Biochimica et Biophysica Acta 894 (1987) 120–126
Elsevier

BBA 42637

Aerobic dissimilatory reduction of nitrite by cells of *Paracoccus denitrificans*: the role of nitric oxide

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(Received 28 April 1987)

Key words: Nitrite reduction; Nitric oxide generation; Nitrite transport; Oxidase inhibition; (*P. denitrificans*)

With anaerobically grown cells of *Paracoccus denitrificans* it was previously found (Kučera, I. and Dadák, V. (1983) Biochem. Biophys. Res. Commun. 117, 252–258) that, in the presence of an uncoupler, nitrite as terminal acceptor was preferred to oxygen, the consumption of which was simultaneously inhibited. In the present study it is shown that besides an increased inhibition of terminal oxidases brought about by NO_2^- anion another potent inhibitor originating in the course of nitrite reductase reaction affects the division of electron flow between oxygen and nitrite. The inhibitor, the creation of which is accompanied by the aerobic nitrite reduction, is formed, even in the absence of an uncoupler, only as a result of a slight inhibition of oxygen respiration exhibited by hydroxylamine addition. From the comparison of the inhibitory effect of the intermediate on aerobically grown cells and membrane vesicles derived from them, it was proved that at neutral pH this substance does not carry an electric charge. A complex absorbing at 563 nm was formed due to the interaction of the inhibitor (generated from nitrite in the presence of uncoupler) with ferricytochrome *c* from bovine heart. From these findings we were led to conclude that it was most probably nitric oxide (NO).

Introduction

At least two enzymes participate in the dissimilatory nitrite reduction in anaerobically grown cells of *Paracoccus denitrificans*, that is, nitrite reductase and nitrous oxide reductase, which are known to be situated in the periplasmic space of the bacterium [1–3]. The reduction of nitrite carried out by intact cells is stopped by oxygen, which is preferred as a terminal acceptor for respiration [4]. However, aerobic reduction of nitrite occurs with permeabilised cells and cells treated with an uncoupler [5,6] (cf. also Ref. 7). Under these conditions the electron flow to oxygen is strongly inhibited, due to the intervention at the terminal part of the respiratory chain [5,6]. In explaining the effect of the uncoupler we assumed that the drop in membrane potential affects the distribution of the nitrite anion between the cell and the medium and that in this way also the inhibition of the active sites of oxidases at the cytoplasmic aspect of the membrane is enhanced [5,6].

Recently, we have shown [8] that an increment

Abbreviations: TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; TPP^+, tetraphenylphosphonium; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CCM, Czechoslovak Collection of Microorganisms.

Symbols: I_{50}, the concentration of given inhibitor which allows an enzyme-catalyzed reaction to proceed at half of the maximum rate; Δp, protonmotive force; $\Delta\psi$, membrane potential; ΔpH, pH gradient across membrane.

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in the flux of redox equivalents to nitrite reductase gives rise to the formation of a potent oxidase inhibitor, probably nitric oxide (NO), which may play a role in switching the electron flow to nitrite [8,9]. Parsonage et al. [7] were led by their studies to a similar conclusion. However, in their opinion it is a permeant negatively charged anion of nitroxyl (NO^-) that exerts the inhibitory effect. Further progress in understanding the mechanism of the complex event which is evoked owing to the presence of an uncoupler may be attained through a definitive identification of the transiently formed inhibitor and by the decision of what the nature of the species is through which nitrite and the inhibitory intermediate cross the cytoplasmic membrane to arrive at the site of the inhibitory action. The present paper is concerned with distinguishing among possible alternatives.

Materials and Methods

Growth conditions. P. denitrificans NCIB 8944 obtained as CCM 982 was grown on succinate as the major carbon source. Crude membrane fraction was derived as described [10].

Measurement of terminal acceptor consumption. Rates of oxygen consumption by cells and vesicles were measured using a Clark-type oxygen electrode. In some experiments nitrite consumption was followed simultaneously in a way described earlier [11]. The rate of nitrate reduction by cells was measured from the increment of nitrite after 30 min incubation in the presence of 1 μg mucidin per mg dry weight of cells [12]; 0.1 M sodium phosphate was the reaction medium in all cases.

Monitoring the changes of membrane potential. The energization of the bacterial membrane was qualitatively followed by means of the fluorescence of 13-ethylberberine cation [13,14]. The electrode, sensitive to the tetraphenylphosphonium cation (TPP^+) was prepared as described by Kamo et al. [15]. From the decrease in the TPP^+ concentration in the medium found on the addition of cells the size of the membrane potential was calculated, the internal volume of 2.7 μl per mg dry weight of anaerobically grown cells determined earlier [16] was taken.

Spectroscopic measurements. The interaction between cytochrome *c* and NO was followed using difference spectroscopy by means of Cary 118 C spectrophotometer.

Chemicals. Mucidin was obtained from Dr. Musílek (Institute of Microbiology, ČSAV, Praha, Czechoslovakia); 13-ethylberberine chloride was a gift from Dr. Mikeš from our institute; nitric oxide (NO) was generated from sodium nitrite mixed with ferrous sulphate [17]. The other chemicals were purchased commercially.

Results

Interaction of nitrite with the respiratory chain and the effect of uncoupler

To understand the mechanism of overall processes proceeding in switching the electron flow between O_2 and NO_2^- [5,6], of importance is a clear distinction of effects connected with the action of nitrite on the components of the respiratory chain (e.g., the inhibition of terminal oxidases) from the effects linked to the course of the nitrite reductase reaction (mutual competition of terminal pathways to O_2 and NO_2^-, the formation of the intermediate which is inhibitory to the oxidases). This differentiation can be reached in two ways: (1) by using membrane vesicles derived from cells of *P. denitrificans* that do not contain periplasmic and relatively loosely bound nitrite reductase [2] and, (2) by studying *P. denitrificans* cells in the presence of a suitable bc_1 inhibitor, such as mucidin, which completely stops the flow of redox equivalents from the substrate to nitrite [18].

The established inhibitory action of nitrite on oxidase activities of the vesicles given in I_{50} values was 1.7 and 7.5 mM for NADH oxidase and succinate oxidase, respectively, that is, at an order of millimolar concentrations. A clearly higher sensitivity towards nitrite observed with NADH oxidase does not express the preferential junction of NADH dehydrogenase with a terminal oxidase more sensitive to nitrite, but, as shown recently [19], it is due to a higher influx of redox equivalents into the ubiquinone region of the respiratory chain. By decreasing the rate of NADH oxidase by rotenone to the level of the succinate oxidase, the I_{50} value increased in the expected way.

Intact cells of *P. denitrificans* in the saturating

inhibitory concentration of mucidin can reduce two terminal acceptors: oxygen and nitrate [18]. The respiratory nitrate reductase and the oxidase, which is evidently of the cytochrome *o* type, withdraw redox equivalents from the ubiquinone region of the respiratory chain [18,20]. For the aim of our study it was of importance to know whether membrane potential is generated in the presence of mucidin. This was found true with cells respiring succinate, on the one hand, by following the response of 13-ethylberberine as a fluorescent probe, and, on the other hand, by means of TPP^+ distribution measurements with the ion-selective electrode. Quantification of the measurements led to mean values which were only by about 10% lower in the presence of mucidin (−145 mV) than in its absence (−157 mV), and were sensitive to protonophore addition. The finding was employed for studying the effect of $\Delta\psi$ on the inhibition of the oxidase activity of the cells with nitrite, on condition that the parallel course of the nitrite reductase reaction does not interfere. However, before a conclusion on the effect of a drop in $\Delta\psi$ can be drawn, it should be proved whether the change in sensitivity to NO_2^- does not result from a stimulation of respiration due to the uncoupling. For this purpose another inhibitor of terminal oxidase, hydroxylamine, was used [21]. Unlike nitrite, which at pH 7.3 is found mostly in the form of anion (pK_a of HNO_2 is 3.4), hydroxylamine acts as an electroneutral molecule which is protonated only to a minor part to the ion NH_3OH^+ ($pK_a = 5.8$). The comparison of the change in the inhibitory efficiency of the two inhibitors is given in Table I. It is evident that on the drop in Δp the sensitivity of the oxidase activity to nitrite increases ten times. In contrast, to nitrite, the inhibitory action of hydroxylamine is not depenent on the presence of the uncoupler.

With *P. denitrificans* it has already been shown [22] that through the electron flow from succinate to nitrate membrane potential is created. In order to be able to compare at least qualitatively the permeability of the cytoplasmic membrane of *P. denitrificans* for NO_2^- with that for some typical permeant ion, the inhibition of nitrate reductase activity was examined with thiocyanate in an analogous way. In that case, the decline in I_{50} value observed on uncoupling was somewhat more pronounced than was found with nitrite (see Table I). The results of the effect of uncoupling, as summarized in Table I, show that the permeability of cytoplasmic membrane for the NO_2^- ion, and thus even the effect of $\Delta\psi$ on the distribution of nitrite between the cells and the medium cannot be neglected.

TABLE I

THE EFFECT OF UNCOUPLING ON THE INHIBITORY ACTION OF HYDROXYLAMINE, NITRITE AND THIOCYANATE

The reaction medium (2 ml) was 0.1 M sodium phosphate (pH 7.3) with 5 mM sodium succinate. For titrating the oxidase activity 1.5 mg dry weight of anaerobically grown cells and 5 μg mucidin were added. From the initial velocity of oxygen consumption the activity was estimated (100% equals 41.4 nmol O_2 and 46.8 nmol O_2/min per mg dry weight without and with the uncoupler, respectively). In the case of nitrate reductase the anaerobic reaction medium was incubated 30 min at 25 °C and the reaction stopped by the addition of 1 ml of saturated solution of uranyl acetate. 100% activity equals 1.5 and 0.9 μmol NO_2^-, respectively (per 30 min with 1 mg dry weight of cells). Values are I_{50} (mM).

Additions	Mucidin-insensitive oxidase		Nitrate reductase
	NH_2OH	NO_2^-	SCN^-
–	0.5	50	110
2.5 μM CCCP	0.5	5	5

Inhibition of terminal oxidases leads to the reduction of nitrite under aerobic conditions

In an earlier paper [21] it was mentioned that the inhibitory effect of oxygen on the reduction of nitrate by the cells of *P. denitrificans* can be removed by a slight inhibition of terminal oxidases with hydroxylamine. The advantage of using hydroxylamine as an oxidase inhibitor was that, in comparison with cyanide and other well-known oxidase inhibitors, hydroxylamine was not inhibitory to enzymes participating in nitrate respiration. In the medium where nitrogeneous terminal acceptors were lacking a 33 μM hydroxylamine brought about 15% inhibition of the oxidase activity of cells. Fig. 1 documents the fact that an analogous situation arises also in the case of nitrite consumption. In the absence of hydroxylamine, oxygen as the terminal acceptor was strictly preferred to nitrite, whose reduction could only take

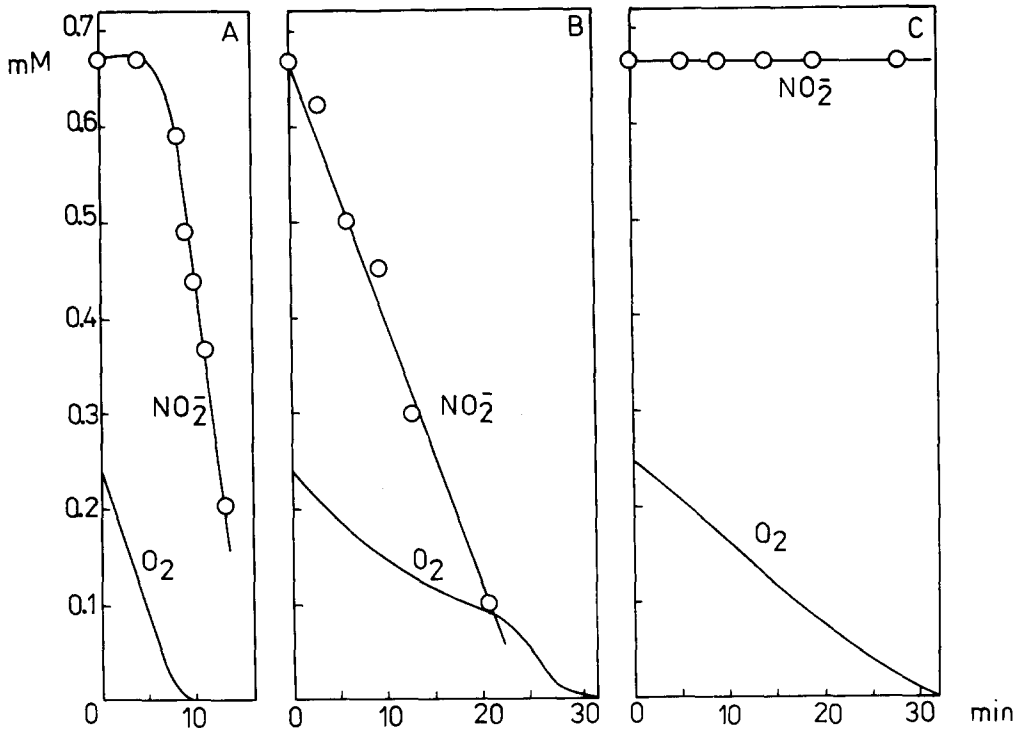

Fig. 1. Aerobic reduction of nitrite by cells of *P. denitrificans* in the presence of hydroxylamine. In the vessel of an oxygraph 3 ml of reaction mixture were held at 25 °C. The composition was: 0.1 M sodium phosphate (pH 7.3), 6.7 mM sodium succinate and 0.67 mM $NaNO_2$. After the addition of anaerobically grown cells (0.7 mg dry weight) consumption of oxygen and nitrite were measured in parallel (compare Materials and Methods). The additions were in experiment B 33 μM $NH_2OH \cdot HCl$ and in experiment C 33 μM $NH_2OH \cdot HCl$ plus 1 μg of mucidin.

place after anaerobiosis had been established (Fig. 1(A)). If the reaction medium contained hydroxylamine at a concentration of 33 μM, the consumption of the two terminal acceptors took place concomitantly (Fig. 1(B). Fig. 1(C) represents a control experiment which by demonstrating the inhibitory effect of mucidin on the aerobic reduction of NO_2^- excludes the possibility that the decrease in nitrite concentration depicted in Fig. 1(B) is due to the non-enzyme reaction of NO_2^- with hydroxylamine.

In another experiment, the concentration dependence of the inhibition of oxidase activity of cells by hydroxylamine was examined (not shown). It was obvious that in the case when the medium contained nitrite, which resulted in formation of the inhibitor of oxidase activity, the inhibition commenced already at lower hydroxylamine concentration (the value of I_{50} equalled 0.1 mM compared to 0.6 mM in the absence of nitrite). Thus, as it stands, the effect of hydroxylamine in this case is very similar to that observed in the presence of an uncoupler [5,6,9].

The inhibitor of oxidases arising in the nitrite reductase reaction is an electroneutral agent

Recent communications [7–9] have already shown that in the dissimilatory reduction of nitrite by the cells of *P. denitrificans* a hitherto unidentified nitrogen intermediate is transiently formed which strongly inhibits terminal oxidase of the respiratory chain. Insufficient stability and low concentration of the inhibitor make its exact chemical determination considerably difficult. The discrepancy concerning its character between electroneutral [8] and ionic [7] can, however, be easily tested experimentally. To resolve the conflicting evidence, we compared the effect of the generated inhibitor on the oxidase activity of aerobically grown cells (not containing nitrite reductase) or

membrane vesicles derived from these cells. Since in intact cells the inhibitor — diffusing to the active centre of oxidases — must overcome the transmembrane difference of electrical potential, it can be expected that only an electroneutral substance will have the same inhibitory potency with membrane vesicles which were mainly of inside-out orientation.

The results of the experiments are summarized in Fig. 2. We titrated with hydroxylamine the oxidase activity of anaerobically grown cells, the activity of testing systems (as explained before), and, finally, the activity of the testing systems in a mixture with anaerobically grown cells. Since the solution contained nitrite in all cases, hydroxylamine evoked the formation of the intermediate inhibiting the testing systems. In the upper part of the figures the degrees of inhibition of the two testing systems vs. hydroxylamine concentration are plotted. It is evident that both testing systems showed a similar sensitivity towards the inhibitor generated by anaerobically grown cells, which witnesses the fact that the molecules of the inhibitor diffusing across the cytoplasmic membrane do not carry an electric charge. Besides that, it is obvious from the zero inhibitory values in Fig. 2 that anaerobically grown cells did not generate the inhibitor of the oxidase activity in the presence of oxygen, unless hydroxylamine was present. Similar phenomena were observed on the addition of the uncoupler (2.5 μM CCCP) that is a strong inhibition of both testing systems (not shown).

Spectroscopic evidence of nitric oxide formation during aerobic nitrite reduction in the presence of uncoupler

It has been known for a long time that a relatively stable complex of nitric oxide and cytochrome *c* (Fe^{3+}) is formed which shows a characteristic absorption maximum at 563 nm [17]. The interaction of both compounds can be conveniently used to an otherwise very difficult ex-

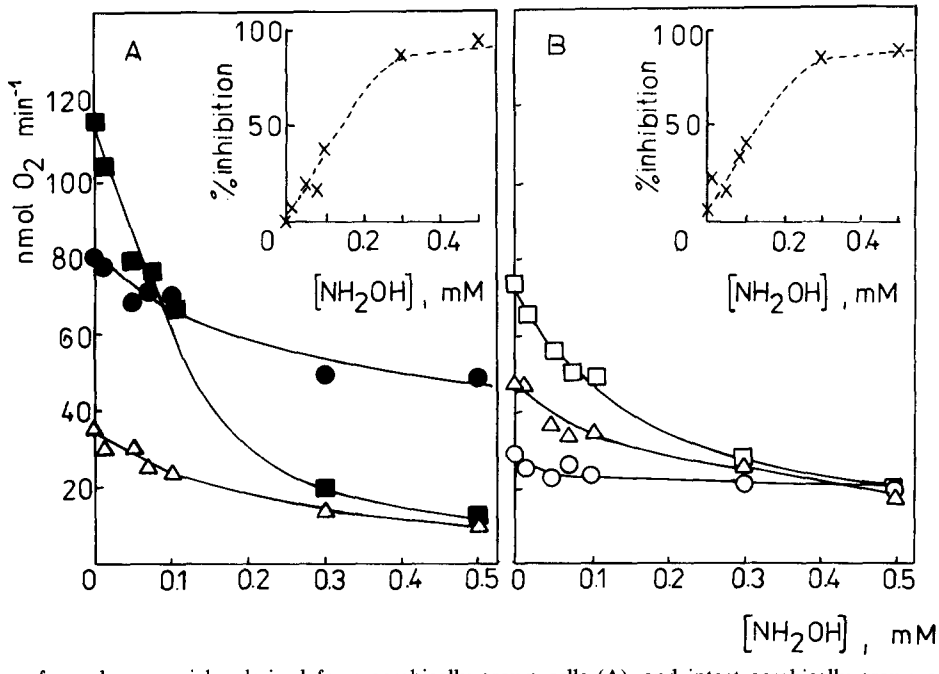

Fig. 2. Inhibtion of membrane vesicles derived from aerobically grown cells (A), and intact aerobically grown cells (B), by the intermediate generated in nitrite reductase reaction. The reaction mixture (2 ml) consisted of 0.1 M sodium phosphate (pH 7.3) containing 5 mM sodium succinate and 0.5 mM $NaNO_2$. In A, 0.6 mg dry weight of anaerobically grown cells (Δ), 1.1 mg protein of vesicles derived from aerobically grown cells ●), or the mixture of both experimental subjects (■) were used. In B, vesicles were substituted by 1.4 mg dry weight of aerobically grown cells (○), or by a mixture of aerobically and anaerobically grown cells [□]. The percentage of inhibition of aerobic cells and vesicles is shown in the insets.

perimental proof of a free NO production in bacterial denitrification. Fig. 3 shows an experiment registering the difference spectra of spectrophotometric cuvettes which were filled with a dilute suspension of anaerobically grown cells of *P. denitrificans* with cytochrome *c* (Fe^{3+}) from bovine heart and nitrite. To the sample cuvette the uncoupler was added. In an independent experiment using a Clark electrode it was proved that oxygen in the cuvettes was not exhausted during registering the spectra. From Fig. 3 it can be seen that aerobic denitrification of NO_2^- in the sample cuvette brought about by the presence of an uncoupler is accompanied by the formation of a substance characterized by a distinct absorption maximum at 563 nm. Due to the fact that the same effect can be achieved by introducing gaseous NO (not shown), this indicates the existence of free nitric oxide in the reaction mixture. After the nitrite was exhausted — in a period lasting about 25 min (not shown) — the cytochrome *c* (Fe^{3+}) NO complex decomposed slowly to cytochrome *c* (Fe^{2+}) showing the absorbance maximum at 550 nm, in accordance with the expected course described in the literature [17].

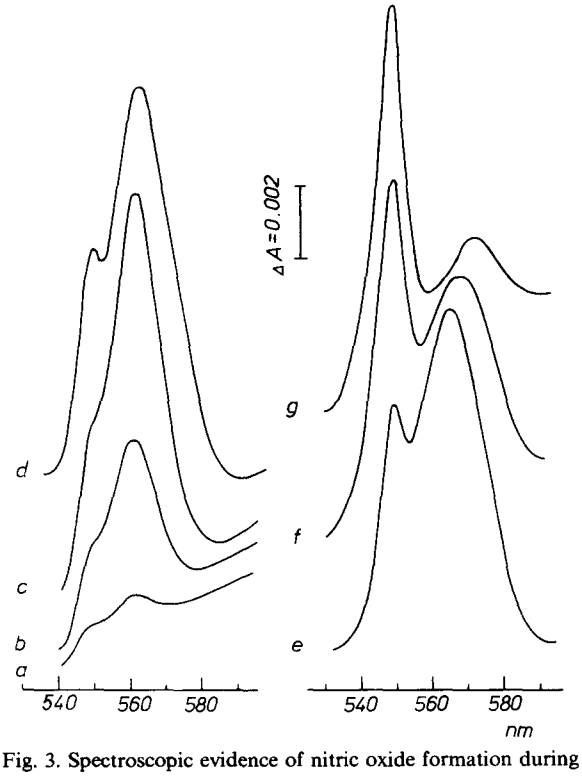

Fig. 3. Spectroscopic evidence of nitric oxide formation during aerobic reduction of nitrite. Contents of the cuvettes were: 3 ml of 0.1 M sodium phosphate (pH 7.3) (25 °C) with the addition of 50 μM ferricytochrome *c* from bovine heart and 0.3 mM sodium nitrite; in the sample cuvette was 3.3 μM CCCP. At zero time 0.6 mg dry weight of anaerobically grown cells was added. The spectra were scanned at a speed of 0.5 nm/s in the following intervals (min): 1 (a), 4 (b), 7 (c), 16 (d), 28 (e), 34 (f), 43 (g).

Discussion

In clarifying the mechanism of aerobic dissimilatory nitrite reduction the question arises of which step is dependent on the decay of membrane potential of the cell. In our previous work [5,6,9], we considered it to be the penetration of the negatively charged NO_2^- anion to the active sites of oxidases situated on the inner aspect of the cytoplasmic membrne. According to this idea, the drop in membrane potential should resulte in increased inhibition of the oxidase by nitrite, in the appearance of an oxidase inhibitor (NO) created in the nitrite reductase reaction [8], and, finally in a complete redistribution of the electron flow from O_2 to NO_2^-. On the other hand, Parsonage et al. [7] assumed that the collapse of the protonmotive force did not practically affect the transmembrane distribution of NO_2^- and, consequently, it did not alter the value of I_{50} for the nitrite inhibition of the oxidase activity. In their opinion an intermediate originating in the nitrite reductase reaction is presumably an electronegatively charged nitroxyl anion (NO^-) which on the addition of an uncoupler crossed the cell membrnae, with subsequent inhibition of terminal oxidases. This suggestion is, however, inconsistent with our findings that the cells of *P. denitrificans* did not generate an inhibitor of terminal oxidases from NO_2^- as far as an uncoupler or hydroxylamine is not added (Figs. 1 and 2) and that the inhibitory species does not carry an electric charge at neutral pH (Fig. 2). Nitroxyl generated by the radiolysis of the acqueous solution of NO exhibited $pK_a = 4.7$ [23], and therefore it may be deduced that its distribution between the cell and its surroundings should be determined predominantly by the ΔpH component of the protonmotive force similarly as in the case of azide ($pK_a = 4.8$) [24]. It is not evident

whether nitroxyl, as an extremely unstable particle, can exist in free state during denitrification and diffuse in the solution from the site of its generation to the site of the assumed inhibitory intervention. For those reasons, and on the basis of the spectroscopic study (Fig. 3), we identify the intermediate inhibitor with electroneutral nitric oxide (NO). Recently we have shown [8] that NO inhibits strongly the activity of TMPD oxidase of the respiratory chain of *P. denitrificans*.

The other point about which conflicing evidence exists is the postulated role of NO_2^- in switching the electron flow from O_2 to NO_2^- According to Ref. 7, NO_2^- cannot evoke the switching, since in membrane vesicles it inhibits the oxidase activity at markedly higher concentrations than in intact cells treated with an uncoupler. In this objection, however, it was not considered that, unlike in membrane vesicles, which do not retain nitrite reductase, reduction of nitrite by the periplasmic enzyme can set in at the same time. The effect is quite obvious, even at the titration of the oxidase activity with hydroxylamine, if the mixture contains extra nitrite. As follows from the experiments with hydroxylamine (Fig. 1), the initial inhibition need not be high, and the effect of switching may operate even at an NO_2^- concentration considerably below the I_{50} value in membrane vesicles. In accordance with this mechanism is the observed decrease in I_{50} of nitrite for the inhibtion of oxidase activity of cells on uncoupling (Table I). The less pronounced difference in I_{50} values with nitrite in comparison with the case of thiocyanate can be due, on the one hand, to a higher occurrence of the protonated form in the solution of the former anion at the pH 7.3 (the pK_a of HNO_2 is 3.4 and that of HSCN is -1.8). On the other hand, the deviations from the Nernst distribution can arise if either the anion is not very permeable and/or the flux of the protonated form is significant. On the basis of the mechanism suggested, it is possible to predict that the aerobic reduction of nitrite will also take place even in untreated cells at an NO_2^- concentration so high that the intracellular NO_2^- level will be sufficient for the inhibition of oxidases. Actually, this effect has been recently described by Japanese authors [25], who observed a steep rise in the rate of nitrite reduction at the increase in NO_2^- concentration from 15 to 50 mM, even in the presence of oxygen.

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