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Electron Transfer during Sulphide and Sulphite Oxidation in *Thiobacillus denitrificans*

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SUMMARY

Cytochromes of the *c*, *a* and *d* types have been detected in crude extracts of *Thiobacillus denitrificans*. In the membrane fraction (P 144), 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 S 144 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. Inhibitor studies on sulphide oxidation linked to either oxygen uptake or nitrite reduction indicated that flavin, a terminal oxidase and copper may be involved. A scheme of electron transport during sulphide and sulphite oxidation is proposed.

INTRODUCTION

The thiobacilli oxidize a variety of sulphur compounds and these reactions appear to be coupled to electron transport via a cytochrome system (Roy & Trudinger, 1970). Cytochromes of the *c* type in the thiobacilli are well documented and several have been purified and characterized. Thus, Trudinger (1961) separated five types of cytochrome *c* from *Thiobacillus neapolitanus*. Milhaud, Aubert & Millet (1958) demonstrated that a *c*-type cytochrome from *T. denitrificans* mediates electron transfer between thiosulphate or sulphite and nitrate. Moriarty & Nicholas (1969) found a CO-combining cytochrome of the *c* type which was involved in sulphide oxidation. According to Aleem (1965), neither flavin nor cytochrome *b* mediates electron flow between thiosulphate and oxygen in *T. novellus*.

The oxidation of sulphite in *Thiobacillus thio-oxidans* (Kodama & Mori, 1968), thiosulphate in *T. novellus* (Aleem, 1965) and in *T. neapolitanus* (Saxena & Aleem, 1973), and sulphide in *T. concretivorus* (Moriarty & Nicholas, 1969) are all inhibited by CO, and these effects are photoreversible. Cytochrome a_3 and cytochrome *o* have been proposed as terminal oxidases in *T. denitrificans* (Peeters & Aleem, 1970). Moriarty & Nicholas (1970) reported that two electron-transfer pathways exist in *T. concretivorus*. One operates during sulphide oxidation and involves cytochromes *b*, *c* and *d* and copper, whereas the other is associated with sulphite oxidation which involves cytochromes *b*, *c* and *a*.

We have shown that in *Thiobacillus denitrificans* sulphide oxidation may be linked to oxygen, nitrate or nitrite as terminal acceptors (Aminuddin & Nicholas, 1973). We have also reported (Aminuddin & Nicholas, 1974) that a particulate sulphite oxidase uses either oxygen or nitrate as terminal acceptors in the same bacterium. In this paper we present data on the electron transfer systems which mediate these reactions.

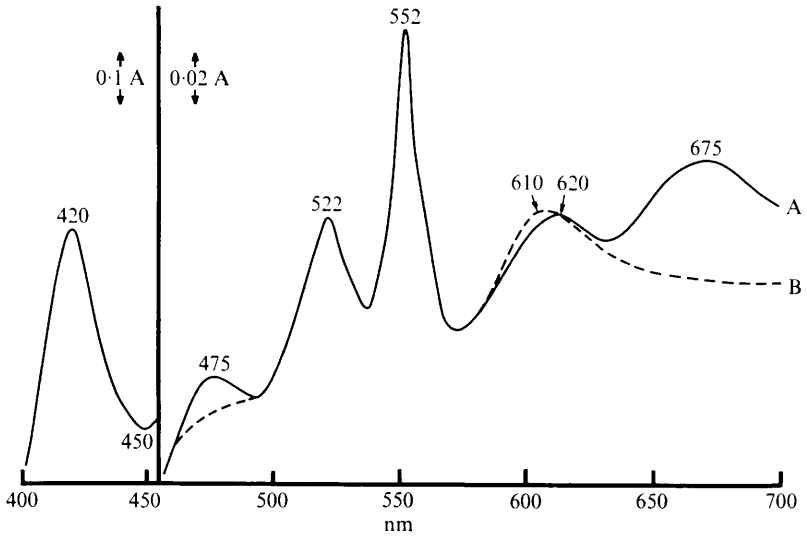


Fig. 1. Reduced versus oxidized difference spectra of crude extract (S 10). One cm cuvettes contained 0·5 ml crude extract (10 mg protein) in 50 mM-phosphate buffer (pH 7·5). A few crystals of either Na_2S or Na_2SO_3 were added to the sample cuvette. (A), Na_2S as reductant; (B) Na_2SO_3 as reductant.

METHODS

Culture of organism. Thiobacillus denitrificans ('Oslo' strain) was grown and harvested as described previously (Aminuddin & Nicholas, 1974).

Preparation of extracts. Extracts were prepared from bacteria suspended in 50 mM-phosphate buffer with 0·2 mM-Na-EDTA, and disrupted in a French pressure cell as described previously (Aminuddin and Nicholas, 1974).

O_2 measurement. O_2 uptake was determined by an electrode method (Aminuddin & Nicholas, 1973; 1974).

Spectrophotometry. A Shimadzu multi-purpose recording spectrophotometer (Model MPS-50L) was used to record the absorption spectra of components of the respiratory chain.

Nitrite reductase assay. Nitrite reductase, using sulphide as an electron donor, was assayed in stoppered tubes (7·5 × 1 cm). The reaction mixture, containing 0·1 ml enzyme, 2μ-mol Na_2S and 1 μmol $NaNO_2$ in 50 mM-phosphate buffer (pH 7·5), was preincubated for 5 min at 30 °C before adding the electron donor. After 10 min the reaction was terminated by adding 1·0 ml zinc acetate 10%, w/v. After centrifuging at 2000 *g* for 5 min, nitrite was determined in a sample of the supernatant fraction by the Griess and Ilosvay colorimetric method (Hewitt & Nicholas, 1968).

Adenosine 5'-sulphatophosphate (APS) reductase assay. A modification of the method of Peck (1961) was employed (Aminuddin & Nicholas, 1973).

Sulphite oxidase assay. This was determined as described previously (Aminuddin & Nicholas, 1974).

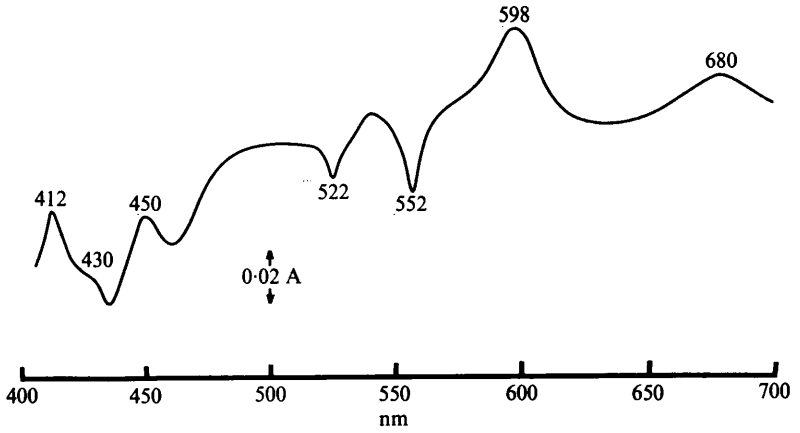


Fig. 2. CO-sulphide minus sulphide-reduced difference spectra of crude extract (S 10). One cm cuvettes contained 0·5 ml crude extract (10 mg) protein in 50 mM-phosphate buffer (pH 7·5) reduced first with a few crystals of Na_2S. CO was bubbled through the sample cuvette for 2 min.

RESULTS

Cytochrome components

Crude extracts (S 10). The difference spectra of the crude extract (S 10), sulphide reduced versus oxidized, indicated that the following types of cytochromes were present: cytochrome *c* (420, 522 and 552 nm) and cytochrome *d* (475 and 675 nm) (Fig. 1). In addition, there was a broad absorption band around 600 to 620 nm which may have been 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 suggested that flavin was also present. The cytochromes reduced by sulphide were reoxidized by oxygen or under anaerobic conditions by nitrite. Nitrate, however, was ineffective.

With sulphite as the reductant instead of sulphide, the reduced minus oxidized difference spectra of S 10 showed that cytochromes of the *c* and *a* types were reduced but not cytochrome *d*, since under these conditions the 475 nm and 675 nm absorption bands were not recorded (Fig. 1). The 610 nm absorption band was associated with an *a*-type cytochrome. These cytochromes reduced by sulphite were reoxidized by either O_2 or nitrate but not by nitrite.

The CO-sulphide reduced versus sulphide reduced difference spectra exhibited an absorption band at 595 nm, which suggested an *a*-type cytochrome (Fig. 2). In addition there were absorption bands at 450 nm and 680 nm which may have been associated with cytochrome *d*. The CO-combining effects were reversed by light.

Supernatant fraction (S 144). The reduced versus oxidized difference spectra of the supernatant fraction S 144 (Fig. 3) indicated the presence of cytochromes of the *c* (420, 522, 552 nm) and *a* (610 nm) types. These cytochromes reduced by sulphide were not reoxidized by O_2, nitrite or nitrate. This agreed with the finding that the sulphide-oxidizing activity was located in the pellet (P 144) fraction (Aminuddin & Nicholas, 1973). However, cytochromes in the S 144 fraction reduced by sulphite were reoxidized by O_2 but not by either nitrate or nitrite.

The CO-sulphite reduced minus sulphite reduced difference spectra of the S 144 fraction exhibited an absorption band at 595 nm (cytochrome *a* type) as found in the crude extract

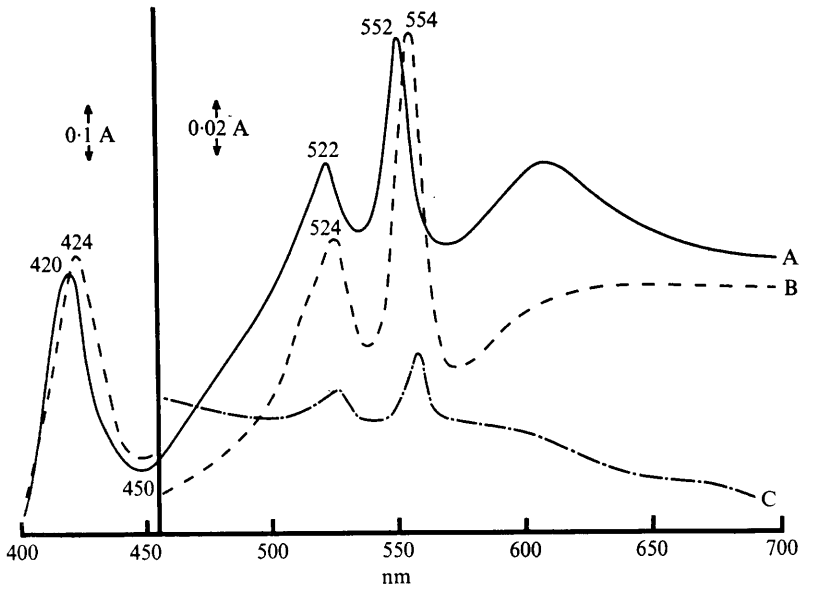


Fig. 3. Reduced minus oxidized difference spectra of the S 144 and P 144 fractions. One cm cuvettes contained either 2·0 ml S 144 or 0·5 ml P 144 (20 mg protein in each) in 50 mM-phosphate buffer (pH 7·5). A few small crystals of reductant were added to the sample cuvettes. (A), S 144 fraction reduced with either Na_2S or Na_2SO_3; (B), P 144 fraction reduced with Na_2SO_3; (C), as (B), with a few crystals of $NaNO_3$ added.

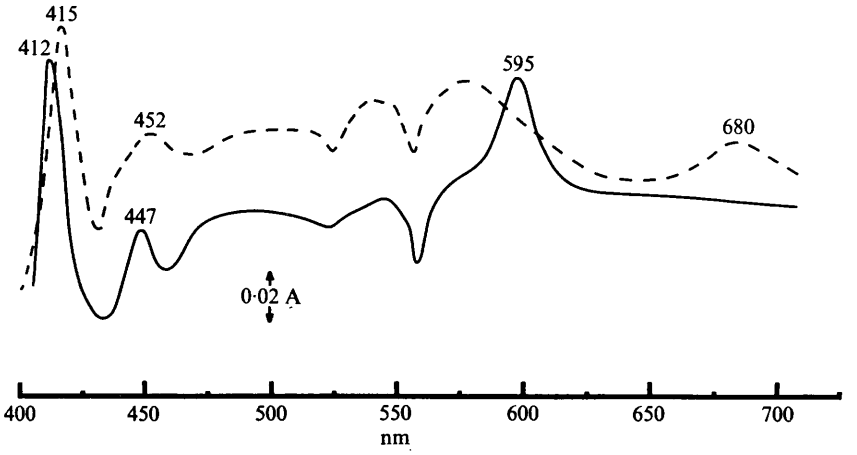


Fig. 4. CO-dithionite minus dithionite reduced difference spectra of S 144 and P 144 fractions. Conditions as in Fig. 2 except that dithionite was used as reductant.

S 10 (Fig. 4). There was also an absorption band at 447 nm which may have been the Soret band of cytochrome *a* that does not bind CO. Thus, the S 144 fraction may contain the cytochrome a-a_3 complex.

Membrane fraction (P 144). When this fraction was treated with either sulphide or sulphite, the reduced versus oxidized difference spectra obtained were markedly different from those of the S 144 fraction. Cytochromes *c* and *d* reduced by sulphide were reoxidized under

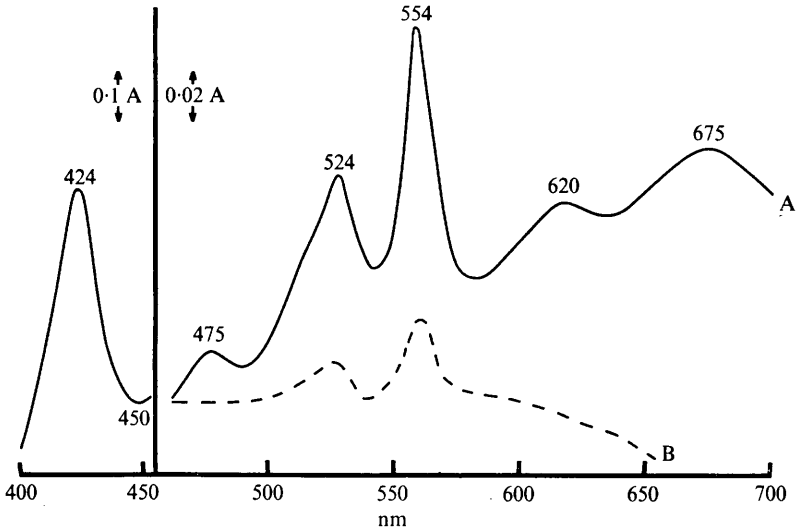


Fig. 5. Sulphide reduced minus oxidized spectra of P 144 fraction. One cm cuvettes with side arm, designed for anaerobic studies, contained 0·5 ml P 144 fraction (10 mg protein) in 50 mM-phosphate buffer (pH 7·5). Na_2S (2·5 μmol) was added to the sample cuvette, which was then rigorously evacuated to 10^{-5} mm Hg. (A), reduced versus oxidized difference spectra; (B), after readmitting air and shaking briefly or tipping in 0·2 ml 10 mM-$NaNO_2$ from the side arm.

Table 1. *Distribution of cytochromes in the various fractions of Thiobacillus denitrificans*

Reductant	Extract*	Cytochromes (λ in nm)	Effective terminal acceptor: (O_2, NO_2^- or NO_3^-)
S^{2-}	S 10	*c* (420,522,552) *a* (610) *d* (475,620,675)	O_2 or NO_2^-
	S 144	*c* (420,522,552) *a* (610)	All ineffective
	P 144	*c* (424,524,554) *d* (475,620,675)	O_2 or NO_2^-
SO_3^{2-}	S 10	*c* (420,522,552) *a* (610)	O_2 or NO_3^-
	S 144	*c* (420,522,552) *a* (610)	O_2 only
	P 144	*c* (424,524,554)	NO_3^- or O_2

* For description see text.

anaerobic conditions by either oxygen or nitrite (Fig. 5). Cytochrome of the *a* type was not detected in the pellet fraction. Sulphite only reduced cytochrome *c* in this fraction. This indicates that cytochrome *d* is involved specifically in sulphide oxidation. In contrast to the cytochromes reduced by sulphide, those reduced by sulphite were reoxidized by nitrate only (Fig. 3); nitrite was ineffective and reoxidation by O_2 was slow.

After treatment of the dithionite-reduced P 144 fraction with pyridine and alkali to form haemochromogens (Falk, 1964), the reduced versus oxidized difference spectra showed maxima at 523 and 554 nm (cytochrome *c*). A broad absorption band with maxima at 625 to 627 nm and an absorption band at 473 nm was probably associated with haem *d*.

Table 2. *Effect of inhibitors on either O_2 uptake or nitrite reduction during sulphide oxidation by P 144 fractions*

Inhibitor	Final concentration (mM)	Inhibition (%)	
		O_2 uptake	Nitrite reduction
Amytal	1·0	35	20
Rotenone	0·1	15	10
Antimycin A	1·0	7	10
HOQNO	1·0	5	3
NaN_3	1·0	3	2
KCN	0·1	89	93
Iodoacetamide	1·0	45	36
Arsenite	1·0	77	75
DIECA	1·0	88	83
Bathocuproin	1·0	92	94
CO	Bubbled through for 5 min	—	96
CO	Exposed to tungsten light for 10 min	—	21

Oxygen uptake and nitrite reduction were determined as described previously (Aminuddin & Nicholas, 1973). The inhibitor was preincubated with the enzyme and buffer for 5 min before adding the substrates. The specific activities for the control reactions (without inhibitor) were 29 nmol oxygen utilized/min/mg protein for the oxygen uptake and 102 nmol nitrite reduced/10 min/mg protein for the nitrite reductase.

The CO-sulphide reduced versus sulphide reduced difference spectra of the P 144 fraction exhibited maxima at 452 and 680 nm, indicating a cytochrome of the *d* type (Fig. 4).

The cytochromes involved in sulphide and sulphite oxidation with the respective terminal electron acceptors in the various cell fractions are summarized in Table 1.

Inhibitors of sulphide oxidation. The effects of various inhibitors on sulphide oxidation, as determined by oxygen uptake or by nitrite reduction in P 144 fraction, are shown in Table 2.

Rotenone, antimycin A, 2-heptyl-4-hydroxyquinoline-*N*-oxide (HOQNO), NaN_3 and 2-2′-dipyridyl were ineffective, but amytal, *o*-phenanthroline and iodoacetamide reduced activity by 15 to 30 % at 1·0 mM. The most potent inhibitors were KCN, dithiol, parachloromercuribenzoate (*p*CMB), arsenite, sodium diethyldithiocarbamate (DIECA) and bathocuproin. The reversal by light of the CO inhibition of sulphide-linked nitrite reductase suggests that a terminal oxidase is involved. At the concentrations shown in Table 2, the inhibitors listed had no effect on the chemical determination of nitrite.

DISCUSSION

Sulphide oxidation. The formation from sulphide of the 300 to 350 nm-absorbing polysulphide (Aminuddin & Nicholas, 1973) and the reduction of nitrite by sulphide in the P 144 fraction were inhibited by CO. The latter effect, which was reversed by light, suggests that a terminal oxidase is involved. Reduced and CO-reduced spectra indicated that this terminal cytochrome was probably of the *d*-type. Milhaud *et al.* (1958) found an *a*-a_3-type cytochrome in *Thiobacillus denitrificans* which Peeters & Aleem (1970) suggested was involved in sulphide oxidation linked to either O_2 or nitrate as a terminal acceptor. In our experiments, however, the *a*-a_3 complex was not detected in the P 144 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 suggested that cytochrome *o* may be involved in sulphide oxidation but we were unable to detect it in our preparations.

The α-bands of the CO-reduced haem *d* are similar to those found in other bacteria (Lam & Nicholas, 1969; Newton, 1969; Yamanaka & Okunuki, 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 our preparation.

A dissimilatory nitrite reductase from *Pseudomonas aeruginosa* (Yamanaka & Okunuki, 1963) and *Micrococcus 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 P 144 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 supported 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 *Thiobacillus concretivorus* (Moriarty & Nicholas, 1969), DIECA did not inhibit the reduction of cytochromes by sulphide, indicating that the site of action of this metal is at the terminal end of the respiratory chain. Nitrite reductases from other bacteria (Yamanaka, 1964; Radcliffe & Nicholas, 1968) have been shown to contain copper.

Trudinger (1967) has postulated that the initial reaction of sulphide oxidation involves a membrane-bound thiol. In our experiments, iodoacetamide and *p*CMB inhibited the reduction of cytochromes by sulphide. These effects are difficult to interpret because sulphide reacts chemically with these inhibitors.

Sulphite oxidation. We have reported (Aminuddin & Nicholas, 1974) that oxygen uptake during sulphite oxidation is affected by inhibitors of electron transport. Our results agree quite closely with those of Adams, Warnes & Nicholas (1971), 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 we found that HOQNO inhibited oxygen uptake during sulphite oxidation, which may suggest the involvement of cytochrome *b*, we have not been able to detect it in spectra of cell extracts. Difference spectra of P 144 fraction showed that sulphite reduced only flavin and cytochrome *c*. Milhaud *et al.* (1958) suggested that cytochrome a-a_3 mediates electron flow between sulphite or thiosulphate and nitrate, but our results indicated 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 in purified fractions (Adams *et al.* 1971) and the fact that cytochromes in the P 144 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 (Aminuddin & Nicholas, 1974).

Although both sulphite-linked nitrate reductase and sulphide-linked nitrite reductase occur in the P 144 fraction, the electron donor (SO_3 or S^{2-}) is specific for the inorganic nitrogen acceptor (NO_3^{2-} or NO^{2-}) as shown in the scheme below. We have recently extracted

the SO_3^{2-}-linked nitrate reductase from the P 144 membrane fraction, leaving behind the S^{2-}-linked NO_2^- reductase. The sequence of electron flow for the SO_3^{2-} linked nitrate reductase is as follows:

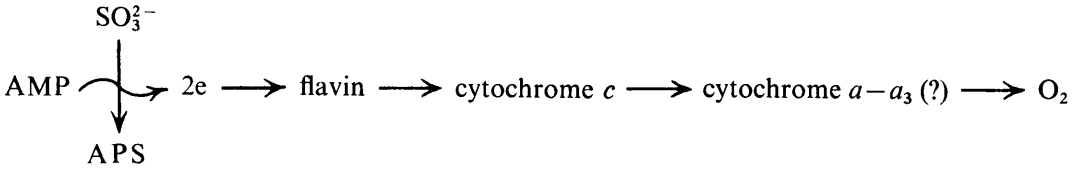
$$SO_3^{2-} \rightarrow FAD \rightarrow \text{cytochrome } c \rightarrow NO_3^-$$

Thus at the present time our data suggest that there are two reducing systems involved.

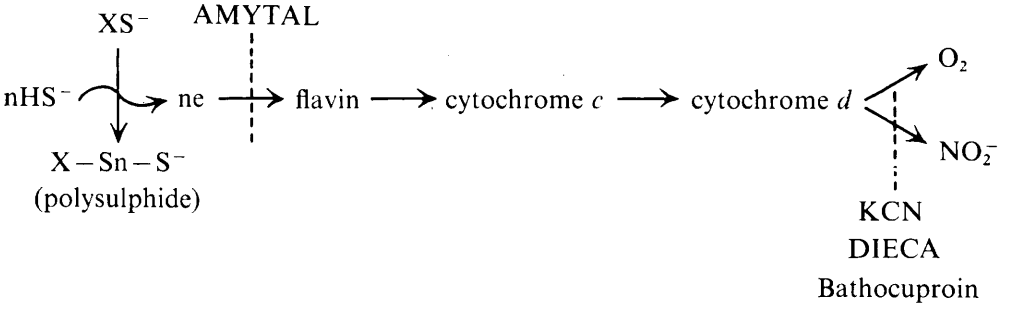
In the soluble fraction (S 144), where the APS reductase is located, the cytochromes reduced by sulphite were reoxidized by oxygen. Bowen, Happold & Taylor (1966) have shown that purified APS reductase from *Thiobacillus denitrificans* synthesized APS in the absence of any added acceptor, presumably because of the activity of the enzyme with oxygen. However, other workers have been unsuccessful in linking the APS reductase to electron acceptors other than ferricyanide.

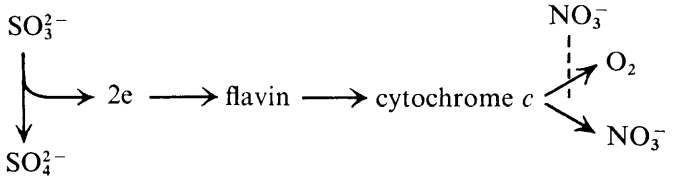
A tentative scheme for electron transfer during the oxidation of sulphide and sulphite in *T. denitrificans* is as follows:

Supernatant fraction (S 144)



Membrane fraction (P 144)





X = acceptor protein.

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