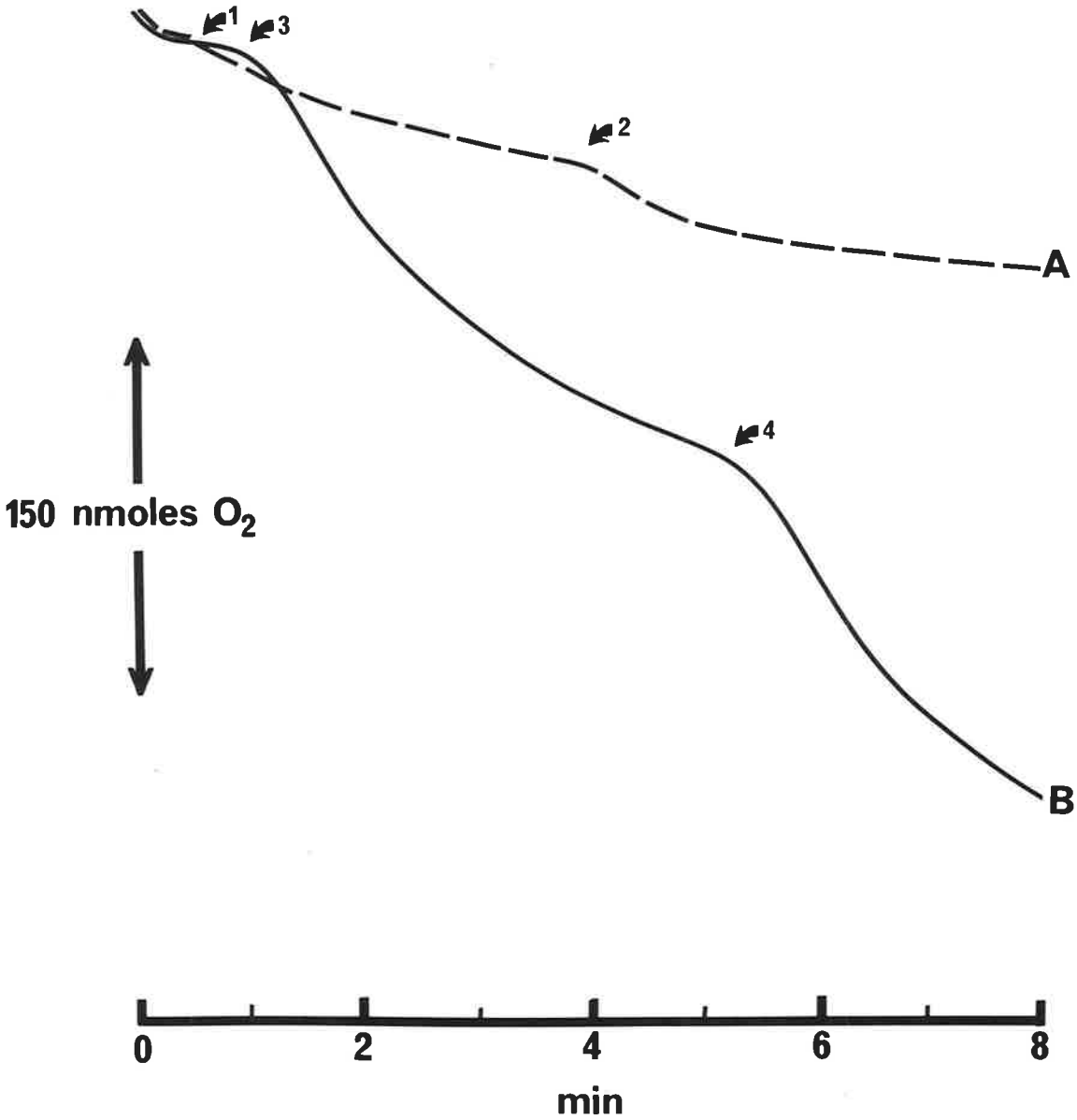


Figure 6. Effect of pH on oxygen uptake and nitrite reduction during sulphide oxidation in the P144 fraction.

Oxygen uptake was measured as described in Section 4.3.2 and nitrite reduction with sulphide as an electron donor as described in Section 4.2.5.2. 50 mM Tris-HCl and phosphate buffers were used.

Oxygen uptake : ●-----● phosphate buffer

o------o Tris-HCl buffer

Nitrite reduction : ▲——▲ phosphate buffer

Δ——Δ Tris-HCl buffer

Figure 6

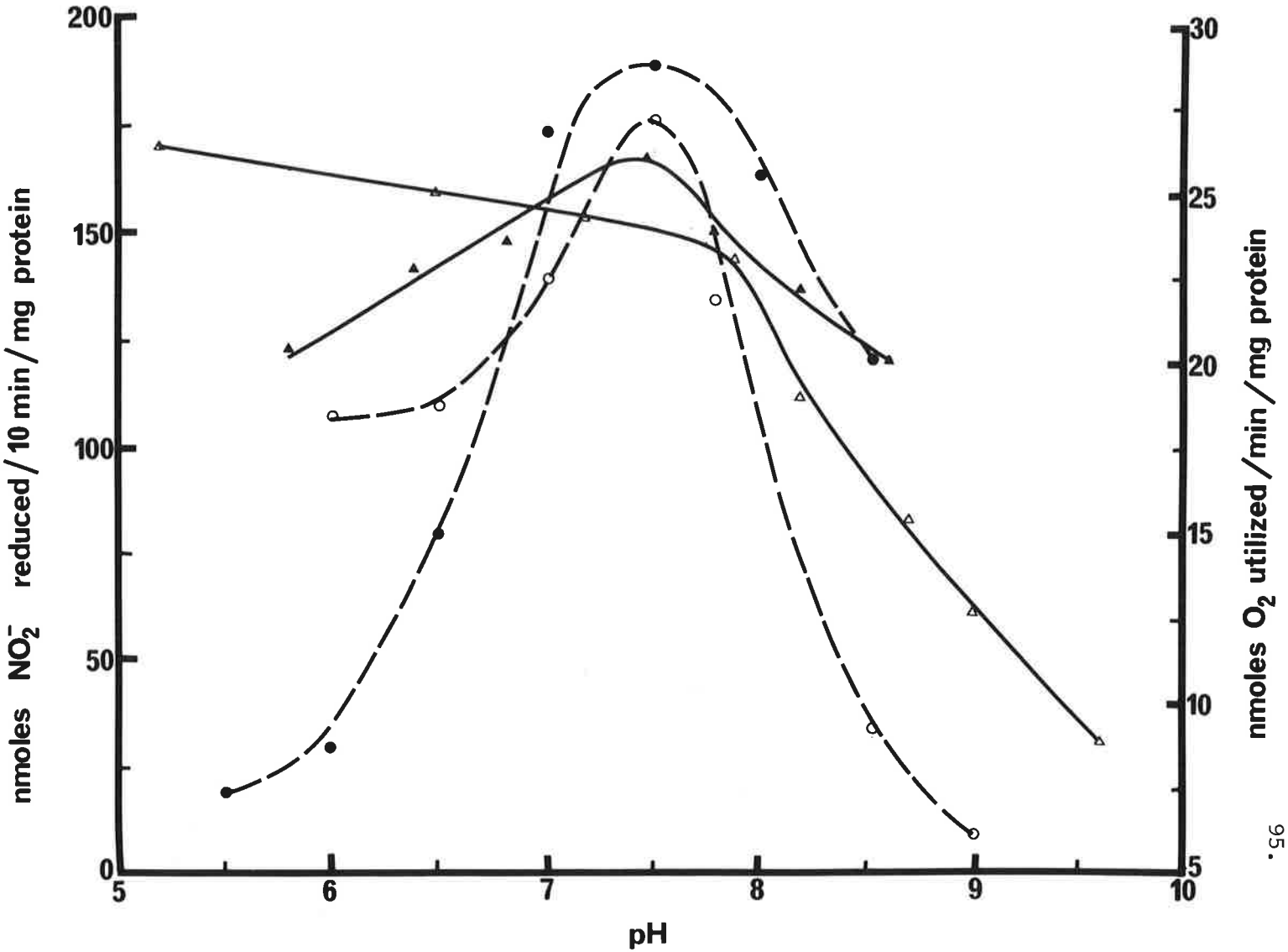

200
150
100
50
0
nmoles NO_2^- reduced / 10 min / mg protein
30
25
20
15
10
5
nmoles O_2 utilized / min / mg protein
5
6
7
8
9
10
pH

Table 3. Stoichiometry of sulphide oxidized and oxygen utilized.

The reaction mixture contained 0.25 mM sulphide in a final volume of 3 ml made up with 50 mM Tris-HCl buffer (pH 7.5) with 0.2 mM Na-EDTA. The initial oxygen concentration was found to be 0.25 mM. The reaction mixture was placed in a vessel designed for oxygen uptake studies (Section 4.3.2). At zero time the P144 fraction (4 mg protein) was added and oxygen uptake measured polarographically (Section 4.3.2). At specified time intervals an aliquot was withdrawn from the reaction mixture and the sulphide concentration determined by the sulphide electrode (Section 4.3.1). Thus, the amounts of oxygen utilized and sulphide oxidized can be calculated.

Time (min)	$S^=$ oxidized (μM)	O_2 utilized (μM)	$S^= : O_2$
1	86	38	2.23
2	158	75	1.98
3	172	97	1.76
4	181	110	1.68
5	190	123	1.46

in air with nitrate and sulphide, nitrite was produced (Figure 7). Under anaerobic conditions, however, nitrite production was either very low or not detectable. The rate of nitrite production was dependent on the relative amounts of sulphide and the crude extract used (Figure 8).

Neither the S144 nor the P144 fraction linked the oxidation of sulphide to nitrate reduction but this activity, restored on recombining the two fractions (Table 4), was dependent on the relative amounts of each fraction added. Thus, when the P144 fraction was fixed at 1 mg protein and the S144 fraction varied, there was a linear relation between the rate of nitrate reduction and the amount of S144 fraction added (Figure 9). Similar results were obtained when the S144 fraction was fixed and the P144 fraction varied (Figure 9). There was no increase in enzyme activity, however, when a boiled preparation of either the S144 or P144 fraction was used in the recombined mixture.

5.1.1.3 Nitrite

When cells were incubated with nitrite and sulphide under anaerobic conditions, the NO, N_2O and N_2 gases produced were detected in the mass spectrometer (Table 5).

Nitrite was reduced concomitantly with the oxidation of sulphide in the crude extract (S10) or the P144 fraction. The S144

Figure 7. Effect of incubation time on sulphide-linked nitrate reduction in crude extracts (S10).

Enzyme activity was determined as described in Section 4.2.5.1. The anaerobic assay was conducted in Warburg flasks as described for the NADH-linked nitrate reductase (Section 4.2.5.1). Activity is expressed in nmoles nitrite produced/mg protein. Boiled control preparations were included in each series of experiments.

Δ——Δ 5 µmoles sulphide

▼----▼ 2.5 µmoles sulphide

●----● 2.5 µmoles sulphide under anaerobic conditions

Figure 8. Effect of sulphide concentration on nitrate reduction in crude extracts (S10).

Nitrate reductase was determined as described in Section 4.2.5.1 except that sulphide was adjusted to a final concentration as specified in the figure.

●——● 5 mg protein S10

Δ-----Δ 2 mg protein S10

Figure 7

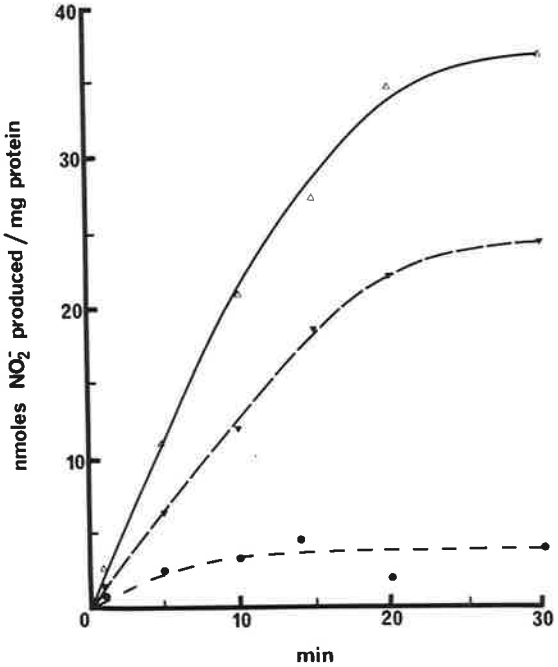


Figure 8

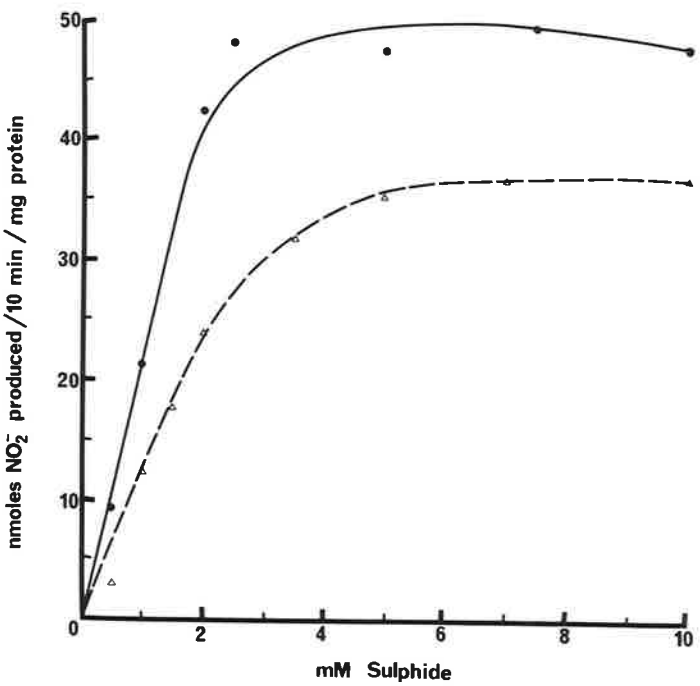


Table 4. Distribution of sulphide-linked nitrate reductase in cell fractions.

The various cell fractions (S10, S144 and P144 fractions) were prepared in 50 mM Tris-HCl buffer (pH 7.5) containing 0.2 mM Na-EDTA as described in Section 4.2.1. Nitrate reductase activity with sulphide as donor was determined as described in Section 4.2.5.1.

Fraction	Procedure	Specific activity
I	French Pressure Cell homogenate	95
II	Crude extract - supernatant fraction (S10) obtained by centrifuging I at 10,000 x *g* for 30 min	127
III	Supernatant fraction (S144) left after centrifuging II at 144,000 x *g* for 90 min	10
IV	Pellet fraction (P144) left from III	14
V	S144 and P144 fractions recombined (ratio 1 : 1 mg protein)	92

Specific activity: nmoles nitrite produced/10 min/mg protein.

Figure 9. Effect of varying the amounts of S144 and P144 fractions on sulphide-linked nitrate reductase activity.

Sulphide-linked nitrate reductase was determined as described in Section 4.2.5.1 except that the S144 and P144 fractions were varied as follows:

●———● P144 fixed at 1 mg protein; S144 varied as shown

o———o P144 fixed at 1 mg protein; S144 (boiled preparation) varied

▲-----▲ S144 fixed at 2 mg protein; P144 varied

Δ-----Δ S144 fixed at 2 mg protein; P144 (boiled preparation) varied

Figure 9

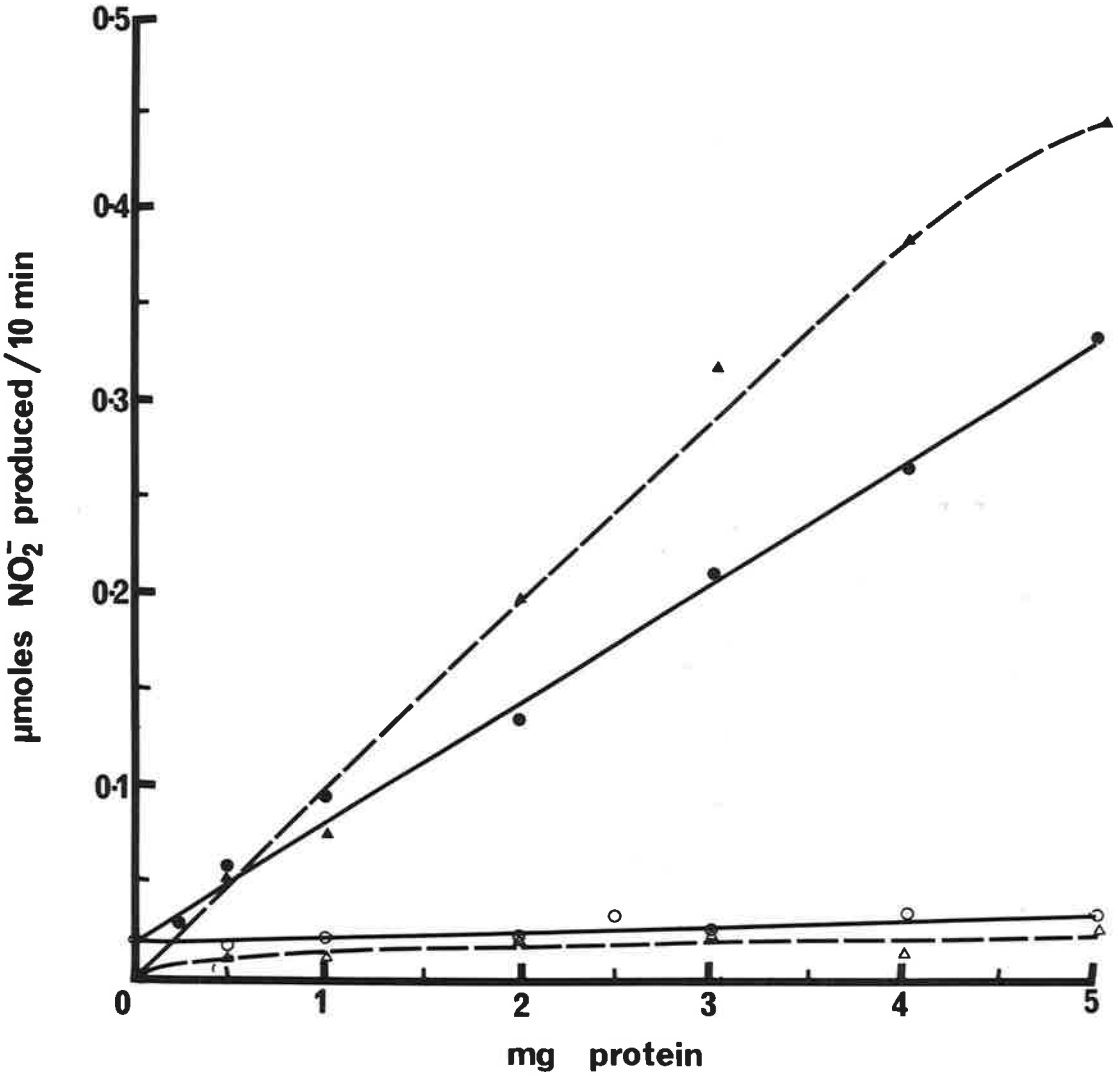


Table 5. Products of nitrite reduction by cell suspensions.

The reaction mixture contained 2 µmoles $NaNO_2$ and 5 µmoles of the appropriate electron donor; 0.1 ml of cell suspension (25%, w/v) was used. The reaction, conducted as described in Section 4.3.5, was incubated for 3 hr at 30°. The gases formed were analysed in an A.E.I. MS-2 mass spectrometer.

Gas production is expressed as relative peak heights (proportional to concentrations) corrected for controls using boiled preparations.

Mass No.	Compound	Electron donor			
		Na_2S	Na_2SO_3	$Na_2S_2O_3$	NADH/PMS
23	N_2	6.6	2.1	13.5	5.8
30	NO	3.2	2.5	2.8	0.9
44	N_2O	21.3	10.0	13.2	24.1

fraction, however, had little or no sulphide-linked nitrite reductase activity (Figure 10). When assayed anaerobically this activity in either the S10 or the P144 fraction was about 25% higher than that recorded in an aerobic assay.

The rate of nitrite reduction was dependent on the relative amounts of sulphide, nitrite and the extract used. When the concentration of nitrite was fixed at 1 mM, the apparent Km for sulphide was 0.8 mM (Figure 11).

The optimum pH for sulphide-linked nitrite reductase activity in the P144 fraction was 7.5 (Figure 6). One mole of sulphide was oxidized per mole of nitrite reduced (Table 6).

The effects of inhibitors on sulphide oxidation coupled to nitrite reduction are presented in Section 5.5.3.2.

5.1.2 Products of sulphide oxidation

5.1.2.1 Experiments with (^{35}S)-sulphide

Inorganic sulphur compounds were separated by high-voltage paper electrophoresis at pH 5.0, and their relative mobilities are shown in Figure 12. In this system elemental sulphur and membrane-bound sulphur compounds remained at the origin. Sulphide and sulphite were treated with N-ethylmaleimide (NEM) prior to their application to the paper and were run as N-ethylmaleimide

Figure 10. Effect of incubation times on sulphide-linked nitrite reductase in various cell fractions.

The reaction was carried out in stoppered tubes as described in Section 4.2.5.2. Each extract (2 mg protein) was incubated for times varying from 0 to 30 min.

Δ-----Δ Crude extract (S10)

o———o P144 fraction

∇-----∇ S144 fraction

Figure 11. Effect of varying sulphide concentration on nitrite reductase activity in the P144 fraction.

Enzyme activity was determined as described in Section 4.2.5.2. The P144 fraction (5 mg protein) was incubated with various amounts of sulphide as shown in the figure. The results are expressed as a Lineweaver-Burk plot. The apparent Km for sulphide was 0.8 mM.

Figure 10

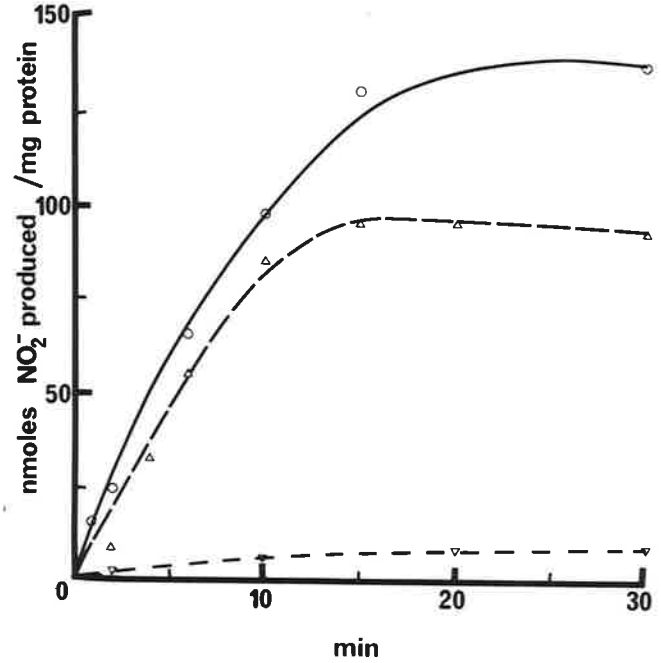


Figure 11

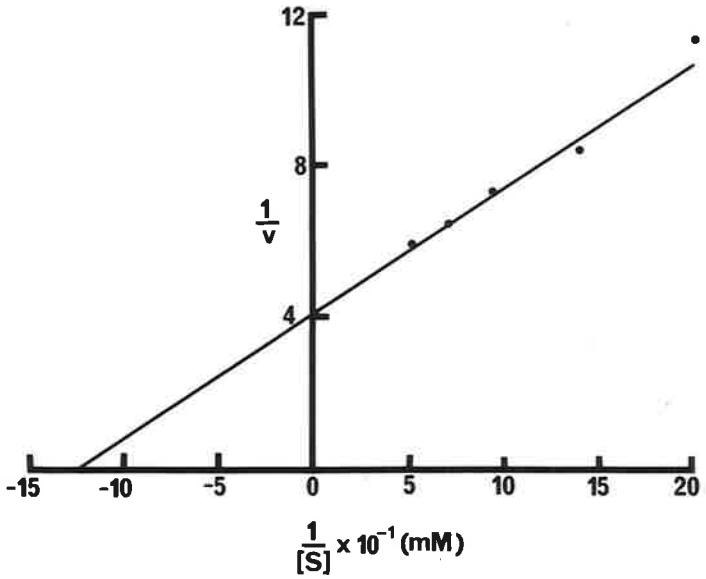


Table 6. Stoichiometry of sulphide oxidized and nitrite reduced.

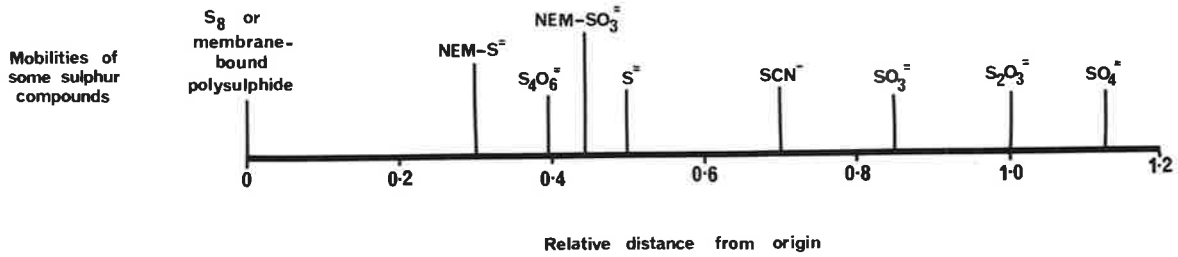
The reaction mixture contained in a total volume of 2 ml in 50 mM phosphate buffer (pH 7.5) with 0.2 mM Na-EDTA, 2 µmoles each of Na_2S and $NaNO_2$ and P144 fraction (4 mg protein). The reaction, conducted under anaerobic conditions, was started by adding sulphide and terminated after 30 or 60 min as described in Section 4.2.5.2. The mixture was then centrifuged at 2,000 x *g* for 5 min to sediment precipitated proteins. Aliquots of the supernatant fraction were withdrawn for determining sulphide and nitrite as described in Section 4.4.4 and Section 4.4.2 respectively.

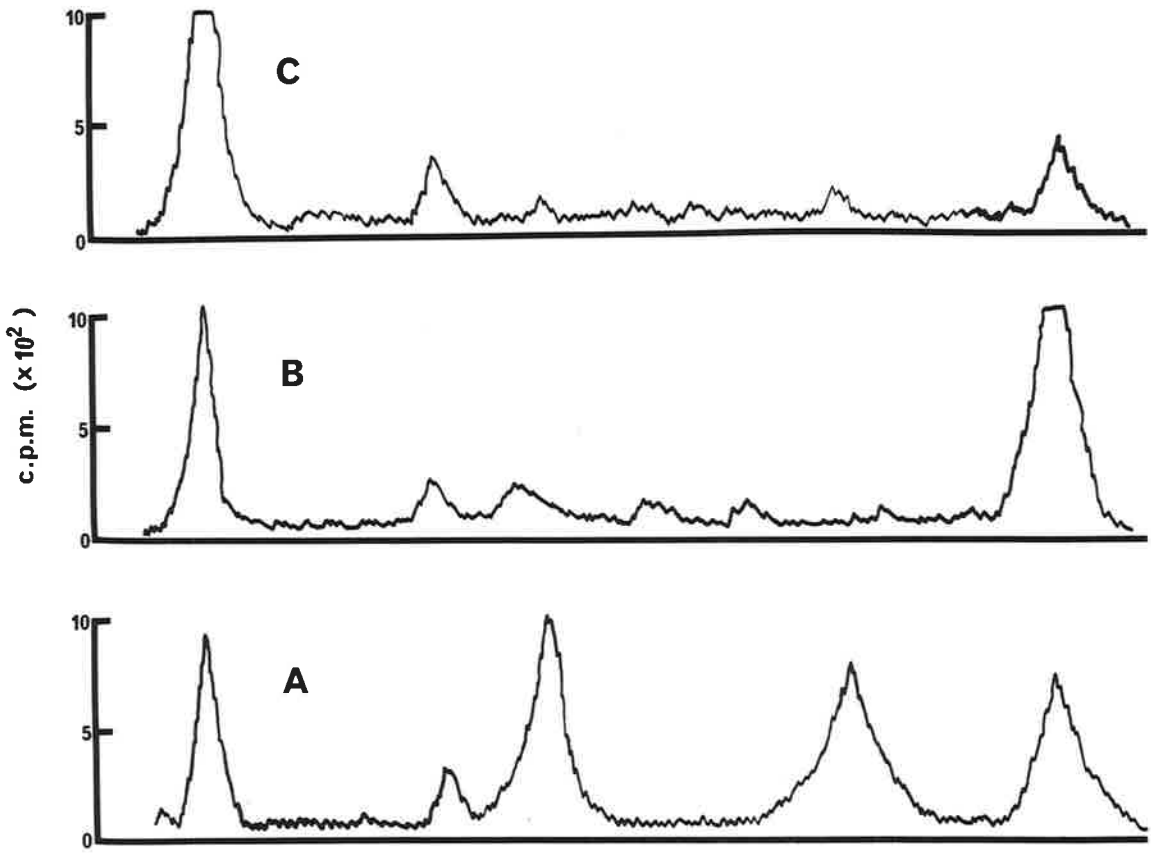
Time (min)	$S^=$ oxidized (nmoles)	NO_2^- reduced (nmoles)	$S^=$: NO_2^-
30	118	125	0.95
60	277	281	0.99

Figure 12. Electrophoresis of the products of sulphide oxidation.

The reaction mixture in (A) contained 50 mM Tris-HCl buffer (pH 7.5) with 0.2 mM Na-EDTA, 0.4 µC $Na_2{}^{35}S$ and 5 µmoles Na_2S carrier in a total volume of 0.2 ml; (B) as in (A) plus 5 µmoles $NaNO_3$; (C) as in (A) plus 5 µmoles $NaNO_2$. In each case 2.5 mg protein of the crude extract (S10) was used. (A) and (B) were incubated in air and (C) anaerobically, all for 15 min. The reaction was terminated with 50 µl 50 mM N-ethylmaleimide. Aliquots (20 µl) of each mixture were then spotted on to a Whatmann 3 MM paper and subjected to high voltage electrophoresis as described in Section 4.3.4. Radioactive areas were detected by running the dried chromatogram through a Packard 7201 radiochromatogram scanner (Section 4.3.6.1).

Figure 12


Mobilities of some sulphur compounds
S_8 or membrane-bound polysulphide
NEM-$S^=$
$S_4O_6^=$
NEM-$SO_3^=$
$S^=$
SCN^-
$SO_3^=$
$S_2O_3^=$
$SO_4^=$
0
0·2
0·4
0·6
0·8
1·0
1·2
Relative distance from origin


c.p.m. (x 10^2)
10
5
0
C
B
A

complexes (Ellis, 1968).

The crude extract (S10), incubated in air for 15 min with (^{35}S)-sulphide with or without nitrate, was treated with N-ethyl-maleimide and the products of the reaction then separated by high-voltage electrophoresis. In the presence of nitrate the products were (^{35}S)-sulphate and (^{35}S)-labelled material which remained at the origin. When nitrate was excluded from the reaction mixture, (^{35}S)-sulphide was oxidized primarily to (^{35}S)-sulphite as shown in Figure 12. The (^{35}S)-labelled material at the origin and some (^{35}S)-sulphate were also produced.

When the crude extract was incubated anaerobically for 15 min with (^{35}S)-sulphide in the presence of nitrite, the bulk of the (^{35}S)-labelled material remained at the origin of the electrophoretogram (Figure 12C).

When these reaction mixtures, after the 15-min incubation period, were treated with iodine before application on to the paper, only 10% of the total radioactivity was located at the origin; the rest of the tracer was primarily associated with polythionates.

When the crude extract (S10) was incubated for 2 min with (^{35}S)-sulphide and then centrifuged at 224,000 x *g* for 1 hr, between 60-75% of the radioactivity was found on the pellet fraction (P224) (Table 7). This suggests that the sulphur compound produced

Table 7. The enzymic binding of ^{35}S-sulphide to membrane fractions (P144) under various experimental conditions.

The reaction mixture was the same as that described in Figure 12(A) except that 0.8 µC of ^{35}S-sulphide was used and the total volume was 1 ml. After pre-equilibration of the enzyme at 30° for 5 min the reaction was started by adding ^{35}S-sulphide followed immediately by 5 µmoles carrier Na_2S. After 2 min incubation the reaction vessel (stoppered test tubes, 7.5 x 1 cm) was plunged into an ice-salt mixture. The contents in each tube were then transferred to a pre-chilled centrifuge tube and centrifuged at 224,000 x *g* for 1 hr at 2° in a Spinco Model L ultracentrifuge (Type Ti50 rotor). Aliquots of the supernatant fraction (S224) and pellet fraction (P224, after re-suspending in 50 mM Tris-HCl buffer, pH 7.5) were assayed for ^{35}S in a liquid scintillation spectrometer, as described in Section 4.3.6.2.

Unless otherwise stated, the reactions were carried out in air. Carbon monoxide was flushed through the reaction mixture for 3 min prior to adding ^{35}S-sulphide.

Each figure represents an average of two determinations. The total counts in a control mixture (enzyme omitted) was taken as 100%.

Experimental conditions	% radioactivity in the P224 fraction
Complete	67
Complete (anaerobic)	25
Boiled enzyme	25
Complete plus NO_3^-	66
Complete plus NO_3^- (anaerobic)	45
Complete plus NO_2^-	73
Complete plus NO_2^- (anaerobic)	57
Complete, flushed with CO for 3 min	37

Complete = S10 + ^{35}S-sulphide.

binds with the membrane fraction. However, when the crude extract was kept under anaerobic conditions and then incubated with the radiotracer as above only 25% of the radioactivity was detected in the pellet. On adding nitrite under strictly anaerobic conditions and incubating the reaction mixture for a further 2 min, the percentage of radioactivity recovered in the pellet increased to around 60%. In the presence of nitrate the degree of binding of (^{35}S)-sulphide to the membrane fraction was higher when incubated in air than under anaerobic conditions (Table 7). This is in accord with the observation that sulphide-linked nitrate reductase activity was low when assayed anaerobically. Carbon monoxide inhibited the binding of (^{35}S)-sulphide to the membrane fraction since only about 35% of the radioactivity was recovered in the pellet when the crude extract was flushed for 3 min with CO before adding (^{35}S)-sulphide.

5.1.2.2 Absorption spectra of polysulphide-type compounds

When sulphide was incubated with either the crude extract (S10) or the P144 fraction, there was an increase in absorbance around 300-350 nm with a maximum at 315 nm (Figure 13). This suggests a polymeric sulphur compound as found by Moriarty & Nicholas (1969) in *T. concretivorus*. The S144 fraction had little

Figure 13. Difference spectra of various cell fractions, reduced with sulphide *versus* oxidized.

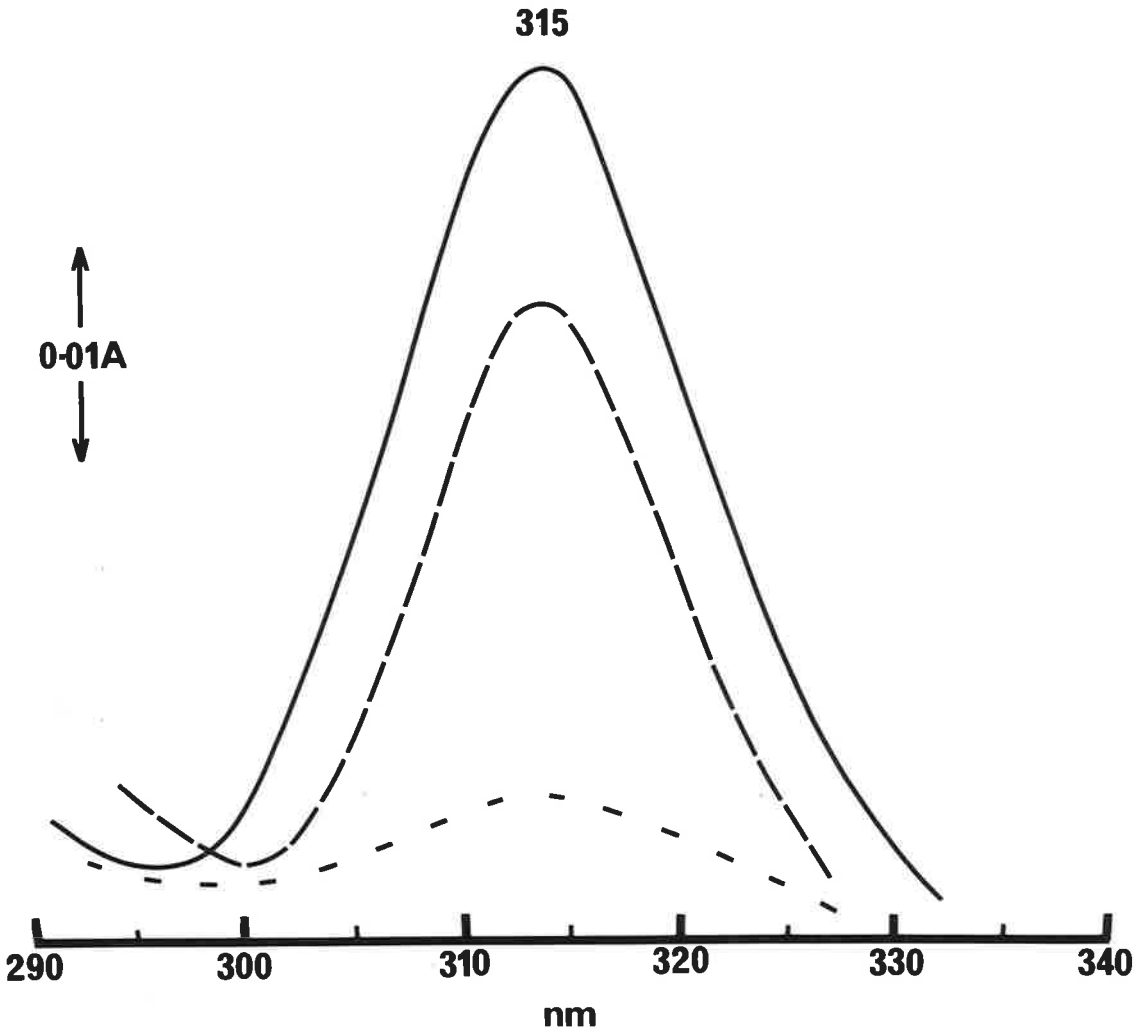
Reference and sample cuvettes (quartz, 1 cm path) contained cell extract (3 mg protein) in 3 ml 50 mM phosphate buffer (pH 7.0) with 0.2 mM Na-EDTA. To the sample cuvette was added 5 µmoles Na_2S and the difference spectra recorded in a Shimadzu Multipurpose Recording Spectrophotometer (Section 4.5.1).

—— —— —— Crude extract (S10)

———————— P144 fraction

- - - - - - - - S144 fraction

Figure 13



activity and boiled preparations of either the crude extract or the P144 fraction did not produce this polymeric sulphur compound. Under anaerobic conditions it was only produced when nitrite was present (Figure 14), but nitrate, however, was ineffective.

When the P144 fraction, which had been treated with deoxycholate (0.2 mg/mg protein) is centrifuged at 105,000 X *g* for 30 min the supernatant fraction (S105) thus obtained did not produce the 300-350 nm band on adding sulphide. This indicates that the sulphide oxidizing enzyme is tightly bound to the membrane fraction. Crude extracts of other bacteria, e.g. *Azotobacter vinelandii* and *Nitrosomonas europaea* did not form this absorption band at 315 nm.

The absorbance at 315 nm is dependent on the amounts of the extract and sulphide added. The Km for sulphide based on this absorbance was 0.3 mM, which is similar to those obtained by other methods - 0.4 mM (sulphide electrode), 0.1 mM (oxygen uptake) and 0.8 mM (nitrite reduction).

The formation of the 315 nm absorbing material was inhibited by CO and this effect was reversed by light. When sulphide was added to the P144 fraction which had been previously treated with CO, there was only a very small increase in absorbance at 315 nm, but after exposing to a bright tungsten light for 5 min about 85% of the

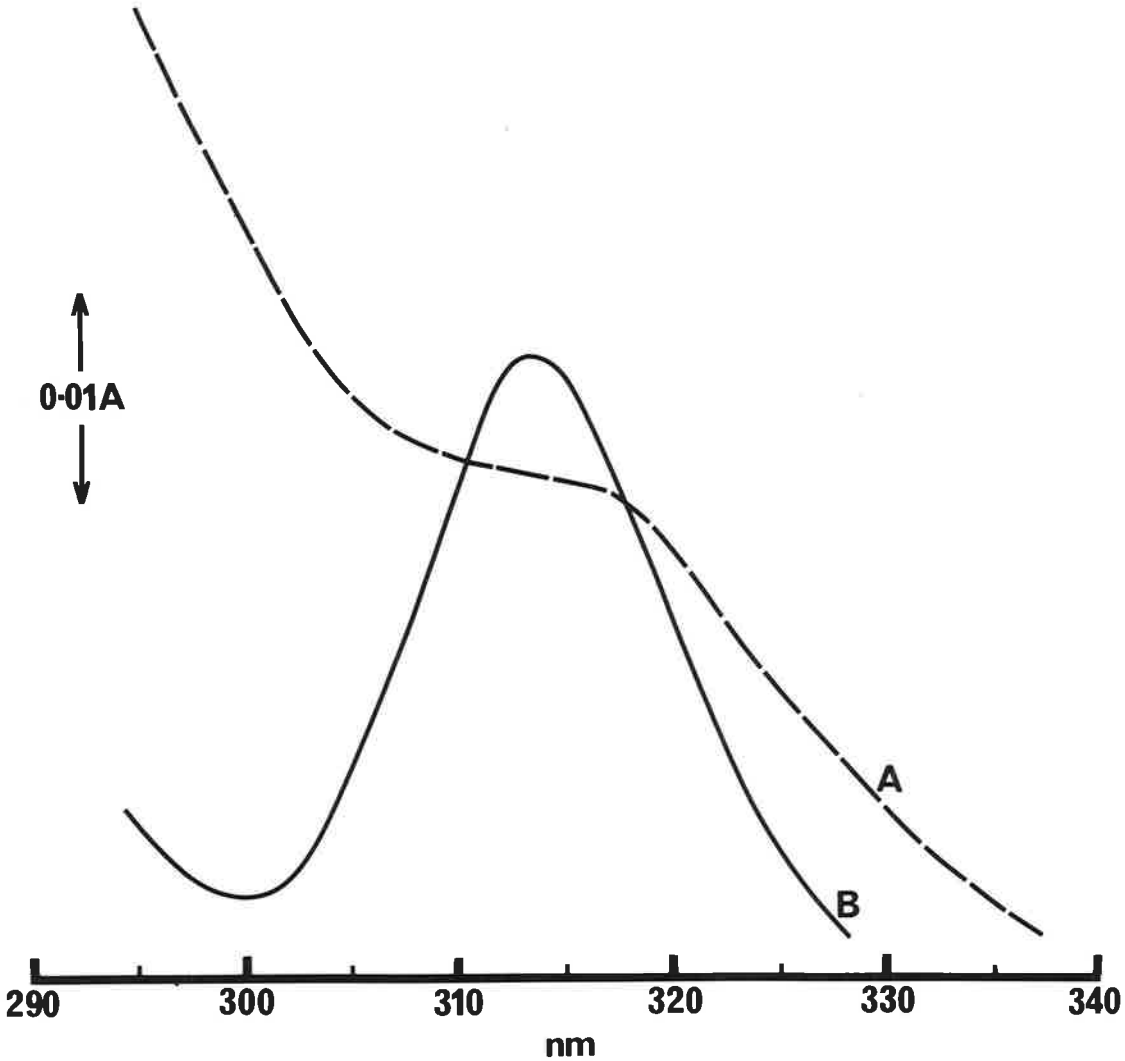
Figure 14. Effect of nitrite on the oxidation of sulphide to polysulphide in the P144 fraction.

The formation of polysulphide was followed by its characteristic absorption spectrum (300 - 350 nm). The sample cuvette (1 cm path), designed for anaerobic studies, contained the P144 fraction (10 mg protein) in 50 mM phosphate buffer (pH 7.0) with 0.2 mM Na-EDTA in the main compartment. The side arm contained either 2.5 µmoles Na_2S or 2.5 µmoles Na_2S plus 5 µmoles $NaNO_2$. The reference cuvette (normal type) contained the same components as the main compartment of the sample cuvette. The latter was evacuated and flushed several times with O_2-free N_2 gas. The contents of the side arm were then tipped into the main compartment and mixed thoroughly. The difference spectra were then recorded.

A : Sulphide reduced *versus* oxidized difference spectra without nitrite, under anaerobic conditions

B : As in A but with nitrite

Figure 14



original 315 nm band was restored (Figure 15).

When a preparation of colloidal sulphur (S_8) was suspended in a solution of inert protein such as bovine serum albumin (0.1 mg sulphur/mg protein) the difference spectra (albumin plus sulphur *versus* albumin) exhibited a broad absorption band between 280-320 nm with a maximum at 305 nm (Figure 16). Similar spectra were recorded when the elemental sulphur was suspended in equivalent protein concentrations of either the crude extract (S10) or the P144 fraction.

After incubating the P144 fraction with sulphide for 1 min the 315 nm band was formed and on adding 0.1M iodine in 0.1M KI there was a shift in absorption maximum to 305 nm, suggesting that the polymeric sulphur compound had been oxidized to elemental sulphur (Figure 17). When cyanide was added to a similar preparation, there was no shift in the absorption maximum but the absorbance at 315 nm decreased (Figure 17). This reaction indicates a typical nucleophilic displacement from a polymeric sulphur compound. Although sulphite is a nucleophilic agent, it only produced this effect after treating the reaction mixture with acetone to release the polymeric sulphur compound from the membrane (Figure 18).

5.1.2.3 Production of sulphite

Large amounts of sulphite accumulated when sulphide was incubated with the crude extracts in air (Figure 19A).

Figure 15. Effect of CO on the difference spectra of the products of sulphide oxidation (polysulphide) in the P144 fraction.

Sample and reference cuvettes (1 cm path) contained the P144 fraction (10 mg protein) in 3 ml 50 mM phosphate buffer (pH 7.0) with 0.2 mM Na-EDTA.

A : After treatment of the contents of the sample cuvette with CO for 3 min

B : After adding 5 µmoles Na_2S to A

C : 10 min after exposing B to tungsten light

Figure 16. Absorption spectra of elemental sulphur and bovine serum albumin (BSA).

Elemental sulphur was prepared by the method of Roy & Trudinger (1970). The final concentrations in 3 ml 50 mM phosphate buffer (pH 7.0) containing 0.2 mM Na-EDTA in quartz cuvettes (1 cm path) were: BSA, 2 mg protein/ml; elemental sulphur, 0.2 mg dry weight/ml.

—— —— —— : Absolute spectra of BSA (BSA *versus* buffer)

———————— : Difference spectra, BSA plus sulphur *versus* BSA alone

--------- : Absolute spectra of elemental sulphur (sulphur *versus* buffer)

Figure 15

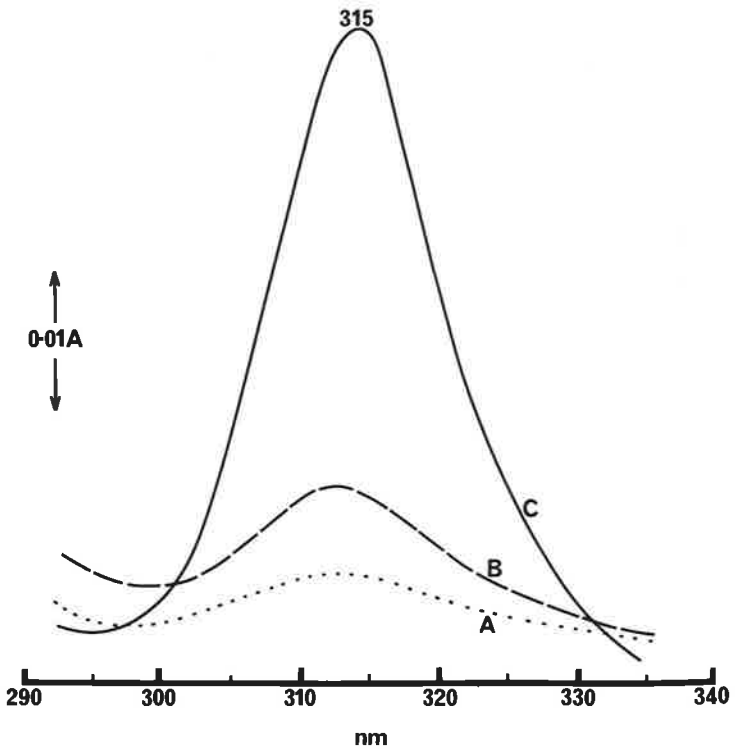


Figure 16

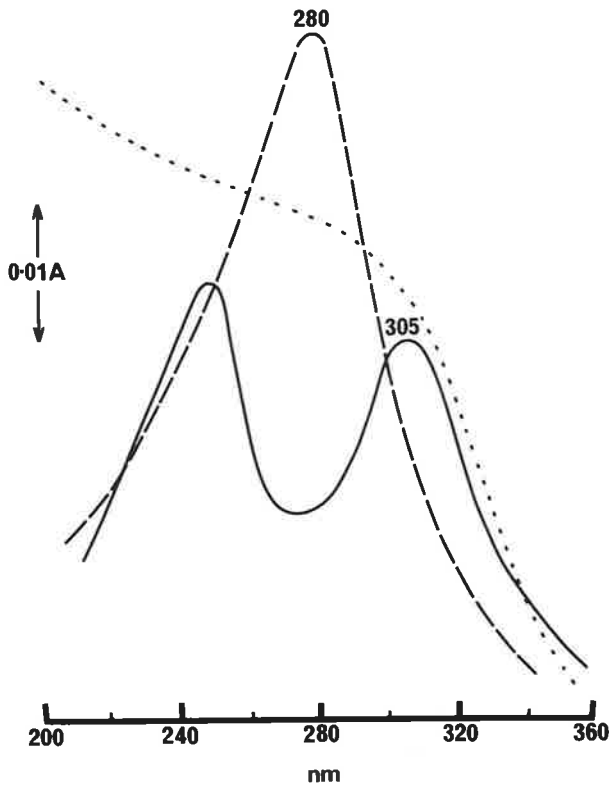


Figure 17. Effect of cyanide and iodine on the difference spectra of the products of sulphide oxidation (polysulphide) in the P144 fraction.

Sample and reference cuvettes contained the P144 fraction (10 mg protein) in 3 ml 50 mM phosphate buffer (pH 7.0) with 0.2 mM Na-EDTA.

A : After adding 5 µmoles Na_2S to sample cuvette

B : After adding 20 µl 0.1 M I_2 to A

C : After adding a small crystal of KCN to A

Figure 18. Effect of sulphite on the difference spectra of the products of sulphide oxidation (polysulphide) before and after acetone treatment.

Sample and reference cuvettes contained the P144 fraction (10 mg protein) in 3 ml 50 mM phosphate buffer (pH 7.0) with 0.2 mM Na-EDTA.

A : After adding 5 µmoles Na_2S to the sample cuvette

B : After adding a few small crystals of Na_2SO_3 to A

C : After adding 0.1 ml acetone to A

D : After adding a few small crystals of Na_2SO_3 to C

Figure 17

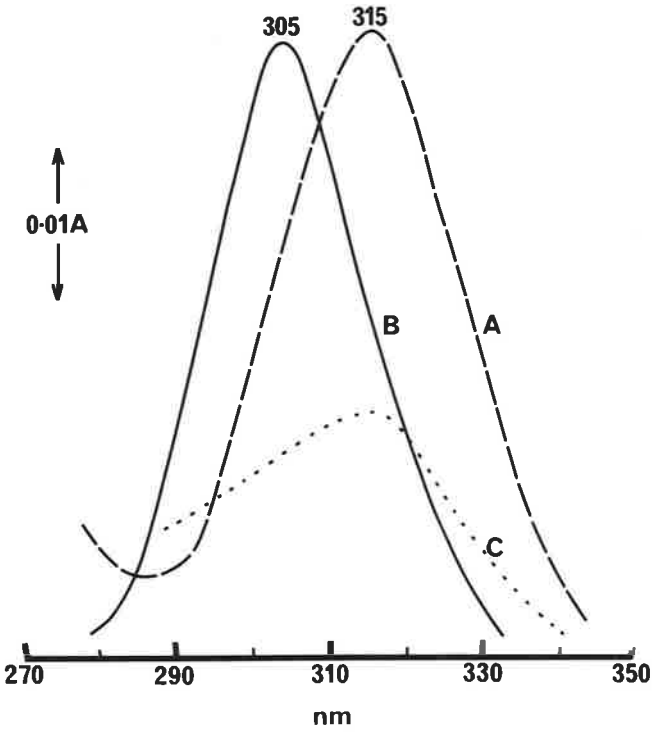


Figure 18

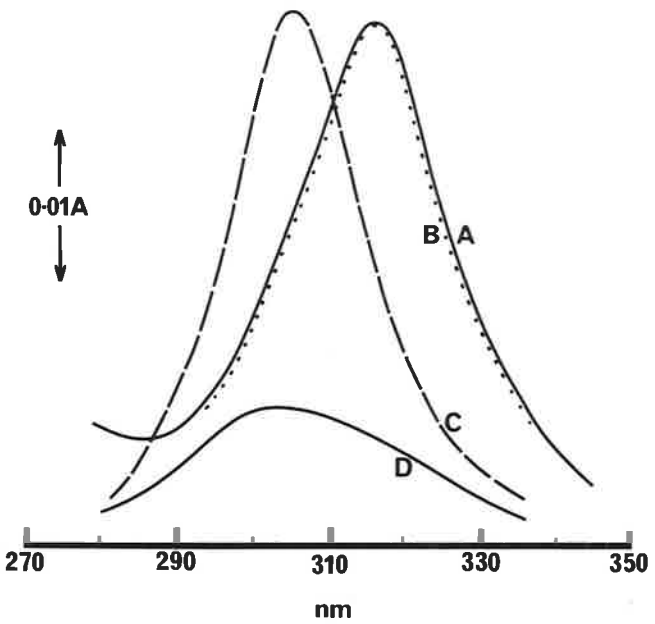


Figure 19. Effect of incubation times on the production of sulphite from sulphide in the presence and absence of nitrate in the crude extract (S10).

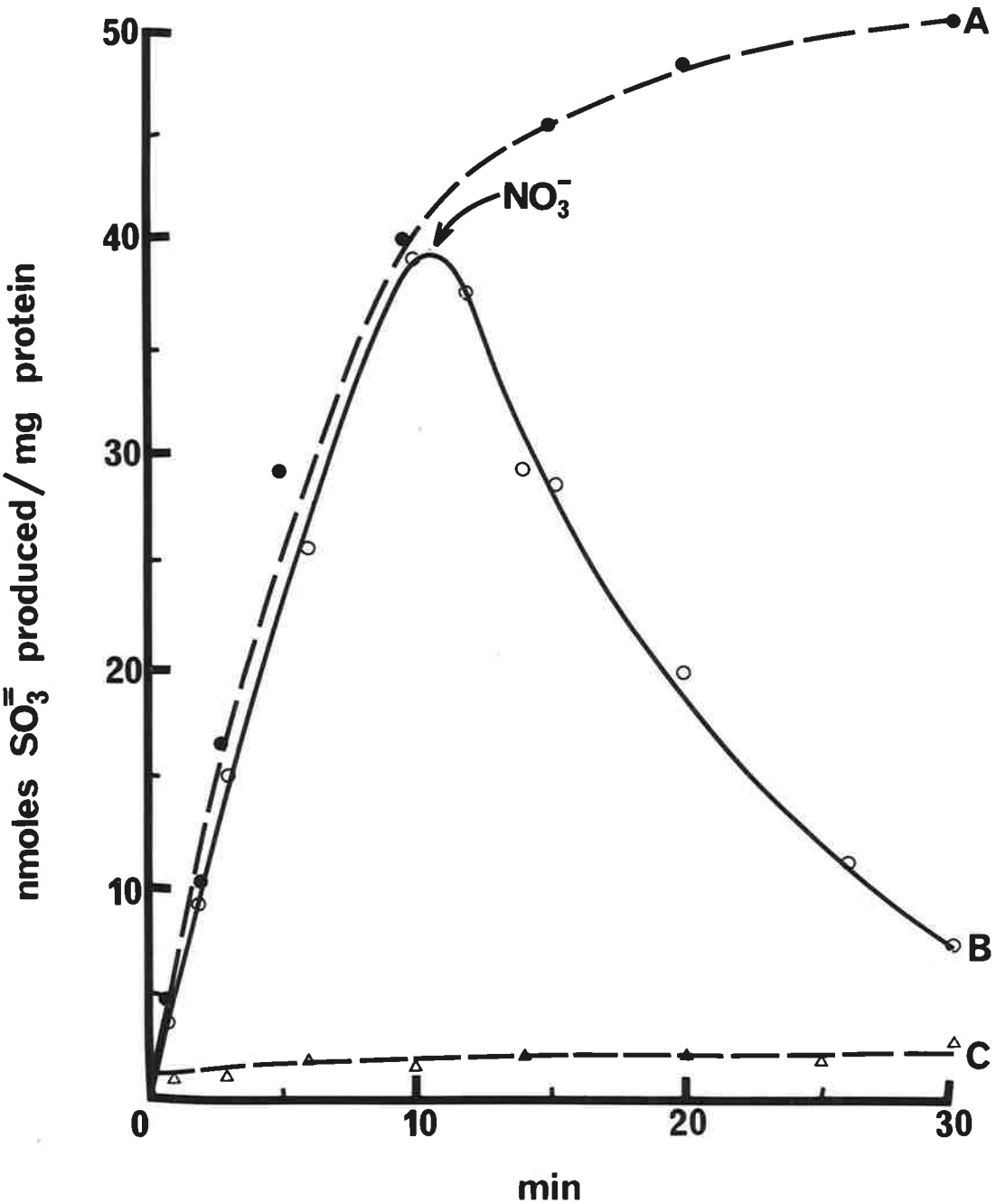
The enzymic production of sulphite from sulphide in the crude extract (S10) was determined as described in Section 4.2.5.3 for various incubation times.

A : Without nitrate

B : Nitrate added 10 min after starting the reaction

C : Nitrate added at zero time

Figure 19


50
40
30
20
10
0
10
20
30
min
nmoles $SO_3^=$ produced / mg protein
NO_3^-
A
B
C

The rate of sulphite formation was used to follow the oxidation of sulphide. Neither the S144 nor the P144 fraction alone oxidized sulphide to sulphite. But the oxidizing system was reconstituted on recombining the two fractions (Table 8).

Sulphite was not detected when the crude extract was incubated with sulphide in the presence of nitrate (Figure 19C). When nitrate was added 10 min after the start of the reaction the level of sulphite decreased sharply (Figure 19B). Nitrite, however, did not affect the production of sulphite from sulphide.

5.1.3 Stability of sulphide oxidizing enzyme

The sulphide oxidizing enzyme was found to be relatively unstable. Thus, after storing the P144 fraction at 0^{o} for 24 hr about a half of the activity remained, as measured by either nitrite reduction or oxygen uptake. The enzyme was most active in extracts of freshly harvested cells.

5.2 Oxidation of Polysulphide and Elemental Sulphur

5.2.1 Oxidation of polysulphide

When sulphide was added to the P144 fraction the 300-350 nm band, associated with polysulphide, was formed as mentioned previously (Section 5.1.2.2). On adding the S144 fraction to the

Table 8. The production of sulphite from sulphide in various cell fractions.

Crude extracts (S10), S144 and P144 fractions were prepared as described in Section 4.2.1. The enzymic sulphite production from sulphide was determined as described in Section 4.2.5.3.

Fraction	Procedure	Specific activity
I	Crude extract, supernatant fraction (S10) obtained by centrifuging cell homogenate at 10,000 x *g* for 30 min	58
II	Supernatant fraction (S144) left after centrifuging I at 144,000 x *g* for 90 min	17
III	Pellet fraction from II	14
IV	S144 and P144 fractions recombined (ratio 1 : 1 mg protein)	67

Specific activity: nmoles sulphite produced/10 min/mg protein.

reaction mixture, the absorbance at 315 nm decreased markedly (Figure 20). This was further decreased on adding GSH. Boiled preparations of the S144 fraction had no such effect on the absorbance even in the presence of GSH. This suggests that an enzyme present in the S144 fraction catalyses the oxidation of polysulphide. The observation is in accord with the finding that O_2 uptake by the P144 fraction was stimulated on adding the S144 fraction (Figure 5).

5.2.2 Oxidation of elemental sulphur

When elemental sulphur was suspended in bovine serum albumin (BSA) the difference spectra recorded (BSA *versus* BSA plus sulphur) indicate an increase in absorption at 305 nm as shown previously (Section 5.1.2.2, Figure 16). When the S144 fraction was added to such a preparation there was a slight decrease in absorbance, which became more marked on adding GSH (Figure 21).

5.2.2.1 Linked to oxygen uptake

There was an increase in oxygen uptake when the S144 fraction was incubated with GSH and elemental sulphur, but neither substrate alone utilized oxygen. The rate of oxygen uptake in the P144 fraction was less than that in the S144 fraction (Table 9). This agrees with the spectrophotometric data that a

Figure 20. Effect of S144 fraction and GSH on polysulphide formation during sulphide oxidation by the P144 fraction.

Sample and reference cuvettes contained the P144 fraction (10 mg protein) in 3 ml 50 mM phosphate buffer (pH 7.0). Difference spectra were recorded after the following additions:

A : 5 µmoles Na_2S to the sample cuvette

B : Boiled S144 fraction (5 mg protein) to reference and sample cuvettes and 1 µmole GSH to sample cuvette in A

C : S144 fraction (5 mg protein) to sample and reference cuvettes in A

D : 1 µmole GSH to the sample cuvette in C

Figure 21. Effect of S144 fraction and GSH on elemental sulphur suspended in bovine serum albumin.

Elemental sulphur was prepared by the method of Roy & Trudinger (1970). Sample and reference cuvettes (1 cm path) contained bovine serum albumin (5 mg protein) in 50 mM phosphate buffer (pH 7.0). Difference spectra were recorded after the following additions:

A : 0.1 ml elemental sulphur (20 mg) to sample cuvette

B : S144 fraction (5 mg protein) to reference and sample cuvettes in A

C : 1 µmole GSH to sample cuvette in B

Figure 20

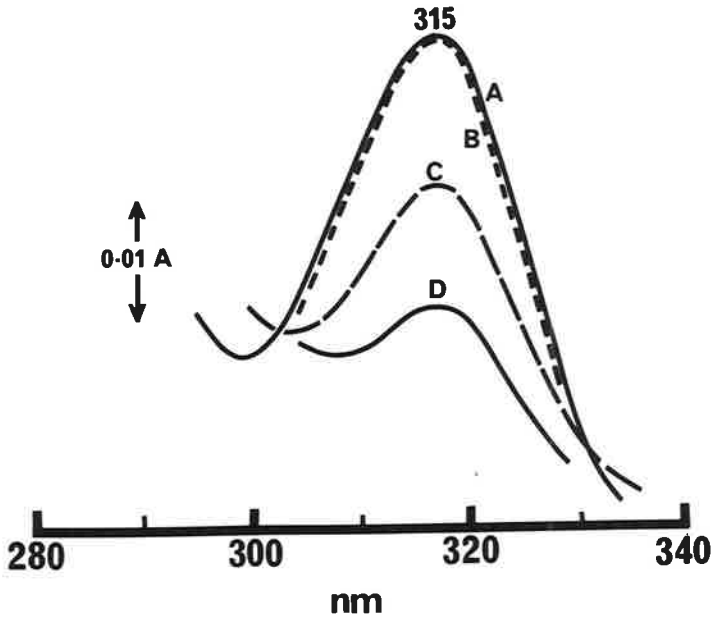


Figure 21

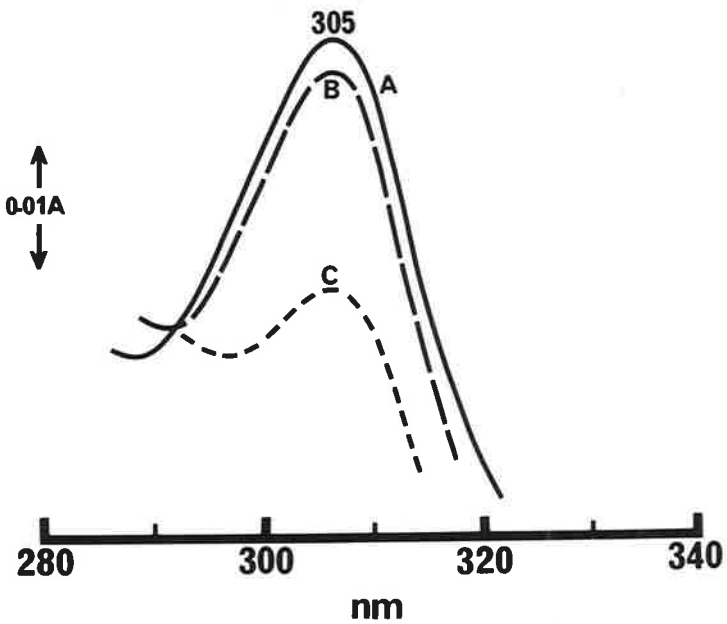


Table 9. Distribution of sulphur oxidizing enzyme in various cell fractions.

Enzyme activity was determined either by oxygen uptake or by the reduction of nitrate to nitrite. The S144 and P144 fractions were prepared as described in Section 4.2.1.3. Oxygen uptake was determined polarographically as described in Section 4.3.2. The reaction mixture in a total volume of 2.5 ml contained, phosphate buffer (pH 7.5), 120 µmoles; Na-EDTA, 0.4 µmoles; elemental sulphur, 40 mg; GSH, 5 µmoles and enzyme. The reaction conducted at 30^{o} was started by adding GSH.

Sulphur-linked nitrate reductase was determined as described in Section 4.2.5.1.

Fraction	Specific activity	
	Nitrate reductase	O_2 uptake
Crude extract (S10)	244	48
S144 fraction	33	30
P144 fraction	102	18
S144 + P144 (ratio 1 : 1 mg protein)	382	-

Specific activities:

Nitrate reductase: nmoles nitrite produced/10 min/mg protein.

Oxygen uptake: nmoles oxygen utilized/min/mg protein.

sulphur oxidizing enzyme system is located in the S144 fraction. Boiled preparations of the S144 fraction did not utilize oxygen (Figure 22). The rate of O_2 uptake decreased when the S144 fraction was dialysed against three changes of 50 mM phosphate buffer (pH 7.0) over a 9 hr period. However, when a boiled preparation of undialysed S144 fraction (Section 4.2.3) was added to the dialysed S144 fraction there was a marked increase in oxygen consumption (Figure 22).

5.2.2.2 Linked to nitrate reduction

Nitrate was reduced to nitrite when the crude extract (S10) was incubated with GSH and elemental sulphur. The activity was linear for the first 10 min of reaction (Figure 23). Sulphur oxidation coupled to nitrate reduction occurred only when GSH and active enzyme were present (Table 10). There was no activity associated with boiled enzyme. Enzyme activity was not affected by adding GSH first, then sulphur or *vice versa*. Mercaptoethanol or cysteine did not substitute for reduced glutathione in the reaction. Kinetic studies show that high concentrations of GSH or elemental sulphur inhibit enzyme activity (Figure 24).

The rate of nitrate reduction in either the S144 or P144 fractions was lower than that obtained with the crude extract (Table 9). However, when these fractions (S144 and P144) were recombined

Figure 22. Oxygen uptake during the oxidation of elemental sulphur by the S144 fraction.

Oxygen uptake was determined as described in Section 4.3.2. The reaction mixture was the same as in Table 9 except that the S144 fraction (1.6 mg protein), was used.

A : Boiled S144 fraction (dialysed or undialysed)

B : Dialysed S144 fraction

C : Undialysed S144 fraction

D : Dialysed S144 fraction with 0.1 ml of a heat-stable extract prepared from undialysed S144 fraction as described in Section 4.2.3.

Figure 22

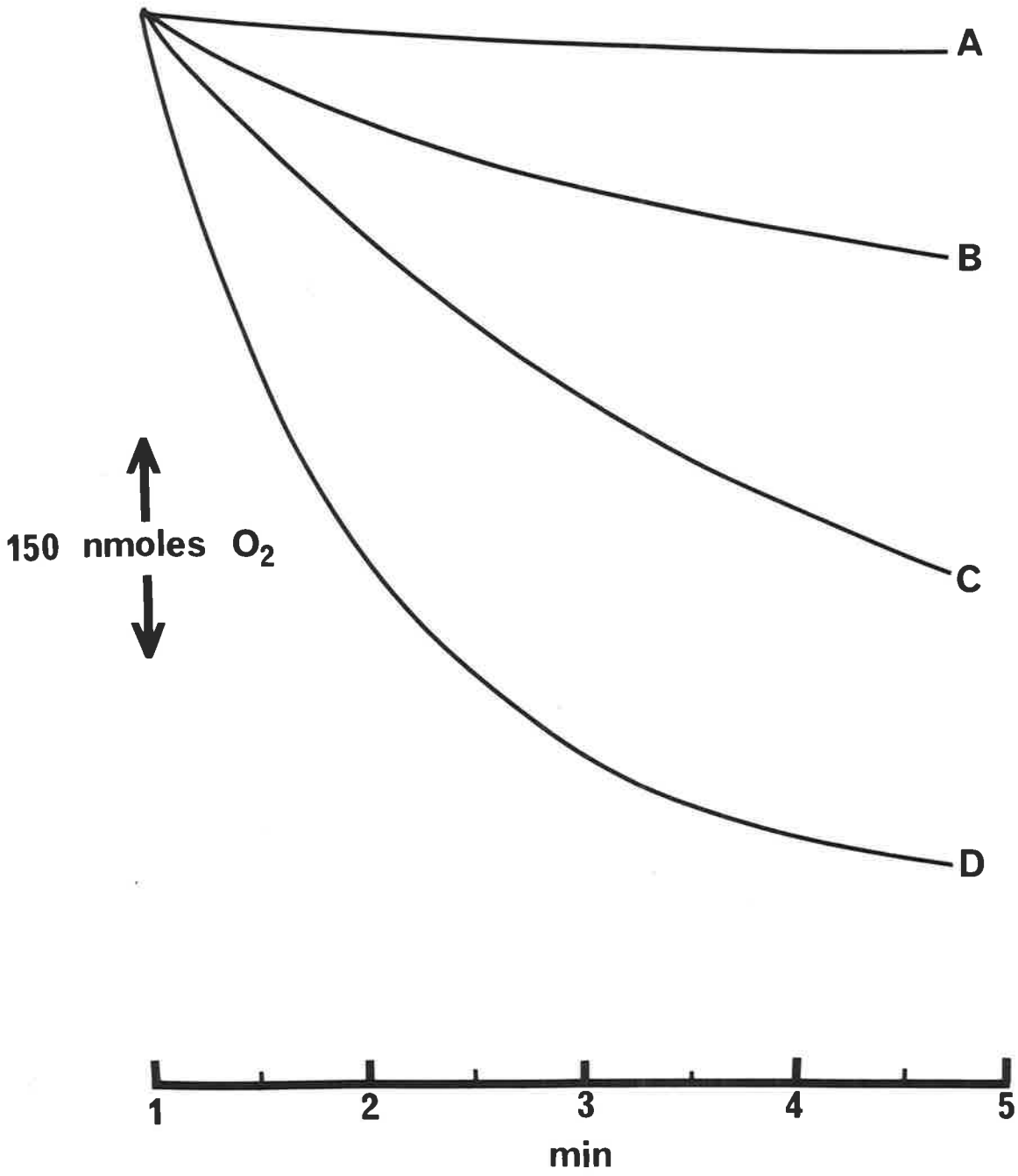


Figure 23. Effect of incubation times on the oxidation of elemental sulphur linked to nitrate reduction in the crude extract (S10).

The reaction mixture was the same as that described in Table 10 except that the reaction was carried out for times varying from 0 to 30 min.

Figure 23

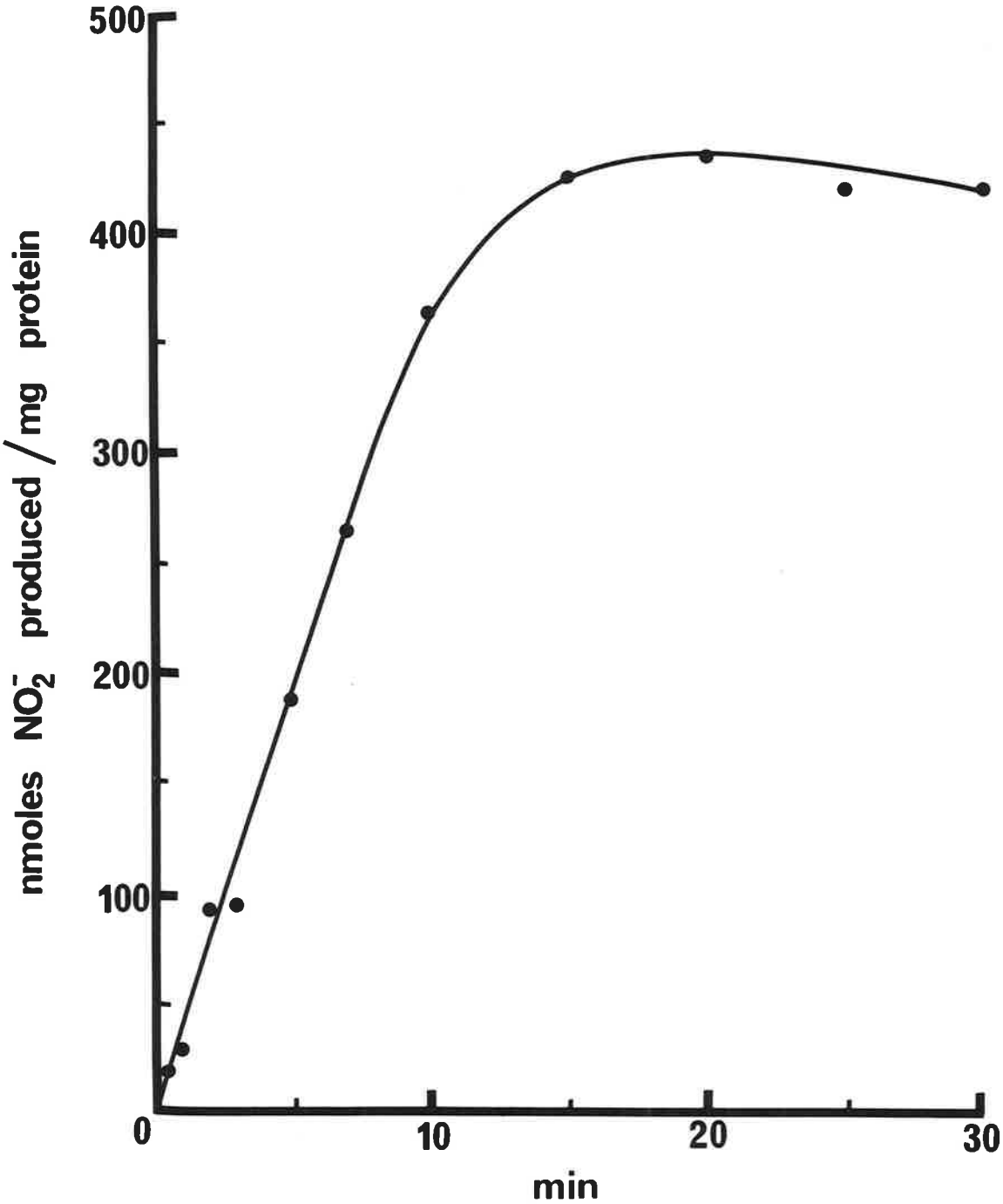

500
400
300
200
100
0
10
20
30
min
nmoles NO_2^- produced / mg protein

Table 10. Requirements for the oxidation of elemental sulphur linked to nitrate reduction in crude extracts (S10).

The complete reaction mixture in a final volume of 2 ml contained, phosphate buffer (pH 7.5), 80 µmoles; Na-EDTA, 0.3 µmoles; $NaNO_3$, 5 µmoles; GSH, 2 µmoles; elemental sulphur, 20 mg and crude extract (S10), 5 mg protein. The reaction was conducted as described in Section 4.2.5.1. In boiled preparations the crude extract was boiled in the buffer for 3 min prior to adding the other reactants.

Assay conditions	Specific activity
Complete	44
Boiled enzyme	0
GSH omitted	2
Sulphur omitted	4
Enzyme omitted	0
Sulphur and GSH omitted	2

Specific activity: nmoles nitrite produced/10 min/mg protein.

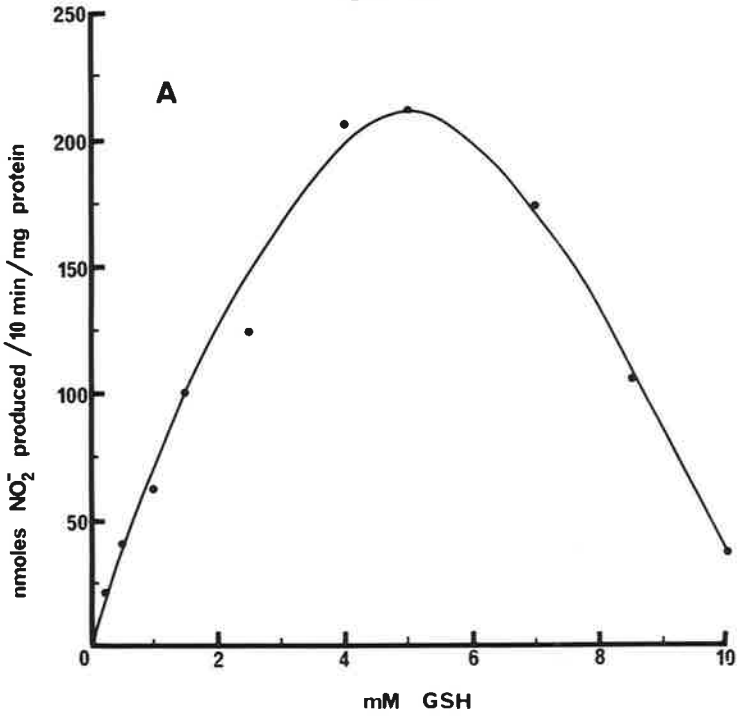
Figure 24. Effects of varying the concentrations of elemental sulphur and GSH on nitrate reduction in the crude extract (S10).

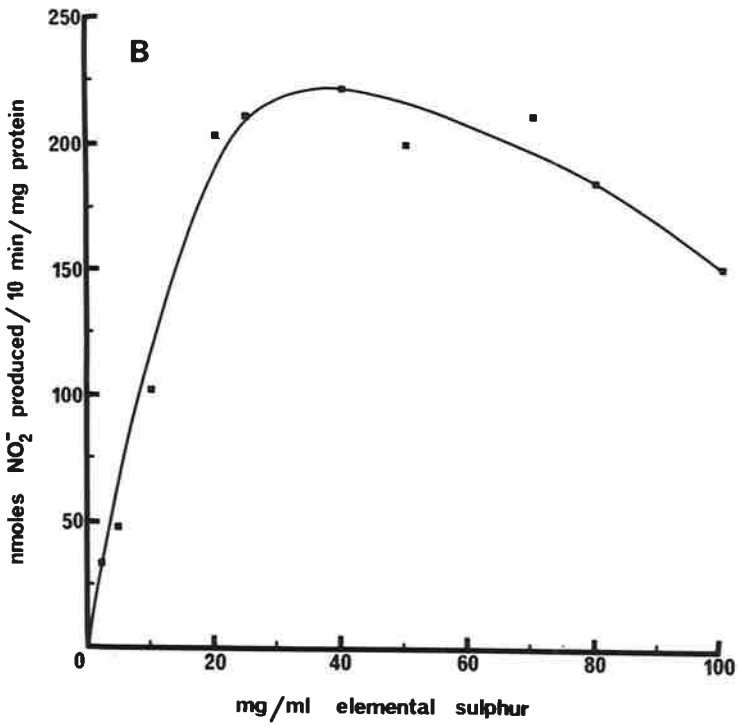
The reaction mixture was the same as that described in Table 10 except that the concentrations of elemental sulphur and GSH were adjusted to a level specified in the figures.

A : Effect of GSH concentration at a fixed level of elemental sulphur (10 mg/ml)

B : Effect of elemental sulphur; GSH fixed at 2.5 mM

Figure 24


A
250
200
150
100
50
0
2
4
6
8
10
nmoles NO_2^- produced /10 min/mg protein
mM GSH


B
250
200
150
100
50
0
20
40
60
80
100
nmoles NO_2^- produced/ 10 min/mg protein
mg/ml elemental sulphur

the activity increased sharply (Table 9). When the concentration of undialysed S144 fraction was fixed as shown in Figure 25, the sulphur-linked nitrate reductase activity was dependent on the amount of the P144 fraction added to it. There was no difference in activity when either dialysed or undialysed P144 fraction was used. However, when the S144 fraction was varied, while P144 was maintained at a fixed level, the rate of nitrate reduction was markedly influenced on adding the S144 fraction. The undialysed S144 fraction was found to be more effective than the dialysed fraction (Figure 26). However, the rate was much higher when the dialysed S144 fraction was used in the presence of a heat-stable extract (Section 4.2.3) prepared from undialysed S144 fraction (Figure 26). These results confirm those for O_2 uptake that the oxidation of elemental sulphur is catalysed by a soluble enzyme which requires a heat-stable factor in addition to GSH.

5.2.2.3 Product of sulphur oxidation

Sulphite was formed when the S144 fraction was incubated with GSH and elemental sulphur (Table 11). In order to demonstrate that sulphite was initially produced, the reaction mixture was incubated in the presence of formaldehyde which serves to trap the sulphite formed. After stopping the reaction, the formaldehyde-bisulphite complex was dissociated by treatment with 1M NaOH and the free sulphite separated by high voltage electrophoresis

Figure 25. Effect of varying P144 fraction (at a fixed amount of the S144 fraction) on the oxidation of elemental sulphur linked to nitrate reduction.

The reaction mixture contained, phosphate buffer (pH 7.5), 80 µmoles; GSH, 5 µmoles; $NaNO_3$, 5 µmoles; elemental sulphur, 40 mg; S144 fraction (3.6 mg protein) and P144 fraction (ranging from 0 to 3 mg protein). The reaction was conducted as described in Section 4.2.5.1.

o——o Untreated P144 fraction

●----● Boiled P144 fraction

Figure 25

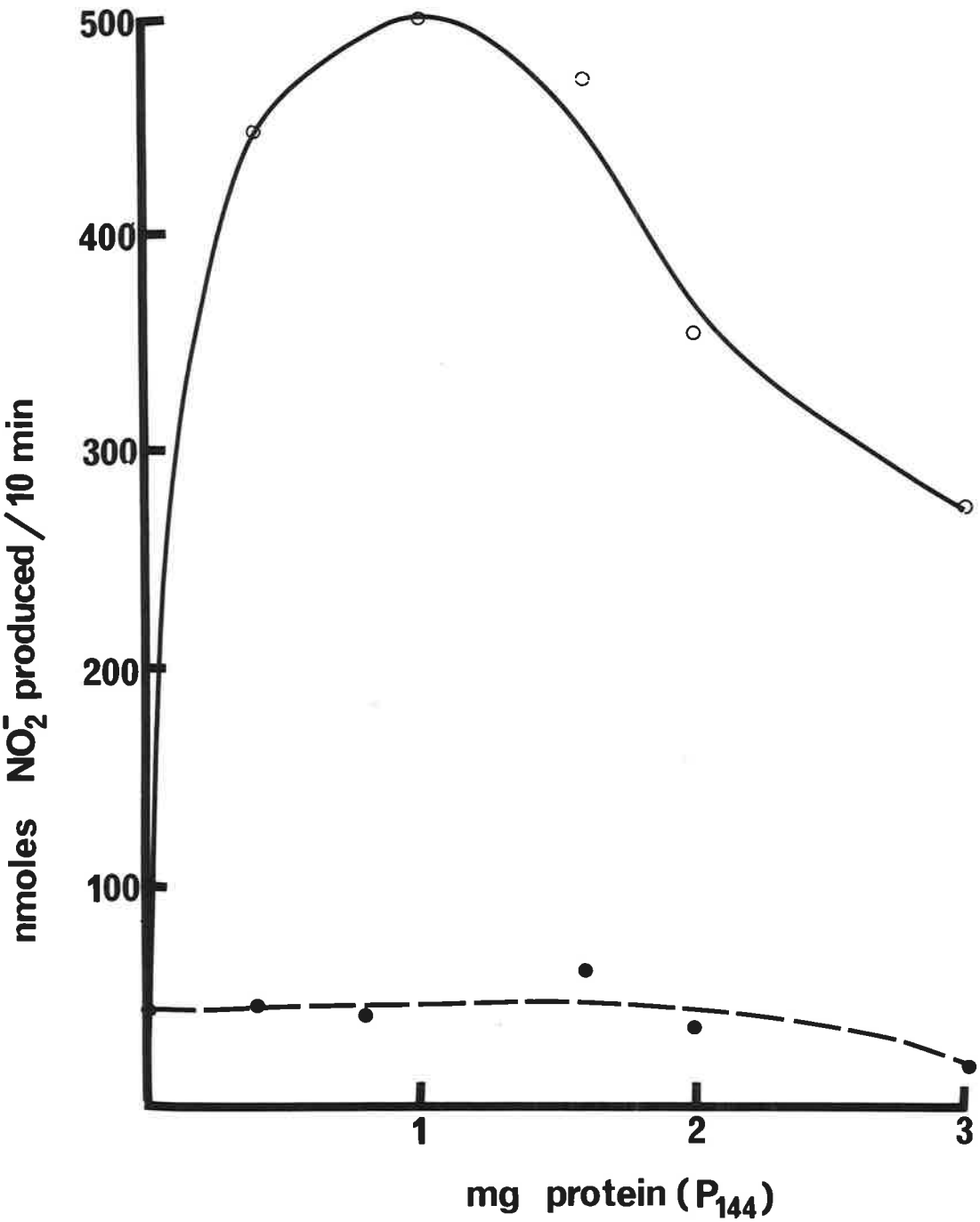

500
400
300
200
100
nmoles NO_2^- produced / 10 min
1
2
3
mg protein (P_{144})

Figure 26. Effect of varying S144 fraction (at a fixed amount of the P144 fraction) on the oxidation of elemental sulphur linked to nitrate reduction.

The reaction mixture was the same as that described for Figure 25 except that the P144 fraction was fixed at 2 mg protein and the S144 fraction varied as indicated.

A : Undialysed S144 fraction which had been boiled for 3 min

B : Dialysed S144 fraction

C : Undialysed S144 fraction

D : Dialysed S144 fraction plus 0.5 ml of a heat-stable extract prepared from the undialysed S144 fraction as described in Section 4.2.3.

Figure 26

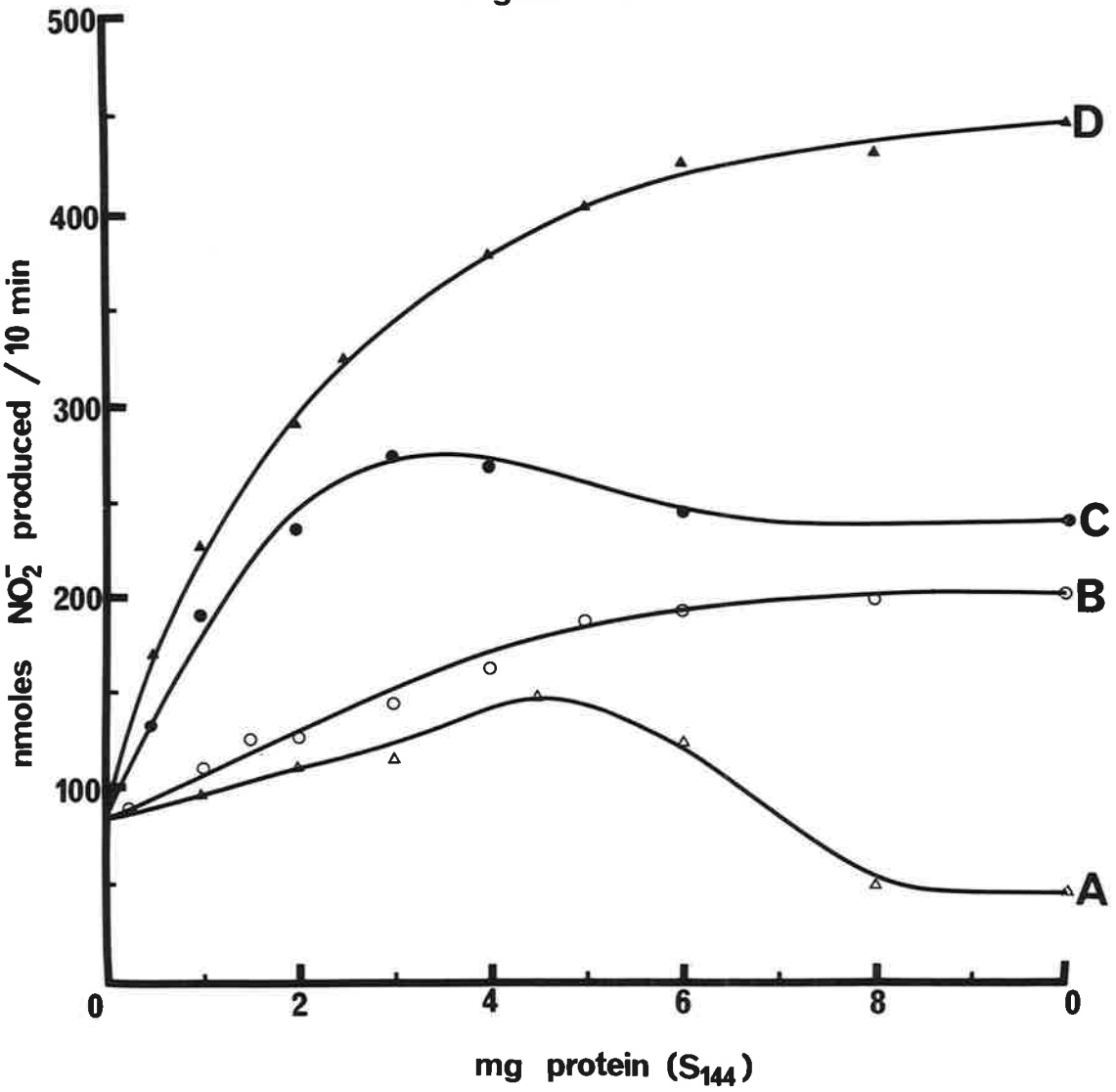

500
400
300
200
100
nmoles NO_2^- produced / 10 min
D
C
B
A
0
2
4
6
8
0
mg protein (S_{144})

Table 11. Comparison of the electrophoretic mobility of the product of sulphur oxidation with that of authentic sulphite.

I : The reaction mixture contained undialysed S144 fraction (5 mg protein), 5 µmoles GSH, 50 mg elemental sulphur and 0.1 ml of 40% (v/v) formaldehyde in a total volume of 1 ml made up with 50 mM phosphate buffer (pH 7.5). The reaction, started by adding elemental sulphur, was incubated for 10 min at 30°. The reaction was stopped with 0.5 ml of 10% (w/v) zinc acetate. After centrifuging at 3,000 x *g* for 5 min, 0.1 ml 1 M NaOH was added to the supernatant fraction and the mixture left at room temperature for 30 min to dissociate the formaldehyde-bisulphite complex. An aliquot of the mixture was then spotted on to Whatman 3 MM chromatographic paper and subjected to high voltage electrophoresis as described in Section 4.3.4. Sulphite was detected on the dried chromatogram as described in Section 4.3.4.1.

II : Na_2SO_3 standard.

III : Na_2SO_3 standard mixed with a boiled extract of the S144 fraction prior to spotting on to the paper. The extract itself did not contain sulphite.

Mobility is expressed as the distance moved relative to ^{35}S-sulphate.

Reaction mixture	R_f
I	0.85
II	0.84
III	0.85

(Section 4.3.4.1). Sulphite was not detected when formaldehyde was omitted from the reaction mixture. Thiosulphate and some polythionates were also formed and these were identified as described in Section 4.3.4.1.

5.3 Sulphite Oxidation

5.3.1 Breakage of cells

The French Pressure Cell technique was selected after a comparison of several methods had shown that it yielded the maximum amounts of both the sulphite oxidizing enzymes, namely APS-reductase and sulphite-linked nitrate reductase (Table 12).

5.3.2 Sulphite oxidation in cell extracts

The crude extract (S10) oxidized sulphite as measured by the reduction of either nitrate or ferricyanide, or by oxygen uptake (Table 13). In the pellet fraction (P144) enzyme activity assayed by all the three methods was not significantly affected by AMP. In the supernatant fraction (S144), however, enzyme activity, measured by reduction of ferricyanide, increased on adding AMP. Sulphite oxidation measured in the absence of AMP by all three methods is much greater in the P144 than in the S144 fraction, suggesting that an AMP-independent enzyme system is associated with

Table 12. Comparison of three procedures for the extraction of APS-reductase and sulphite-linked nitrate reductase.

Cells suspended (20%, w/v) in 50 mM Tris-HCl buffer (pH 7.5) containing 0.2 mM Na-EDTA were disrupted (a) in a glass homogenizer, (b) by means of an ultrasonic probe and (c) in a French Pressure Cell as described in Section 4.2.1.1. The homogenates thus prepared were centrifuged at 10,000 x *g* for 30 min and enzyme activities determined in the supernatant fraction (S10). The assay procedure for APS-reductase was as described in Section 4.2.5.5, while sulphite-linked nitrate reductase was determined by following nitrite production (Section 4.2.5.1). Protein was measured by the method of Lowry *et al.* (1951) (Section 4.4.1).

Procedure	Protein concentration (mg/ml)	Specific activity	
		APS-reductase	Nitrate reductase
(a) Glass homogenizer	4.7	16	93
(b) Ultrasonic probe	13.6	107	236
(c) French Pressure Cell	18.0	285	270

Specific activities:

APS-reductase: µmoles ferricyanide reduced/hr/mg protein.

Nitrate reductase: nmoles nitrite produced/10 min/mg protein.

Table 13. Distribution of sulphite oxidizing enzyme in various cell fractions.

Cell-free extracts (S10, S144 and P144 fractions) were prepared in 50 mM phosphate buffer (pH 7.0) containing 0.2 mM Na-EDTA as described in Section 4.2.1. Enzyme activity was measured by (a) oxygen uptake (Section 4.2.5.4), (b) the reduction of ferricyanide (Section 4.2.5.4), and (c) the reduction of nitrate (Section 4.2.5.1). In each case an extract containing 4 mg protein was used. AMP (final concentration, 2.5 mM) was mixed with the extract and buffer prior to adding the other reactants; sulphite was added last. Controls included incubations with boiled preparations of the corresponding cell-free extract.

Table 13. Distribution of sulphite oxidizing enzymes in various cell fractions

Assay method	Specific activity (rate/hr/mg protein)		Cell-extract		
			S10	S144	P144
Oxygen uptake	nmoles O_2 utilized	with AMP	755	162	990
		without AMP	900	162	975
Ferricyanide reduction	µmoles ferricyanide reduced	with AMP	6.3	85	12.2
		without AMP	8.7	4	13.5
Nitrate reduction	µmoles nitrite produced	with AMP	0.32	0.02	0.8
		without AMP	0.28	0.01	0.8

cell particles.

5.3.3 AMP-independent sulphite oxidation (sulphite oxidase)

5.3.3.1 Linked to oxygen uptake

In cell-free extracts there was a concomitant uptake of O_2 during sulphite oxidation. Thus, in the crude extract (S10) and in the P144 fraction there was a gradual utilization of oxygen reaching a maximum after 8 min. The S144 fraction, however, had only 15% of this activity. There was very little oxygen uptake on adding either sulphite or cell extract (S10 or P144) to the reaction mixture and the rate was dependent on the relative amounts of the reductant and the enzyme used. AMP did not affect oxygen uptake in any of the fractions.

Nitrate inhibited the uptake of oxygen during sulphite oxidation. The effect of other inhibitors on O_2 uptake is presented in Section 5.5.3.2. The inhibition by nitrate was found to be of the non-competitive type (Figure 27).

5.3.3.2 Linked to ferricyanide reduction

(a) Purification of enzyme

The ferricyanide assay procedure was used to monitor the purification of the enzyme. As a comparison enzyme activity, measured by nitrate reduction, was carried out at all stages

Figure 27. Effect of various concentrations of nitrate on oxygen uptake during sulphite oxidation in the P144 fraction.

Oxygen uptake was determined by the oxygen electrode as described in Section 4.3.2. The P144 fraction (3 mg protein) in 50 mM Tris-HCl buffer (pH 8.3) containing 0.2 mM Na-EDTA was incubated at 30^{o} with nitrate for 5 min prior to adding sulphite. For each level of nitrate used the concentration of sulphite was adjusted as specified in the Figure. The results are presented as double reciprocal plots.

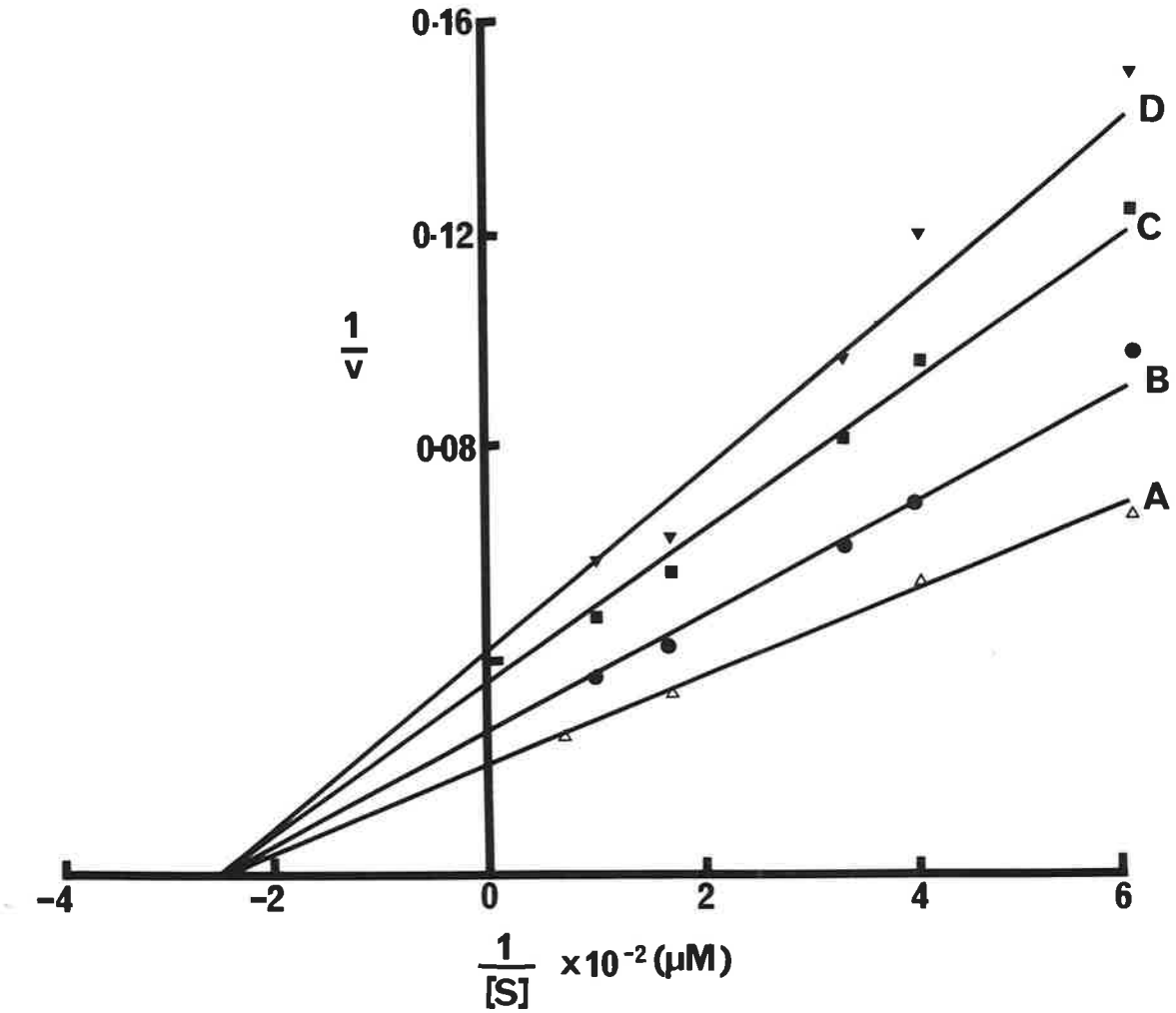
Concentrations of nitrate : A - 0

B - 0.1 mM

C - 1 mM

D - 5 mM

Figure 27



of purification. The buffer used for the entire purification procedure was 50 mM phosphate (pH 7.0) containing 0.2 mM Na-EDTA.

The P144 fraction, prepared as described in Section 4.2.1.3 was washed with buffer and resuspended with the aid of a glass homogenizer. It was then diluted with buffer until a protein concentration of 30 mg/ml was achieved. Sodium deoxycholate (0.2 mg/mg protein) was added to the suspension after the pH was adjusted to 8.0 with 0.1M NaOH. After incubating at 30^{o} for 30 min the extract was centrifuged at 105,000 X *g* for 30 min (Spinco preparative centrifuge rotor type 30) and the supernatant fraction (Fraction III) dialysed extensively against buffer to remove residual deoxycholate. The resultant solution was pink in colour.

A Sepharose-6B column (50 X 2 cm) prepared as described in Section 4.3.7.3 (void volume, 50 ml, determined by elution of blue dextran) was equilibrated with at least two column volumes of buffer. The solubilized pellet (Fraction III) was applied carefully to the top of the gel surface and the column then developed with the equilibrating buffer with an average flow rate of 20 ml/hr. Fractions (10 ml each) were collected in the cold using an LKB-Ultravac fraction collector. The enzyme was eluted in the void volume (Figure 28). The active fractions of the eluate were pooled and concentrated by ultrafiltration under N_2 as described in Section 4.3.8, thus obtaining Fraction IV. Purification of sulphite oxidase

Figure 28. Elution profile of sulphite oxidase from a Sepharose-6B column.

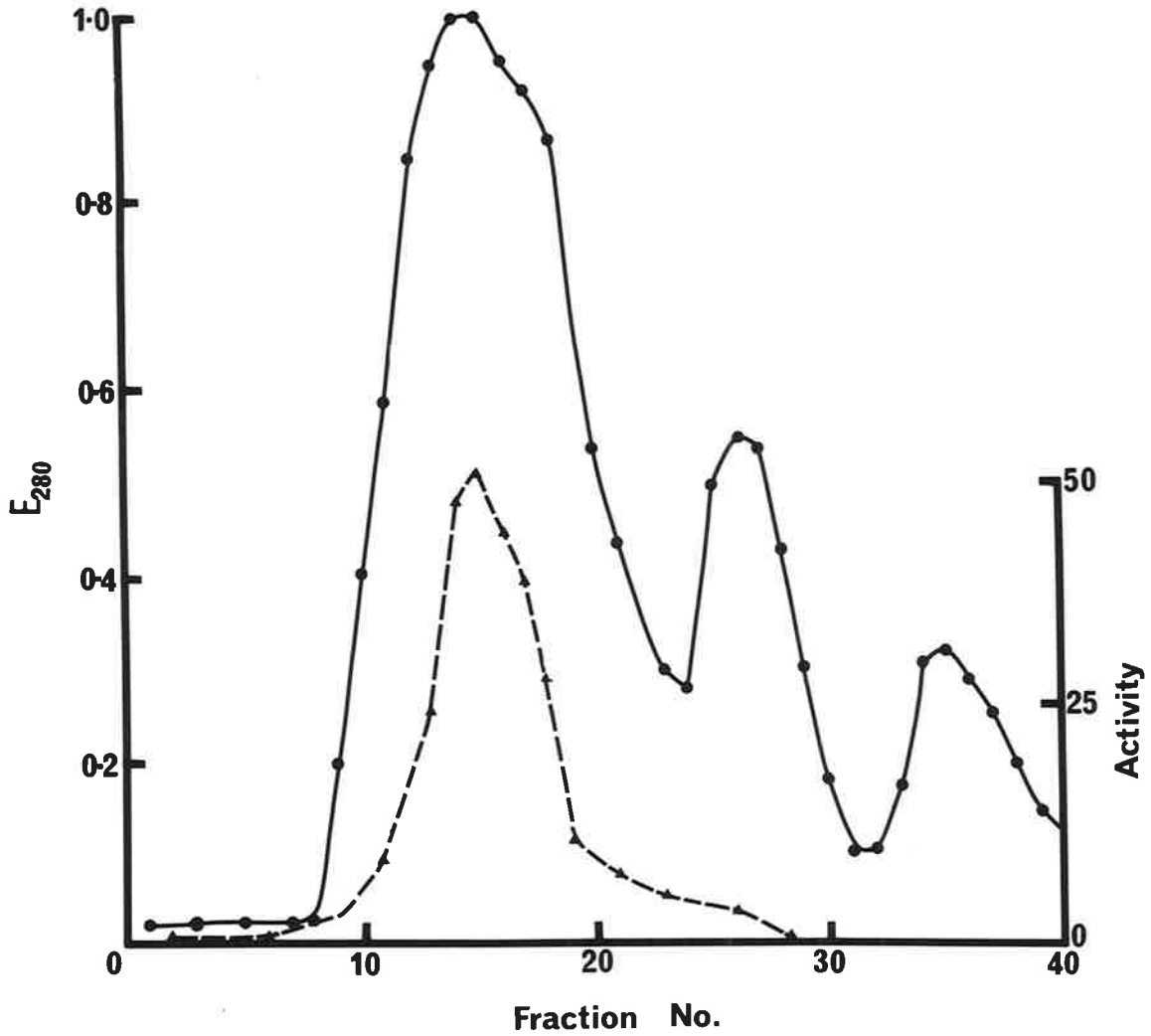
A Sepharose-6B column (50 x 2 cm) was prepared as described in Section 4.3.7.3. The column was equilibrated with 50 mM phosphate buffer (pH 7.0) containing 0.2 mM Na-EDTA. The P144 fraction, treated with deoxycholate (Fraction III, Table 14), was loaded on to the column and the enzyme was eluted with the same buffer. Effluent fractions (5 ml each) were collected and analysed for sulphite oxidase activity using the ferricyanide assay procedure described in Section 4.2.5.4

Protein was measured by extinction at 280 nm.

●——● E_{280}

▲- - - -▲ Sulphite oxidase activity (µmoles ferricyanide reduced/ml fraction/hr)

Figure 28



on the basis of molecular size resulted in approximately two-fold increase over the previous step with about 70% recovery of activity.

A DEAE-32 cellulose column (3.2 X 14 cm) was prepared as described in Section 4.3.7.1. The column was equilibrated with 50 mM phosphate buffer (pH 7.0) containing 0.2 mM Na-EDTA until the pH of the effluent was the same as that of the equilibrating buffer. The enzyme preparation from the previous step (Fraction IV) was then loaded on to the column and a linear phosphate gradient (0.05M to 0.3M, pH 7.0) used to develop the column. Fractions (5 ml each) were collected at 2^{o} using an automatic fraction collector (Paton Industries, Beaumont, South Australia) at a flow rate of 60-80 ml/hr. The enzyme activity appeared in the effluent when the salt concentration was around 0.11M (Figure 29). The active fractions were pooled and dialysed extensively against phosphate buffer. There was a 3-fold purification from the previous step with a recovery of approximately 60%.

The dialysed fraction V was concentrated by slowly adding ammonium sulphate until 35% saturation was achieved. The suspension was then centrifuged at 20,000 X g for 20 min and the pellet which contained about 5% of the total activity was discarded. The supernatant fraction was then taken up to 50%. The second

Figure 29. Elution profile of sulphite oxidase from a DEAE-cellulose column.

Fraction IV (Table 14) obtained by Sepharose-gel filtration chromatography was loaded on to a DEAE-32 cellulose column (14 x 3.2 cm) which had been first equilibrated with 50 mM phosphate buffer (pH 7.0) containing 0.2 mM Na-EDTA. The column was then eluted with a linear gradient, 0.05 M - 0.3 M phosphate buffer (pH 7.0). The total volume of the gradient solution was 300 ml and 5 ml fractions were collected in an automatic fraction collector.

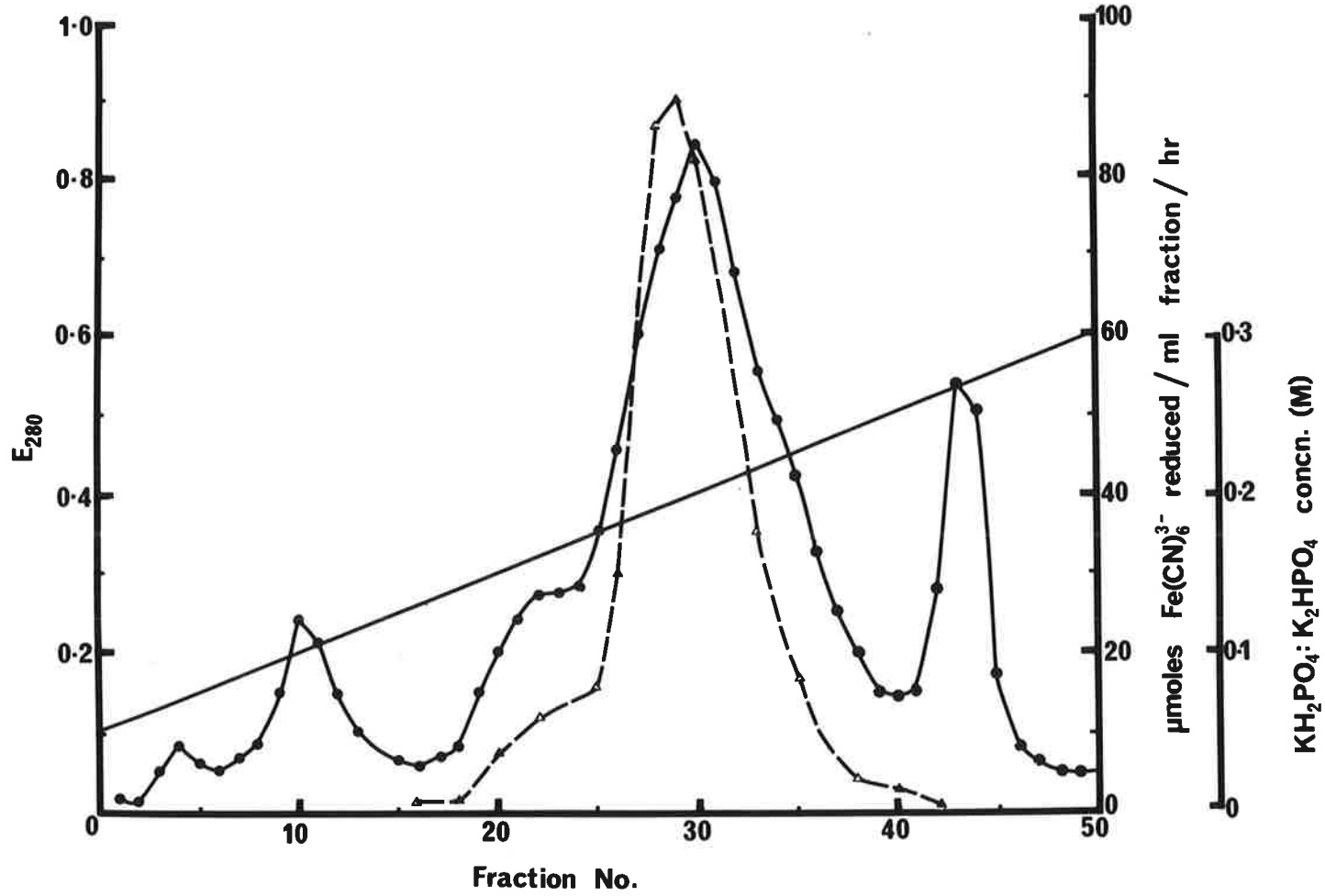
Sulphite oxidase activity was determined in 0.5 ml aliquots of the appropriate fractions as described in Section 4.2.5.4. The protein concentration was determined by abosrbance at 280 nm.

●———● E_{280}

Δ------Δ Sulphite oxidase activity

——— Concentration of eluting buffer

Figure 29


1·0
0·8
0·6
0·4
0·2
0
E_{280}
100
80
60
40
20
0
µmoles $Fe(CN)_6^{3-}$ reduced / ml fraction / hr
0·3
0·2
0·1
0
KH_2PO_4: K_2HPO_4 concn. (M)
10
20
30
40
50
Fraction No.

supernatant fraction was discarded since the 35-50% pellet now contained all the activity. The pellet was dissolved in the buffer and dialysed for 9 hr against 3 l of the same buffer.

A summary of the purification procedure is set out in Table 14 with a final overall purification of around 50-fold.

(b) Properties of the purified enzyme

Electron acceptors. The purified sulphite oxidase did not utilize nitrate or O_2 as an electron acceptor. During the purification process, however, fractions III (solubilized pellet) and IV (Sepharose-6B eluate) retained activity with nitrate as an electron acceptor, although the specific activity was lower than that of crude extracts (Table 15). The activity of the purified enzyme with either mammalian or yeast cytochrome *c* was only about 10% of that observed with ferricyanide.

Effect of enzyme and buffer concentrations. The relationship between activity and enzyme concentration was linear up to a protein concentration of 90 µg (Figure 30). AMP had no effect on the enzyme. Unlike the sulphite oxidases from *T. novellus* (Charles & Suzuki, 1966*B*) and *T. thioparus* (Lyric & Suzuki, 1970*A*) and in agreement with the enzyme from *T. neapolitanus* (Hempfling, Trudinger & Vishniac, 1967), the enzyme from *T. denitrificans* was not inhibited by Tris-HCl or phosphate buffers

Table 14. Purification of sulphite oxidase.

Enzyme activity was determined by the reduction of ferricyanide as described in Section 4.2.5.4. Washed cells were suspended in 50 mM phosphate buffer (pH 7.0) containing 0.2 mM Na-EDTA and disrupted in a French Pressure Cell (Section 4.2.1.1). The crude extract (S10), obtained by centrifuging the homogenate at 10,000 x *g* for 30 min was used as the starting material for the fractionation of the enzyme. For details of fractionation procedures see Section 5.3.3.2.(a).

Total activity: μmoles ferricyanide reduced/hr

Specific activity: μmoles ferricyanide reduced/hr/mg protein

Table 14. Purification of sulphite oxidase.

Fraction	Procedure	Total activity	Specific activity	Recovery (%)	Fold Purification
I	Crude extract (S10), supernatant fraction left after centrifuging cell homogenate (prepared in French Pressure Cell) at 10,000 x g for 30 min.	18,230	3.8	100	1
II	Particulate fraction left after centrifuging I at 144,000 x g for 90 min.	16,760	6.5	92	1.7
III	Fraction II treated with deoxycholate, centrifuged at 105,000 x g for 60 min and the supernatant fraction obtained dialysed against 50 mM phosphate buffer (pH 7.0).	11,570	19.3	64	5.7
IV	Fraction III eluted from a Sepharose-6B column and concentrated by ultrafiltration	9,190	40	50	10.6
V	Fraction IV eluted from a DEAE-cellulose column with a linear gradient of 0.05 - 0.3 M phosphate buffer (pH 7.0).	530	88.2	29	23
VI	Fraction V precipitated with $(NH_4)_2SO_4$ between 35-50% saturation	200	183	11	48

Table 15. Activity of sulphite-linked nitrate reductase at various stages of purification of sulphite oxidase.

Sulphite-linked nitrate reductase activity was determined by following the production of nitrite as described in Section 4.2.5.1. Fractions I - VI are the same fractions described in Table 14. For details of the purification procedure, see Section 5.3.3.2.(a).

Fraction	Purification stage	Specific activity
I	Crude extract (S10)	305
II	P144 fraction	788
III	P144 fraction treated with deoxycholate	226
IV	Eluate from Sepharose-6B column	207
V	Eluate from DEAE-cellulose column	21
VI	Ammonium sulphate precipitate	10

Specific activity: nmoles nitrite produced/10 min/mg protein.

Figure 30. Effect of enzyme concentration on sulphite oxidase activity.

Enzyme activity was determined by the ferricyanide-linked assay as described in Section 4.2.5.4 using purified sulphite oxidase (Fraction VI, Table 14).

Figure 31. Effect of sulphite concentration on sulphite oxidase activity.

Fraction VI (Table 14) was used as the source of the enzyme. Activity was determined by following ferricyanide reduction in a reaction mixture described in Section 4.2.5.4 except that sulphite was adjusted to a final concentration as specified in the Figure. The results are presented as a Lineweaver-Burk plot and the Km for sulphite was found to be 0.5 mM.

Figure 30

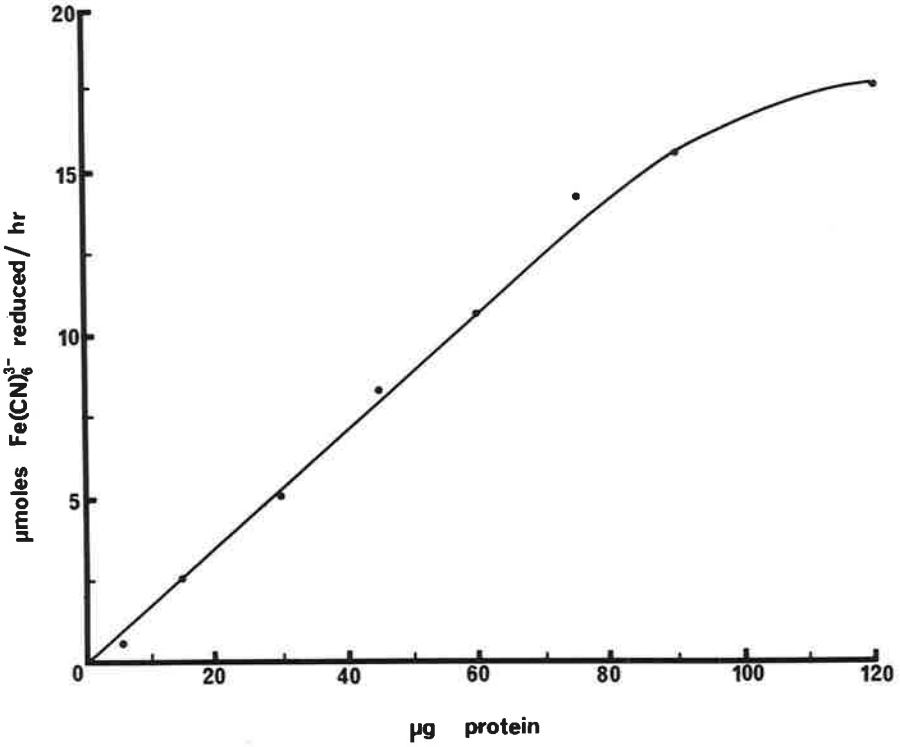
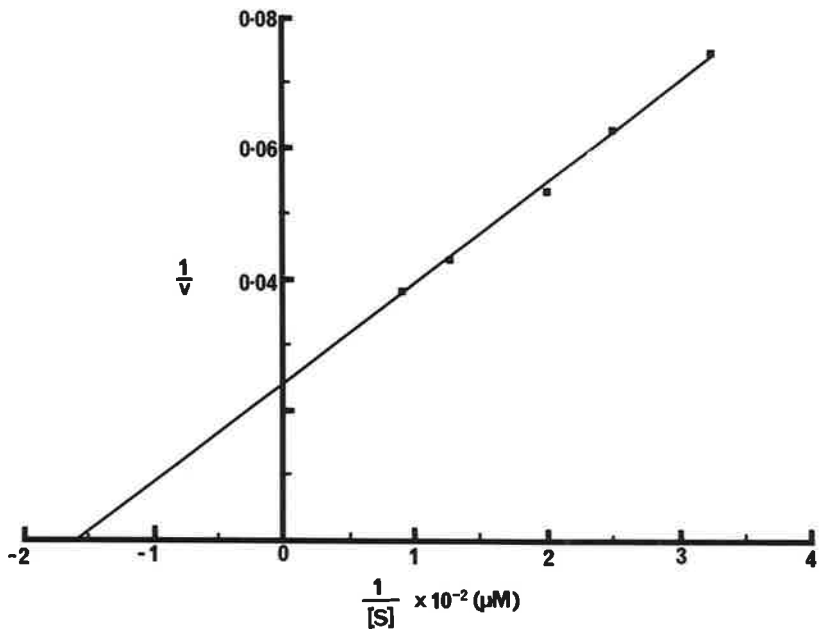


Figure 31



(pH 8.3) at concentrations of up to 75 mM.

Effect of substrate concentration. A plot of various concentrations of enzyme activity exhibits a typical substrate-concentration curve. The Km for sulphite was found to be 0.5 mM for the purified enzyme using the ferricyanide assay (Figure 31) as compared with 0.4 mM for the P144 fraction by the oxygen uptake method (Figure 27).

Effect of pH. The pH optimum of the enzyme reaction was around 8.3 (Figure 32). This was observed both with the purified enzyme using the ferricyanide assay and with the P144 fraction assayed by O_2 uptake. Adams, Warnes & Nicholas (1971) found that the optimal pH for sulphite oxidation linked to nitrate reduction was 8.5.

Stoichiometry. Between 1.7 to 2.3 moles of $Fe(CN)^{3-}$ were reduced for each mole of sulphite oxidized (Table 16). This shows good agreement with the theoretical value of 2:1.

Effect of thiol-binding reagents. As shown in Table 17 -SH binding reagents were potent inhibitors of the enzyme. This effect was completely reversed by dithiothreitol.

Figure 32. Effect of pH on sulphite oxidase activity.

50 mM Tris-HCl, phosphate and borate-phosphate buffers, each containing 0.2 mM Na-EDTA were prepared with pH values ranging from 5.8 to 9.5 according to the method of Gomori (1955).

Graph A : Oxygen uptake. This was determined as described in Section 4.3.2 using the P144 fraction (3.5 mg protein)

Graph B : Ferricyanide reduction. This was measured as described in Section 4.2.5.4 using the purified enzyme (Fraction VI, Table 14).

●——● Phosphate buffer

▲——▲ Borate-phosphate buffer

o-----o Tris-HCl buffer

Figure 32

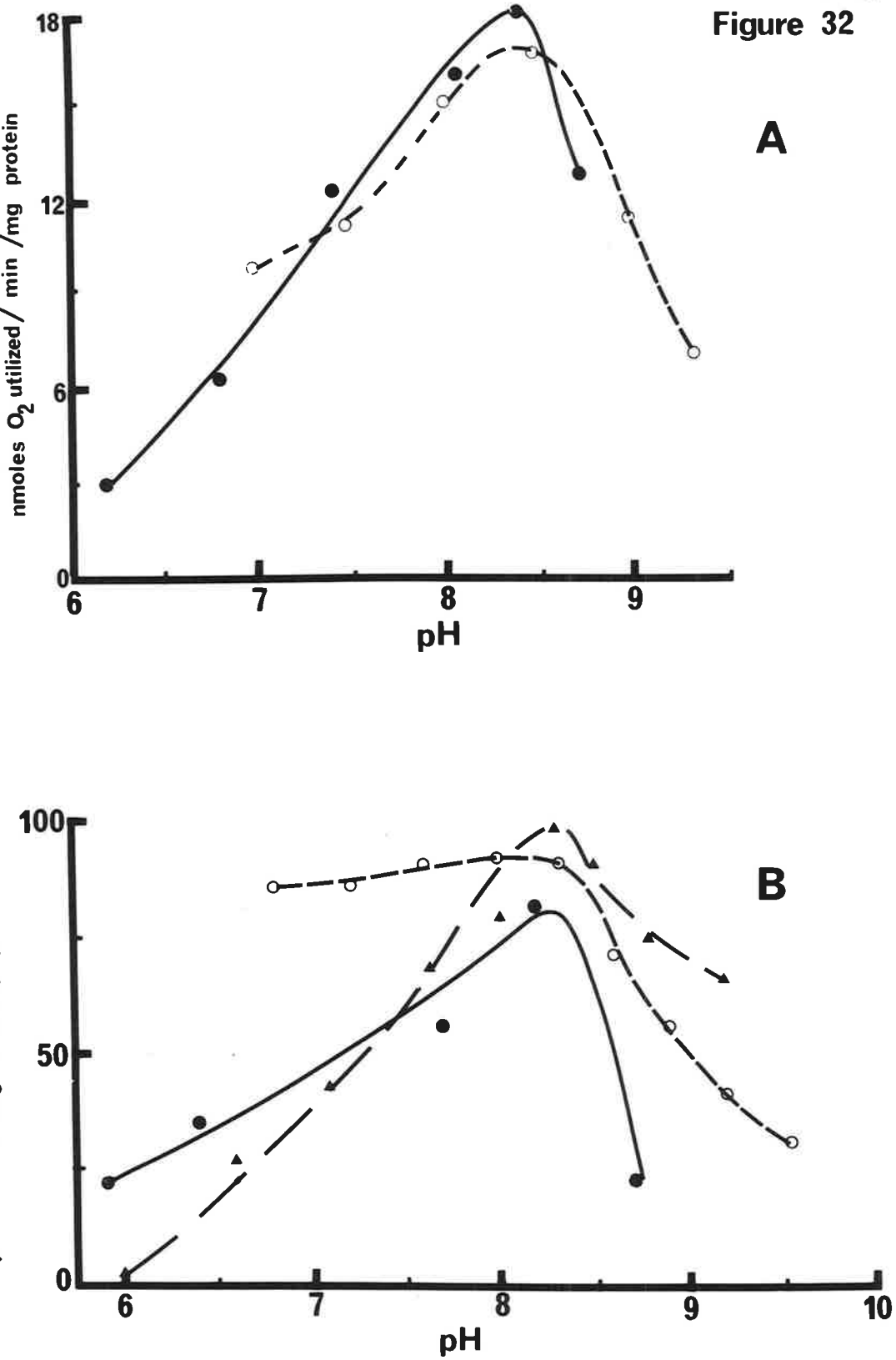

A
nmoles O_2 utilized / min /mg protein
18
12
6
0
6
7
8
9
pH
B
µmoles $Fe(CN)_6^{3-}$ reduced/hr/mg protein
100
50
0
6
7
8
9
10
pH

Table 16. Stoichiometry of sulphite oxidation and ferricyanide reduction by purified sulphite oxidase.

The reaction mixture consisted of 5 µmoles each of Na_2SO_3 and potassium ferricyanide with 0.1 ml of purified sulphite oxidase (Fraction VI, Table 14) in a total volume of 3 ml made up with 50 mM Tris-HCl buffer (pH 8.3), containing 0.2 mM Na-EDTA. The reaction, carried out at 30^{o}, was initiated by adding sulphite. At the specified times shown, 20 µl aliquots were withdrawn from the reaction mixture for the determination of sulphite as described in Section 4.4.3. The concentration of ferricyanide was calculated from a standard curve of absorbance at 420 nm *versus* graded amounts of ferricyanide.

Time (min)	µmoles ferricyanide reduced	µmoles sulphite oxidized	Ferricyanide : Sulphite
1	0.35	0.15	2.3
3	0.89	0.42	2.1
5	1.22	0.60	2.0
7	1.40	0.85	1.7

Table 17. Effect of sulphydryl inhibitors on sulphite oxidase activity.

Purified sulphite oxidase (Fraction VI, Table 14) was incubated for 10 min with the inhibitor (final concentration as indicated) before initiating the reaction. Activity was measured by following the reduction of ferricyanide (Section 4.2.5.4). Where indicated dithiothreitol (final concentration 1 mM) was added 5 min after the reaction was started.

Enzyme activity is expressed as a percentage of the rate of reaction in a control incubation (without inhibitor) which was 127 μmoles ferricyanide reduced/hr/mg protein.

Compound added	Final concentration (mM)	% activity
N-ethylmaleimide	0.1	88
	1.0	66
pCMB	0.1	10
	1.0	0
Iodoacetamide	0.1	57
	1.0	25
pCMB plus dithiothreitol	1.0 (each)	115

Enzyme storage and heat stability. The purified enzyme (Fraction VI, Table 14) was relatively stable at -15°, maintaining full activity after at least 2 months. At 2° about 65% of the original activity was lost after a week. The P144 fraction was more stable than the purified enzyme and one such P144 preparation retained 80% of the initial activity after a month at 2°. Freezing and thawing had no effect on the purified enzyme but boiling for 1 min inactivated it completely.

At higher temperatures the enzyme was relatively unstable. About 95% of the activity was lost after a 5 min incubation at 75° and only 30% remained after 10 min at 60° (Figure 33).

5.3.4 AMP-dependent sulphite oxidation (APS-reductase)

5.3.4.1 Formation of APS

Crude extracts (S10) were incubated with ^{35}S-sulphite and AMP in the presence of various electron acceptors. The products of the reaction were separated by DEAE-cellulose column chromatography. The elution of nucleotides was followed at 260 nm while ^{35}S in the various fractions was measured in the liquid scintillation spectrometer (Section 4.3.6.2). APS in the eluates was further separated and identified by high voltage paper electrophoresis (Section 4.3.4.2).

Figure 33. Time course for the inactivation of sulphite oxidase by heating at 37°, 60° and 75°.

Three aliquots of purified sulphite oxidase (Fraction VI, Table 14) were heated at 37°, 60° and 75° respectively. Samples were periodically removed for determination of activity by the ferricyanide assay (Section 4.2.5.4).

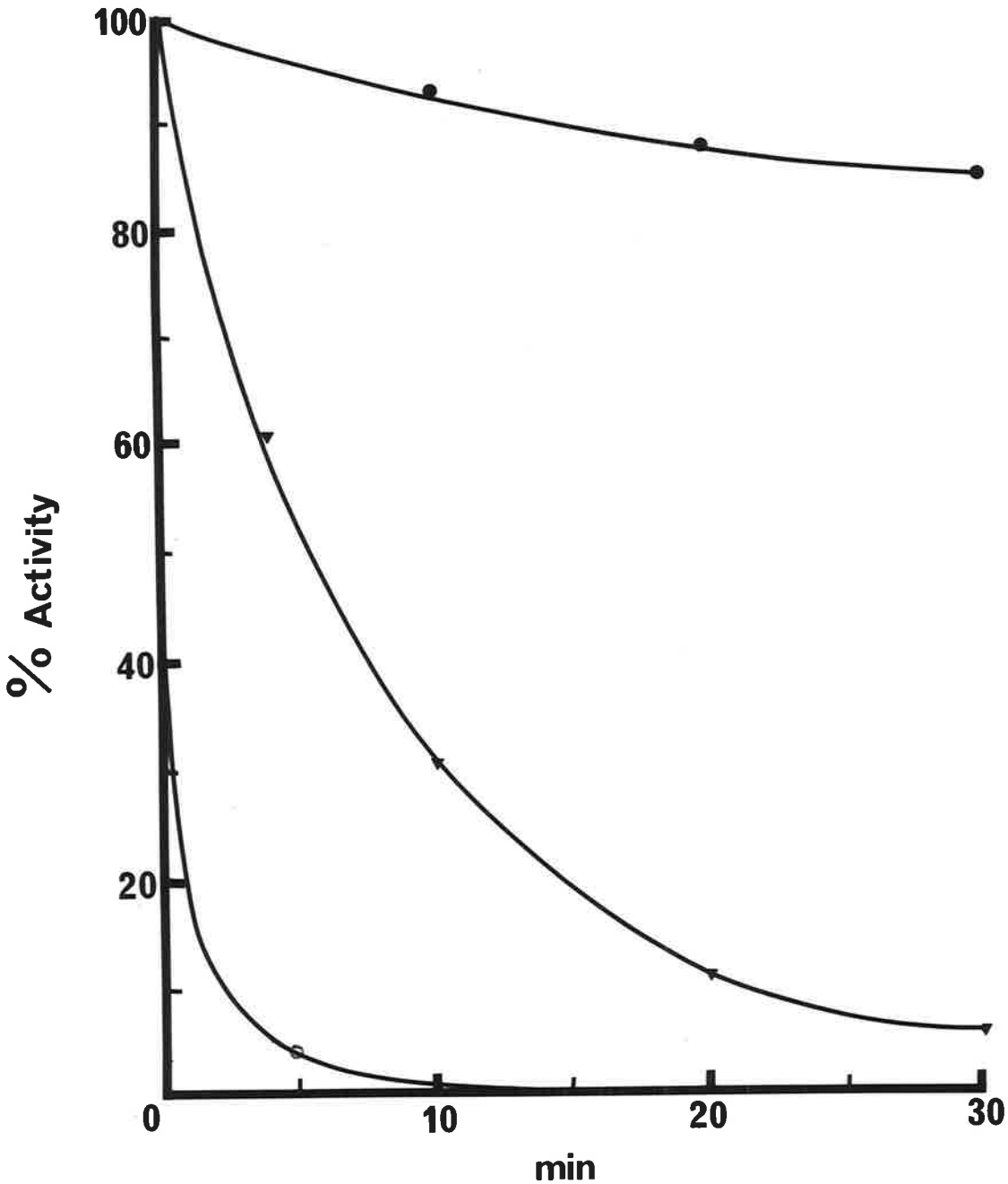
Enzyme activity is expressed as a percentage of the activity before heating, which was 64 μmoles ferricyanide reduced/hr.

●——● Heated at 37°

▼——▼ Heated at 60°

o——o Heated at 75°

Figure 33



In the presence of ferricyanide, sulphite and AMP readily formed APS in crude extracts (Figure 34A). When ferricyanide was replaced by other electron acceptors such as NO_2^-, O_2 or cytochrome *c* (mammalian or yeast) no APS was formed. However, nitrate in the presence of FMN under anaerobic conditions produced APS in the crude extract, although the yield was only about 10% of that obtained with ferricyanide (Figure 34C). Purified APS-reductase formed APS only with ferricyanide as an electron acceptor.

5.3.4.2 Purification of APS-reductase

Crude homogenates, prepared as described in Section 4.2.1.1 were centrifuged at 144,000 X *g* for 90 min and the supernatant fraction (S144) was retained (fraction II, Table 18). Solid ammonium sulphate was stirred slowly into this fraction at 4^o with the aid of a magnetic stirrer. The precipitate which formed between 35-60% saturation was collected, resuspended in 50 mM phosphate buffer (pH 7.0) containing 0.2 mM Na-EDTA, and dialysed for 12 hr against 2 l of the same buffer. The buffer was changed 3 times over this period. The dialysate (fraction III) was loaded on to a DEAE-cellulose column (DE-11; 2.5 X 20 cm), previously equilibrated with the same buffer. The column was developed with a linear phosphate gradient (0.05M to 0.25M, pH 7.0). APS-reductase was eluted when the salt concentration was around 0.2M

Figure 34. Separation of the products of APS-reductase by DEAE-cellulose chromatography.

A : The reaction mixture contained in a final volume of 0.32 ml, AMP, 5 µmoles; $Na_2{}^{35}SO_3$, 0.89 µCi (plus 5 µmoles carrier Na_2SO_3); potassium ferricyanide, 5 µmoles; Tris-HCl buffer (pH 7.5), 10 µmoles and 0.2 ml S10 (3.5 mg protein in 50 mM Tris-HCl buffer containing 0.2 mM Na-EDTA). The reaction was started by adding ferricyanide and terminated after 5 min by boiling in a water bath for a further 2 min. After centrifuging at 2,000 x *g* for 5 min 0.1 ml aliquot of the supernatant fraction was loaded on to a DEAE-11 cellulose column (8 x 1.7 cm) prepared as described in Section 4.3.7.2. The column was then developed by a linear gradient generated from 150 ml 0.2 M NH_4HCO_3 and 150 ml double-distilled water. Effluent fractions (4 ml each) were collected by an automatic fraction collector.

Radioactivity was measured in 0.1 ml aliquot of each fraction, the remainder of which was used to determine nucleotide concentration by measuring at 260 nm. The nucleotides eluted from the column were identified by high voltage electrophoresis as described in Section 4.3.4.2.

B : The reaction mixture was the same as that in (A) except that ferricyanide was omitted. The products of the reaction were separated and analysed as described in (A).

C : The reaction mixture was the same as in (A) except that 5 µmoles each of FMN and $NaNO_3$ were substituted for ferricyanide. The products were separated and analyzed as in (A).

●———● Absorbance at 260 nm

Δ------Δ Radioactivity (^{35}S)

Figure 34

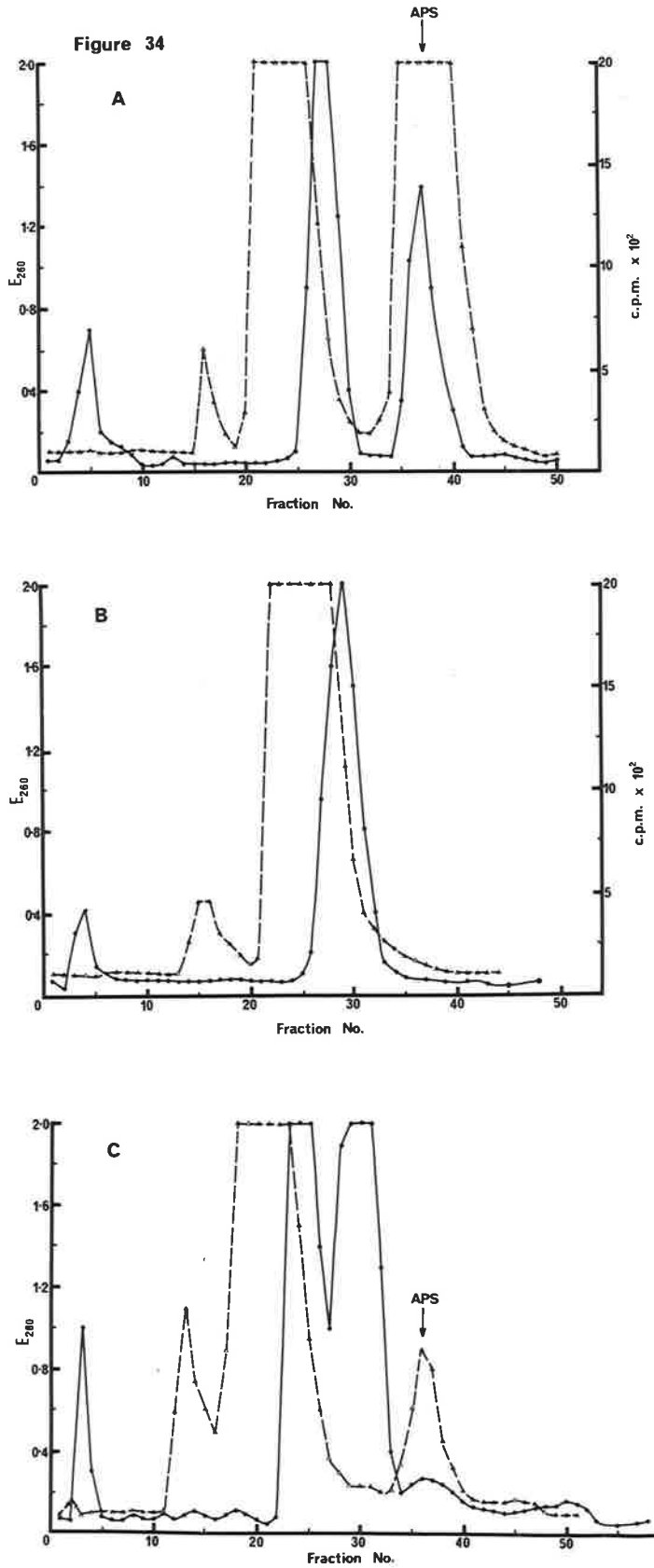

A
APS
E_{260}
c.p.m. x 10^2
Fraction No.
B
E_{260}
c.p.m. x 10^2
Fraction No.
C
APS
E_{280}
c.p.m. x 10^2
Fraction No.

Table 18. Purification of APS-reductase.

APS-reductase activity was determined as described in Section 4.2.5.5.

Washed cells, suspended with the aid of a glass homogenizer in 50 mM phosphate buffer (pH 7.0) containing 0.2 mM Na-EDTA were disrupted in a French Pressure Cell (Section 4.2.1.1); the homogenate thus obtained was used as the starting material for enzyme purification (Fraction I).

Table 18. Purification of APS-reductase.

Fraction	Procedure	Total activity	Specific activity	Recovery %	Fold purification
I	Cells disrupted in French Pressure Cell	9,894	26	100	1
II	S144 (supernatant fraction left after centrifuging at 144,000 x *g* for 90 min)	9,374	81	94	3.1
III	Fraction II precipitated with ammonium sulphate between 35% and 60% saturation and dialysed against 10 mM phosphate buffer (pH 7.5)	5,048	237	51	8.9
IV	Fraction III eluted from a DEAE-cellulose column with a linear gradient of 0.05 to 0.25 M phosphate buffer (pH 7.5) and concentrated by ultra-filtration	3,135	650	38	25.0
V	Fraction IV eluted from a Sephadex G-200 column with buffer equivalent to 1.5 - 2.0 times the void volume	2,688	960	27	36.3

Total activity: µmoles ferricyanide reduced/hr

Specific activity: µmoles ferricyanide reduced/hr/mg protein

(Figure 35). The eluted enzyme was yellow in colour. The DEAE-eluates containing enzyme were pooled and concentrated by membrane ultrafiltration (Section 4.3.8), thus giving fraction IV. This was then passed through a Sephadex G-200 column (Figure 36).

A final purification of about 35-fold was achieved (Table 18).

5.3.4.3 Properties of purified APS-reductase

Most of the studies on APS-reductase reported herein agree with the data of Bowen, Happold & Taylor (1966). Thus, the enzyme has an optimum pH of 7.5, is tightly bound to a flavin moiety and its activity is directly proportional to the enzyme concentration, over the range 0-90 µg protein.

APS-reductase (Fraction IV, Table 18) was strongly inhibited by thiol-binding reagents and this effect may be reversed partially by dithiothreitol. Inhibitors of electron transport have no effect on the enzyme except for mepacrine (Table 19). The Km for sulphite was 0.5 mM (Figure 37).

Unlike sulphite oxidase, APS-reductase was more heat-stable. Thus, at 60^{o} and 75^{o} only 5% and 10% of the initial activity, respectively, was lost after 10 min incubation (Figure 38).

5.3.5 Purity of sulphite oxidase and APS-reductase

A starch-gel electrophoretogram of the purified sulphite

Figure 35. Elution profile of APS-reductase from a DEAE-cellulose column.

Dialysed Fraction III (Table 18; Section 5.3.4.2) containing 22 mg protein was loaded on to a column of DEAE-cellulose (DE-11; 2.5 x 20 cm) which had been first equilibrated with 20 mM phosphate buffer (pH 7.5) containing 0.2 mM Na-EDTA. The enzyme was eluted with a linear gradient of phosphate buffer (pH 7.5) between 0.05 M and 0.25 M of the same buffer. The total volume of the gradient was 500 ml and 5 ml fractions were collected in an automatic fraction collector at 2^{o}. APS-reductase activity was determined in 0.5 ml aliquots of the appropriate fractions as described in Section 4.2.5.5. The protein concentration was determined by measuring at 280 nm.

●——● E_{280}

Δ------Δ APS-reductase activity

——— Concentration of phosphate buffer

Figure 35

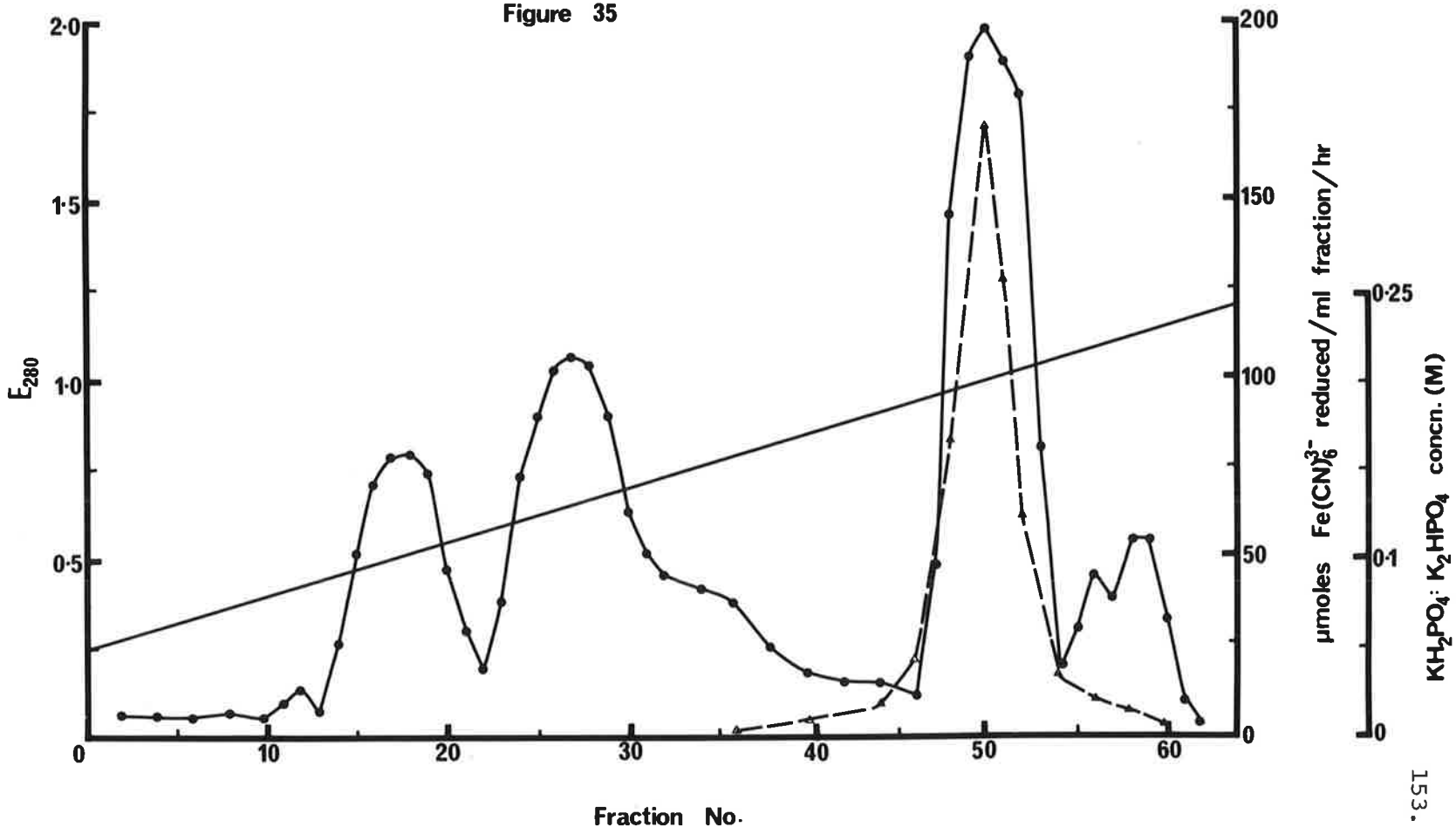

2·0
1·5
1·0
0·5
0
E_{280}
200
150
100
50
0
0·25
0·1
0
10
20
30
40
50
60
Fraction No.
µmoles $Fe(CN)_6^{3-}$ reduced/ml fraction/hr
KH_2PO_4: K_2HPO_4 concn. (M)

Figure 36. Elution profile of APS-reductase from a Sephadex G-200 column.

A Sephadex G-200 column (2.5 x 45 cm) was prepared as described in Section 4.3.7.3. The column was equilibrated with 50 mM phosphate buffer (pH 7.5) containing 0.2 mM Na-EDTA until the pH of the effluent was the same as that of the equilibrating buffer.

Fraction IV (Table 18) containing 5 mg protein was loaded on to the column and eluted with the same buffer. Effluent fractions (10 ml each) were collected and analysed for APS-reductase activity. Protein concentration was measured at 280 nm.

●———● E_{280} .

Δ------Δ APS-reductase activity

Figure 36

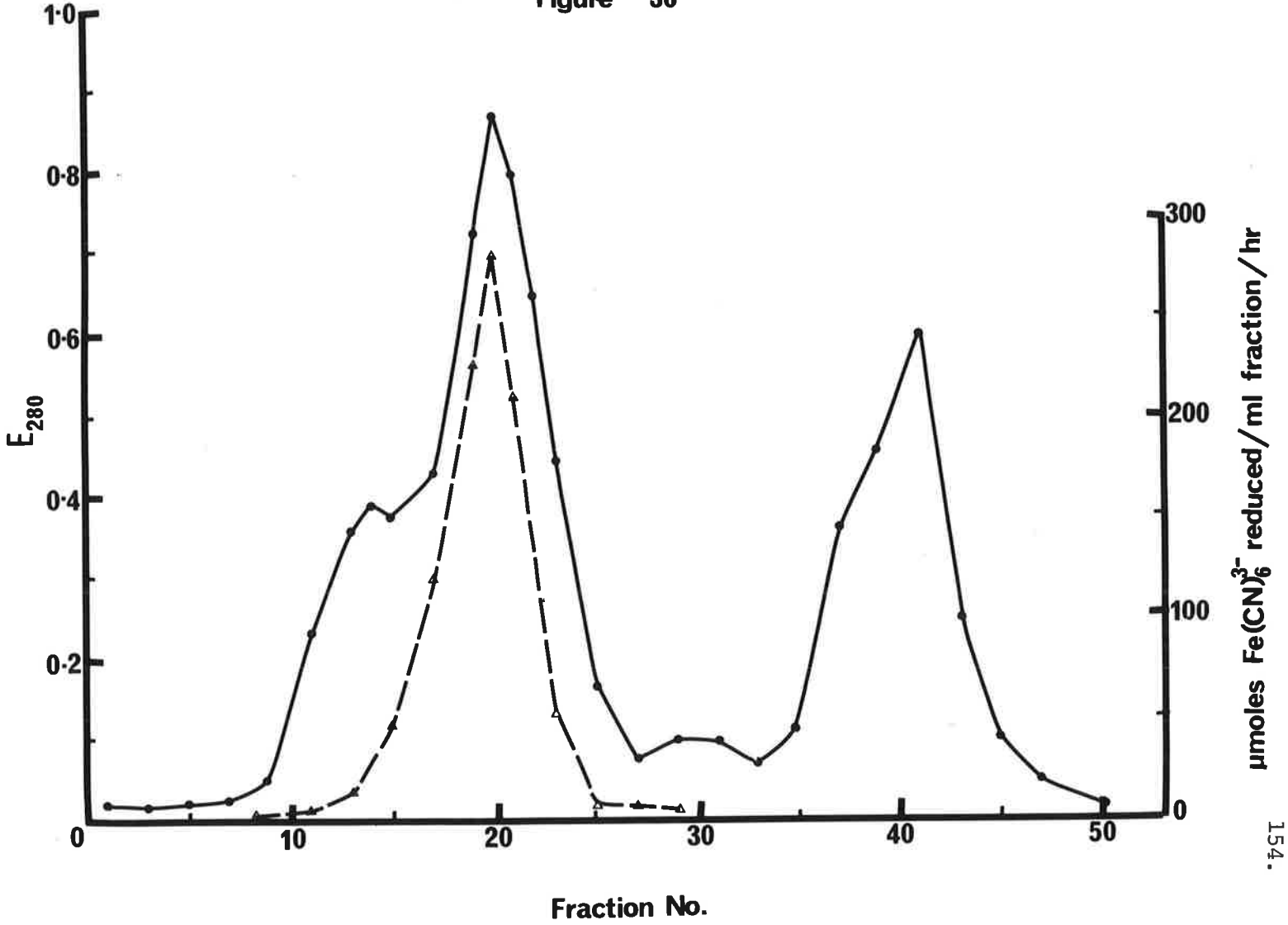

1·0
0·8
0·6
0·4
0·2
0
E_{280}
10
20
30
40
50
Fraction No.
300
200
100
0
µmoles $Fe(CN)_6^{3-}$ reduced/ml fraction/hr

Table 19. Effect of inhibitors on APS-reductase.

The reaction mixture was the same as that described in Section 4.2.5.5. The enzyme (Fraction IV, Table 18) was pre-incubated with the inhibitor (final concentration as shown) for 5 min before initiating the reaction. Where indicated, dithiothreitol (final concentration 1 mM) was added 5 min after the start of the reaction.

Enzyme activity is expressed as a percentage of inhibition of a normal reaction (without inhibitor) with a specific activity of 630 µmoles ferricyanide reduced/hr/mg protein.

Inhibitor	Final concentration (mM)	% inhibition
pCMB	1.0	90
	0.1	65
Iodoacetamide	1.0	87
	0.1	75
N-ethylmaleimide	1.0	45
	0.1	15
Iodoacetamide plus dithiothreitol		15
Arsenite	1.0	0
HOQNO	1.0	0
Mepacrine	1.0	21
Azide	1.0	6
Cyanide	1.0	0
DIECA	1.0	0
o-phenanthroline	1.0	0

Figure 37. Effect of sulphite concentration on APS-reductase activity.

APS-reductase (Fraction IV, Table 18) was determined by the method described in Section 4.2.5.5 except that sulphite was adjusted to a final concentration as shown in the figure.

The apparent Km was found to be 1 mM.

Figure 38. Time course for the inactivation of APS-reductase by heating at 60° and 75°.

Two aliquots of APS-reductase (Fraction IV, Table 18) were heated at 60° and 75° respectively. Samples were periodically removed to determine APS-reductase as described in Section 4.2.5.5

Enzyme activity is expressed as a percentage of the activity present before heating which was 590 μmoles ferricyanide reduced/hr/mg protein.

o———o Heated at 60°

Δ———Δ Heated at 75°

Figure 37

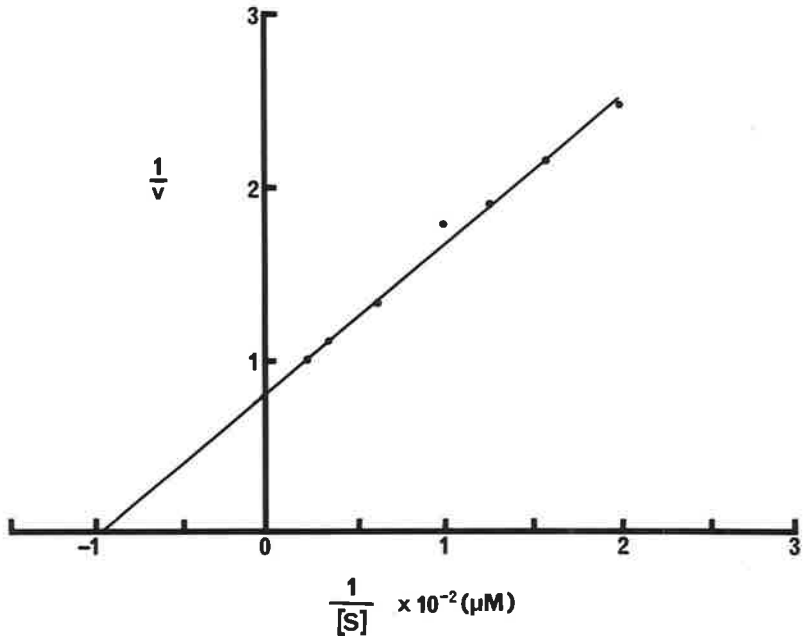
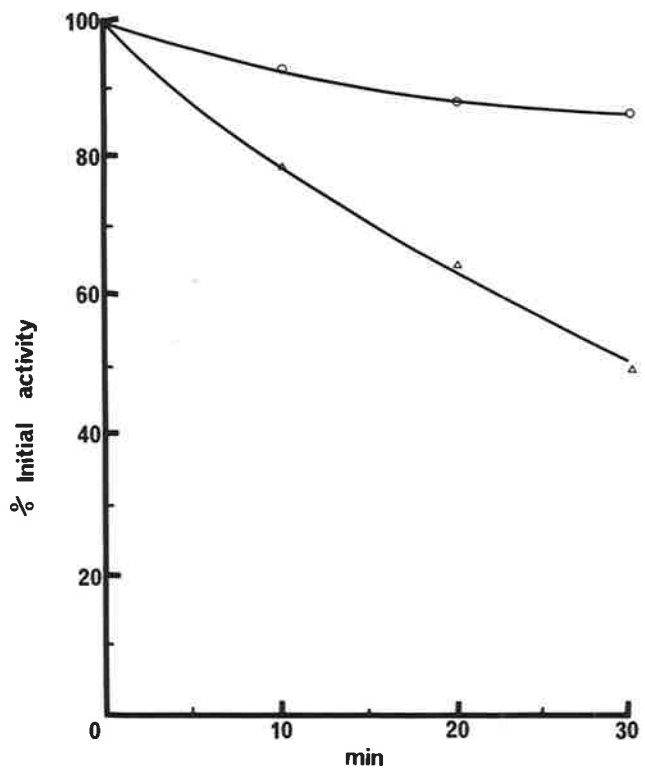


Figure 38



oxidase (Fraction VI, Table 14) indicates one major fast-moving protein band and two slow-moving minor bands, while with APS-reductase (Fraction V, Table 18) there was only one minor band, in addition to a major, slow-moving band (Figure 39).

5.3.6 Molecular weight determinations

The apparent molecular weights of APS-reductase and sulphite oxidase were determined by Sephadex G-200 gel-filtration as described in Section 4.2.7 . Assuming that both enzymes are approximately globular in shape, the estimated molecular weight was found to be 250,000 for APS-reductase and 94,000 for sulphite oxidase with a standard error of ± 5,000 (Figure 40).

5.4 Thiosulphate Oxidation

5.4.1 Linked to oxygen uptake

Thiosulphate stimulated the uptake of oxygen in cells of *T. denitrificans* suspended in 50 mM citrate buffer (pH 5.0). There was no oxygen uptake, however, in cell extracts on adding thiosulphate, but, in the presence of GSH and thiosulphate, the S10, S144 and P144 fractions rapidly utilized oxygen. GSH alone does not stimulate oxygen uptake. The rate of oxygen consumption was much higher in the P144 than in the S144 fraction (Table 20).

Figure 39. Starch-gel electrophoresis of sulphite oxidase and APS-reductase.

Aliquots of the fractions listed below were absorbed on to small 3 MM chromatography paper sections and inserted into the gel at the origin. Electrophoresis was carried out for 1.5 hr (30 V/cm) in 0.1 M Tris-citrate buffer (pH 8.1) as described in Section 4.3.3, after which the gel was sliced horizontally and stained with nigrosine : amido black to detect proteins as described in Section 4.3.3.

A : P144 fraction treated with deoxycholate (Fraction III, Table 14)

B : Purified sulphite oxidase (Fraction VI, Table 14)

C : S144 fraction (Fraction II, Table 18)

D : Purified APS-reductase (Fraction V, Table 18)

Enzyme activities were located in the major bands of B and D.

Figure 39

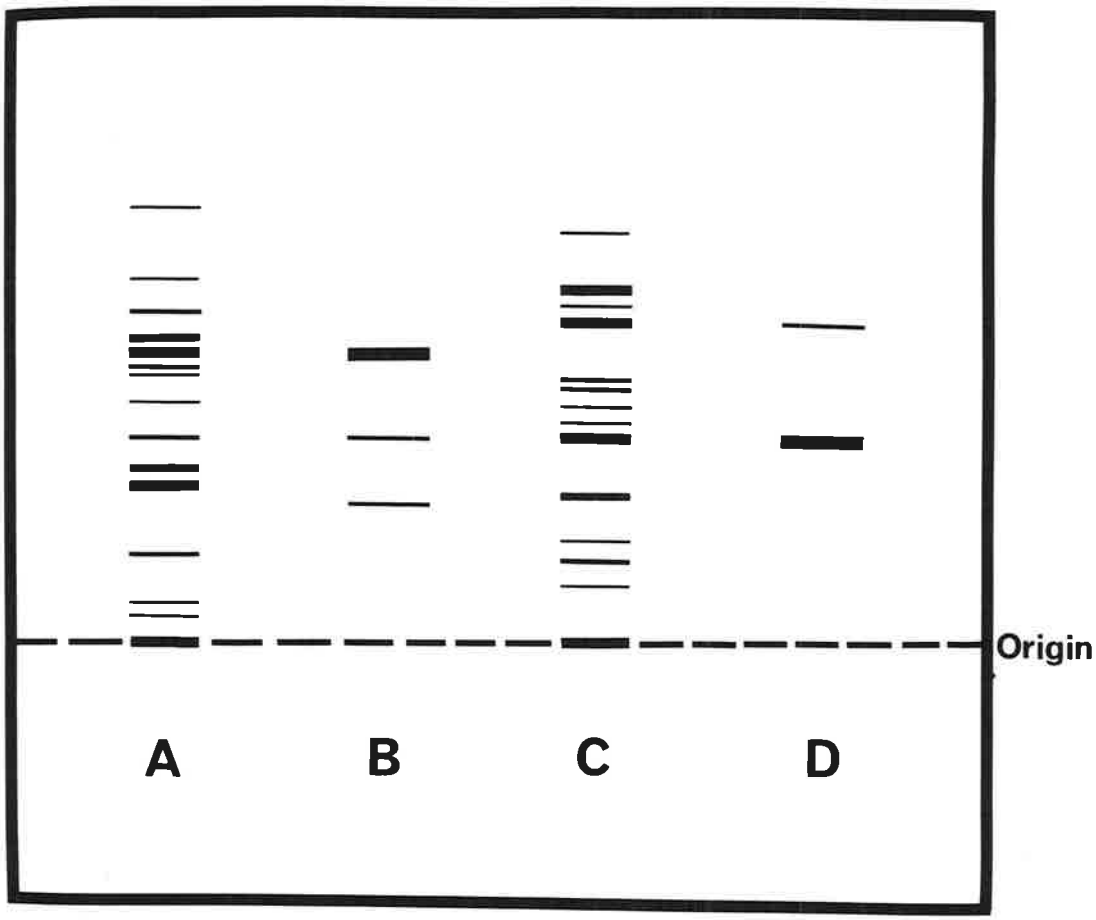


Figure 40. Determination of the molecular weights of ATP-sulphurylase, APS-reductase and sulphite oxidase.

Sephadex G-200 was calibrated as described in Section 4.2.7 using standard proteins of known molecular weights as well as blue dextran and ^{35}S-sulphate. The distribution coefficient, Kd, was calculated as shown in Section 4.2.7.

Marker protein or enzyme	M.W.	Kd	Symbols used
Cytochrome *c*	12,700	0.61	A
Bovine serum albumin	67,000	0.40	B
Lactate dehydrogenase	140,000	0.26	C
γ-globulin	160,000	0.26	D
Apoferritin	460,000	0.11	E
Sulphite oxidase (Fraction V, Table 14)		0.33	F
APS-reductase (Fraction IV, Table 14)		0.19	G
ATP-sulphurylase (Fraction IV, Table 38)		0.175	H

Figure 40

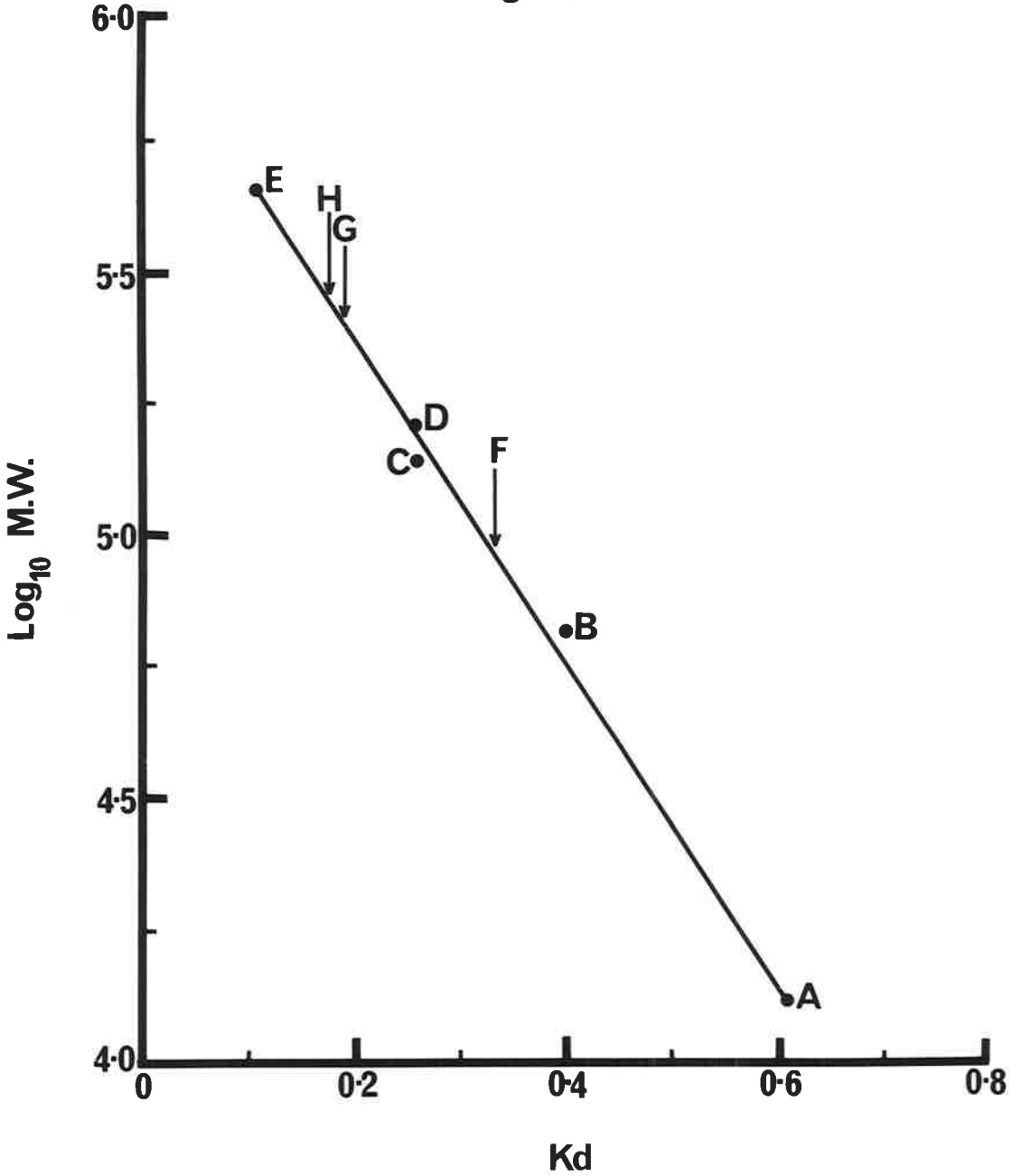


Table 20. Oxygen uptake during thiosulphate oxidation in various cell fractions.

Oxygen uptake was determined as described in Section 4.3.2. The reaction mixture contained in a total volume of 2.5 ml sodium citrate buffer (pH 5.0), 100 µmoles; $Na_2S_2O_3$, 5 µmoles; GSH, 5 µmoles and cell extract (S10, S144 or P144 fraction), 3 mg protein.

Extract	Conditions	Specific activity
S10	Complete	21
S10	Omit $S_2O_3^{=}$	0
S10	Omit GSH	0
S144	Complete	15
P144	Complete	24

Specific activity: nmoles oxygen utilized/min/mg protein.

5.4.2 Linked to nitrate reduction

Thiosulphate reduced nitrate to nitrite in crude extracts (S10). After centrifuging the crude extract at 144,000 X *g* for 90 min, the activity was detected mainly in the P144 fraction (Table 21). Enzyme activity *versus* incubation time shows that the reaction is linear for the first 10 min (Figure 41). The optimum pH for thiosulphate-linked nitrate reductase is 5.0 with little or no activity detected below pH 4.0 or above pH 7.0 (Figure 47, page 174).

5.4.3 Production of sulphite from thiosulphate

When crude extracts (S10) were incubated with thiosulphate in the presence of GSH, large amounts of sulphite were formed (Figure 42). The formation of sulphite was maximal at pH 5.0 (Figure 43) and was dependent on GSH, $S_2O_3^{2-}$ and enzyme (Table 22). GSH may be replaced by other -SH compounds; dithiothreitol was the most effective (Table 23).

When thiosulphate, GSH and nitrate were incubated with the P144 fraction at pH 5.0 the amounts of thiosulphate utilized, sulphite produced and nitrate reduced were in the ratio of 3:1:1 (Table 24).

5.4.4 Experiments with $^{35}S\text{-}SO_3^=$ and $S\text{-}^{35}SO_3^=$

When crude extracts (S10) were incubated with ^{35}S-

Table 21. Distribution of rhodanese, thiosulphate-linked nitrate reductase and the enzymic production of sulphite from thiosulphate.

Cell-free extracts (S10, S144 and P144 fractions) were prepared in 50 mM phosphate buffer (pH 7.0) containing 0.2 mM Na-EDTA as described in Section 4.2.1.

Rhodanese activity was determined as described in Section 4.2.5.8; thiosulphate-linked nitrate reductase as in Section 4.2.5.1 and the enzymic production of sulphite from thiosulphate as in Section 4.2.5.7.

Enzyme system	Specific activity (rate/10 min/mg protein)	Cell fraction		
		S10	S144	P144
Rhodanese	µmoles KCNS produced	6.7	0.63	5.0
Thiosulphate-linked nitrate reductase	nmoles NO_2^- produced	12.2	1.8	18.8
Production of sulphite from thiosulphate	nmoles $SO_3^=$ produced	35	11	41

Figure 41. Effect of incubation times on the oxidation of thiosulphate linked to nitrate reduction in the P144 fraction.

The reaction mixture was the same as that described in Section 4.2.5.1 except that the enzyme source was the P144 fraction (4.5 mg protein). The reaction was carried out for times varying from 0 to 35 min.

Figure 41

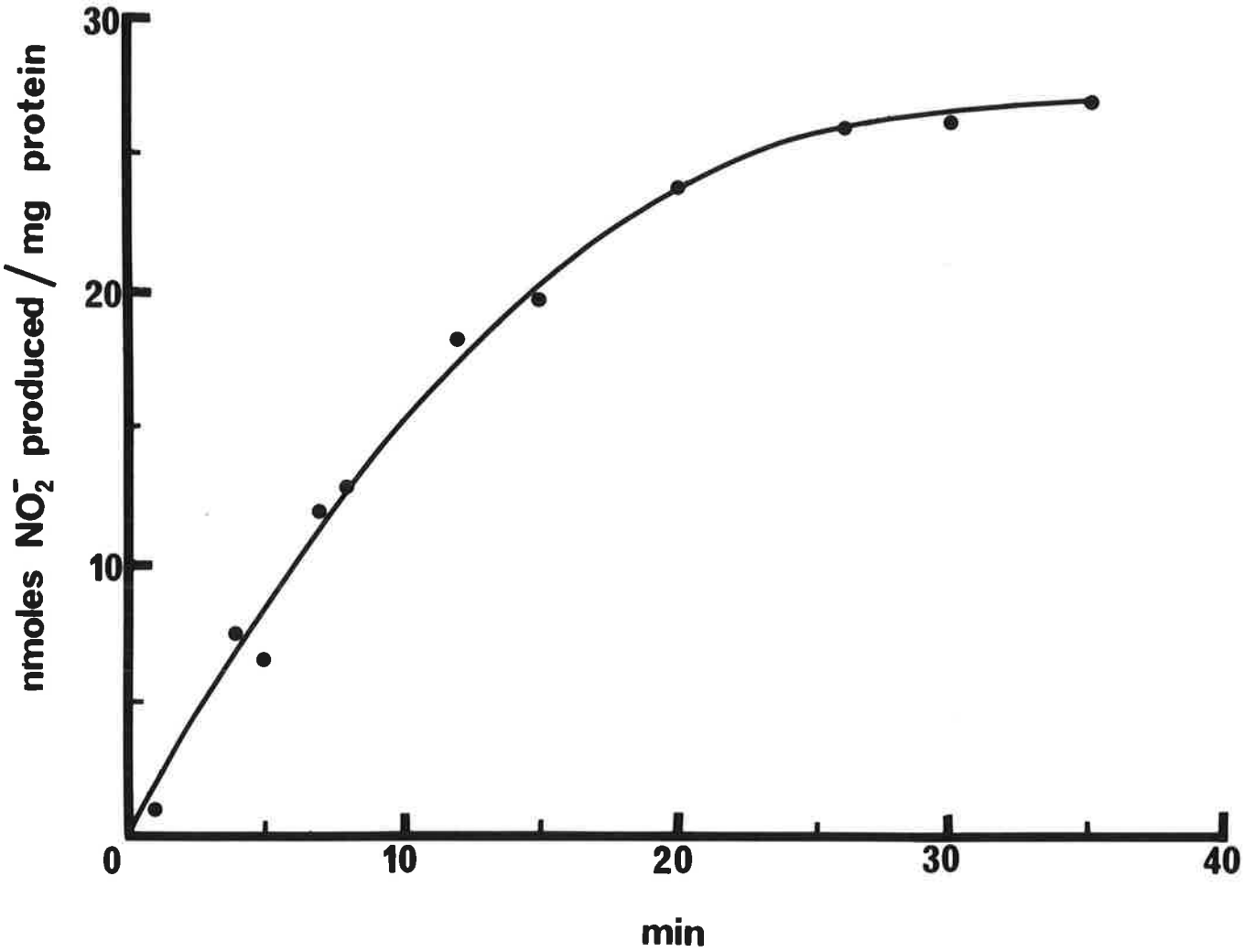


Figure 42. Effect of incubation times on the production of sulphite from thiosulphate.

Enzyme activity was measured by the production of sulphite in a reaction mixture described in Table 22 except that the reaction was carried out for times varying from 0 to 30 min. The enzyme source was the P144 fraction.

Figure 43. Effect of pH on the production of sulphite from thiosulphate in the P144 fraction.

50 mM citrate and citrate-phosphate buffers were prepared with pH values ranging from 3 to 7. Enzyme activity was measured by the production of sulphite as described in Section 4.2.5.7.(b), except that the buffers used were at a pH specified in the figure.

o———o Citrate buffer

Δ------Δ Citrate-phosphate buffer

Figure 42

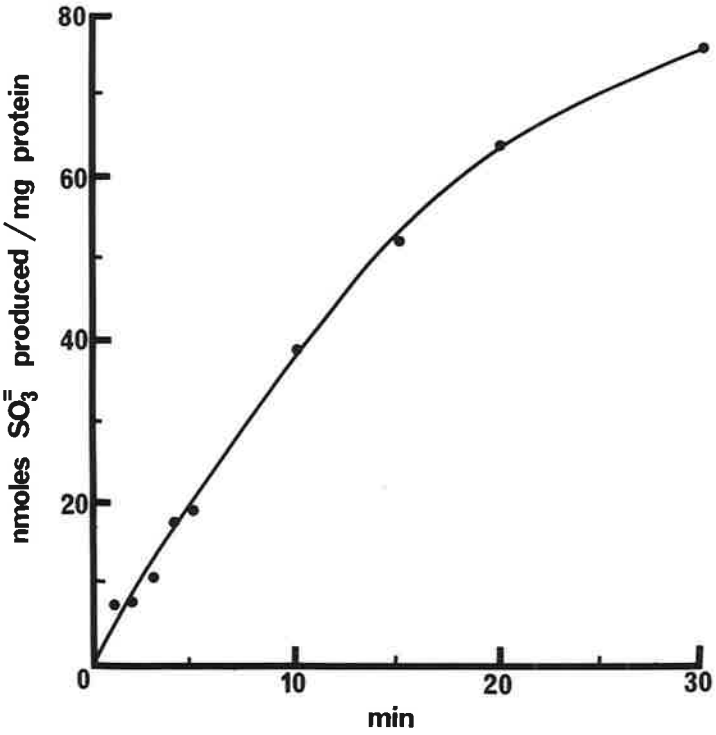


Figure 43

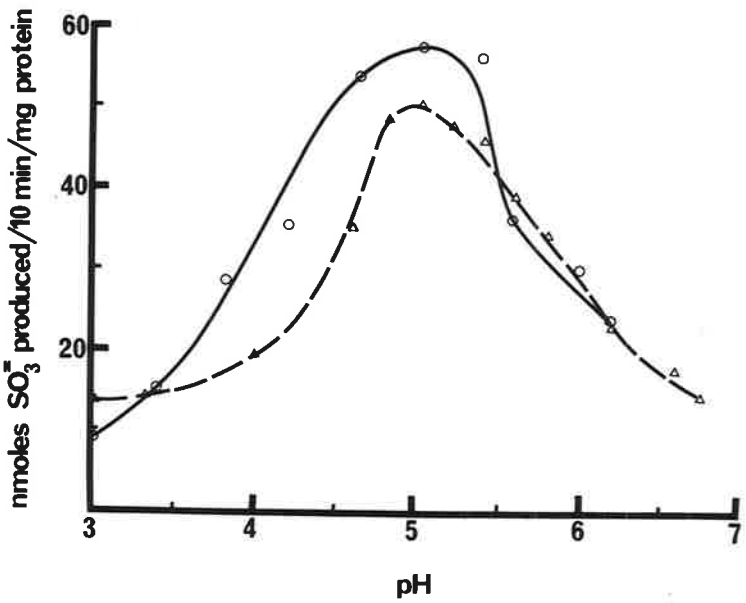


Table 22. Assay requirements for the production of sulphite from thiosulphate.

The reaction mixture contained sodium citrate buffer (pH 5.0), 85 µmoles; $Na_2S_2O_3$, 5 µmoles; GSH, 2 µmoles and the crude extract (S10), 4.2 mg protein in a total volume of 2 ml. The reaction was conducted in air for 10 min at 30^{o} as described in Section 4.2.5.7.(b). At the end of the reaction the mixture was centrifuged at 2,000 x *g* for 5 min and an aliquot of the supernatant fraction was taken for sulphite determination by the fuchsin method as described in Section 4.4.3.

Control samples were prepared by boiling the extract for 3 min with buffer, then cooling prior to adding the other reactants.

Conditions	Specific activity
Complete	47
Omit GSH	11
Omit $Na_2S_2O_3$	0
Omit $Na_2S_2O_3$ and GSH	0
Boiled enzyme	0

Specific activity: nmoles $SO_3^{=}$ produced/10 min/mg protein.

Table 23. Effect of -SH compounds on the production of sulphite from thiosulphate.

The reaction mixture in a total volume of 2 ml contained sodium citrate buffer (pH 5.0), 85 µmoles; $Na_2S_2O_3$, 4 µmoles and the P144 fraction (3.7 mg protein). The -SH compound (final concentration as specified) was incubated with the enzyme for 5 min prior to adding thiosulphate.

-SH compound	Concentration (mM)	Specific activity
-	-	9
GSH	0.5	38
Cysteine	0.5	72
Mercaptoethanol	0.5	39
BAL	0.5	94
Dithiothreitol	0.5	145

Specific activity: nmoles sulphite produced/10 min/mg protein.

Table 24. Stoichiometry of thiosulphate utilized and sulphite and nitrite produced.

The reaction mixture in a total volume of 2 ml contained $Na_2S_2O_3$, 5 µmoles; $NaNO_3$, 5 µmoles; GSH, 2 µmoles and the P144 fraction (5 mg protein). At intervals of 5, 15, 30 and 60 min, aliquots of the mixture were withdrawn and thiosulphate, sulphite and nitrite determined as described in Sections 4.4.5, 4.4.3 and 4.4.2, respectively.

Time (min)	Thiosulphate utilized (nmoles)	Sulphite produced (nmoles)	Nitrite formed (nmoles)	Ratio ($S_2O_3^{=}$: $SO_3^{=}$: NO_2^{-})
0	0	9	11	-
5	200	116	118	1.7 : 1 : 1
15	680	268	292	2.5 : 1 : 1
30	1,150	417	385	2.9 : 1 : 1
60	1,200	458	401	3 : 1 : 1

thiosulphate (outer sulphur atom labelled, $^{35}SSO_3^{=}$) in the presence of GSH and nitrate the radioactive products detected in the standard electrophoretic system (Section 4.3.4.1) were sulphide (trapped as the NEM-sulphide complex), polysulphide, tetrathionate and sulphate (Figure 44A). Under anaerobic conditions, in the presence of nitrate, the products were the same except that more sulphide and polysulphide were formed (Figure 44B). However, when crude extracts were incubated in air with ^{35}S-thiosulphate (inner sulphur atom labelled, $S^{35}SO_3^{=}$) and GSH the main product was sulphite (Figure 45A). On replacing GSH with nitrate the products were tetrathionate and sulphate (Figure 45B).

5.5 Electron Transport

5.5.1 Terminal acceptors

5.5.1.1 Nitrate

Sulphide, sulphite, NADH and thiosulphate reduced nitrate to nitrite in cell suspensions (Figure 46). Adams, Warnes and Nicholas (1971) have shown by mass spectrometry that nitrogenous gases were produced when cells were incubated with nitrate and inorganic sulphur compounds.

In crude extracts the reduction of nitrate may be linked to the oxidation of sulphide, sulphite, thiosulphate, elemental sulphur,

Figure 44. Products of thiosulphate (^{35}S-$SO_3^=$) oxidation by crude extracts (S10).

The reaction mixture in (A) consisted of 0.3 µCi ^{35}S-thiosulphate (outer sulphur atom labelled) with 5 µmoles $Na_2S_2O_3$ carrier, 2 µmoles $NaNO_3$, 5 µmoles GSH and the crude extract (2.2 mg protein) in a total volume of 0.34 ml made up with 50 mM phosphate buffer (pH 5.0). The reaction mixture in (B) was the same as in (A) except that nitrate was omitted. (A) was incubated in air and (B) anaerobically, both for 15 min. The reaction was terminated with 60 µl 0.5 M N-ethylmaleimide. 20 µl aliquots of each mixture were then spotted on to Whatmann 3 MM paper and subjected to high voltage electrophoresis as described in Section 4.3.4.1. Radioactive areas were detected by running the dried chromatogram through a Packard radioscanner (Section 4.3.6.1). The relative mobilities of some sulphur compounds are shown in Figure 12.

Figure 44

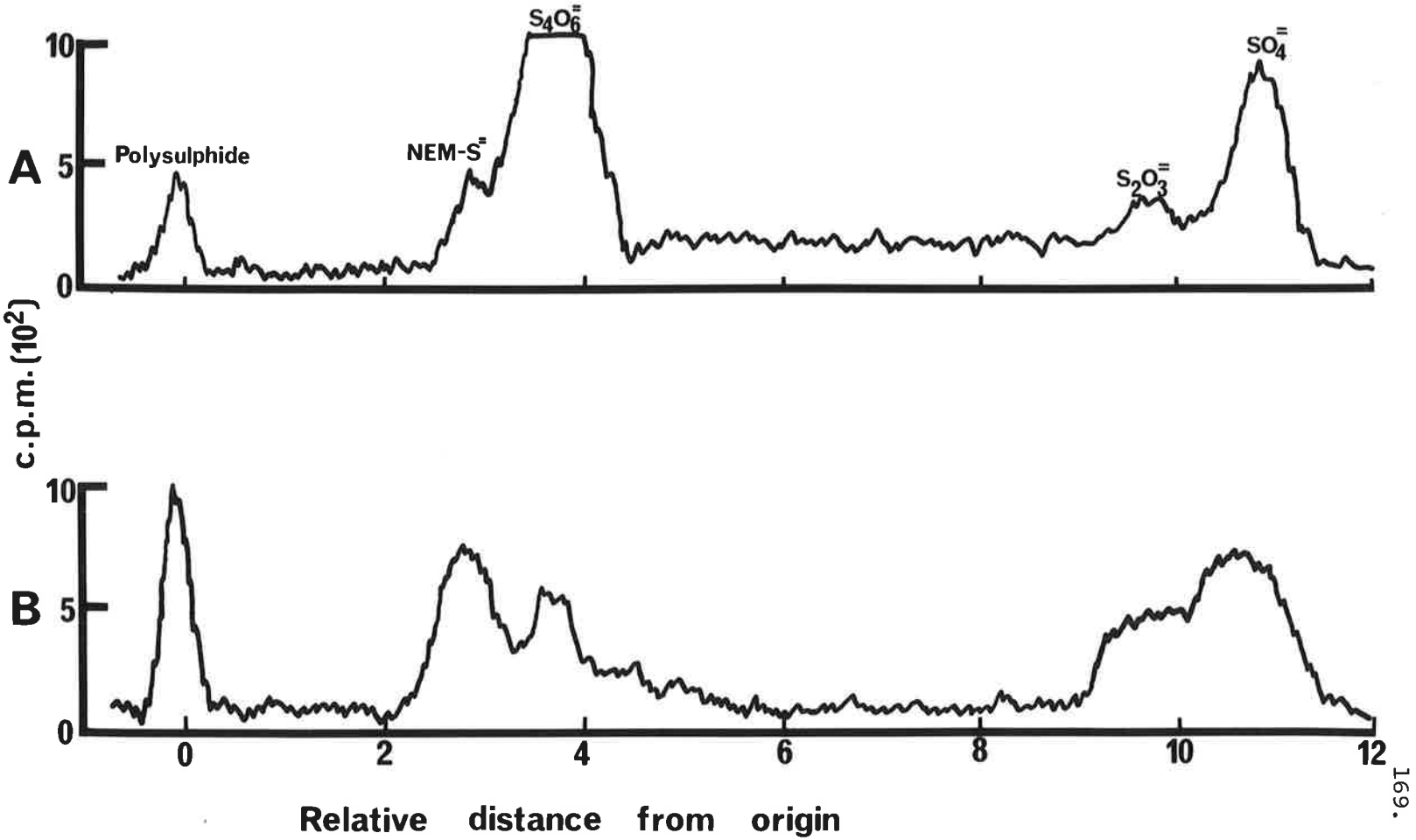

A
B
10
5
0
Polysulphide
NEM-S=
S4O6=
S2O3=
SO4=
c.p.m. (10²)
0
2
4
6
8
10
12
Relative distance from origin

Figure 45. Products of Thiosulphate ($S-^{35}SO_3^=$) oxidation.

The reaction mixture in (A) consisted of 0.3 µCi ^{35}S-thiosulphate (inner sulphur atom labelled) with 5 µmoles $Na_2S_2O_3$ carrier, 5 µmoles GSH and the crude extract (2.2 mg protein) in a total volume of 0.34 ml made up with 50 mM citrate buffer (pH 5.0). The reaction mixture in (B) was the same as in (A) except that 2 µmoles $NaNO_3$ was substituted for GSH. The reaction, conducted in air for 15 min was terminated with 60 µl 0.5 M N-ethylmaleimide. An aliquot of the mixture was separated at the end of the reaction by high voltage electrophoresis as for Figure 12 (Section 4.3.6.1).

Figure 45

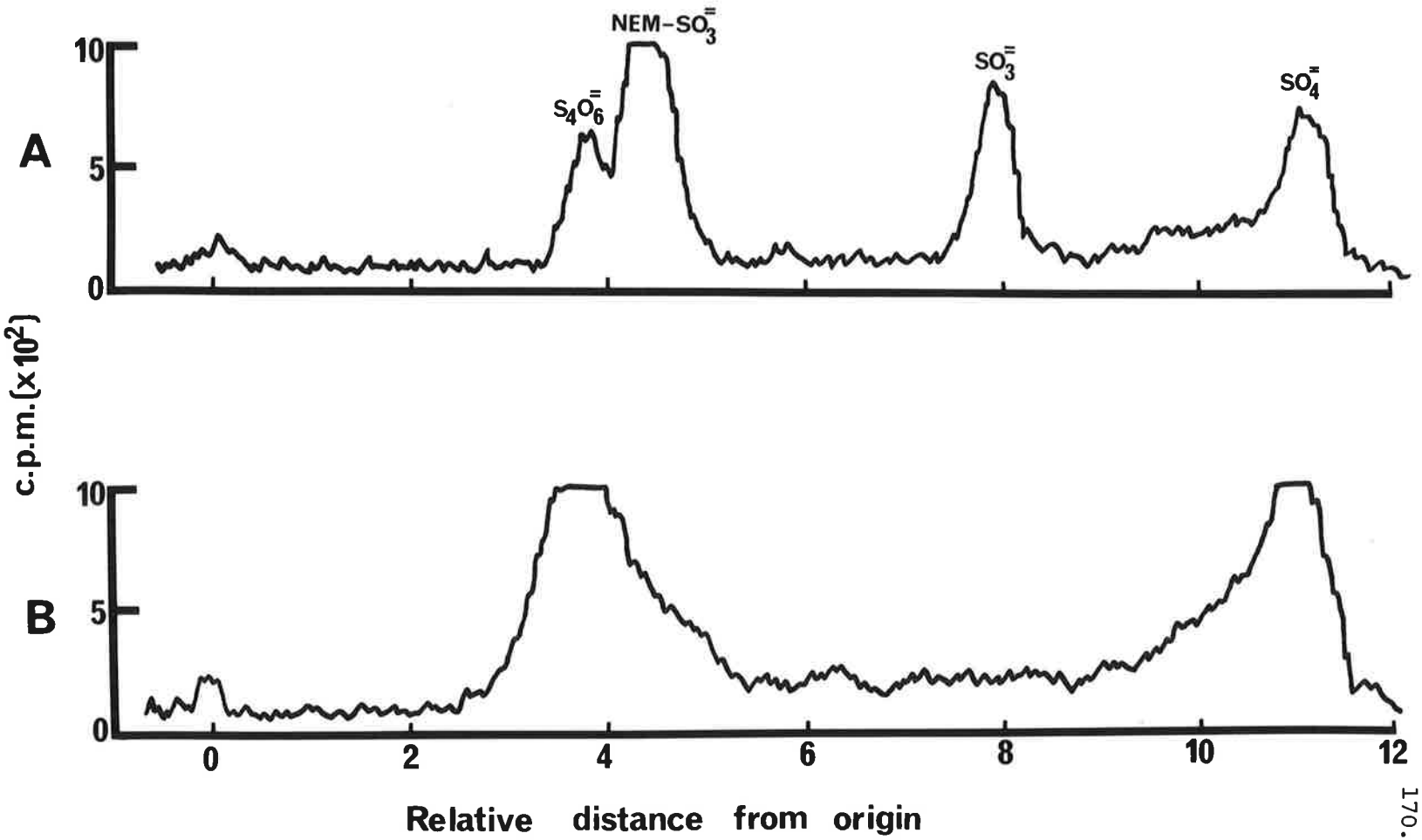

NEM-$SO_3^=$
$S_4O_6^=$
$SO_3^=$
$SO_4^=$
A
B
10
5
0
c.p.m.(x10²)
0
2
4
6
8
10
12
Relative distance from origin

Figure 46. The reduction of nitrate by cell suspension with various electron donors.

The reaction mixture in a total volume of 2 ml contained Tris-HCl buffer (pH 7.5), 85 µmoles; Na-EDTA, 0.4 µmoles; $NaNO_3$, 5 µmoles; electron donor, 5 µmoles and 0.2 ml cell suspension (25%, w/v, equivalent to 31 mg bacterial dry weight). With thiosulphate as an electron donor sodium citrate buffer (pH 5.0) was used instead of Tris-HCl. The reaction was conducted at 30^{o} for various time intervals indicated as described in Section 4.2.5.1.

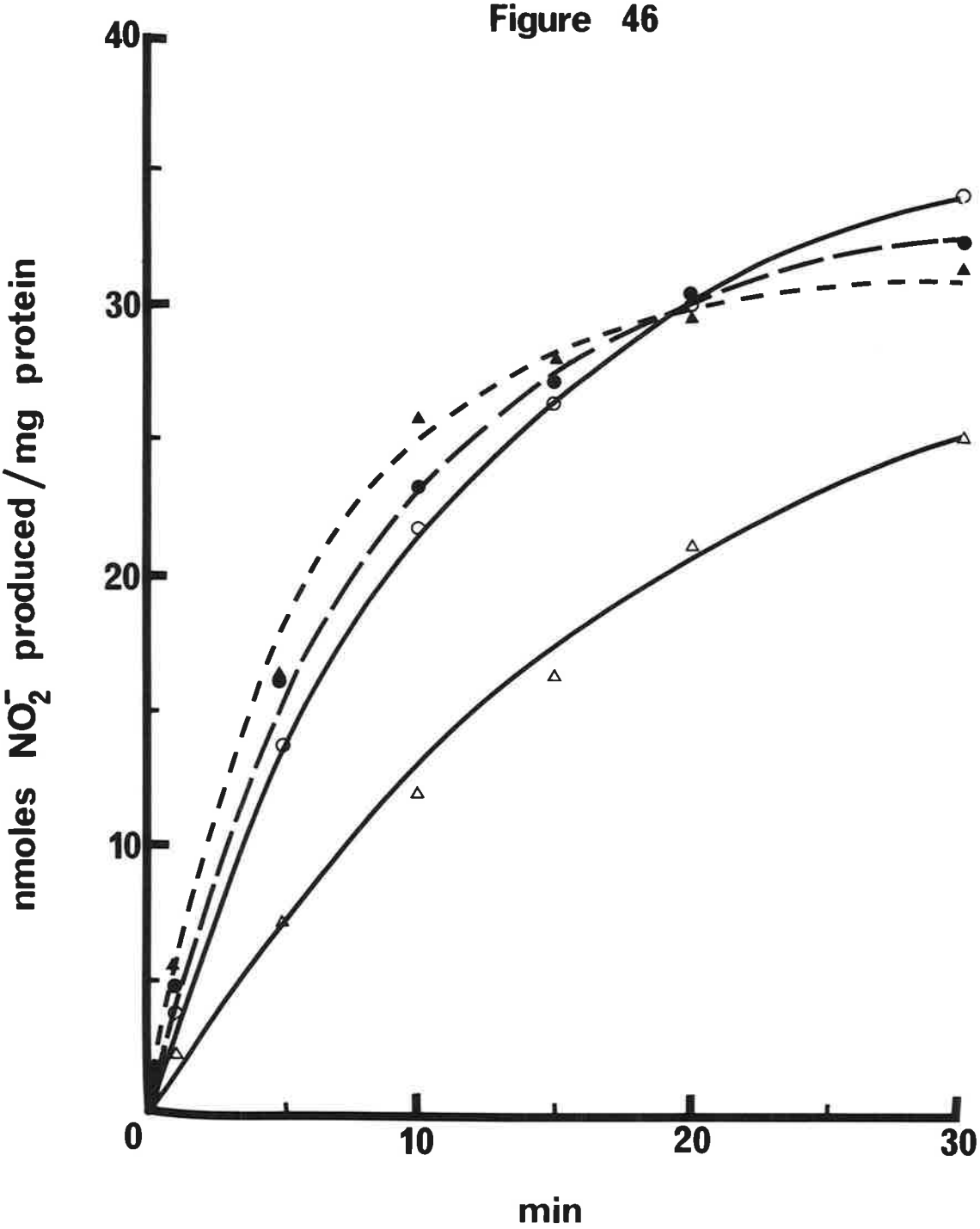
o———o Sulphide

Δ———Δ Thiosulphate

Δ------Δ Sulphite

●– – – –● NADH

Figure 46



dithionite, NADH or NADPH. Sulphite was the most effective electron donor (Table 25). In the P144 fraction, however, only sulphite, NADH and thiosulphate reduced nitrate to nitrite. It has been shown that the oxidation of elemental sulphur and sulphide linked to nitrate reduction requires the recombination of the S144 and P144 fractions (Section 5.1.1.2 and Section 5.2.2.2 respectively).

The effect of pH on nitrate reductase with sulphite, NADH and thiosulphate as electron donors is shown in Figure 47. With thiosulphate the pH optimum was 5.0. Sulphite-linked nitrate reductase has an optimum of pH 8.5 as reported by Adams, Warnes & Nicholas (1971*A*). When NADH was the electron donor the pH optimum was 7.0.

5.5.1.2 Nitrite

As shown previously (Section 5.1.1.3) nitrite is effectively reduced to NO, N_2O and N_2 by cell suspensions when sulphide, sulphite and thiosulphate were the electron donors. Although these sulphur compounds reduced nitrite in cell suspensions (Figure 48), in cell extracts sulphide and NADH were the only effective donors.

Table 25. Comparison of the effectiveness of various electron donors for nitrate reduction in crude extracts (S10).

The reaction mixture was the same as that described for Figure 46, except that 5 mg protein of the crude extract (S10) was used. The reaction was conducted at 30° as described in Section 4.2.5.1.

Electron donor	Specific activity
Sulphite	265
Sulphide	127
Thiosulphate	15
Elemental sulphur (+ GSH)	144
Dithionite	176
NADH	92
NADPH	45

Specific activity: nmoles nitrite produced/10 min/mg protein.

Figure 47. Comparison of the pH optima for nitrate reductase with sulphite, thiosulphate and NADH, respectively, as electron donors.

The buffers (concentration of each 50 mM) mentioned below were prepared with different ranges of pH values. In addition each buffer contained 0.2 mM Na-EDTA. Nitrate reductase was assayed as described in Section 4.2.5.1 using the P144 fraction as the enzyme source.

▼———▼ Citrate buffer with thiosulphate as the electron donor

o-----o Citrate-phosphate buffer with thiosulphate as the electron donor

Δ———Δ Phosphate buffer with thiosulphate as the electron donor

x———x Borate-phosphate buffer with NADH as the electron donor

▲———▲ Phosphate buffer with sulphite as the electron donor

●———● Tris-HCl buffer with sulphite as the electron donor

Figure 47

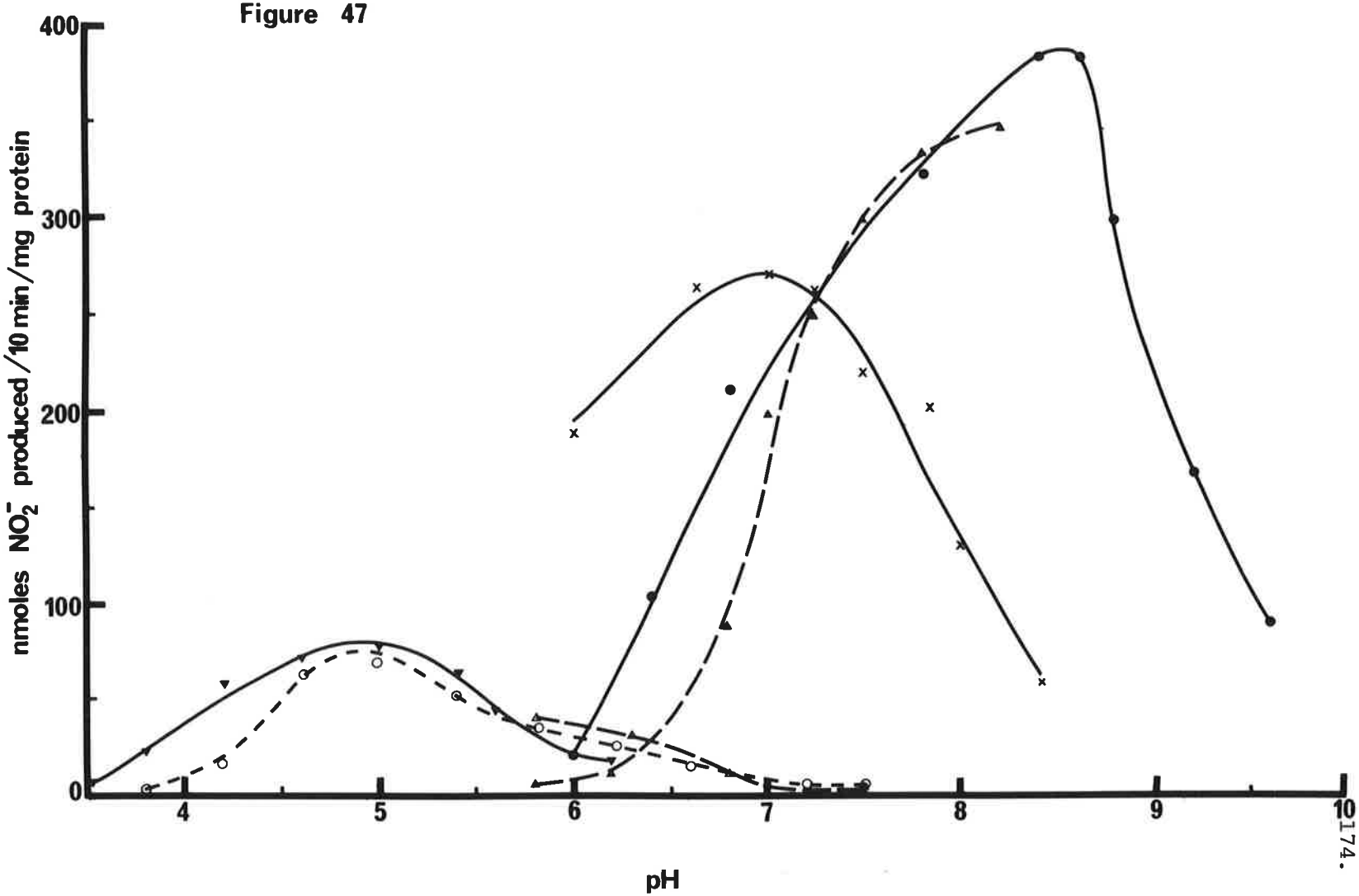

400
300
200
100
0
nmoles NO_2^- produced /10 min/mg protein
4
5
6
7
8
9
10
pH

Figure 48. The reduction of nitrite by cell suspension with various electron donors.

The reaction mixture contained in a total volume of 2 ml Tris-HCl buffer (pH 7.5), 85 µmoles; $NaNO_2$, 5 µmoles; 0.2 ml cell suspension (25%, w/v, equivalent to 28 mg bacterial dry weight) and 5 µmoles of one of the following electron donors: Na_2SO_3, Na_2S, $Na_2S_2O_3$ and NADH (with 2 µmoles PMS). The reaction, carried out in Warburg flasks under anaerobic conditions (Section 4.2.5.2), was started by tipping the electron donor from the side arm into the main compartment of the flask.

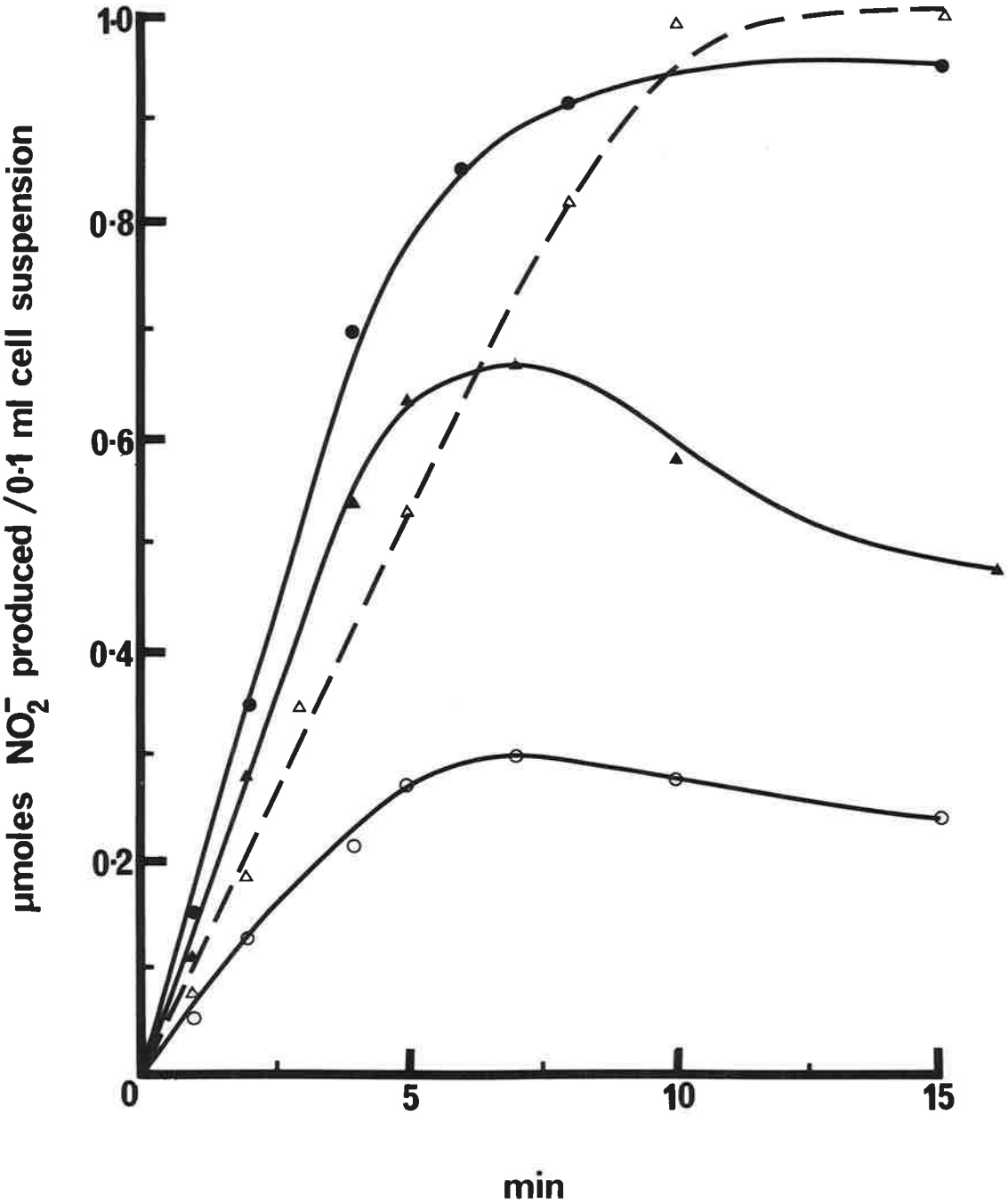
Δ- - - -Δ Thiosulphate

●——● Sulphide

o——o Sulphite

▲——▲ NADH/PMS

Figure 48


1·0
0·8
0·6
0·4
0·2
0
5
10
15
µmoles NO_2^- produced /0·1 ml cell suspension
min

5.5.2 Cytochrome components of the electron transport systems

The difference spectra of crude extracts (S10), dithionite-reduced *versus* oxidized indicate that the following types of cytochromes are present: cytochrome *c* (420, 522 and 552 nm), and cytochrome *d* (475 and 675 nm) (Figure 49). In addition there is also a broad absorption band around 610 - 620 nm which may be due to the α-absorption bands of cytochromes of the *a* or *d* types or both. Cytochrome *b* was not detected. A trough at 450 nm suggests that flavin is also present.

The dithionite-reduced *versus* oxidized difference spectra of the supernatant fraction (S144) illustrated in Figure 50 indicate the presence cytochromes of the *c* (420, 522 and 552 nm) and *a* (610 nm) types. The difference spectra of P144, dithionite-reduced *versus* oxidized, was markedly different from those of the S144 fraction. Cytochrome *c* (422, 524 and 554 nm) and *d* (475, 620 and 675 nm) but not cytochrome *a* were detected (Figure 50). When the P144 fraction solubilized with deoxycholate (fraction III, Table 14) was reduced with dithionite, the reduced minus oxidized difference spectra indicate that only flavin and cytochrome *c* were present (Figure 50).

When compared on equivalent protein basis the S144 fraction

Figure 49. Reduced *versus* oxidized difference spectra of the crude extract (S10).

Reference and sample cuvettes contained the crude extract (20 mg protein) in 3 ml 50 mM phosphate buffer (pH 7.5). A few small crystals of either Na_2S, $Na_2S_2O_4$ or Na_2SO_3 were added to the sample cuvette and the difference spectra then recorded in a Shimadzu recording spectrophotometer (Section 4.5.1).

A : Either Na_2S or $Na_2S_2O_4$ as a reductant

B : Na_2SO_3 as a reductant

Figure 50. Reduced *versus* oxidized difference spectra of the S144 and P144 fractions.

The S144 and P144 fractions were prepared as described in Section 4.2.1.3. The P144 fraction was treated with deoxycholate (Fraction III, Table 14) as described in Section 5.3.3.2(a).

Each of the above extracts (20 mg protein) was mixed with 3 ml 50 mM phosphate buffer (pH 7.5) in the reference and sample cuvettes. A few small crystals of $Na_2S_2O_4$ were added to the sample cuvette and the difference spectra recorded (Section 4.5.1).

A : S144 fraction

B : P144 fraction

C : P144 fraction solubilized with deoxycholate (Fraction III, Table 14).

Figure 49

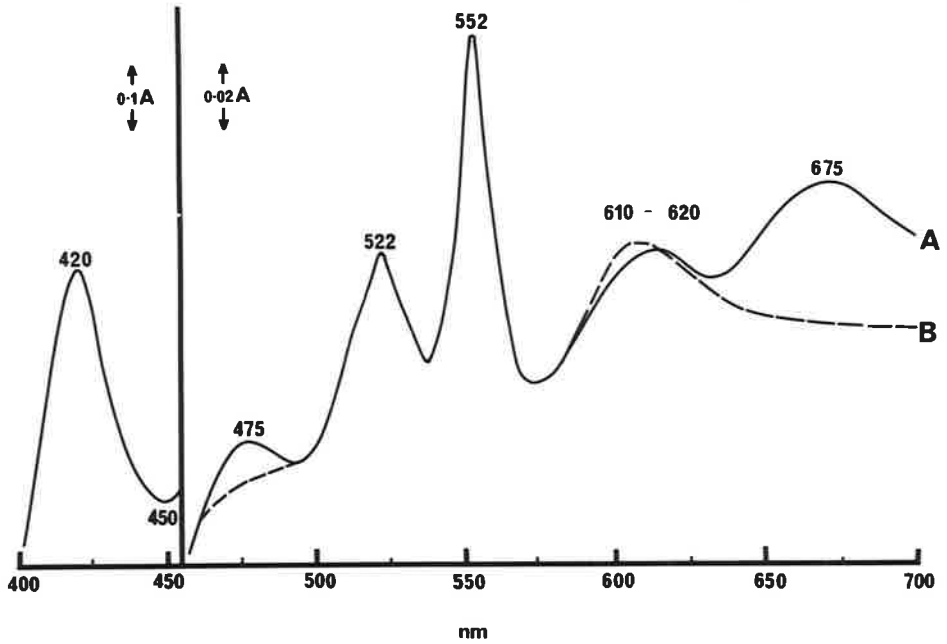
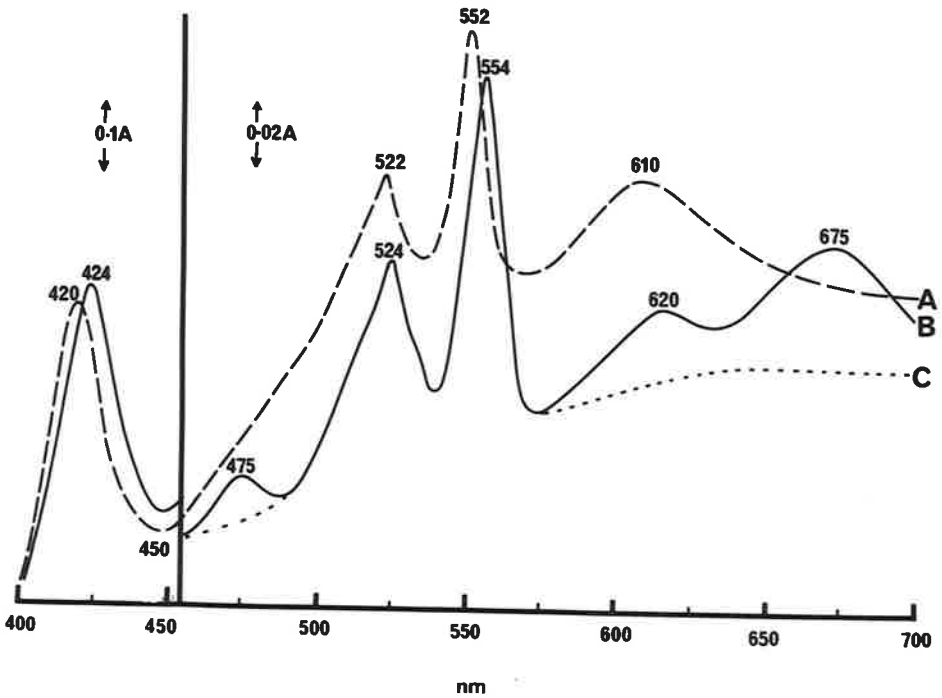


Figure 50



contained about 40% more flavoprotein than the P144 fraction (Table 26). This is in accordance with the finding that APS-reductase, a flavoprotein, is located mainly in the S144 fraction. There is almost equal distribution of cytochrome *c* in both fractions. There is slightly more cytochrome *a* in the S144 fraction than in the P144 fraction. Conversely the P144 fraction contained more cytochrome *d* than the S144 fraction.

Equivalent amounts of reduced sulphur compounds were added to the P144 fraction to determine their effectiveness in reducing the cytochromes as compared to dithionite. Based on the absorbance of cytochrome *c* at 554 nm, sulphide was found to be as strong a reductant as dithionite. Sulphite reduced about 90% of cytochrome *c* but only 20% of the flavoprotein. Elemental sulphur and thiosulphate reduced the cytochromes only when GSH was present. NADH was a weak reductant as it reduced only 10% of cytochrome c_{554}.

After treating the dithionite-reduced P144 fraction with pyridine and alkali to form the haemochromogen as described by Falk (1964), the reduced *versus* oxidized difference spectra showed absorption maxima at 523 and 554 nm (cytochrome *c*) (Figure 51). A broad absorption band with a maximum at 620-625 nm and an absorption band at 473 nm is probably associated with haem *d*. When the S144 fraction was subjected to the same treatment, the reduced minus oxidized difference spectra indicate that haem *c* (551 nm) and

Table 26. Cytochromes and flavoproteins in the S144 and P144 fractions.

The amounts of cytochromes and flavoproteins in the S144 and P144 fractions were determined spectrophotometrically from difference spectra (dithionite reduced minus oxidized) by the method of Asano & Brodie (1964) as described in Section 4.4.8. Cytochrome *d* was measured by the method of Newton (1969) based on the 620 nm absorption of the pyridine haemochromogen (Figure 51).

Compound	Concentration (nmoles/mg protein)	
	S144	P144
Flavoprotein	9.7	6.1
Cytochrome *c*	3.5	3.5
Cytochrome *a*	3.7	2.3
Cytochrome *d*	0.8	7.1

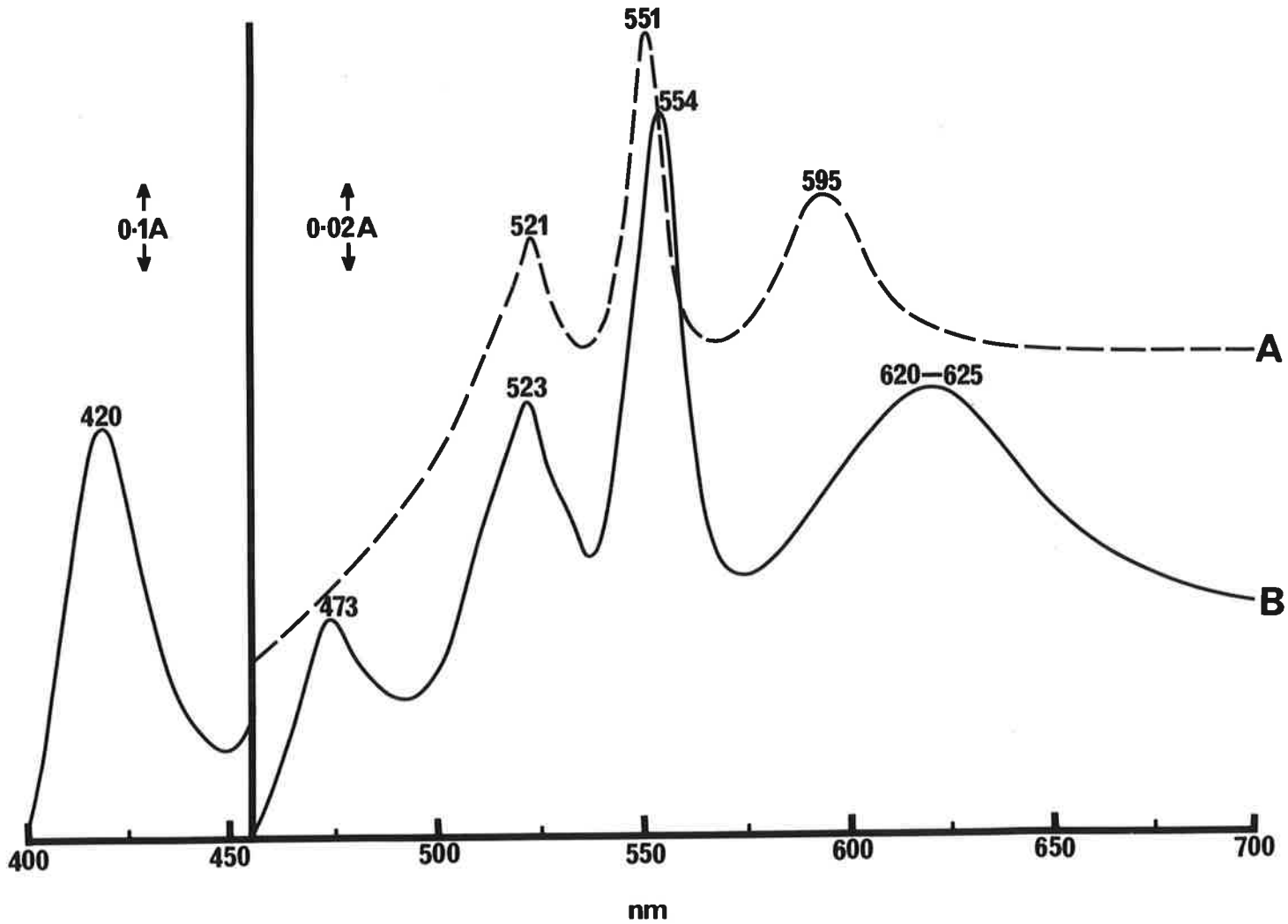
Figure 51. Difference spectra of alkaline pyridine haemochromogens of the S144 and P144 fractions.

The method of Falk (1964) for the formation of haemochromogens was used. The S144 or P144 fraction (10 mg protein) in 50 mM phosphate buffer (pH 7.5) was mixed with an equal volume of a mixture containing 2 M pyridine and 0.2 N sodium hydroxide. The sample was equally divided and pipetted into two cuvettes. Sodium dithionite was added to one cuvette and the difference spectra then recorded (Section 4.5.1).

A : S144 fraction

B : P144 fraction

Figure 51


551
554
595
0·1A
0·02A
521
A
620—625
523
420
473
B
400
450
500
550
600
650
700
nm

haem *a* (595 nm) were present (Figure 51).

The CO-sulphide-reduced *versus* sulphide-reduced difference spectra of the crude extract (S10) exhibited an absorption band at 598 nm which suggests an *a* type cytochrome (Figure 52). In addition there are absorption bands at 450 nm and 680 nm which may be associated with cytochrome *d*. The CO-sulphite reduced minus sulphite-reduced difference spectra of the S144 fraction exhibit an absorption band at 595 nm (cytochrome *a*) (Figure 53) as found in the crude extract (S10). There is also an absorption band at 447 nm which may be the Soret band of cytochrome *a* that does not bind CO. Thus, the S144 fraction may contain the cytochrome a-a_3 complex. The CO-sulphide *versus* sulphide reduced difference spectra of the P144 fraction exhibit maxima at 452 and 680 nm, indicating a cytochrome of the *d* type (Figure 53). The effects of CO mentioned above were reversed by light.

5.5.3 Characterization of the overall electron transport system

5.5.3.1 Sulphide oxidation

The cytochromes in the P144 fraction reduced by sulphide were reoxidized by oxygen or under anaerobic conditions by nitrite (Figure 54). When sulphide was used to reduce

Figure 52. CO-sulphide reduced *versus* sulphide reduced difference spectra of the crude extract (S10).

Sample and reference cuvettes contained the crude extract (10 mg protein) and a few crystals of sodium dithionite in 50 mM phosphate buffer (pH 7.5). CO was bubbled through the sample cuvette for 5 min and the difference spectra recorded.

Figure 53. CO-sulphite reduced *versus* sulphite reduced difference spectra of the S144 and P144 fractions.

Conditions were as in Figure 52 except that for the S144 fraction Na_2SO_3 was used as the reductant.

A : P144 fraction

B : S144 fraction

Figure 52

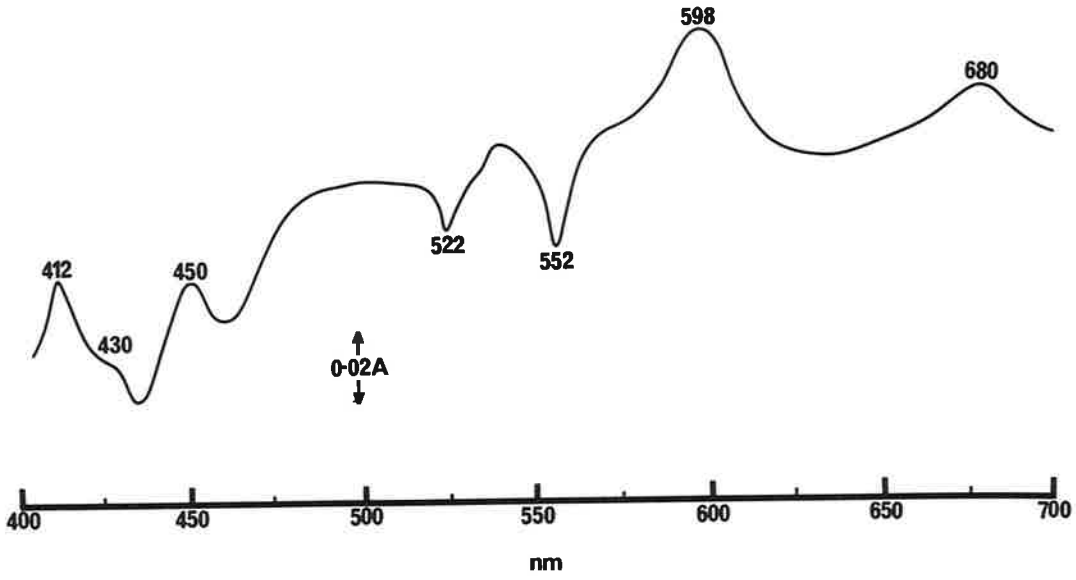


Figure 53

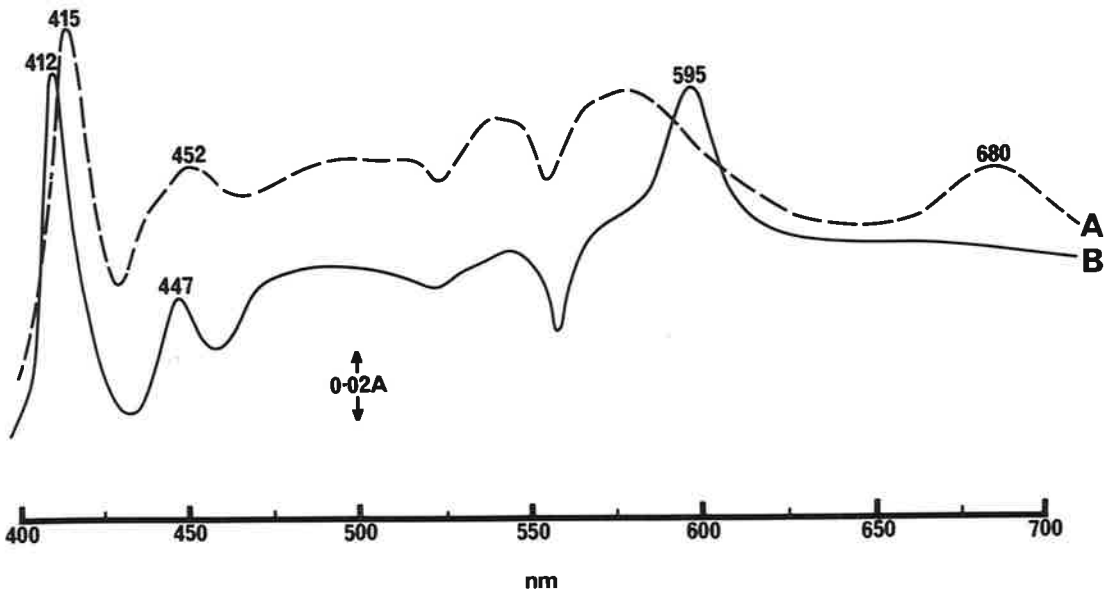


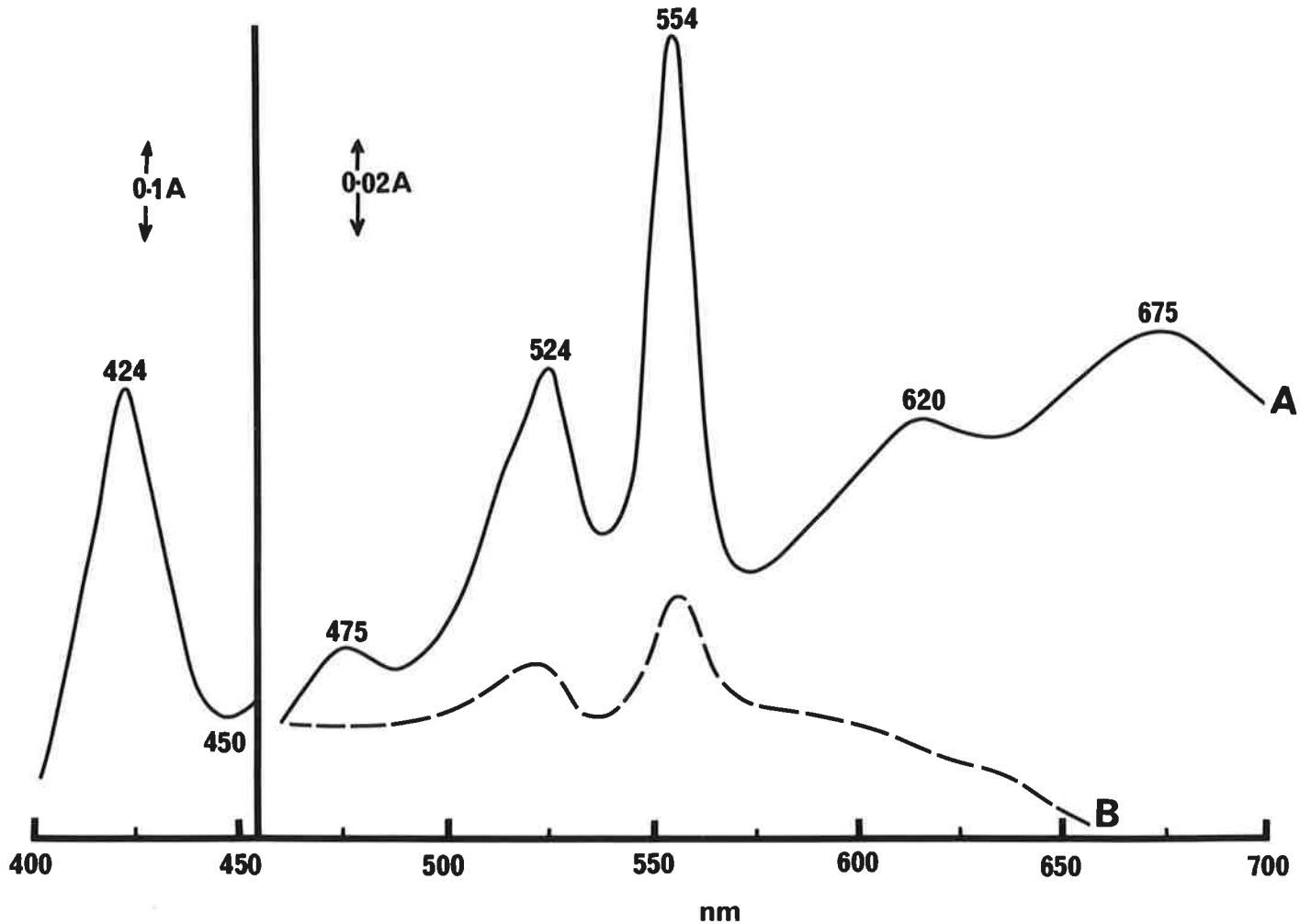
Figure 54. Sulphide reduced *versus* oxidized difference spectra of the P144 fraction.

The sample cuvette (1 cm path), designed for anaerobic studies, contained the P144 fraction (10 mg protein) in 50 mM phosphate buffer in the main compartment. The side arm contained 0.2 ml 10 mM $NaNO_2$. The reference cuvette (normal type) contained the same components as in the main compartment of the sample cuvette. After adding 2.5 µmoles Na_2S to the main compartment, the sample cuvette was flushed several times with O_2-free N_2 gas. The difference spectra were then recorded.

A : Reduced *versus* oxidized difference spectra.

B : After either readmitting air and shaking briefly or tipping in the $NaNO_2$ from the side arm.

Figure 54


554
0·1A
0·02A
675
524
424
620
A
475
450
B
400
450
500
550
600
650
700
nm

cytochromes in the deoxycholate-treated P144 fraction (Fraction III, Table 14) cytochrome *d* was not detected, which suggests that it was still bound to the membrane fraction. Furthermore, the cytochromes which were reduced by sulphide in the deoxycholate-treated fraction were not reoxidized by either oxygen or nitrite. This indicates that cytochrome *d* is specifically involved with sulphide oxidation linked to oxygen or to nitrite. Sulphide also reduced cytochromes in the S144 fraction, but these were not reoxidized by either oxygen, nitrate or nitrite. This agrees with the finding that the sulphide oxidizing enzyme was located in the P144 fraction.

The effects of various inhibitors on sulphide oxidation as determined by oxygen uptake or nitrite reduction in the P144 fraction are shown in Table 27. Rotenone, antimycin A, HOQNO, NaN_3 and 2,2'-dipyridyl were ineffective but amytal, o-phenanthroline and iodoacetamide at 1 mM reduced activity by 15 to 30%. The most potent inhibitors were KCN, dithiol, pCMB, arsenite, DIECA, and bathocuproin. The reversal by light of the CO inhibition of sulphide-linked nitrite reductase suggests that a haem type terminal oxidase is involved. At the concentrations shown in Table 27, the inhibitors listed had no effect on the chemical determination of nitrite.

Table 27. Effect of inhibitors on either oxygen uptake or nitrite reduction during sulphide oxidation by the P144 fraction.

Oxygen uptake was determined by the oxygen electrode as described in Section 4.3.2. Nitrite reductase with sulphide as an electron donor was assayed as in Section 4.2.5.2. The P144 fraction (4 mg protein), in 50 mM Tris-HCl buffer (pH 7.5), containing 0.2 mM Na-EDTA, was incubated at 30^{o} with each inhibitor for 5 min prior to adding sulphide to start the reaction. The specific activities for the control reactions (without inhibitor) were 29 nmoles oxygen utilized/min/mg protein for the oxygen uptake and 102 nmoles nitrite reduced/10 min/mg protein for the nitrite reductase.

Table 27. Effect of inhibitors on either oxygen uptake or nitrite reduction during sulphide oxidation by the P144 fraction.

Inhibitor	Final concentration (mM)	% inhibition NO_2^- reduction	% inhibition O_2 uptake
Amytal	1.0	20	35
Rotenone	0.1	10	15
Antimycin A	1.0	10	7
HOQNO	1.0	3	5
NaN_3	1.0	2	3
KCN	0.1	93	89
Dipyridyl	1.0	7	-
o-phenanthroline	1.0	14	-
Dithiol	1.0	84	-
pCMB	1.0	56	-
Iodoacetamide	1.0	36	-
Arsenite	1.0	75	77
CO	Bubbled through for 5 min	96	-
	Exposed to tungsten light for 10 min	21	-
Bathocuproin	1.0	94	92
DIECA	1.0	83	88

5.5.3.2 Sulphite oxidation

The cytochromes in the P144 fraction or in the deoxycholate-treated P144 fraction, reduced by sulphite were reoxidized by oxygen or by nitrate (Figure 55). Reoxidation of the cytochromes by oxygen was much slower than by nitrate. Nitrite, however, was ineffective. In the S144 fraction cytochromes reduced by sulphite were reoxidized by oxygen but not by nitrate or nitrite (Figure 55).

Thiol-binding reagents strongly inhibited oxygen uptake coupled to sulphite oxidation in the P144 fraction (Table 28) indicating the involvement of -SH groups. Inhibition by KCN and NaN_3 suggests that a heavy metal is involved. The inhibition of HOQNO suggests the involvement of cytochrome *b*, contrary to spectral evidence since cytochrome *b* was not detected. A notable feature is the inhibition of oxygen uptake by nitrate (see Section 5.3.3.1). The -SH inhibitors pCMB and iodoacetamide inhibited the reduction of cytochromes by sulphite indicating that the site of action of the -SH groups is before the cytochrome *c* component of the electron transport chain.

The cytochromes involved in sulphide and sulphite oxidation with the respective terminal electron acceptors in the various cell fractions are summarised in Table 29.

Figure 55. Sulphite reduced *versus* oxidized difference spectra of the P144 and S144 fractions.

Conditions were as in Figure 60 except that sulphite was used as the reductant.

A : Sulphite reduced *versus* oxidized difference spectra of the P144 fraction or the solubilized P144 fraction (Section 5.3.3.2.(a)).

B : Sulphite reduced *versus* oxidized difference spectra of the S144 fraction.

C : After adding a few crystals of $NaNO_3$ to the sample cuvette in A OR after bubbling oxygen through the sample cuvettes in either A or B.

Figure 55

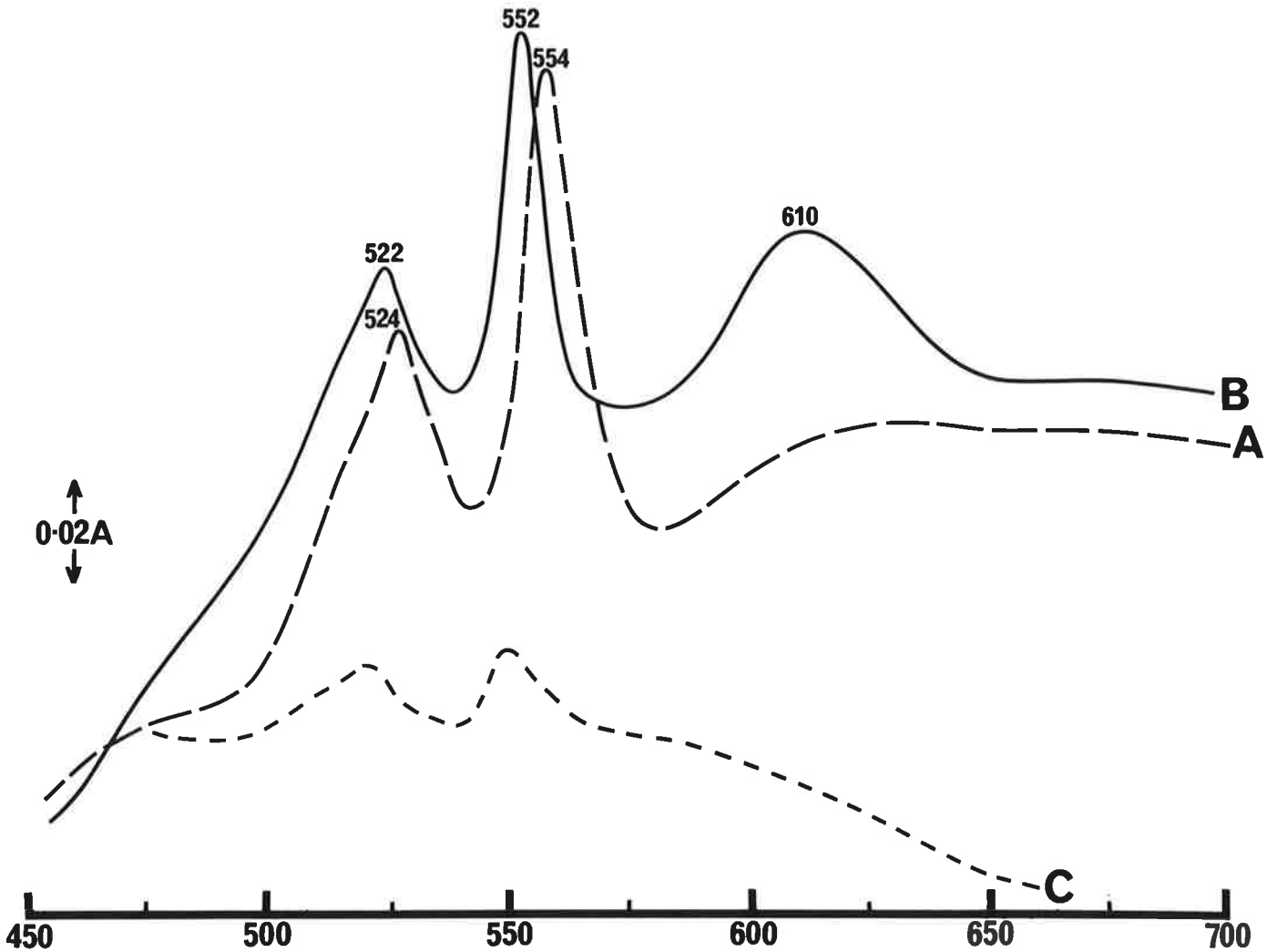


Table 28. Effect of inhibitors on oxygen uptake during sulphite oxidation by the P144 fraction.

Oxygen uptake was determined as described in Section 4.3.2. The P144 fraction (3 mg protein), in 50 mM Tris-HCl buffer (pH 8.3), containing 0.2 mM Na-EDTA, was incubated at 30° with each inhibitor for 5 min before adding sulphite (final concentration 1 mM). The specific activity of the control reaction (without inhibitor) was 21 nmoles oxygen utilized/min/mg protein.

Inhibitor	Final concentration (mM)	% inhibition
pCMB	0.1	69
	1.0	96
Iodoacetamide	1.0	79
KCN	0.1	90
NaN_3	1.0	77
Arsenite	1.0	74
Amytal	1.0	20
HOQNO	0.01	87
DIECA	1.0	0
o-phenanthroline	1.0	5
Bathocuproin	1.0	0
Nitrate	0.1	23
	1.0	43
	5.0	55
Nitrite	1.0	0
Hydroxylamine	1.0	3

Table 29. Distribution of cytochromes in various cell fractions.

The data presented are extracted from Figures 49, 54 and 55.

Reductant	Cell extract	Cytochromes (λ in nm)	Effective terminal acceptors (O_2, NO_3^- or NO_2^-)
Sulphide	S10	*c* (420, 522, 552) *a* (610) *d* (475, 620, 675)	O_2 or NO_2^-
	S144	*c* (420, 522, 552) *a* (610)	All ineffective
	P144	*c* (424, 524, 554) *d* (475, 620, 675)	O_2 or NO_2^-
Sulphite	S10	*c* (420, 522, 552) *a* (610)	O_2 or NO_3^-
	S144	*c* (420, 522, 552) *a* (610)	O_2 only
	P144	*c* (424, 524, 554)	NO_3^- or O_2

5.5.4 Effect of growth conditions on respiratory enzyme and electron transport components

When *T. denitrificans* was grown anaerobically with nitrate as described in Section 4.1.3, the cell yield after 3 days' growth was about 1 g (wet weight) per litre of medium. There was no significant difference in growth between nitrate-grown cultures which have been sparged with O_2-free N_2 and those left as still cultures. However, when the bacterium was grown without nitrate and sparged with O_2 (twelve subcultures) (Section 4.1.4), the cell yield was low (0.5 gm wet weight/1 medium). The bacterium did not grow anaerobically when nitrate was either omitted or substituted with nitrite in the medium.

The S144 and P144 fractions were prepared from aerobically (without nitrate, sparged with O_2) grown cells as described in Section 4.2.1.3. The components of the electron-transport chain and the activities of some enzymes in these cell fractions were compared with those in the S144 and P144 fractions of cells grown with nitrate anaerobically.

The dithionite-reduced minus oxidized difference spectra of the P144 fraction from O_2 grown-cells (without nitrate) indicate that cytochrome *b* (560 nm) was present, as well as cytochromes *c* and *d* (Figure 56). Cytochrome *b* was not detected in nitrate-grown

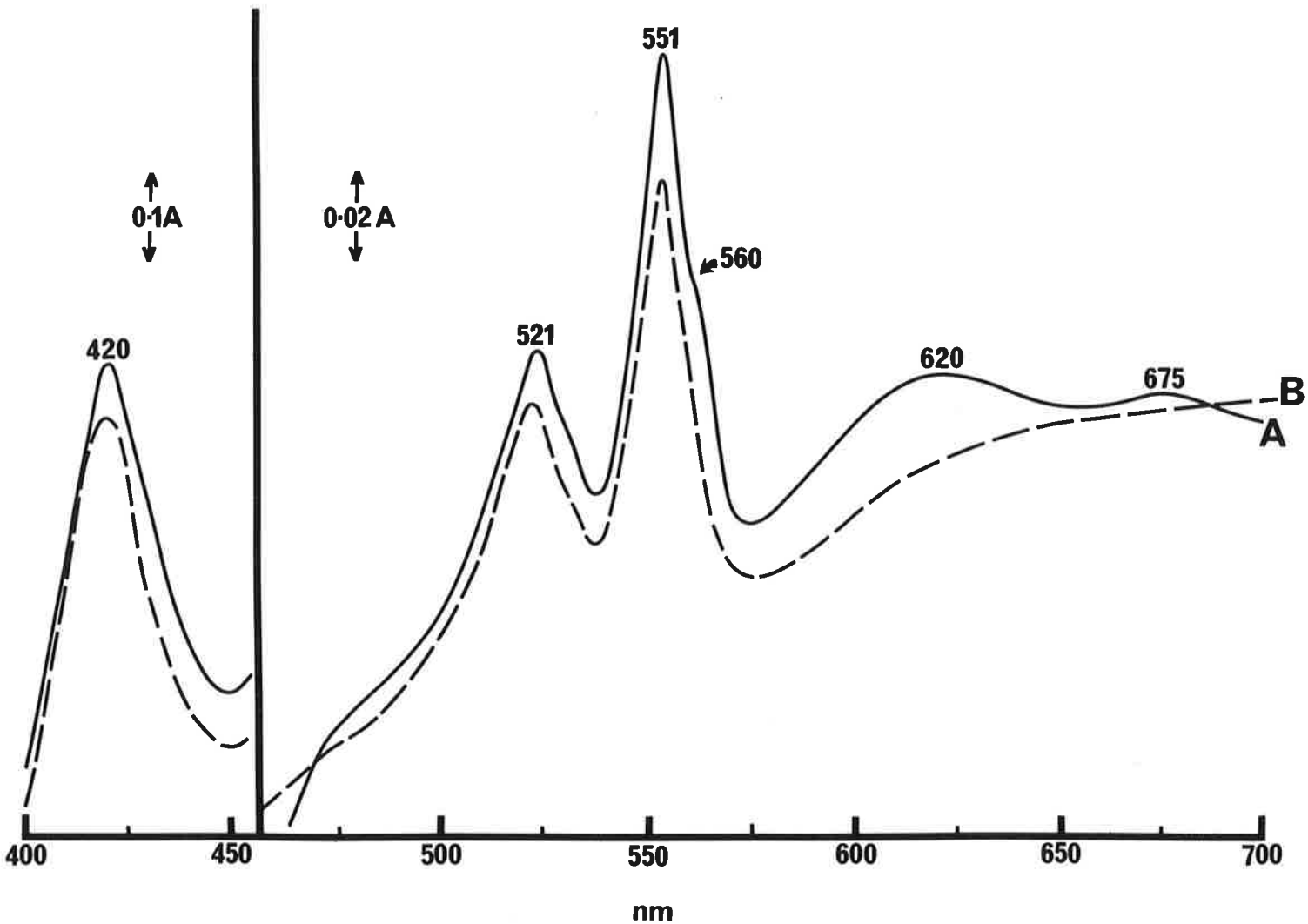
Figure 56. Dithionite reduced *versus* oxidized difference spectra of the S144 and P144 fractions from aerobically grown cells.

Cells were grown aerobically (without nitrate) as described in Section 4.1.4. The P144 and S144 fractions were prepared as described in Section 4.2.1.3. The reference and sample cuvettes contained either the S144 fraction (7 mg protein) or the P144 fraction (8 mg protein) in 50 mM Tris-HCl buffer (pH 7.5). A few small crystals of $Na_2S_2O_4$ were added to the sample cuvette and the difference spectra then recorded (Section 4.5.1).

A : P144 fraction

B : S144 fraction

Figure 56


0·1A
0·02 A
420
521
551
560
620
675
B
A
400
450
500
550
600
650
700
nm

cells. Furthermore, cytochrome *a* was not detected in the S144 fraction of O_2-grown (aerobic) cells (Figure 56). The total cytochrome content of crude extracts (S10) of anaerobically grown cells was 20% higher than that in crude extract of O_2-grown cells.

Table 30 lists the activities of some enzymes in cell fractions prepared from cells grown aerobically (O_2) and anaerobically (NO_3^-). Nitrate reductase with sulphite, NADH or thiosulphate as donors is markedly reduced in aerobically grown cells. Nitrite reductase using either sulphide or NADH/PMS as electron donors was not detected in O_2-grown cells. Oxygen uptake during sulphide and sulphite oxidation and sulphite oxidase linked to ferricyanide reduction was similar in extract from either O_2 or NO_3^- grown cells. APS-reductase activity was slightly higher in aerobically grown cells than in those grown with nitrate.

5.6 ATP-generating Systems

5.6.1 ATP production coupled to sulphite oxidation

ATP was produced during sulphite oxidation either from AMP in the S144 fraction or from ADP in the P144 fraction (Figure 57). Ferricyanide was used as the electron acceptor. There was no ATP formed when ADP was replaced by AMP in the particulate fraction. The production of ATP from ADP in the S144 fraction is due to adenylate kinase which is located mainly in this fraction

Table 30. Comparison of the activities of some enzymes in fractions of cells grown aerobically (with oxygen) or anaerobically (with nitrate).

The activities of the enzymes listed below were determined using the P144 fraction as described in Section 4.2.5 except for APS-reductase which was assayed in the S144 fraction.

Enzyme system	Specific activity	
	O_2 grown	NO_3^- grown
Nitrate reductase		
NADH as an electron donor	92	310
Sulphite as an electron donor	154	690
Thiosulphate as an electron donor	28	78
Nitrite reductase		
NADH as an electron donor	0	155
Sulphide as an electron donor	0	170
Sulphite production from thiosulphate	35	36
Sulphite oxidation (O_2 uptake)	18	15
Sulphite oxidation (O_2 uptake) in the presence of nitrate)	17	7
Sulphide oxidation (O_2 uptake)	42	40
APS-reductase	2,150	1,770

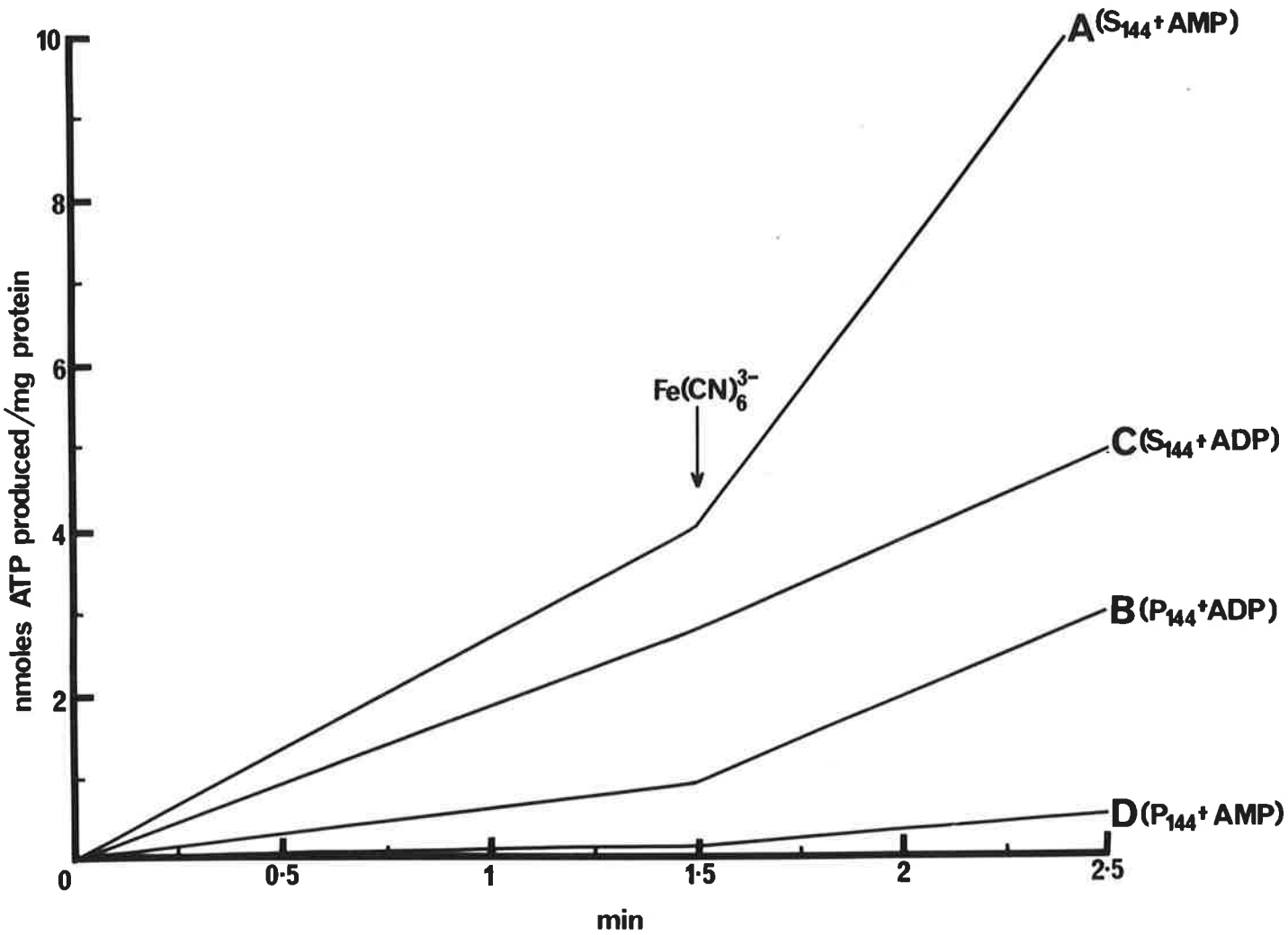
Specific activity: nmoles substrate utilized or product released/10 min/mg protein.

Figure 57. Effect of AMP and ADP on phosphorylation linked to the oxidation of sulphite in the S144 and P144 fractions.

The reaction mixture for (A) contained the following in a vial: 10 µmoles phosphate buffer (pH 7.5), 0.1 µmole AMP, 0.5 µmole $MgCl_2$, 50 µmoles arsenate-HCl buffer (pH 7.5), S144 fraction (5 mg protein) and 50 µl firefly extract made to a final volume of 3 ml with distilled water. The reaction carried out at 20^{o} in a Packard liquid scintillation spectrometer was initiated by adding 1 µmole Na_2SO_3. After incubating for 1.5 min 1 µmole $K_3(FeCN)_6$ was added as indicated.

(B) as in (A) except that 0.1 µmole ADP was substituted for AMP and the P144 fraction (5 mg protein) instead of the S144 fraction; (C) as in (A) except that 0.1 µmole ADP was substituted for AMP: (D) as in (A) except that the P144 fraction (5 mg protein) was substituted for the S144 fraction.

Figure 57


A (S144 + AMP)
C (S144 + ADP)
B (P144 + ADP)
D (P144 + AMP)
Fe(CN)6 3-
nmoles ATP produced/mg protein
10
8
6
4
2
0
0·5
1
1·5
2
2·5
min

(Table 36). Furthermore, fericyanide did not stimulate ATP formation from ADP in the S144 fraction.

Cyanide, 2,4-dinitrophenol, oligomycin and HOQNO inhibited the formation of ATP from ADP in the P144 fraction indicating that phosporylation is coupled to sulphite oxidation via an electron transport system (Table 31). However, these inhibitors had only a slight effect on ATP production from AMP in the S144 fraction which suggests that ATP was generated through substrate phosphorylation.

5.6.2 Oxidative phosphorylation

Cells and cell extracts catalyse the formation of ATP from ADP and P_i when an oxidizable substrate was added. Thus, in the P144 fraction a rapid formation of ATP accompanied the oxidation of NADH, sulphite, sulphide and thiosulphate (Figure 58). NADH was the most effective electron donor, followed by sulphite, sulphide and thiosulphate. ATP production was dependent on the concentrations of enzyme, ADP, and electron donor used.

Crude extracts (S10) were more effective in phosphorylating ADP to ATP than the P144 fractions with sulphide as the best electron donor. No significant loss of activity was recorded when the crude extract was dialysed against 50 mM Tris-HCl buffer (pH 7.5) for 4 hr but after an 18-hr dialysis only about 20% of the activity remained.

Table 31. Effect of inhibitors of electron transport and uncouplers of oxidative phosphorylation on ATP production coupled to sulphite oxidation in the S144 and P144 fraction.

Phosphorylation in the S144 fraction (AMP-dependent) was carried out as for Figure 57 (A) and that in the P144 fraction (ADP-dependent) as for Figure 57 (B). Inhibitors or uncouplers were incubated with the appropriate cell fractions before adding the other reactants. The specific activities of the control reactions (without inhibitor) were 3.3 and 1 nmoles ATP produced/min/mg protein in the S144 and P144 fractions respectively.

Inhibitor	Final concentration (mM)	% inhibition	
		S144 (AMP-dependent)	P144 (ADP-dependent)
HOQNO	0.01	0	46
KCN	0.1	5	67
DNP	0.1	12	40
Oligomycin	0.1	12	49

Figure 58. Phosphorylation of ADP to ATP during the oxidation of various sulphur compounds and NADH in the P144 fraction.

ATP production from ADP was determined as described in Section 4.2.5.14.(b). At zero time 10 pmoles of ATP internal standard was added. At point (1) 1 nmole ADP and at point (2) 1 µmole of the following electron donor were added:

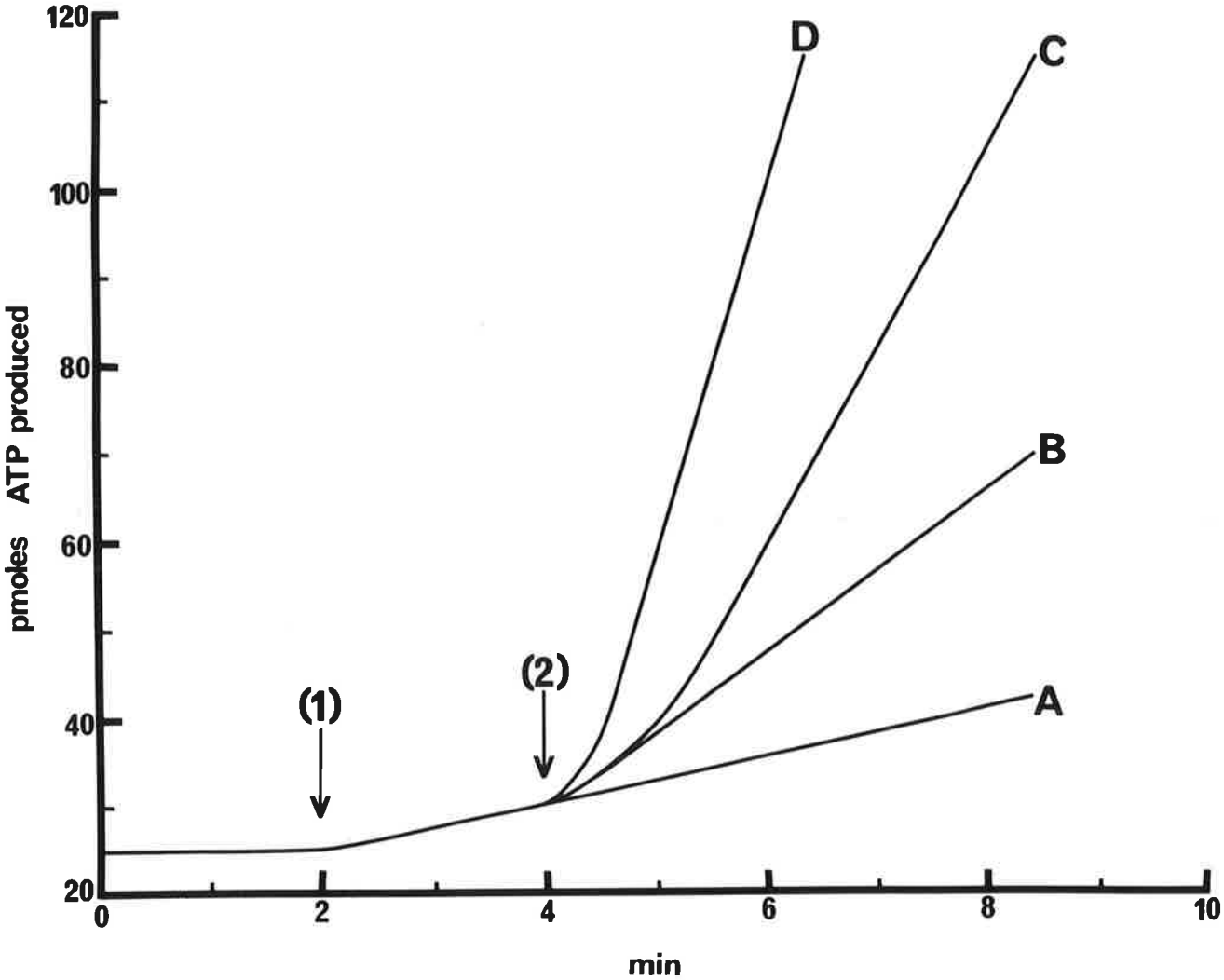
A : Thiosulphate and GSH

B : Sulphide

C : Sulphite

D : NADH

Figure 58



The P/O ratios obtained with crude extracts were relatively low and range from 0.08 for sulphide to 0.6 for sulphite. Furthermore, the presence of a very active adenylate kinase in crude extracts with a specific activity 29 nmoles ATP formed/min/mg protein made assessment of ATP production by oxidative phosphorylation difficult.

The effects of inhibitors on ATP production coupled to the oxidation of sulphide, sulphite and NADH are presented in Table 32.

Under anaerobic conditions ATP production coupled to sulphite oxidation is stimulated by nitrate in cells and in the P144 fraction (Table 33). However, the activity in the P144 fraction is only about 15% of that in whole cells. ATP is also produced in whole cells during sulphide oxidation under anaerobic conditions with nitrite as a terminal acceptor (Table 34). Attempts to achieve ATP production coupled to the oxidation of sulphide in cell extracts (S10, S144 or P144) were not successful. The P/2e ratios were 0.6 for sulphite linked nitrate reductase and 0.5 for sulphide linked nitrite reductase in whole cells.

5.6.3 Substrate level phosphorylation

When the S144 fraction was incubated with APS and P_i there was a rapid production of ATP which reached a maximum after 2 min followed by a gradual decrease in activity (Figure 59).

Table 32. Effect of inhibitors on oxidative phosphorylation with either NADH, sulphite or sulphide as electron donors in the P144 fraction.

Oxidative phosphorylation was measured by the continuous method described in Section 4.2.5.14.(b). The inhibitor (final concentration as indicated) was incubated with the enzyme for 5 min prior to adding the other reactants. The specific activities of the control reactions (without inhibitor) were 260, 125 and 55 nmoles ATP produced/min/mg protein with NADH, sulphite and sulphide as electron donors respectively.

Inhibitor	Final concentration (mM)	% inhibition		
		NADH	Sulphite	Sulphide
KCN	0.1	75	50	30
	1.0	90	75	60
NaN_3	1.0	90	70	65
HOQNO	0.01	75	90	60
Mepacrine	1.0	80	50	65
Antimycin A	0.1	60	70	45
DNP	0.1	40	50	40
Arsenite	1.0	0	30	40
Oligomycin	0.1	60	50	30

Table 33. Effect of nitrate on phosphorylation during sulphite oxidation in cell suspensions and in the P144 fraction.

The production of ATP from ADP and P_i was determined by the sampling technique described in Section 4.2.5.14.(b) except that 5 µmoles nitrate was also added. The anaerobic assay was conducted in Warburg flasks as described in Section 4.2.5.2.

Experimental conditions	Specific activity			
	Cell suspension		P144 fraction	
	aerobic	anaerobic	aerobic	anaerobic
Complete	3.5	4.2	0.60	0.50
Omit nitrate	3.2	2.8	0.57	0.42
Omit sulphite	2.1	2.4	0.44	0.40
Omit ADP	0.6	1.3	0.06	0.02
Omit enzyme	0.2	0.3	0.12	0.10

Specific activity: nmoles ATP produced/min/mg protein.

Table 34. Effect of nitrite on phosphorylation during sulphide oxidation in cell suspensions.

ATP production in air and under anaerobic conditions was determined as described in Table 33.

Conditions	ATP produced	
	Aerobic	Anaerobic
Complete	4.5	5.3
Omit nitrite	4.3	4.9
Omit sulphide	4.0	4.2
Omit ADP	1.3	1.1
Omit enzyme	0.4	0.4
Omit all substrates	0.4	0.4

ATP produced: nmoles/min/mg protein.

Figure 59. Effect of incubation times on the generation of ATP from APS and P_i in various cell fractions.

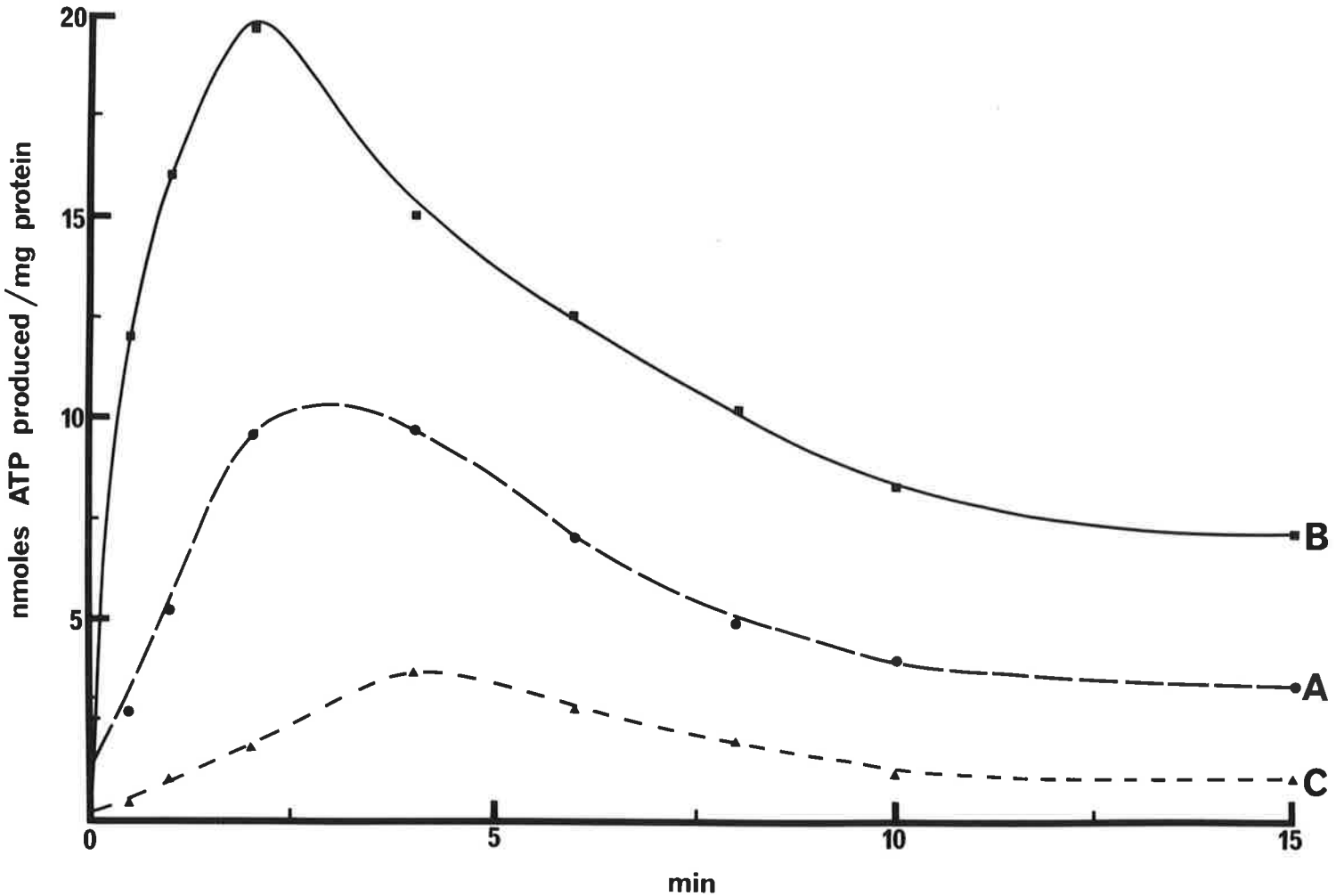
The generation of ATP from APS and P_i was determined as described in Section 4.2.5.14.(a). The crude extract (S10), S144 and P144 fractions, prepared in 50 mM Tris-HCl buffer (pH 7.5) as described in Section 4.2.1, were incubated with the substrates for various times.

A : Crude extract (S10)

B : S144 fraction

C : P144 fraction

Figure 59



The undialysed P144 fraction had only 10% of the activity of the S144 fraction. No ATP was produced from APS and P_i after washing the P144 fraction once with buffer.

ATP production in the S144 fraction was dependent upon APS, P_i, Mg^{++} and enzyme (Table 35). There was no ATP formed when the enzyme preparation was heated for 3 min in boiling water.

Separation of reaction products by high voltage electrophoresis showed that $^{32}P_i$ was incorporated into both ADP and ATP after 10 min incubation (Figure 60).

The optimum pH for ATP production was 8.0 with Tris-HCl and 8.5 with Tris-maleate buffers (Figure 61). The reaction was sensitive to pH; little ATP was produced below 7.0 or above 9.0.

A survey of the enzymes in the various cell fractions showed that in the crude extract (S10) inorganic pyrophosphatase, APS-reductase, ATP-sulphurylase and adenylate kinase were very active but there was considerably less ADP-sulphurylase activity (Table 36). These enzymes were located mainly in the S144 fraction.

Iodoacetamide, pCMB and sulphate strongly inhibited ATP-sulphurylase but the effect on adenylate kinase was relatively slight (Table 37). On the other hand NaF inhibited adenylate kinase but had little effect on ATP-sulphurylase. The effect of these inhibitors on ATP production from APS and P_i parallel those on ATP-sulphurylase.

Table 35. Assay conditions for the production of ATP from APS and P_i in the S144 fraction.

The reaction mixture contained Tris-HCl buffer (pH 8.0), APS, $MgCl_2$, P_i and the S144 fraction as described in Section 4.2.5.14.(a).

Reaction mixture	ATP produced (nmoles/min/mg protein)
Complete	9.7
Boiled enzyme	0
Omit APS	0.3
Omit $MgCl_2$	1.3
Omit P_i	0
Omit S144	0

Figure 60. Separation of reaction products of substrate phosphorylation by high voltage electrophoresis.

The reaction mixture contained APS, $MgCl_2$, $^{32}P_i$ and the S144 fraction as described for the ADP-sulphurylase assay (Section 4.2.5.10).

Figure 61. Effect of pH on the production of ATP from APS and P_i.

ATP production in the S144 fraction was determined as described in Section 4.2.5.14.(a). Tris-HCl and Tris-maleate buffers (50 mM) were used.

Δ-----Δ Tris-HCl buffer

o———o Tris-maleate buffer

Figure 60

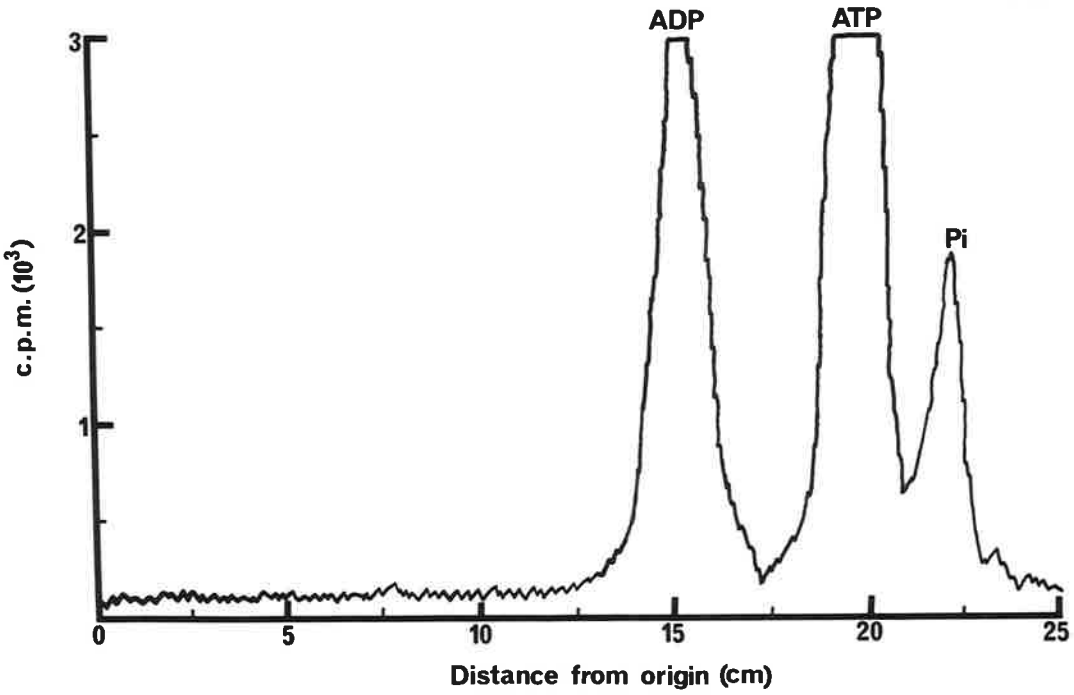


Figure 61

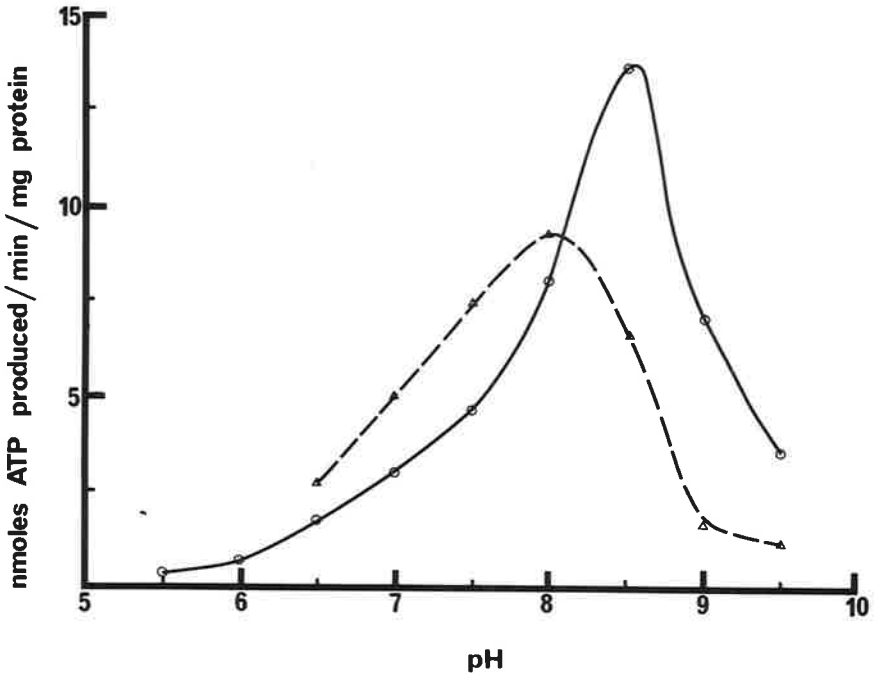


Table 36. Activity of various enzymes in cell fractions.

Enzyme activities were determined as described in Section 4.2.5.

Enzyme	Specific activity (rate/hr/mg protein)	Cell fraction		
		S10	S144	P144
ADP-sulphurylase	µmoles ADP produced	0.11	0.13	0.01
Adenylate kinase	µmoles ATP produced	0.59	1.76	0.048
ATP-sulphurylase	µmoles ATP produced	0.69	2.32	0.012
Inorganic pyrophosphatase	µmoles P_i released	0.96	15.0	3.6
APS-reductase	µmoles ferricyanide reduced	63.0	85.8	12.2

Table 37. Effect of inhibitors on ATP production by (i) partially purified adenylate kinase, (ii) purified ATP-sulphurylase and (iii) APS/P_i system in the S144 fraction.

The S144 fraction was prepared as described in Section 4.2.1.3. Adenylate kinase was partially purified as described in Section 4.2.4. Fraction V, Table 38 was used for the ATP-sulphurylase.

ATP production from APS and P_i was determined as in Section 4.2.5.14.(a), adenylate kinase as in Section 4.2.5.11 and ATP-sulphurylase as in 4.2.5.9.(b). The inhibitor was incubated with each enzyme system for 5 min at 30° before adding the other substrates.

Inhibitor	Final concentration (mM)	% inhibition		
		ATP production from APS and P_i	Adenylate kinase	ATP-sulphurylase
pCMB	0.1	85	18	76
Iodoacetamide	1.0	39	15	19
Molybdate	1.0	4	-	-
Sulphate	1.0	35	0	24
Azide	1.0	7	2	9
Cyanide	10	16	10	32
Fluoride	1.0	0	31	13

There was no increase in ATP production from APS and P_i when purified preparations of adenylate kinase and ATP-sulphurylase were added to the S144 fraction. Commercially available inorganic pyrophosphatase (Section 3.3.2) did not produce ATP from APS and P_i when it was combined with purified ATP-sulphurylase. Attempts to purify inorganic pyrophosphatase from *T. denitrificans* were not successful.

5.7 ATP-sulphurylase

5.7.1 Purification

ATP-sulphurylase was purified from cells of *T. denitrificans* as described below. All operations were carried out at 2^{o}.

Washed cells suspended (25%, w/v) in 50 mM Tris-HCl buffer (pH 7.5) was disrupted in a French Pressure Cell as described in Section 4.2.1.1. The homogenate was then centrifuged at 10,000 x *g* for 30 min and the supernatant fraction (S10) used as the starting material for the fractionation of the enzyme (Fraction I, Table 38). The supernatant fraction (S144, Fraction II) left after further centrifuging the S10 extract at 144,000 x *g* for 90 min was treated with ammonium sulphate as follows: solid ammonium sulphate was added slowly to the S144 fraction until 50% saturation

Table 38. Purification of ATP-sulphurylase.

Washed cells, suspended in 50 mM Tris-HCl buffer (pH 7.5) were disrupted in the French Pressure Cell (Section 4.2.1.1). The crude extract (S10) prepared as described in Section 4.2.1.2 was used as the starting material (Fraction I).

Enzyme activity was determined by the continuous bioluminescence method as in Section 4.2.5.9.(a).

Table 38. Purification of ATP-sulphurylase.

Fraction	Procedure	Protein (mg/ml)	Total activity	Specific activity	Recovery (%)	Fold purification
I	Crude extract (S10) left after centrifuging disrupted cells at 10,000 x *g* for 30 min	29	41	11.8	100	1
II	Supernatant fraction left after centrifuging I at 144,000 x *g* for 90 min	14.5	36.6	33.4	89	2.8
III	Fraction II precipitated with ammonium sulphate (50-65% saturation) and dialysed against 50 mM Tris-HCl buffer (pH 7.5)	18.2	28.9	322	70	27.2
IV	Fraction III loaded on to a DEAE-cellulose column, eluted with a linear gradient (0.05-0.25 M) Tris-HCl buffer (pH 7.5) and concentrated by membrane ultrafiltration	2.9	20	630	49	53.5
V	Fraction IV loaded on to a Sephadex G-200 column and eluted with 50 mM Tris-HCl buffer (pH 7.5)	0.1	13.3	2,958	32	254

Total activity: μmoles ATP produced/min.

Specific activity: nmoles ATP produced/min/mg protein

was achieved. The suspension was allowed to stand for 15 min before centrifuging at 12,500 X *g* for 20 min and the residue was discarded. Solid ammonium sulphate was added to the supernatant fraction until 65% saturation was reached and the mixture allowed to stand for another 15 min. The precipitate, obtained after the second centrifuging, was then dissolved in a minimal volume of cold 50 mM Tris-HCl buffer. The dialysed fraction (Fraction III) was then loaded on to a DEAE-cellulose column (DE-11; 1.5 X 24.5 cm) which had been pre-equilibrated with 50 mM Tris-HCl buffer (pH 7.5). The column was then eluted with a linear gradient of 150 ml each of 0.05M and 0.25M Tris-HCl buffer (pH 7.5). The elution profile of the enzyme is shown in Figure 62. Fractions (5 ml each) containing ATP-sulphurylase were collected, pooled and concentrated by membrane ultrafiltration as described in Section 4.3.8. This yielded fraction IV, which was further purified by Sephadex G-200 gel chromatography. The enzyme eluted with 50 mM Tris-HCl buffer (pH 7.5) was collected in 5 ml fractions and then bulked to give fraction V (Figure 63).

The data for the entire fractionation are presented in Table 38. The enzyme was purified about 250-fold over the crude (S10) extract.

Figure 62. DEAE-cellulose chromatography of ATP-sulphurylase.

A DEAE-cellulose column (DE 11; 1.5 x 24.5 cm) was equilibrated with 50 mM Tris-HCl buffer (pH 7.5) at 0^{o}. Fraction III (Table 38) was loaded on to the column and eluted with a linear gradient of 150 ml each of 0.05 M and 0.25 M Tris-HCl buffer (pH 7.5) at a rate of 250-300 ml/hr; 5 ml fractions were collected in an automatic fraction collector.

Protein was determined by absorbance at 280 nm and ATP-sulphurylase activity determined as in Section 4.2.5.9.(a). The active fractions were pooled and concentrated by membrane ultrafiltration (Section 4.3.8) to give Fraction IV.

●———● E_{280}

Δ-----Δ ATP-sulphurylase activity

——— Concentration of Tris-HCl buffer

Figure 62

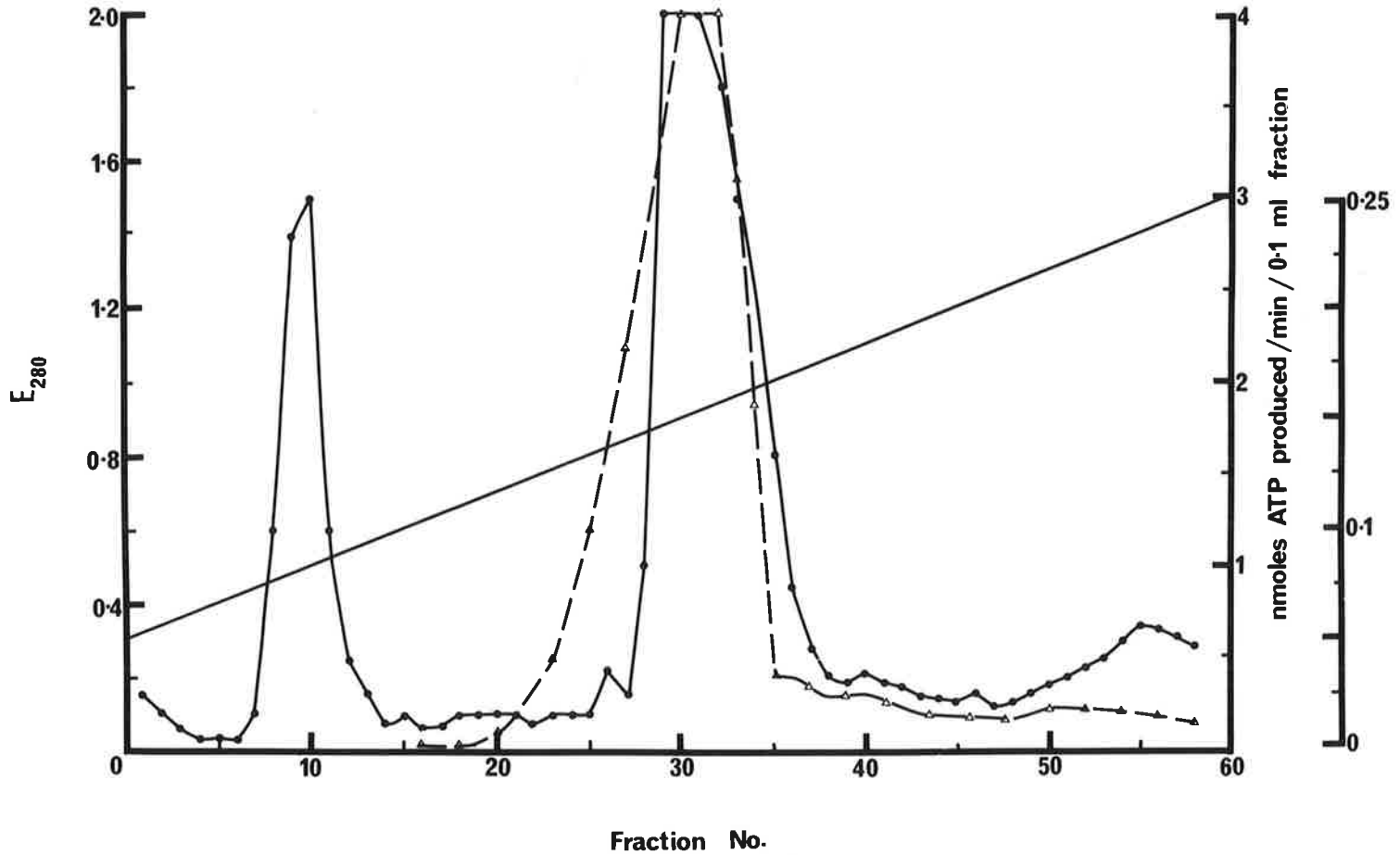

2·0
1·6
1·2
0·8
0·4
0
E_{280}
0
10
20
30
40
50
60
Fraction No.
4
3
2
1
nmoles ATP produced /min / 0·1 ml fraction
0·25
0·1
0
Tris-HCl concentration

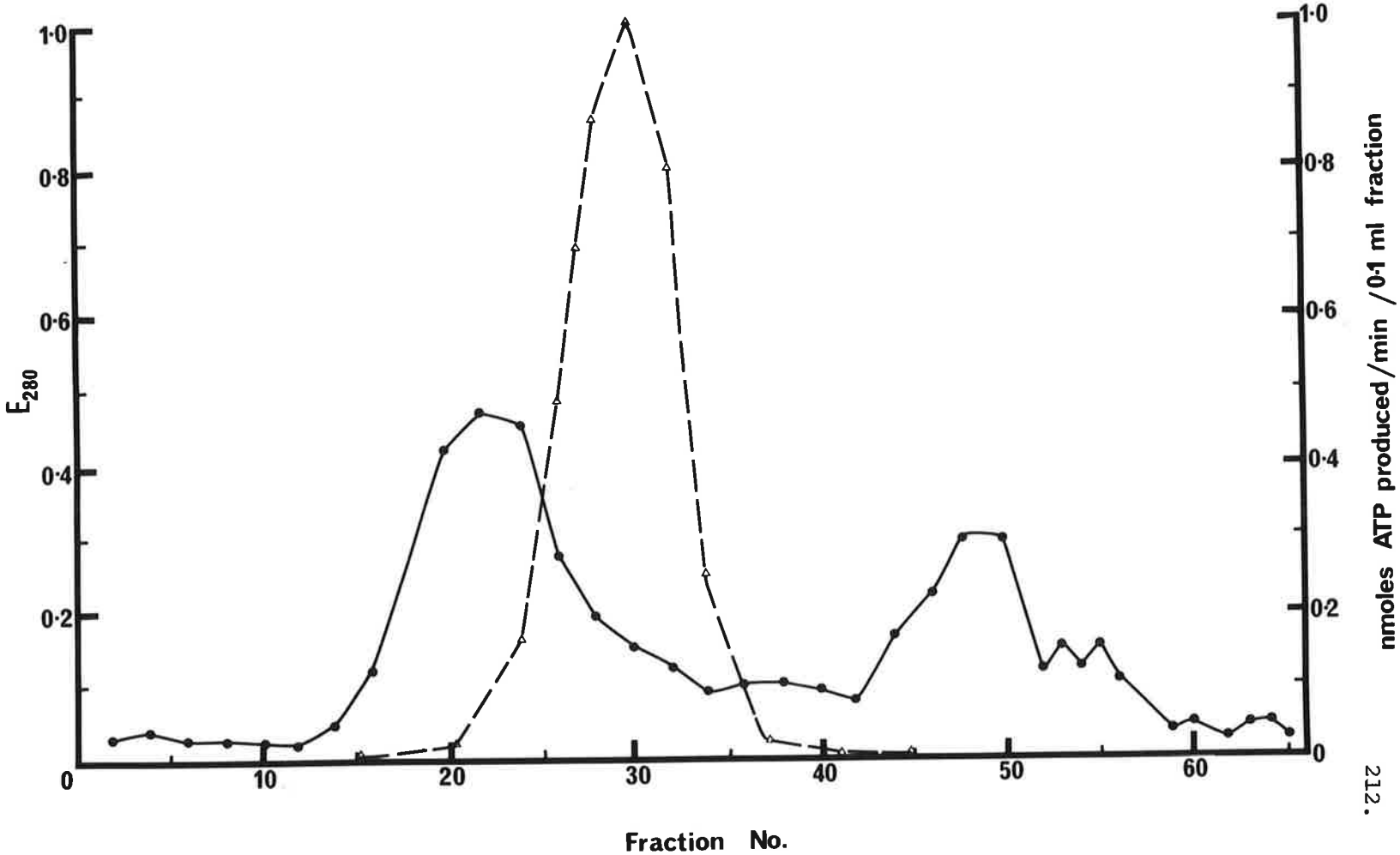
Figure 63. Sephadex G-200 gel-chromatography of ATP-sulphurylase.

Fraction IV (Table 38) was loaded on to a Sephadex G-200 column (45 x 2.5 cm) which had been equilibrated with 50 mM Tris-HCl buffer (pH 7.5). The enzyme was eluted with the same buffer at a rate of 30-35 ml/hr at a pressure head of 25 cm; 5 ml fractions were collected. Protein and enzyme activity were determined as for Figure 62.

●———● E_{280}

Δ-----Δ ATP-sulphurylase activity

Figure 63


1·0
0·8
0·6
0·4
0·2
0
E_{280}
10
20
30
40
50
60
Fraction No.
nmoles ATP produced/min /0·1 ml fraction

5.7.2 Properties

5.7.2.1 Distribution

All the activity was found in the supernatant fraction, after centrifuging the crude extract (S10) at 144,000 x *g* for 90 min (Table 36). ATP-sulphurylase was not detected in the P144 fraction which had been washed once with 50 mM Tris-HCl buffer (pH 7.5).

5.7.2.2 Purity of the enzyme

The enzyme obtained from DEAE-cellulose chromatography (Fraction IV, Table 38) was invariably green in colour. On reducing the enzyme with $Na_2S_2O_4$, a distinct band at 600 nm was formed (Figure 64A). The $Na_2S_2O_4$ reduced *versus* oxidized and the CO-$Na_2S_2O_4$ reduced *versus* $Na_2S_2O_4$ reduced difference spectra indicate that the green colour was due to cytochrome *a* (Figure 64B and C) as found in the S144 fraction (Section 5.5.2; Figure 51A and Figure 53B). However, this cytochrome was dissociated from the ATP-sulphurylase after Sephadex G-200 gel chromatography. The absolute spectrum of the purified enzyme (Fraction V, Table 38) indicates only one major absorption band at 280 nm. The purified enzyme was also devoid of APS-reductase, adenylate kinase and inorganic pyrophosphatase activities.

Figure 64. Absolute and difference spectra of partially purified ATP-sulphurylase (Fraction IV, Table 38).

Absorption spectra were recorded as described in Section 4.5.1.

A : Absolute spectrum, reduced with $Na_2S_2O_4$

B : Difference spectra, $Na_2S_2O_4$ reduced *versus* oxidized

C : Difference spectra, CO-$Na_2S_2O_4$ reduced *versus* $Na_2S_2O_4$ reduced.

Figure 65. Effect of incubation time on ATP-sulphurylase activity.

ATP-sulphurylase was assayed by the sampling bioluminescence procedure as described in Section 4.2.5.9.(b). Purified ATP-sulphurylase (Fraction V, Table 38) was incubated with the substrates for times ranging from 0 to 10 min.

Figure 64

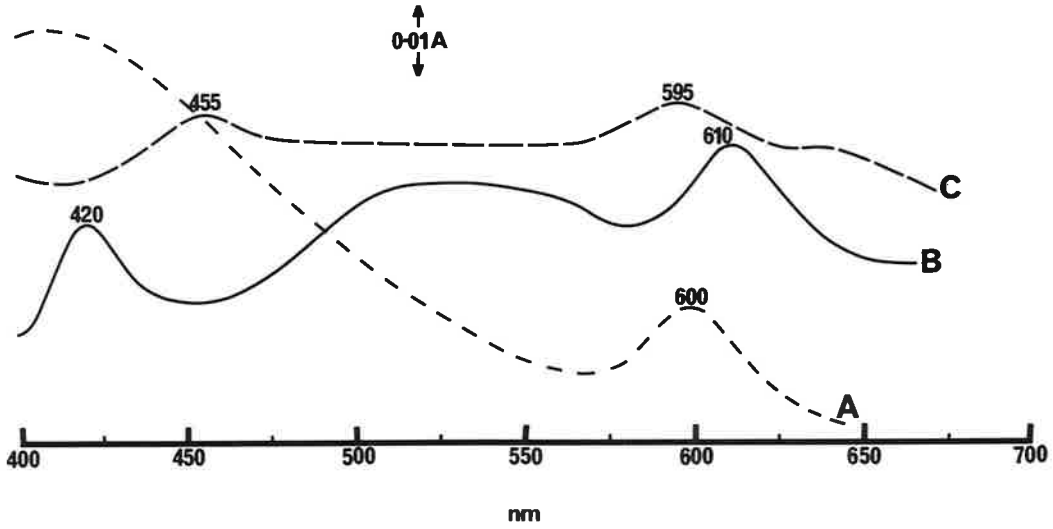
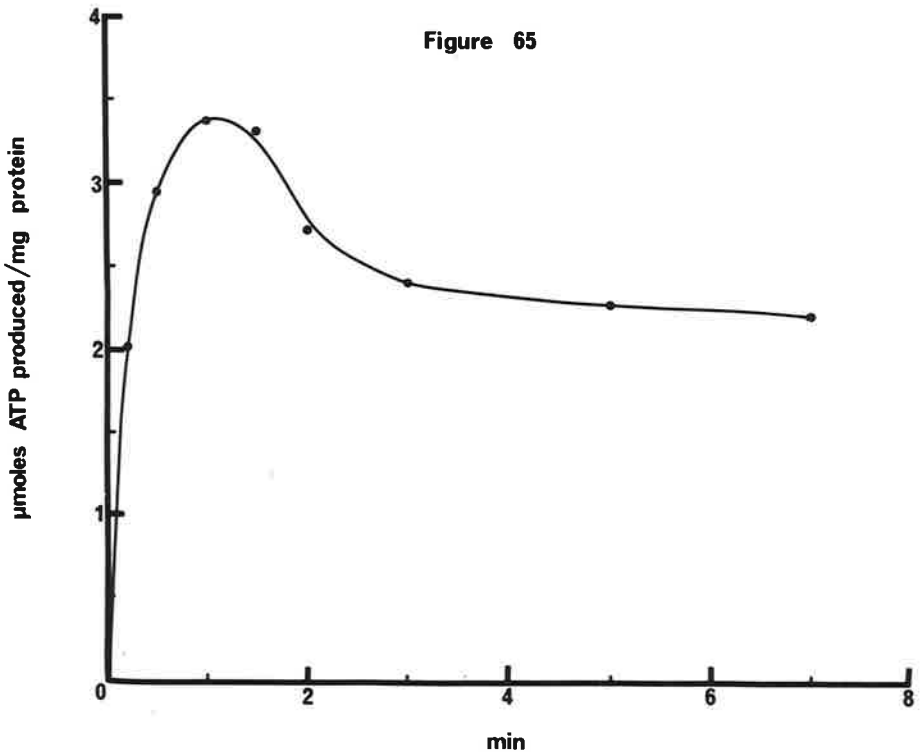


Figure 65



5.7.2.3 Effect of incubation time and composition of reaction mixture

The effects of periods of incubation on ATP sulphurylase measured by the sampling technique (Section 4.2.5.9(b)) is shown in Figure 65. The reaction was linear for the initial 0.5 min and reached a maximum after 1 min. When incubated for periods longer than 1 min there was a steady decline of enzyme activity. Thus, a one-minute incubation was used routinely to assay the enzyme.

Little or no ATP was produced in the absence of either Mg^{++}, PP_i or APS (Table 39). The specific activity obtained by the sampling bioluminescence method was 1,120 nmoles ATP formed/min/mg protein compared to 990 nmoles/min/mg protein determined by the continuous bioluminescence assay (Section 4.2.5.9(a)).

5.7.2.4 Effect of enzyme concentration, pH, temperature and inhibitors

The effect of enzyme concentration on the rate of ATP formation measured either by the continuous method or the sampling technique is shown in Figure 66.

ATP-sulphurylase is relatively insensitive to pH change with 50 mM Tris-HCl buffer since the activity was the same at pH 6.3 and 9.2 and was only 30% lower than that at the optimum pH 7.5

Table 39. Assay conditions for ATP-sulphurylase.

The reaction mixture contained 0.25 µmoles APS, 0.2 µmoles sodium pyrophosphate, 0.5 µmoles $MgCl_2$ and the purified ATP-sulphurylase (Fraction V, Table 38) made up to a final volume of 1 ml with 50 mM Tris-HCl buffer (pH 7.5). The reaction was conducted as described in Section 4.2.5.9.(b). In boiled preparations the enzyme was immersed in a boiling water bath for 3 min and then cooled down before adding the other reactants.

Conditions	Specific activity
Complete	2.1
Boiled enzyme	0.03
Omit enzyme	0
Omit $MgCl_2$	1.1
Omit PP_i	0.03
Omit APS	0
P_i substituted for PP_i	0.02

Specific activity: nmoles ATP produced/min/mg protein.

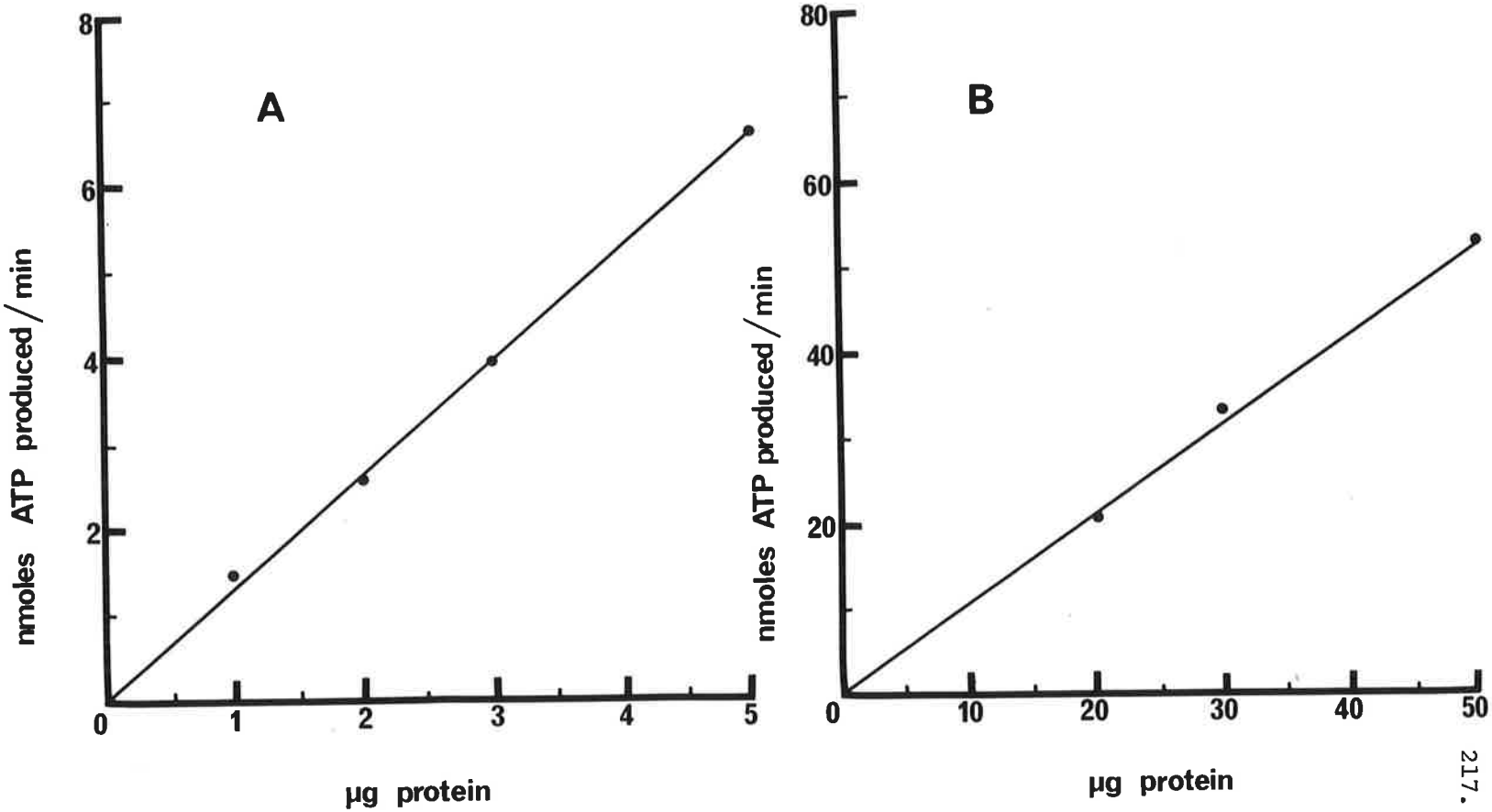
Figure 66. Effect of enzyme concentration on ATP-sulphurylase activity.

ATP-sulphurylase (Fraction V, Table 38) was determined by the continuous as well as the sampling techniques (Section 4.2.5.9).

A : Continuous method

B : Sampling method

Figure 66


A
B
nmoles ATP produced / min
nmoles ATP produced / min
µg protein
µg protein
0
1
2
3
4
5
10
20
30
40
50
2
4
6
8
20
40
60
80

(Figure 67).

The effect of temperature on enzyme activity is shown in Figure 68. The optimum temperature was around 30-40°. The enzyme was inactivated after 3 min incubation at 85°.

The effect of some inhibitors on the purified enzyme has been considered in Section 5.6.3 (Table 37).

5.7.2.5 Effect of APS and PP_i

The rate of production of ATP was measured at various concentrations of PP_i and APS using the purified ATP-sulphurylase (Fraction V, Table 38). The results show normal Michaelis-Menten kinetics and in each case there is a linear relation between ATP production and substrate concentration in the lower ranges (up to 10 µM with APS and 1 µM with PP_i). High levels of PP_i inhibited the enzyme, e.g. at 10 µM PP_i, the activity was about 50% of that obtained with 1 µM PP_i. However, high levels of APS had little effect on the enzyme up to a concentration of 100 µM. The calculated Km values are 5.5 µM for APS and 0.2 µM for PP_i (Figure 69, Figure 70).

5.7.2.6 Molecular weight determination

The apparent molecular weight of ATP-sulphurylase was determined by gel-filtration using Sephadex G-200 following the method of Andrews (1965) (Section 4.2.7). The

Figure 67. Effect of pH on ATP-sulphurylase activity.

Tris-HCl buffer (50 mM) at pH values ranging from 6.3 to 9.2 was used for the assay of ATP-sulphurylase (Fraction V, Table 38) by the sampling bioluminescence technique (Section 4.2.5.9.(b)).

Specific activity: nmoles ATP produced/min/mg protein.

Figure 68. Effect of temperature on ATP-sulphurylase activity.

The sampling bioluminescence technique was used to determine ATP-sulphurylase activity (Section 4.2.5.9.(b)). The enzyme (Fraction V, Table 38) was incubated for 3 min at the temperature specified prior to starting the reaction. The buffer, 50 mM Tris-HCl, was adjusted to pH 7.5 for each temperature.

Specific activity: nmoles ATP produced/min/mg protein.

Figure 67

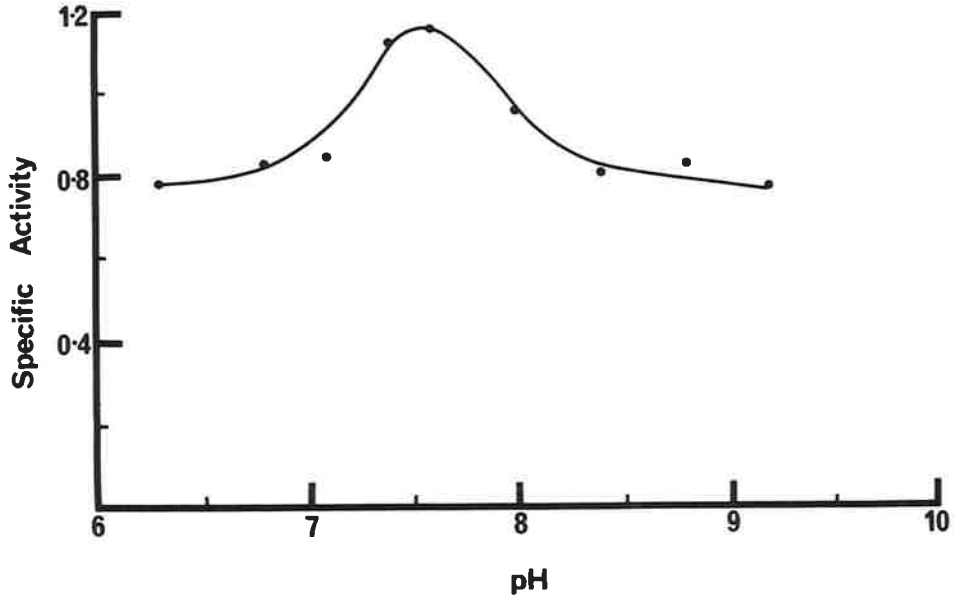


Figure 68

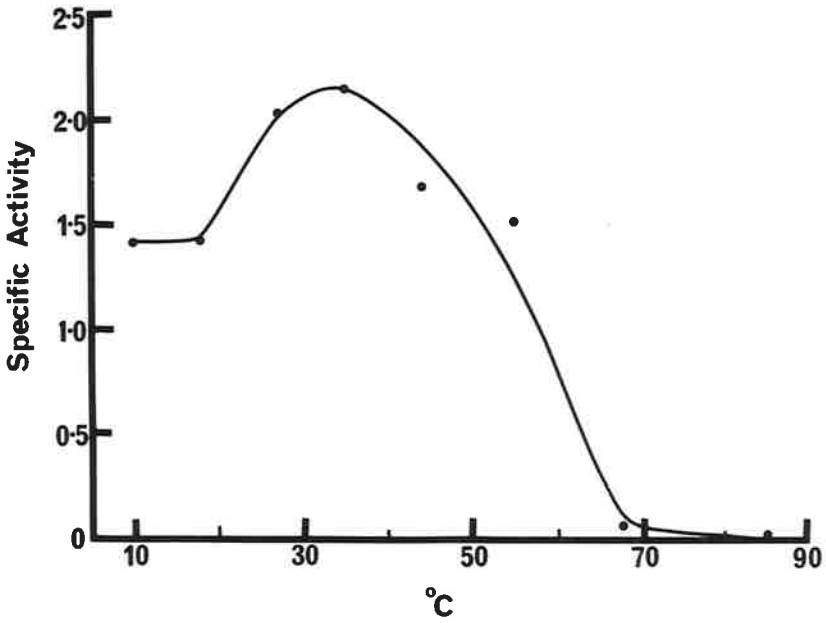


Figure 69. Effect of inorganic pyrophosphate concentration on ATP-sulphurylase activity.

The activity of ATP-sulphurylase (Fraction V, Table 38) was determined in the presence of varying amounts of inorganic pyrophosphate as described in Section 4.2.5.9.(a). The level of APS was fixed at 20 µM.

The Km for pyrophosphate was found to be 0.2 µM.

Figure 70. Effect of APS concentration on ATP-sulphurylase activity.

ATP-sulphurylase was determined as described in Section 4.2.5.9.(a). The purified enzyme (Fraction V, Table 38) was incubated with APS at the concentration indicated. Inorganic pyrophosphate was fixed at 2 µM.

The Km for APS was found to be 5.5 µM.

Figure 69

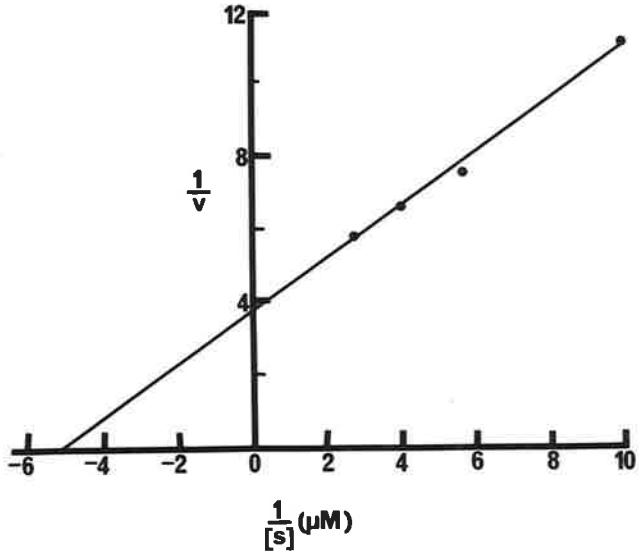
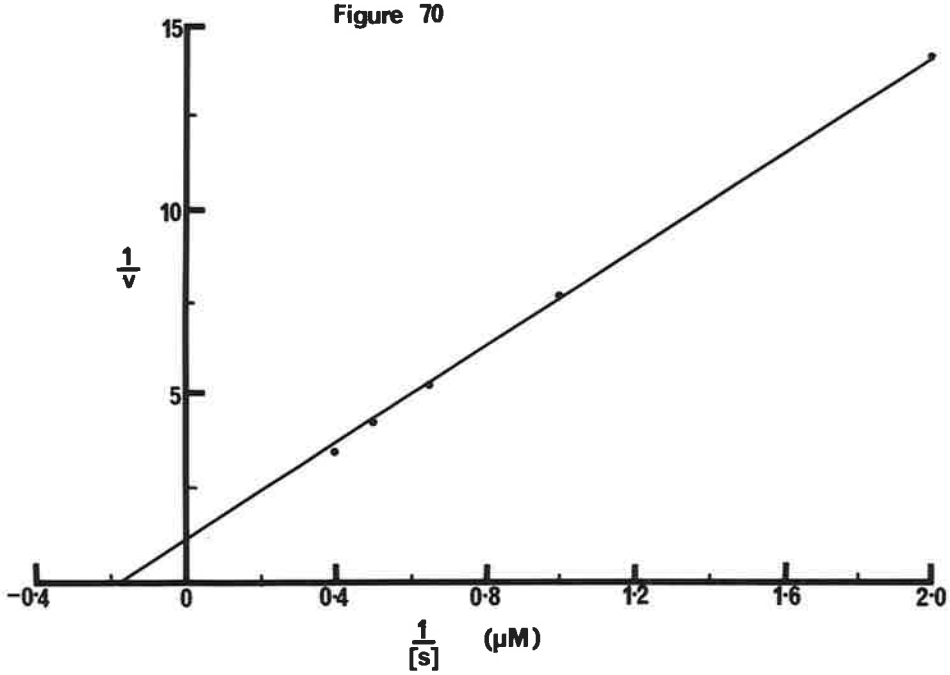


Figure 70



distribution coefficient of the enzyme (Kd) calculated according to the formula given in Section 4.2.7 was found to be 0.175 (Figure 40). This gave a molecular weight of 285,000 ± 5,000.

5.7.2.7 Stability of enzyme

The purified enzyme (Fraction V, Table 38) was comparatively unstable at 0^{o}. Only 5% of the activity remained after 2 weeks. No activity was detected after 4 weeks at 0^{o}. The enzyme was inactivated less rapidly when stored at -15^{o}; half of its activity remaining after 4 weeks at this temperature.

6. DISCUSSION

6.1 The Oxidation of Sulphide to Sulphite

That sulphide oxidation in *Thiobacillus denitrificans* is mediated by enzymes is supported by several lines of evidence including kinetic data and the inactivity of boiled preparations. This is in accord with the observations of Moriarty & Nicholas (1969) in *T. concretivorus*, but contrasts with those of Adair (1966) who reported a heat-stable sulphide oxidizing system in *T. thioparus* and *T. thiooxidans*. Unlike *T. concretivorus* (Moriarty & Nicholas, 1969), oxygen uptake in crude extracts and particulate fractions of *T. denitrificans* continued after all sulphide had disappeared indicating that the products of sulphide oxidation were further oxidised by enzymes.

The Km for sulphide measured by various methods ranged from 0.1 mM to 0.8 mM. This range of values is higher than that obtained with *T. concretivorus* (Moriarty & Nicholas, 1969). A possible explanation for this difference is that in the present study the P144 fraction was used whereas in experiments with *T. concretivorus* a crude SO_{20} preparation was the enzyme source.

The pH optimum for sulphide oxidation is 7.5 as determined by the uptake of O_2 and the reduction of nitrite. Based on the

pK_2 of H_2S

$$HS^- \rightleftharpoons H^+ + S^{2-}, \quad pK_2 = 11.96$$

(Weast & Selby, 1966)

and applying the Handerson-Hasselback equation, it is known that HS^- is the predominant ionic species; this suggests that HS^- may be the form of the reductant for nitrite reductase.

The early product of sulphide oxidation is probably bound to the lipoprotein membrane fraction. This is suggested from the results of experiments with ^{35}S-sulphide. Moriarty & Nicholas (1970*A*) found a considerable degradation of ^{35}S-sulphur when it was run in the standard electrophoretic system. The fact that the ^{35}S-labelled material formed by cell extracts of *T. denitrificans* was stable on electrophoresis indicates that elemental sulphur was unlikely to be a product.

It is known that the absorption bands of polysulphides shift to longer wavelengths as the number of conjugated sulphur atoms increases (Baer & Carmack, 1949). The observation that the products of sulphide oxidation absorb at longer wavelengths (λ_{max} 315 nm) than does colloidal sulphur (λ_{max} 305 nm) suggests that the number of sulphur atoms polymerised to form polysulphides exceeds that in colloidal sulphur. The probable formation of

elemental sulphur on oxidizing the membrane-bound polysulphides with iodine, resulting in a shift in the absorption band, is in accord with the proposal that polysulphides are formed from sulphide by the membrane fraction (P144). This membrane-bound intermediate also reacts with cyanide to give products expected from reactions between a nucleophilic reagent and a polymeric sulphur compound. The decrease in absorbance between 300 and 350 nm when cyanide reacts with the oxidation product of sulphide also supports the view that the spectra are those of polymeric sulphur compounds. Sulphite, though a strong nucleophilic reagent, reacted with the membrane-bound polysulphide intermediate only after it was dissociated from the membrane fraction by treatment with acetone. This effect, which was not observed in *T. concretivorus* (Moriarty & Nicholas, 1969), has physiological significance since sulphite is an intermediate during the oxidation of sulphide in *T. denitrificans*. Sulphite, however, is not unequivocally established as an intermediate during sulphide oxidation in *T. concretivorus* and the sulphite oxidizing enzymes, namely APS-reductase and sulphite-linked nitrate reductase, were not detected in this bacterium (Moriarty, 1969).

The cyanolysis of elemental sulphur involves an initial, rate-limiting nucleophilic attack by CN^- on the S_8 ring followed

by a series of fast displacements of the weakly nucleophilic thiocyanate ion by cyanide (Foss, 1950; Bartlett & Davis, 1958). This reaction is rapid in organic solvents. However, in aqueous solutions and at room temperature, i.e. under experimental conditions of the present study, the scission of the S_8 ring does not readily occur with CN^-. A straight chain polymeric sulphur compound is much more reactive. Since the membrane-bound polymeric sulphur intermediate formed during the enzymic oxidation of sulphide reacts rapidly with cyanide, it is more likely to be a linear chain of polymeric sulphur, i.e. polysulphide, rather than elemental sulphur.

The oxidation of sulphide to polysulphide was inhibited by CO and this effect was reversed by light. Furthermore, O_2 uptake and nitrite reduction coupled to sulphide oxidation were inhibited by compounds that adversely affect electron transport (see Section 6.4). These observations suggest that an electron transport chain with either O_2 or nitrite as the terminal electron acceptor is required for the formation of the u.v. light-absorbing, membrane-bound, polymeric sulphur compound.

The nitrite reductase, which utilizes sulphide as an electron donor, was found to be associated with the membrane fraction, P144. This agrees with the finding that the sulphide-oxidizing system, as determined by the sulphide and O_2 electrode

methods, is also located in the membrane fraction. Other sulphur compounds did not serve as donors for nitrite reductase, but, under anaerobic conditions, NADH, in the presence of PMS, was effective. A nitrite reductase from *M. denitrificans*, which utilizes NADH as an electron donor, was found to have a cytochrome oxidase activity (Lam & Nicholas, 1969*C*). The fact that in *T. denitrificans*, sulphide is oxidized to polymeric sulphur compounds in air or under anaerobic conditions in the presence of nitrite suggests that the sulphide-linked nitrite reductase may also function as a cytochrome oxidase.

Nicholas (unpublished results) has recently solubilized the sulphide-linked nitrite reductase system of *T. denitrificans* by lowering the pH of the P144 fraction to around 5. Thus, the way is now open for a purification and characterization of this system.

The use of the sulphide and oxygen electrode as well as spectrophotometric techniques in studies reported herein are superior to the manometric method for oxygen uptake used to investigate sulphide oxidation by previous workers (London & Rittenberg, 1964; Adair, 1966; Suzuki, 1965 *A*, *B*; Silver & Lundgren, 1968; Taylor, 1968) since these methods measure single enzymic reactions (i.e. sulphide $\longrightarrow$ polysulphide). Furthermore, the unique feature of *T. denitrificans* in utilizing

reduced sulphur compounds as reductants for the denitrification process has a particular advantage since the oxidation of sulphide and sulphur may be followed by linking them to the reduction of nitrate or nitrite.

The oxidation of polysulphide and elemental sulphur in *T. denitrificans* is apparently catalysed by the same enzyme system located mainly in the soluble fraction of the cell.

The increased rate of oxygen uptake when the S144 fraction was added to the P144 fraction during sulphide oxidation may be attributed to the oxidation of the intermediate polysulphide. Saxena & Aleem (1973) have reported that in *T. neapolitanus* sulphide oxidation proceeds in two stages but unlike in *T. concretivorus* (Moriarty & Nicholas, 1969) the second stage of oxygen uptake, associated with the oxidation of polysulphide is catalysed by the S144 fraction.

The absolute requirement for GSH during the oxidation of elemental sulphur indicates that polysulphide is indeed the substrate for the sulphur oxidizing enzyme in *T. denitrificans* as suggested by Suzuki for *T. thiooxidans* (1965*A*). In addition to GSH, a soluble, low molecular weight, heat-stable factor was also essential for sulphur oxidation. Kodama (1969) has found that in *T. thiooxidans* this factor may be replaced by NAD or NADP.

Adair (1966) reported that the sulphur oxidizing enzyme in *T. thiooxidans* is particulate in nature. On the contrary, other workers have shown that the enzyme is located in the soluble fraction (Suzuki & Silver, 1966; Tano & Imai, 1968), as indeed is the case in *T. denitrificans*. Roy & Trudinger (1970) suggest that the soluble enzyme might arise by degradation of a co-ordinated particulate enzyme complex catalysing the complete oxidation of sulphur to sulphate.

The initial product of sulphur oxidation in *T. denitrificans* is sulphite, which was also shown to be so in *T. thiooxidans* (Suzuki & Silver, 1966). Thus, in *T. denitrificans* the oxidation of sulphide and elemental sulphur is linked to nitrate reduction via sulphite. When crude extracts (S10) were incubated with sulphide and nitrate under aerobic conditions, sulphide was initially and almost completely converted to polysulphide because of the presence of O_2. This has been shown to be a fast reaction (about 2 min) utilizing most of the sulphide. Polysulphide is then oxidized at a slower rate to sulphite, which then reduces nitrate to nitrite enzymically. Nitrite accumulates and cannot be reduced further even though an active nitrite reductase is present because the electron donor (S^{2-}) has been depleted. Nitrate reductase using sulphide as an electron donor cannot be detected when the experiment is conducted under anaerobic

conditions because O_2 is now not available for the first step ($S^{2-} \longrightarrow$ polysulphide). Consequently, sulphite is not formed and nitrate is not reduced to nitrite. Further evidence that sulphite is indeed the intermediate comes from the observation that in the presence of nitrate, sulphite was not produced from sulphide.

Trudinger (1967) postulated that sulphide is initially bound to a thiol group prior to its oxidation. This may also be the case in *T. denitrificans* since thiol-binding reagents inhibited O_2 uptake and nitrite reduction coupled to the oxidation of sulphide. Furthermore, these sulphydryl inhibitors affected the reduction of cytochromes by sulphide.

Thus, the overall oxidation of sulphide may be represented as follows:

$$nHS^- + X\text{-}SH + \frac{n}{2} O_2 \longrightarrow X - Sn - SH + nOH^- \qquad (1)$$

where X-SH is the acceptor protein.

When coupled to nitrite as an electron acceptor the reaction may be expressed as:

$$nHS^- + nNO_2^- + X\text{-}SH + nH_2O \longrightarrow 2nOH^- + n[NOH] + X\text{-}Sn\text{-}SH \qquad (2)$$

This would explain the observed stoichiometry of one mole of sulphide oxidized per mole of nitrite reduced and one mole of oxygen utilized for every two moles of sulphide oxidized.

The mechanism for oxidation of elemental sulphur postulated by Suzuki (1965 *A*, *B*) and Suzuki & Silver (1966) may also function in *T. denitrificans* as follows:

$$GSH + S_8 \longrightarrow G - S_8 - SH \quad (3)$$

$$G - S_8 - SH + O_2 \longrightarrow G - S_8 - SO_2H \quad (4)$$

$$G - S_8 - SO_2H + OH^- \longrightarrow G - S_7 - SH + HSO_3^- \quad (5)$$

First, GSH reacts non-enzymically with elemental sulphur to form a polysulphide (equation 3). This then becomes indistinguishable from the membrane-bound polysulphide produced by the oxidation of sulphide (equations 1 and 2) and may thus be oxidized by the same enzyme. A sulphonate group is formed at the end of the polysulphide chain (equation 4) which is then split off as sulphite (equation 5). Sulphite may then be oxidized by the two sulphite oxidizing enzymes (Section 6.2).

6.2 Sulphite Oxidation

Both AMP-dependent and AMP-independent sulphite-oxidizing enzymes are present in *T. denitrificans*. They are located in different parts of the cell; thus sulphite oxidase is associated with membrane fractions whereas APS-reductase is found in the more soluble fractions.

The particulate sulphite oxidase is linked to electron transport with nitrate or oxygen as a terminal acceptor. Evidence for this is provided in studies with inhibitors of electron transport which agree well with the results of Adams, Warnes & Nicholas (1971*A*). The inhibition of O_2 uptake by nitrate further indicates that the two terminal electron acceptors are associated with the same electron transport chain.

Some properties of the purified sulphite oxidase from *T. denitrificans* were found to be similar to those from *T. thioparus* (Lyric & Suzuki, 1970*A*) and *T. novellus* (Charles & Suzuki, 1966*B*). The enzyme from *T. denitrificans* differed from those from the other two thiobacilli in that it was associated with cell particles, it linked more effectively to ferricyanide than to mammalian or yeast cytochrome *c* and was not inhibited by either Tris-HCl or phosphate buffers. The strong inhibition by thiol-binding reagents and the complete reversal of this effect by dithiothreitol suggests the involvement of -SH groups. A comparison of the properties of sulphite oxidase from various thiobacilli is presented in Table 40.

Trudinger (1969) proposed a mechanism involving the formation of an enzyme-sulphite complex which could react either with water to produce sulphate or with AMP to form APS. He further suggests that sulphite oxidase is a modified APS-reductase

Table 40. AMP-independent sulphite oxidizing enzymes from various thiobacilli.

Unless otherwise stated, enzyme activity was determined by the ferricyanide method.

Source	Distribution	Effect of AMP	Electron acceptors	Fold purification	Km for $SO_3^=$	Reference
T. thioparus	Soluble	Inhibited by 21% (cyt. *c* coupled)	cyt. *c* ferricyanide	160 (cyt. *c* coupled)	88 µM	1
T. thiooxidans	Particulate	-	O_2	not purified	-	2, 3, 4
T. novellus	Soluble	Inhibited by 25% (cyt. *c* coupled)	O_2 ferricyanide cyt. *c*	74 (cyt. *c* coupled)	20 µM 40 µM (cyt. *c* coupled)	5
T. neapolitanus	Soluble	Stimulated by 80%	O_2	100 (O_2 uptake)	-	6
T. concretivorus	Particulate	Not affected	O_2 ferricyanide	100	1 mM 0.3 mM (O_2 uptake)	7
T. ferrooxidans	Soluble	Not affected	ferricyanide cyt. *c*	7.3	0.54 mM 0.58 mM (cyt. *c*)	8
T. denitrificans	Particulate	Not affected	O_2 ferricyanide nitrate	48	0.5 mM 0.4 mM (O_2 uptake)	Present study

References: (1) Lyric & Suzuki (1970*B*); (2) London & Rittenberg (1964); (3) Adair (1968); (4) Kodama & Mori (1968); (5) Charles & Suzuki (1966*B*); (6) Hempfling, Trudinger & Vishniac (1967); (7) Moriarty (1969); (8) Vestal & Lundgren (1971).

which has lost the ability to react with AMP. Some evidence for this idea is provided by Hempfling, Trudinger & Vishniac (1967), who purified sulphite oxidase from *T. neapolitanus* and found that the relative activities with and without AMP remained fairly constant throughout the purification procedure. These authors concluded that the same enzyme is responsible for both the AMP-dependent and AMP-independent activities. However, the results presented in this thesis and those of Lyric & Suzuki (1970 *A*, *B*) show unequivocally that APS-reductase and sulphite oxidase are distinct enzymes.

Lyric & Suzuki (1970*B*) have found that APS-reductase comprises about 3% of the cell protein in *T. thioparus* while the reported value for *T. denitrificans* is 4-5% (Bowen, Happold & Taylor, 1966). The presence of large amounts of the enzyme strongly suggests that it has an important function in the energy metabolism of the thiobacilli (see Section 6.5). Indeed, the high activity of the enzyme has been exploited for the large scale production of APS (Adams, Warnes & Nicholas, 1971*B*).

Purified APS-reductase from *T. denitrificans* utilized only ferricyanide as an electron acceptor although cytochrome *c* has been shown to be effective also in *T. thioparus* (Lyric & Suzuki, 1970*B*). However, crude extracts of *T. denitrificans* synthesized APS from AMP

and sulphite in the presence of nitrate, although the activity was only about 10% of that with ferricyanide.

Although Bowen, Happold & Taylor (1966) have reported that thiol-binding reagents do not inhibit APS-reductase, the present work confirms that these sulphyldryl inhibitors affect the enzyme as shown by Lyric & Suzuki (1970*B*) in *T. thioparus*. A comparison of the properties of purified sulphite oxidase with that of purified APS-reductase from *T. denitrificans* is presented in Table 41.

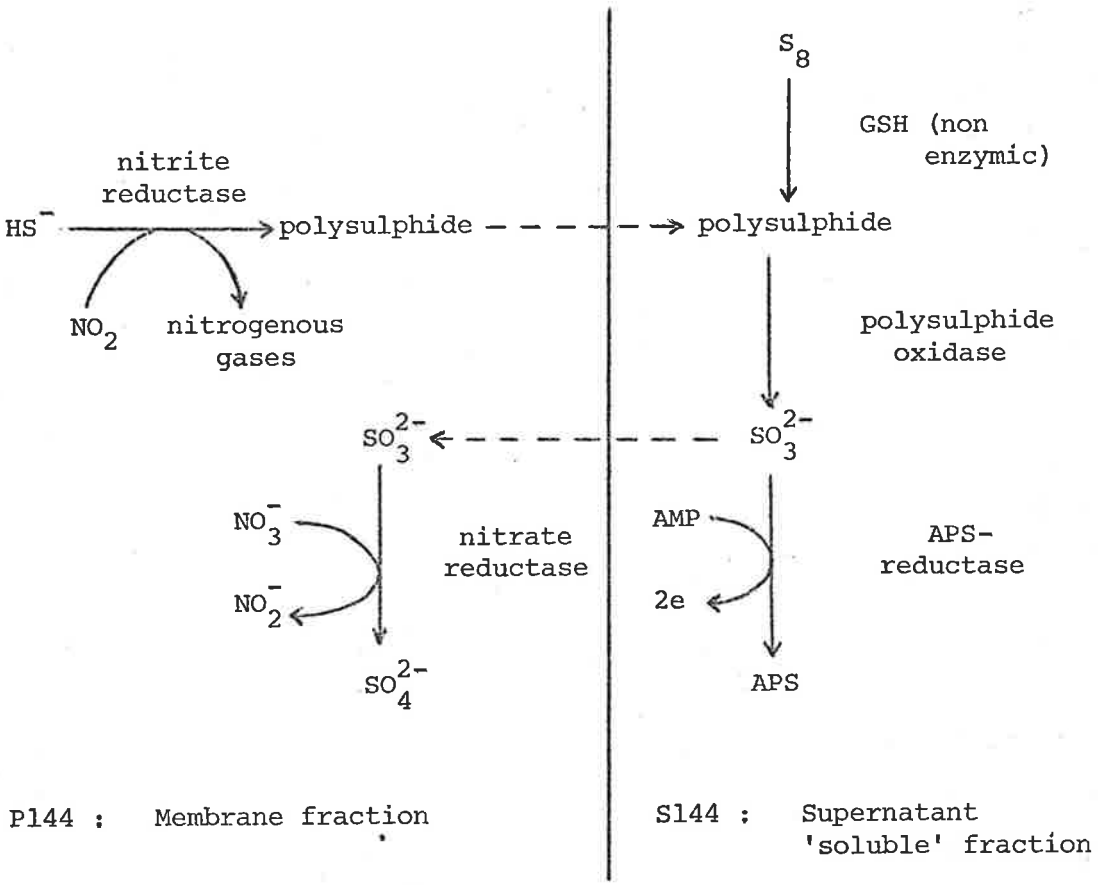
The physiological significance of APS-reductase and sulphite oxidase in *T. denitrificans* in this work is unclear. Lyric & Suzuki (1970*C*) considered that two enzymes operating at different pH optima (7.5 and 9.5) would be more effective in oxidizing sulphite in *T. thioparus*. In *T. denitrificans* each enzyme is closely linked to an ATP-generating system (Section 6.5). The association of sulphite oxidase with membrane fractions may be of particular significance since the enzyme is closely associated with a sulphide oxidizing enzyme, a thiosulphate-cleaving enzyme and the denitrifying enzymes, nitrate and nitrite reductases, all of which are membrane-bound. On the other hand, the enzymes with which APS- reductase is closely involved in the production of ATP by substrate level phosphorylation are located in the soluble

Table 41. Comparison of APS-reductase and sulphite oxidase from *Thiobacillus denitrificans*.

Properties	APS reductase	Sulphite oxidase
Distribution	Soluble	Particulate
Product	APS	Sulphate
Specific activity (µmoles ferricyanide reduced/hr/mg protein)	498	183
pH optimum	7.5	8.3
Km for sulphite	1.5 mM	0.5 mM
Molecular weight	250,000	94,000
Stability to heat	Comparatively heat-stable	Comparatively heat-labile

fraction (S144).

The complete oxidation of sulphide, elemental sulphur and sulphite is summarized in the following scheme:



6.3 Thiosulphate Oxidation

Oxygen was readily utilized by cells of *T. denitrificans* when thiosulphate was the electron donor. Upon breaking the cells, however, oxygen consumption was extremely low. Similar

results were also obtained with thiosulphate-linked nitrate reductase. This may not be due simply to the loss of structural integrity of the membrane since very little of the sulphide and sulphite oxidizing enzyme systems was impaired upon cell breakage. In cell-free extracts thiosulphate oxidation measured by O_2 uptake and sulphite production required the addition of GSH or other thiol reagent. It is likely then that the loss in thiosulphate oxidizing activity upon cell breakage results from the oxidation of membrane-bound thiol groups which are essential for the cleavage of thiosulphate to sulphide and sulphite. Fragmentation of the membrane fraction would tend to expose the thiol groups more to oxidizing agents.

Le John *et al.* (1967) found that the enzymes involved in thiosulphate metabolism in the thiobacilli can be simultaneously induced and repressed and concluded that the thiosulphate oxidizing and cleaving enzymes are not involved in the same pathway. Supporting evidence for this idea in the present study was obtained from experiments with differentially labelled ^{35}S-thiosulphate. In the presence of nitrate tetrathionate was formed from thiosulphate, labelled either in the inner or outer sulphur atom. This may be explained in the following equation:

$$2S_2O_3^{=} + NO_3^{-} \longrightarrow S_4O_6^{=} + NO_2^{-} \qquad (6)$$

When GSH was substituted for nitrate, the inner (oxidized) sulphur atom of thiosulphate was oxidized mainly to sulphite while the outer (reduced) sulphur atom was converted to sulphide, as shown in the following equation:

$$^{35}S - SO_3^{=} \xrightarrow{GSH} {}^{35}S^{=} + SO_3^{=} \qquad (7)$$

The sulphide formed may then be oxidized to polysulphide. Thus, the reduction of nitrate to nitrite with thiosulphate as an electron donor (GSH not required) may be taken as a measure of thiosulphate oxidizing enzyme whereas the production of sulphite from thiosulphate (GSH required) may be used to assay the thiosulphate-cleaving enzyme. Equations 6 and 7 may also explain the observed stoichiometry of 3 moles of thiosulphate utilized for each mole of sulphite and nitrite produced, assuming that the two enzymes act independently of each other.

The optimum pH for thiosulphate oxidation in *T. thioparus* (Lyric & Suzuki, 1970*C*), and *T. concretivorus* (Moriarty, 1969) was reported to be around 5. Both the oxidizing and cleaving enzymes for thiosulphate in *T. denitrificans* were also maximal at pH 5. Although sulphite was produced from thiosulphate in the presence of nitrate at pH 5, the latter was not likely to be reduced to nitrite by sulphite since the sulphite-linked nitrate

reductase, which has an optimum pH of 8.5, was not detectable at pH 5.0.

Various mechanisms have been advanced to 'unify' the two hypotheses for thiosulphate oxidation (Lees, 1960; Vishniac & Trudinger, 1962; Trudinger, 1967, 1969). However, the present study indicates that thiosulphate oxidation and thiosulphate cleavage may well be two distinct events.

Thiol groups are essential for thiosulphate cleavage. This may be generated by GSH or other sulphyldryl reagents as follows:

$$E\text{-}S\text{-}S\text{-}E + GSH \longrightarrow 2E\text{-}SH \qquad (8)$$

where E is the cleaving enzyme. The outer (reduced) sulphur atom of thiosulphate becomes attached to the enzyme while the inner (oxidized) sulphur atom is released as sulphite:

$$E\text{-}SH + {}^{-}\overset{*}{S}\text{-}SO_3^{-} \longrightarrow E\text{-}S\text{-}\overset{*}{S}H + SO_3^{=} \qquad (9)$$

Sulphide may then be released from the enzyme

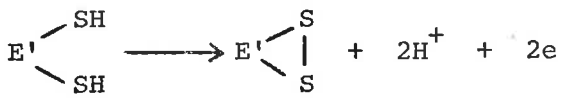
$$E\text{-}S.\overset{*}{S}H + E\text{-}SH \longrightarrow E\text{-}S\text{-}S\text{-}E + H_2\overset{*}{S} \qquad (10)$$

However, it is more likely that a polysulphide (formed from sulphide oxidation) may accept the sulphur atom, thus lengthening the polysulphide chain:

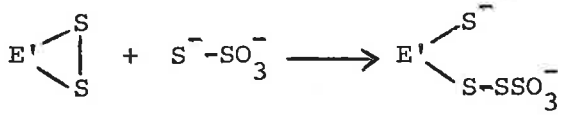
$$E\text{-}S.\overset{*}{S}H + X\text{-}Sn\text{-}SH \longrightarrow E\text{-}SH + X\text{-}S_{n+1}\text{-}\overset{*}{S}H \qquad (11)$$

The scheme proposed in equations 8-11 for thiosulphate cleavage is similar to the more elaborate scheme proposed for the rhodanese enzyme (Bowen, Butter & Happold, 1966).

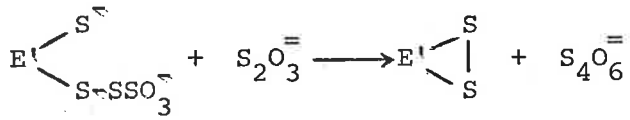
The mechanism for thiosulphate oxidation may be explained by a hypothesis originally proposed by Lees (1960) if it is assumed that an oxidizing agent (such as NO_3^- or ferricyanide) oxidizes the enzyme thiol groups to a disulphide. A possible series of reactions is as follows:



(12)



(13)



(14)

where E' is the thiosulphate oxidizing enzyme.

All these reactions are, of course, speculative and further work is required to identify and characterize the enzyme systems

and the intermediates involved.

6.4 Electron Transfer Systems

The formation of the 300-350 nm absorbing polysulphide from sulphide and the reduction of nitrite by sulphide in the P144 fraction are inhibited by CO. The latter effect, which was reversed by light, suggests that a terminal oxidase is involved. Reduced and CO-reduced spectra indicate that this terminal cytochrome is probably of the *d* type. Milhaud, Aubert & Millet (1958) found an a-a_3-type cytochrome which Peeters & Aleem (1970) suggest is involved in sulphide oxidation linked to either O_2 or nitrate as a terminal acceptor in *T. denitrificans*. In the present study, however, the a-a_3 complex was not detected in the P144 fraction which contained the sulphide-oxidizing activity. Peeters & Aleem (1970) did not report a cytochrome of the *d* type although a maximum at 475 nm, which is the Soret band of this cytochrome, was present in their reduced minus oxidized difference spectra. Unfortunately, absorbance above 600 nm was not recorded by them where the α-bands of cytochrome *d* occur (620 and 675 nm). These authors also suggest that cytochrome *o* may be involved in sulphide oxidation but this cytochrome was not detected in the studies reported in this thesis.

The α-bands of the CO-reduced haem *d* are similar to those found in other bacteria (Lam & Nicholas, 1969*C*; Newton, 1969; Yamanaka & Okinuki, 1963). Yamanaka & Okunuki (1963) showed that the Soret bands of cytochrome *d* in *Pseudomonas aeruginosa* had a relatively low absorption compared with other cytochromes. This may account for the failure to detect it in cell-free extracts of *T. denitrificans*.

A dissimilatory nitrite reductase from *Ps. aeruginosa* (Yamanaka & Okunuki, 1963) and *M. denitrificans* (Lam & Nicholas, 1969), which also functions as a cytochrome oxidase, contains cytochromes of the *c* and *d* types, probably in one complex. The fact that these cytochromes in the P144 fraction of *T. denitrificans* are reduced by sulphide and reoxidized by either oxygen or nitrite suggests that they are associated with a nitrite reductase which also has a cytochrome oxidase activity. Inhibitor studies support the view that sulphide oxidation is linked to O_2 or nitrite via a respiratory chain with the cytochrome *cd* complex as a terminal oxidase.

The effectiveness of bathocuproin and DIECA in inhibiting sulphide oxidation suggests that copper may be involved in sulphide oxidation. However, unlike the system found in *T. concretivorus* (Moriarty & Nicholas, 1970*B*), DIECA did not inhibit the reduction

of cytochrome by sulphide, indicating that the site of action of this metal is at the end of the respiratory chain. It is of interest that nitrite reductases from other bacteria (Yamanaka, 1964) have been shown to contain copper.

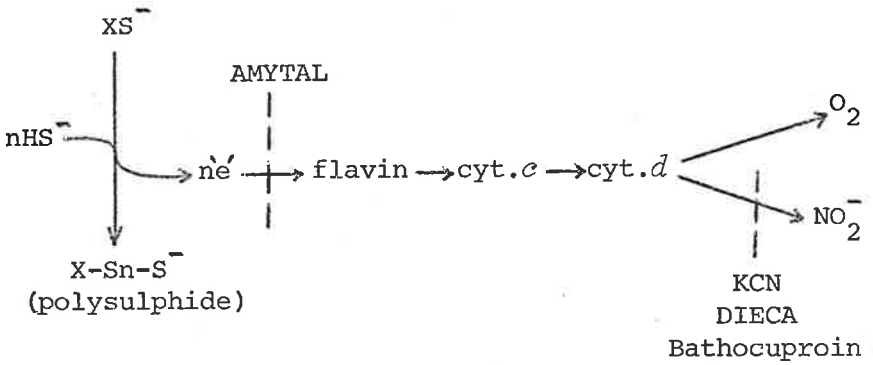
Trudinger (1967) has postulated that the initial reaction of sulphide oxidation involves a membrane-bound thiol. In the present investigation iodoacetamide and pCMB inhibited the reduction of cytochromes by sulphide. These effects are difficult to interpret because sulphide reacts chemically with these inhibitors.

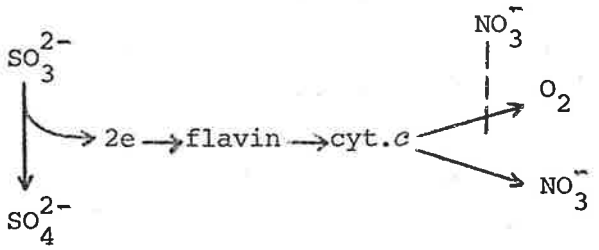
Oxygen uptake during sulphite oxidation is affected by inhibitors of electron transport. The results reported herein agree quite closely with those of Adams, Warnes & Nicholas (1971*A*), who suggested that cytochromes of the *b* and *c* types and possibly a cytochrome oxidase are involved in membrane-bound sulphite oxidase linked to nitrate reduction. Although HOQNO inhibited oxygen uptake during sulphite oxidation, which may suggest the involvement of cytochrome *b*, this cytochrome was not detected in spectra of cell extracts. Difference spectra of P144 fraction showed that sulphite reduced flavin and cytochrome *c* only. Milhaud, Aubert & Millet (1958) suggest that cytochrome a-a_3 mediates electron flow between sulphite or thiosulphate and nitrate but the present results indicate that this may not be the case, since cytochrome a-a_3 was not detected in the membrane fraction where sulphite oxidase is located.

The observed 1:1 stoichiometry for sulphite oxidized and nitrate reduced (Adams, Warnes & Nicholas, 1971) and the fact that cytochromes in the P144 fraction reduced by sulphite were reoxidized by nitrate even in air suggests that nitrate is preferentially reduced when both nitrate and oxygen are available as terminal electron acceptors. Indeed, nitrate inhibited the uptake of oxygen during sulphite oxidation.

Although both sulphite-linked nitrate reductase and sulphide-linked nitrite reductase occur in the P144 fraction, it is noteworthy that the electron donor ($SO_3^=$ or $S^=$) is specific for the inorganic nitrogen acceptor (NO_3^- or NO_2^-). Nicholas (unpublished results) has recently extracted the sulphite-linked nitrate reductase from the P144 membrane fraction, leaving behind the sulphide-linked nitrite reductase.

A tentative scheme for electron transfer during the oxidation of sulphide and sulphite in *T. denitrificans* is as follows:





X = acceptor protein

6.5 Phosphorylation

The coupling of ATP production to sulphite oxidation in soluble and membrane fractions of *T. denitrificans* agree closely with the results for the enzymes and electron transport for the oxidation of sulphite (Section 6.4). Thus, in the S144 fraction, where APS-reductase is mainly located, ATP is formed from AMP and sulphite via APS. Sulphite oxidase, located in the membrane fraction is coupled to an electron transport chain with either

nitrate or oxygen as the electron acceptor. ATP production from ADP and P_i is associated with the membrane fraction. The contrasting effects of DNP and oligomycin on the generation of ATP by the two systems above suggest that there are two distinct levels for phosphorylation during sulphite oxidation.

The results reported herein also confirm that ATP production is associated with sulphide oxidation (Kelly & Syrett, 1966; Saxena & Aleem, 1973).

Both nitrate and nitrite are effective terminal acceptors for coupled oxidative phosphorylation in *M. denitrificans* (Naik & Nicholas, 1966). It was shown that the oxidation of sulphide and sulphite in *T. denitrificans* may be linked to the reduction of nitrite and nitrate respectively. In the present study it is clear that under anaerobic conditions ATP can be generated during sulphide oxidation coupled to nitrite reduction or sulphite oxidation linked to the reduction of nitrate.

ATP can be generated from APS and P_i by a soluble cell-free extract (S144) of *T. denitrificans* without the involvement of electron transfer. Peck (1960, 1962) has suggested a scheme whereby APS and P_i are first converted to ADP by ADP-sulphurylase and then to ATP by adenylate kinase. However, the low level of ADP-sulphurylase and the results of inhibitor studies suggest

that adenylate kinase may not be the main route for the production of ATP.

The high level of ATP-sulphurylase activity in *T. denitrificans* indicates that this enzyme participates in ATP production. Indeed Peck (1960) has shown that this enzyme in *T. thioparus* is 10 to 15 times more active than in crude extracts of yeast. This enzyme is usually involved in the activation of sulphate prior to its reduction:

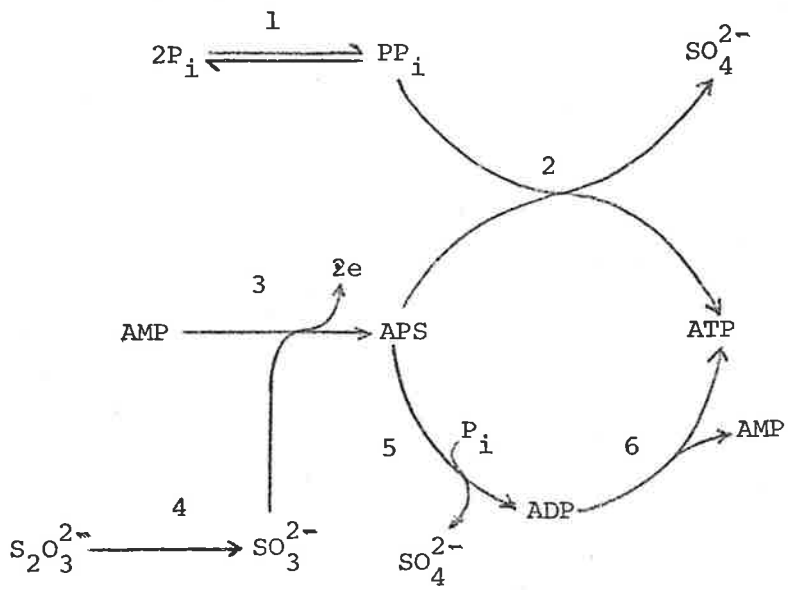
$$ATP + SO_4^{2-} \rightleftharpoons APS + PP_i \qquad (15)$$

However, the reverse reaction is favoured on thermodynamic grounds since the standard free energy change is -11 kcal (Robbins & Lipmann, 1958). The results reported herein confirm those of Bowen, Happold & Taylor (1966) that *T. denitrificans* contains a highly active APS-reductase which synthesizes APS from AMP and sulphite. Although APS is a high energy compound it does not readily participate in energy-requiring reactions of the cell as does ATP. However, it can be directly converted to ATP if inorganic pyrophosphate is present. Model systems have been reported which couple the endergonic formation of PP_i from P_i to exergonic oxidation-reduction reactions, and can thus function in conserving biological energy (Clark, Kirby & Todd, 1958;

Baltrop, Grub & Hesp, 1963). The reversal of inorganic pyrophosphatase has been demonstrated in *Rhodospirillum* chromatophores by several workers (Keister & Yike, 1967 *A*, *B*; Baltscheffsky, von Stedingk, Heldt & Klingenberg, 1966; Guillory & Fisher, 1972).

The similarity of the effects of inhibitors on ATP-sulphurylase and on ATP production from APS and P_i suggests that ATP-sulphurylase rather than adenylate kinase is probably a key enzyme in substrate level phosphorylation.

A tentative scheme for substrate phosphorylation in *T. denitrificans* is outlined below:



Enzymes: (1) Inorganic pyrophosphatase

(2) ATP-sulphurylase

(3) APS-reductase

(4) Thiosulphate-cleaving enzyme

(5) ADP-sulphurylase

(6) Adenylate kinase

The relative importance of the two phosphorylating systems is difficult to assess. The low P/O and P/2e ratios recorded and the high activity of the enzymes involved in substrate level phosphorylation suggest that the latter mechanism may be more important in the energy metabolism of *T. denitrificans*.

6.6 ATP-sulphurylase

ATP-sulphurylase is found in the soluble fraction of the cell (S144) along with APS-reductase. The coupled action of these two enzymes results in the synthesis of ATP from sulphite and AMP as follows:

$$SO_3^{=} + AMP \xrightarrow{\text{APS-reductase}} APS + 2e \qquad (16)$$

$$APS + PP_i \xrightarrow{\text{ATP-sulphurylase}} ATP + SO_4^{=} \qquad (17)$$

The high activity of ATP-sulphurylase in *T. denitrificans*

compared to that from other organisms suggests that the enzyme is primarily involved in the production of ATP. Furthermore the low Km values for APS and PP_i coupled with the highly exergonic nature of the reaction strongly suggests that these substrates (APS and PP_i) are indeed geared for ATP synthesis.

Sulphudryl groups appear to be required for ATP sulphurylase activity in *T. denitrificans* in contrast to the lack of inhibition by pCMB of the enzyme from higher plants (Adams & Johnson, 1968; Ellis, 1969). In agreement with previous work with ATP-sulphurylase the present data confirm a requirement for Mg^{++}. The enzyme in *T. denitrificans* remained active over a wide pH range (7-9) which is in accord with previous studies on the enzyme from yeast (Robbins, 1962; Hawes & Nicholas, 1973), spinach leaves (Balharry & Nicholas, 1970; Shaw & Anderson, 1972), and from *Penicillium chrysogenum* (Tweedie & Segel, 1971*A*).

The properties of purified ATP-sulphurylase from *T. denitrificans* are compared with those from a variety of organisms in Table 42.

6.7 General Discussion and Conclusions

Based on the results reported in this thesis, a scheme for the possible pathways for the oxidation of sulphide, sulphite,

Table 42. Comparison of the properties of purified ATP-sulphurylase from various organisms.

Source	Relative purity	Assay method	Specific activity	Inhibitors	Km for APS (M)	Km for PP_i (M)	Molecular weight	Reference
P. chrysogenum		Molybdolysis	-	APS, $SO_4^=$	7.1×10^{-6}	7.7×10^{-5}	425,000	1, 2
Spinach leaves	53	Bioluminescence	58		4.7×10^{-7}	3×10^{-6}		3
Rat liver	1,000	"	750	APS, PP_i pCMB	2.5×10^{-4}	3.7×10^{-5}	900,000	4
N. agilis	820	Radioassay	68	pCMB, Group VI anions	2.5×10^{-5}	1.2×10^{-4}	700,000	5
Baker's yeast	140	Molybdolysis	69	APS, $SO_4^=$			200,000	6, 7
T. denitrificans	250	Bioluminescence	2,958	$SO_4^=$, PP_i pCMB	5.5×10^{-6}	2.0×10^{-7}	285,000	Present study

Specific activity: nmoles substrate utilized/min/mg protein.

References: (1) Tweedie & Segel (1971*A*); (2) Tweedie & Segel (1971*B*); (3) Balharry & Nicholas (1970); (4) Varma & Nicholas (1971); (5) Levi & Wolf (1969); (6) Hawes (1973); (7) Hawes & Nicholas (1973).

thiosulphate and elemental sulphur and the reduction of nitrate and nitrite is presented in Figure 71.

T. denitrificans grew well under anaerobic conditions with nitrate as the terminal acceptor and thiosulphate as the energy and sulphur source. It is likely that cleavage of the bond between the two sulphur atoms of thiosulphate occurs first rather than its oxidation to tetrathionate. The outer sulphur atom of thiosulphate probably forms a polysulphide while the inner sulphur atom is rapidly oxidized to sulphate or APS. Polysulphide, which is also formed from elemental sulphur may be stored in the cell for subsequent oxidation. It is of interest that the thiosulphate cleaving enzyme, sulphide oxidizing enzyme and AMP-independent sulphite oxidase are all associated with membranes. This close association would result in an effective and a rapid way of oxidizing thiosulphate. Furthermore, the membrane-bound electron transfer system is an integral part of the sulphide and sulphite oxidizing systems. This respiratory chain generates ATP as well as reducing power (NADH). Substantial amounts of ATP are also produced by substrate level phosphorylation.

The denitrifying process in *T. denitrificans*, which is probably similar to that reported for other denitrifiers, involves the reduction of nitrate to nitrite and eventually to nitrogenous gases. A unique feature of the metabolism of *T. denitrificans*

Figure 71. Pathways of inorganic sulphur and nitrogen metabolism in *Thiobacillus denitrificans*.

Enzymes :

1. Thiosulphate cleaving enzyme
2. Rhodanese
3. Thiosulphate-linked nitrate reductase
4. Sulphide-linked nitrite reductase
5. Sulphite-linked nitrate reductase
6. Polysulphide oxidase
7. APS-reductase
8. ADP-sulphurylase
9. Adenylate kinase
10. ATP-sulphurylase
11. Inorganic pyrophosphatase

- - - - - Non-enzymic

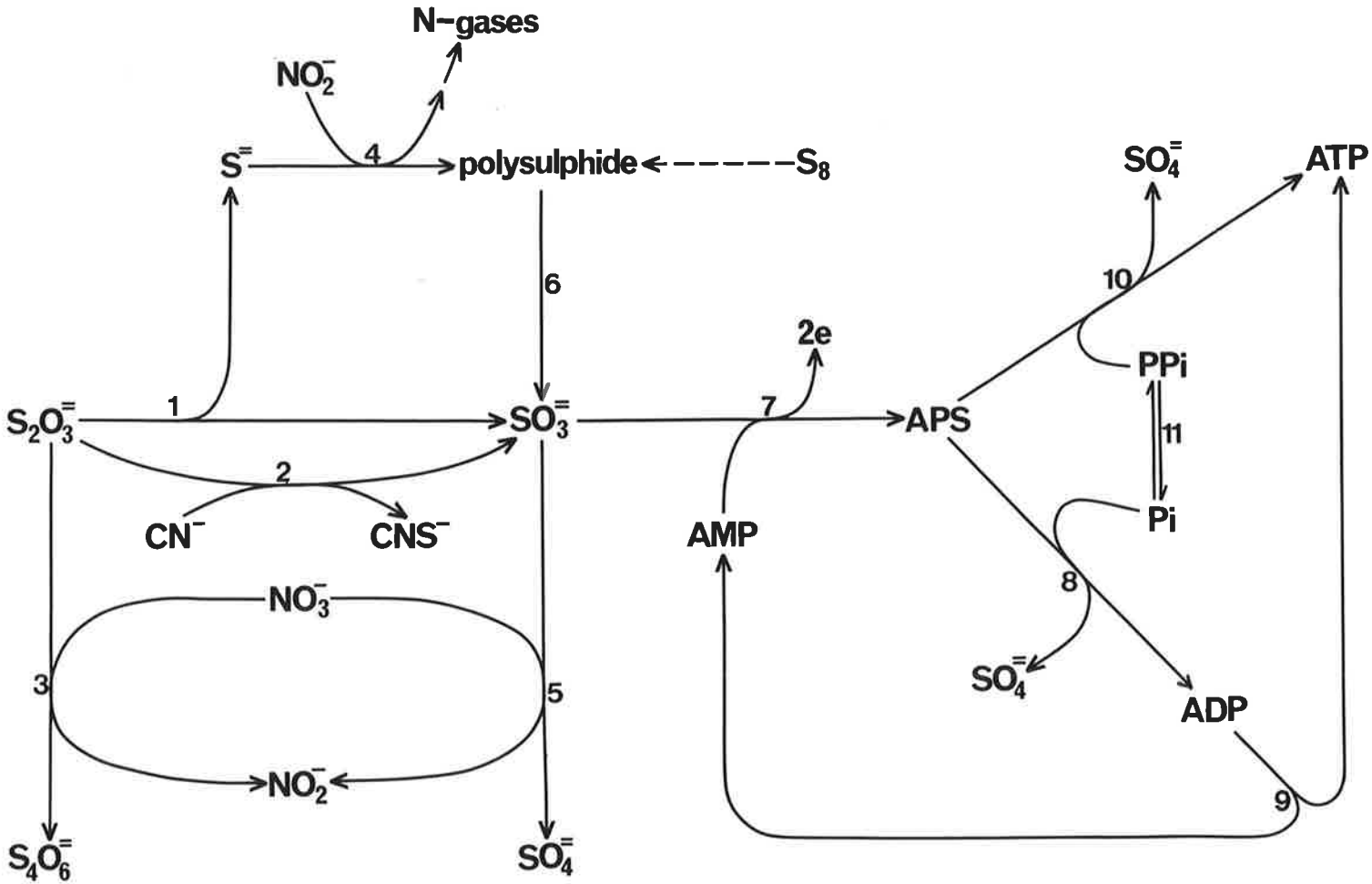

N-gases
NO_2^-
$S^=$
4
polysulphide
S_8
$SO_4^=$
ATP
10
6
2e
PPi
$S_2O_3^=$
1
$SO_3^=$
7
APS
11
2
Pi
CN^-
CNS^-
AMP
NO_3^-
8
3
5
$SO_4^=$
ADP
NO_2^-
9
$S_4O_6^=$
$SO_4^=$

Figure 71

is that the denitrification process is closely linked to the oxidation of reduced inorganic sulphur compounds.

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