Pertanika 2(1), 21–27 (1979)

The Oxidation of Elemental Sulphur by *Thiobacillus denitrificans*

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Key words: *Thiobacillus denitrificans*; **Elemental Sulphur**

RINGKASAN

Pengoksidan sulfan unsor oleh Thiobacillus denitrificans *adalah dimangkinkan oleh enzim yang didapati di dalam pecahan S_{144}; GSH dan satu faktor yang stabil terhadap haba adalah diperlukan untuk tindakbalas. Di dalam pecahan S_{144} pengoksidan sulfan boleh dikaitkan kepada pengambilan oksigen dan di dalam pecahan S_{10} pengoksidan dikaitkan kepada penurunan nitrat. Hasil permulaan pengoksidan sulfan ialah sulfit.*

SUMMARY

The oxidation of elemental sulphur in Thiobacillus denitrificans *is catalysed by an enzyme present in the S_{144} fraction; GSH and a heat-stable factor are required for the reaction. Sulphur oxidation may be linked to oxygen uptake in the S_{144} fraction and to nitrate reduction in the crude extract (S_{10}). The initial product of sulphur oxidation is sulphite.*

INTRODUCTION

Thiobacillus denitrificans is a facultative anaerobe utilizing, under anaerobic conditions, nitrate instead of oxygen as the terminal electron acceptor. It is a chemoautotroph since it derives energy for growth by oxidizing reduced forms of inorganic sulphur compounds and it fixes CO_2 via a pentose pathway (Trudinger, 1956).

The ability of the thiobacilli to oxidize elemental sulphur is induced by growth on sulphur (Margalith, *et al.* 1966; Silver and Lundgren, 1968). Suzuki and Werkman (1959) and Silver and Lundgren (1968) showed that the oxidation of sulphur by extracts of *Thiobacillus thiooxidans* necessitates the addition of substrate concentrations of reduced glutathione (GSH) and they suggested that sulphur is reduced to sulphide prior to oxidation. Reduced glutathione is regenerated by glutathione reductase (Suzuki and Werkman, 1960).

Suzuki (1965) 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. However, further studies indicated that sulphite was in fact the initial product of the enzyme reaction (Suzuki and Silver, 1966; Silver and Lundgren, 1968), and that thiosulphate arises from a non-enzymatic condensation of sulphur and sulphite. Aminuddin and Nicholas (1973) have presented evidence indicating that sulphide is oxidized to sulphite via a polysulphide in *T. denitrificans*.

Cell-free systems from *Thiobacillus neapolitanus* (Taylor, 1968) and *T. thiooxidans* (Tano and Imai, 1968) which oxidize sulphur to sulphate have been described. These systems differ from that described by Suzuki (1965) in that GSH is not required. 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 *et al.* (1967).

In this paper the oxidation of elemental sulphur in cell-free extracts of *T. denitrificans* is described.

METHODS AND MATERIALS

Culture and harvest of organism

T. denitrificans ('Oslo' strain) was grown anaerobically as described by Adams *et al.* (1971). The culture was maintained at pH 7.0 by titrating sterile 25% (w/v) K_2CO_3 into the culture by means of a pH stat unit. After 3 days' growth the cells were collected in a Sorvall RC-2 refrigerated centrifuge fitted with a continuous flow-rotor at 2°C. They were washed twice with 50 mM phosphate buffer (pH 7.5).

Preparation of cell extracts

Washed cells suspended (25%, w/v) in 50 mM phosphate buffer (pH 7.5) containing 0.2 mM EDTA (sodium salt) were passed twice through a French pressure cell at 20,000 lb/inch^2 at 2°C. The crude homogenate was centrifuged at 10,000 $\times$ *g* for 30 min and the supernatant fraction (S_{10}) was used as the crude extract. This fraction was further centrifuged at 144,000 $\times$ *g* for 90 min (Spinco ultra-centrifuge Model L) resulting in a supernatant fraction (S_{144}) and a particulate fraction (P_{144}). The latter was re-suspended (10 ml/gm wet wt.) in 50 mM phosphate buffer (pH 7.5). Both the fractions (S_{144} and P_{144}) were extensively dialysed against at least two changes of the same buffer at 2°C.

Preparation of heat-stable extract

The supernatant fraction (S_{144}) obtained on centrifuging the undialysed crude extract (S_{10}) at 144,000 $\times$ *g* for 90 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 $\times$ *g* for 10 min and the resulting supernatant fraction referred to as the heat-stable extract.

Oxygen uptake

Oxygen uptake was measured polarographically with a Beckman oxygen electrode fitted with an adaptor box and connected to a Beckman oxygen analyser and a Beckman recorder. The electrode was standardized with 50 mM phosphate buffer (pH 7.5) containing 0.2 mM Na-EDTA by the method of Dixon and Kleppe (1966). The reaction mixture in a total volume of 2.5 ml contained phosphate buffer (pH 7.5) 120 μmoles; Na-EDTA, 0.4 moles; elemental sulphur, 40 mg (dry weight); GSH, 5 μmoles and 0.1 ml cell extract. The reaction conducted at 30°C was started by adding either GSH or elemental sulphur. Enzyme activity is expressed in μmoles oxygen utilised/min/mg protein.

Separation and identification of sulphur compounds

Inorganic sulphur compounds were separated on Whatmann 3 MM chromatography paper in 0.1 M sodium citrate buffer (pH 5.0) at 1500 V for 1 hr. The apparatus used was that of Tate (1968) for routine high voltage electrophoresis. In each run either $^{35}SO_4^{=}$ or thiosulphate was used as a marker compound. Polythionates, thiosulphate and sulphide were detected by dipping the electrophoretogram in a $AgNO_3$ solution. Sulphite was detected by basic Fuchsin.

Absorption spectra

Absorption measurements were made in a Shimadzu Multipurpose Recording spectrophotometer, Model MPS-50L. Spectra at room temperature were recorded using a 1 cm path cuvette.

Nitrate reductase assay

Nitrate reductase assay using elemental sulphur as an electron donor was carried out as follows: the reaction mixture in a total volume of 2 ml contained phosphate buffer (p H 7.5), 85 moles; $NaNO_3$, 5 μ moles; GSH, 5 μ moles; elemental sulphur, 40 mg (dry weight) and 0.1 ml enzyme extract (S_{10}, S_{144}, P_{144}). The reaction initiated by adding elemental sulphur and incubating for 10 min at 30°C was terminated with 1 ml 10% (w/v) zinc acetate. The mixture was then centrifuged at 3,000 $\times$ *g* for 5 min and aliquots of the supernatant taken for the chemical determination of nitrate as described by Hewitt and Nicholas (1968). Enzyme activity is expressed in nmoles nitrite produced/10 min/mg protein.

Protein determination

The Folin method of Lowry *et al.* (1951) was used with bovine serum albumin as standard.

Preparation of elemental sulphur

Elemental sulphur, prepared by the method of Roy and Trudinger (1970) was washed twice with 10 mM phosphate buffer (pH 7.0) before use. A suspension of the prepared sulphur was used for experiments.

RESULTS

When a preparation of colloidal sulphur (S_8) was suspended in a solution of 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 (Fig. 1). Similar spectra were recorded when the elemental sulphur was suspended in equivalent protein concentrations of either the crude extract (S_{10}), the S_{144} fraction or the P_{144} fraction.

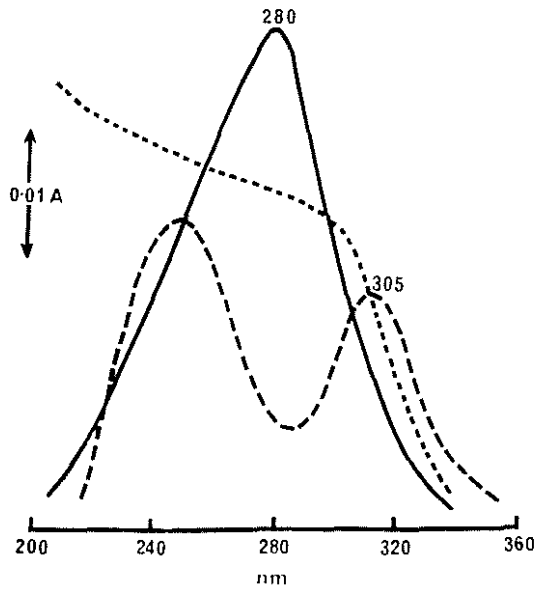

Fig. 1 Absorption spectra of elemental sulphur and bovine serum albumin (BSA).
——— : *Absolute spectra of BSA (BSA* versus *buffer)*
– – – – : *Difference spectra, BSA plus sulphur* versus *BSA*
..... : *Absolute spectra of elemental sulphur (sulphur* versus *buffer)*

When the S_{144} fraction was added to a preparation of sulphur in bovine serum albumin there was a slight decrease in absorbence, which became more marked on adding GSH (Fig. 2).

Sulphur oxidation linked to oxygen uptake

There was an increase in oxygen uptake when the S_{144} fraction was incubated with GSH and elemental sulphur. The rate of oxygen uptake in the P_{144} fraction was less than that in the S_{144} fraction (Table 1). This agrees with the spectrophotometric data that sulphur utilization occurs in the S_{144} fraction. Boiled preparation of the S_{144} fraction did not utilise oxygen (Fig. 3). The rate of oxygen uptake decreased when the S_{144} 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 S_{144} (see Methods and Materials) was added to the dialysed S_{144} fraction there was a marked increase in oxygen consumption (Fig. 3).

Sulphur oxidation linked to nitrate reduction

Nitrate was reduced to nitrite when the crude extract (S_{10}) was incubated with GSH and elemental sulphur. The activity was linear for

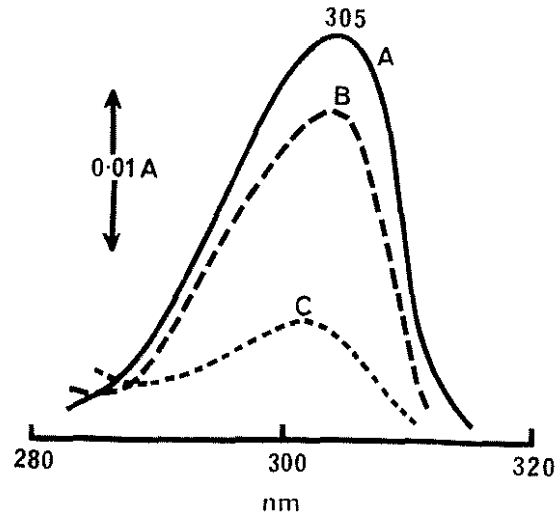

Fig. 2: Effect of S_{144} fraction and GSH on elemental sulphur suspended in bovine serum albumin.

Sample and reference cuvette contained bovine serum albumin (5 mg protein) in 50 mM phosphate buffer (pH 7.0). Difference spectra were recorded after the following additions:
A: Elemental sulphur in sample cuvette
B: S_{144} fraction (5 mg protein) added to reference and sample cuvette in A
C: GSH added to sample cuvette in B

TABLE 1

Distribution of sulphur oxidizing enzyme in various cell fractions

Fraction	Specific activity*	
	Nitrate reductase	O_2 uptake
Crude extract (S_{10})	244	48
S_{144} fraction	33	30
P_{144} fraction	102	18
S_{144} + P_{144} (ratio 1 : 1 mg protein)	382	Not determined

*Specific activities:
Nitrate reductase: nmoles nitrite produced/10 min/mg protein
Oxygen uptake : nmoles oxygen utilised/min/mg protein

the first 10 min of reaction (Fig. 4). Sulphur oxidation coupled to nitrate reduction occurred only when GSH and an active enzyme were present (Table 2). There was no activity asso-

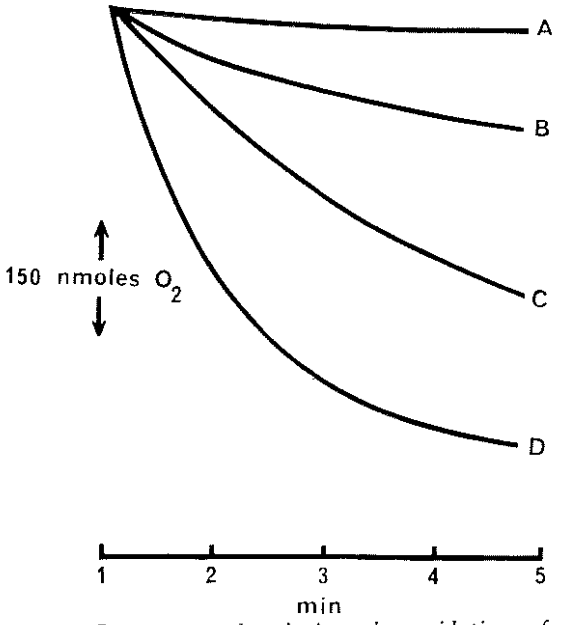

Fig. 3: Oxygen uptake during the oxidation of elemental sulphur by the S_{144} fraction.
A: Boiled S_{144} fraction (dialysed or undialysed)
B: Dialysed S_{144} fraction
C: Undialysed S_{144} fraction
D: Dialysed S_{144} fraction with 0.1 ml of a heat-stable extract prepared from undialysed S_{144} fraction

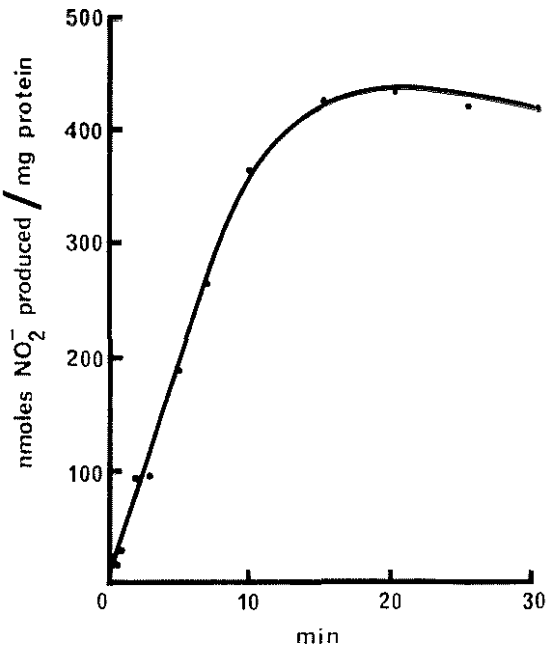

Fig. 4: Effect of incubation time on the oxidation of elemental sulphur linked to nitrate reduction in the crude extract (S_{10}).

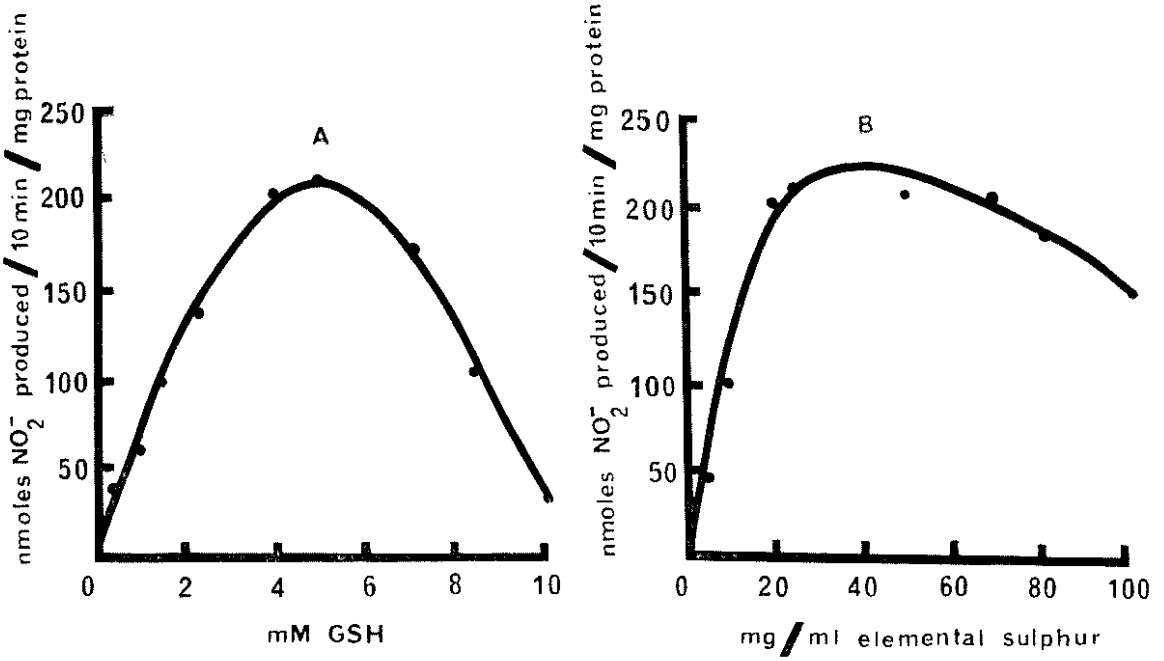

Fig. 5: Effect of varying the concentrations of elemental sulphur and GSH on nitrate reduction in the crude extract (S_{10}).
A: Effect of GSH concentration at a fixed level of elemental sulphur (10 mg/ml)
B: Effect of elemental sulphur; GSH fixed at 2.5 mM

ciated 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 (Fig. 5).

TABLE 2

Requirements for the oxidation of elemental sulphur linked to nitrate reduction in the crude extract (S_{10})

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

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

The rate of nitrate reduction in either the S_{144} or P_{144} fractions was lower than that obtained with the crude extract (Table 1). However, when these fractions (S_{144} and P_{144}) were recombined the activity increased sharply (Table 1). When the concentration of undialysed S_{144} fraction was fixed as shown in Fig. 6, the sulphur linked nitrate reductase activity was dependent on the amount of P_{144} fraction added to it. There was no difference in activity when either dialysed or undialysed P_{144} fraction was used. However, when the S_{144} fraction was varied, while P_{144} was maintained at a fixed level, the rate of nitrate reduction was markedly influenced on adding the S_{144} fraction. The undialysed S_{144} fraction was found to be more effective than the dialysed fraction (Fig. 7). However, the rate was much higher when the dialysed S_{144} fraction was used in the presence of a heat-stable extract prepared from undialysed S_{144} fraction (Fig. 7). These results confirmed those for oxygen uptake that the oxidation of elemental sulphur is catalysed by a soluble enzyme which requires a heat-stable factor in addition to GSH.

Product of sulphur oxidation

Sulphite was formed when the S_{144} fraction was incubated with GSH and elemental sulphur (Table 3). 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 com-

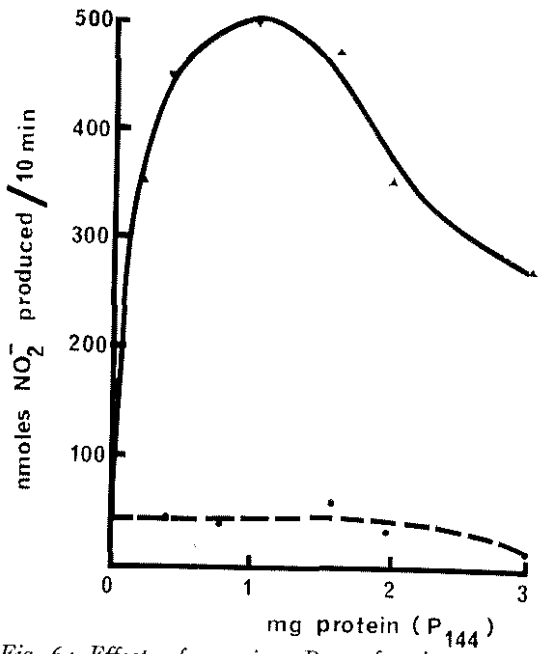

Fig. 6: Effect of varying P_{144} fraction on the oxidation of elemental sulphur linked to nitrate reduction. S_{144} fixed at 3.6 mg protein.

——— Untreated P_{144} fraction

– – – – – Boiled P_{144} fraction

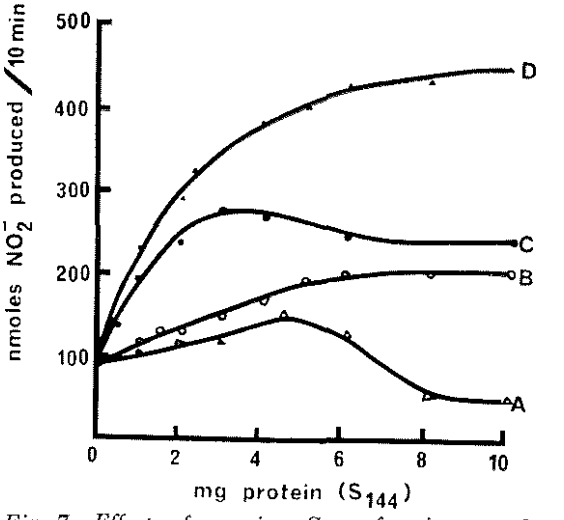

Fig. 7: Effect of varying S_{144} fraction on the oxidation of elemental sulphur linked to nitrate reduction. P_{144} fixed at 2 mg protein.

A: Undialysed S_{144} fraction which had been boiled for 3 min

B: Dialysed S_{144} fraction

C: Undialysed S_{144} fraction

D: Dialysed S_{144} fraction plus 0.5 ml of a heat- stable extract

TABLE 3

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

Reaction mixture	Rf
I	0.85
II	0.84
III	0.85

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

I: Aliquot from complete reaction mixture
II: $Na_2S_2O_3$ standard
III: $Na_2S_2O_3$ standard mixed with a boiled extract of the P_{144} fraction prior to spotting on to the paper.

plex was dissociated by treatment with 1M NaOH and the free sulphite separated by high-voltage electrophoresis. Sulphite was not detected when formaldehyde was omitted from the reaction mixture. Thiosulphate and some polythionates were also formed.

DISCUSSION

The oxidation of elemental sulphur in *T. denitrificans* is catalysed by an enzyme system located mainly in the soluble fraction of the cell. The requirement for GSH during the oxidation of elemental sulphur indicates that polysulphide is indeed the substrate for the sulphur oxidizing enzyme in *T. denitrificans* (Aminuddin and Nicholas, 1973). In addition to GSH, a soluble, 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 and Silver, 1966; Tano and Imai, 1968) as is the case in *T. denitrificans*. Roy and 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 the product of sulphide oxidation (Aminuddin and Nicholas, 1973). Thus, in *T. denitrificans* the oxidation of sulphide and elemental sulphur is linked to nitrate reduction via sulphite. Studies on the recombination of the S_{144} and P_{144} fraction indicate that for the complete oxidation of sulphur to sulphate both the soluble and particulate fractions are necessary.

The mechanism for oxidation of elemental sulphur postulated by Suzuki (1965) and Suzuki and Silver (1966) may also function in *T. denitrificans* as follows:

$$GSH + S_8 \rightarrow G - S_8 - SH \quad (1)$$

$$G - S_8 - SH + O_2 \rightarrow G - S_8 - SO_2H \quad (2)$$

$$G - S_8 - SO_2H + OH^- \rightarrow G - S_7 - SH + HSO_3^- \quad (3)$$

First, GSH reacts non-enzymically with elemental sulphur to form polysulphide (equation 1). This then becomes indistinguishable from the membrane-bound polysulphide (Aminuddin and Nicholas, 1973). A sulphonate group is formed at the end of the polysulphide chain (equation 2) which is then split off as sulphite (equation 3). Sulphite may then be oxidized further by the two sulphite oxidizing enzymes (Bowen, *et al.*, 1966; Aminuddin and Nicholas, 1974).

ACKNOWLEDGEMENT

The author wishes to thank Professor D. J. D. Nicholas of the Department of Agricultural Biochemistry and Soil Science, Waite Agricultural Research Institute, University of Adelaide.

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(Received 25 September 1978)