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CHARACTERIZATION OF THE PARTICULATE NITRITE OXIDASE AND ITS COMPONENT ACTIVITIES FROM THE CHEMOAUTOTROPH *NITROBACTER AGILIS**

JOSEPH C. O'KELLEY**, GEORGE E. BECKER*** AND ALVIN NASON
The McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Md. 21218 (U.S.A.)

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SUMMARY

1. Difference spectra, at room and liquid N_2 temperatures, of $S_2O_4^{2-}$-, and NO_2^--reduced intact cells and cell-free preparations of *Nitrobacter agilis* demonstrated the presence of cytochromes of the *c*- and *a*-types. Reduction of cytochromes by succinate, and to a limited extent, by NADPH also occurred, provided KCN (0.1 mM) was also present.

2. A particulate, heat-labile nitrite oxidase having an absolute requirement for O_2 was prepared from *N. agilis* cells using sonic oscillation and differential centrifugation. The particles also possessed NADH oxidase, succinoxidase, formate oxidase and traces of NADPH oxidase activity. The stoichiometry of the nitrite oxidase reaction approached the theoretical value of 2 moles of NO_2^- consumed per mole of O_2 consumed. The pH optimum of the nitrite oxidase system shifted to progressively more alkaline values as the NO_2^- concentration was increased, changing from a pH value of 6.8 at 0.6 mM KNO_2 to pH 8.0 at 0.01 M KNO_2 with apparent K_m's of 0.2 and 1.2 mM NO_2^-, respectively. Computations of the HNO_2 concentrations present under the above conditions showed an approx. 500-fold greater affinity for HNO_2 which was independent of pH, suggesting the involvement of HNO_2 as both a substrate and an inhibitor (at higher concentrations) of the nitrite oxidase system. The marked inhibition by NaN_3, NaCN and Na_2S, as well the light-reversible inhibition by CO, indicated the presence of cytochrome oxidase which was subsequently characterized. NO_2^- proved to be a competitive inhibitor of the nitrite oxidase system.

3. The particulate preparation also possessed a heat-labile nitrite–cytochrome *c* reductase activity which was energy independent and routinely measured under anaerobic conditions. As in the case of nitrite oxidase, the affinity of the enzyme for NO_3^- increased as the pH was lowered, but the pH optimum remained unaffected. In terms of calculated HNO_2 concentration an approximately constant K_m of about 0.2 μM was estimated at the several pH's examined. The inhibition by NO_3^- was

Abbreviation: PHMB, *p*-hydroxymercuribenzoate.

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** Special Fellow, National Institute of General Medical Sciences, U.S. Public Health Service. Present address: Department of Biology, University of Alabama, Tuscaloosa, Ala.

*** Postdoctoral Fellow, National Institutes of Health Training Grant. Present address: Department of Microbiology, University of Iowa, Iowa City, Iowa.

shown to be competitive. The marked sensitivity of the reductase to several metal-binding agents implicated a metal component in the electron transport chain at the site prior to cytochrome *c*.

4. The membrane-like composition of the nitrite oxidase system is indicated.

INTRODUCTION

The chemoautotroph *Nitrobacter agilis* derives its energy from the oxidation of NO_2^- to NO_3^- according to the overall equation:

$$NO_2^- + 1/2\ O_2 \longrightarrow NO_3^- + 17.8\ \text{kcal}$$

Until recent years studies of the physiology and biochemistry of the organism have been limited because of the earlier difficulties in obtaining large numbers of cells. However, when ALEEM AND ALEXANDER[1] in 1958 devised liquid culture methods of growing large quantities of the organism, the way was opened for studies with cell-free preparations.

ALEEM AND NASON[2] demonstrated that the NO_2^- oxidizing activity of *N. agilis* resides in a cytochrome-containing particule designated as nitrite oxidase. Spectral evidence suggested that nitrite involved the enzymatic transfer of electrons from NO_2^- to O_2 *via* cytochrome *c*- and *a*-like components according to the following sequence:

$$NO_2^- \xrightarrow{\text{cytochrome } c \text{ reductase}} \text{cytochrome } c \rightarrow \text{cytochrome } a_1 \xrightarrow{\text{cytochrome oxidase}} O_2$$

The presumed significant energy required for the transfer of electrons from NO_2^- to cytochrome *c* has been the concern of subsequent studies by KIESOW[3] and by VAN GOOL AND LAUDELOUT[4]. On the basis of spectrophotometry and the use of uncouplers and inhibitors ALEEM[5] claimed that NO_2^- enters the electron transport chain in Nitrobacter at the level of cytochrome a_1, and suggested that the reduction of cytochrome *c* involves an energy-dependent reversal of electron transfer from cytochrome a_1.

The enzymatic oxidation of NO_2^- by O_2 in *N. agilis* has been shown to be coupled to the formation of ATP[6–8]. KIESOW[9] and ALEEM *et al.*[10] reported that the oxidation of NO_2^- to NO_3^- is also coupled to the reduction of NAD^+; the latter authors presented evidence that the NAD^+ reduction is ATP-dependent and results from a reverse flow of electrons from cytochrome *c* to NAD^+. STRAAT AND NASON[11] reported on a respiratory nitrate reductase present in *N. agilis*; and KIESOW[3] proposed NO_2^- oxidation in a cycle that involves NADH and ATP in the complete cycle.

The present paper describes investigations of the particulate nitrite oxidase system from *N. agilis* as well as its component nitrite–cytochrome *c* reductase (which is energy independent) and cytochrome oxidase activities. Characterization of the above systems, including inhibitor studies and detailed spectral properties are reported. Evidence is also presented indicating that the entry site of NO_2^- into the electron transport is at or prior to cytochrome *c*; and that HNO_2 probably acts as both a substrate and an inhibitor (at higher concentrations) of the nitrite oxidase system. A second paper[12], which immediately follows, describes the properties of the formate oxidase activity apparently present in the same particles.

EXPERIMENTAL PROCEDURE

Culture methods

Cells of *Nitrobacter agilis* (ATCC 9482) were grown in the inorganic culture solution first used by ALEEM AND ALEXANDER[1] and were harvested and stored using procedures described by STRAAT AND NASON[11] with yields of about 3 g of cells, wet weight, per carboy containing 15 l of nutrient solution.

Cofactors and other substances

NADH, NADPH, FAD, FMN and horse-heart cytochrome *c* (Type III) were obtained from the Sigma Chemical Co. The horse-heart cytochrome *c* was reduced as a 5 % solution in 0.1 M Tris buffer (pH 7.5) with palladium asbestos and H_2 gas[13]. Sulfanilic acid and *N*-(1-naphthyl)ethylenediamine·2 HCl for NO_2^- assay were obtained from the Baker Chemical Co. and Eastman Organic Chemicals, respectively. Crystalline bovine albumin, used as a standard for protein determination, was provided by Mann Research Laboratories.

Preparation of cell-free extracts

1 g (wet wt.) of *N. agilis* cells suspended in 25 ml of 0.1 M Tris buffer (pH 7.5) was subjected to sonic oscillation for 20 min in a Raytheon 10 kcycles sonic oscillator. Unbroken cells and debris were removed by centrifugation at 10000 × *g* for 10 min and the supernatant solution centrifuged at 110000 or 144000 × *g* for 1 h. All or a major portion of the nitrite oxidase activity was found in the resulting 110000 or 144000 × *g* pellet, which was resuspended for use with a Ten Broeck tissue homogenizer in sufficient 0.1 M Tris buffer (pH 7.5) (usually 5 ml) to give a protein concentration of approx. 7–10 mg/ml. Several preparations of the above particulate fraction were used for the studies of nitrite oxidase, nitrite–cytochrome *c* reductase and cytochrome oxidase reported in this paper.

Spectral measurements

Room temperature difference spectra of cell-free preparations were determined with the Cary Model 14 recording spectrophotometer, using the sensitive slide wire (0–0.1 A) and 1.0-ml volumes in cuvettes of 1 cm light path. Several reducing agents were employed, including $S_2O_4^{2-}$, NO_2^-, NADH, NADPH, succinate, and sodium formate.

Low-temperature spectra were determined in cooperation with Dr. Ronald Estabrook of the Johnson Foundation, University of Pennsylvania. Whole cells and a 110000 × *g* particulate preparation were reduced in 0.1 M Tris buffer (pH 7.5) with solid $S_2O_4^{2-}$, or with NO_2^- solution at a final concentration of 5 mM NO_2^-. Enough glycerine was then added to give a 50 % glycerine–buffer mixture. When NO_2^- served as the reducing agent, stirring to mix glycerine and the reduced preparation was carried out under N_2. The reference cells contained a 50 % glycerine–buffer mixture. Samples were frozen in liquid N_2 until vitrified, then warmed until devitrified, and finally recooled to liquid N_2 temperature before recording the spectra of the reduced preparations.

Protein determination

Protein in enzyme preparations was determined by the Lowry method as described (LAYNE[14]) with bovine serum albumin as the standard.

Standard assay procedures

Nitrite oxidase by NO_2^- disappearance. Nitrite oxidase activity was assayed by measuring either the decrease in NO_2^- concentration or the uptake of O_2. The decrease in NO_2^- concentration was determined colorimetrically in aerated 0.5-ml reaction mixtures incubated at room temperature. Aeration was achieved by incubating the reaction mixtures consisting of 0.1 ml of 3 mM KNO_2, 0.35 ml of 0.1 M potassium phosphate buffer (pH 7.0) and 0.05 ml of enzyme preparation (approx. 100 μg protein) in test tubes of approx. 1.5 cm diameter in a test tube holder rotating vertically at 60 rev./min. The reaction was started by addition of enzyme, allowed to proceed for 15 min, and terminated by addition of 1 ml of 1 % (w/w) sulfanilic acid in 20 % (v/v) HCl. Following the addition of 20 ml of distilled water, 1 ml of 0.02 % naphthylethylenediamine reagent was added, and after 15 min the resulting color measured on the Klett–Summerson colorimeter with a No. 54 green filter (500–600 mμ). According to a standard curve utilizing KNO_2, 10 Klett units are equivalent to 8.95 mμmoles of NO_2^-.

Nitrite oxidase by O_2 consumption. The rate of O_2 consumption was determined manometrically by both the "direct method" of Warburg, using constant volume respirometers[15], and by the Clark oxygen electrode, utilizing the method of KIELLEY[16] which measures and records O_2 in solution in a closed reaction chamber. The manometric reaction mixtures consisted of 0.1 M potassium phosphate buffer (pH 8.0), 0.01 M KNO_2, and approx. 3 mg protein (enzyme preparation) in a total volume of 2.8 ml.

The oxygen electrode reaction was started by adding from a syringe 0.2 ml enzyme preparation (approx. 1.5 mg protein) to a mixture containing 0.15 ml 3 mM KNO_2 and 0.1 M potassium phosphate (pH 7.0) to give a total volume of 3.0 ml. For experiments conducted in a smaller reaction chamber, the mixtures were scaled down by one-half. The manometric and oxygen electrode reactions were run for 10–15 min, with the change during the first 10 min usually taken as a measure of enzymatic activity.

Nitrite–cytochrome c reductase. Nitrite–cytochrome *c* reductase was measured anaerobically in spectrophotometric Thunberg cuvettes. The reaction mixture (final volume 3 ml) consisted of 0.3 ml of 2 % cytochrome *c*, 0.1 M Tris buffer (pH 8.0) and enzyme containing approx. 200 μg protein. Reactions were started by the addition of 0.15 ml of 1.0 M KNO_2, and the rate of increase in absorbance at 550 mμ measured either in the Gilford Model 2000 absorbance photometer attached to a Beckman DU monochromator or at 15-sec intervals for 90 sec in Beckman DU spectrophotometer. The change during the 30–90-sec interval is taken as a measure of enzymatic activity.

Cytochrome oxidase. Cytochrome oxidase was assayed by measuring the rate of oxidation of reduced cytochrome *c*. The reaction mixture consisted of the enzyme preparation (approx. 10 μg of protein), 0.1 % reduced cytochrome *c*, and 0.1 M phosphate buffer (pH 6.0) to give a final volume of 1.0 ml. The reaction was started by the addition of reduced cytochrome *c*, and the decrease in absorbance at 550 mμ measured either at 30-sec intervals for 2 min in a Beckman DU spectrophotometer or in the above Gilford instrument.

Other enzyme activities. NADH oxidase and NADPH oxidase activities of the nitrite oxidase particles were measured in 0.1 M potassium phosphate buffer (pH 7.5)

by following the rate of decrease in absorbance at 340 mμ starting with substrate concentrations of 5 mM. Succinoxidase and formate oxidase activities were assayed for under the same conditions of pH and substrate concentration by measuring the rate of O_2 uptake with the Clark oxygen electrode. NADH–, NADPH– and succinate–cytochrome *c* reductase activities of the nitrite oxidase particles were determined as described for nitrite–cytochrome *c* reductase except that the assays were conducted aerobically, 1 mM KCN was included in the reaction mixture and final concentrations of 3 mM NADH, 3 mM NADPH, and 0.01 M sodium succinate were used in place of NO_2^-.

RESULTS

Nitrite oxidase

Distribution of nitrite oxidase activity. The distribution of nitrite oxidase activity during the preparation of a typical 110000 × *g* pellet is summarized in Table I. Sonicated cells showed approx. 13 % of the activity of whole cells, of which a little more than half occurred in the subsequent 10000 × *g* supernatant fraction. The nitrite oxidase activity of the 10000 × *g* pellet was probably due to unbroken cells and large cellular fragments. After centrifuging the 10000 × *g* supernatant fraction at 110000 × *g* or 144000 × *g* for 4 h, the activity was found solely in the resulting pellet, representing about 4 and 34 % of the total activity of the intact cells and sonicated cells, respectively, with 4-fold increase in specific activity as compared to that of the sonicated cells.

Cytochrome components. Room temperature difference spectra ($S_2O_4^{2-}$-treated or NO_2^--treated *minus* untreated) of the nitrite oxidase system (144000 × *g* pellet) demonstrated the presence of several cytochromes (Fig. 1). The extent of reduction by NO_2^- was considerably less than that by $S_2O_4^{2-}$. The absorption maxima at about 550, 520 and 420 mμ are representative of the α-, β-, and γ-peaks of a cytochrome of the *c* type. The peak at 590 and 438 mμ are indicative of a cytochrome a_1-like component, and the shoulder at 605 mμ of cytochrome *a*. Difference spectra of more concentrated particulate preparations (not shown) clearly exhibited a small but defi-

TABLE I

DISTRIBUTION OF NITRITE OXIDASE ACTIVITY UPON PREPARATION OF PELLET FRACTION FROM WHOLE CELLS

A unit of nitrite oxidase is defined as the amount of enzyme which results in the uptake of 1 mμmole O_2 per min at room temperature in 0.1 M Tris buffer (pH 7.5) with initial NO_2^- concentration of 5 mM.

Fraction	*Total enzyme units*	*Total protein (mg)*	*Specific activity (enzyme units/ mg protein)*	*% Recovery (from whole cell activity)*
Unbroken cells	325 000	—	—	—
Sonicated cells	42 880	722	58.1	12.9
10000 × *g* (10 min) pellet	14 040	606	23.1	4.4
10000 × *g* (10 min) supernatant solution	23 100	248	93.2	7.0
110000 × *g* (4 h) pellet	14 216	55	258.0	4.4
110000 × *g* (4 h) supernatant solution	0	114	0	—

nite peak or shoulder at 605 mμ (cytochrome *a*) and a shoulder at 530 mμ. Spectra similar to those shown for NO_2^- and $S_2O_4^{2-}$ were observed under similar experimental conditions with 0.01 M NADH. They were also seen with 0.01 M succinate or NADPH, providing KCN (0.1 mM) was also present.

Spectra displaying the above major absorption peaks were also obtained with NO_2^-- or $S_2O_4^{2-}$-reduced whole cells (not shown). The peaks were lower than those observed with the cell-free particles, but the degree of reduction by NO_2^- was nearly as great as by $S_2O_4^{2-}$ in contrast to the results with cell-free nitrite oxidase preparations.

The high-speed supernatant solutions (110000 or 144000 × *g*, 1 h) exhibited cytochrome *c* and small cytochrome *a*-like absorption peaks when reduced with $S_2O_4^{2-}$. The cytochrome *a*-like, but not the cytochrome *c* components, could be completely sedimented by prolonged high-speed centrifugation (110000 or 144000 × *g*, 4 h). The cytochrome *c* remaining in the supernatant solution, free of cytochrome *a*-like components, failed to undergo reduction by NO_2^-, apparently because the nitrite oxidase system had been removed.

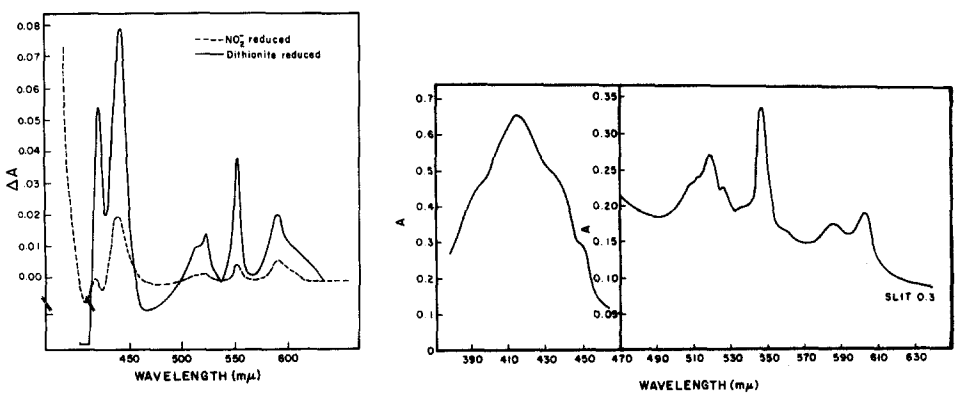

Fig. 1. Difference spectra of cell-free, nitrite oxidase preparations. The above spectra were obtained on the Cary model 14 recording spectrophotometer by adding a few crystals of $Na_2S_2O_4$ or $NaNO_2$ to a 144000 × *g* pellet (0.2 mg protein) obtained after disruption of cell suspensions in a Raytheon 10-kcycles sonicator as described under EXPERIMENTAL PROCEDURE. The reference cuvette in each case contained an identical cell extract, without added reducing agent.

Fig. 2. Low-temperature spectrum of $S_2O_4^{2-}$-reduced whole cells, 12 mg fresh wt. in 2.5 ml 0.1 M Tris buffer (pH 7.5) and glycerol (50%). The preparation was frozen in liquid N_2 until vitrified, warmed until devitrification occurred, and recooled to liquid N_2 temperature. The reference cuvette contained glycerol and buffer.

Low-temperature spectra of $S_2O_4^{2-}$-reduced whole cells (Fig. 2) clearly confirmed the presence of cytochrome *a* in addition to cytochrome a_1 by resolving the room temperature assymetric peak at 590 mμ into two maxima at 604 mμ (α-peak cytochrome *a*) and 586 mμ (α-peak cytochrome a_1) with the two shoulders at 450 and 437 mμ presumably corresponding to the γ-peaks of cytochrome *a* and a_1, respectively. The low-temperature spectrum also indicates a high concentration of *c*-type cytochrome (α-, β- and γ-peaks at 547, 519 and 415 mμ). NO_2^--reduced 110000 × *g* pellet preparations (nitrite oxidase) yield similar absorption spectra (not shown), but the proportion of cytochrome *c* was lower than in whole cells.

Stability. The nitrite oxidase system is heat-labile, losing 36 % of its activity after 2 min at 50° and all activity after 2 min at 100°. Pellet samples (pH 8.5) stored at −15° lost nitrite oxidase activity to 60 % of their original levels during the first 5 days but remained stable for the next 2 weeks. A similar pattern of decrease in activity occurred during storage of samples at pH's 7.0, 7.5 and 8.0, with the pellets stored at pH 7.0 showing the most deterioration.

Proportionality and time-course. The rate of nitrite oxidase activity is directly proportional to enzyme concentration and is linear with time up to 30 min as measured by NO_2^- disappearance or O_2 consumption.

pH optimum and NO_2^- affinity. The present experiments indicate the pH optimum of the particulate nitrite oxidase system to be related to NO_2^- concentration in such a way that the optimum shifts to more alkaline values as the substrate concentration is increased. For example, with 0.1 M phosphate buffer the optimum pH is 6.8 for 0.6 mM KNO_2, 7.4 for 2.5 mM KNO_2 and 8.0 for 0.01 M KNO_2 (Fig. 3). The same shift is observed with Tris and other suitable buffers and with $NaNO_2$ as substrate in place of KNO_2. An examination of the effect of different NO_2^- concentrations

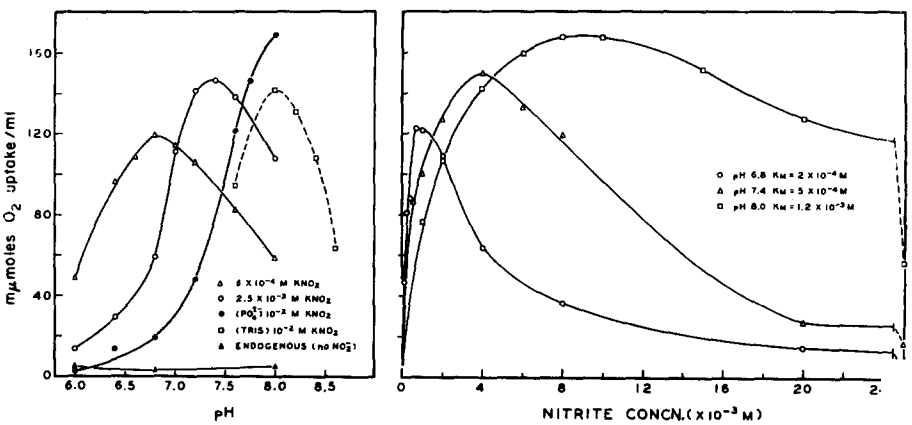

Fig. 3. Influence of NO_2^- concentration on the pH optimum of nitrite oxidase. Conditions of standard assay using oxygen electrode and 144000 × *g* pellet. Potassium phosphate buffer (0.1 M) was used at all pH's except with the 0.01 M KNO_2 levels as indicated in the figure.

Fig. 4. Influence of pH on affinity of NO_2^- for nitrite oxidase. Conditions of standard assay using oxygen electrode and 144000 × *g* pellet.

at the above three pH's (Fig. 4) showed that the affinity of NO_2^- for the enzyme increased as the pH was lowered, since the apparent K_m's for NO_2^- as estimated from the saturation curves was 1.2 mM at pH 8.0, 0.5 mM at pH 7.4, and 0.2 mM at pH 6.8.

The apparently greater affinity of the enzyme system for NO_2^- at lower pH's raised the possibility that HNO_2 (and not NO_2^-) is the substrate for the nitrite oxidase system. Moreover, the observation that higher concentrations of NO_2^- are inhibitory, with the enzyme becoming progressively more sensitive to NO_2^- as the pH is lowered (Figs. 3 and 4), suggested that HNO_2 might also be an inhibitor as well as a substrate for the nitrite oxidase system. By utilizing the ionization constant K for HNO_2 ($\rightleftarrows H^+ + NO_2^-$) of $4.6 \cdot 10^{-4}$ at 25° the concentrations of HNO_2 present in the above

NO_2^- solutions of known pH were calculated. A replot of the data of Fig. 4 in terms of HNO_2 concentrations gave a more unified family of curves (Fig. 5) for substrate saturation (and inhibition). The affinity for HNO_2 (K_m of 50 nM estimated from Fig. 5) is independent of pH, in contrast to that for NO_2^-, and considerably greater (by at least 500-fold) than that for the NO_2^-. In addition, the inhibitory action of HNO_2 beyond its optimal substrate effect (at about 0.1 μM) is also more or less independent of pH, becoming progressively greater with increasing HNO_2 concentration. A semi-reciprocal plot of activity against the calculated HNO_2 concentration (not shown) confirms the occurrence of inhibition by excess substrate with a dissociation constant K_s' of about 0.25 μM. Finally, Fig. 5 indicates that of the three different pH conditions examined, optimal nitrite oxidase activity with reference to the calculated HNO_2 concentrations occurs at the highest pH value tried, namely 8.0.

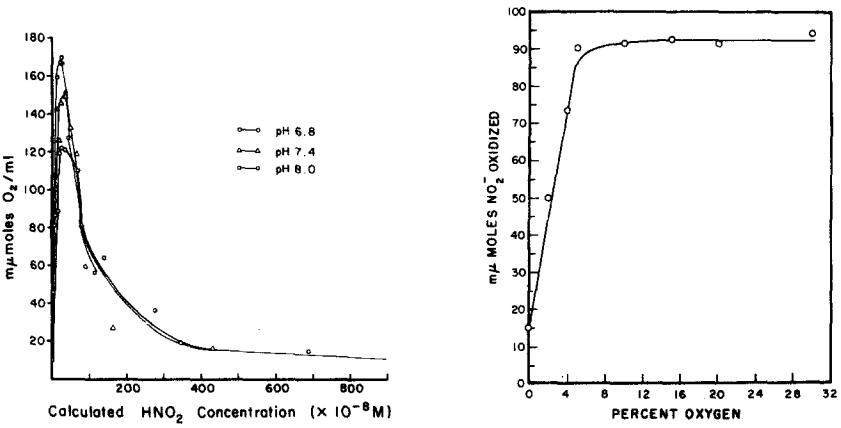

Fig. 5. Effect of calculated HNO_2 concentration on nitrite oxidase. Replot of Fig. 4 using HNO_2 concentrations calculated by means of the ionization constant for the acid and the known concentrations of NO_2^- and H^+ as indicated in text.

Fig. 6. Effect of O_2 tension on nitrite oxidase activity as measured by NO_2^- disappearance. Conditions of standard assay were employed as described in EXPERIMENTAL PROCEDURE except that the reactions were carried out in Thunberg tubes containing the indicated concentrations of O_2 (mixed with appropriate amounts of He gas to give a total pressure of 1 atm).

O_2 dependence. The effect of O_2 tension on nitrite oxidase activity is shown in Fig. 6. The apparent K_m for O_2, presumably for the terminal oxidase portion of the NO_2^--oxidizing chain, as estimated from Fig. 6 is a partial pressure of 2.2 % (based on the partial pressure of O_2 over the reaction mixture and not on the concentration of O_2 dissolved at this partial pressure). The small amount of activity observed in an atmosphere presumed to be 100 % He was probably due to traces of O_2 since no activity could be detected in other experiments under anaerobic conditions.

Specificity of nitrite oxidase pellet. The 110000 × *g* pellet exhibits significantly lower levels of NADH oxidase, succinoxidase and formate oxidase activities than nitrite oxidase activity and almost no NADPH oxidase activity. At pH 7.5 and with a substrate concentration of 5 mM, NADH oxidase activity was 40 %, succinoxidase activity 25 % and formate oxidase activity 20 % of the nitrite oxidase activity. NADPH oxidase activity was present at only about 2 % of the nitrite oxidase activity.

TABLE II

STOICHIOMETRY OF NITRITE OXIDASE ACTIVITY

Initial NO_2^- concentration of reaction mixture 665 mμmoles/ml. O_2 consumption was measured in a closed reaction vessel using the Clark oxygen electrode and particulate enzyme as described under EXPERIMENTAL PROCEDURE. After the reaction had proceeded for 30 min the vessel was opened, a sample removed and immediately assayed for NO_2^- in the usual manner.

pH of reaction mixture	*NO_2^- loss (mμmoles/ml)*	*O_2 consumed (mμmoles/ml)*	*Ratio NO_2^-/O_2*
7.2	189	97	1.95
8.6	115	55	2.09

TABLE III

EFFECT OF INHIBITORS ON NITRITE OXIDASE AND ITS COMPONENT ACTIVITIES

Nitrite oxidase activity was measured by O_2 consumption as described in EXPERIMENTAL PROCEDURE by means of the Clark oxygen electrode except where noted with an asterisk (*) to indicate assay by NO_2^- disappearance. Conditions of standard assay for NO_2^- disappearance using 110000 × *g* pellet. The compounds tested were dissolved in the buffer of the standard assay or in ethanol and added at the start of the reaction. Nitrite–cytochrome *c* reductase and cytochrome oxidase were determined under the conditions of standard assay as described in EXPERIMENTAL PROCEDURE, except that in the cytochrome *c* reductase assay after evacuation of the Thunberg tubes the reaction was started by the addition of enzyme from the side arm.

Compound	*Final concn. (mM)*	*Inhibition (%)*		
		Nitrite oxidase	*Nitrite–cytochrome c reductase*	*Cytochrome oxidase*
N_3^-	0.05	100*	70	28
CN^-	0.05	—	51	100
	0.01	45*	—	—
S^{2-}	0.10	54*	85	100
CO** (darkness)	—	47	—	—
(after illumination)	—	0	—	—
8-Hydroxyquinoline	5	58	21	—
2,4-Dinitrophenol	1	41*	12	—
o-Phenanthroline	5	8	77	—
Salicylaldoxime	5	0	29	—
Amytal	1	15	0	0
Antimycin A	80 μg/ml	75*	—	—
2-*n*-Heptyl-4-hydroxy-quinoline-*N*-oxide	10 μg/ml	65	—	—
PHMB	5	85	30	0
Quinacrine·HCl	1	63	15	27
Quinacrine + FAD	1 + 1	34***	15	38
$NaNO_2$	5	57	58	—
	1	39*	17	—

** CO was bubbled through the enzyme preparation for 30 min prior to assay; illumination of the CO-treated enzyme was achieved by exposing the enzyme at 0–4° to sunlight for 10 min following the CO treatment.

*** Same results whether with simultaneous addition of quinacrine and FAD, or with addition of FAD 10 min after adding quinacrine. FMN had neither a preventive nor a restorative effect.

Stoichiometry. The stoichiometry for the nitrite oxidase reaction was determined by measuring NO_2^- disappearance and O_2 consumption in the same reaction mixture. The results gave NO_2^-/O_2 ratios that approached the theoretically expected value of 2 (Table II).

Inhibitors. The effects of various inhibitors on nitrite oxidase (and its component nitrite–cytochrome *c* reductase and cytochrome oxidase activities) are shown in Table III. The marked inhibition by NaN_3, NaCN and Na_2S as well as the light-reversible inhibition by CO suggest the presence of cytochrome oxidase already indicated by the cytochrome *a* peaks (Figs. 1 and 2). The properties of the component cytochrome oxidase activity of the nitrite oxidase particles are presented in a later section of this paper. Of the metal chelators tested, 8-hydroxyquinoline at a final concentration of 5 mM caused substantial inhibition of nitrite oxidase activity. The inhibitory effect by the uncoupling agent 2,4-dinitrophenol can be attributed to its properties as a metal chelator. A metal component appears to be involved in the early portion of the electron transport sequence in view of the marked sensitivity of the nitrite–cytochrome *c* reductase activity of the preparation to several metal binding agents such as N_3^-, CN^-, S^{2-}, and *o*-phenanthroline.

Sodium amytal had little or no effect. Antimycin A inhibited at concentrations considerably higher than are required to inhibit mammalian respiratory systems. Similarly, inhibition by 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide was observed at concentrations of 10 μg/ml and higher. The possibility that sulfhydryl groups may be necessary for nitrite oxidase activity is suggested by the inhibition with *p*-hydroxymercuribenzoate (PHMB). Since NO_2^- is chemically reduced by glutathione or β-mercaptoethanol, the reversibility of the inhibition could not be tested.

Quinacrine·HCl inhibited nitrite oxidase significantly at relatively high concentrations (1 mM and more) but had only a small effect on nitrite–cytochrome *c* reductase. The addition to the reaction mixture of 1 mM FAD simultaneously with, or 10 min after adding, 1 mM quinacrine partially prevented or reversed the inhibition. FMN was without effect.

NO_3^- as the product of nitrite oxidase activity proved to be inhibitory as measured either by NO_2^- disappearance or by O_2 consumption. Lineweaver–Burk plots (not shown) indicated the inhibition to be competitive.

The separate addition of the metal ions Fe^{2+}, Fe^{3+}, Co^{2+}, Zn^{2+}, MoO_4^{2-}, B^-, Wo^{2-}, Mn^{2+} and Mg^{2+} (1 mM, final concn.) to the reaction mixture did not stimulate nitrite oxidase activity. Cu^{2+} and Ni^{2+} at 1 mM inhibited the enzyme approx. 50 %.

Nitrite–cytochrome c reductase of the particulate nitrite oxidase system

Stability, proportionality, time-course, and pH optimum. Nitrite–cytochrome *c* reductase loses 55 % of its activity after 2 min at 50° and is completely destroyed after 2 min at 100°. The activity is linear with time for about the first 90 sec and is proportional at low concentrations of enzyme, leveling off rather abruptly at higher enzyme concentrations. Under the conditions of standard assay with NO_2^- at final concentrations of 0.05 M, nitrite–cytochrome *c* reductase has a sharp pH optimum at approx. 8.4 in 0.1 M Tris or 0.1 M boric acid–borate buffers and a broader optimum of 7.8–8.5 in 0.1 M barbital buffer (Fig. 7). The activity is seemingly energy-independent since it is routinely assayed under anaerobic conditions with no apparent energy input.

Cytochrome and NO_2^- affinities. At pH 8.0 nitrite–cytochrome *c* reductase activity is proportional to cytochrome *c* concentration until about 80 μM, attaining maximal activity at 160 μM. The K_m for mammalian cytochrome *c*, as estimated from the saturation curve (not shown) is 32 μM. At concentrations of cytochrome *c* greater than 160 μM cytochrome *c* reductase activity falls off very sharply reaching zero at 320 μM cytochrome *c*.

In view of the proposed HNO_2 effect on nitrite oxidase activity, the combined influence of NO_2^- concentration and pH on nitrite–cytochrome *c* reductase were examined. Fig. 8a shows that, like nitrite oxidase, the affinity for NO_2^- increases as

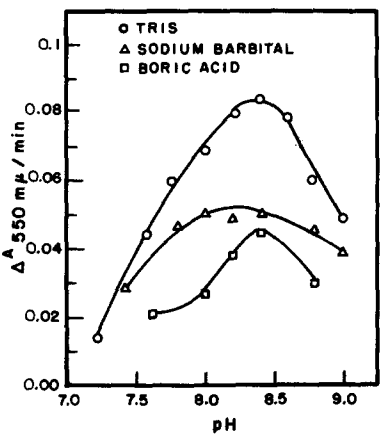

Fig. 7. Effect of pH on nitrite–cytochrome *c* reductase. Conditions of standard assay. ○—○, 0.1 M Tris–HCl buffer; □—□, 0.1 M boric acid–borate buffer; △—△, 0.1 M barbital buffer.

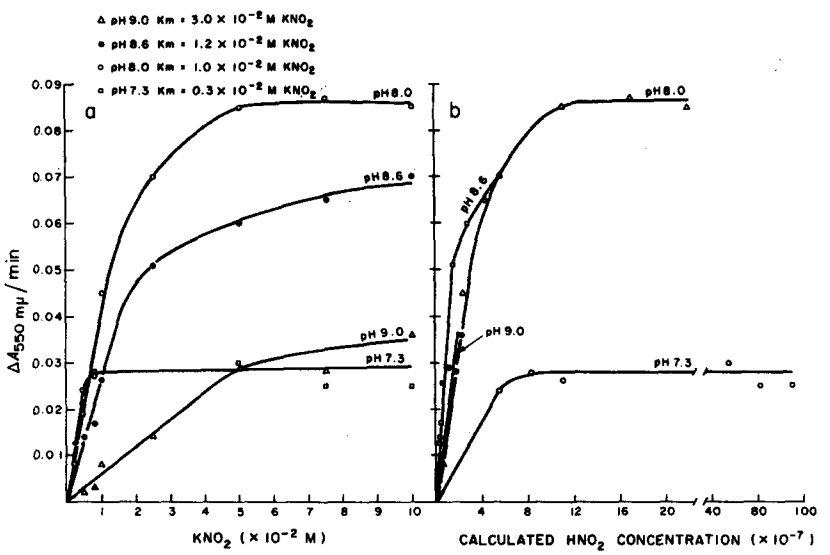

Fig. 8. a. Influence of pH on affinity of NO_2^- for nitrite–cytochrome *c* reductase activity. Conditions of standard assay as described in EXPERIMENTAL PROCEDURE. b. Effect of calculated HNO_2 concentration on nitrite–cytochrome *c* reductase activity. Replot of Fig. 8a using HNO_2 concentrations calculated by means of the ionization constant for the acid and the known concentrations of NO_2^- and H^+ (as indicated for Fig. 4).

the pH is lowered. The apparent K_m for NO_2^- as estimated from the saturation curves extends from 30 mM at pH 9.0 to 3 mM at pH 7.3. NO_2^- concentrations greater than 0.2 M, particularly at the lower pH's, caused progressively greater inhibition (not shown). However, unlike nitrite oxidase, nitrite–cytochrome *c* reductase activity has a pH optimum which is unaffected by as much as a 20-fold change in NO_2^- concentration (from 5 to 100 mM KNO_2). The enzymatic activity simply experienced a decrease at the lower levels of NO_2^- (not shown). If the data of Fig. 8a are replotted in terms of HNO_2 concentrations (calculated from the ionization constant), a more unified family of curves (Fig. 8b) is attained at the higher pH's (8.0, 8.6 and 9.0) with approximately the same K_m values for HNO_2, about 0.2 μM. The considerably lower nitrite–cytochrome *c* reductase activity at pH 7.3 is probably due to a pH effect on one or more ionizable groups of the enzyme. In general, the affinity of NO_2^- (or HNO_2) for the nitrite oxidase system is about 10-fold greater than that of NO_2^- for the nitrite–cytochrome *c* reductase.

Electron acceptor specificity and other enzymatic activities. The particulate fraction can catalyze the transfer of electrons from NO_2^- to $K_3Fe(CN)_6$ in place of cytochrome *c* but not to 2,3,6-trichloroindophenol (0.1 mM), methylene blue (0.005 %), menadione (0.5 mM) or phenazine methosulfate (1 mM). Optimal conditions with $K_3Fe(CN)_6$ as an electron acceptor were essentially the same as with cytochrome *c* except that excess $K_3Fe(CN)_6$ was not inhibitory. Appreciable NADH–cytochrome *c* reductase activity, and to a lesser extent succinate–cytochrome *c* reductase were present. At most only traces, if at all, of NADPH–cytochrome *c* reductase activity could be detected. Despite the presence in the particles of formate oxidase, as described in the paper which follows[12] formate–cytochrome *c* reductase could not be consistently demonstrated.

Inhibitors. The activity of nitrite–cytochrome *c* reductase was significantly inhibited by CN^-, N_3^-, S^{2-}, and *o*-phenanthroline (Table III), suggesting involvement of a metal component. By contrast the NADH–cytochrome *c* reductase activity present in the preparation was unaffected by any of the metal-binding agents tested (S^{2-} was not tried) at final concentrations ranging to 0.01 M. Amytal and PHMB inhibited nitrite–cytochrome *c* reductase to a small extent or not at all at final concentrations ranging from 1 to 5 mM. Quinacrine·HCl inhibited about 15 % at 1 mM and about 50 % at 2 mM.

$NaNO_3$ at 5 mM caused an inhibition of about 60 %. A further examination of this effect together with Lineweaver–Burk plots of the resulting data indicated the inhibition by NO_3^- to be competitive (Fig. 9).

Cytochrome oxidase of particulate nitrite oxidase system

Stability, proportionality, time-course and pH optimum. The cytochrome oxidase loses 55 and 100 % of its activity after 2 min at 50 and 100°, respectively. Activity is directly proportional to enzyme concentration, is linear with time, and is optimal in the range of about pH 5.0–6.0, falling off gradually above and below these values.

Cytochrome affinity and O_2 requirement. Cytochrome oxidase activity is proportional to reduced cytochrome *c* concentration until 40 μM, reaching maximal activity at 80 μM and falling off sharply at higher concentrations. The K_m for reduced mammalian cytochrome *c*, as estimated from the saturation curve (not shown), is 24 μM.

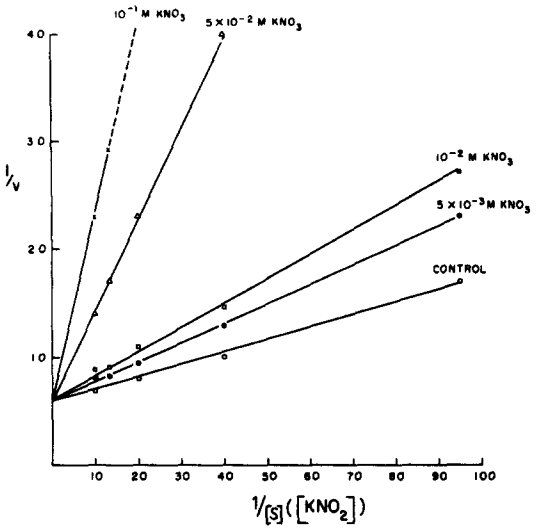

Fig. 9. Lineweaver–Burk plot showing competitive inhibition between NO_3^- and NO_2^- in the nitrite–cytochrome *c* reductase reaction. Conditions of standard assay.

TABLE IV

EFFECT OF NO_3^- AND NO_2^- ON THE CYTOCHROME OXIDASE ACTIVITY OF PARTICULATE NITRITE OXIDASE

Conditions of standard assay except for modifications where indicated.

Expt. No.	*pH*		*Oxidation of reduced cytochrome c ($-\Delta A_{550\ m\mu}$ per 2 min)*	
			Enzymatic (plus nitrite oxidase pellet)	*Non-enzymatic (minus nitrite oxidase pellet)*
1	6.0	Control	0.175	—
		+ 10 mM KNO_3	0.312	0.004
		+ 1 mM KNO_3	0.239	0.002
2	6.0	Control	0.058	—
		+ 100 mM KNO_3	0.102	—
		+ 50 mM KNO_3	0.076	0.000
3	6.0	Control	0.034	—
		+ 100 mM KNO_2	0.239	0.230
4	7.2	Control	0.099	—
		+ 100 mM KNO_3	0.181	0.000
		+ 25 mM KNO_2	0.080	0.003
5	6.0	+ 100 mM KNO_2	—	0.239
		+ 25 mM KNO_2	—	0.035
	7.8	+ 100 mM KNO_2	—	0.000
		+ 250 mM KNO_2	—	0.000

A requirement for O_2 was demonstrated by the complete loss of activity under anaerobic conditions, when no other electron acceptor was provided.

Inhibitors. Cytochrome oxidase is markedly sensitive to CN^- and S^{2-} and to a lesser extent to N_3^- (Table III). Neither amytal, nor PHMB, nor a variety of chelating agents affected enzyme activity. Quinacrine·HCl at 1 mM inhibited slightly; FAD at 1 mM also inhibited slightly (approx. 15 %), and quinacrine and FAD together produced an inhibition that was approximately additive (Table III).

Effect of NO_3^- and NO_2^-. Table IV shows the effects of added NO_3^- and NO_2^- on the cytochrome oxidase activity of the nitrate oxidase system. NO_3^- at 1 and 100 mM stimulated enzymatic oxidation of reduced cytochrome by 50 and 100 %, respectively, presumably by serving as an enzymatic acceptor of electrons from reduced cytochrome *c*. The effect appears to be similar to the respiratory nitrate reductase phenomenon already reported for this system[11]. NO_2^- at lower pH's (*i.e.* pH 6.0) was responsible for the non-enzymatic oxidation of reduced cytochrome *c*, and at higher pH's (7.2 and 7.8), where the non-enzymatic reaction was absent or negligible, inhibited in part the enzymatic oxidation of reduced cytochrome *c*.

Nitrite oxidase composition. The partial composition of nitrite oxidase particles (Table V), particularly their phospholipid content as determined by standard procedures[17,18], suggests that they are part of a membrane system, belonging probably to the highly organized membranous intrusions of the plasma membrane at the poles of the cell[19]. The nitrite–cytochrome *c* reductase activity of the particles is about 2–3 times as great per electron transferred as that of the nitrite oxidase (Table V).

TABLE V

COMPOSITION OF NITRITE OXIDASE PARTICLES

Nitrite oxidase particles were isolated according to the procedure described in EXPERIMENTAL PROCEDURE except that Nitrobacter cells were disrupted by French pressure cell disruption at 20000 lb/inch^2 instead of by sonication. Total lipids were extracted from 10.4 mg dry wt. of nitrite oxidase particles (previously washed twice with 5 % trichloroacetic acid) by mixing 3 times with 2 ml each of chloroform–methanol (1:1, v/v). The total extract was dried on aluminum planchettes to constant weight at 90°. Phospholipids were determined by measuring the phosphorous content of the hydrolyzed total lipids according to the method of BARTLETT[17]. 1 mg of phosphorous was taken to be equivalent to 25 mg phospholipid[18]. Protein, cytochrome *c*, nitrite oxidase and nitrite–cytochrome *c* reductase were determined as described in EXPERIMENTAL PROCEDURE.

Components	*Dry wt.* (%)
Protein	41.5
Total lipid	40.4 ± 5.0
Phospholipid	11.4
Cytochrome *c*	0.78
Nitrite oxidizing activity	
Nitrite oxidase	27.9 mμmoles NO_2^- oxidized per min per mg protein
Nitrite–cytochrome *c* reductase	142.8 mμmoles cytochrome *c* reduced per min per mg protein

DISCUSSION

That the pathway of the nitrite oxidase electron transport system includes cytochromes of both the *c*- and *a*-types has previously been shown[2,4,5]. However,

the sequence of utilization of the cytochromes during the course of NO_2^- oxidation, and the involvement of additional electron carriers such as flavin nucleotide, have not been unequivocally established. On the basis of thermodynamically unfavorable redox potentials (E_0') for the NO_2^-/NO_3^- (ref. 20) ($E_0' = +0.43$ V, pH 7.0) and mammalian ferrocytochrome *c*/ferricytochrome *c* (ref. 21) ($E_0' = +0.26$ V, pH 7.0) couples, LEES[22], KIESOW[3] and VAN GOOL AND LAUDELOUT[4] emphasized the need for a significant energy input in the transfer of electrons from NO_2^- to cytochrome *c*. This viewpoint is supported by the recent characterization from *N. agilis* of a cytochrome *c* with an E_0' of $+0.282$ V at pH 7.0 and an unfavorable energy span of 7.2 kcal calculated from the difference in E_0' between the Nitrobacter ferrocytochrome *c*/ferricytochrome *c* and the NO_2^-/NO_3^- couples[23]. However, the present work demonstrating the occurrence in nitrite oxidase particles of nitrite–cytochrome *c* reductase activity which proceeds readily (and in fact more rapidly than nitrite oxidase, Table V) in the absence of any added or generated energy source is in conflict with the above thermodynamic consideration. The possibility of an intermediate in the biological oxidation of NO_2^- to NO_3^- having a nitrogen atom with an oxidation number of $+4$ by contrast to that of $+5$ and $+3$ for NO_3^- and NO_2^-, respectively, may eventually provide an explanation to reconcile the above apparent contradiction.

The claim by ALEEM[5] that cytochrome *c* reduction in the Nitrobacter electron transport chain is an energy-linked process involving electron reversal from cytochrome a_1 is open to other interpretations. His observation that NO_2^- failed to reduce cytochrome *c* in the presence of 0.5 mM CN^-, which he attributes to a block in energy generation by inhibiting cytochrome oxidase, is more readily explained by the present study demonstrating that the nitrite–cytochrome *c* reductase activity of the nitrite oxidase particles is extremely sensitive to several metal-binding agents (indicative of a metal component in this portion of the electron transport chain at a site prior to cytochrome *c*). Moreover, ALEEM's view that since ascorbate reduced cytochrome *c*, a_1, and a_3 when the terminal oxidase was blocked by CN^-, the same absorption spectra should have been obtained with NO_2^- (if the latter entered the electron transport chain at the cytochrome *c* level) is inconsistent with the facts. First, ascorbate rapidly reduces cytochrome *c* (and other cytochromes) nonenzymatically whether or not CN^- is present. Secondly, the demonstrated sensitivity of nitrite–cytochrome *c* reductase to CN^- (and other metal-binding agents) accounts for the failure to obtain in the presence of CN^- the same absorption spectra as with ascorbate. Finally, his claim as evidence of an energy-dependent reduction of cytochrome *c* that preincubation with 2,4-dibromophenol, an uncoupler of oxidative phosphorylation, caused no change in the steady-state reduction of cytochrome a_1 and a_3 but "markedly suppressed" the steady-state reduction of cytochrome *c* is refuted by his observation in the same paper showing the appearance of an almost identical difference spectrum 5 min after addition of NO_2^- in the absence of the uncoupling agent.

The more recent report by SEWELL AND ALEEM[24] purporting to show the ATP-dependent reversal of electron transfer from cytochrome a_1 to cytochrome *c* must be criticized on two major grounds: (a) all experiments were performed with a crude cell-free Nitrobacter extract (10000 $\times$ *g* supernatant solution) and monitored solely by following rates of change in absorbance of cytochromes *c* and a_1; and (b) several important control experiments are lacking, in particular a reaction mixture with added ATP but without the substrate NO_2^-. The latter control experiment is of

special importance in view of the apparent initial stimulation by ATP of cytochrome *c* reduction in the complete reaction mixture. Does a similar effect occur without the addition of NO_2^-?

Whether flavin is also involved in the nitrite oxidase system, is not certain. The inhibition of nitrite oxidase and nitrite–cytochrome *c* reductase activities by inordinately high quinacrine concentrations only, and the slight, if any, reversal by added FAD are inconclusive in implicating a flavin in the NO_2^- oxidation system. VAN GOOL AND LAUDELOUT[4] working with cell-free preparations of *Nitrobacter winogradskyi* reported a 60 % inhibition of NO_2^- oxidizing activity by 60 μM final concentration of quinacrine. Their claim for reversal of quinacrine inhibition by addition of FAD is questionable on two counts. First, there is no control experiment to show the effect of added FAD on the uninhibited preparation, an important point in view of their data showing greater than 100 % restoration of quinacrine inhibition by FAD. Secondly, the published details of their experimental protocol would indicate prevention rather than reversal of quinacrine inhibition. Direct spectrophotometric evidence for flavin involvement in the electron transport chain of NO_2^- oxidation is also inconclusive. The tendency to attribute the pronounced trough at 450 and 465 mμ in the difference spectra of nitrite oxidase preparations to the bleaching of a flavin component[4,24], must be viewed with caution. The trough between 450 and 465 mμ in difference spectra of nitrite oxidase preparations is also present in purified preparations of Nitrobacter cytochrome *c* and in highly purified horse-heart cytochrome *c*[23]. Thus, it is possible that the absorption minimum observed at 465 mμ in difference spectra of whole cells and extracts of *N. winogradskyi*[4], and the decrease in absorbance at 450 mμ of crude cell-free preparations of *N. agilis*[24] may not be due to the flavin component suggested by these authors.

Two unusual features of the nitrite oxidase complex tending to implicate HNO_2 as a substrate and, at higher concentrations as an inhibitor of the enzyme system are (a) the shift in pH optimum to more alkaline values with increasing NO_2^- levels, with the enzyme becoming progressively less sensitive to the inhibitory effects of NO_2^- as the pH is raised, and (b) the apparently greater affinity of the enzyme system for NO_2^- at lower pH's. Computations of the HNO_2 concentrations present under the above conditions showed that the affinity for HNO_2 (K_m of 50 μM) which was at least 500-fold greater than that for NO_2^- as well as the concentrations of HNO_2 for maximal enzymatic activity were independent of pH in contrast to that for NO_2^-. The inhibitory action of HNO_2 beyond its optimal substrate effect was also more or less independent of pH. BOON AND LAUDELOUT[25] on the basis of a kinetic study of NO_2^- oxidation using *N. winogradskyi*, also concluded that the apparent inhibitory action of NO_2^- on its own oxidation is due to undissociated HNO_2. They reported that NO_3^- non-competitively inhibited the oxidation of NO_2^-, which is at odds with the present study demonstrating a competitive inhibitory effect by NO_3^- of both nitrite oxidase and nitrite–cytochrome *c* reductase activities. Although the presently proposed interpretation that HNO_2 is a substrate as well as an inhibitor of nitrite oxidase appears to fit the data, several other possible explanations have not been ruled out. For example, the influence of H^+, NO_2^- and HNO_2 concentrations on possible conformational changes and on dissociable groups of one or more protein components of the electron transport chain could also be responsible for the observed phenomena, without invoking the idea that HNO_2 is the enzyme substrate. Finally,

it should be noted that HNO_2, which is unstable and cannot be isolated in solution, is in equilibrium with several different forms[26]. Therefore, although the above proposed relationship of HNO_2 to nitrite oxidase appears to fit the data, it cannot be stated with certainty that HNO_2 is the reactive species.

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