Sulphite- and NADH-dependent Nitrate Reductase from *Thiobacillus denitrificans*

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(Received 8 November 1976)

SUMMARY

A membrane-bound nitrate reductase from *Thiobacillus denitrificans* can utilize either sulphite or NADH as an electron donor. The sulphite-dependent nitrate reductase activity was released from the membrane by treatment with sodium deoxycholate. Cytochrome *c* and FAD were separated from the solubilized enzyme by heat treatment and subsequent chromatography on DEAE-cellulose. The bacterial cytochrome *c* and a preparation of horse-heart cytochrome *c* served as electron mediators to the solubilized sulphite-dependent nitrate reductase activity with apparent K_m values of 1.5 and 1.3 μM respectively. The NADH-linked enzymic activity, which was unstable during storage, was re-activated with reduced glutathione. It was also inactivated after treatment with deoxycholate but this effect was reversed by menadione. A possible scheme for electron transport for the sulphite- and NADH-dependent enzyme is proposed.

INTRODUCTION

Thiobacillus denitrificans derives energy for growth from the oxidation of sulphur compounds and, in the absence of oxygen, nitrate functions as an alternative terminal electron acceptor (Beijerinck, 1904; Baalsrud & Baalsrud, 1954; Delwiche, 1956; Peeters & Aleem, 1970). Baalsrud & Baalsrud (1954) reported that, under anaerobic conditions, the oxidation of elemental sulphur and thiosulphate was linked to the reduction of nitrate to nitrite and nitrogen gas. It was subsequently demonstrated that sulphite and sulphide served as reductants for nitrate and nitrite reductases, respectively (Adams, Warnes & Nicholas, 1971; Aminuddin & Nicholas, 1973).

A sulphite-dependent nitrate reductase from *T. denitrificans* has been partially purified and the enzyme was found to catalyse the reduction of nitrate by a Ping-Pong mechanism (Adams *et al.*, 1971). The involvement of cytochromes *b* and *c* in electron transport from sulphite to nitrate has been demonstrated (Milhaud, Aubert & Millet, 1958; Peeters & Aleem, 1970; Adams *et al.*, 1971; Aminuddin & Nicholas, 1974*a*). In addition to sulphite, NADH is also an effective electron donor for nitrate reductase, but the relationship between these two electron donors has not been examined fully. This paper reports on the solubilization and properties of sulphite-dependent nitrate reductase activity and its relation to the NADH-linked enzyme activity.

METHODS

Culture and harvesting of the organism. *Thiobacillus denitrificans* (9547, NCIB strain AB5) was grown and harvested as described by Aminuddin & Nicholas (1974*b*). All subsequent procedures were carried out at 4 °C.

Preparation of cell-free extracts. The washed cells were suspended (25 %, w/v) in 0.05 M-Tris/HCl buffer, pH 7.5, and passed twice through an Aminco French pressure cell at 140 MPa. Unbroken organisms and debris were removed by centrifuging at 10000 *g* for 30 min. The supernatant fraction (S_{10}) was further centrifuged at 100000 *g* for 2 h. Nitrate reductase was almost exclusively located in the final pellet fraction (P_{100}).

Solubilization of nitrate reductase. The P_{100} fraction was suspended in 0.05 M-Tris/HCl buffer, pH 7.5, and homogenized in a Teflon tissue grinder. The suspension, diluted in 0.05 M-Tris/HCl buffer, pH 7.5, to a final protein concentration of 30 mg ml⁻¹, was ultrasonicated for 30 s (MSE Ultrasonicator; 60 W output). It was then incubated with sodium deoxycholate [0.06 mg (mg protein)⁻¹] for 30 min at 30 °C in a shaking water bath (100 oscillations min⁻¹), and centrifuged at 100000 *g* for 30 min. The supernatant fraction [S_{100} -(deoxy)] was passed through a Sephadex G-25 column (1.25 × 15 cm) which had been equilibrated with 0.05 M-Tris/HCl buffer, pH 7.5. The resulting preparation is referred to as the solubilized enzyme.

*Separation and detection of flavin and cytochrome *c* from the solubilized enzyme.* The solubilized enzyme was placed in a boiling water bath for 15 min. The precipitated proteins were removed by centrifuging at 100000 *g* for 30 min. The supernatant fraction was loaded on to a DEAE-cellulose column (DE 32; 3 × 12 cm) which had been equilibrated with 0.05 M-Tris/HCl buffer, pH 7.5. The column was eluted sequentially at room temperature with two 100 ml batches of 0.05 M-Tris/HCl buffer, pH 7.5, the first containing 0.1 M-NaCl and the second, 0.2 M-NaCl. Cytochrome *c* was eluted with 0.1 M-NaCl and the flavin fraction with 0.2 M-NaCl. The fractions containing cytochrome *c* and flavin were pooled separately, desalted by passing through a Sephadex G-25 column (1.25 × 15 cm), and concentrated in a rotary vacuum evaporator at 30 °C.

The concentrated sample containing flavin was spotted, together with FAD and FMN standards, on to 3MM Whatman paper for descending paper chromatography. The chromatogram was developed for 30 h using t-butanol/water (3:2, v/v) as solvent. Flavins were detected under u.v. light. The apparatus described by Tate (1968) was also used to identify flavins by high-voltage electrophoresis. After spotting the samples on to Whatman 3MM paper, the electrophoresis was done in 0.1 M-sodium citrate buffer, pH 5.0, at 1500 V for 60 min. Authentic FAD and FMN samples were used as standards.

Spectrophotometry. Absorption spectra of cytochrome *c* and the P_{100} fraction were recorded using a multipurpose recording spectrophotometer (model MPS-50L; Shimadzu, Kyoto, Japan).

*Estimation of molecular weight of cytochrome *c*.* This was determined by gel chromatography in a Sephadex G-75 column as described by Andrews (1964), using bovine serum albumin, horse-heart cytochrome *c*, myoglobin (horse-heart) and ovalbumin as standard proteins of known molecular weight.

*Oxidation-reduction potential of cytochrome *c*.* This was determined by the procedure of Davenport & Hill (1952), except that 1 cm open glass cuvettes were used. The E'_0 value for the ferro-ferricyanide couple used in the Nernst equation was +423 mV as determined by O'Reilly (1973). All reactions were carried out in 0.05 M-Tris/HCl buffer, pH 7.5, at room temperature.

Assay of nitrate reductase. The enzyme activity was determined by following the rate of nitrite production with either NADH or Na₂SO₃ as an electron donor. The reaction mixture contained (final vol. 2 ml): KNO₃, 1 μmol; Na₂SO₃ or NADH, 1 μmol; Tris/HCl buffer, pH 7.5, 90 or 80 μmol (for the SO₃²⁻ or NADH systems respectively); and 0.01 ml enzyme preparation. The tubes were equilibrated at 30 °C for 2 min in a reciprocating water bath

(100 oscillations min^{-1}) and the reaction was started by adding either Na_2SO_3 or NADH. For assays of the SO_3^{2-} -linked enzyme, the reaction was stopped after 15 min by adding 1 ml 1% (w/v) sulphanilamide in 1 M-HCl followed by 1 ml 0.01% (w/v) *N*-1-naphthylethylenediamine dihydrochloride (Hewitt & Nicholas, 1964). For assays of the NADH-linked enzyme, the reaction was terminated after 15 min by adding 0.1 ml 1 M-acetaldehyde and 0.1 ml crystalline alcohol dehydrogenase (0.3 mg protein ml^{-1} in 0.05 M-Tris/HCl buffer, pH 7.5) to oxidize the residual NADH. The amount of nitrite produced in the reaction mixture was determined colorimetrically by the sulphanilamide method described above. After leaving the mixture to stand for 20 min, E_{540} was measured in a Shimadzu QV-50 spectrophotometer. Enzyme activity is expressed as nmol NO_2^- produced in 15 min.

For assays using cytochrome *c* as an electron mediator, the assay mixture and experimental procedure were as described above, except that 0.1 ml of the solubilized enzyme was used. Cytochrome *c* prepared from *T. denitrificans* as described above and horse-heart cytochrome *c* were reduced by adding excess solid sodium dithionite and then passed through a Sephadex G-25 column (1.25×15 cm).

Protein determinations. The micro-biuret method of Itzhaki & Gill (1964) was used to determine protein in the enzyme preparations, using bovine serum albumin as a standard.

Chemicals. Analytical-grade chemicals were dissolved in double-distilled water. Solutions of sodium sulphite and NADH were prepared just before use. NADH, FAD, FMN, ovalbumin (grade V), bovine serum albumin (fraction V), horse-heart cytochrome *c* (type VI), dithiothreitol, reduced glutathione (GSH), 2-heptyl-4-hydroxyquinoline-*N*-oxide (HOQNO), antimycin A, alcohol dehydrogenase [338 units (mg protein) $^{-1}$], *p*-chloromercuribenzoic acid, and Tris were obtained from Sigma; sodium diethyldithiocarbamate (DIECA) from Merck; bathocuproin from Fluka, Buchs, Switzerland; atabrine from Imperial Chemical Industries; Whatman DEAE-cellulose (DE 32) and 3MM chromatography paper were from H. Reeve Angel, London EC4; and Sephadex G-75, G-25 and Blue Dextran from Pharmacia.

RESULTS

Comparison of sulphite- and NADH-linked nitrate reductase activities

Sulphite and NADH as electron donors. Although the SO_3^{2-} - and NADH-dependent nitrate reductase activities were similar in the S_{10} and P_{100} preparations (Table 1), solubilization of the enzyme from the P_{100} fraction with sodium deoxycholate resulted in an almost complete loss of the NADH-dependent activity, whereas the specific activity of the SO_3^{2-} -linked nitrate reductase was increased. The activity of the NADH-linked enzyme in the deoxycholate-treated P_{100} preparation was enhanced more than twofold by including phenazine methosulphate in the reaction mixture (Table 1) but neither horse-heart cytochrome *c* nor FMN had any effect.

The NADH-dependent nitrate reductase activity in the solubilized preparation was fully restored by adding 2.5 mM-menadione (Fig. 1), but this inhibited the untreated P_{100} fraction. Menadione had no effect on the SO_3^{2-} -linked nitrate reductase activity.

When both NADH and SO_3^{2-} were added to the reaction mixture containing the P_{100} fraction, the amount of nitrite produced was equivalent to the sum of nitrite formed by each reductant separately (Table 2). This effect was observed even when SO_3^{2-} and NADH were present at optimum concentrations.

Effect of glutathione. The NADH-dependent nitrate reductase activity in the P_{100} fraction was labile during storage whereas the SO_3^{2-} -linked activity was stable. Over 50% of the NADH-dependent activity of the P_{100} fraction was lost after 5 days at 0 °C; this was restored

Table 1. *Comparison of sulphite and NADH as electron donors for nitrate reductase activity in fractions of T. denitrificans*

Nitrate reductase was purified and assayed as described in Methods. Where indicated, 1 μ mol phenazine methosulphate (PMS) was added to the assay mixture. Enzyme activities are expressed as nmol NO_2^- produced $(15 \text{ min})^{-1} (\text{mg protein})^{-1}$.

Fraction	Electron donor ...	Enzyme activity		
		SO_3^{2-}	NADH	NADH + PMS
S ₁₀ . Supernatant fraction after centrifuging the broken cells at 10000 g for 30 min		117	117	22
S ₁₀₀ . Supernatant fraction after centrifuging S ₁₀ at 100000 g for 2 h		23	9	10
P ₁₀₀ . Pellet fraction after centrifuging S ₁₀ at 100000 g for 2 h		300	340	54
Fraction P ₁₀₀ incubated with sodium deoxycholate [0.06 mg (mg protein) ⁻¹] at 30 °C for 30 min		368	31	73
S ₁₀₀ (deoxy). Supernatant fraction after centrifuging P ₁₀₀ treated with sodium deoxycholate at 100000 g for 30 min		453	24	69
Solubilized enzyme. Fraction S ₁₀₀ (deoxy) passed through a Sephadex G-25 column		428	16	141

Fig. 1. Restoration of NADH-linked nitrate reductase activity with menadione in the deoxycholate-treated P₁₀₀ fraction. Samples of the untreated P₁₀₀ fraction (●) and the sodium deoxycholate-treated P₁₀₀ fraction (○) were pre-incubated with various concentrations of menadione for 10 min at 4 °C before the enzyme activity was determined.

almost completely by pre-incubating with 5 mM-GSH for 15 min at 4 °C. GSH did not stimulate the SO_3^{2-} -linked enzyme and had a small inhibitory effect above 5 mM.

pH optima. The maximum activities for NADH and SO_3^{2-} were at pH 7.0 and 8.0 respectively (Fig. 2). The SO_3^{2-} -linked enzyme activity was markedly inhibited in 0.05 M-phosphate buffer over the range pH 5.8 to 8.0 but the NADH-linked activity was unaffected.

Effect of substrate concentration. The apparent K_m values for SO_3^{2-} and NO_3^- in the P₁₀₀ fraction were 110 μ M and 41.6 μ M respectively, and those for NADH and NO_3^- were 330 μ M and 83.3 μ M respectively.

Table 2. Combined effects of sulphite and NADH as electron donors for nitrate reductase activity

The enzyme activity was determined (as described in Methods) in the P_{100} fraction at different concentrations of sulphite, either alone or in the presence of 0.5 mM or 1.0 mM-NADH. Activities are expressed as nmol NO_2^- produced $(15 \text{ min})^{-1} (\text{mg protein})^{-1}$.

Na_2SO_3 in assay (mM)	Enzyme activity		
	Without NADH	With 0.5 mM-NADH	With 1.0 mM-NADH
0	0	317	330
0.125	121	417	434
0.25	152	434	445
0.5	178	478	460
0.75	171	439	456
1.00	169	434	456
1.25	165	417	445
1.50	160	417	408

Fig. 2. Effect of pH on the enzyme activity in the P_{100} fraction. (a) Sulphite-linked nitrate reductase activity; (b) NADH-linked nitrate reductase activity. Buffers were: $\circ$, 0.05 M-phosphate; $\bullet$, 0.05 M-Tris/maleic acid; $\triangle$, 0.05 M-Tris/HCl.

Temperature stability. The NADH- and SO_3^{2-} -dependent enzymes were inactivated by 14 % and 50 % respectively by heating the P_{100} fraction at 45 °C for 15 min.

Absorption spectra. Difference spectra (reduced-minus-oxidized) of the P_{100} fraction, with either NADH or SO_3^{2-} as reductants, showed absorption maxima at 524 and 554 nm, consistent with the presence of cytochrome *c*. The reduced cytochrome was rapidly oxidized in both systems on adding nitrate (Fig. 3). This suggests that cytochrome *c* is involved in the reduction of NO_3^- by either electron donor.

Effects of various potential inhibitors

Sulphydryl reagents. The greater sensitivity of the NADH-linked nitrate reductase activity to *p*-chloromercuribenzoate and iodoacetamide indicates that this system has a higher

Fig. 3. Difference spectra of the P_{100} fraction. The spectra were examined under anaerobic conditions in Thunberg cuvettes (1 cm light path) fitted with a side arm. The main compartment contained 0.3 ml P_{100} fraction (9 mg protein) and 0.05 M-Tris/HCl buffer, pH 7.5, in a final volume of 2 ml. Sulphite or NADH ($2 \mu\text{mol}$) was added to the sample cuvette and a crystal of potassium ferricyanide was added to the reference cuvette. Nitrate ($2 \mu\text{mol}$) was placed in the side arm of the sample cuvette. (a) Reduced-minus-oxidized spectrum; (b) spectrum after addition of nitrate.

Table 3. *Effect of various sulphhydryl inhibitors on sulphite- and NADH-linked nitrate reductase activities*

The enzyme reactions were carried out as described in Methods. The inhibitor was incubated with the P_{100} fraction for 15 min before adding nitrate and the electron donor. The specific activities in the control reactions without inhibitor were 330 and 390 nmol NO_2^- produced $(15 \text{ min})^{-1}$ (mg protein) $^{-1}$ for NADH and SO_3^{2-} respectively.

Inhibitor	Final concn (mM)	GSH (mM)	Electron donor	...	% Inhibition	
					SO_3^{2-}	NADH
<i>p</i> -Chloromercuribenzoate	0.01	0			—	100
	0.01	2			—	37
	1.0	0			58	100
	1.0	2			68	84
Iodoacetamide	5.0	0			49	84
	5.0	5			49	84
<i>N</i> -Ethylmaleimide	1.0	0			88	62
	1.0	2			10	20

requirement for sulphhydryl groups than the SO_3^{2-} -dependent system (Table 3). The inhibition of the SO_3^{2-} -dependent nitrate reductase activity by *N*-ethylmaleimide (NEM) was probably associated with the formation of a SO_3^{2-} -NEM complex.

Flavoprotein inhibitors. Amytal at 5 mM inhibited NADH-linked nitrate reductase activity by about 22 %, but the SO_3^{2-} -dependent activity was inhibited by about 70 %. In contrast, the effect of atabrine was more pronounced on the NADH- than on the SO_3^{2-} -linked enzyme. The inhibition of NADH-dependent nitrate reductase activity by atabrine was almost completely relieved by including 5 mM-menadione in the reaction mixture (Table 4). Both enzyme activities were inhibited by rotenone. Again the NADH-linked nitrate reductase

Table 4. *Inhibition of sulphite- and NADH-linked nitrate reductase activities by antimycin A, HOQNO, dicumarol, flavin and metal inhibitors*

The reaction conditions were as described in Table 3. The specific activities in the control reactions with NADH and SO_3^{2-} as electron donors were, respectively, 342 and 380 nmol NO_2^- produced $(15 \text{ min})^{-1} (\text{mg protein})^{-1}$ for experiments with amytal, atabrine or rotenone as inhibitor, and 230 and 260 nmol NO_2^- produced $(15 \text{ min})^{-1} (\text{mg protein})^{-1}$ for all other experiments.

Inhibitor	Final concn (mM)*	Electron donor ...	% Inhibition	
			SO_3^{2-}	N ADH
Amytal	2.0		38	11
	5.0		69	22
Amytal + menadione	5.0 + 5.0		69	52
Atabrine	0.5		0	59
	2.0		23	76
Atabrine + menadione	2.0 + 5.0		23	15
Rotenone	0.5		45	60
Rotenone + menadione	0.5 + 1.0		45	36
HOQNO	0.1 μM		42	0
	0.5 μM		69	0
HOQNO + coenzyme Q_{10}	0.5 μM + 1 mM		52	0
HOQNO	5.0 μM		97	29
HOQNO + menadione	5.0 μM + 1 mM		97	3
HOQNO + coenzyme Q_{10}	5.0 μM + 0.25 mM		97	17
Antimycin A	0.05		60	33
	0.1		70	45
Antimycin A + menadione	0.1 + 1.0		70	2
Antimycin A + coenzyme Q_{10}	0.1 + 0.25		60	36
Dicumarol	1.0		72	86
Azide	0.05		86	71
KCN	0.5		58	34
KSCN	0.5		64	47
8-Hydroxyquinoline	5.0		2	4
Sodium diethyldithiocarbamate	5.0		51	49
2,2'-Bipyridyl	5.0		18	3
Bathocuproin	5.0		65	57
Urethane	5.0		17	11
Dithiol	5.0		77	44

* Except where noted

activity was restored by adding menadione, but menadione was ineffective in overcoming the inhibition of the SO_3^{2-} -linked enzyme.

Other electron transport carriers. The SO_3^{2-} -dependent activity was inhibited at lower concentrations of antimycin A and HOQNO than the NADH-linked nitrate reductase system. The inhibition of the NADH-linked nitrate reductase activity by these inhibitors was almost completely relieved by menadione, but coenzyme Q_{10} was not as effective (Table 4). Both enzyme activities were inhibited by dicumarol.

Metal chelators. Both the NADH- and SO_3^{2-} -dependent activities were inhibited by KSCN (0.5 mM), bathocuproin (5 mM), azide (0.05 mM) and cyanide (0.5 mM). However urethane, 2,2'-bipyridyl and 8-hydroxyquinoline did not inhibit either of these activities to a significant extent (Table 4).

Chlorate. Chlorate inhibited the reduction of nitrate with either NADH or SO_3^{2-} as electron donors. The NADH-dependent activity was inhibited by chlorate competitively with respect to NO_3^- while it showed mixed-type inhibition when SO_3^{2-} was used as an electron donor (Fig. 4a, b).

Fig. 4. Double-reciprocal plots of the rate of nitrate reduction in the P_{100} fraction with various concentrations of nitrate and chlorate. (a) NADH-linked nitrate reductase activity; (b) sulphite-linked nitrate reductase activity. Chlorate concentrations were: ○, 1 mM; △, 0.5 mM; ●, none.

Some properties of the solubilized nitrate reductase

Cytochrome *c* was isolated from the solubilized nitrate reductase as described in Methods. The reduced-minus-oxidized spectrum of this cytochrome *c* had maxima at 554, 524 and 417 nm. The oxidation-reduction potential at pH 7.5 was +0.223 V and its molecular weight, estimated by gel chromatography through Sephadex G-75, was 13 000. This bacterial cytochrome *c* and the horse-heart cytochrome *c* both served as effective electron mediators to the solubilized SO_3^{2-} -dependent nitrate reductase. The apparent K_m values for the bacterial cytochrome *c* and horse-heart cytochrome *c* were $1.53 \mu\text{M}$ and $1.33 \mu\text{M}$ respectively.

The yellow fraction eluted from the DEAE-cellulose column was analysed by paper chromatography and FAD was identified as the native flavin in the fraction. On incubating this flavin with snake venom pyrophosphatase, FMN was produced and this was identified by high-voltage electrophoresis. These results were confirmed by paper chromatography.

DISCUSSION

The nitrate-reducing system able to use either SO_3^{2-} or NADH as an electron donor is localized in the membrane fractions of *T. denitrificans*. This respiratory-type enzyme is usually associated with membranes, as in *Escherichia coli* (Iida & Taniguchi, 1959), *Pseudomonas aeruginosa* (Fewson & Nicholas, 1961), *P. denitrificans* (Lam & Nicholas, 1969) and

Fig. 5. Proposed scheme for the transfer of electrons from SO_3^{2-} and NADH to nitrate.

Micrococcus denitrificans (Radcliffe & Nicholas, 1970). The particulate SO_3^{2-} -linked nitrate reductase activity from *T. denitrificans* was solubilized by treatment with sodium deoxycholate. As with other particulate nitrate reductases, treatment with detergent resulted in the inactivation of the NADH-linked enzyme activity (Lam & Nicholas, 1969; Radcliffe & Nicholas, 1970; Kiskiss & Downey, 1972*a*). This loss in activity has been attributed to disruption of electron flow from NADH to nitrate (Kiskiss & Downey, 1972*b*) and in *T. denitrificans* we have shown that the loss may be restored by adding menadione.

Inhibitor studies reported here indicated that sulphydryl groups are more important for the NADH-linked nitrate reductase activity. The inhibition of both the SO_3^{2-} - and the NADH-dependent activities by various inhibitors of the electron transport chain is similar to the results of Peeters & Aleem (1970) and Adams *et al.* (1971). The present studies confirm that cytochromes *b* and *c* participate in both NADH- and SO_3^{2-} -linked enzyme activities.

The identification of cytochrome *c* and FAD in the solubilized nitrate reductase indicates a possible role for them in electron transfer to nitrate. The participation of cytochrome *c* is further supported by the observation that the cytochrome *c* reduced in the P_{100} fraction by either SO_3^{2-} or NADH was readily oxidized by nitrate. Moreover, reduced cytochrome *c* isolated either from the solubilized enzyme or from a horse-heart preparation served as an electron mediator for nitrate reduction. It is of interest that cytochrome *c* separated from the solubilized enzyme had a similar redox potential (+0.223 V) and molecular weight (13000) to the purified horse-heart cytochrome *c*. Aubert *et al.* (1958) also separated cytochrome *c* from *T. denitrificans*: its E_0' is +0.27 V and it mediated electron flow between thiosulphate or sulphite and nitrate (Milhaud *et al.*, 1958).

Although the SO_3^{2-} -linked nitrate reductase from *T. denitrificans* has been known to utilize NADH, most of the earlier studies have been confined to the former donor. In this investigation, comparison of SO_3^{2-} - and NADH-linked nitrate reductase activities indicated several differences in their properties. These include different stabilities to storage at 0 °C and after deoxycholate treatment, different pH optima, and different sensitivities to various inhibitors. The inhibition of the SO_3^{2-} -linked nitrate reductase by phosphate buffer noted here for *T. denitrificans* has also been found for the sulphite:cytochrome *c* oxidoreductase from *T. thioparus* by Lyric & Suzuki (1970). These workers suggested that phosphate inhibits the enzyme activity by acting as a pseudo-end product because of its chemical similarity with sulphate.

The observed differences between the SO_3^{2-} - and NADH-linked nitrate reductase activities indicate that the electrons from these two reductants are donated to different sites of the enzyme complex. Similar inhibition by the quinone antagonist, dicumarol, and the metal-chelating agents, and the involvement of cytochrome *c* in both the SO_3^{2-} - and the NADH-linked nitrate reductase activities, suggest that the components of the electron transfer chain after the dehydrogenases are common to both systems. The electrons from SO_3^{2-} and NADH

are likely to be transferred to the enzyme complex via different flavoproteins and dehydrogenases and subsequently passed to coenzyme Q of the main electron transport chain. Such a mechanism would explain the selective loss and lability of NADH-dependent enzyme activity.

The reversal of inhibitory effects of atabrine, rotenone, antimycin A or HOQNO on the NADH-dependent nitrate reductase activity by menadione (but not on the SO_3^{2-} -linked system) suggests that NADH:menadione reductase mediates the transfer of electrons from NADH to cytochrome *c*. On the basis of these observations, we propose the scheme in Fig. 5 for the transfer of electrons from SO_3^{2-} and NADH respectively to nitrate in *T. denitrificans*.

REFERENCES

- ADAMS, C. A., WARNES, G. M. & NICHOLAS, D. J. D. (1971). A sulphite-dependent nitrate reductase from *Thiobacillus denitrificans*. *Biochimica et biophysica acta* **235**, 398–406.
- AMINUDDIN, M. & NICHOLAS, D. J. D. (1973). Sulphide oxidation linked to the reduction of nitrate and nitrite in *Thiobacillus denitrificans*. *Biochimica et biophysica acta* **325**, 81–93.
- AMINUDDIN, M. & NICHOLAS, D. J. D. (1974*a*). Electron transfer during sulphide and sulphite oxidation in *Thiobacillus denitrificans*. *Journal of General Microbiology* **82**, 115–123.
- AMINUDDIN, M. & NICHOLAS, D. J. D. (1974*b*). An AMP-dependent sulphite oxidase from *Thiobacillus denitrificans*: purification and properties. *Journal of General Microbiology* **82**, 103–113.
- ANDREWS, P. (1964). Estimation of molecular weights of proteins by Sephadex gel-filtration. *Biochemical Journal* **91**, 222–233.
- AUBERT, J. P., MILHAUD, G., MONCEL, C. & MILLET, J. (1958). Existence, isolement et propriétés physico-chimiques d'un cytochrome *c* de *Thiobacillus denitrificans*. *Comptes rendus hebdomadaires des séances de l'Académie des sciences* **246**, 1616–1619.
- BAALSRUD, K. & BAALSRUD, K. S. (1954). Studies on *Thiobacillus denitrificans*. *Archiv für Mikrobiologie* **20**, S34–62.
- BEIJERINCK, M. W. (1904). Phénomènes de réduction produits par les microbes. *Archiv für Néerland Science, Exactes et Naturelles, Série 2* **9**, 131.
- DAVENPORT, H. E. & HILL, R. (1952). The preparation and some properties of cytochrome *f*. *Proceedings of Royal Society B* **139**, 327–345.
- DELWICHE, C. C. (1956). Denitrification. In *Symposium on Inorganic Nitrogen Metabolism*, pp. 233–256. Edited by W. D. McElroy and B. H. Glass. Baltimore: Johns Hopkins Press.
- FEWSON, C. A. & NICHOLAS, D. J. D. (1961). Nitrate reductase from *Pseudomonas aeruginosa*. *Biochimica et biophysica acta* **49**, 335–349.
- HEWITT, E. J. & NICHOLAS, D. J. D. (1964). Enzymes of inorganic nitrogen metabolism. In *Modern Methods of Plant Analysis*, vol. 7, pp. 67–172. Edited by Y. F. Linskens, B. D. Sanwal and M. V. Tracey. Berlin: Springer Verlag.
- IIDA, K. & TANIGUCHI, S. (1959). Studies on nitrate reductase system of *Escherichia coli*. I. Particulate electron transport system to nitrate and its solubilization. *Journal of Biochemistry* **46**, 1041–1055.
- ITZHAKI, R. F. & GILL, D. M. (1964). A micro-biuret method for estimating proteins. *Analytical Biochemistry* **9**, 401–410.
- KISZKISS, D. F. & DOWNEY, R. J. (1972*a*). Localization and solubilization of the respiratory nitrate reductase of *Bacillus stearothermophilus*. *Journal of Bacteriology* **109**, 803–810.
- KISZKISS, D. F. & DOWNEY, R. J. (1972*b*). Physical aggregation and functional reconstitution of solubilized membranes of *Bacillus stearothermophilus*. *Journal of Bacteriology* **109**, 811–819.
- LAM, Y. & NICHOLAS, D. J. D. (1969). A nitrate reductase from *Micrococcus denitrificans*. *Biochimica et biophysica acta* **178**, 225–234.
- LYRIC, R. M. & SUZUKI, I. (1970). Enzymes involved in metabolism of thiosulphate by *Thiobacillus thio-parus*. I. Survey of enzymes and properties of sulphite:cytochrome *c* oxidoreductase. *Canadian Journal of Biochemistry* **48**, 334–343.
- MILHAUD, G., AUBERT, J. P. & MILLET, J. (1958). Rôle physiologique du cytochrome *c* de la bactérie chimio-autotrophe *Thiobacillus denitrificans*. *Comptes rendus hebdomadaires des séances de l'Académie des sciences* **246**, 1766–1769.
- O'REILLY, J. E. (1973). Oxidation–reduction potential of the ferro–ferricyanide systems in buffer solutions. *Biochimica et biophysica acta* **292**, 509–515.
- PEETERS, T. & ALEEM, M. I. H. (1970). Oxidation of sulphur compounds and electron transport in *Thiobacillus denitrificans*. *Archiv für Mikrobiologie* **71**, 319–330.
- RADCLIFFE, B. C. & NICHOLAS, D. J. D. (1970). Some properties of a nitrate reductase from *Pseudomonas denitrificans*. *Biochimica et biophysica acta* **205**, 273–287.
- TATE, M. E. (1968). Separation of myoinositol pentaphosphates by moving paper electrophoresis (MPE). *Analytical Biochemistry* **23**, 141–149.