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The high affinity of *Paracoccus denitrificans* cells for nitrate as an electron acceptor. Analysis of possible mechanisms of nitrate and nitrite movement across the plasma membrane and the basis for inhibition by added nitrite of oxidase activity in permeabilised cells

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(1) The apparent K_m for nitrate of the electron-transport system in intact cells of *Paracoccus denitrificans* was less than 5 μM. In contrast the apparent K_m for nitrate of inverted membrane vesicles oxidising NADH was greater than 50 μM. When azide, a competitive inhibitor, was present, the apparent K_m observed with the vesicles was raised to 0.64 mM, consistent with values previously reported for purified preparations of the reductase. In membrane vesicles the nitrate reductase is probably not rate-limiting for NADH-nitrate oxido-reductase activity, and thus a lower limit for K_m (NO_3^-) is obtained. It is suggested that the very low K_m (NO_3^-) in intact cells must arise from either a transport process or a nitrate-specific pore that allows access of nitrate directly to the active site of its reductase from the periplasm. (2) The swelling of spheroplasts has been studied under both aerobic and anaerobic conditions to probe possible mechanisms of nitrate and nitrite transport across the plasma membrane of *P. denitrificans*. Nitrate reductase was inhibited by azide to prevent reduction of internal nitrate. No evidence for operation of either nitrate-nitrite antiport or proton-nitrate symport was obtained. (3) Measurements from the fluorescence intensity of 8-anilino-naphthalene-1-sulphonate of the rates of decay of diffusion potentials generated by addition of potassium salts to valinomycin-treated plasma membrane vesicles from *P. denitrificans* showed that the permeability of the membrane to anions is $SCN^- > NO_3^-$, NO_2^-, pyruvate, acetate $> Cl^- > SO_4^{2-}$. In the presence of a protonophore the rate of decay of the diffusion potential was considerably enhanced with potassium acetate or potassium nitrite, but not with potassium salts of nitrate, chloride or pyruvate. This result indicates that HNO_2 and CH_3COOH can rapidly and passively diffuse across the cell membrane. This finding suggests that transport systems for nitrite are in general probably not required in bacteria. The failure of a protonophore to enhance the dissipation of the diffusion potential generated by potassium nitrate is evidence against the operation of a proton-nitrate symporter. (4) Low concentrations of added nitrite very strongly inhibit electron flow to oxygen in anaerobically grown cells, provided that they have been treated with Triton X-100 or an uncoupler. This inhibition is not observed with aerobically grown cells. It is concluded that the inhibitory species is a reaction product or an intermediate of the nitrite reductase reaction. The requirement

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Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; ANS, 8-anilinonaphthalene sulphonate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

for collapse of protonmotive force by uncoupler or permeabilising the plasma membrane suggests that any such species could be negatively charged. Nitroxyl anion (NO^-) can be considered, as its conjugate acid is a postulated intermediate between nitrite and nitrous oxide; nitroxyl anion can bind to heme centres to give nitrosyl derivatives. (5) The basis for the ability of permeabilised, but not intact, cells of *P. denitrificans* to reduce oxygen and nitrate simultaneously is discussed.

Introduction

The use of nitrate and nitrite as electron acceptors for anaerobic respiration by *Paracoccus denitrificans*, and presumably also by other denitrifying bacteria, raises intriguing questions about possible mechanisms of nitrate and nitrite transport across the bacterial plasma membrane. It is thought that reduction of nitrate to nitrite occurs on the cytoplasmic surface of the plasma membrane. The principal basis for this view is that in the intact cell the active site of nitrate reductase is inaccessible to chlorate, which is readily reduced by this enzyme in inverted membrane vesicles from *P. denitrificans* and by purified preparations of the enzyme [1–3]. Evidence that the α subunit of the analogous respiratory nitrate reductase of *Escherichia coli* has a catalytic role and is largely, if not exclusively, found on the cytoplasmic face of the membrane [4] strengthens this view. The probable location of the nitrate reductase active site thus implies that there is a mechanism for nitrate entry into the cell that overcomes the tendency of the membrane potential (approx. 150 mV, negative inside [5]) to restrict the intracellular concentration to approx. 1/1000 of the extracellular concentration. With this point in mind, objectives of the work reported in the present paper included probing for a putative transport system for nitrate by comparisons of the minimum concentrations of nitrate that could support the full rate of nitrate reduction by both cells of *P. denitrificans* and inverted membrane vesicles from the same bacterium.

The nitrite produced by the action of nitrate reductase in cells is reduced in the periplasm [6,7]. This means that nitrite must move outwards through the cell membrane. Possible mechanisms of nitrate entry and nitrite exit have been considered for some time. A nitrate-nitrite antiporter is an obvious candidate and although direct tests for its operation in *E. coli* gave negative results [8], the ability of added nitrite to reduce the duration of a lag period before the onset of electron flow from hydrogen to nitrate in *P. denitrificans* has been taken as an indication for the operation of an antiporter [9]. A proton-nitrate symporter has also been suggested by Boogerd et al. [9], who propose that such a system is responsible for the initiation of nitrate respiration when there is no nitrite present to activate the putative antiporter.

Earlier experiments designed to detect the operation of either the antiporter or a proton-nitrate symporter have tested the effects of a variety of ionophores on the ability of solutions of nitrate salts that are isotonic with the cell cytoplasm to provide osmotic support to suspensions of spheroplasts prepared from *P. denitrificans* and *E. coli* [8,10]. The present paper reports experiments of a similar design, except that the range of conditions tested was extended in order to evaluate possible explanations for previous failures to observe a pattern of swelling consistent with either a nitrate-nitrite antiporter or a nitrate-proton symporter.

The movement of nitrate and nitrite across the bacterial plasma membrane does not only occur in association with electron flow to nitrate and nitrite, but is also believed to occur in *Nitrobacter*, which oxidises nitrite to nitrate to provide energy for growth, and also during the assimilation of nitrate and nitrite when they act as the nitrogen sources for growth. In all cases the transport processes are poorly characterised. The present paper describes a method that can be used to test for relative permeabilities of nitrite, nitrate and nitrous acid as well as for the possible operation of a proton-nitrate symport.

Cells of *P. denitrificans* do not reduce nitrate when oxygen is available [1], except in the presence of certain electron transport inhibitors [11,12], or unless the plasma membrane is permeabilised by Triton X-100 [7,11] or other treatments [12]. Kučera et al. [12] have argued that one of the

reasons for the diversion of electron flow from oxygen to nitrate in such conditions is that nitrite, formed by reduction of nitrate, has a potent inhibitory effect on electron-transport pathways to oxygen. An inhibitory effect of low concentrations of nitrite is only observed after permeabilisation or collapse of the membrane potential [12,13], suggesting that nitrite might be a less potent inhibitor with intact cells owing to the membrane potential driving its exclusion from the cytoplasm [12,13]. Earlier work [5] as well as results described in the present paper indicates that the nitrite anion is not readily moved across the plasma membrane of *P. denitrificans*. This paper presents evidence that the inhibitory effect of added nitrite upon oxygen reduction by cells treated with an uncoupler or detergent is not a direct effect of nitrite, but rather of a product of nitrite reduction which is possibly an intermediate on the denitrifying pathway from nitrite to nitrous oxide.

Materials and Methods

P. denitrificans (NCIB 8944) was grown in a 2-1 batch culture with succinate as substrate and nitrate as terminal electron acceptor as described by Burnell et al. [14]. The cells were harvested at the late logarithmic stage of growth ($A_{550} = 1.7–1.8$ in a 1 cm path length cuvette in a Unicam SP 500 spectrophotometer) by centrifugation for 20 min at 4°C and 5000 × *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.

Reduction of nitrate by cells was measured using the electrode described by Alefounder et al. [15]. Nitrite was determined colorimetrically [7]. Rates of oxygen reduction by cells were measured using a Clark-type oxygen electrode.

Type-1 spheroplasts were prepared by the procedure of Alefounder and Ferguson [7]. Osmotic swelling of spheroplasts was followed spectrophotometrically in a cuvette of 1 cm light-path by recording the absorbance at 550 nm in a Unicam SP 1800 spectrophotometer.

Membrane vesicles were prepared as described elsewhere [14] except that all acetate salts were replaced by chlorides for those experiments in which the effects of anions on diffusion potentials were examined.

Nitrate reduction by vesicles was measured by following spectrophotometrically at 340 nm the oxidation of NADH in a cuvette which was fitted with a closure through which nitrogen gas was passed to maintain anaerobic conditions.

The fluorescence of 8-anilino-naphthalene sulphonate (ANS) was measured as described by Parsonage and Ferguson [16].

Results

Comparison of affinities for nitrate of intact cells and inverted membrane vesicles

Studies on purified and partially purified respiratory nitrate reductases from a number of different bacteria have shown that these enzymes have a K_m value for nitrate in the range from 0.3 to 1.5 mM [2,3,17]. These data were obtained using non-physiological substrates as the reductant, including benzyl viologen and flavin mononucleotide. Inspection of previously published records of the rate of nitrate reduction by cells of *P. denitrificans* [1,11,15] showed no sign of the rate of reduction decreasing as the nitrate concentration fell into the range of reported K_m values for nitrate reductases. This observation led us to investigate whether a range of nitrate concentrations could be found over which the initial rates of nitrate reduction progessively decreased owing to an apparent K_m for nitrate being reached. The nitrate electrode was capable of detecting nitrate concentrations as low as 5 μM, but drift of the baseline made the usual procedure of calibration of the electrode with sequential additions of nitrate, followed by addition of cells and measurement of the rate of nitrate reduction, very difficult to perform. Consequently, the procedure shown in Fig. 1 was used, in which different low concentrations of nitrate were sequentially added to an anaerobic suspension of cells. The amount of cells used was chosen so that the rate of nitrate reduction was slow enough to allow the electrode response to the additions of nitrate to be almost complete. Fig. 1 shows that using this procedure linear rates of nitrate reduction were recorded at concentrations of nitrate at least as low as 5 μM. These findings therefore show that the apparent

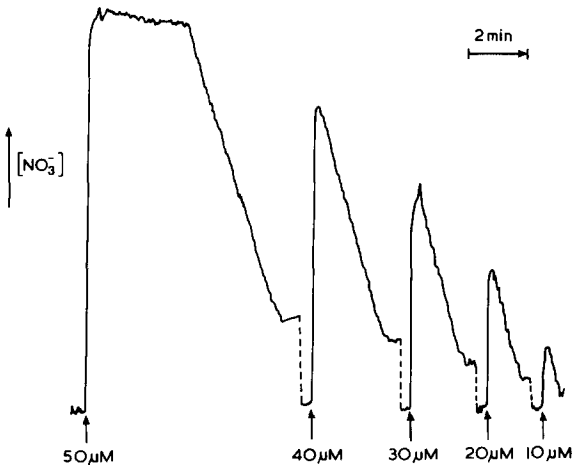

Fig. 1. Linear rates of reduction of low concentrations of nitrate by cells of *P. denitrificans*. The reaction chamber was fitted with nitrate and reference electrodes and contained at 30°C 5 ml 100 mM Na^+-Hepes (pH 7.5)/10 mM sodium succinate and cells, 9.3 mg dry weight. Nitrate was added as shown. Nitrogen was continuously blown over the surface of the reaction mixture. The lag before commencement of reduction of the initial 50 μM addition of nitrate corresonds to the time required for reduction of residual dissolved oxygen.

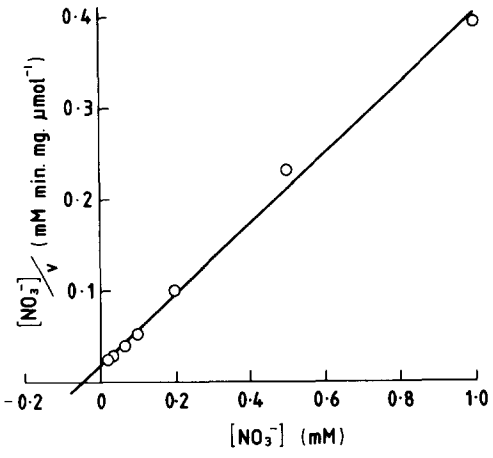

Fig. 2. Determination of apparent K_m for nitrate reduction by inverted membrane vesicles from *P. denitrificans*. The initial rate of nitrate reduction was determined from the rate of oxidation of NADH as described in Materials and Methods. The reaction medium was 3 ml 20 mM Tris-acetate (pH 7.3)/0.25 mM NADH/45 μg vesicle protein supplemented with 3 μg gramicidin D + 30 mM ammonium acetate to dissipate any protonmotive force and thus ensure that maximal and uncontrolled rates of electron transfer were observed.

K_m for nitrate reduction by intact cells is less than 5 μM.

The very low value found for the K_m (NO_3^-) in intact cells might be indicative of a high-affinity transport system for nitrate. Alternatively, the higher values for K_m obtained in studies with preparations of nitrate reductases might have arisen from the use of non-physiological reductants and/or purified preparations of nitrate reductase, in which the catalytic site might have been perturbed. Consequently, we attempted to determine K_m (NO_3^-) for nitrate reductase in inverted membrane vesicles using NADH as a physiological reductant. Unfortunately this approach, although physiologically relevant, will give the true value for K_m only if nitrate reductase is clearly the rate-limiting step in the sequence [18] NADH → ubiquinone → nitrate reductase. This condition may not be satisfied. The rate of nitrate reduction by membrane vesicles with nitrate as substrate followed approximate Michaelis-Menten kinetics (Fig. 2) with a value for K_m of 50 μM, although the value of K_m varied between preparations. It fell in the range 40 μM to 75 μM. As it was not possible to determine whether nitrate reductase was the rate-limiting step these values should be regarded as lower limits.

Characteristics of inhibition by azide of nitrate reduction by inverted membrane vesicles

Azide has been reported to be a competitive inhibitor of nitrate reductases [2,3,17]. When the kinetics of nitrate reduction by membrane vesicles were studied in the presence of azide, competitive inhibition was observed (Fig. 3) with $K_i(N_3^-)$ of 0.8 μM, close to values that have been reported for the purified enzyme [2,3,17]. Analysis of the kinetics of the reductase measured in the presence of azide showed that the K_m for nitrate was rather higher than in its absence. A value of 0.64 mM was obtained for the experiment shown in Fig. 3. These results suggest that in the presence of the azide concentrations tested, nitrate reductase became rate limiting, and hence K_m approached its true value. Nitrate concentrations of 2 and 10 mM were chosen for the experiments shown in Fig. 3, so that the anticipated 'true' K_m for nitrate was always exceeded. In this way complications arising from a shift in apprent K_m (NO_3^-) as the presence of azide made the nitrate reductase more rate limiting were anticipated to be minimised.

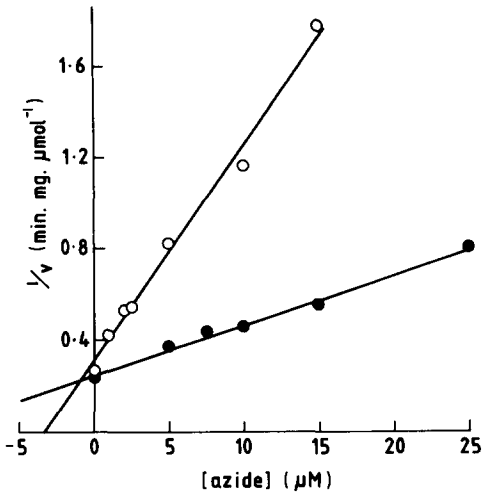

Fig. 3. Competitive inhibition of nitrate reduction by azide in *P. denitrificans* vesicles. Conditions were as described in the legend to Fig. 2, except that 0.122 mg vesicle protein was used. The two concentrations of nitrate used were 2 mM (○) and 10 mM (●).

Swelling of spheroplasts as an indicator of ion movement across the plasma membrane of P. denitrificans

Several investigators have used the rate of swelling of spheroplasts as a probe for investigating possible mechanisms of nitrate and nitrite transport in both *E. coli* [8] and *P. denitrificans* [10]. In none of these works were measures taken to test either whether the spheroplast suspensions were

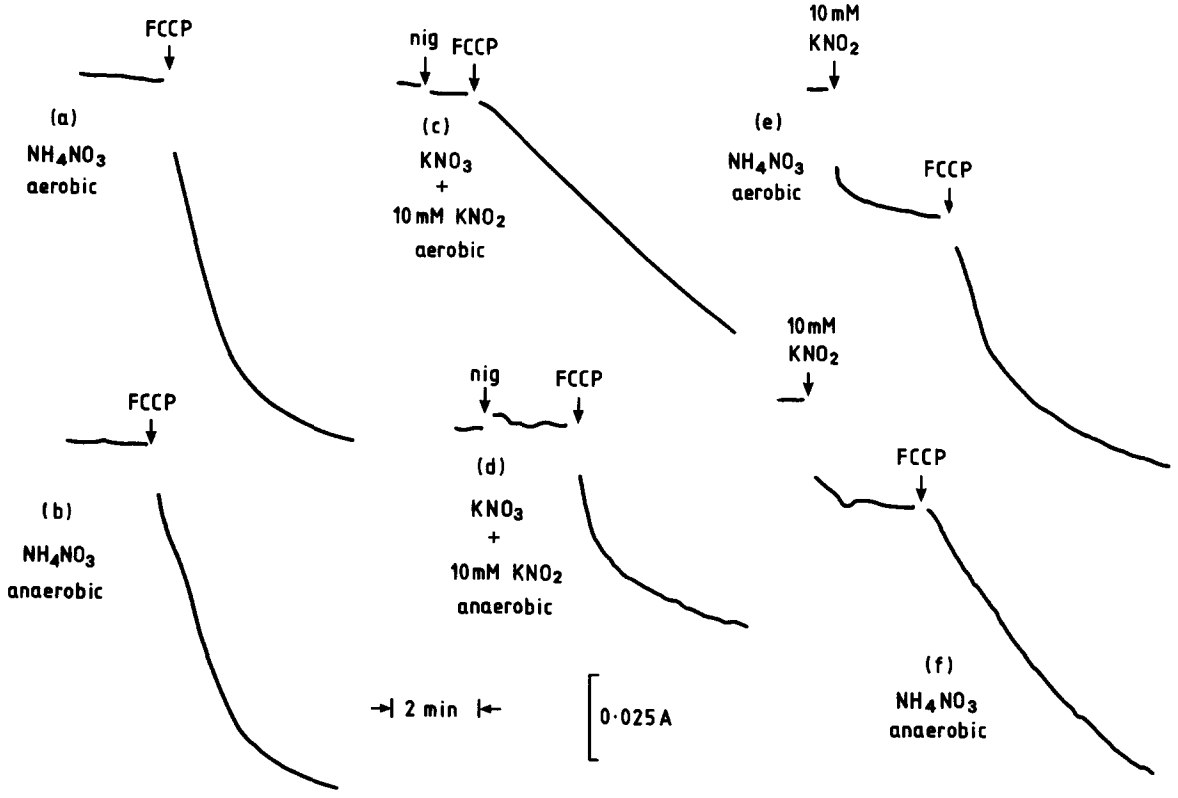

Fig. 4. Osmotic swelling of *P. denitrificans* spheroplasts in isotonic nitrate salts. For aerobic experiments, 10 μl of spheroplast suspension was added to 2.5 ml of air-saturated, isotonic solution of the indicated salt, incubated at 30°C. For anaerobic experiments, 10 μl of spheroplast suspension was added to 2.5 ml of N_2-saturated isotonic solution containing 10 mM sodium succinate and incubated for 30 min at 30°C to ensure anaerobiosis. The initial absorbance was approx. 0.4. The anaerobic medium was gassed with N_2. In addition to the isotonic solution of the relevant salt, the reaction medium contained 10 mM Hepes/NaOH (pH 7.3). 0.1 mM azide was included in all experiments to prevent any nitrate reduction – verified in a parallel experiment. If nitrite was included in the experiments (c, d, e, f), antimycin A (5 μg) was added to prevent any reduction of nitrite. FCCP (2 μM) and nigericin (2.5 μg) were added as ethanolic solution.

anaerobic or whether nitrate or nitrite reduction was occurring. Control of these aspects might be important because some experimental evidence [7,11] suggests that the movement of nitrate across the *P. denitrificans* plasma membrane could be inhibited when pathways of electron flow to oxygen are available. Rapid reduction of internal nitrate followed by exit of nitrite could also prevent swelling in isotonic nitrate solutions. Consequently, we have reinvestigated the swelling of *P. denitrificans* spheroplasts under conditions of either aerobiosis, or anaerobiosis in the presence of azide to inhibit nitrate reductase.

If nitrate were to move across the membrane in symport with a proton, as has been proposed by other workers [19,20], swelling of a suspension of spheroplasts in ammonium nitrate is expected. Fig. 4 shows that under either aerobic or anaerobic conditions in the presence of 100 μM azide, which was shown to inhibit very effectively nitrate reductase activity under these conditions, no swelling of spheroplasts suspended in ammonium nitrate was observed until proton permeability was enhanced by addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). Under these conditions nitrate was entering the cell either by passive diffusion or uniport, but the rate of such nitrate movement was similar in aerobic and anaerobic conditions, as judged by the kinetics of swelling.

A nitrate-nitrite antiporter is a possible candidate for the route for supplying nitrate to the cytoplasm and transferring the nitrite to its site of reduction in the periplasmic space [7,9]. The operation of such a system would cause swelling of spheroplasts in the presence of KNO_3, KNO_2 and nigericin as a result of nitrous acid influx, nitrite exit via the putative antiporter in exchange for nitrate entry, and import of external K^+ together with export of internal H^+ by the action of nigericin, giving in combination net influx of potassium nitrate [8]. However, Fig. 4 shows that under neither aerobic nor anaerobic conditions in the presence of azide was such swelling observed. Only when a protonophore (FCCP) was added did swelling occur owing to the effective influx of potassium nitrate through the combined effects of potassium entry in exchange for proton exit via nigericin, proton entry via FCCP and nitrate entry via a uniport mechanism. The slower rate of swelling following addition of FCCP under aerobic conditions is not considered significant as the effect was not reproducible, and also the rate of nitrate influx via uniport or passive diffusion was not accelerated by anaerobiosis under other conditions (records a, b, e and f in Fig. 4).

The strategy shown in records c and d of Fig. 4 for seeking to detect a nitrate-nitrite antiport might fail if the concentration of K^+ within the spheroplasts were high. Thus a second approach is useful for testing whether a nitrate-nitrite antiporter is operational. When spheroplasts are suspended in isotonic ammonium nitrate the addition of a low concentration of potassium nitrite will induce swelling if entry of nitrate in exchange for nitrite via the putative antiporter is followed by influx of ammonia to give net influx of ammonium nitrate. Fig. 4 (records e and f) shows that only limited swelling was observed following addition of potassium nitrite under anaerobic or aerobic conditions and that FCCP, just as in the experiments shown in records a and b, was required for complete swelling. A similar, although less extensive, effect of potassium nitrite was also observed in analogous experiments with *E. coli* spheroplasts by Garland et al. [8]. This effect of potassium nitrite is attributed to the movement of ammonia plus nitrous acid across the spheroplast membrane because an identical drop in apparent absorbance was observed if potassium acetate, but not chloride or sulphate salts, was added in place of nitrite. Ammonia plus acetic acid movement across membranes by passive diffusion is well established (e.g. Ref. 8) (and see the next subsection). It is concluded that the experiments shown in records e and f in Fig. 4 provide no evidence for a nitrate-nitrite antiporter, and thus reinforce the same conclusion drawn from the experiments shown in records c and d of the same figure.

Rates of decay of potassium diffusion potentials as indicators of possible mechanisms of nitrite and nitrate movement across the plasma membrane of P. denitrificans

When a K^+-diffusion potential is generated between the lumen of membrane vesicles and the external aqueous phase, the fluorescence of the probe ANS is enhanced with the extent of the

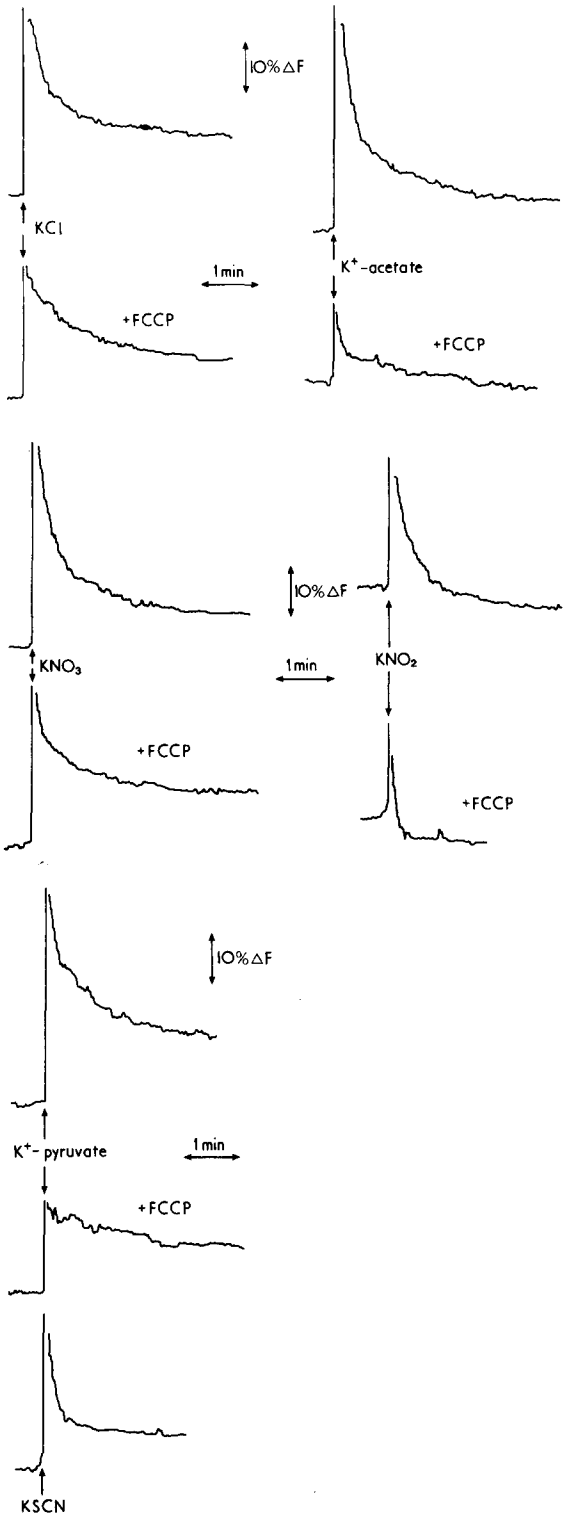

Fig. 5. The decay of K^+-diffusion potentials induced in *P. denitrificans* membrane vesicles. Measurements were carried out at 30°C, in 3 ml of reaction medium comprising 20 mM Tris-HCl (pH 7.3), *P. denitrificans* vesicles (0.49 mg protein) and 0.5 μg valinomycin. ANS (5 μM) was added to the cuvette and after a steady level of fluorescence was established, the potassium salt (5 mM K^+) was added. Where indicated, 1 μM FCCP was present. ΔF is the relative change in fluorescence upon adding a K^+ salt.

enhancement increasing linearly with log [K^+] added [21,22]. The maximum observed enhancement decreases and its decay rate increases as the permeability of the added anion increases. Fig. 5 shows the enhancements of ANS fluorescence that were observed when a number of different K^+ salts were added to a suspension of *P. denitrificans* vesicles that had been treated with valinomycin. From these data SCN^- is clearly the most permeable anion while pyruvate and chloride appear least permeable. Diffusion potentials with K_2SO_4 (not shown) were larger and decayed more slowly than those with KCl. Sulphate, therefore, is taken to be the least permeable of the anions tested. Nitrate, nitrite and acetate evidently have an intermediate permeability.

A K^+ diffusion potential can be dissipated by both the movement of anions and the counter movement of other cations. In the presence of a protonophore K^+ diffusion potentials can in principle be rapidly dissipated by efflux of protons from vesicles. In practice such efflux is normally limited by the consequent rise in the internal pH and therefore of the transmembrane pH gradient. When, however, a permeable weak acid is present, the supply of internal protons can be replenished by inward diffusion of the uncharged protonated acid. Thus K^+ diffusion potentials are dissipated in both submitochondrial particles [23] and in *P. denitrificans* vesicles (Fig. 5) when potassium acetate is the added salt in the presence of FCCP as protonophore. An analogous effect of potassium acetate, valinomycin and FCCP upon the swelling of mitochondria and synthetic phospholipid vesicles has been reported [24].

The collapse of K^+ diffusion potential in the presence of a protonophore can therefore be used as a test for the permeability of weak acids. According to this criterion, HNO_2 must rapidly cross the vesicle membrane, but the rate of movement of pyruvic acid must be significantly slower, because the diffusion potential following addition

of KNO_2 is rapidly dissipated by FCCP but with potassium pyruvate the effect of FCCP is less. This result is of interest because pK_a HNO_2 is 3.4 and that of pyruvic acid 2.5. Thus two factors can contribute to the relatively slower decay of the diffusion potential in the presence of K^+ pyruvate: the 10-fold lower steady-state concentration of pyruvic acid compared with HNO_2 and a possible higher intrinsic permeability of the latter compound compared with the former.

FCCP only partially dissipated the K^+-diffusion potential generated by KCl or KNO_3 addition (Fig. 5). This is understandable, since the steady-state concentrations of HNO_3 or HCl would have been too small to contribute to the supply of internal protons. This result also suggests that an active proton-nitrate symport system is not operating in the vesicles. Such a system would cause dissipation of the K^+ diffusion potential in the presence of a protonophore. The vesicles used in the experiments of Fig. 5 are known [25] to be a mixture of right-side-out and inside-out vesicles, and thus a proton-nitrate symporter involved in the import of nitrate into cells might only be detected in the right-side out fraction unless the putative transport system is symmetrical. Nevertheless, even taking this factor into account does not alter the conclusion that the data shown in Fig. 5 provide no evidence for a proton-nitrate symporter in the vesicle membranes.

Comparison of the effects of chlorate and nitrate on electron flow to oxygen in Triton-treated cells

The nitrate reductase of cells of *P. denitrificans* will reduce chlorate only after the plasma membrane has been sufficiently disrupted by low concentrations of detergents, including Triton X-100, to permit chlorate access to the active site of the enzyme at the cytoplasmic surface of the membrane [1]. When chlorate is added to Triton-treated cells under aerobic conditions electron transport is inhibited by approx. 50% owing to competition for

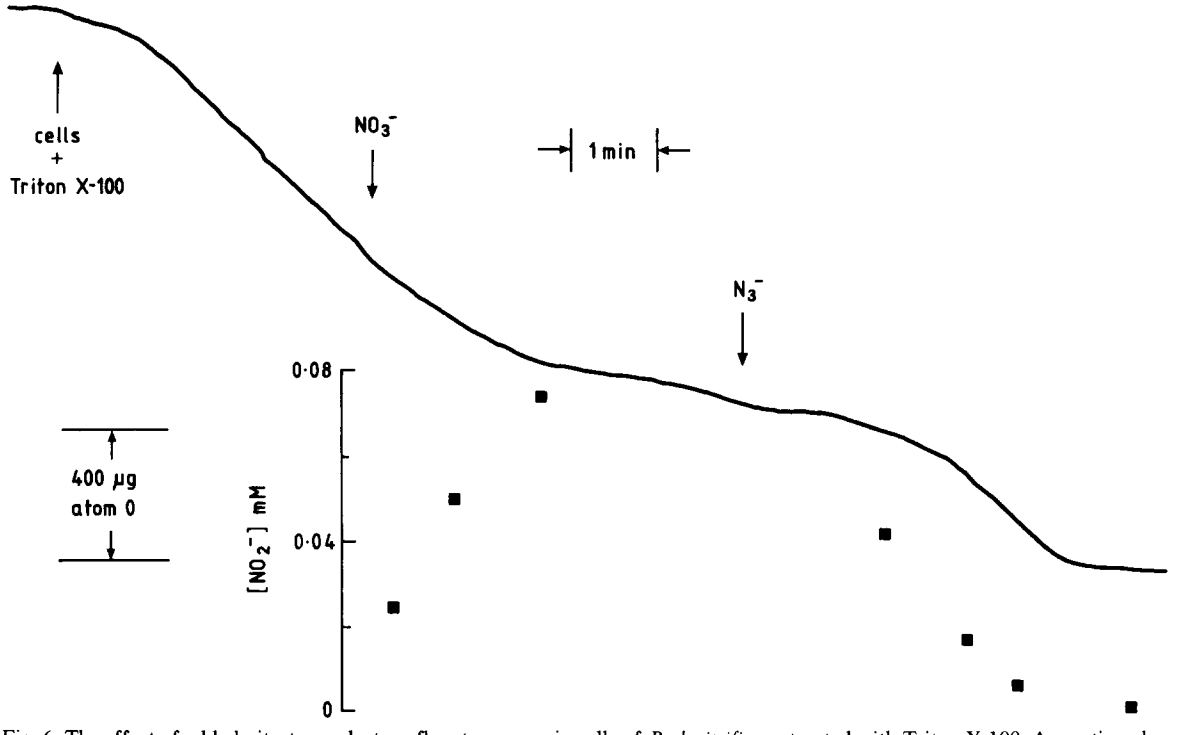

Fig. 6. The effect of added nitrate on electron flow to oxygen in cells of *P. denitrificans* treated with Triton X-100. A reaction chamber fitted with a Clark-type oxygen electrode was completely filled (2 ml) with 0.1 M Hepes-NaOH (pH 7.3), 10 mM sodium succinate containing cells, 5.9 mg dry wt, plus 0.03% (v/v) Triton X-100. 1 mM KNO_3 and 0.1 mM NaN_3 were added as shown. Samples were withdrawn and assayed for nitrite at the indicated times. The temperature was 30°C.

electrons from the nitrate reductase pathway [11]. Addition of 0.1 mM azide either before or after introduction of chlorate prevents inhibition of electron flow to oxygen as a consequence of its strong inhibitory effect upon chlorate reduction by nitrate reductase [11]. When the physiological substrate, nitrate, was added to Triton-treated cells, a more complex pattern of behavior was observed. The extent of inhibition of electron flow to oxygen progressively increased beyond 50% and the addition of azide did not immediately restore the full rate of electron flow to oxygen (Fig. 6) in contrast to when chlorate was present (see Fig. 8 in Ref. 11). However, the rate of oxygen reduction was restored to close to its original value several minutes after the addition of azide (Fig. 6). Addition of azide before nitrate prevented the inhibition of the rate of oxygen reduction.

Inhibition of electron flow to oxygen by added nitrite in cells treated with Triton X-100 or FCCP

The increasing inhibition of electron flow to oxygen following addition of nitrate to Triton-treated cells suggested that the product of nitrate reduction, nitrite, might be influencing the rate of electron transport to oxygen, as foreseen previously [11]. That the presence of nitrite was indeed causing inhibition of electron transport to oxygen was shown in two ways. First, samples were taken from the chamber of the oxygen electrode for analysis of nitrite. The restoration of the rate of oxygen reduction clearly corresponded to the disappearance of nitrite from the reaction medium (Fig. 6). Second, the effect on Triton-treated cells of adding similar concentrations of nitrite to those encountered during the inhibited phase of oxygen reduction in Fig. 6 was tested. It was found that addition of, for example, 0.2 mM nitrite caused an immediate and almost complete inhibition in the rate of oxygen reduction by Triton-treated cells in the presence of succinate. The inhibition was reversed upon disappearance of detectable nitrite from the medium.

These findings with Triton-treated cells contrast with the effects of comparable concentrations of nitrite upon untreated cells, since previous work has shown that concentrations of nitrite as high as 10 mM only inhibit electron transport to oxygen by approx. 20% [5]. Intact cells do not usually reduce nitrite under aerobic conditions with physiological substrates [11] in contrast to Triton-treated cells (Fig. 6) (but see Ref. 13). A possible interpretation of the effect of Triton X-100 was that it permitted nitrite access to the cytoplasmic surface of the plasma membrane and thereby exerted its inhibitory effect on the reduction of oxygen, c.f. Ref. 13. Elsewhere in this paper it is argued that HNO_2 is sufficiently permeable that the nitrite concentration in the cytoplasm could in principle reach the same level as the external concentration. Thus consideration was given to the possibility that the cytoplasmic concentration of nitrite was held at a low value owing to a membrane potential-dependent extrusion of the nitrite anion from the cells. To test this proposal the consequences of dissipating the membrane potential through the addition of the uncoupler FCCP were tested. Fig. 7 shows that in the presence of FCCP nitrite was a potent inhibitor of electron transport to oxygen by cells supplied with succinate as added substrate. Just as when Triton X-100 was added, the inhibitory effect of nitrite persisted until its concentration had fallen below the limit of detectability.

Further experiments were performed to identify the site(s) at which added nitrite exerted its inhibitory effect. Triton-treated cells do not respire in the absence of added reductants for the respiratory chain [11]. Hence, when ascorbate plus *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) is added to Triton-treated cells the sole operational pathway of electron flow should be from cytochrome *c* to terminal oxidases because cytochrome *c*-deficient mutants cannot oxidise ascorbate plus TMPD [26]. Figs. 8 and 9 show that a low concentration of nitrite strongly inhibited the reduction of oxygen by either Triton-treated cells or cells in the presence of FCCP when ascorbate plus TMPD was the substrate. In each case the inhibition was reversed upon the disappearance of nitrite from the medium.

As experiments with Triton-treated and uncoupled cells indicated that the inhibitory site of action of nitrite could be on the cytoplasmic surface of the plasma membrane, the effect of nitrite on electron transport reactions of inside-out membrane vesicles was tested. It was found that with NADH as substrate, concentrations of nitrite in the millimolar range were required for significant

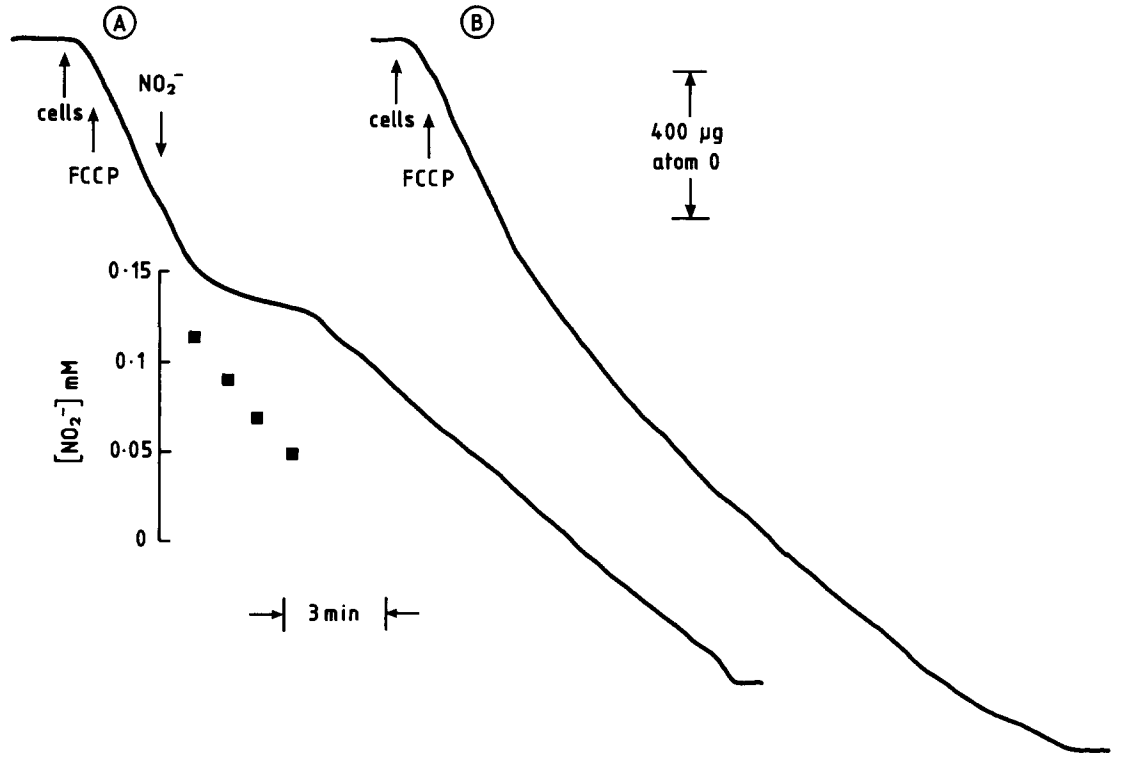

Fig. 7. Inhibition of electron flow to oxygen in cells of *P. denitrificans* following addition of nitrite in the presence of FCCP. A reaction chamber fitted with an oxygen electrode contained 2 ml 100 mM Hepes/NaOH (pH 7.3)/10 mM sodium succinate at 30°C. Cells, 2.7 mg dry wt, 4 µM FCCP and 0.15 mM nitrite were added as shown. Record B shows the non-linear rate of oxygen consumption in the absence of added nitrite as a basis for comparison with the experiment in which nitrite was added (record A).

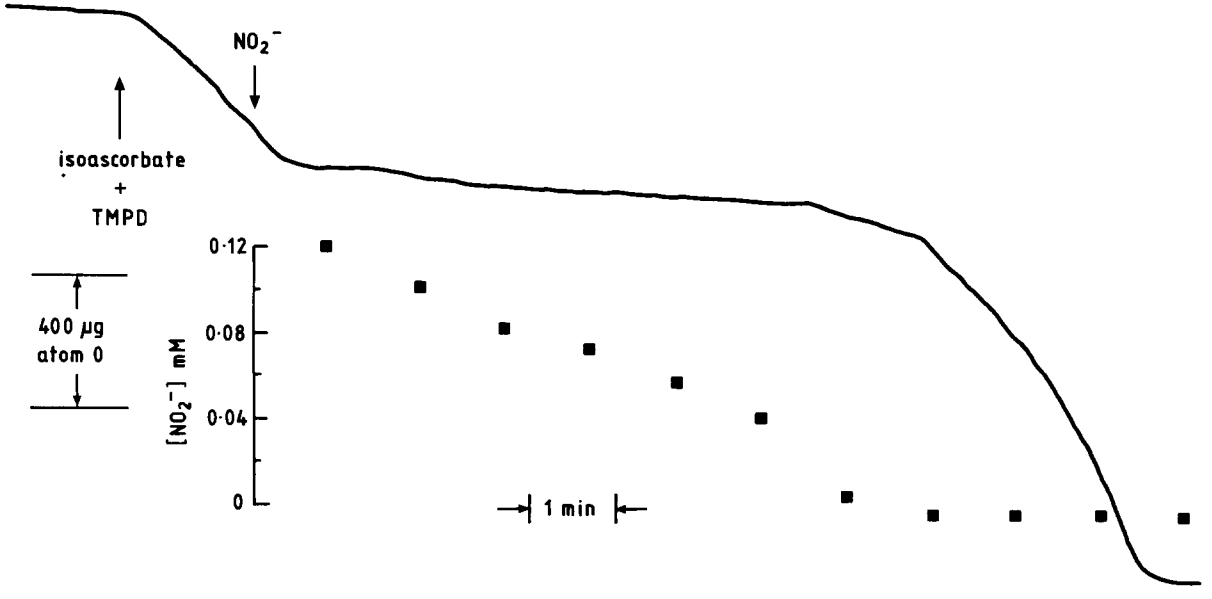

Fig. 8. Inhibition by added nitrite of aerobic oxidation of ascorbate plus TMPD by cells of *P. denitrificians* in the presence of Triton X-100. A reaction chamber fitted with an oxygen electrode was completely filled with 2 ml of 100 mM Hepes/NaOH (pH 7.3) 0.03% (v/v) Triton X-100 and 4.7 mg dry wt of cells at 30°C. 10 mM sodium isoascorbate plus 0.1 mM TMPD and 0.2 mM KNO_2 were added as shown. Samples were withdrawn and assayed for nitrite at the times indicated.

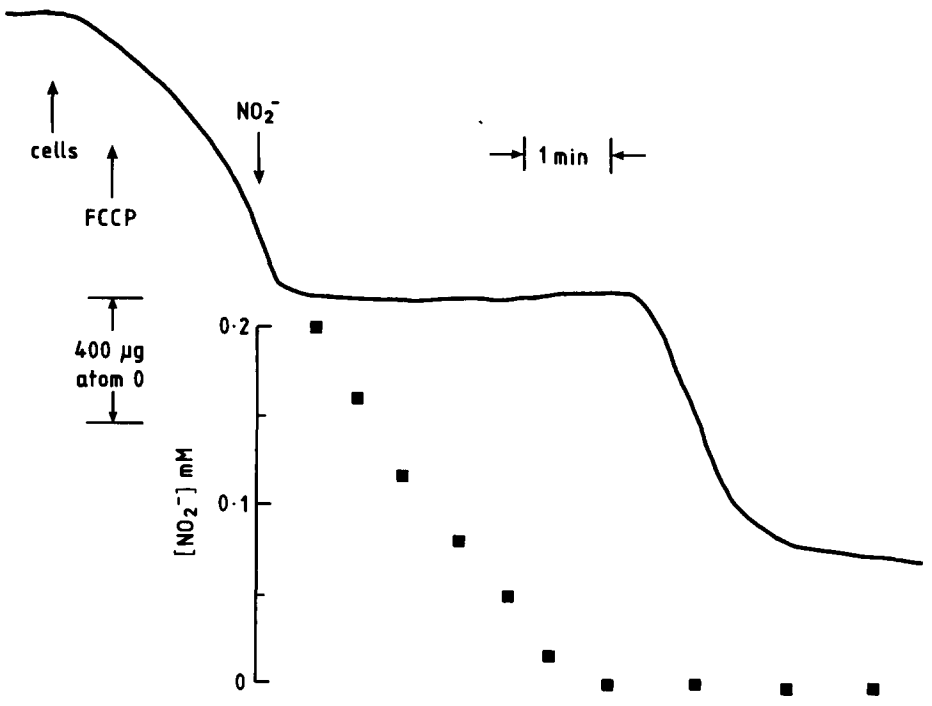

Fig. 9. Inhibition by added nitrite of aerobic oxidation of ascorbate plus TMPD by cells of *P. denitrificans* in the presence of FCCP. A reaction chamber fitted with an oxygen electron was completely filled with 2 ml of 100 mM Hepes/NaOH (pH 7.3) and 10 mM sodium isoascorbate plus 0.1 mM TMPD at 30°C. Cells, 1.3 mg dry weight, 4 µM FCCP and 0.2 mM KNO_2 were added as shown. Samples were withdrawn and assayed for nitrite at the times indicated.

inhibition of electron transport to oxygen, irrespective of whether or not gramicidin plus ammonium acetate was added as uncoupler [27]. Similar results were obtained with succinate or ascorbate plus TMPD as substrate. These findings were inconsistent with the interpretation that nitrite was a potent inhibitor of electron flow to oxygen only in FCCP- or Triton-treated cells owing to its higher concentration at the cytoplasmic surface of the membrane under these conditions. This surface is in direct contact with added nitrite in the vesicles that have the inside-out configuration. These form approx. 40% of the total vesicle population and are solely responsible for oxidising NADH and probably also succinate [25]. Thus if nitrite itself was responsible for the inhibitory effects observed with intact cells (Figs. 6–9), electron flow to oxygen catalysed by inverted vesicles should have been severely inhibited at concentrations of nitrite indicated in Figs. 6–9.

The contrast between the inhibitory effects of low concentrations of added nitrite on intact cells in the presence of either Triton or FCCP, and the absence of comparable effects on inside out vesicles, prompted consideration of whether the inhibitory effect might be exerted not by nitrite, but rather by a product formed as a result of nitrite reduction. This view was supported by the observation that with cells grown aerobically the inhibitory effect on oxygen respiration of low concentrations of nitrite was absent, even in the presence of FCCP or Triton X-100. This result was valid for cells grown either to early or late logarithmic phase. In each case the cells lacked nitrite reductase activity. The cells harvested from late logarithmic cultures did possess nitrate reductase activity, and it was of interest to note that the effect of adding nitrate to such cells after treatment with Triton X-100 was indistinguishable from the effect of adding chlorate. This contrasts with the effects of nitrate and chlorate on anaerobically grown cells after treatment with Triton X-100 (compare Fig. 6 in this paper with Fig. 8 in Ref. 11). All these results indicated that it was the

catalytic activity of nitrite reductase that was responsible for generating a species that caused the potent inhibition of electron flow to oxygen. This species was freely diffusable because in mixtures of aerobically and anaerobically grown cells the effect of added nitrite in the presence of Triton was to inhibit at least substantially the respiration of the aerobically grown cells as well as that of the anaerobically grown cells.

Discussion

Interpretation of $K_m(NO_3^-)$ values for intact cells and inverted membrane vesicles

For purified or semi-purified preparations of dissimilatory nitrate reductases $K_m(NO_3^-)$ values of 0.3–0.55 mM (*P. denitrificans*) [2], 0.5–1.5 mM (*E. coli*) [3] and 0.3 mM (*Pseudomonas aeruginosa*) [17] have been reported. These values are strikingly higher than the apparent K_m for NO_3^- reduction by intact cells of *P. denitrificans* (Fig. 1). This difference might have several underlying causes. First the K_m value found in purified preparations of nitrate reductase might be misleadingly high owing to either the use of non-physiological substrates including benzyl viologen, as the reductant in the assay, or the perturbation of the active site of the enzyme structure upon purification. To test this possibility we measured apparent $K_m(NO_3^-)$ in inverted membrane vesicles using the NADH dehydrogenase of the respiratory chain as a physiological source of reductant for nitrate reductase. A difficulty is that unless the reductase is the rate-limiting enzyme in the sequence, this approach is liable to provide an underestimate for K_m. That the value of 50 μM obtained by this procedure was probably an underestimate was indicated by our finding that in the presence of azide, a well-characterised competitive inhibitor of purified nitrate reductases from a variety of organisms, including *P. denitrificans* ($K_i = 1$ μM [2]), inhibition by azide fitted to competitive kinetics with $K_i = 0.8$ μM and K_m (NO_3^-) = 0.64 mM. Under these conditions nitrate reductase is considered to have become close to rate-limiting, and thus the K_m value obtained is suggested to reflect more closely its real value.

Although a comparison of the apparent K_m values obtained for nitrate reduction by cells and inverted membrane vesicles has seemingly not been explicitly made hitherto, Garland et al. [8] did note that the maximum initial rate of H^+ translocation by the respiratory chain of *E. coli* was supported by only 20 μM nitrate whereas K_m (NO_3^-) for the partially purified reductase was much higher. It was also observed that 40 μM nitrate was sufficient to give the maximum rate of nitrate reduction, albeit as judged by an indirect method depending on a 'cycle time' of cytochrome *b* oxidation [8]. Such earlier observations on *E. coli* thus complement the more direct measurements reported here for *P. denitrificans*.

Accepting that the real K_m (NO_3^-) of membrane-bound nitrate reductase is of the order of at least 500 μM when the respiratory chain supplies the reductant, the question arises as to the explanation for the very low K_m (NO_3^-) observed in cells (Fig. 1). Two extreme explanations can be contemplated. The first is that in intact cells the nitrate reductase is so far from being rate limiting that the apparent K_m is two orders of magnitude lower than the real K_m. As nitrate reductase follows Michaelis-Menten kinetics, a simple calculation shows that this would require V_{max} to be approx. 100-times the observed catalytic rate. Experimental evidence against this explanation is that nitrate reduction by cells is accompanied by simultaneous reduction of nitrite and nitrous oxide, but that inhibition of the latter reactions with antimycin diverts only 25% of the inhibited electron flow to nitrate reductase even when the nitrate concentration is as high as 0.5 mM [11,15]. This observation shows that for intact cells there is not a large excess overall catalytic capacity for nitrate reduction [11,15]. Thus low K_m (NO_3^-) in cells probably has its basis in a catalytic process distinct from events at the catalytic site of the nitrate reductase enzyme. A candidate for this process is either a transport system for nitrate or a binding step, whereby nitrate can reach the active site of its reductase via a different route in cells than in purified enzyme or inverted membrane vesicles. For the latter a nitrate-specific pore as an integral part of the nitrate reductase complex might be suggested (c.f. Garland et al. [8]). The operation of such a pore rather than a distinct transport system might account for the failure of spheroplast swelling experiments to indicate mediated movement of

nitrate across the plasma membrane.

The low K_m (NO_3^-) observed with intact cells presumably reflects the requirement for cells to grow in the presence of low concentrations of nitrate in natural habitats. It also means that nitrate can be used in experiments analogous to the 'oxygen-pulse' method for measuring proton translocation linked to nitrate reduction in cells [8,19,20].

Possible transport mechanisms for nitrate and nitrite

The osmotic swelling technique has been used successfully to follow the transport of galactosides in the original application [29] of the method, and more recently, for example, electrogenic sodium ion-proton antiport in *Desulfovibrio vulgaris* [30]. The failure therefore to detect from the experiments shown in Fig. 5 the nitrate-nitrite antiporter or nitrate proton-symporter is puzzling because it stands contrary to the indirect evidence presented by Boogerd et al. [9] for the operation of such transport systems (but see discussion in previous section).

The results of the present work have eliminated two plausible reasons, inhibition of transport by electron flow to oxygen or anaerobic reduction of nitrate, for previous failures to observe swelling of spheroplasts consistent with the operation of either of these transport systems. The failure of the swelling experiments cannot be attributed to a loss of periplasmic binding proteins for nitrate because intact spheroplasts denuded of periplasmic proteins retain high rates of nitrate reduction [7]. Measurements of ANS fluorescence in response to potassium diffusion potentials also failed to detect the operation of a proton-nitrate symporter. These negative results caused consideration of whether the failure of intact cells to reduce chlorate might be a reflection of a lower intrinsic permeability of chlorate rather than evidence for a nitrate transport system that discriminates against chlorate. Evidence against this interpretation was that diffusion potentials generated by addition of potassium nitrate or chlorate to valinomycin-treated vesicles decayed with very similar kinetics, implying that the permeabilities of the two anions are similar. Work is required in the direction of identification of polypeptides that participate in one or more transport systems associated with respiratory nitrate reduction in *P. denitrificans*. A point to keep in mind is that the putative nitrate-nitrite antiporter could be bypassed by movement of nitrous acid.

The experiments with ANS also showed that even at pH 7 the movement of HNO_2 across the bacterial plasma membrane is rapid enough to equalise within the measurement time nitrite concentrations on its two sides, whereas the movement of pyruvic acid equalised the pyruvate concentrations more slowly. The comparison of the permeabilities of pyruvic acid and HNO_2 is of interest because it relates to the question of whether bacteria require transport systems for nitrite, which in turn is reminiscent of the old question of whether pyruvate entered mitochondria by passive diffusion of the protonated acid form. It is now known that the low concentration of pyruvic acid present at pH 7 cannot sustain a sufficiently rapid flux of pyruvate into the mitochondrion, and that facilitated transport of the pyruvate anion occurs [31]. Nitrite transport by bacteria, at least for purposes of assimilation, may not pose such a problem because the required flux of this species into bacteria is likely to be very much less than that of pyruvate into mitochondria, and the rate of movement of HNO_2 across the *P. denitrificans* membrane is fast relative to pyruvate (Fig. 5). Thus, except perhaps under conditions of very high external pH, very low nitrite concentration, or very high flux as in the case of *Nitrobacter* which oxidises nitrite [32], a transport system for nitrite uptake into bacteria is probably not required.

Effects of the presence of nitrite on rates of electron flow to oxygen in cells treated with Triton or a protonophore

The observations reported here concerning the considerable enhancement of the inhibitory effect of nitrite on oxygen respiration by treatment of cells with Triton X-100 or FCCP parallel similar observations recently reported by Kucera et al. [12,13]. Their interpretation has been that in untreated cells nitrite distributes across the plasma membrane according to the Nernst equation, and thus an inhibitory concentration of nitrite is only found at the cytoplasmic surface after collapse of the membrane potential following treatment with FCCP or Triton X-100 [12,13]. Several features of

the present work are inconsistent with that interpretation. (1). Very low concentrations of added nitrite caused inhibition of oxygen respiration catalysed by Triton- or FCCP-treated cells. (2). The inhibitory effect of nitrite was absent from aerobically grown cells even in the presence of FCCP or Triton X-100. (3). Millimolar concentrations of nitrite were required to cause even 50% inhibition of oxygen respiration by inverted vesicles. (4). Experiments measuring the fluorescence of ANS indicated that flux of nitrous acid across the plasma membrane was rapid and therefore that a distribution of nitrite across the plasma membrane in accordance with the Nernst equation would not be expected. If nitrite anions were freely permeable then potassium nitrite would act as an uncoupler, contrary to observation [5]. The corollorary from these considerations is that observed potent inhibition by added nitrite must be caused by a species generated by the action of nitrite reductase on its substrate. The nature of any such species is conjectural at present although consideration can be given to NO and NO^- (nitroxyl anion), both of which have been considered as products or intermediates of nitrite reduction [33,34] and can inhibit electron transport by binding to heme centres. Two considerations argue against NO being the inhibitory species. First, it is not expected that the plasma membrane would present a significant permeability barrier to NO and thus the requirement for Triton X-100 or FCCP is not accounted for. Second, nitric oxide reacts relatively rapidly with oxygen, although not instantaneously [35], yet during the inhibited phase there was essentially no consumption of oxygen (Figs. 8, 9). If nitrite disappearance correlated with synthesis of NO (Figs. 6, 8, 9), a steady consumption of oxygen owing to the chemical reaction of NO with O_2 to give NO_2 would have been expected, unless NO was converted to nitrous oxide by an NO reductase activity that was functional in the presence of oxygen.

Garber et al. [33] have reported data that are consistent with HNO or NO^- being an intermediate in the conversion of nitrite to nitrous oxide by *P. denitrificans*. NO^- is believed to react with metal centres including hemes [36] to give nitrosyls. The requirement for Triton or FCCP could arise because NO^- is excluded from the cell by the negative internal membrane potential. For this explanation to be valid the reaction of NO^- with oxygen must be slow to account for the period of essentially zero oxygen consumption in Figs. 8 and 9. Furthermore, dimerisation and dehydration of the conjugate acid, HNO, to give N_2O [34], must not be too effective in removing NO^-. The rates of reactions that consume NO^-, or another inhibitory species, might vary from one cell preparation to another as we have on occasion observed less inhibition than shown in Figs. 8 9.

Whatever the molecular nature of the species responsible for the observed inhibition of oxygen respiration (Figs. 6, 8, 9) it must act on cytochrome aa_3 or another cytochrome that can accept electrons from cytochrome *c* which is required for oxidation of asorbate plus TMPD [26].

The effects, identified in the present work, of a product of nitrite reduction on oxygen reduction also help clarify the basis for the simultaneous reduction of oxygen and nitrate by cells of *P. denitrificans* that have been treated with Triton X-100. When nitrate is added to so-treated aerobically grown cells there is a diversion of electron flow from oxygen to nitrate reductase which can be reversed by adding azide to inhibit nitrate reductase. A similar result is obtained when chlorate is added to anaerobically grown cells in the presence of Triton X-100 [11]. When, however, nitrate rather than chlorate is added to the latter, a more complicated pattern ensues and oxygen respiration is increasingly also inhibited by a product formed by further reduction of the nitrite generated by nitrate reductase (Fig. 6). The important point is that rendering the plasma membrane permeable to small ions by several treatments [7,11,12] is sufficient to allow considerable activity of nitrate reductase under aerobic conditions in which it is generally not functional [1,7,11]

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