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SULPHIDE OXIDATION LINKED TO THE REDUCTION OF NITRATE AND NITRITE IN *THIOBACILLUS DENITRIFICANS*

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SUMMARY

1. Whole cells of *Thiobacillus denitrificans* reduce nitrite to NO, N_2O and N_2 when sulphide is the electron donor.

2. Crude extracts catalyse the oxidation of sulphide, which may be coupled to either oxygen, nitrate or nitrite as terminal acceptors.

3. The initial product of sulphide oxidation is a membrane-bound polymeric sulphur compound which is probably a polysulphide. This step is inhibited by CO.

4. In the absence of either nitrate or nitrite, sulphide is oxidized to polysulphide and sulphite. When nitrate is present, sulphide is oxidized to sulphate with a concomitant reduction of nitrate to nitrite. Under anaerobic conditions and in the presence of nitrite, sulphide is oxidized to polysulphide only.

5. Sulphite is oxidized to either adenosine 5'-phosphosulphate in the soluble fraction (S_{144}) or to sulphate by the membrane fraction (P_{144}).

6. A scheme for the oxidation of sulphide is proposed.

INTRODUCTION

Thiobacillus denitrificans is a facultative anaerobe utilizing, under anaerobic conditions, nitrate instead of O_2 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[1,2].

Parker and Prisk[3] demonstrated the uptake of O_2 during sulphide oxidation and proposed that elemental sulphur is an intermediate, whereas Vishniac and Santer[4], London and Rittenberg[5] and Peck and Fisher[6] suggested that thiosulphate and polythionates are the intermediary products. Recently Moriarty and Nicholas[7] found that a membrane-bound polysulphide was an early intermediate of sulphide oxidation in *Thiobacillus concretivorus*. Contrary to Adair's[8] suggestion that sulphide oxidation is a non-enzymic event, these authors[9] further demonstrated that it is mediated by oxidative enzymes *via* the respiratory chain to O_2 as a terminal acceptor.

Little work has been done on the relation between denitrification and the oxidation of inorganic sulphur compounds in *T. denitrificans*. Baalsrud and Baalsrud[10],

Abbreviations: PMS, phenazine methosulphate; APS, adenosine 5'-phosphosulphate.

working with whole cells of this bacterium, found that the oxidation of elemental sulphur and thiosulphate was coupled to nitrate and nitrite reduction. More recently, Adams *et al.*[11] reported a particulate nitrate reductase which utilizes sulphite as an electron donor.

In this paper it is shown that the oxidation of sulphide to polysulphide is linked to nitrite reduction and the complete oxidation of sulphide to sulphate is linked to nitrate reduction *via* sulphite as an intermediate.

MATERIALS AND METHODS

Culture and harvest of organism

T. denitrificans ('Oslo' strain) was grown anaerobically as described by Adams *et al.*[11]. The culture was maintained at pH 7.0 by titrating sterile 25% (w/v) K_2CO_3 into the cultures 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 with 2 lots of 400 ml of 50 mM Tris–HCl buffer (pH 7.5).

Preparation of cell extracts

Washed cells, suspended (25%, w/v) in 50 mM Tris–HCl buffer (pH 7.5) containing 0.2 mM EDTA (sodium salt), were passed twice through a French pressure cell at 20000 lb/inch^2 at 4 °C. The crude homogenate was centrifuged at $10000 \times g$ for 30 min and the supernatant fraction (S_{10}) was used as the crude extract. This fraction was further centrifuged at $144000 \times g$ for 90 min (Spinco, Model L, rotor type Ti50) resulting in a supernatant fraction (S_{144}) and a pellet fraction (P_{144}). The latter was resuspended in 50 mM Tris–HCl buffer (pH 7.5). Both the fractions, S_{144} and P_{144}, were extensively dialysed against several changes of the same buffer at 4 °C.

Sulphide measurement

A Beckman sulphide ion electrode (Model 39610) connected to a Beckman H5 pH meter and recorder was used to determine the concentration of sulphide in the reaction mixtures[12]. 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 mV per unit time. The molar rate was obtained from a calibration curve of sulphide standard solutions.

Oxygen uptake

Oxygen uptake was measured polarographically with a Beckman oxygen electrode (Model 39065) fitted with an adaptor box (Model 96260) and a Beckman recorder. To 3 ml of 50 mM Tris–HCl buffer (pH 7.5) containing 0.2 mM EDTA (sodium salt) in the reacting vessel was added 0.1 ml each of 10 mM Na_2S and cell extract (S_{10}, S_{144} or P_{144}) with the aid of micro syringes. The oxygen electrode was standardized by the method of Dixon and Kleppe[13].

Separation and identification of sulphur compounds

Inorganic sulphur compounds were separated on Whatman 3 MM chromato-

graphy paper in 0.1 M sodium citrate buffer (pH 5.0) at 1500 V for 1 h. The apparatus used was that of Tate[14] for routine high voltage electrophoresis. In each run either $^{35}SO_4^{2-}$ or thiosulphate was used as a marker compound. Polythionates, thiosulphate and sulphide were detected by dipping the electrophoretogram in a $AgNO_3$ solution (8 g in 10 ml water and 90 ml acetone). Sulphite was detected by basic fuchsin (1%, w/v) in 10 ml water and 90 ml acetone and thiocyanate by $Fe(NO_3)_3$ (10 g) in 2 M HNO_3 (10 ml) and acetone (90 ml).

Radioactivity measurements

Radioactive areas on the electrophoretograms were detected either by running the paper through a Packard 7201 radiochromatogram scanner or by cutting it into small parts (1.5 cm × 2 cm) and counting in a toluene solution of 0.5% (w/v) PPO and 0.3% POPOP in a Packard Tri-Carb liquid scintillation spectrometer (Model 3375).

Spectrophotometry

A Shimadzu Multipurpose Recording Spectrophotometer (Model MPS-50L) was used to record absorption spectra.

Mass spectrometry

The reaction mixture in a Rittenberg tube[15] contained 2.0 μmoles $NaNO_2$ and 5 μmoles of either Na_2S, Na_2SO_3 or $Na_2S_2O_3$ in 0.2 ml in one of the arms. The other limb of the tube contained 1.7 ml 50 mM Tris–HCl buffer (pH 7.5) containing 0.2 mM EDTA (sodium salt) and 0.1 ml of 25% (w/v) cell suspension in the buffer. A roll of filter paper soaked in 10 M KOH was placed inside the cap of the tube to absorb any CO_2 produced. The tubes were rigorously evacuated to $1 \cdot 10^{-5}$ mmHg and the contents were then mixed and incubated for 3 h at 30 °C. The gases formed were then transferred under high vacuum from the Rittenberg tubes into an A.E.I. MS-2 mass spectrometer for analysis.

Enzyme assays

Nitrate reductase assay. The enzyme was assayed with either Na_2SO_3 or Na_2S as an electron donor. With Na_2SO_3 the reaction mixture contained in μmoles: Tris–HCl buffer (pH 8.5), 85.0; $NaNO_3$, 5.0; Na_2SO_3, 5.0, and 0.1 ml enzyme in a final volume of 2.0 ml. The reaction was initiated by adding the donor and incubating for 10 min in open test tubes (10 cm × 1.3 cm) at 30 °C and terminated with the nitrite reagents[16], *i.e.* 1.0 ml 1% (w/v) sulphanilamide in 1.0 M HCl and 1.0 ml 0.1% (w/v) *N*-(1-naphthyl)-ethylenediamine·HCl. After 15 min the absorbance of these solutions was measured at 540 nm. When Na_2S was the electron donor, the reaction mixture contained in μmoles: Tris–HCl buffer (pH 7.5), 85; Na_2S, 4; $NaNO_3$, 5; and 0.1 ml enzyme in a final volume of 2.0 ml. The reaction, conducted in glass tubes (7.5 cm × 1.0 cm) stoppered with rubber septa, was initiated and incubated as in the previous assay but it was terminated with 1.0 ml 10% (w/v) zinc acetate. After centrifuging at 2000 × *g* for 5 min, nitrite was determined in an aliquot of the supernatant fraction[16]. Enzyme activity is expressed in nmoles nitrite formed in 10 min per mg protein.

Nitrite reductase assay. With NADH as electron donor, enzyme activity

was measured in 10 ml Warburg flasks after evacuating and refilling them with O_2-free N_2. The following reaction mixture was used for the assay: 0.1 ml enzyme, 2 μmoles NADH, 1 μmole phenazine methosulphate (PMS) and 1 μmole $NaNO_2$ in 50 mM phosphate buffer (pH 7.5) in a total volume of 1.0 ml. The reaction mixture was preincubated for 5 min at 30 °C before adding the electron donor. After 10 min the residual nitrite was determined as described previously. When Na_2S was the electron donor, the reactions were conducted in stoppered tubes (7.5 cm × 1.0 cm) which contained 0.1 ml enzyme, 2 μmoles Na_2S and 1 μmole $NaNO_2$ in 50 mM phosphate buffer (pH 7.5) in a total volume of 1.0 ml. The residual nitrite was determined as described for the sulphide-linked nitrate reductase. Nitrite reductase activity is expressed in nmoles nitrite reduced in 10 min per mg protein.

Adenosine 5'-phosphosulphate (APS) reductase. The assay employed was a modification of that described by Peck[17]. The reaction mixture contained in μmoles: Tris–HCl (pH 7.5), 150; Na_2SO_3, 10; $K_3Fe(CN)_6$, 5.0; AMP, 5.0, and enzyme in a final volume of 3.0 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. Absorbance changes at 420 nm were followed in a Unicam SP-800 recording spectrophotometer equilibrated at 30 °C. Activity is expressed in μmoles ferricyanide reduced per h per mg protein.

Production of sulphite from sulphide. The reaction mixture contained in a total volume of 1.0 ml, 4 μmoles Na_2S, 50 mM Tris–HCl buffer ((pH 7.5) and 0.1 ml enzyme. The reaction was initiated, incubated and terminated as described for the sulphide-linked nitrite reductase. The sulphite formed was determined by the basic fuchsin method of Grant[18]. An aliquot diluted to 1.0 ml with water was mixed with 4.0 ml of colour reagent. After 10 min the absorbance at 570 nm was measured. The basic fuchsin was freshly prepared daily. Enzyme activity is expressed in nmoles sulphite formed in 10 min per mg protein.

Protein determination

The Folin method of Lowry *et al.*[19] was used with bovine serum albumin as a standard.

Chemicals

Sodium sulphide solution was freshly prepared by dissolving washed crystals of $Na_2S \cdot 9\ H_2O$ in O_2-free double-distilled water[20]. Bovine serum albumin, NADH and AMP were purchased from Sigma Chemical Co., U.S.A.; sulphur and potassium tetrathionate were made by the methods of Roy and Trudinger[21] and Trudinger[22], respectively. Radioactive chemicals were purchased from the Radiochemical Centre, Amersham, England. All other reagents were of analytical grade.

RESULTS

Sulphide oxidation in relation to terminal acceptors

Sulphide was rapidly utilized by whole cells and crude extracts (S_{10}), as shown in Fig. 1. Since all sulphide was oxidized within 3 min, little of it was lost to the atmosphere. The membrane fraction (P_{144}) also oxidized sulphide at a rate equivalent to that of the crude extract. Neither the S_{144} fraction nor the boiled

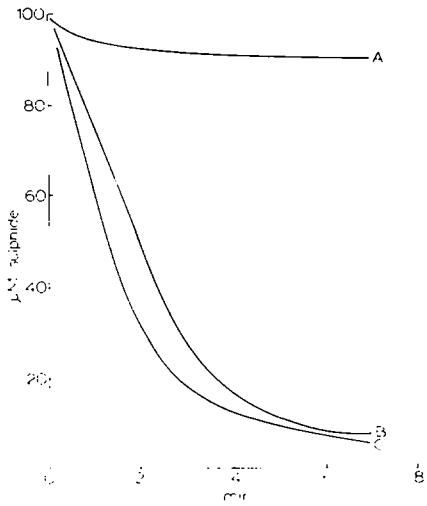


Fig. 1. Sulphide utilization by whole cells and cell fractions. The reaction mixture in a total volume of 5.0 ml contained 50 mM Tris–HCl buffer (pH 7.5) and either 4 mg protein or 0.2 ml cell suspension (25%, w/v) equivalent to 3.0 mg bacterial dry wt. At zero time 50 μl of 10 mM Na_2S were added. A, S_{144} fraction; B, cell suspension; C, crude extract (S_{10}) or P_{144} fraction.

P_{144} preparation utilized sulphide. When incubated under an atmosphere of nitrogen, neither the cell suspension nor the cell extracts oxidized sulphide.

A plot of varying concentrations of sulphide against its rate of oxidation exhibits a typical substrate concentration curve. The K_m for sulphide, determined by a double-reciprocal plot, was found to be 0.4 mM.

Oxygen as acceptor. When sulphide was added to either the crude extract (S_{10}) or the P_{144} fraction there was a gradual uptake of O_2 (electrode method). Although sulphide disappeared after 3 min, O_2 was still being utilized and this reached a maximum in 8 min. The initial rate of O_2 uptake, however, was directly dependent on the relative amounts of enzyme (S_{10} or P_{144}) and sulphide added. Little O_2 was utilized when either was omitted from the reaction mixture. When the S_{144} fraction was used the rate of O_2 uptake during sulphide oxidation was only 5% of that catalysed by either S_{10} or P_{144}.

Sulphide was effectively oxidized by the membrane fraction over the pH range 6 to 9, with an optimum at 7.5 as determined by the uptake of O_2. The stoichiometry between sulphide oxidized and O_2 utilized was 1:2 for the first 3 min of reaction time.

Nitrate as acceptor. When crude extracts (S_{10}) were incubated in air with nitrate and sulphide, nitrite was produced. Nitrite production was not detected under anaerobic conditions.

Neither the S_{144} nor the P_{144} fraction linked the oxidation of sulphide to nitrate reduction but this activity, restored on recombining the two fractions (Table I), was dependent on the relative amounts of each. Thus, when P_{144} was fixed at 1 mg and the S_{144} fraction varied, there was a linear relation between the rate of nitrate reduction and the amounts of S_{144} added (Fig. 2). Similar results

TABLE I

NITRATE AND NITRITE REDUCTASE ACTIVITIES IN CELL FRACTIONS

The NO_3^- and NO_2^- reductase assays using S^{2-} as electron donor were employed. Assay conditions were as described in Materials and Methods. Specific activity for nitrate reductase is expressed in nmoles nitrite formed/10 min per mg protein; for nitrite reductase, nmoles nitrite reduced/10 min per mg protein.

Fraction	*Nitrate reductase*	*Nitrite reductase*
French pressure cell extract	95	120
Crude extract (S_{10})	127	154
Supernatant fraction (S_{144})	10	0
Pellet fraction (P_{144})	14	229
$S_{144}+P_{144}$ (ratio 1:1, mg)	92	-

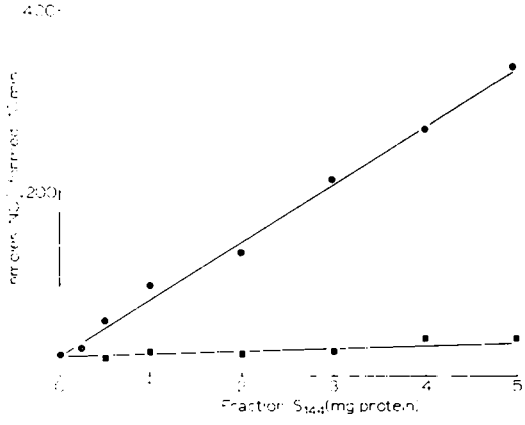


Fig. 2. Effect of varying the levels of S_{144} on nitrate reductase activity using sulphide as an electron donor. P_{144} was fixed at 1 mg. Assay conditions as in Materials and Methods. ●—●, S_{144}; ■—■, boiled S_{144}.

TABLE II

PRODUCTS OF NITRITE REDUCTION BY WHOLE CELLS

The assay conditions are given in Materials and Methods. Gas production is expressed as relative peak height (proportional to concentration) corrected for controls using boiled cell suspensions.

Mass No.	*Compound*	*Electron donor*		
		Na_2S	*Na_2SO_3*	*$Na_2S_2O_3$*
28	N_2	6.6	2.1	13.5
30	NO	3.2	2.5	2.8
44	N_2O	21.3	10.0	13.2

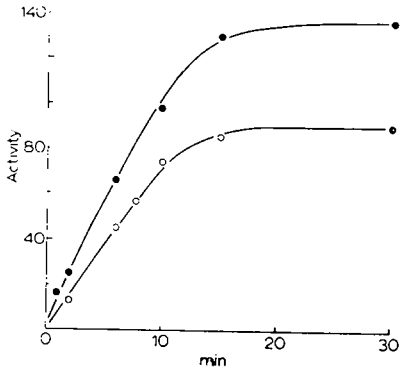


Fig. 3. Reduction of nitrite by membrane fractions (P_{144}). Assay conditions as in Materials and Methods. ●—●, sulphide as donor; ○—○, NADH and PMS as donor. Activity: nmoles nitrite reduced/mg protein.

were obtained when S_{144} was fixed and P_{144} varied. There was no increase in enzyme activity when a boiled preparation of either S_{144} or P_{144} was used.

Nitrite as acceptor. When whole cells were incubated with nitrite and sulphide

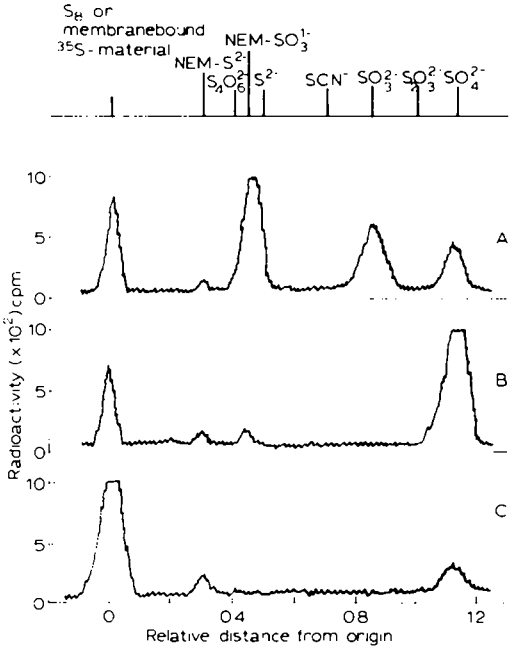


Fig. 4. Electrophoresis of the products of sulphide oxidation. Reaction mixture in A contained 50 mM Tris–HCl buffer (pH 7.5), 0.4 μCi $Na_2{}^{35}S$ and 5 μmoles Na_2S carrier in a total volume of 0.2 ml. (B) As in (A) *plus* 5 μmoles nitrate. (C) As in (A) *plus* 5 μmoles nitrite. A and B were incubated in air and C anaerobically, all for 15 min. The reaction was terminated with 50μl 0.05 M *N*-ethylmaleimide (NEM). 20-μl aliquots of each mixture were then spotted on to Whatman 3 MM paper. Details of electrophoresis are given in Materials and Methods. Radioactive areas were detected by running the chromatogram through a Packard 7201 radiochromatogram scanner.

under anaerobic conditions, the NO, N_2O and N_2 gases produced were detected in the mass spectrometer (Table II). Thiosulphate was also an effective electron donor for gas production while sulphite was relatively ineffective.

Nitrite was reduced concomitantly with the oxidation of sulphide by cell suspensions, crude extracts (S_{10}) or the P_{144} fraction. The S_{144} fraction had no sulphide-linked nitrite reductase activity (Table I). NADH (in the presence of PMS) also served as a donor for nitrite reduction in the P_{144} fraction (Fig. 3, ○—○). Thiosulphate, sulphite and tetrathionate were all ineffective as donors for nitrite reduction in the S_{10} or P_{144} fraction.

The pH optimum for sulphide-linked nitrite reductase activity in the P_{144} fraction was 7.5, which agrees with the value obtained by the oxygen-electrode method. One mole of sulphide was oxidized for each mole of nitrite reduced.

Products of sulphide oxidation

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 Fig. 4. In this system, elemental sulphur and membrane-bound sulphur compounds remained at the origin[7]. Sulphide and sulphite were treated with *N*-ethylmaleimide prior to their application to the paper and were run as *N*-ethylmaleimide complexes.

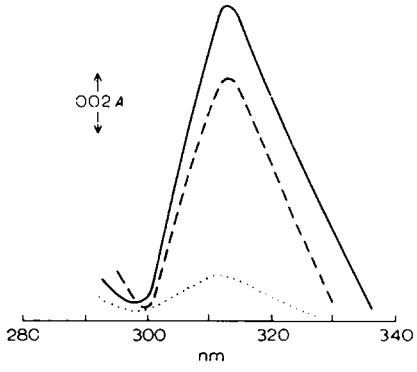
Crude extracts (S_{10}), incubated in air for 15 min with [^{35}S]sulphide with or without nitrate, were treated with *N*-ethylmaleimide and the products of the reaction separated by high-voltage electrophoresis. In the presence of nitrate the products were [^{35}S]sulphate and ^{35}S-labelled material 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 Fig. 4. The ^{35}S-labelled material at the origin and some [^{35}S]sulphate were also produced.

When crude extracts were 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.

When these reaction mixtures after the incubation periods were treated with iodine before application on to 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 (S_{10}) was incubated for 2 min with [^{35}S]sulphide and then centrifuged at 244000 × *g* for 1 h, between 60–75% of the radioactivity was found in the pellet (P_{224}). This suggests a binding of the sulphur compound produced 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 strict anaerobic conditions and incubating the reaction mixture for a further 2 min, however, the percentage of radioactivity recovered in the pellet increased to around 60%. CO inhibited the binding of [^{35}S]sulphide to the membrane fraction since only about 35% of the radioactivity was detected in the pellet when the crude extract was flushed with CO for 3 min before adding [^{35}S]sulphide.

Absorption spectra of polysulphide-type compounds. When sulphide was incubated with either crude extracts (S_{10}) or the P_{144} fraction, there was an increase in absorbance around 300–350 nm with a maximum at 315 nm (Fig. 5). This suggests



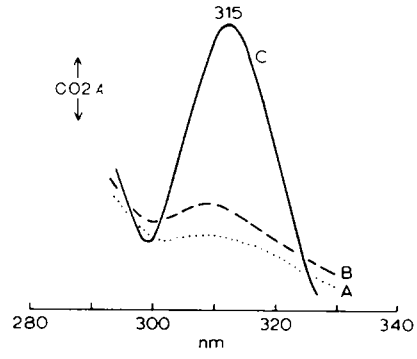


Fig. 5. Difference spectra of various cell fractions (3 mg protein/ml) oxidized *vs* reduced with sulphide, determined in a Shimadzu multipurpose recording spectrophotometer (Model MPS-50L) using 1-cm quartz cuvettes. —, Fraction P_{144}; ----, crude extract (S_{10}); . . . ., S_{144} fraction.

Fig. 6. Effect of CO on the difference spectra of the P_{144} fraction, sulphide reduced *vs* oxidized. Sample and reference cuvettes (1 cm) contained 3.0 ml P_{144} fraction (10 mg) in 50 mM phosphate buffer (pH 7.0). A, after treatment of sample cuvette with CO for 3 min; B, after addition of 5 μmoles Na_2S to A; C, 10 min after exposing B to tungsten light.

a polymeric sulphur compound[7]. The S_{144} fraction had little activity and boiled preparations of either a crude extract or a P_{144} fraction did not produce this polymeric sulphur compound. Under anaerobic conditions it was only produced when nitrite was present, but nitrate however was ineffective.

The absorbance at 315 nm depends on the amounts of extract and sulphide added. The K_m for sulphide based on this absorbance was 0.3 mM, which is close to that obtained by the sulphide-electrode method (0.4 mM).

The formation of the 315 nm absorbing material was inhibited by CO and this effect was reversed by light (Fig. 6). When sulphide was added to the P_{144} fraction which had been previously treated with CO, there was only a very small increase in absorbance at 315 nm, but after exposing to tungsten light for 5 min about 85% of the original 315 nm band was restored.

When a preparation of colloidal sulphur (S_8) (0.2 mg/ml) was suspended in a solution of inert protein such as bovine serum albumin, the difference spectra (albumin *plus* sulphur *vs* albumin) exhibited a broad absorption band between 280–320 nm with a maximum at 305 nm. A similar spectrum was recorded when the elemental sulphur was suspended in equivalent protein concentrations of the crude extract (S_{10}) or the P_{144} fraction.

After incubating the P_{144} fraction with sulphide for 1 min the 315 nm band was formed and on adding 0.1 M iodine in 0.1 M KI there was a shift in absorption maximum to 305 nm, suggesting that the polymeric sulphur compound had been oxidized to elemental sulphur (Fig. 7). When cyanide was added to a similar preparation, there was no shift in the absorption maximum but the absorbance at 315 nm decreased (Fig. 7). 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 (Fig. 8).

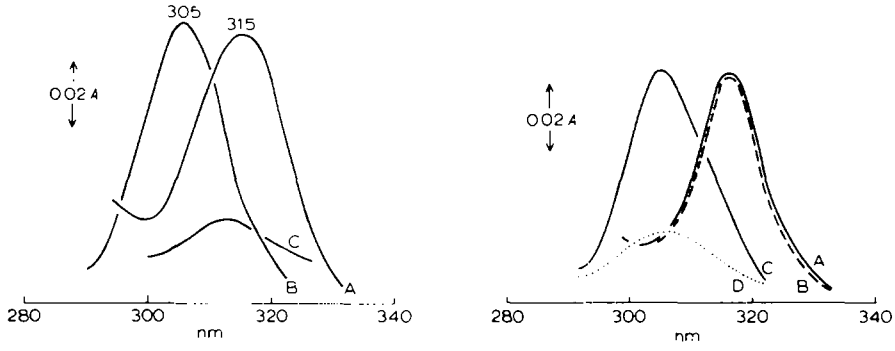


Fig. 7. Effect of cyanide and I_2 on the difference spectra of products of sulphide oxidation. Sample and reference cuvette contained 10 mg P_{144} in 3.0 ml 50 mM phosphate buffer (pH 7.0). A, after adding 5 μmoles Na_2S to sample cuvette; B, after adding 20 μl 0.1 M I_2 to A; C, after adding a small crystal of KCN to A.

Fig. 8. Effect of sulphite on the difference spectra of products of sulphide oxidation before and after acetone treatment. A, sulphide reduced *vs* oxidized difference spectra of P_{144} fraction (4 mg/ml); B, after adding a few crystals of Na_2SO_3 to A; C, after adding 0.1 ml acetone to A; D, after adding a few crystals of Na_2SO_3 to C.

Production of sulphite. Large amounts of sulphite accumulated when sulphide was incubated in air with crude extract (S_{10}). The rate of sulphite formation was used to follow sulphide oxidation. Neither the S_{144} nor the P_{144} fraction alone oxidized sulphide to sulphite. However, by recombining them the oxidizing system was reconstituted (Table III). When NO_3^- was added to the reaction mixture SO_3^{2-} was not detected.

Sulphite oxidation

The two sulphite-oxidizing systems, namely sulphite-coupled nitrate reductase and APS reductase, were separated by differential centrifugation. The latter enzyme was found in the soluble fraction (S_{144}) while sulphite-dependent nitrate reductase was located in the pellet fraction (P_{144}) (Table IV).

TABLE III

PRODUCTION OF SULPHITE FROM SULPHIDE BY CELL PREPARATIONS

Assay conditions are given in Materials and Methods.

Fraction	*Specific activity (nmoles SO_3^{2-} formed/10 min per mg protein)*
Crude extract (S_{10})	58
Supernatant fraction (S_{144})	17
Membrane fraction (P_{144})	14
$S_{144}+P_{144}$ (ratio 1:1, mg)	67

TABLE IV

DISTRIBUTION OF SULPHITE-OXIDIZING SYSTEMS

Assay conditions are given in Materials and Methods. Specific activity for the sulphite-linked nitrate reductase is expressed in nmoles nitrite formed/10 min per mg protein; for APS reductase, in μmoles ferricyanide reduced/h per mg protein.

Fraction	*Sulphite-linked nitrate reductase*	*APS reductase*
Crude extract (S_{10})	270	12.3
Supernatant fraction (S_{144})	18	25.6
Pellet fraction (P_{144})	780	2.8

DISCUSSION

Unlike *T. concretivorus*[12], O_2 uptake in *T. denitrificans* continued after all sulphide had been oxidized, probably because the products of sulphide oxidation are themselves being oxidized by enzymes. The K_m for sulphide in *T. denitrificans* is higher than that reported for *T. concretivorus*[12] since in the former a P_{144} fraction was used whereas crude S_{20} preparation was used in studies with *T. concretivorus*.

The early product of sulphide oxidation is probably bound to the lipoprotein membrane fraction. This is suggested by experiments with radioactive sulphide. Moriarty and Nicholas[7] found a considerable degradation of ^{35}S 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 not a likely product.

It is known that the absorption bands of polysulphides shift to longer wavelength as the number of conjugated sulphur atoms increases[23]. 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 in membrane fractions 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 by membrane fractions (P_{144}). 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, did not react with the membrane-bound sulphur intermediate but did so only after it was dissociated from the membranes by treatment with acetone. This effect which was not observed in *T. concretivorus*[12] 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 in *T. concretivorus* and the sulphite oxidizing enzymes, namely APS reductase and sulphite-linked nitrate reductase, are absent in this bacterium.

The formation of the 300–350 nm absorbing material was inhibited by CO

and this effect was reversed by light. CO also restricted the binding of [^{35}S]sulphide by membrane fractions. Under anaerobic conditions sulphide is bound only in the presence of nitrite. 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 this ultraviolet light-absorbing polymeric sulphur compound which is membrane-bound.

The membrane-bound polysulphide was the only intermediate of sulphide oxidation detected by Moriarty and Nicholas[7] in *T. concretivorus*. Other workers[3-6] have proposed that elemental sulphur, thiosulphate and polythionates are intermediates in the process. In addition to the membrane-bound polysulphide, we find that sulphite was produced from sulphide by crude extracts of *T. denitrificans*. The oxidation of sulphide in this bacterium is linked to nitrate reduction *via* the sulphite-dependent nitrate reductase since, in the presence of nitrate, sulphite was not detected.

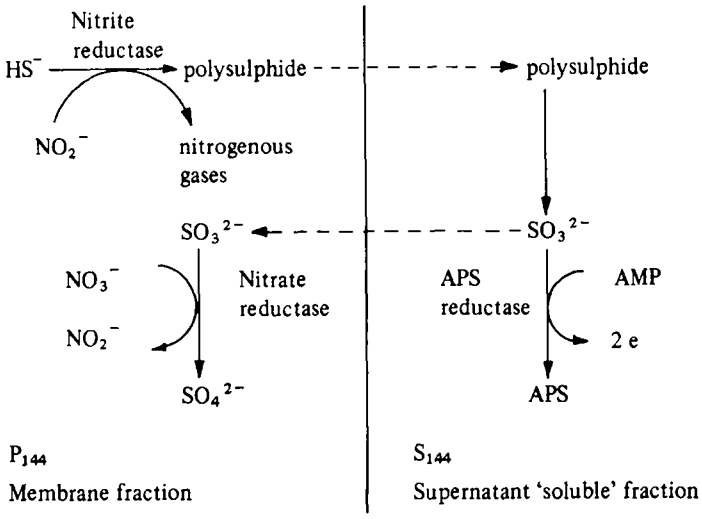
When crude extracts (S_{10}) are incubated with S^{2-} and NO_3^- under aerobic conditions, S^{2-} is initially and almost completely converted to polysulphide because of the presence of O_2. This has been shown to be a fast reaction utilizing most of the sulphide. Polysulphide is then oxidized at a slower rate to SO_3^{2-}, which then reduces NO_3^- to NO_2^- enzymically. NO_2^- could not be reduced further even though an active NO_2^- reductase is present because the donor (S^{2-}) has been depleted. Hence, NO_2^- accumulates and can thus be detected. This would also explain why NO_3^- reductase using S^{2-} as donor cannot be detected when the experiment is conducted under anaerobic conditions because O_2 is now not available and therefore the first step (S^{2-} -> polysulphide) cannot occur. Consequently SO_3^{2-} is not formed and NO_3^- is not reduced to NO_2^-.

The production of NO, N_2O and N_2 from nitrite by cell suspensions confirms that the bacterium is a denitrifier utilizing reduced sulphur compounds as electron donors. Adams *et al.*[11] have shown that in whole cells sulphide, sulphite and thiosulphate were all effective in reducing nitrate to nitrogenous gases.

The nitrite reductase, which utilizes sulphide as a donor, was found to be associated with the membrane fraction P_{144}. 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 were ineffective as donors for nitrite reductase in this fraction, but NADH, in the presence of PMS, was effective under anaerobic conditions. A nitrite reductase from *Micrococcus denitrificans*[24] which utilizes NADH as an electron donor was found to have a cytochrome oxidase activity. 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-dependent nitrite reductase may also function as a cytochrome oxidase.

The pH optimum for sulphide oxidation is 7.5 as determined by the uptake of oxygen and the reduction of nitrite. Based on the pK_2 (ref. 25) for H_2S and applying the Handerson–Hasselback equation, it is known that between pH 6 and 9 the HS^- is the predominant ionic species, indicating that this may be the form utilized by the nitrite reductase.

Based on the results reported herein, we propose Scheme 1 for the oxidation of sulphide in *T. denitrificans*.



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