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SELECTION AND ORGANISATION OF DENITRIFYING ELECTRON-TRANSFER PATHWAYS IN *PARACOCCUS DENITRIFICANS*

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(1) Under anaerobic conditions the respiratory chain in cells of *Paracoccus denitrificans*, from late exponential cultures grown anaerobically with nitrate as electron acceptor and succinate as carbon source, has been shown to reduce added nitrate via nitrite and nitrous oxide to nitrogen without any accumulation of these intermediates. (2) Addition of nitrous oxide to cells reducing nitrate strongly inhibited the latter reaction. The inhibition was reversed by preventing electron flow to nitrous oxide with either antimycin or acetylene. Electron flow to nitrous oxide thus resembles electron flow to oxygen in its inhibitory effect on nitrate reduction. In contrast, addition of nitrite to an anaerobic suspension of cells reducing nitrate resulted in a stimulation of nitrate reductase activity. Usually, addition of nitrite also partially overcame the inhibitory effect of nitrous oxide on nitrate reduction. The reason why added nitrous oxide, but not nitrite, inhibits nitrate reduction is suggested to be related to the higher reductase activity of the cells for nitrous oxide compared with nitrite. Explanations for the unexpected stimulation of nitrate reduction by nitrite in the presence or absence of added nitrous oxide are considered. (3) Nitrous oxide reductase was shown to be a periplasmic protein that competed with nitrite reductase for electrons from reduced cytochrome *c*. Added nitrous oxide strongly inhibited the reduction of added nitrite. (4) Nitrite reductase activity of cells was strongly inhibited by oxygen in the presence of physiological reductants, but nitrite reduction did occur in the presence of oxygen when isoascorbate plus *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine was the reductant. It is concluded that competition for available electrons by two oxidases, cytochrome aa_3 and cytochrome *o*, severely restricted electron flow to the nitrite reductase (cytochrome *cd*). For this reason it is unlikely that the oxidase activity of this cytochrome is ever functional in cells. (5) The mechanism by which electron flow to oxygen or nitrous oxide inhibits nitrate reduction in cells has been investigated. It is argued that relatively small changes in the extent of reduction of ubiquinone, or of another component of the respiratory chain with similar redox potential, critically determine the capacity for reducing nitrate. The argument is based on: (i) the response of an anthroyloxystearic acid fluorescent probe that is sensitive to changes in the oxidation state of ubiquinone; (ii) consideration of the total rates of electron flow through ubiquinone both in the presence of oxygen and in the presence of nitrate under anaerobic conditions; (iii) use of relative extents of oxidation of *b*-type cytochromes as an indicator of ubiquinone redox state, especially the finding that *b*-type cytochrome of the antimycin-sensitive part of the respiratory chain is more oxidised in the presence of added nitrous

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Abbreviations: TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; Hepes, *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulphonic acid.

oxide, which inhibits nitrate reduction, than in the presence of added nitrite which does not inhibit. Arguments against *b*- or *c*-type cytochromes themselves controlling nitrate reduction are given. (6) In principle, control on nitrate reduction could be exerted either upon electron flow or upon the movement of nitrate to the active site of its reductase. The observations that inverted membrane vesicles and detergent-treated cells reduced nitrate and oxygen simultaneously at a range of total rates of electron flow are taken to support the latter mechanism. The failure of an additional reductant, durohydroquinone, to activate nitrate reduction under aerobic conditions in the presence of succinate is also evidence that it is not an inadequate supply of electrons that prevents the functioning of nitrate reductase under aerobic conditions. (7) In inverted membrane vesicles the division of electron flow between nitrate and oxygen is determined by a competition mechanism, in contrast to cells. This change in behaviour upon converting cells to vesicles cannot be attributed to loss of cytochrome *c*, and therefore of oxidase activity, from the vesicles because a similar change in behaviour was seen with vesicles prepared from cells of a cytochrome *c*-deficient mutant.

Introduction

When grown anaerobically with nitrate as added terminal electron acceptor, *Paracoccus denitrificans* synthesises reductases for nitrate, nitrite and nitrous oxide. The sequential operation of these reductases thus converts nitrate to nitrogen gas, the process known as denitrification. The organisation of the electron-transfer chain is such that whereas electrons flow from ubiquinone to nitrate reductase, probably via a specific *b*-type cytochrome, electrons destined for nitrite or nitrous oxide reductases flow through the antimycin-sensitive cytochrome b-c_1 complex and cytochrome *c* [1–5]. There are two constitutive oxidase activities [2,6]: cytochrome aa_3, for which cytochrome *c* is the immediate reductant, and an *o*-type cytochrome which is considered to receive electrons from the ubiquinone region of the chain [4,5,7].

One of the best known features of electron transport in denitrifying bacteria such as *P. denitrificans* is that reduction of nitrate is inhibited when oxygen is present [8]. Recent studies have shown that this is not a consequence of a direct inhibitory effect of molecular oxygen [2,9]. The inhibitory effects of oxygen on nitrate reduction are partially relieved upon inhibition of oxidase pathways by either antimycin [2,9] or hydroxylamine [9]. Ferricyanide also inhibits reduction of nitrate under anaerobic conditions, but upon addition of antimycin reduction of nitrate is restored [2,9]. These observations indicate that reduction of nitrate is inhibited by oxygen or ferricyanide when the extent of oxidation of a component of the electron-transfer chain attains a certain level which is not reached in the presence of antimycin or hydroxylamine. Ubiquinone could be this component as it is the last known common component before the respiratory chains to oxygen and nitrate diverge [2,5].

The inhibition of nitrate reduction in the presence of oxygen is not necessarily a consequence of restriction on electron flow to the nitrate reductase. An alternative possibility is that the inhibition is exerted on the access of nitrate to the active site of its reductase at the cytoplasmic surface of the plasma membrane [10]. Two lines of evidence support this view. First, inside-out membrane vesicles, in which nitrate would have direct access to its reductase, can reduce oxygen and nitrate simultaneously and at similar rates [8]. Second, the nitrate reductase in cells of *P. denitrificans* can only reduce chlorate, a substrate analogue, after a permeability barrier has been disrupted [8]. Significantly, the titre of detergent that permits the appearance of chlorate reduction also removes the control by oxygen on nitrate reduction [10]. The present paper reports on further experiments that were designed to distinguish between control on electron flow to nitrate reductase and control on nitrate movement to the reductase. Additionally, evidence has been sought for a change in the redox state of ubiquinone at the point of anaerobiosis when nitrate reduction begins.

Nitrite and nitrous oxide both accept electrons from the respiratory chain on the oxidising side of the point at which the electron-transfer pathways to oxygen and nitrate diverge [5]. Two questions

therefore arose. First, would electron flow to nitrite and/or nitrous oxide resemble electron flow to oxygen or ferricyanide and exert an inhibition on nitrate reduction? Second, and related to the first problem, was the question as to whether, during reduction of nitrate, nitrite and/or nitrous oxide accumulate, or whether all three species are reduced simultaneously. This was of particular interest because there have been indications that nitrite and nitrous oxide are reduced sequentially by denitrifying bacteria [11,12], and consideration has been given to the possibility that nitrite reduction is inhibited by nitrate [13]. Our recent introduction of a nitrous oxide electrode [3] enabled us to study these problems more readily than hitherto.

Apart from the issue of the factors that control nitrate reduction in a denitrifying bacterium, the question of whether and how nitrite reduction might be controlled by oxygen and nitrous oxide has also been investigated. Nitrous oxide reduction has already been shown to be inhibited by oxygen [3,14]. The nitrite reductase enzyme, a *cd*-type cytochrome, has been shown to be located in the periplasmic space in *P. denitrificans* [10,15], and therefore probably receives electrons directly from cytochrome *c*. In order to understand any effects of nitrous oxide on nitrite reductase activity and vice versa, it would be helpful to know if nitrous oxide reductase has a similar location. Consequently, a study was also made of this point, which is also important for understanding the bioenergetics of electron flow to nitrous oxide [5,16].

Preliminary accounts of parts of this work have been presented [17,18].

Materials and Methods

Unless otherwise indicated the experimental organism was *P. denitrificans* NCIB 8944. This was grown in a 2-l batch culture with succinate as substrate and nitrate as terminal electron acceptor as described by Burnell et al. [19]. The cells were harvested at the late logarithmic stage of growth ($A_{550} = 1.7$ in a 1 cm path length curvette in a Unicam SP 500 spectrophotometer) by centrifugation for 20 min at 4°C and $5000 \times g$. The cells were washed once with 400 ml of cold (4°C) 0.1 M Hepes-NaOH, pH 7.3, and then resuspended to a volume of 5–10 ml in the same medium. The cells were stored on ice for up to 4 h before use. Membrane vesicles from strain NCIB 8944 were prepared from cells grown on succinate and nitrate according to the procedures in Refs. 8 and 19.

A cytochrome *c*-deficient mutant (HUUG 25) was obtained from Dr. H.W. Van Verseveld (Biologisch Laboratorium, Vrije Universiteit, 1007 MC Amsterdam, The Netherlands). Such mutants cannot easily be grown anaerobically with nitrate as lack of cytochrome *c* prevents reduction of nitrite which can have a toxic effect. Consequently, in order to obtain cells with nitrate reductase activity, the cells were grown in 700 ml of the medium described by Burnell et al. [19], but under aerated conditions in 2-l flasks on an orbital incubator (200 rpm) until A_{550} was between 1.7 and 1.9. Cells grown in this way were either used for experiments or converted into vesicles by the same procedure that was employed with wild-type cells. In either case the cells were checked to ensure that significant reversion to wild-type phenotype had not occurred. This was done by measuring ascorbate/TMPD oxidase activity or ferricyanide reductase activity as cytochrome *c*-deficient mutants are lacking in both these activities [2,7,20].

Reduction of nitrate by cells was measured using the electrode described by Alefounder et al. [2]. Nitrite was determined colourimetrically [21]. Dissolved nitrous oxide concentrations were determined using the silver cathode electrode described by Alefounder and Ferguson [3]. Rates of oxygen reduction by cells and vesicles were measured using a Clark-type oxygen electrode.

The oxidation and reduction of *b*-type cytochrome was measured at 575–560 nm with a Perkin-Elmer 356 dual-wavelength spectrophotometer. The cell suspension in the cuvette was continuously stirred, and argon was continually flushed over the surface to maintain anaerobiosis.

The fluorescence of 12-(9-anthroyloxy)stearic acid (obtained from Sigma Chemical Co.) was measured using an excitation wavelength of 370 nm and an emission wavelength of 450 nm. For these experiments cells were incubated at room temperature under anaerobic conditions, but in the absence of nitrate, with 12-(9-anthroyloxy)stearic acid for up to 2 h to allow equilibration of the fluorescent probe with the cell membranes.

Durohydroquinone was purchased from K&K

Laboratories (via Kodak Ltd., Liverpool L33 7UF, U.K.) and a stock 100 mM solution in dimethyl sulphoxide [22] was prepared immediately before use.

Results

Nitrite and nitrous oxide can be reduced simultaneously with nitrate

It was established that under anaerobic conditions reduction of nitrate to nitrite was accompanied by simultaneous conversion of nitrite to nitrous oxide and of the latter to nitrogen. The evidence was that during reduction of 1 mM nitrate neither nitrite nor nitrous oxide accumulation was detected. An example of this behaviour is illustrated on the left-hand side of Fig. 1 which shows that neither nitrite nor nitrous oxide could be detected during reduction of nitrate until exogenous nitrous oxide was added to the reaction mixture. In previous work [2,9] it has been noted that addition of antimycin stimulated the rate of anaerobic reduction of nitrate. An explanation for this finding is now available because it was found that in the presence of sufficient antimycin to inhibit electron flow through the cytochrome bc_1 segment of the electron-transfer chain, nitrite accumulated almost stoicheiometrically as nitrate was reduced. Thus, the stimulation of nitrate reduction in the presence of antimycin [2,9] is at least in part a consequence of the prevention of electron flow to nitrite and nitrous oxide that would otherwise occur.

The combined rate of electron flow to endogenously produced nitrite and nitrous oxide thus only partially inhibits the rate of nitrate reduction in contrast to the effect of oxygen. With a view to determining how changes in the rate of electron flow to nitrite and/or nitrous oxide were reflected in the nitrate reduction rate, the effects of acetylene, a specific inhibitor of nitrous oxide reductase [23,24], and of partial titres of antimycin were examined. The presence of sufficient acetylene to inhibit nitrous oxide reduction, which would block one-third of the total electron flow to nitrite and nitrous oxide (for each four electrons flowing to two nitrite ions to produce one nitrous oxide molecule, two electrons are required to reduce nitrous oxide to nitrogen), had scarcely any stimulatory effect on the nitrate reduction rate. This can be seen from the right-hand side of Fig. 2A where it is shown that equal rates of nitrate reduction are observed after reduction of added nitrous oxide (Expt. a) and in the presence of acetylene (Expt. b). The antimycin titrations were slightly variable, but in general it was found that more than one-third of the total electron flow through the cytochrome bc_1 segment had to be blocked before an increase in the rate of nitrate reduction was observed.

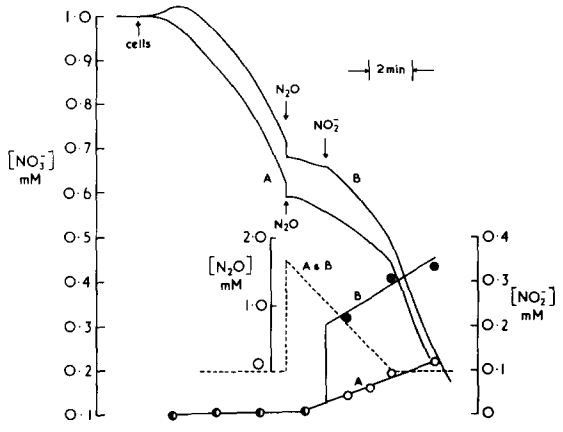

Fig. 1. Simultaneous measurements of nitrate, nitrite and nitrous oxide during anaerobic electron flow in *P. denitrificans*, and the effects of added nitrous oxide and nitrite on the rate of nitrate reduction. A and B refer to two separate experiments in which concentrations of nitrate, nitrite and nitrous oxide were each measured. In both experiments 17 μmol N_2O were added as shown and in experiment B 2 μmol $NaNO_2$ were also added subsequently. The reaction chamber was fitted with both nitrate and nitrous oxide electrodes and contained 10 ml 0.1 M Hepes-NaOH, pH 7.3, 10 mM sodium succinate. The nitrate electrode was calibrated by stepwise additions of KNO_3 in increments of 0.1 mM to a final concentration of 1 mM. 38 mg dry wt. cells were added as shown. Throughout the experiments nitrogen was blown over the surface of the reaction mixture. The temperature was 20°C. Samples were withdrawn for assay of nitrite (Expt. A (○———○); Expt. B (●———●)) (see Materials and Methods). (———) Nitrate concentration; (------) nitrous oxide concentration.

Added nitrous oxide but not nitrite inhibits nitrate reduction

When nitrous oxide was introduced to an anerobic suspension of cells that was reducing nitrate there was an immediate inhibition of nitrate reduction (Figs. 1 and 2). This effect of nitrous oxide was prevented when either antimycin or

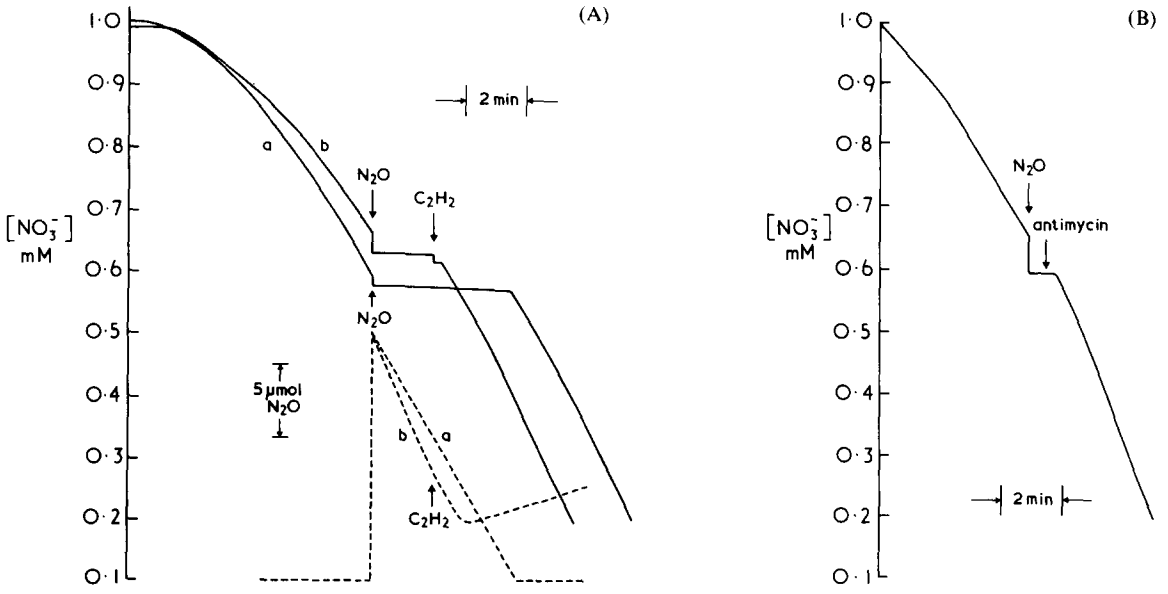

Fig. 2. Acetylene or antimycin reverse the inhibition by nitrous oxide of nitrate reduction. (A) Conditions as in Fig. 1 except that 36 mg dry wt. cells were used. 17 μmol nitrous oxide and 15 μmol acetylene were added as shown. (———) Nitrate concentration, (- - - - - -) nitrous oxide concentration. (B) The reaction chamber contained 12 ml of the reaction mixture described in Fig. 1 together with 28 mg dry wt. cells under an atmosphere of nitrogen. 58 μmol N_2O and 5 μg antimycin were added as shown. The temperature was 20°C. There was a dilution effect upon adding nitrous oxide.

acetylene were present as inhibitors of electron flow to nitrous oxide (Fig. 2A and B). In the experiments of Fig. 2A nitrous oxide resembled oxygen [2,8,9] and caused a complete inhibition of nitrate reduction, which persisted for as long as dissolved nitrous oxide was detectable. Slightly different behaviour is evident in Fig. 1 (Expt. A) in which the extent of inhibition by nitrous oxide gradually decreased. Over a large number of experiments a range of behaviour between the patterns shown in Figs. 1 and 2 was observed. As the K_m of nitrous oxide reductase for its substrate is very low [3,25] these observations were not a consequence of a declining rate of electron flow to nitrous oxide as the concentration of nitrous oxide fell below K_m. Fig. 1 also shows that nitrite began to accumulate after addition of nitrous oxide. When additional nitrite was introduced into the cell suspension the rate of nitrate reduction increased further (Expt. B, Fig. 1). Thus, accumulation of small amounts of nitrite from nitrate occurred after addition of nitrous oxide. The nitrite produced increased the rate of nitrate reduction, which in turn raised the concentration of nitrite with the consequence of a steadily increasing rate of nitrate reduction in the presence of added nitrous oxide (Fig. 1).

Whereas electron flow to added nitrous oxide clearly inhibited reduction of nitrate, added nitrite, up to a concentration of 2 mM, did not inhibit the rate of nitrate reduction under anaerobic conditions. In contrast, there was usually a slight stimulation of the rate of nitrate reduction following addition of nitrite (Fig. 3). This was not considered to be a consequence of an uncoupling effect of nitrite because concentrations of nitrite that stimulated nitrate reduction had no effect on the rate of oxygen reduction, and earlier measurements of the membrane potential did not reveal any uncoupling effect of nitrite [26].

The specific rate of reduction of added nitrite by cells was generally very similar to the rate of nitrate reduction when nitrate was the sole added electron acceptor. This meant that the catalytic capacity of nitrite reductase was only sufficient to match the rate of nitrite production from nitrate. Consequently, increases in the rate of nitrate reduction following addition of nitrite (Figs. 1 and

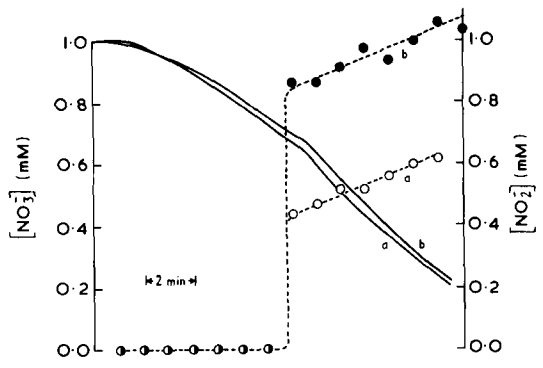

Fig. 3. Stimulation by nitrite of nitrate reduction by *P. denitrificans* cells. The 12-ml reaction mixture at 20°C and under nitrogen was as specified in the legend to Fig. 2B. 23 mg dry wt. cells were used. $NaNO_2$ was added as shown to a final concentration of 0.4 mM (a) or 0.8 mM (b). (———) Nitrate concentration; (○———○) and (●———●) nitrite concentrations determined as in Fig. 1.

3) could be expected to be accompanied by an increase with time of the nitrite concentration, just as is shown in Figs. 1 and 3. The rate of accumulation of nitrite was, however, slightly greater than the increase in the rate of nitrite formation from nitrate. This means that in the presence of added nitrite there is some inhibition of electron flow to nitrite reductase via cytochrome *c*.

The effect of added nitrite on the rate of nitrate reduction was also examined in the presence of acetylene to prevent concomitant reduction of nitrous oxide. Under these conditions nitrite again stimulated the rate of nitrate reduction, and data similar to those shown in Fig. 3 were obtained. The lack of effect of acetylene agreed with the observation described earlier that the rate of reduction of nitrate was not altered after selective inhibition of the reduction of nitrous oxide produced from added nitrate (Fig. 2A, Expts. a and b).

Competition for electrons between two periplasmic enzymes, nitrite and nitrous oxide reductases

Fig. 4 shows that reduction of nitrite by cells was strongly inhibited by nitrous oxide, both when a physiological substrate, succinate, and when a non-physiological substrate, isoascorbate plus TMPD, was used. The effects of nitrous oxide were abolished when acetylene was added to inhibit nitrous oxide reductase activity. In the experiment with isoascorbate as substrate the added nitrous oxide had been reduced approx. 7 min after its addition, corresponding to the point at which the rate of nitrite reduction began to accelerate, whereas with succinate as substrate nitrous oxide could still be detected at the end of the experiment.

Nitrite had a much smaller inhibitory effect on the rate of reduction of nitrous oxide. A 30% decrease in the rate of nitrous oxide reduction, calculated after allowance for the rate of nitrous oxide production from nitrite, was the maximum effect observed. However, in the presence of nitrite the total rate of electron flow to both nitrite and nitrous oxide was usually lower than the rate when nitrous oxide was the sole electron acceptor. This finding adds to the earlier instance of nitrite appearing to act as an inhibitor of electron flow to cytochrome *c*.

Nitrite reductase has been demonstrated to have a periplasmic location in *P. denitrificans* [10,15], and is most probably reduced directly by cytochrome *c* [27,28]. In order to add to the understanding of the factors that control the relative

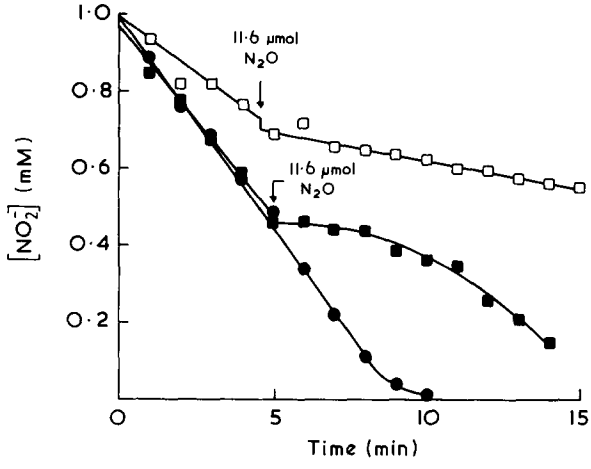

Fig. 4. Inhibition by nitrous oxide of nitrite reduction by cells of *P. denitrificans*. The reaction chamber was fitted with a nitrous oxide electrode and completely filled with 6 ml of an anaerobic solution of 0.1 Hepes-NaOH, pH 7.3, and 1 mM $NaNO_2$ plus: (●———●) 3 mM sodium isoascorbate, 0.2 mM TMPD and cells (7.2 mg dry wt.); (■———■) as previous experiment except that 12 μmol nitrous oxide were added as shown; (□———□) 10 mM sodium succinate, cells (14 mg dry wt.) and 12 μmol nitrous oxide added as shown. The temperature was 20°C.

flow of electrons to nitrous oxide and nitrite, a study was made to determine whether nitrous oxide reductase is also a periplasmic enzyme. Application of a procedure described previously for preparing spheroplasts that are depleted of nitrite reductase activity [10] gave a preparation in which nitrous oxide reductase activity was essentially undetectable (Table I). To provide a basis for comparison between the original cells and the spheroplasts the nitrous oxide reductase activities have been expressed as a percentage of the oxidase activity. The virtual absence of nitrous oxide reductase activity in the presence of either succinate or isoascorbate plus TMPD is not necessarily evidence that the reductase is absent from the spheroplasts. An alternative explanation is loss of either cytochrome *c* or of another unknown component that is required to mediate transfer of electrons from either of these substrates to nitrous oxide reductase. Loss of cytochrome *c* cannot have been very substantial because the spheroplasts retained a high isoascorbate plus TMPD oxidase activity [10], which is dependent on cytochrome *c* [7,10]. Additionally, nitrous oxide reduction by spheroplasts was not stimulated by addition of reduced benzyl viologen which has been reported to be a direct electron donor to nitrous oxide reductase [25]. In contrast to the experiments with spheroplasts, when reduced benzyl viologen was added to anaerobic cells conversion to the colourless oxidised form was clearly seen after addition of nitrous oxide, but the reduced form reappeared after the nitrous oxide had been consumed. Thus, the most reasonable interpretation of the observations with spheroplasts summarised in Table I is that nitrous oxide reductase is a periplasmic and water-soluble enzyme, and is therefore lost from this type of spheroplast which is believed to have had its cell wall extensively degraded [10].

Effect of oxygen on reduction of nitrite

Oxygen inhibits not only nitrate reduction but also nitrite reduction [8]. Even less is known of the mechanisms of the latter effect than of the former. Fig. 5 shows that the addition of oxygen to an anaerobic suspension of cells that was reducing nitrite, in the presence of succinate, caused an almost complete inhibition of nitrite reduction. In contrast, nitrite reduction was not completely inhibited when electrons were fed to cytochrome *c* at a faster rate via TMPD from isoascorbate. The restoration of the initial rate of nitrite reduction with either added substrate corresponded to the suspension of cells reestablishing anaerobiosis. These results suggest that the nitrite reductase enzyme itself is not inactivated in the presence of oxygen but rather starved of electrons when physiological reductants are used.

Correlation of changes in redox states of ubiquinone and cytochrome b with control of nitrate reduction

It was mentioned in the Introduction that the extent of reduction of ubiquinone might control the rate of reduction of nitrate. Unfortunately, this is difficult to investigate experimentally as the difference in absorbance in the region 275–290 nm between the oxidised and reduced forms of ubiquinone cannot be readily measured in cells, owing to light scattering and the high background absorbance from other components. In principle, an alternative method would be to use solvent extraction of ubiquinone from cells [29], but we have found this procedure to be insufficiently sensitive. Consequently, we investigated the use of a

TABLE I

LOSS OF NITROUS OXIDE REDUCTASE ACTIVITY FROM SPHEROPLASTS

Measurements were made at 20°C in 0.1 M Hepes-NaOH, pH 7.3.

Added electron donor	Rate N_2O reduction/rate O_2 reduction		
	Cells	Spheroplasts	Spheroplasts/cells (%)
10 mM sodium succinate	4.8	< 0.04	< 1
3 mM isoascorbate + 0.1 mM TMPD	2.7	< 0.17	< 7

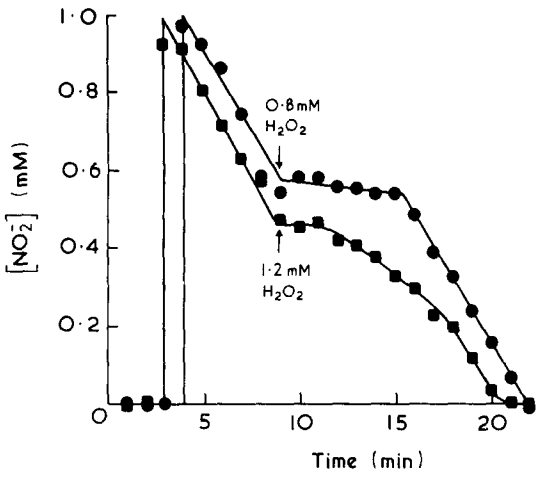

Fig. 5. Effect of oxygen on reduction of nitrite by cells of *P. denitrificans*. The reaction chamber was completely filled with 6 ml of 0.1 M Hepes-NaOH, pH 7.3, and 38 μmol acetylene, and was fitted with an oxygen electrode; temperature 20°C. Further additions were: (●———●) 10 mM sodium succinate and 15 mg dry wt. cells; (■———■) 3 mM sodium D-isoascorbate plus 0.2 mM TMPD and 4 mg dry wt. cells. In each case the cells were allowed to respire until the electrode indicated that anaerobiosis had been attained. 1 mM $NaNO_2$ was then added.

spectroscopic probe, DL-12-(9-anthroyloxy)stearic acid, in which the fluorescence of the anthroyl moiety has previously been shown to be more effectively quenched by oxidised than by reduced ubiquinone in mitochondrial membranes [30].

When cells of *P. denitrificans* were incubated with anthroyloxystearic acid under anaerobic conditions, there was a slow rise in fluorescence as the probe was incorporated into the cell membrane. This continued for about $1\frac{1}{2}$–2 h before an approximately steady-state final level of fluorescence was reached. When a mixture of nitrate and H_2O_2, from which oxygen was generated via the action of the catalase activity of the cells, was added to the cells the fluorescence intensity decreased, consistent with an increased oxidation of ubiquinone (Fig. 6). The important feature was that at the time when the dissolved oxygen concentration fell to zero and nitrate reduction began, the fluorescence of anthroyloxystearic acid increased slightly (Fig. 6), indicating an extra reduction of ubiquinone. The correlation with a switch from use of oxygen to use of nitrate was established by a separate experiment in which nitrate reduction was measured in cells that had been preincubated with anthroyloxystearic acid. In addition, the period before the small increase in anthroyloxystearic acid fluorescence depended linearly on the quantity of H_2O_2 that was added with the nitrate. The changes in anthroyloxystearic acid fluorescence were insensitive to the uncoupler carbonyl cyanide *p*-trifluoromethoxypenylhydrazone at sufficient concentration to collapse the proton electrochemical gradient. Thus, by analogy with studies on mitochondria [30], it is concluded that the changes in probe fluorescence intensity reflect changes in the oxidation state of ubiquinone.

The *b*-type cytochromes in anaerobically grown *P. denitrificans* have not been fully characterised, but probably there are at least three types. These are the *b*-type cytochromes associated with the antimycin-sensitive segment of the respiratory chain, a cytochrome *b* associated with nitrate reductase and a *b*-type oxidase known as cytochrome *o* [4,5]. This means that although the *b*-type cytochromes as a whole are clearly more oxidised in the presence of oxygen than in the presence of nitrate (Fig. 7), it is difficult to correlate the extent of oxidation of a particular *b*-type cytochrome with the control of nitrate reduction. For reasons outlined in the Discussion, ubiquinone is consid-

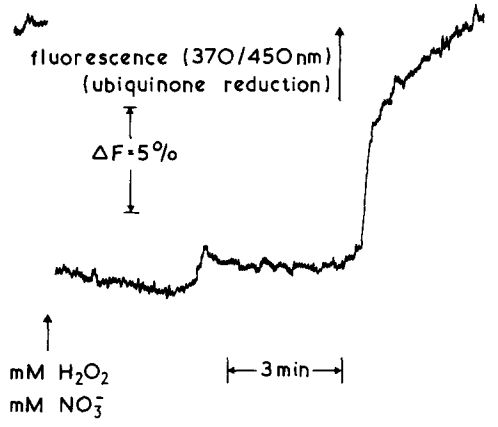

Fig. 6. Changes in fluorescence of anthroxyloxystearic acid during aerobic and anaerobic electron transport in cells of *P. denitrificans*. The reaction mixture contained, in a total volume of 2.5 ml, 0.1 M Hepes-NaOH, pH 7.3, 10 mM sodium succinate, 10 μM anthroxyloxystearic acid plus cells (7 mg dry wt.). This was incubated for 2 h at 0°C and then for $1\frac{1}{2}$ h at room temperature (approx. 20°C) which was also the temperature at which the fluorescence was measured.

ered a better candidate than *b*-type cytochrome for a component of the respiratory chain that might control nitrate reduction. The relative extent of oxidation of *b*-type cytochromes should also reflect the extent of oxidation of ubiquinone because the latter immediately precedes *b*-type cytochromes in the respiratory chain [5]. Thus, the increased oxidation of *b*-type cytochromes in the presence of oxygen can be taken as an additional indication to that from the fluorescent probe study (Fig. 6) that ubiquinone is more oxidised by addition of oxygen than by addition of nitrate.

When nitrite or nitrous oxide is added to the suspension of cells only the *b*-type cytochrome associated with the antimycin-sensitive ubiquinol-cytochrome *c* reductase part of the electron-transfer chain should be oxidised [1,5]. Fig. 7 shows that addition of nitrous oxide caused a greater oxidation of *b*-type cytochrome than did addition of nitrite, which is consistent with the rate of nitrous oxide reduction being greater than the rate of nitrite reduction, as can be calculated from Fig. 7. These results are related to the observations that added nitrous oxide but not nitrite inhibits the reduction of nitrate (Figs. 1–3) because if, as in mitochondria [31], the oxidation/reduction state of the pool of ubiquinone is closely related to the redox poise of the cytochrome *b* in the ubiquinol-cytochrome *c* reductase complex, then the relatively small change in the extent of oxidation of cytochrome *b* seen upon adding nitrous oxide instead of nitrite (Fig. 7) can be taken as evidence that only a slight increase in the extent of ubiquinone oxidation is sufficient to cause essentially complete inhibition of nitrate reduction.

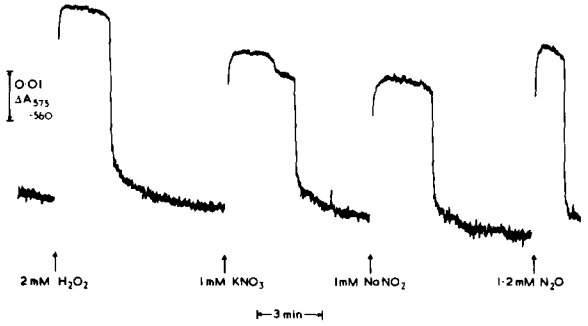

Fig. 7. Spectrophotometric measurement of the oxidation of *b*-type cytochrome by different electron acceptors. The cuvette contained, in a total volume of 2 ml, 0.1 M Hepes-NaOH, pH 7.3, 10 mM sodium succinate and 30 mg dry wt. cells. Argon gas was blown over the stirred reaction mixture throughout, temperature 20°C. The record is discontinuous because the photomultiplier shutter was closed when additions were made to the cuvette.

Effect of limited treatment of cells with detergent on control of nitrate reduction by oxygen

In previous work it was shown that low concentrations of Triton X-100, a non-ionic detergent, rendered cells able to reduce nitrate and oxygen simultaneously [10]. The loss of the control by oxygen correlates with the disappearance of a permeability barrier that in intact cells prevents chlorate from reaching the active site of nitrate reductase [10]. To investigate further whether the effect of detergent in releasing control by oxygen was related to an increase in the permeability of the plasma membrane, two additional detergents, cetyltrimethylammonium bromide, which is cationic, and lauryldimethylamine oxide, which is zwitterionic, have been tested. With both these agents it was also found that the titres necessary, approx. 0.01% in each case, for permitting reduction of chlorate released the control on nitrate reduction by oxygen.

The effects of Triton X-100 on electron-transfer reactions in cells were investigated in more detail. Chlorate rather than nitrate was used as the substrate for nitrate reductase in these experiments because the product of nitrate reduction, nitrite, can be further reduced thus possibly complicating analysis of the patterns of electron flow. Fig. 8 (Expt. 1) shows that in the absence of Triton an addition of chlorate did not cause any inhibition of the rate of oxygen reduction. Inspection of the record of Expt. 1 in Fig. 8 suggests that chlorate stimulated the rate of oxygen reduction, but this was not the case because a similar non-linear rate that increased with time was also observed in the absence of chlorate, presumably reflecting an activation of one or more reactions in the cells. Addition of a sufficient amount of Triton to permit the cells to reduce chlorate also stimulated, by approx. 2-fold, the rate of oxygen reduction (Fig. 8, Expt. 2). In the presence of Triton the rate of oxygen reduction became linear. The ability of Triton-treated cells to reduce chlorate was reflected by the inhibition of electron flow to oxygen fol-

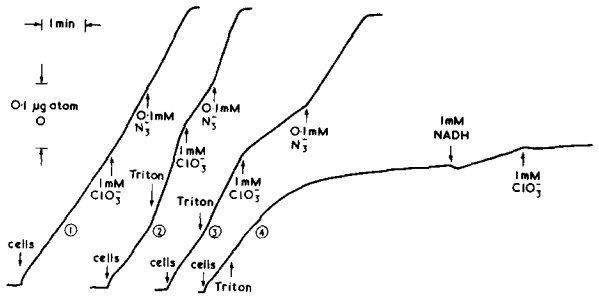

Fig. 8. The effect of low concentrations of Triton X-100 on electron flow to oxygen and chlorate in cells of *P. denitrificans*. A reaction chamber fitted with a Clark-type oxygen electrode was completely filled with 0.1 M Hepes-NaOH, pH 7.3, at 30°C. In Expts. 1–3 25 mM sodium succinate was present and in Expt. 3 1 mM sodium malonate was also added. Cells, approx. 4.5 mg dry wt., and chemicals were added as shown. The final concentration of Triton X-100 was 0.02% (v/v).

lowing addition of chlorate, and the relief of this inhibition when azide was added to block the competing electron flow via nitrate reductase to chlorate (Fig. 8, Expt. 2). The amount of azide added selectively inhibits nitrate reductase [8,32] and has no effect on oxidase activities (Fig. 8, Expt. 1).

The observation that Triton not only permitted chlorate reduction, but also stimulated the rate of electron flow to oxygen, raised the possibility that the capacity for Triton-treated cells for reducing nitrate in the presence of oxygen might be related to the stimulation of electron flow rather than to the effect of Triton on the ionic permeability of the cell membrane. Discrimination between these two possibilities was sought in the following experiment. Sufficient malonate was added to reduce the rate of electron flow to oxygen in the presence of Triton to the rate observed in the absence of Triton. Under these conditions the addition of chlorate (or nitrate – data not shown) still resulted in an inhibition of the rate of oxygen reduction (Fig. 8, Expt. 3) owing to diversion of electron flow to nitrate reductase, although for unknown reasons azide was not quite as effective as restoring electron flow to oxygen under these conditions (cf. Expts. 2 and 3 in Fig. 8). The operation of nitrate reductase in the presence of oxygen, but under conditions of partially inhibited electron flow (Fig. 8, Expt. 3), is the expected result if it is the action of Triton in rendering the cell membrane permeable to nitrate and chlorate that is responsible for permitting the reduction of these ions under aerobic conditions.

Expt. 4 in Fig. 8 shows that when succinate was omitted from the reaction mixture, the rate of respiration slowly declined almost to zero after addition of Triton but was partially restored by addition of NADH. An interpretation of this result is that in the presence of Triton, NAD and NADH leaked from the cells so that endogenous substrates could no longer be used. Thus, in the presence of succinate and Triton (e.g., Expts. 2 and 3 in Fig. 8) electron flow must almost exclusively originate from succinate dehydrogenase.

Fig. 8 also shows that even at the low rates of electron flow that are observed in the presence of Triton and NADH (Expt. 4) it was still possible for chlorate to be reduced. This result supports the conclusion that the functioning of nitrate reductase under aerobic conditions in the presence of Triton is a consequence of the enhanced permeability of the cell membrane. The action of Triton, and the other two detergents tested, is apparently restricted to this aspect, because examination by electron microscopy of cells treated with the appropriate quantities of these detergents did not reveal any detectable morphological changes.

Triton-treated cells also oxidised durohydroquinone in the absence of succinate, and electron flow again partitioned between nitrate or chlorate and oxygen in proportions similar to those when succinate was the substrate.

Investigation of durohydroquinone as a reductant for nitrate reductase under aerobic conditions

The finding that durohydroquinone could act as an electron donor to nitrate reductase in Triton-treated cells prompted study of whether the presence of this non-physiological reductant, in addition to added succinate and endogenous substrates, would result in reduction of nitrate by untreated cells under aerobic conditions. Before attempting such an experiment two criteria had to be satisfied. First, it had to be demonstrated that durohydroquinone was a reductant for nitrate reductase in untreated cells. This was established by inhibiting with rotenone the electron flow from endogenous substrates by 80%, and then showing

that the rate of anaerobic nitrate reduction was considerably stimulated by addition of 1 mM durohydroquinone. In these experiments nitrate reduction was measured from the appearance of nitrite, with antimycin present to inhibit nitrite reduction. This procedure was adopted because durohydroquinone interfered with the nitrate electrode. The second criterion was the necessity to demonstrate that durohydroquinone acted as an electron donor to the respiratory chain whilst NADH (generated from endogenous substrates) and added succinate were also being oxidised. This requirement was met because the total rate of electron flow to oxygen in the presence of endogenous substrates plus 25 mM succinate and 1 mM durohydroquinone was 25% greater than in the absence of durohydroquinone. Thus, durohydroquinone was oxidised via the respiratory chain simultaneously with the other substrates.

When 1 mM durohydroquinone was added to an aerobic suspension of cells containing endogenous substrates in the presence of 25 mM succinate and 1 mM nitrate there was no detectable reduction of nitrate as judged by the absence of any formation of nitrite. In a separate experiment it was shown that nitrite was not reduced under these conditions and that therefore an assay for nitrate reduction based on detecting the appearance of nitrite was valid. The conclusion from the failure of durohydroquinone to stimulate nitrate reduction under aerobic conditions is that merely providing an additional potential source of electrons to the electron-transfer chain is not sufficient to overcome the inhibition of nitrate that is exerted in the presence of oxygen.

Partition of electron flow between nitrate and oxygen in membrane vesicles from wild-type and cytochrome c-deficient cells

Inside-out vesicles from *P. denitrificans* reduce O_2 and NO_3^- simultaneously [8], and in our experiments addition of nitrate generally caused a 50–60% reduction in the rate of electron flow to oxygen, in agreement with the findings of John [8]. In order to investigate the basis for the distribution of electrons between nitrate reductase and oxidase pathways in vesicles, the rates of electron flow to oxygen and to nitrate were measured over a range of total rates of electron flow. Fig. 9 shows that rotenone inhibited with very similar titration profiles both NADH oxidase activity and NADH-nitrate oxidoreductase activity, the latter being measured during simultaneous electron flow to oxygen. A similar percentage inhibition of oxidase activity after addition of nitrate was observed over the complete range of rotenone concentrations shown in Fig. 9. These measurements were made in the absence of an uncoupler and so electron flow was subject to respiratory control, with maximum rates of oxygen reduction of 280 nmol O/min per mg protein before, and 160 nmol O/min per mg protein after addition of nitrate. The maximum rate of nitrate reduction was 170 nmol/min per mg protein. Addition of an uncoupler stimulated electron flow by up to 6-fold, so that even before rotenone was added the electron-transfer chain was operating below its maximal rate in the experiments shown in Fig. 9. Therefore, it is concluded from Fig. 9 that nitrate reductase activity in vesicles does not decrease more sharply than oxidase activity even when the

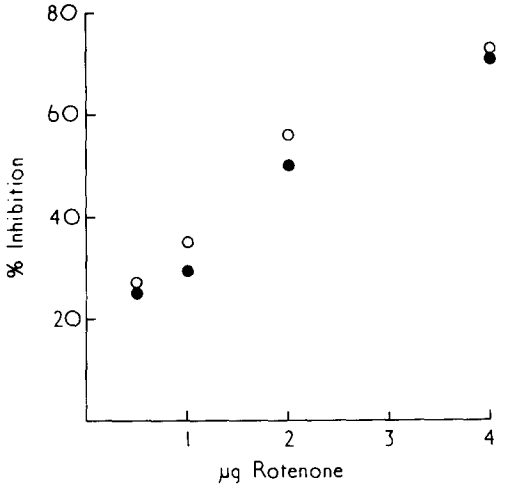

Fig. 9. Inhibition of NADH oxidase activity (●), measured in the absence of nitrate, and of nitrate reductase activity (○) measured in the presence of oxygen in membrane vesicles from wild-type *P. denitrificans*. A reaction chamber fitted with an oxygen electrode was filled with 2 ml of a medium at 30°C containing 10 mM P_i-Tris, 5 mM magnesium acetate, 1% ethanol, 250 μg yeast dehydrogenase, vesicles (0.55 mg protein) and the appropriate amount of rotenone. Respiration was started by addition of 0.6 mM NAD. After a linear rate had been established 1 mM KNO_3 was added, and 0.05-ml samples of the reaction mixtures were withdrawn at 2-min intervals for assay of production of nitrite. The rates of nitrite production and thus of nitrate reduction were linear for 12 min.

electron-transfer chain is relatively oxidised at the low range of rates of electron flow studied in Fig. 9. The simplest interpretation of Fig. 9 is that whatever the rate of electron flow from NADH dehydrogenase, electrons partition by a simple competition mechanism between pathways to nitrate and oxygen, and that there is no evidence for the sharp 'switch-off' of nitrate reduction that is observed in cells [2,8,9]. Further evidence that division of electron flow in vesicles between nitrate and oxygen was determined by a competition mechanism came from studies using 1 mM durohydroquinone as reductant. Electron flow partitioned between oxygen and nitrate in proportions similar to those when NADH was the reductant.

An explanation to be considered for the lack of stringent control on nitrate reduction by oxygen in vesicles is that a relatively large loss of oxidase activity, perhaps owing to loss of cytochrome *c*, occurs during the preparation of the vesicles [33]. In other words, vesicles might behave like antimycin- or hydroxylamine-treated cells. To investigate whether this explanation is valid we have studied the effect of oxygen on nitrate reduction by cells of a cytochrome *c*-deficient mutant, and also in vesicles obtained from these cells. In agreement with previous results [7] it was found that the cells of this type of mutant did not reduce nitrate in the presence of oxygen. Aerobic nitrate reduction was not observed following the addition of antimycin, in contrast to the wild-type cells [2,9]. This was expected because electron transport is insensitive to antimycin in these mutants [7]. Vesicles prepared from the mutant cells did, however, reduce nitrate and oxygen simultaneously and addition of nitrate inhibited electron flow to oxygen by 50%, as in vesicles from wild-type cells. In these experiments the cells from which the vesicles were prepared were checked to ensure that reversion had not occurred (see Materials and Methods). Electron flow to oxygen from reduced ubiquinone in these mutants is believed to involve only cytochrome *o* [5,7], which as far as is known is an integral membrane protein. Therefore, in the case of the mutant the appearance of simultaneous reduction of nitrate and oxygen in the vesicles cannot be attributed to the loss of the water-soluble component of the respiratory chain such as the cytochrome *c* that is found in the wild-type cells.

Discussion

Factors influencing the relative rates of anaerobic electron flow to reductases for nitrate, nitrite and nitrous oxide

Previous work [2,9] had shown that the flow of electrons away from ubiquinone, either to oxygen or to ferricyanide via cytochrome *c*, inhibited the reduction of nitrate. These findings in turn raised the question of whether the physiological acceptors from cytochrome *c*, nitrite and nitrous oxide, would also inhibit nitrate reduction. The present work has shown that in cells grown on succinate and nitrate and harvested at the late logarithmic phase of growth exogenous nitrous oxide, but not exogenous nitrite, resembles oxygen or ferricyanide and inhibits nitrate reduction. The difference between nitrite and nitrous oxide correlates with the higher maximum rate of reduction of the latter. Typical values with succinate as added substrate at 20°C and pH 7.3 were 110 nmol N_2O/min per mg dry wt. and 40 nmol NO_2^-/min per mg dry wt. The difference between these two rates is not, however, the difference between the total rates of electron flow via cytochrome *c* because during nitrite reduction the nitrous oxide produced is simultaneously reduced to nitrogen. Therefore, the rate of electron flow in the presence of added nitrous oxide was usually approximately only twice that in the presence of added nitrite. This observation, together with the evidence that a slight inhibition with antimycin of electron flow to oxygen permits aerobic nitrate reduction [2,9], indicates that relatively small changes in the rate of electron flow to terminal electron acceptors play a key role in regulating nitrate reduction. The relationship between this rate and the rate of nitrate reduction does not appear to be simple because whereas antimycin can accelerate the rate of nitrate reduction by up to 50% as a result of inhibiting electron flow to nitrite and nitrous oxide, no acceleration can be detected when nitrous oxide reduction alone is blocked with acetylene.

The finding in the present work, and independently by Kučera et al. [34], that added nitrous oxide inhibits the reduction of nitrate contradicts the previous view [11,12] that gaseous nitrogen oxides do not inhibit the reduction of the ionic oxides. However, nitrous oxide will not necessarily

always inhibit nitrate reduction. The results in the present work suggest that if the activity of nitrous oxide reductase were approx. 50% lower (i.e., similar to the activity of nitrite reductase), then inhibition of nitrate reduction by nitrous oxide would not be expected. On the other hand, inhibition of nitrate reduction by added nitrite would be expected if nitrite reductase activity were higher. This could be the explanation why Kučera et al. [34] have recently found that nitrite inhibits reduction of nitrate in washed suspension of *P. denitrificans* cells that had been grown under different conditions from those used in the present work.

In general, inhibition of nitrate reduction by denitrifying bacteria after addition of nitrite or nitrous oxide, and the associated question of whether nitrite and/or nitrous oxide accumulate during reduction of nitrate [12,13], can be expected to depend on the relative catalytic capacities of the reductases for nitrate, nitrite and nitrous oxide. As nitrite and nitrous oxide accept electrons from the oxidising side of the point at which electrons leave the electron-transfer chain for nitrate reductase, the chain can be finely adjusted so that reduction of nitrate to nitrite is restricted to a rate at which it is accompanied by simultaneous reduction of nitrite to nitrous oxide and of the latter to nitrogen.

It has been thought that there is a sequential use by denitrifying bacteria of nitrate followed by nitrite and then nitrous oxide [12]. Together with the recent results of others [13,34], our observations on not only simultaneous reduction of nitrate, nitrite and nitrous oxide (Fig. 1), but also inhibition of both nitrate and nitrite reduction by added nitrous oxide (Figs. 1, 2 and 4), show that sequential use of nitrate, nitrite and nitrous oxide is not a general phenomenon. Indeed, taking account of the properties of the denitrifying electron-transfer chain discussed in the present paper, the latter type of behaviour could only be expected in rather restricted circumstances. Accumulation of either nitrite or nitrous oxide could be disadvantageous to a bacterium because both these species might have toxic effects [26], in the case of nitrous oxide perhaps related to its well characterised action as a general anaesthetic.

There are several puzzling effects of nitrite reported in the present paper. The rate of nitrate reduction is stimulated by addition of nitrite, and the inhibition by exogenous nitrous oxide of nitrate reduction is partially reversed by addition of nitrite. After addition of extra nitrite and concomitant stimulation of nitrate reductase activity, nitrite reductase activity is no longer sufficient to match the rate of production of nitrite and consequently nitrite accumulates (Fig. 3). A curious feature is that the rate of nitrite accumulation is greater than the difference in rates of nitrate reduction before and after nitrite addition, and thus there is presumably a decrease in the rate of electron flow to nitrite reductase via cytochrome *c*.

Two possible explanations for these effects of nitrite can be considered. The first is that nitrite might stimulate a nitrate/nitrite antiport system, following the report by Boogerd et al. [35] that nitrite stimulates the initial rate of nitrate reduction by cells. At first sight, the addition of exogenous nitrite might be expected to block exit of nitrite from, and entry of nitrate into, the cell. However, as the pK_a of HNO_2 is 3.4, the intracellular concentration of nitrite could be raised by movement of HNO_2 across the cell membrane, with the result that the rates of antiporter-mediated nitrite efflux and nitrate influx might rise and therefore allow an increase in the rate of nitrate reduction at the cytoplasmic surface [8,10] of the cell membrane (Boogerd, F.C., personal communication). Such a mechanism would require that the putative antiporter is rate limiting under conditions where nitrate alone is added. The decrease in rate of nitrite reduction could then be accounted for if the stimulation of nitrate reductase activity by added nitrite tilted a competition between nitrate and nitrite reductases for available electrons from dehydrogenases in favour of nitrate reductase. A second explanation is that nitrite inhibits the rate of electron flow via cytochrome *c* to nitrite reductase. This would permit a greater rate of electron flow to nitrate, just as is seen when antimycin inhibits electron flow to cytochrome *c*.

Cellular location of nitrous oxide reductase

The conclusion that nitrous oxide reductase has a periplasmic location agrees with the observation that the protons required for reduction of nitrous oxide are taken from the periplasmic side of the

plasma membrane [16], a finding that was also taken as evidence that the nitrous oxide reductase is found at the periplasmic surface of the cell membrane [16]. Kristjansson and Hollocher [25] found that a preparation of spheroplasts from *P. denitrificans* retained nitrous oxide reductase activity. They suggested that the higher K_m for nitrous oxide reported for intact cells compared with a soluble enzyme preparation arose because the intracellular concentration of nitrous oxide was lower than the extracellular concentration. The implication from the latter points was that nitrous oxide reductase is located on the cytoplasmic surface of the membrane. However, these observations [25] are not inconsistent with a periplasmic location because periplasmic proteins are only lost from spheroplasts from *P. denitrificans* that have been prepared by extensive digestion of the cell wall [10], and using an electrode for nitrous oxide the K_m in cells has been found to be of similar magnitude to that reported for the soluble enzyme [3]. Thus, the available evidence is consistent with a nitrous oxide reductase being associated with the periplasmic surface of the membrane. The periplasmic locations now demonstrated for both nitrite and nitrous oxide reductases, taken together with the higher specific activity of the latter enzyme (see earlier in Discussion), means that the inhibition of nitrite reduction by nitrous oxide (Fig. 4) is most probably a reflection of the rate of electron flow at the periplasmic surface of the membrane being faster from cytochrome *c* to nitrous oxide reductase than to nitrite reductase.

Basis for inhibition by oxygen of nitrite reduction

Several factors may underlie the observation that oxygen is an effective inhibitor of nitrite reduction. The first of these is that the nitrite reductase enzyme is able to reduce not only nitrite but also oxygen [27,28]. For the purified enzyme $K_m(O_2)$ is 80 μM whereas for nitrite the K_m value is 6 μM [28]. Therefore, the nitrite reductase enzyme ought to be capable of reducing nitrite in the presence of approximately equimolar oxygen. However, when electrons were fed into the electron-transfer chain by physiological substrates, oxygen very strongly inhibited nitrite reduction, even when the concentration of oxygen had fallen below its K_m value for the nitrite reductase. Nitrite reduction recommenced fairly sharply only as the oxygen concentration fell to zero (Fig. 5). When ascorbate plus TMPD was used to feed electrons into the respiratory chain at the level of cytochrome *c* nitrite reduction continued, albeit at a reduced rate, in the presence of oxygen. This observation suggests that the basis of the inhibition of nitrite reduction by oxygen seen with physiological substrates is that the supply of electrons to the nitrite reductase enzyme is severely limited owing to competition for electrons from the two oxidases present, cytochrome *o* and cytochrome aa_3 [2,6]. The inhibition of nitrous oxide reductase by oxygen reported before [3,14] is likely to have a simpler basis because the enzyme is inactivated by oxygen, reversibly in the short term [3], and partially irreversibly in the long term [36].

Does cytochrome cd (nitrite reductase) function as an oxidase in cells and play a role in regulating nitrate reductase?

Kučera et al. [9] have demonstrated aerobic nitrate reduction in the presence of antimycin or hydroxylamine which both selectively block electron flow to oxygen. In interpreting their data they assumed that cytochrome aa_3 was absent from the cells, although evidence presented elsewhere [2,6] indicates that batch cultures of cells grown on succinate plus nitrate possess high levels of this cytochrome. Consequently, the ability of antimycin, and also hydroxylamine, to permit aerobic reduction of nitrate was attributed to this inhibitor blocking electron flow to oxygen via nitrite reductase (cytochrome *cd*), and the latter enzyme was suggested to play an important role in the control of nitrate reduction [9]. However, three considerations make any specific role for cytochrome *cd* improbable. First, spheroplasts depleted in cytochrome *cd* retain a strong control by oxygen on nitrate reduction [10]. Second, $K_m(O_2)$ for nitrite reductase is 80 μM [28] so that if nitrite reductase were specifically involved in the control an increasing release of the control would be predicted as the dissolved oxygen concentration fell below 80 μM. Yet, it is observed (e.g., see Ref. 8) that oxygen concentrations as low as 20 μM are effective at completely inhibiting nitrate reduction, and that nitrate reduction begins sharply as the dissolved oxygen concentration falls to zero. The

third consideration is that, as demonstrated in the present paper, and by Willison and John [7], a mutant that lacks cytochrome *cd* retains a fully operational control by oxygen on nitrate reduction.

The observations that rates of oxygen reduction do not decline as the oxygen concentration falls below 80 μM, and that spheroplasts depleted in cytochrome *cd* retain a high oxidase activity [10], also demonstrate that in the cell cytochrome *cd* does not normally function as an oxidase. Furthermore, the effect of oxygen on the rate of nitrite reduction with succinate as substrate indicates that competition for electrons by cytochromes aa_3 and *o* most probably renders cytochrome *cd* almost completely inactive under aerobic conditions with physiological electron donors.

Analysis of possible relation between extent of reduction of ubiquinone and control of nitrate reduction

A principal objective of the work reported in the present paper was to investigate further the mechanism whereby nitrate reduction is switched off in the presence of either oxygen [2,8,9], ferricyanide [2,9] or added nitrous oxide. Before proceeding further it is appropriate to review why the redox state of ubiquinone rather than that of *b*-type cytochrome (cf. Fig. 7) has been suggested [2,17] to be directly involved in controlling flux through nitrate reductase. Present knowledge of the respiratory chain of *P. denitrificans* indicates that ubiquinone is the last common component of the respiratory chain before pathways of electron flow to oxygen and to nitrate diverge [2,5]. The reduced form of ubiquinone is thought to be the substrate for the nitrate reductase complex, and thus must directly interact with the complex. Such interactions could easily have both control and catalytic functions. The nature of a direct role for *b*-type cytochrome in regulating nitrate reductase activity is much harder to envisage. Essentially two types of *b*-type cytochrome can be considered; those associated with the antimycin-sensitive segment of the electron-transfer chain (analogous to Complex III in mitochondria), and the *b*-type cytochrome that is thought to act as an oxidase and is usually called cytochrome *o*. How could the redox state of these *b*-type cytochromes be directly involved in regulating flux through nitrate reductase? As these cytochromes occur in the respiratory chain after the branch point to nitrate reductase [5] one would have to postulate some kind of complex between nitrate reductase and the *b*-type cytochrome so that nitrate reductase could be sensitive to the redox state of the cytochrome. There are two difficulties with a mechanism of this kind. First, the current view of electron-transfer complexes in membranes is that they exist as independent diffusing species and that electron transfer is achieved by collisions with relatively small and highly mobile electron carriers such as ubiquinone and cytochrome *c* [37]. Second, any direct role for *b*-type cytochrome cannot be specific because nitrate reduction is inhibited both when electron flow is exclusively through the *b*-type heme of the antimycin-sensitive segment of the electron-transport chain (e.g., in the presence of exogenous nitrous oxide) and when only the cytochrome *o* is functional (e.g., the cytochrome *c*-deficient mutant in the presence of oxygen [7]). The demonstration of control by oxygen on nitrate reduction in the cytochrome *c*-deficient mutant [7] also renders unlikely any specific role for cytochromes *c* or c_1 in regulating nitrate reduction. The difficulty in formulating a mechanism whereby flux through nitrate reductase could be under the direct control of a *b*-type cytochrome also applies to any postulated control function for *c*-type cytochrome. Additional arguments against a specific role for cytochrome *cd* were given earlier.

Unfortunately, we have so far been able only to obtain indirect evidence that ubiquinone becomes more reduced when electron flow to oxygen ceases and the reduction of nitrogen oxides begins. The observation that the *b*-type cytochrome of the antimycin-sensitive cytochrome *b*-c_1 complex of the respiratory chain is more oxidised in the presence of added nitrous oxide than in the presence of added nitrite correlates with the inhibition of nitrate reduction by nitrous oxide but not by nitrite. In the mitochondrial respiratory chain, to which that of *P. denitrificans* shows many resemblances [4,5,38–40], increasing oxidation of this *b*-type cytochrome is usually accompanied by a comparable extent of increased oxidation of the bulk ubiquinone pool [31]. Thus, by analogy with mitochondria it is considered that electron flow to nitrous oxide results in a higher extent of oxida-

tion of ubiquinone than electron flow to nitrite, thus accounting for the inhibition of nitrate reduction by nitrous oxide. It is suggested that the extent of oxidation of ubiquinone in the presence of oxygen is at least as great as in the presence of nitrous oxide. Hence, initiation of nitrate reduction following a transition from aerobic to anaerobic conditions should be accompanied by an increased reduction of ubiquinone, as indeed was found qualitatively with the fluorescent probe anthroyloxystearic acid (Fig. 6).

Ubiquinone is usually considered to function as a homogeneous pool in mitochondria [31]. The kinetics of dehydrogenase activity have been shown to fit an equation of the form:

$$V_D \frac{[Q]}{[Q]+[QH_2]} \quad (1)$$

where V_D is the maximum rate of dehydrogenase activity, [Q] the concentration of oxidised ubiquinone and $[QH_2]$ the concentration of reduced ubiquinone. By analogy, a similar equation might be anticipated to describe electron transfer to ubiquinone in *P. denitrificans*, although there is uncertainty as to whether ubiquinone is present in as great an excess over the respiratory chain complexes [41,42] as is found for mitochondria [31]. Evidence for a pool function has come from the finding that NADH oxidase activity in membrane vesicles becomes less sensitive to 2-(*n*-heptyl)-4-hydroxyquinoline *N*-oxide, which acts on the oxidising side of ubiquinone [7], as the rate of electron flow from NADH is reduced by titration with rotenone [43]. On the other hand, John [44] has questioned whether all the aspects of electron transport in vesicles from *P. denitrificans* are compatible with the operation of a homogeneous ubiquinone pool. Nevertheless, consideration of Eqn. 1 is worthwhile, because it should at least act as a guide to the extent of the changes in the oxidation state of ubiquinone that can be expected to accompany the transition from use of oxygen to use of nitrate and its reduction products as electron acceptors. Table II shows that this transition is sometimes accompanied by an increase in the total rate of electron flow, whilst in some other examples quoted the total rate of electron flow under anaerobic conditions is slightly less than in the presence of oxygen. As electron flow always originates from the dehydrogenases Eqn. 1 shows that the changes in total electron-flow rates given in Table II would have to be accompanied by relatively small changes in the oxidation state of ubiquinone, in agreement with the experimental data described here. In the present paper evidence has been given that the onset of nitrate reduction is accompanied by an increase in the extent of reduction of ubiquinone, yet Eqn. 1 would suggest that increases in electron-flow rate from the dehydrogenases accompanying increases in the total electron-flow rate during nitrate reduction (Table II) would require an increase in the extent of ubiquinone oxidation, assuming that V_D values do not change upon the aerobic–anaerobic transition. This apparent inconsistency may indicate that when, as with intact cells, two or more dehydrogenases are operating together, the concept of a homogeneous ubiquinone pool needs modification, as suggested by Gutman [45] for mitochondria.

The kinetics of ubiquinone oxidation in mitochondria follow an equation of the form [31]:

$$V_{OX} \frac{[QH_2]}{[Q]+[QH_2]} \quad (2)$$

where V_{OX} is the maximum rate of respiratory chain activity on the oxidising side of the ubiquinone pool and [Q] and $[QH_2]$ have the same meaning as before. In an extension of this model, De Troostembergh and Nyns [46] have shown that a composite equation with different values of V_{OX} for two alternative oxidase pathways can account for the partition of electron flow between the two oxidases in yeast mitochondria. It is instructive to consider whether such a model, expanded to include not only the two oxidase pathways present in cells of *P. denitrificans* [4,5,7] but also the nitrate reductase pathway, could account for the zero or close to zero [2,8,9] rate of electron flow through nitrate reductase under aerobic conditions. According to a model of this kind the rate of nitrate reduction would be given by Eqn. 2 except that V_{OX} would be replaced by $V_{NO_3^-}$, the maximum rate of the branch of the respiratory chain that leads through nitrate reductase. Thus, to account for the very large increase [2,8,9] in the rate of nitrate reduction upon exhaustion of oxygen the fraction of ubiquinone in the reduced form would similarly have to increase very substantially.

TABLE II

COMPARISON OF RATES OF OXYGEN REDUCTION WITH RATES OF ELECTRON FLOW TO AVAILABLE ACCEPTORS IN THE PRESENCE OF ADDED NITRATE UNDER ANAEROBIC CONDITIONS BY CELLS OF *P. DENITRIFICANS*

Reference and reaction conditions	Rate of oxygen reduction	Rate of NO_3^- reduction	Total rate [a] of electron flow to NO_3^-, NO_2^- and N_2O	Rate [a] of electron flow to NO_2^- and N_2O
b	360 nmol O/min per mg dry wt.	114 nmol/min per mg dry wt.	285 nmol/min mg dry wt.	171 nmol/min per mg dry wt.
c	6.3 μg atom O/min	4.2 μmol/min	10.5 μmol/min	6.3 μmol/min
d	130 nmol O/min per mg dry wt.	40 nmol/min per mg dry wt.	100 nmol/min per mg dry wt.	60 nmol/min per mg dry wt.
e	34 nmol O/min per mg dry wt.	25 nmol/min per mg dry wt.	62.5 nmol/min per mg dry wt.	37.5 nmol/min per mg dry wt.
f	56 nmol O/min per mg dry wt.	37 nmol/min per mg dry wt.	92.5 nmol/min per mg dry wt.	55.5 nmol/min per mg dry wt.

[a] Calculated on the basis that NO_2^- and N_2O do not accumulate during anaerobic reduction of nitrate. Expressed in nmol NO_3^-, NO_2^- and N_2O reduced. Recall that for each N_2O reduced to N_2, two NO_3^- are reduced via NO_2^- to N_2O.

[b] John [8], 200 mM sucrose, 20 mM Tris-HCl, pH 8.0, 30°C.

[c] John and Whatley [40], 10 mM potassium phosphate, pH 8.0, 30°C, quantity of cells used not given.

[d] Alefounder et al. [2], 200 mM sucrose, 20 mM Tris-HCl, pH 8.0, 30°C. Note that the rate of NO_3^- reduction given is in the absence of antimycin. Contrast calculation given in Ref. 53 where the rate in the presence of antimycin is taken from Ref. 2. The latter calculation is inappropriate because reduction of both NO_2^- and N_2O is blocked by antimycin.

[e] and [f]. Present work, 0.1 M Hepes-NaOH, pH 7.3, 20°C.

Both the experimental evidence reported here, and the discussion of the kinetics of electron flow from dehydrogenases to ubiquinone, argue against the inhibition of nitrate reduction by oxygen being a simple consequence of a reaction mechanism described by a modified version of Eqn. 2, in which V_{OX} is replaced by $V_{NO_3^-}$, because the increase in the fraction of ubiquinone in the reduced form is thought to be small.

Acceptance that ubiquinone is more highly oxidised during electron flow to oxygen than during electron flow to nitrogen oxides raises the question of how the rate of electron flow from ubiquinone to oxygen can be similar to, or greater than, the total rate of electron flow via the cytochrome b-c_1 complex to nitrite and nitrous oxide (Table II), when Eqn, 2 suggests that it might be less if values of V_{OX} are comparable. However, it should be recalled that cells grown as described here have at least two oxidase pathways, one via cytochrome bc_1 to cytochrome aa_3, the other being the cytochrome o pathway which probably uses ubiquinone as the electron donor [2,5,6]. The simultaneous operation of these two oxidases will permit a given ratio of reduced to oxidised ubiquinone to support a higher rate of electron flow to oxygen than if only one oxidase pathway were operating (cf. Ref. 46). Recognition of this point may provide at least one explanation why *P. denitrificans* has two alternative oxidase pathways, but only single routes of electron flow to nitrogen oxides. The two oxidase pathways can provide a device for ensuring that oxygen is used as an electron acceptor in preference to nitrogen oxides. The reason for this very strict preference is not altogether clear because the stoicheiometries of proton and charge translocation associated with electron flow to oxygen via cytochrome o and to the nitrogen oxides are similar although not identical [16,47,48]. A factor to consider is that nitrate and nitrite can be used as a source of nitrogen for growth and therefore it is advantageous to the cell not to use these ions as electron acceptors unless it is unavoidable.

On the basis of the work discussed in the present paper it is proposed that relatively small

changes in the oxidation state of ubiquinone critically control the functioning of nitrate reductase. An additional line of experimentation to test this proposition might be investigation of the effect on nitrate reductase activity of reducing electron flow from the dehydrogenases. At lower rates of electron flow ubiquinone should be more oxidised and nitrate reductase activity is predicted to be inhibited to a greater extent than oxidase activity. In preliminary experiments on the effect of rotenone on cells of *P. denitrificans* such a result has been obtained, but the interpretation is complex because inhibition by rotenone is only partial, and simultaneous electron flow to nitrite and nitrous oxide occurs.

From current knowledge of the respiratory chain of *P. denitrificans* [4,5] ubiquinone is the most plausible candidate for a role in regulating reduction of nitrate. An alternative possibility that there exists an unidentified component of the respiratory chain with this role, perhaps analogous to the low-potential flavoprotein that has been suggested to operate a switch on electron transfer in plant mitochondria [49], cannot yet be definitely excluded.

Is control of nitrate reduction achieved by restricting movement of electrons or nitrate?

As explained in the Introduction, control on nitrate reductase activity in cells could in principle be achieved by preventing movement of either nitrate or electrons to the reductase. Several observations reported in the present paper are consistent with the control being exerted on the access of nitrate to its reductase. In particular, the finding that titres of three different detergents that allowed chlorate to be reduced by nitrate reductase in cells also permitted aerobic nitrate reduction argues against a detergent having a coincidental deleterious effect on a putative mechanism for controlling the flow of electrons to nitrate reductase. This consideration, taken together with both the evidence from the electron microscope that the detergents had a very limited disruptive effect on the cells, and the finding that in the presence of Triton and oxygen electron flow via nitrate reductase was observed at a wide range of total rates of electron flow, strengthens the evidence in favour of a mechanism in which nitrate reductase activity is controlled by restricting access of nitrate to the active site.

If nitrate reduction by cells were to be inhibited by oxygen because of preferential flow to oxygen of electrons from dehydrogenases, then the availability to the respiratory chain of an additional source of electrons might promote reduction of nitrate under aerobic conditions. In the present work durohydroquinone was shown to be an electron donor to the respiratory chain of *P. denitrificans* cells even when other substrates (NADH and succinate) were being utilised. The exact point at which durohydroquinone feeds electrons into the chain is uncertain, but there is evidence that it does not reduce ubiquinone in membranes [50]. Thus, durohydroquinone probably acts as a surrogate reduced ubiquinone and therefore is a direct reductant for nitrate reductase, cytochrome *o* and the antimycin-sensitive segment of the respiratory chain. The finding in the present work that addition of durohydroquinone did not result in nitrate reduction under aerobic conditions can therefore be taken as another indication that it may not be the failure of electrons to reach the reductase that prevents the reduction of nitrate in the presence of oxygen.

A question that arises from the use of durohydroquinone is whether its presence diminishes the rate at which the respiratory chain can oxidise the reduced form of ubiquinone, and thus increases the fraction of ubiquinone that is present in the reduced state. In the absence of reliable direct measurements of the redox state of ubiquinone this cannot be answered, but ubiquinone would only become more reduced if pathways for ubiquinol oxidation were rate limiting in the overall process of electron transfer from dehydrogenases to electron acceptors. In the mitochondrial membrane such pathways are frequently not rate limiting [31], thus setting a precedent that could be applicable to other systems. Hence, an interpretation for the failure of durohydroquinone to stimulate nitrate reduction under aerobic conditions is that as durohydroquinone donates electrons to the respiratory chain after the branch point to nitrate reductase, the extent of oxidation of the component that controls nitrate reduction remains constant and thus nitrate movement to its reductase is blocked. Although this component is suggested to

be ubiquinone the same argument would apply to any other component that is located in the respiratory chain on the reducing side of the point at which durohydroquinone feeds electrons into the respiratory chain.

Inverted membrane vesicles from *P. denitrificans* reduce nitrate and oxygen simultaneously [8]. An explanation for this behaviour, consistent with the conclusions made in the previous paragraphs, was given in the Introduction. An alternative view is that the vesicles are sufficiently depleted in oxidase activity relative to cells, owing to loss of cytochrome *c* during preparation, that a preferential flow of electrons to oxygen suggested to occur in intact cells is lost [33]. The experiments with vesicles from cytochrome *c*-deficient cells reported in the present paper are taken as evidence against this view. Furthermore, advocation of loss of oxidase activity as the reason for the appearance of aerobic nitrate reduction is not consistent with the behaviour of Triton-treated cells in which a stimulation of the rate of electron flow to oxygen is observed.

Jones et al. [33] concluded that competition for electrons between nitrate reductase and oxidases could account for the partition of electron flow from NADH to either oxygen or nitrate observed with inverted membrane vesicles. The experiments shown in Fig. 9 support this conclusion and differ from those of Jones et al. [33] in that nitrate reductase activity was measured from the rate of appearance of nitrite rather than deduced from the decrease in the rate of oxygen reduction when nitrate was added to respiring vesicles. In vesicles the simultaneous rates of nitrate and oxygen reduction are similar. Thus, if it is to be argued that the control on nitrate reduction by oxygen in cells is a consequence of a considerably more effective operation of the competition mechanism identified in vesicles, it must also be argued that in cells oxidase activity is at least 10-times greater than nitrate reductase activity, and that nine-tenths of this oxidase activity is lost upon preparing vesicles. This is considered improbable especially as the vesicles have a high specific oxidase activity [40]. In agreement with earlier parts of the Discussion, it is suggested that a competition mechanism between nitrate and oxygen does not operate in cells because nitrate is not freely available to its reductase, unlike the situation in vesicles.

On the basis of the results discussed in this section, and those published earlier [10], a good case can be made for the control of nitrate reduction being exerted on movement of nitrate rather than on the transfer of electrons to nitrate reductase. However, studies on the mechanism of this control are at an early state and further experiments will be required before a final decision between control of nitrate or electron movement, or even a combination of both these factors, can be made.

It is important to appreciate that two separate facets of the control of nitrate reduction can be identified: (i) which component(s) of the respiratory chain switches the nitrate reducing system on and off; (ii) what is the mechanism of this switching? From the foregoing discussion it should be clear that distinct sets of experiments relate to these two questions and to that extent the issues are independent. Nevertheless, the two questions are obviously related and combination of results in the present and recent papers [2,10] suggests that the redox state of ubiquinone could control the movement of nitrate to its reductase. Such a regulatory role for the ubiquinone would be novel, but it is of related interest that recently the redox state of plastoquinone in thylakoids has been implicated in controlling the activity of a membrane-bound enzyme, a protein kinase [51]. Phosphorylation of a light-harvesting protein by this kinase has been shown to control the distribution of quanta between the two photosystems. By analogy, a similar phosphorylation mechanism might be involved in controlling nitrate reduction in *P. denitrificans*, although the speed of the transition from use of oxygen to use of nitrate [2,8] can be taken as preliminary evidence against this type of mechanism. A mechanism for controlling nitrate reduction that can be eliminated is one involving any role for the proton-motive force [2], in contrast to inhibition of electron flow to oxygen by light in photosynthetic bacteria [52]. Confirmation and identification of the proposed role for ubiquinone in controlling nitrate reduction will probably require experiments with purified and reconstituted components of the *P. denitrificans* respiratory chain.

A recent extended version of the work of Kučera

et al. [34] has been published [53]. There are useful points of comparison to be made between the latter and the present paper.

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