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METABOLIC PATHWAYS FOR NITRATE REDUCTION IN *ESCHERICHIA COLI*

J. A. COLE* AND J. W. T. WIMPENNY
Group for Microbial Structure and Function, Department of Microbiology, University College, Cardiff (Great Britain)

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SUMMARY

1. An assay for the production of formate from pyruvate has been described. The presence of the enzyme involved in this reaction has been established for *Escherichia coli* K_{12} grown anaerobically in the presence of nitrate.

2. Formate, lactate, pyruvate and NADH were effective donors for nitrate reduction.

3. Cell membrane preparations catalysed the reduction of nitrate to nitrite when NADH or formate was the electron donor. Reduction by lactate required both "soluble" and "membrane-bound" enzymes and cofactors. The NADH–nitrite reductase also present in these extracts was soluble.

4. Increasing concentrations of nitrate supplied during anaerobic growth in a chemostat were paralleled by increased activities of NADH–nitrate reductase (EC 1.6.6.2) and NADH oxidase activity. In contrast, cytochrome c_{552} and a_2 synthesis and NADH–nitrite reductase activity (EC 1.6.6.4) were highest at low concentrations of nitrate.

5. The oxidation and reduction of cytochromes in whole cells with various substrates and inhibitors has been followed spectrophotometrically. Cytochrome c_{552} was oxidised by both nitrite (azide insensitive) and nitrate. Other cytochromes were oxidised only by nitrate (azide sensitive).

6. The possible involvement of cytochrome c_{552} in nitrite or nitrate reduction has been discussed.

INTRODUCTION

In a previous paper[1] it was reported that *Escherichia coli* and *Escherichia aurescens* synthesised more cytochrome c_{552} anaerobically than aerobically, and more anaerobically in the presence of nitrate than in its absence. Further work on the effect of nitrate on glucose metabolism in *E. coli*[2] showed that higher levels of cytochrome c_{552} were found in cells grown in media containing 10 mM nitrate than at higher concentrations. This paper investigates the possibility that *E. coli* can synthesise more than one enzyme system for nitrate reduction[3], and that nitrate reduction by different

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* Present address: Bacteriology Department, U.C.L.A., Los Angeles, Calif. 90024, U.S.A.

electron donors is favoured by different growth conditions[4]. The possible involvement of cytochrome c_{552} in one of these systems has also been investigated[1].

Aconitase (EC 4.2.1.3) and fumarase (EC 4.2.1.1) activities are extremely low in extracts of cells grown anaerobically in the presence of nitrate[2]. It was concluded, therefore, that nitrate cannot simply substitute for O_2 as a terminal electron acceptor for the reoxidation of NADH produced during glucose oxidation by Embden–Meyerhof and Krebs cycle reactions. The question arises whether compounds such as formate, ethanol, succinate and lactate, which accumulate only when glucose is fermented in the absence of an added inorganic terminal electron acceptor[5,6], can themselves reduce nitrate when this acceptor is available. The physiological importance of these reactions can only be estimated when the effect of nitrate on the synthesis and activity of enzymes producing these fermentation products has been determined. Although the reduction of nitrate by formate has already been fully investigated[7–10], this paper provides evidence for the first time that extracts of *E. coli* grown in the presence of nitrate can produce formate from pyruvate, and hence can use pyruvate as an electron donor for nitrate reduction.

MATERIALS AND METHODS

Organisms and media

E. coli strain K_{12} was maintained on nutrient agar slants. Inocula for large fermentors were grown in 20-ml quantities of tryptone soya broth shaken for 10 h at 37°.

The synthetic medium, before adding the carbon source, contained per l: 100 ml 0.5 M NH_4Cl, 4 ml 1 M KH_2PO_4, 36 ml 1 M K_2HPO_4 and 5 ml mineral salts. The stock solution of mineral salts contained per l: 10 g $MgSO_4 \cdot 7\ H_2O$, 1 g $MnCl_2 \cdot 4\ H_2O$, 0.4 g $FeSO_4 \cdot 7\ H_2O$ and 0.1 g $CaCl_2$. It was stabilised by adjusting the pH to 2.0.

The complex medium contained per l: 14 g casamino acids (Difco), 1 ml 1 M KH_2PO_4, 4 ml 1 M K_2HPO_4 and 5 ml mineral salts. Potassium nitrate, (2 g/l) was added to either medium when required before sterilisation, and sterile glucose (final concentration 0.4 %, w/v) introduced immediately before inoculation.

Growth of organisms and preparation of extracts

These were as described in previous papers of this series[11,12], except that cells were harvested in an M.S.E. "Mistral" refrigerated centrifuge.

The chemostat used for part of this work was the model described by ELSWORTH, CAPELL AND TELLING[13].

Enzymic and chemical estimations

Hydrogenase (EC 1.12.1.1) was determined manometrically[1], and the oxidation of reduced benzyl viologen by nitrate or fumarate was followed spectrophotometrically at 580 mμ, as previously described[2].

Cytochromes were assayed spectrophotometrically in a Cary Model 14 recording spectrophotometer. Peak heights at 630, 590, 559 and 552 mμ for cytochromes a_2, a_1, b_1 and c_{552} were compared on a protein basis, and expressed as absorbance units/g protein, multiplied by 10.

A partially purified sample of cytochrome c_{552} was prepared from a high-speed supernatant extract by ammonium sulphate fractionation. Proteins precipitated at 0° by 30 % and 40 % satd. ammonium sulphate solutions were discarded, but the 40–60 % satd. fraction contained the bulk of the cytochrome c_{552}. This precipitate was redissolved in 50 mM phosphate buffer (pH 7.4), dialysed overnight against a large volume of 1 mM phosphate buffer, and then concentrated to a small volume by leaving the solution in sealed dialysis tubing surrounded by Ficoll (Pharmacia) powder.

NADH-linked nitrate reductase (EC 1.6.6.2)[14,15] was estimated by following pyridine nucleotide oxidation at 340 mμ in anaerobic cuvettes containing nitrate. Volumes of 2.0 ml in the test cuvette contained 1.5 μmoles NADH, 200 μmoles potassium phosphate buffer (pH 7.4) and 10 μmoles sodium nitrate placed in the side arm. The cuvette was repeatedly evacuated and flushed with high-purity N_2 (Air Products) until no further NADH oxidation could be detected. The rate of NADH oxidation after tipping in the nitrate, expressed as μmoles NADH oxidised per mg per h, was taken as the activity of nitrate reductase. For NADH–nitrite reductase (EC 1.6.6.4)[16] nitrite (10 μmoles) replaced nitrate as oxidant.

The NADPH-dependent nitrate and nitrite reductases were estimated under identical conditions as above, except that NADPH (1.5 μmoles) replaced NADH.

The reduction of nitrate to nitrite by electron donors other than pyridine nucleotides or benzyl viologen was determined by incubating nitrate (10 μmoles), donor (10 μmoles), phosphate buffer (200 μmoles, pH 7.4) and enzymes in deep tubes at 30°. After a suitable period of time samples were taken for nitrite estimation.

Nitrite was assayed by the method of SNELL AND SNELL[17] and protein concentrations by the method of LOWRY *et al.*[18].

RESULTS AND DISCUSSION

The enzymes and cofactors required for the reduction of nitrate by formate have been partially purified and studied in considerable detail. Formate production from pyruvate is catalysed by the phosphoroclastic enzyme during anaerobic growth, but this activity appears to be absent aerobically[19]. The physiological importance of formate as a donor for nitrate reduction therefore depends upon the presence of the phosphoroclastic enzyme in cells growing with nitrate. An assay for determining formate production from pyruvate has been developed which involves measuring the gas evolved when formate is oxidised to CO_2. Cell-wall membranes prepared from *E. coli* grown aerobically in a complex medium contain an active formate–methylene blue oxidoreductase. These membranes catalyse CO_2 evolution from formate but not from pyruvate. The phosphoroclastic activity of crude cell extracts can therefore be determined by first incubating the test sample at 30° under N_2 with pyruvate, precipitating protein with 30 %, w/v, perchloric acid after a suitable time interval, neutralising the sample with KOH solution and determining the formate concentration of the supernatant. Unfractionated extracts of cells grown anaerobically in the presence and absence of nitrate in the complex medium were tested in this way. Both showed conversion of pyruvate to formate. It was concluded, therefore, that the reduction of nitrate by formate was probably of physiological importance in cells growing with glucose as an added carbon source.

The previous paper in this series established that the presence of nitrate during anaerobic growth depressed fumarate reductase activity[2]. This has now been confirmed by adding either nitrate or fumarate to batch cultures of *E. coli* growing anaerobically: samples of log phase cells harvested before and after the appropriate addition were broken in the French pressure cell and fumarate and nitrate reductase activities assayed with reduced benzyl viologen as electron donor. Table I shows that addition of fumarate little affected nitrate reductase activity, but stimulated fumarate reductase. Nitrate apparently induced nitrate reductase, but fumarate reductase decreased. Since succinate synthesis by both Krebs cycle and reductive pathway[23] activities is small during anaerobic growth in the presence of nitrate, it is reasonable to propose that succinate is not an active donor for nitrate reduction.

TABLE I

THE EFFECT OF NITRATE AND FUMARATE ON NITRATE REDUCTASE AND FUMARATE REDUCTASE

E. coli was grown anaerobically in the defined medium with glucose as carbon source. A sample of cells was harvested at the middle of logarithmic growth, and either nitrate of fumarate (final concentration 20 mM) added to the remainder of the culture. Nitrate and fumarate reductase activities were measured for crude extracts of cells harvested at the end of logarithmic growth (about 2 h later), and for extracts from a control experiment in which no addition was made during growth. The volumes of extract added were 0.01–0.2 ml (0.2–4 mg protein).

Growth	*Growth conditions*	*Nitrate reductase**	*Fumarate reductase**
Control	Glucose only	16	34
	2 h later	13	39
Expt. 1	Glucose only	31	28
	Glucose and fumarate	47	90
Expt. 2	Glucose only	17	36
	Glucose and nitrate	476	4

* Units are $\Delta A_{580\,m\mu}$/mg protein per min, measured by following the reoxidation of reduced benzyl viologen.

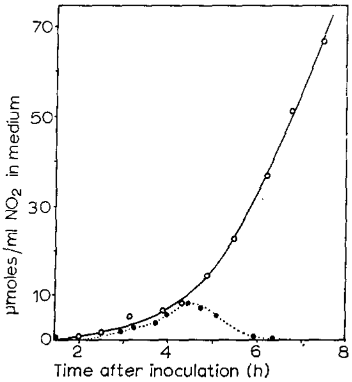


Fig. 1. Nitrite accumulation from nitrate during growth. *E. coli* was grown anaerobically in the complex medium supplemented with either 10 mM or 100 mM potassium nitrate. The concentration of nitrite which accumulated in the medium was measured throughout the growth cycle. ● · · · ●, initial NO_3^- concentration 10 mM; ○ — ○, initial NO_3^- concentration 100 mM.

Nitrate reduction with different donors

Fig. 1 shows that nitrite accumulated in the medium during anaerobic growth in the presence of nitrate. Since cytochrome c_{552} synthesis was influenced by the concentration of nitrate in the medium[2], two different concentrations, 10 mM and 100 mM were used. At the low nitrate concentration, nitrite was released into the medium during the early logarithmic phase of growth, but subsequently disappeared. At the high concentration, nitrite continued to accumulate throughout the growth cycle. These results clearly reflect the differences in rates of production of nitrite from nitrate by nitrate reductase and its subsequent removal by nitrite reductase.

TABLE II

OXIDATION OF REDUCED PYRIDINE NUCLEOTIDES BY NO_2^- AND NO_3^-

The anaerobic oxidation of reduced pyridine nucleotides by nitrite or nitrate was followed spectrophotometrically at 340 mμ. Cells were grown anaerobically in a medium containing either 10 mM or 100 mM $NaNO_3$, and crude extracts prepared in the French pressure cell. Units are μmoles nucleotide oxidised per mg protein per h. Concentrations of cytochrome c_{552} in high-speed supernatant extracts are shown for comparison.

Donor	*Oxidant*	*Cells grown with 10 mM NO_3^-*	*Cells grown with 100 mM NO_3^-*
NADH	NO_2^-	17.7*	7.9
NADH	NO_3^-	3.2	12.8
NADPH	NO_2^-	0.12	0.17
NADPH	NO_3^-	0.01	0.07
Cytochrome c_{552} synthesised (units defined in METHODS)		35.4	3.2

* 0.1-ml samples of crude extracts containing 2.0–4.0 ml of protein were assayed in a final volume of 2.0 ml.

TABLE III

DONORS FOR NITRATE REDUCTION

Unfractionated cell extracts from cells grown in media containing 10 or 100 mM nitrate were incubated at 30° in deep tubes containing 4 mM buffered nitrate and different substrates (final concentration 4 mM). Nitrite accumulation was assayed at intervals, and units of nitrate reductase activity calculated as μmoles NO_2^- accumulating per h per mg protein. A slow endogenous rate in the absence of an added electron donor has been deducted in each ease.

Donor	*Nitrate reductase activity (μmoles NO_2^- per h per mg protein)*	
	10 mM NO_3^- cells	*100 mM NO_3^- cells*
Formate	2.10*	2.34**
Lactate	0.42	1.27
Pyruvate	0.23	0.71
Succinate	0.40	0.05
Malate	<0.01	<0.01
Ethanol	<0.01	<0.01

* Final protein concentration, 2.5 mg/ml.
** Final protein concentration, 2.1 mg/ml.

Table II shows that during late logarithmic growth, cells grown with low concentrations of nitrate contained higher activities of nitrite reductase than nitrate reductase. Comparable cells grown in 100 mM nitrate showed higher NADH–nitrate reductase activity but a reduced NADH–nitrite reductase. NADPH was not an effective substrate for nitrate or nitrite reduction under either condition[21].

Many donors are known to be able to reduce nitrate in the presence of *E. coli* extracts[3]. The relative activities of these different pathways were tested in crude extracts of cells grown anaerobically. Table III shows that for cells grown in both high and low nitrate concentrations, nitrite accumulates most rapidly from nitrate when formate is the electron donor. Lactate and pyruvate are more effective donors for cells grown in the higher nitrate concentration. Once again the increased rate of nitrite accumulation from extracts of cells grown at the higher nitrate concentration could be due to a decreased nitrite reductase and or an increased nitrate reductase activity. It is reasonable to propose, however, that the results presented reflect the relative physiological importance of the different substrates as donors for nitrate reduction.

Since nitrate reduction by pyruvate is probably mediated by the cleavage of pyruvate to formate, the slow rate of reduction by pyruvate in these extracts could

TABLE IV

CELLULAR LOCALISATION OF NITRATE-REDUCING ENZYMES

Supernatant and cell-wall membrane fractions were prepared from cells grown anaerobically in the chemostat on a complex medium containing 70 mM sodium nitrate. Nitrate accumulation was followed when 0.2 ml of each sample was incubated at 30° with 200 μmoles of phosphate buffer (pH 7.4), 20 μmoles of sodium nitrate and an electron donor.

Donor	*μmoles added*	*μmoles NO_2^- formed per mg protein per h*		
		Cell-wall membranes	*Supernatant*	*Cell-wall membranes and supernatant*
NADH	9	4.7	0.0	0.0
Formate	20	3.1	0.2	4.0
Lactate	20	0.0	0.0	0.6
Succinate	20	0.0	0.0	0.0
None	—	0.0	0.0	0.0

TABLE V

CELLULAR LOCALISATION OF NADH-DEPENDENT NITRATE AND NITRITE REDUCTASE

The cell-wall membrane and supernatant extracts described for Table IV were tested for the ability to oxidise NADH in the presence of nitrate or nitrite. NADH oxidation in Thunberg cuvettes was followed spectrophotometrically at 340 mμ in a Cary 14 recording spectrophotometer.

Oxidant	*NADH oxidised (μmoles/h per mg protein)*	
	Cell-wall membranes	*Supernatant*
Nitrate	5.0	0.0
Nitrite	0.0	11.9

be due either to a low activity *in vivo* of the phosphoroclastic enzyme, or to inactivation during cell breakages. The second explanation is the more likely, since no activity at all is observed if the unfractionated extracts are stored at —20° and then tested for NO_2^- production from NO_3^- in the presence of pyruvate. Cells disintegrated at high pressures also fail to show this activity.

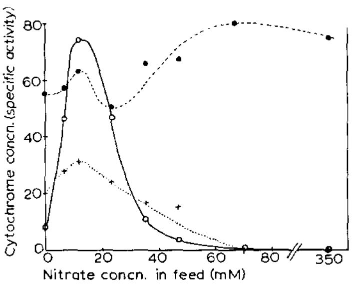
Table IV shows that formate and NADH both reduce nitrate in the presence of a cell-wall membrane preparation. Nitrate reduction by lactate requires both soluble and particulate enzymes, but the soluble fraction prevented nitrite accumulation when NADH was the donor.

Nitrate and nitrite reductase[22,23] activities for the same extracts were measured spectrophotometrically with NADH as the electron donor. Table V shows that the lack of nitrite accumulation when NADH and nitrate were incubated with both cell membranes and a supernatant extract was probably due to further reduction by NADH–nitrite reductase, rather than to an inhibition of the cell-wall membrane nitrate reductase by soluble factors.

Occasionally some cell-wall membrane samples showed very low NADH–nitrate reductase activity: this could be stimulated slightly by adding flavin–adenine dinucleotide (0.1 mg/ml in the assay cuvette), and was restored to the value quoted in Table V by 0.05 mg/ml of vitamin K1.

Effect of NO_3^- concentration during growth

Cells were grown in the complex medium in the continuous culture vessel. Each day the concentration of nitrate in the feed medium was changed. After each 16-h period of equilibration, cells were collected at 0°, washed and Hughes' press extracts



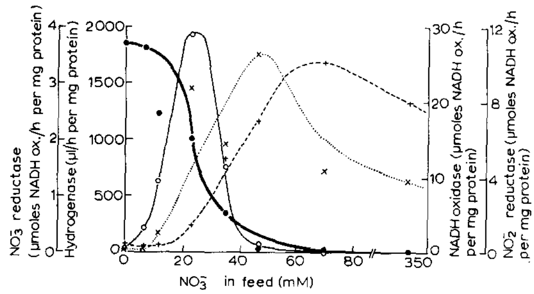


Fig. 2. The effect of nitrate concentration on cytochromes. Cells were grown anaerobically in a chemostat containing the complex medium and 0.4 % glucose. The concentration of nitrate in this medium was varied as indicated. Cells were harvested after equilibrating for 16 h at each nitrate concentration, washed and cell-free extracts prepared with the Hughes' press. Soluble and membrane-bound cytochromes were assayed spectrophotometrically, as described in METHODS. The protein concentrations of unfractionated extracts were in the range 25–40 mg/ml. High-speed supernatant ("soluble") and cell-wall membrane fractions contained 20–40 and 5–15 mg protein per ml, respectively. ●---●, cytochrome b_1 (membrane bound); +···+, cytochrome a_2 (membrane bound); ○—○, cytochrome c_{552} (soluble).

Fig. 3. The effect of nitrate concentration on some enzyme activities. The extracts used here were the same as for the cytochrome determinations in Fig. 2. Hydrogenase, nitrite reductase and nitrate reductase activities were assayed with 0.05 to 0.5-ml samples of unfractionated cell-free extracts. NADH oxidase activity in 0.02–0.05 ml samples of washed membranes is also shown. ●—●, hydrogenase activity; ×···×, nitrate reductase; ○—○, nitrite reductase; +---+, NADH oxidase.

prepared. The changes in cytochrome content of supernatant and cell-wall membrane fractions are shown in Fig. 2. There was an optimum concentration of nitrate in the feed medium required for the formation of cytochrome c_{552}. This nitrate concentration corresponds to peaks in level of two membrane-bound cytochromes, cytochrome b_1 and cytochrome a_2. However, cytochrome b_1 is highest at higher nitrate concentrations. As this was increased still further, cytochrome c_{552} disappeared whilst cytochrome b_1 fell to a steady level which was similar in this experiment to the anaerobic level.

The activities of some enzymes involved in aerobic and anaerobic metabolism were assayed, and the results are shown in Fig. 3. Hydrogenase and nitrite reductase (NADH dependent) activities were absent in cells grown at the highest nitrate concentrations, but NADH oxidase and NADH–nitrate reductase are high. Once again the synthesis of cytochrome c_{552} closely paralleled nitrite reductase, but not nitrate reductase activity. On the other hand synthesis of cytochrome b_1, a known component of nitrate reductase systems[24], varied in the same way as nitrate reductase activity.

Oxidation and reduction of cytochromes in cells and extracts

The oxidation and reduction of cytochromes on addition of various substrates was followed in open cuvettes with the Cary 14 recording spectrophotometer. Cells were grown anaerobically in a batch culture in the complex medium containing 10 mM nitrite. Under these conditions, *E. coli* synthesises large quantities of cytochrome a_2, b_1, and c_{552}. Cells were harvested several hours after reaching the stationary phase of growth, washed and resuspended in 50 mM phosphate buffer (pH 7.4). Difference spectra for test against control cuvettes in the range 650–535 mμ were recorded before and after additions to the test cell suspensions (20 mg dry cell wt. per ml).

When test and control were reduced and oxidised with dithionite and ferricyanide, respectively, peaks at 630, 590 and 555 mμ were observed. The 630- and 590-mμ peaks correspond to cytochrome a_2 and a_1, and the 555-mμ peak was characteristic of cells rich in both cytochrome b_1 and c_{552}. All peaks disappeared when excess nitrate was added to the test cuvette. When the test cuvette contained 5 mM formate, the same difference spectrum was recorded as for dithionite, except that the 555-mμ peak was replaced by a smaller cytochrome b_1 peak at 599 mμ; clearly cytochrome c_{552} remained oxidised in the presence of formate. Addition of nitrate to a concentration of 50 mM to the formate-containing cuvette caused an oxidation of cytochromes a_1 and a_2 after 2 min, and cytochrome b_1 after 8 min. An apparent oxidation rate of formate of at least 1.6 μmoles/h per mg dry cell wt. can be deduced from this experiment, which was run at 20°. Reoxidation of formate-reduced cytochromes could not be detected even after 30 min if nitrate was omitted, or if nitrate was added in the presence of azide (5 mM in the cuvette). Succinate and lactate reduced only 5 and 10 % of the cytochrome b_1 content of the cells, respectively. The complete reduction of cytochrome b_1 by formate was established by recording a dithionite-reduced against formate-*plus*-nitrate-reduced difference spectrum: the only peak was at 552 mμ, corresponding to cytochrome c_{552}.

Finally, an attempt was made to demonstrate cytochrome c_{552} reduction in the presence of glucose. Formate (5 mM) was added to the control cuvette to completely reduce cytochromes a_1, a_2 and b_1 and 5 mM glucose added to the test cuvette. At first no differences in the extent of reduction of any pigment could be detected, but after several minutes the cytochrome c_{552} in the formate-containing cuvette became 75 % reduced. No explanation can be offered for this unexpected observation.

When the reoxidation of cytochromes reduced with either dithionite or formate was studied, further clear distinctions between cytochrome b_1, a_1 and a_2, on the one hand, and cytochrome c_{552}, on the other hand, became apparent. The former were oxidised by nitrate but not by nitrite, whereas cytochrome c_{552} was oxidised by both these acceptors. The oxidation of cytochrome b_1 was inhibited by azide, but the oxidation of cytochrome c_{552} by nitrite was insensitive to this know inhibitor of nitrate reductase[7].

Formate was the most active donor for nitrate reduction in cells containing the largest quantity of cytochrome c_{552}. The possible involvement of the cytochrome in this reaction was investigated. A partially purified cytochrome preparation was degassed in an anaerobic cuvette with various additions, and its spectrum recorded at intervals. A slow reduction of the cytochrome in the presence of both formate and a crude cell-free extract occurred, but controls showed that this was not dependent upon either addition. The crude extract could not catalyse the reduction of NAD^+ by formate, or the reduction of cytochrome c_{552} by lactate or succinate. The addition of acetyl-CoA or ATP either alone or together failed to stimulate the rate of reduction of either NAD^+ or cytochrome c_{552} by formate.

These observations suggest that cytochrome c_{552} does not function as an electron carrier for nitrate reduction with any of the donors tested. In particular the experiments with formate suggest that cytochrome c_{552} is not identical with the factor lost during the purification of the formate–cytochrome b_1–nitrate oxidoreductase complex, as described by ITAGAKI, FUJITA AND SATO[9]. Cytochrome c_{552} synthesis, its cellular localisation and its oxidation and reduction reactions in whole cells and in the presence of various inhibitors rather suggest that the pigment is a component of one of the three nitrite reductase systems in *E. coli*[16]. Since NADH and NADPH were reported to reduce purified cytochrome c_{552} in the presence of a soluble enzyme fraction of *E. coli*[25,26], the possible involvement of cytochrome c_{552} in the NADH-dependent reduction of nitrite has been investigated. The results of these experiments will be reported in a subsequent paper.

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