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AEROBIC AND ANAEROBIC RESPIRATION IN *MICROCOCCUS DENITRIFICANS*

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SUMMARY

The adaptive formation of nitrate and nitrite reductases in *Micrococcus denitrificans* as well as the properties and the relation between the NO_3^- and O_2 respiratory pathways have been investigated.

1. Nitrate and nitrite reductases which are induced by their substrates are repressed by O_2.

2. Increased amounts of cytochromes *b* and *c* and a haemprotein (absorption at 465, 650–680 mμ) were found in cells grown anaerobically with NO_3^- or NO_2^-.

3. Cytochrome-oxidase activity was higher in anaerobically grown cells but NADH oxidase was similar in cells grown with or without air.

4. NO_3^-, NO_2^- and O_2 serve as electron acceptors when lactate, malate, NADH, NADPH and succinate are the donors.

5. O_2 and NO_2^- oxidise all cytochromes in the respiratory chain whereas with a limited concentration of NO_3^- cytochrome *b* was primarily oxidised.

6. NADH oxidase, succinate oxidase and nitrite reductase were inhibited by antimycin A and 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide. These enzymes and nitrate reductase were also inhibited by KCN, amytal, mepacrine, pericidine A and by rotenone when NADH only was the electron donor.

7. NADH oxidase and cytochrome *c* oxidase in extracts from anaerobically grown cells were strongly inhibited by NO_2^- but NADH oxidase only was affected by NO_3^-. NO_3^- and NO_2^- did not affect the same enzymes from cells grown in air.

8. Nitrate reductase was unaffected by O_2 but nitrite-reductase activity was strongly inhibited.

INTRODUCTION

Micrococcus denitrificans (N.C.I.B. 8944) grows aerobically or anaerobically with NO_3^- as a terminal electron acceptor[1,2]. The respiratory system which is associated with membrane fractions contains cytochromes[3,4] *b*, *c*, *a* + a_3 and *o* types as well as flavin and ubiquinone[5]. The NADH-oxidase and succinoxidase activities of aerobically grown cells are sensitive to inhibitors of the electron-transfer chain in

Abbreviations: HQNO, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

animal mitochondria[4,5]. In addition the anaerobically grown cells contain a higher concentration of the *b* and *c* cytochromes and a haemoprotein[7,8] which has similar spectral properties to the cytochrome oxidase of *Pseudomonas aeruginosa*[9]. Bacteria grown anaerobically with NO_3^- contain nitrate and nitrite reductases[10]. These enzymes which convert NO_3^- to N_2 utilize cytochromes as electron carriers[11]. The specific effect of NO_3^- in increasing the cytochrome content under anaerobic conditions is not established since the intermediates formed during the reduction of NO_3^- to N_2 may also affect the respiratory system.

In this paper the induction and properties of the respiratory system in cells grown in air are compared with those from anaerobic cultures. The effects of NO_3^- and NO_2^- on the formation of cytochromes, and the relation between the pathways leading to O_2, NO_3^- and NO_2^- are considered.

MATERIALS AND METHODS

Organisms and culture media

M. denitrificans (N.C.I.B. 8944) was maintained at 37° on agar slopes and then grown in liquid medium. The agar slopes contained the following materials (g/l): glucose, 10; KNO_3, 10; peptone, 1c; $MgSO_4 \cdot 7\ H_2O$, 0.5; $FeCl_3$, 0.002; K_2HPO_4, 1 and agar, 15 (pH 7.0). Liquid cultures contained (g/l): glucose, 10; NH_4Cl, 1; $MgSO_4 \cdot 7\ H_2O$, 0.2; $FeCl_3$, 0.002; Na_2CO_3, 0.45; KH_2PO_4, 0.7 (pH 7.0). Unless otherwise stated 10 g KNO_3 were included in anaerobic cultures.

Culture conditions

For batch culture, inocula from 48-h cultures grown on agar slopes were transferred into 1 l of liquid medium and grown for 18 h at 37° on a reciprocator. This culture was transferred into 9 l of fresh medium. The culture was either aerated or sparged with N_2 through sinter glass units at 500 and 200 ml/min, respectively.

Collection of bacteria and preparation of cell-free extracts

After 18 h growth, cells were collected at 4° in a Sorvall RC-2 refrigerated centrifuge fitted with a continuous-flow attachment and washed with cold 0.05 M phosphate buffer (pH 7.5). The cells were stored as a paste at —15°. Crude extracts were prepared by resuspending 1 portion of cell paste in 2 vol. of cold buffer and passing it through a French pressure cell at 4° (20000 lb·inch^{-2}). The broken cells were treated with deoxyribonuclease (200 μg per 100 ml cell suspension) in the presence of 1 mM $MgCl_2$, at 25° for 1 h. Whole cells and cell debris were removed by centrifuging at 10000 $\times g$ for 15 min. The supernatant fraction (S-10) was used in these experiments.

Adaptive formation of nitrate and nitrite reductases

The organism was sub-cultured through three transfers in liquid media (without KNO_3 or KNO_2) under aerobic conditions. An aliquot (70 ml) from an 18-h culture was transferred into 630 ml of fresh medium containing either KNO_3 or $NaNO_2$ (between 1 and 10 mM) in a conical flask fitted with a pyrex side arm (10 cm $\times$ 1 cm diameter) parallel to the base of the flask. The culture was kept at 37° and gassed with N_2 at 200 ml/min. The rate of growth was determined by measuring changes in turbidity of the cultures in the side-arm at 610 mμ in the Eel colorimeter. An aliquot

of the culture (20 ml) was passed through a Millipore filter (47 mm, 0.2 μ) and washed well with cold buffer. The cells resuspended in fresh buffer were used for enzyme assays. The amounts of NO_3^- and NO_2^- were also determined during growth.

Assay of NADH oxidase

NADH-oxidase activity was determined at 340 mμ in a Unicam SP700 recording spectrophotometer at 25°. The reaction mixture contained 0.01 ml enzyme in 0.05 M phosphate buffer (pH 7.5) in a final volume of 2.9 ml. The reaction was initiated by adding 0.4 μmole NADH which was at the same temperature. NADH-oxidase activity was also measured by a polarographic method described under O_2 uptake.

Assay of nitrate and nitrite reductases

Nitrate reductase was determined in open tubes. Nitrite-reductase activity was measured in Thunberg tubes after evacuation and refilling with O_2-free N_2. Nitrate-reductase activity was followed by determining NO_3^- (ref. 12) and NO_2^- (ref. 13) after enzyme reaction. Nitrite reductase was assayed by measuring residual NO_2^-. The reaction mixtures for these enzymes are as follows: 0.02–0.05 ml enzyme (0.2–0.5 mg protein/ml), 2 μmoles of either KNO_3 or KNO_2, 2 μmoles electron donor (4 μmoles for nitrate-reductase assay) in 0.05 M phosphate buffer (pH 7.5) to a final volume of 3.0 ml. The reaction mixture was preincubated for 5 min at 30° and the reaction started by adding the electron donor and electron acceptor together.

Assay of cytochrome c oxidase

Cytochrome *c*-oxidase activity was measured at 550 mμ. The reaction mixture was as for the NADH oxidase assay except that 200 mμmoles of reduced cytochrome *c* was used instead of NADH. Reduced mammalian cytochrome *c* solution was prepared by adding four times the molar amounts of $Na_2S_2O_4$ and then passing through a Sephadex G-25 (coarse) column equilibrated with 0.05 M phosphate buffer (pH 7.5).

Cytochrome *c* oxidase was also measured polarographically[14] as described for O_2 uptake. Electron donors consisted of 90 μmoles of sodium ascorbate and 0.18 μmole of cytochrome *c* in a final volume of 4.5 ml. These results were corrected for O_2 uptake resulting from non-enzymic oxidation of ascorbate. The cytochrome *c* was maintained at around 85% reduced during the enzymic reaction.

Residual NO_3^- was determined by boiling the reaction mixtures for 10 min and after cooling, aliquots containing between 40 and 80 μmoles of NO_3^- were diluted to 0.5 ml with distilled water. 2 mg of NaCl were added and the solution titrated with 5.0 ml of diphenylamine-*p*-diaminodiphenylsulphone. After incubating at 30° for 3 h, the absorbance of the sample was measured at 610 mμ. The results were compared each time with standards prepared under identical conditions.

The following method was used to determine NO_2^-; 0.1 ml 1 M zinc acetate and 1.9 ml 95% (v/v) redistilled ethanol were added to 1.0 ml of reaction mixture. After 10 min at 0°, the reaction mixture was centrifuged and an aliquot of the supernatant fraction containing not more than 60 mμmoles NO_2^-, 1.0 ml of 1% (w/v) sulphanilamide in 1 M HCl and 0.5 ml 0.02% (w/v) *N*-(1-napthyl)-ethylenediamine di-hydrochloride. The absorbance was measured at 520 mμ.

O_2 uptake

O_2 uptake was measured with the Beckman oxygen sensor (Model 39065) complete with an adaptor box (96260). The reaction mixture contained 0.1 ml enzyme in 0.05 M phosphate buffer (pH 7.5) in a final volume of 4.5 ml. The reaction was started by injecting 4 μmoles electron donor (50 μl) through the substrate inlet by means of a hypodermic needle. The amount of O_2 utilized was monitored by a Beckman recorder unit. The absolute amount of O_2 present was determined by measuring the NADH required to use up all the O_2 in the reaction mixture by the NADH-oxidase system.

Spectra

Absorption spectra were recorded in a Unicam SP800 spectrophotometer. (i) Difference spectra of intact cell suspensions were determined by comparing anaerobic samples containing glucose with aerobic samples saturated with O_2 just prior to measurement. (ii) Difference spectra of cell extracts (S-10) were obtained by comparing an anaerobic sample (reduced by endogenous substrate) with a sample treated with O_2, or under anaerobic conditions with either KNO_3 or KNO_2. (iii) The dialysed cell extracts were reduced with 1 μmole of sodium ascorbate *plus* 0.05 μmole *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) and 1 μmole of NADH under anaerobic conditions.

Determination of protein and total nitrogen

Protein was determined by the Folin method as modified by LOWRY *et al.*[15] using bovine serum albumin as a standard. Total protein nitrogen was determined by the Kjeldahl nitrogen method[16].

Co-factors and chemicals

NADH, NADPH, cytochrome *c* (horse heart Type II), antimycin A, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) and bovine serum albumin were obtained from Sigma Chemical, St. Louis, U.S.A. Ubiquinone (Q_1) was obtained from Merck, Sharp and Dohme, U.S.A. Rotenone was purchased from Aldrich Chemical, Wisc., U.S.A. Pericidine A was a gift from Professor S. Tamura, Department of Agricultural Chemistry, University of Tokyo. Amytal was obtained from Drug Houses of Australia, Australia and mepacrine from Imperial Chemical Industries, England. All other chemicals were of analytical grade and were obtained either from the British Drug Houses, Poole, England or from May and Baker, Dagenham, England.

RESULTS

Formation of nitrate and nitrite reductases

When a 10% inoculum from an 18-h culture of aerobically grown cells was transferred into fresh medium containing NO_3^- and grown under N_2, a lag phase of about 4 h was observed as shown in Fig. 1. Very little nitrate reductase and no nitrite reductase were detected during this period. A rapid increase in nitrate-reductase activity occurred just before the exponential phase of growth (2–4 h). Nitrite-reductase activity was found at the onset of rapid cell division (4–6 h). Identical

cultures grown aerobically showed a lag phase of about 2 h and very little nitrate reductase and no nitrite reductase were found during the 20-h growth period.

The growth with NO_2^- under anaerobic conditions is shown in Fig. 2. The lag phase was affected by the NO_2^- concentration in the medium. Thus at 5 mM $NaNO_2$ it was about 12 h compared with a 20-h period in medium containing 20 mM $NaNO_2$. The size of inoculum also influenced the length of the lag phase. Once cell division commenced, growth was rapid until all NO_2^- was utilized. Low nitrate-reductase but high nitrite-reductase activities were detected in cells collected at the end of growth (30 h).

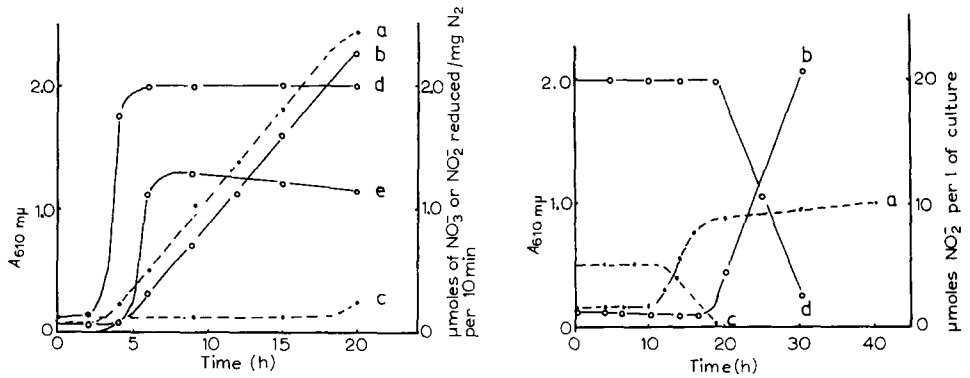


Fig. 1. Formation of nitrate and nitrite reductases under either aerobic or anaerobic conditions in medium containing 0.01 M KNO_3. Cell growth was measured by following increases in absorbance of the cultures in an Eel colorimeter at 610 mμ. Nitrate and nitrite reductases were assayed as described in MATERIALS AND METHODS. Aerobic cultures (– – – –): (a) growth, (c) nitrate reductase; Anaerobic cultures (———): (b) growth, (d) nitrate reductase, (e) nitrite reductase.

Fig. 2. Growth of *M. denitrificans* in medium containing 5 mM KNO_2 (– – –) and 20 mM KNO_2 (———), respectively. a and b, cell growth; c and d, NO_2^- content in cultural medium.

Absorption spectra

Difference spectra (oxidised *vs.* reduced) of cells grown aerobically and those for cells grown anaerobically are given in Fig. 3. Cytochromes of type *b*, *c*, $a + a_3$ (absorption peaks at 560, 550, 605 and 445 mμ, respectively) were found in cells grown under air or N_2. The amounts of cytochromes *b* and *c* were much greater in cells grown without air. Absorption bands at 465 and 650–680 mμ were found exclusively in

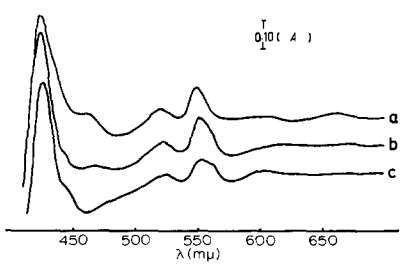


Fig. 3. Difference spectra of cell suspensions incubated with glucose compared with those gassed with O_2. (a) Cells grown anaerobically with KNO_2; (b) cells grown anaerobically with KNO_3; (c) aerobically grown cells. All suspensions (a) and (b) contained 0.40 mg N_2/ml; and (c) contained 0.44 mg N_2/ml.

anaerobically grown cells. The cytochrome content of cells grown in medium containing either NO_3^- or NO_2^- was essentially similar although there was a slight shift in the α peak towards the shorter wavelength and a small decrease in absorption at 560 mμ in cells grown with NO_2^-.

Cytochrome c-oxidase and NADH-oxidase activities

Although the specific activities of the NADH and cytochrome *c* oxidases varied for different batches of cells, the ratio of one enzyme to the other remained fairly constant as shown in Table I. The ratio of cytochrome *c*-oxidase to NADH-oxidase activity was about 3-fold greater in cells grown anaerobically as compared with cells grown in air. It was observed that NADH-oxidase activity was reduced on storage at $-15°$ so that it was essential to use freshly collected cells for the enzyme assays.

TABLE I

CYTOCHROME *c*-OXIDASE AND NADH-OXIDASE ACTIVITY

Cytochrome *c* oxidase and NADH oxidase of cell extracts from aerobic and anaerobic bacteria were assayed polarographically as described in MATERIALS AND METHODS. Reaction mixtures were as follows: cell extracts 0.1 ml, 4 μmoles NADH or 90 μmoles sodium ascorbate and 0.18 μmole cytochrome *c* in final volume of 4.5 ml.

Conditions of growth	*Expt. No.*	*O_2 uptake (μmoles/mg protein per 10 min)*		*Cytochrome oxidase*
		Cytochrome oxidase	*NADH oxidase*	*NADH oxidase*
Aerobic	1	1.9	1.9	1.0
Aerobic	2	1.8	2.0	0.9
Aerobic	3	2.0	1.8	1.1
Anaerobic	1	7.0	2.0	3.5
Anaerobic	2	7.6	2.0	3.8
Anaerobic	3	7.4	2.0	3.7

TABLE II

UTILIZATION OF VARIOUS ELECTRON DONORS

The utilization of various electron donors by cell extracts (S-10) prepared from aerobic and anaerobic bacteria using O_2, KNO_3 and KNO_2 as acceptors were measured as described in MATERIALS AND METHODS. O_2 uptake was measured polarographically. Nitrate and nitrite reductases were determined as described in MATERIALS AND METHODS.

Donor	*Electron acceptor (mμmoles reduced/mg protein per 10 min)*			
	S-10 aerobic culture	*S-10 anaerobic culture*		
	O_2	*O_2*	*NO_3^-*	*NO_2^-*
NADH	2168	2000	410	100
NADPH	872	800	280	40
Acetate	0	0	0	0
Butyrate	0	0	0	0
Formate	98	100	34	0
Glutamate	0	0	4	0
Lactate	490	440	128	12
Malate	360	320	40	8
Succinate	880	800	400	112
Pyruvate	0	0	0	0

Utilization of electron donors

The various types of electron donors oxidised by cell extracts with NO_3^-, NO_2^- and O_2 as terminal electron acceptors are given in Table II. NADH, NADPH, succinate, lactate and malate were the most effective donors in that order. No difference in specificity toward the various electron donors was observed between the three enzyme systems. The nitrate and nitrite reductases were equivalent to approx. 20 and 5% of the oxidase activity.

Oxidation spectra

The electron-transfer chain in crude extracts of bacteria grown anaerobically was reduced by endogenous substrates. The difference spectra for the reduced cell extracts *vs.* those which had been either oxygenated or treated with NO_3^- or NO_2^- are shown in Figs. 4a and 4b. The addition of O_2, NO_3^- or NO_2^- in open cuvettes resulted in a reoxidation of bands at 560, 550 and 610 mμ (corresponding to cytochromes *b*, *c* and *a*, respectively), and also that at 465 mμ and that between 650 and 680 mμ (Fig. 4a). Similar effects were observed when excess NO_3^- or NO_2^- were added to reduced samples under anaerobic conditions. The effects of adding limited amounts of NO_3^- or NO_2^- to similar samples under identical conditions are presented in Fig. 4B.

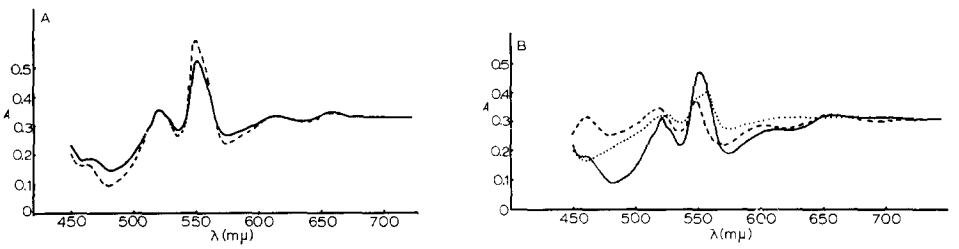


Fig. 4A. (a) Difference spectra of cell extracts reduced by anaerobiosis *vs.* that gassed with O_2 for 1 min (– – – –). (b) Cell extracts reduced by anaerobiosis *vs.* that with excess KNO_3 or KNO_2 (———). Spectrum b was recorded in Thunberg cuvettes filled with O_2-free N_2. The KNO_3 or KNO_2 were then added to the cell extracts from the side-arm. All extracts contained 10 mg protein/ml. Fig. 4B. The difference spectra of cell extracts (reduced by anaerobiosis) *vs.* that with 0.2 μmole of either KNO_3 (· · · · · ·) or KNO_2 (———). The difference spectrum of the cell extracts reduced by anaerobiosis *plus* KNO_3 *vs.* that with KNO_2 is also given (– – – –). Spectra were recorded in Thunberg cuvettes.

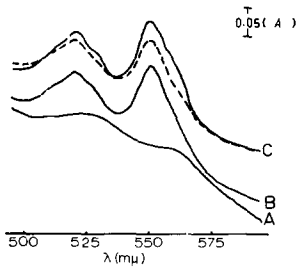


Fig. 5. Spectra were recorded in Thunberg cuvettes which were fitted with serum caps and filled with N_2 after vigorous evacuation. The electron donors and acceptors were prepared under the same conditions and transferred into the cuvettes by means of an air-tight gas syringe in the following order: TMPD, sodium ascorbate, NADH and nitrate. A. Absolute spectrum of dialysed cell extract (S-10) *plus* 0.05 μmole TMPD. B. A *plus* 1 μmole sodium ascorbate. C. ———, B *plus* 1 μmole of NADH; – – – –, obtained after adding 0.5 μmole KNO_3. Cell extract contained 11.5 mg protein/ml.

TABLE III

EFFECT OF INHIBITORS ON THE RESPIRATORY ENZYMES

Details of enzyme assays given in the text; 0.5 mg of protein was used for either O_2 uptake or NO_2^- reduction and 0.2 mg of protein for NO_3^- reduction.

Inhibitors	*Final concn. (mM)*	*Inhibition (%)*					
	Electron acceptor:	O_2		KNO_3		KNO_2	
	Electron donor:	*NADH*	*Succinate*	*NADH*	*Succinate*	*NADH*	*Succinate*
CO	(Enzyme gassed for 3 min)	8	10	0	0	0	0
KCN	1	90	100	55	40	80	70
	0.1	50	48	20	25	58	55
	0.01	5	0	0	0	—	—
Antimycin A**	0.2	100	95	6	4	90	70
	0.02	80	60	0	0	64	30
HQNO**	0.3	60	70	10	5	50	54
	0.03	—	—	6	0	0	0
Amytal**	0.1	60	0	40	0	40	0
Mepacrine	0.33	45	0	35	0	40	0
Pericidin A**	0.06	90*	92	88*	88	89	91
Rotenone	0.06	98	0	88	0	95	0

* 50% reversal of inhibition was recorded when 0.06 mM ubiquinone (Q_1) final concn. was included in the reaction mixture.

** Dissolved in 95% (v/v) ethanol and 5 μl used for each assay; ethanol alone had no effect on enzyme activity.

NO_2^- produced a partial reoxidation of the cytochromes. NO_3^- on the other hand reoxidised primarily the absorption peak at 560 mμ (cytochrome *b*) and only to a much lesser extent at 550 mμ (cytochrome *c*).

The absorption bands at 465 mμ and that between 650 and 680 mμ were reoxidised by NO_2^- only. These can be seen in the difference spectra of samples reoxidised by NO_2^- as compared with samples treated with NO_3^-.

Effects of NO_3^- and NO_2^- when ascorbate and TMPD are donors

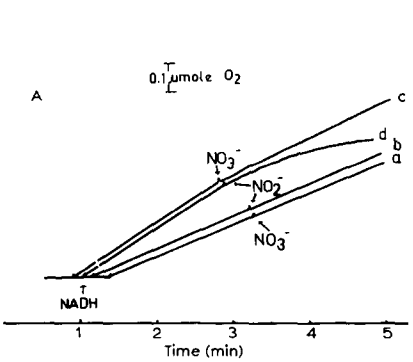
When a dialysed cell-extract was treated with 1 μmole of sodium ascorbate and 0.05 μmole of TMPD under anaerobic conditions, cytochrome *c* (550 mμ) was reduced (Fig. 5). The further addition of 1 μmole of NADH reduced cytochrome *b* (560 mμ). The introduction of 0.5 μmole of NO_3^- reoxidised cytochrome *b* completely but only a very small fraction of cytochrome *c* was affected.

Inhibitor studies

The effects of various inhibitors on the respiratory enzymes when NADH and succinate were the electron donors are shown in Table III. Thus KCN, antimycin A, HQNO, amytal and mepacrine inhibited the oxidation of both NADH and succinate when either O_2 or NO_2^- was the electron acceptor. The extent of inhibition by the various compounds was similar for the oxidase and nitrite reductase. Nitrate reductase however was not affected by antimycin A or HQNO and it was less sensitive to CN^- than either nitrite reductase or NADH oxidase. Pericidin A inhibited all three systems and the effect was reversed by ubiquinone. Rotenone was effective only when NADH was the donor. CO inhibited the oxidase system by about 10% only but it had no effect on either of the reductase systems.

Interaction between different electron acceptors

The NADH-oxidase and cytochrome *c*-oxidase activities of cell extracts, in the presence of NO_3^- or NO_2^-, were studied by following the O_2 uptake and the oxidation



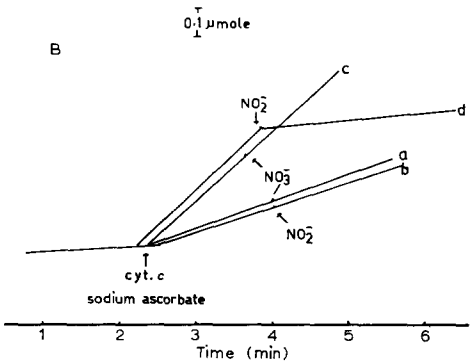


Fig. 6A. The effects of NO_3^- and NO_2^- on the NADH-oxidase activity as measured by O_2 uptake (see MATERIALS AND METHODS). The effects of KNO_3 (a) and KNO_2 (b) on the enzyme from aerobically grown cells; c and d are those for KNO_3 and KNO_2 respectively on the oxidase from anaerobically grown cells.

Fig. 6B. The effects of KNO_3 and KNO_2 on cytochrome *c*-oxidase activity as measured by O_2 uptake (see MATERIALS AND METHODS). The effect of KNO_3 (a) and KNO_2 (b) on the enzyme from aerobically grown cells; (c) and (d) are those for KNO_3 and KNO_2 respectively on the oxidase from anaerobically grown cells.

of substrate. The NADH oxidase and cytochrome *c* oxidase of extracts from cells grown in air were unaffected by NO_3^- or NO_2^- up to 1 mM. The NADH oxidase in extracts of anaerobic cells however was strongly inhibited by NO_2^- at 0.1 mM as measured by O_2 uptake or NADH oxidation. This inhibition increased progressively with time. The cytochrome *c*-oxidase activity from the same extracts was retarded even more than the NADH oxidase. NO_2^- inhibited the oxidation of the electron donor to the same extent as the O_2 uptake. The NADH oxidase was also inhibited by NO_3^- at 0.1 mM when O_2 uptake was measured. Over a 4-min period the extent of inhibition was stoichiometric with the amount of NO_3^- reduced. NADH-oxidase activity measured at 340 mμ and cytochrome *c* oxidase were not affected by NO_3^-.

Nitrate reductase was not inhibited by O_2 or by NO_2^- at 0.1 mM. Nitrite reductase was strongly inhibited by O_2. The comparatively low nitrite-reductase activity compared with the high oxidase level made it impossible to measure nitrite-reductase activity in open tubes.

DISCUSSION

During anaerobic growth in medium containing NO_3^-, nitrate and nitrite reductases are produced by *Micrococcus denitrificans*. However, since NO_2^- is a product of NO_3^- reduction, it is not known whether under these conditions nitrite reductase is produced in response to its own substrate. That this is the case is shown by the production of nitrite reductase in cells grown with NO_2^- as the sole electron acceptor. The fact that this bacterium does not produce nitrate reductase under anaerobic conditions without NO_3^- clearly indicates that this enzyme is formed by a specific induction rather than by de-repression, *i.e.*, removal of O_2. Earlier reports suggest that NO_2^- did not serve as a terminal electron acceptor for anaerobic growth by *M. denitrificans* in autotrophic medium[1]. Subsequent reports confirm that NO_2^- acts as an electron acceptor either in heterotrophic type medium[7] or in autotrophic medium if supplemented with yeast extract[17].

In the current experiments, the bacteria grown anaerobically in medium containing NO_3^- have a much higher cytochrome content than those grown aerobically. This is in agreement with earlier reports[4]. In addition, absorption bands were observed in anaerobically grown cells at 465, 650–680 mμ. These effects were also observed when NO_2^- was the electron acceptor, showing that NO_3^- alone cannot be responsible for the increase of all the cytochromes under anaerobic growth conditions. The haem compound with absorption bands at 465 and between 650–680 mμ appears not to be involved in nitrate reductase since it is also found in cells grown with NO_2^- as terminal electron acceptor when nitrate reductase is not formed.

On the other hand, the α-peaks of cytochromes *b* and *c* from cells grown in medium containing NO_2^- were shifted slightly towards a shorter wavelength, and the absorption at 560 mμ was lower, which may indicate that less cytochrome *b* was formed under these conditions.

Studies with cytochrome *c* oxidase and NADH oxidase in extracts of aerobically and anaerobically grown cells indicate that the ratio of cytochrome *c* oxidase to NADH oxidase is always 3–4 times higher in cells grown without air. This observation does not necessarily imply a net increase in cytochrome *c* oxidase in anaerobically grown cells. It is possible that alterations in the structural arrangement of the electron-

transfer chain may render the cytochrome *c* oxidase more accessible to reduced cytochrome *c* resulting in an increased activity[18,19].

The NO_3^- and O_2 respiratory pathways in extracts from anaerobically grown cells appeared to involve the same type of electron carriers. This view is supported by the observations that the three electron acceptors exhibit the same specificity and efficiency with a variety of electron donors and have similar responses to inhibitors. Further confirmatory evidence comes from spectrophotometric studies which show that the cytochrome components in the extracts were reoxidised either by NO_3^- or NO_2^- under anaerobic conditions or by O_2. These observations however do not differentiate between the following possibilities: (a) that NO_3^- and O_2 respiration systems have some electron carriers in common, or (b) that they have separate electron-transfer chains. The increase in cytochrome content observed to accompany the adaptation to NO_3^- respiration indicates that additional electron carriers are required for NO_3^- respiration. Kinetic studies with excess of NADH as electron donor however, showed that O_2 uptake was partially inhibited by NO_3^- and the inhibition was stoichiometric to the amount of NO_3^- reduced. NADH oxidation measured at 340 mμ was unaffected by NO_3^-. This suggests a competition between NO_3^- and O_2 for the electrons, which would favour Scheme a.

The nitrite reductase of several denitrifying bacteria has been shown to require cytochrome *c* as an electron carrier[20,21]. The same enzyme in *M. denitrificans* is also linked to the electron-transfer chain probably at a site between cytochrome *c* and O_2, because its activity is inhibited by antimycin A and HQNO when NADH or succinate was the electron donor. This is in agreement with spectrophotometric observations of cell extracts which indicate that both cytochromes *b* and *c* were reoxidised by NO_2^-. Absorption bands at 465 mμ and between 650 and 680 mμ remained oxidised when NO_2^- was present even after the addition of excess $Na_2S_2O_4$ suggesting that this haem compound is involved in NO_2^- reduction.

The site at which nitrate reductase operates in the respiratory chain is still uncertain. Earlier reports have suggested that reduced cytochrome *c* is a suitable donor since it was re-oxidised by NO_3^- in the absence of air[11,22]. In these experiments, no account was taken of the possibility that reduced cytochrome *c* could be reoxidised by the nitrite reductase during NO_3^- reduction. The data presented in this paper show that nitrate reductase was relatively insensitive to antimycin A but was inhibited by amytal, mepacrine, pericidin A and rotenone as was NADH oxidase. The fact that O_2 has no detectable effect on nitrate-reductase activity suggests that nitrate reductase functions at a site different from that of the terminal oxidase. Further, the inhibition of O_2 uptake by NO_3^- using NADH as electron donor indicates that the site of nitrate reductase may be closer to the reduced substrate terminal of the respiratory chain, *i.e.* between cytochromes *b* and *c*. This is further supported by the observation that in the absence of air and with a limited amount of NO_3^- present, cytochrome *b* is the major component of the respiratory chain which is reoxidised. Addition of NO_3^- to dialysed cell extracts which had been reduced previously under anaerobic conditions by NADH and ascorbate *plus* TMPD resulted in a reoxidation of cytochrome *b* but there was only a very small effect on cytochrome *c*. This suggests that cytochrome *b* is the site for nitrate-reductase action. However, HQNO, which is reported to be specific in inhibiting electron transport by preventing the oxidation of cytochrome *b* (ref. 23), showed little effect on nitrate-reductase activity.

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