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CYTOCHROME c_{552} AND NITRITE REDUCTION IN *ESCHERICHIA COLI*

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SUMMARY

The influence of growth conditions on the synthesis of cytochrome c_{552} and the activities of NADH–nitrite oxidoreductase (EC 1.6.6.4) and NADH–cytochrome *c* oxidoreductase (EC 1.6.99.3) have been studied in *Escherichia coli* strain K_{12}. Nitrite added to anaerobic cultures stimulated cytochrome c_{552} synthesis as well as nitrite and cytochrome *c* reductase activities. These were decreased by high concentrations of glucose or amino acids, and were low or absent in aerobically grown cells.

When cells were suspended in sucrose and lysozyme (EC 3.2.1.17), and EDTA added, 80% of the cytochrome c_{552} was released into solution. The NADH–nitrite oxidoreductase activity remaining in the osmotically sensitive spheres so formed was the same as in untreated cells. Nitrite reductase activity and cytochrome c_{552} were also separated by gel filtration, and by $(NH_4)_2SO_4$ fractionation. When cytochrome-rich fractions were recombined with nitrite reductase active fractions, no stimulation of the rate of NADH oxidation by nitrite was observed. In every experiment cytochrome *c* reductase and nitrite reductase activities were found in the same fractions in approximately the same ratio.

Both reductases showed substrate inhibition with respect to NADH: the pH optima were 7.9 for nitrite reductase and 8.6 for cytochrome *c* reductase. It is suggested that cytochrome *c* reductase activity is due to a lack of specificity of nitrite reductase for an electron acceptor, and that cytochrome c_{552} is not a physiological intermediate in either reaction. An alternative function for cytochrome c_{552} in nitrite reduction has been suggested.

INTRODUCTION

In a previous paper[1], the possibility that cytochrome c_{552} functions as a component of nitrate reductase (EC 1.6.6.2) in *Escherichia coli* K_{12} was investigated. This cytochrome has a low redox potential, is soluble, and is synthesised only during anaerobic growth[2–5]. Cells grown in the presence of 100 mM nitrate contained little cytochrome, though nitrate reductase pathways were extremely active. The opposite was true for cells grown in media containing 10 mM nitrate, and in this case a high NAD^+-dependent nitrite reductase activity was observed. It was therefore suggested that cytochrome c_{552} might be an electron carrier for nitrite reduction rather than for nitrate reduction.

Three nitrite reductase (EC 1.6.6.4) activities have been demonstrated in *E.*

coli[6]: (i) an $NADP^+$-dependent activity which has since been shown to function physiologically as a sulphite reductase[7], (ii) a little studied particulate activity[6], (iii) a soluble, NAD^+-dependent enzyme[8,9]. Since NADH readily reduces cytochrome c_{552} in the presence of a "soluble" *E. coli* cell-free extract[2,3], the possibility that cytochrome c_{552} is involved in the third nitrite reductase has been studied in detail. Reduced cytochrome c_{552} is readily reoxidised by nitrite[1], thus any enzyme capable of reducing this pigment might be able to function as a nitrite reductase. Therefore properties of the cytochrome *c* reductase of *E. coli* were studied[10]. Substrate quantities of the bacterial cytochrome were unavailable, so mammalian cytochrome *c* was used for these experiments. As the mammalian and bacterial cytochromes differ in many of their properties, careful consideration has been given to the relevance of this *in vitro* mammalian cytochrome *c* reduction to the reduction of cytochrome c_{552} *in vivo*.

MATERIALS AND METHODS

E. coli strain K_{12} was grown and cell extracts were prepared as described in previous papers[1,11,12]. Enzymic and chemical estimations were also as previously described except that the properties of the NADH–nitrite oxidoreductase were determined with very active high speed supernatant preparations. These extracts are rapidly inactivated when diluted, unless stabilised by the addition of bovine serum albumin. This activity was therefore routinely assayed in open cuvettes containing, in 2.0 ml, 0.15 μmole NADH, 5 mg bovine serum albumin (Sigma), 200 μmoles of sodium phosphate buffer (pH 7.4) and 10 μmoles of $NaNO_2$. Observed rates were corrected for NADH oxidase activity by subtracting the slow rate of pyridine nucleotide oxidation in the absence of nitrite. The validity of this procedure was checked by assaying nitrite reductase under N_2 in Thunberg cuvettes from which all O_2 had been expelled. Reaction rates were proportional to the volume of extract added and are expressed as μmoles NADH oxidised per h per mg protein.

Cytochrome *c* reductase was also assayed spectrophotometrically in open cuvettes. Test cuvettes contained 200 μmoles of potassium phosphate buffer (pH 7.4), 5 mg bovine serum albumin; 0.15 μmole NADH and 50 μmoles of cytochrome *c* (horse heart, Boehringer) in a volume of 2.0 ml. Cytochrome reduction was followed at 550 mμ in the Cary-14 recording spectrophotometer, and in every case the enzyme activity quoted refers to the initial rate under the conditions specified. One unit of activity corresponds to the reduction of 1 μmole of cytochrome *c* per h per mg protein.

Spheroplast formation and fractionation

These preparations were essentially the same as those described by FUJITA AND SATO[13]. Freshly harvested cells were washed twice with 0.01 M Tris–HCl buffer (pH 8.0) at 2°, and once with cold 20 % (w/v) sucrose–0.03 M Tris–HCl buffer (pH 8.0). The washed cells were resuspended in the sucrose–Tris buffer to give an absorbance of about 4 at 550 mμ. Na_2 EDTA was added to give a 1 mM solution, then egg white lysozyme (EC 3.2.1.17) (50 mg/ml). The suspension was incubated at 30° for 20 min, and divided into two parts. One part of the suspension was centrifuged at 10000 $\times$ *g* for 20 min at 0° to obtain the supernatant, referred to as "spheroplast medium". The resultant spheroplast pellet was lysed to obtain "spheroplast lysate" by adding a volume of 0.03 M Tris–HCl buffer (pH 8.0) equivalent to the volume of spheroplast

medium. The lysate was homogenised, treated with deoxyribonuclease (EC 3.1.4.5) (20 μg/ml of lysate) at 2° for 15 min to reduce the viscosity, and centrifuged at 150000 × g for 1 h to obtain a high speed supernatant fraction.

The remainder of the original lysozyme suspension was disintegrated in the French press at 2°, and centrifuged for 1 h at 150000 × g to obtain a "French press supernatant".

Control experiments showed that using the concentration of lysozyme quoted above, the quantity of cytochrome c_{552} released per unit dry cell weight was independent of the density of the cell suspension over the range 0–25 mg dry weight/ml. The cell densities used were therefore somewhat greater than those used by FUJITA AND SATO in order that the release of cytochrome c_{552} into the medium could be accurately estimated with 1 cm light path cuvettes.

The quantity of cytochrome released was not greatly increased by incubating cells for longer than 20 min with lysozyme, unless they were left for several hours. In the latter case a considerable release of intracytoplasmic enzymes such as malate dehydrogenase could be detected, suggesting that more extensive damage than dissolution of the cell walls had occurred. Very low activities of malate dehydrogenase were detected in the spheroplast medium during the first hour of incubation at 30° with lysozyme.

pH sensitivity and Michaelis constants

The pH dependencies of nitrite and cytochrome *c* reductase activities were determined in the presence of 100 mM phosphate buffer in the pH range 6.0–8.0; in 100 mM Tris–HCl in the range 7.5–9.3; and in 100 mM glycine–KOH in the range 9.3–10.2.

Initial reaction rates for extracts of cells grown anaerobically in the presence of nitrite were measured as a function of one substrate concentration, the other substrate concentration being held constant. Michaelis constants were calculated from reciprocal plots of reaction velocity against substrate concentration[14].

Fractionation of nitrite reductase with $(NH_4)_2SO_4$

High speed supernatant extracts of cells grown anaerobically in media containing 10 mM KNO_3 were fractionated by $(NH_4)_2SO_4$ precipitation. A saturated salt solution was slowly added at 2° with vigorous stirring, and protein fractions precipitated by 30%, 40%, 50%, and 60% satd. solns. were collected by centrifugation. These were redissolved separately in 50 mM phosphate buffer (pH 7.4), dialysed overnight against the same buffer at 2°, and assayed for their cytochrome content and nitrite and cytochrome *c* reductase activities.

Fractionation of high speed supernatant extracts by gel filtration

Two Sephadex gel columns were equilibrated for 24 h with 20 mM phosphate buffer (pH 7.9) containing 2 mM glutathione and 2 mM dithiothreitol (Cleland's reagent). One column (length 110 cm, internal diameter 2.2 cm) contained G-100; the other (length 75 cm, internal diameter 2.0 cm) contained G-200.

Enzyme preparations were poured into washed dialysis tubing and concentrated by covering them with solid Carbowax (Calbiochem) for 2 h. Samples of approx. 1% of the column volumes were loaded onto the gels, and eluted with 20 mM phosphate

buffer (pH 7.9) containing 2 mM glutathione and 2 mM dithiothreitol. The column eluate was collected in 2.0 ml fractions on an L.K.B. fraction collector at 2°. These were assayed for cytochromes and NADH oxidase, cytochrome *c* reductase and nitrite reductase activities.

RESULTS

Donor specificity for nitrite reduction

Glucose, formate, lactate and succinate were tested for their ability to reduce nitrite in the presence of a crude cell-free extract of cells grown anaerobically in a medium containing nitrite. Formate reduced nitrite very slowly, but the observed rate was less than 1 % of the rate of reduction of nitrite by glucose or NADH. Lactate and succinate did not reduce nitrite.

Cellular localisation of NADH–nitrite and NADH–nitrate reductases

Unfractionated crude extracts of cells grown anaerobically in media containing 10 mM nitrate show high NADH–nitrate and NADH–nitrite reductase activities. Cell-wall membrane preparations as well as the pellet formed during the preparation of high speed supernatant extracts, contain the NADH–nitrate but not the NADH–nitrite system. Both cytochrome b_1 and the nitrate reductase enzyme assayed by the reduced benzyl viologen method[11] are also in the cell-wall membrane, but absent from the supernatant. In contrast, the nitrite reductase activity of crude extracts is a "soluble" system in that it can be completely recovered in high speed supernatants.

Cytochrome c_{552} could not be detected in cell-wall membrane or high speed pellet fractions[6], but the observation of very small quantities would have been masked by the very high content of cytochrome b_1. In contrast, cytochrome b_1 could not be detected in high speed supernatant extracts of cells broken in the Hughes press.

Cells grown anaerobically in the complex medium containing nitrite or nitrate were treated with lysozyme as described under METHODS. The three samples so obtained, supernatants from lysed spheroplasts, the French press preparation, and the spheroplast medium were assayed for cytochrome c_{552}, cytochrome *c* reductase and nitrite reductase (Table I). Although 80 % of the cells' cytochrome c_{552} but very little cytochrome *c* reductase or nitrite reductase were released into the spheroplast medium, the activities of the reductases were as high in the lysed spheroplasts as in the French press supernatant. No stimulation of the nitrite reductase activity of the

TABLE I

CYTOCHROME c_{552} RELEASE FROM WHOLE CELLS

Cells grown anaerobically in the presence of 0.01 M nitrate were treated with lysozyme and EDTA as described in the text. Activities of nitrite and cytochrome *c* reductases, and the cytochrome content of the three samples obtained, are expressed as units/ml of sample (see METHODS).

Sample	*Nitrite reductase*	*Cytochrome* c_{552}	*Cytochrome c reductase*
Supernatant from French press	578	36.6	2850
Supernatant from lysed spheroplasts	577	9.0	2620
Spheroplast medium	8	28.3	64

spheroplasts was observed when this fraction was recombined with the cytochrome c_{552}-rich spheroplast medium. Thus if cytochrome c_{552} is a component of the NADH–nitrite reductase activity, it appears to be present in cells at a far greater concentration than is necessary for the optimum rate of reaction in cell extracts. The release of a large proportion of cytochrome c_{552} by lysozyme suggests that this component is largely localised outside the cell membrane. The apparently high molecular weight of the cytochrome precludes the possibility that lysozyme allows it to leak from the cytoplasm in preference to other "soluble" but smaller enzymes.

This experiment was repeated with *E. coli* strain Yamaguchi, kindly supplied by Professors R. SATO AND T. FUJITA. No significant differences were observed either in cell cytochrome content under comparable growth conditions, or in the enzyme activities of different fractions after lysozyme treatment.

Composition of medium and nitrite reductase

The activities of nitrite and cytochrome *c* reductases, and the synthesis of cytochrome c_{552} were studied in cells grown in the presence of 10 mM nitrite, but different concentrations of amino acids or glucose.

All three activities were higher in cells grown in the defined medium than in the complex medium. Intermediate activities were observed in the semisynthetic medium recommended by FUJITA[3] which contained salts and 0.5 g/l yeast extract powder (Table II).

TABLE II

THE EFFECT OF GROWTH CONSTITUENTS ON NITRITE REDUCTASE AND CYTOCHROME c_{552}

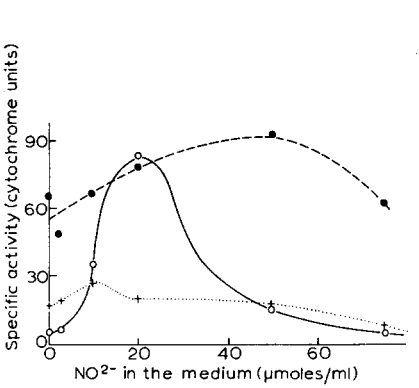
Nitrite reductase, cytochrome *c* reductase and cytochrome c_{552} were assayed in high speed supernatant extracts of cells grown anaerobically in media of different compositions in the chemostat. Glucose (0.4% w/v) was the main carbon source, and in each case 10 mM nitrate was added to the feed medium.

Medium	*Nitrite reductase* (*μmole/h per mg protein*)	*Cytochrome c reductase* (ΔA_{550}/*h per mg protein*)	*Cytochrome* c_{552} (*Specific units*)
Complex	1.1	46	6
Semisynthetic (FUJITA)	1.9	51	14
Defined	4.0	121	27
Glucose 0.1% (w/v)	4.6	101	44
Glucose 2.0% (w/v)	2.2	60	4

Cytochrome c_{552} synthesis was 10 times greater in cells grown in the complex medium with 10 mM nitrite and 0.1% (w/v) glucose than with 2.0% (w/v) glucose. Cytochrome *c* reductase and nitrite reductase were also approximately twice as active in extracts from cells grown at the lower glucose concentration (Table II). Results of these growth experiments agree essentially with those of independent experiments recently published by FUJITA AND SATO[15].

The possibility that nitrite formed from the reduction of nitrate could induce nitrite reductase activity and cytochrome c_{552} synthesis was tested in both batch cultures, and by varying the nitrite concentration in the medium supplied to the chemostat. When nitrite was added to early log phase cells growing anaerobically in the complex medium, cytochrome c_{552} and nitrite reductase activity increased as

expected for inducible enzymes. Variations in enzyme activities and cytochromes as the nitrite concentration in the chemostat feed medium was changed, were also followed. Cytochrome c_{552}, mammalian cytochrome *c* reductase and nitrite reductase increased with nitrite concentration until this appeared to be toxic (Figs. 1 and 2). Cells harvested at the highest nitrite concentration were being diluted out as fresh medium was supplied. Although cytochrome b_1 and nitrate reductase increased throughout this experiment, variations were smaller than variations in cytochrome



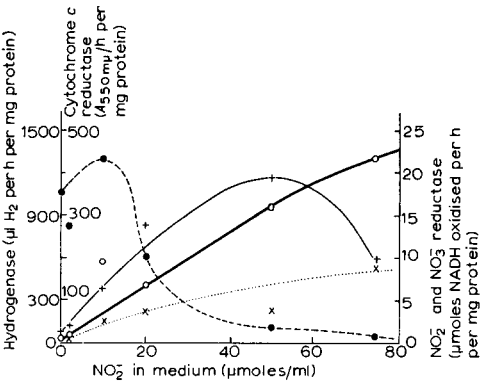


Fig. 1. The effect of nitrite concentration on cytochromes. The nitrite concentration in the feed medium supplied to *E. coli* growing anaerobically in the chemostat was increased daily. The concentrations of cytochromes b_1 and a_2 in cell membranes, and of cytochrome c_{552} in the soluble fraction were determined for each sample, and plotted against the concentration of nitrite supplied to the vessel. ●---●, cytochrome b_1; +·····+, cytochrome a_2; ○——○, cytochrome c_{552}.

Fig. 2. The effect of nitrite concentration on some enzyme activities. The same cell extracts were used here as for the cytochrome determinations shown in Fig. 1. Hydrogenase and nitrate reductase activities of unfractionated extracts and nitrite and cytochrome *c* reductase activities of high speed supernatant fractions are again plotted against the concentration of nitrite in the medium supplied to the chemostat. ○—○, nitrite reductase; +——+, cytochrome *c* reductase; ×····×, nitrate reductase; ●---●, hydrogenase.

TABLE III

THE EFFECT OF OXYGEN ON CYTOCHROMES AND ENZYMES INVOLVED IN AEROBIC OR ANAEROBIC METABOLISM

Cells were grown in the defined medium in the continuous culture vessel. A mixture of air and N_2–CO_2 (95:5, v/v) was supplied to the vessel. The proportion of air in the mixture was varied as indicated, but the total gas flow maintained at 3 l/min. Cell-free extracts were prepared and enzymes and cytochromes assayed as described in METHODS.

Enzymes and cytochromes	*Activity measured (units as previously defined)*							
Air (ml/3 l gas):	*0*	*10*	*20*	*40*	*80*	*200*	*500*	*1000*
Nitrite reductase	2.7	1.7	2.7	0.5	0.0	0.0	0.0	0.0
Nitrate reductase	1.0	1.7	2.5	0.0	0.3	0.0	0.0	0.0
NADH oxidase	0.8	4.3	4.9	7.5	8.2	3.1	3.7	2.9
Isocitrate dehydrogenase	1.7	1.8	4.8	23.8	26.4	28.7	13.2	15.1
Cytochrome b_1	57	72	94	126	72	70	62	36
Cytochrome c_{552}	10	4	6	0	0	0	0	0
Cytochrome a_2	27	36	50	40	13	11	10	6
Hydrogenase	820	172	440	126	3.5	0	0	0
Cytochrome *c* reductase	56	31	39	9	5	11	12	10

c_{552} and nitrite reductase. A further lack of correlation between hydrogenase (EC 1.12.1.1) and cytochrome c_{552} was apparent in this experiment[12,1].

Since cytochrome c_{552} and the benzyl viologen-linked nitrate reductase activity are both inhibited or repressed in aerobically growing cells, the effect of different partial pressures of air supplied to cells growing in the chemostat was investigated. Table III shows that nitrite reductase, mammalian cytochrome *c* reductase, hydrogenase, cytochrome c_{552} and NADH–nitrate reductase decreased as the partial pressure of O_2 increased. In contrast, NADH oxidase, malate dehydrogenase and cytochrome b_1 levels were higher aerobically than anaerobically.

Oxidation and reduction of cytochrome c_{552}

NADH and NADPH both rapidly reduced the cytochrome if either a crude extract or a high speed supernatant was added to the partially purified pigment, prepared by precipitation at high concentrations of $(NH_4)_2SO_4$. The cytochrome alone was reduced extremely slowly with NADH. The reduced cytochrome could be immediately reoxidised by nitrate under nitrogen in the presence of a crude extract or a high speed supernatant from cells broken in the French press. These supernatants can be shown to contain small quantities of cytochrome b_1, and therefore probably small fragments of membrane also. The reduced cytochrome alone was rapidly oxidised when air was admitted to the cuvette, or in the presence of nitrite or hydroxylamine. Nitrate did not oxidise reduced cytochrome *c* in high speed supernatant extracts of cells broken in the Hughes' press. The oxidation of cytochrome c_{552} by nitrate is therefore dependent upon the presence of nitrate reductase activity, and is probably an indirect effect. Cyanide (1 mM) completely inhibited the reoxidation of cytochrome c_{552} by nitrite or hydroxylamine, though no spectral changes were observed.

Stability of nitrite and cytochrome c reductase

The activities of nitrite and cytochrome *c* reductases both decreased by 20 % when a high speed supernatant extract was stored overnight at −20°. Both activities were completely lost after 5 min at 60°, and 90 % of the nitrite reductase and 80 % of the cytochrome *c* reductase were lost after 6 min at 50°. Both activities decreased by 30 % when an extract was diluted to a protein concentration of 1 mg/ml and stored at 0° for 30 min. Undiluted extracts completely retained these activities after 3 h at 0° (but see KEMP AND ATKINSON[9]). Bovine serum albumin stabilised the activities of dilute solutions, though observed activities were independent of albumin concentrations if the protein concentration of the cell extract exceeded 1 mg/ml.

pH sensitivity and Michaelis constants

Both nitrite reductase and cytochrome *c* reductase activities showed sharp maxima at an alkaline pH, cytochrome *c* reductase at pH 8.6, and nitrite reductase at pH 7.9.

Figs. 3 and 4 show that substrate inhibitions by high concentrations of NADH were found for both activities. The Michaelis constants are listed in Table IV. These values for nitrite reductase are somewhat different from those quoted by ZAROWNY AND SANWAL[8].

TABLE IV

MICHAELIS CONSTANTS FOR NADH–NITRITE AND NADH–CYTOCHROME *c* REDUCTASE

Initial reaction rates for extracts of cells grown anaerobically in the presence of 10 mM nitrite were measured as a function of one substrate concentration, the other substrate concentration being held constant. Michaelis constants for the substrate added at different concentrations were calculated from double-reciprocal plots of reaction velocity against substrate concentration.

Enzyme	*Substrate studied*	*Substrate held constant* (μM)		*Michaelis constant* (μM)
Nitrite reductase	NADH	NO_2^-	50	4.6
	NO_2^-	NADH	75	2.5
Cytochrome *c* reductase	NADH	cytochrome *c*	25	8.0
	cytochrome *c*	NADH	75	58

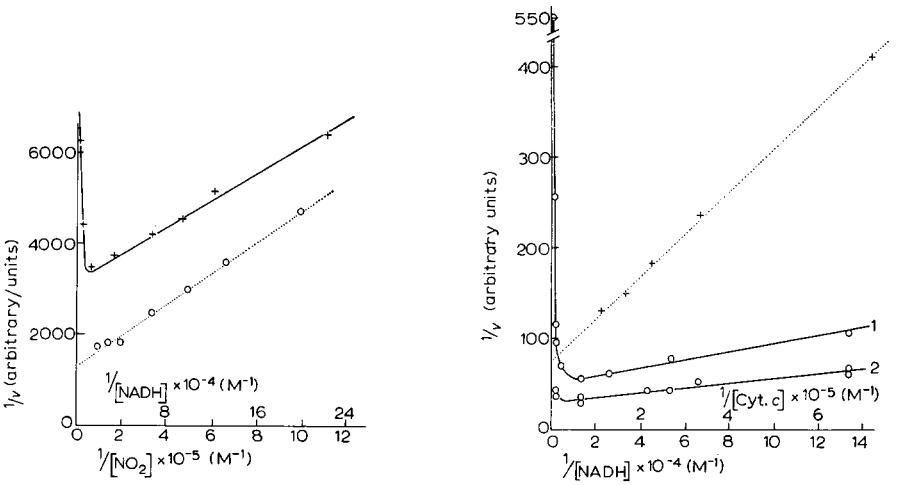


Fig. 3. Affinity of nitrite reductase for NADH and nitrite. The dependence of the initial velocity of nitrite reductase on the concentration of NADH or nitrite was determined. Each substrate concentration was varied independently of the other, and Michaelis constants were calculated from reciprocal plots of initial velocity against substrate concentration. +——+, NADH dependence; O·····O, nitrite dependence.

Fig. 4. Affinity of NADH–cytochrome *c* oxidoreductase for NADH and cytochrome *c*. The dependence of the initial velocity of cytochrome *c* reductase on the concentration of NADH or oxidised mammalian cytochrome *c* was determined. Michaelis constants were again calculated from the reciprocal plots shown in this figure. +·····+, cytochrome *c* dependence; O——O, NADH dependence, (1) cytochrome *c* concentration = 12.5 μM, (2) cytochrome *c* concentration = 25 μM.

The variation of nitrite reductase activity at different initial concentrations of nitrite was particularly troublesome to determine, since at least three possible sources of error had to be avoided. Reaction rates only varied significantly when the initial concentration of nitrite was very low (of the order of 10^{-6} M). In order to record an initial rate accurately, only small quantities of enzyme could be added. Thus either errors due to pipetting very small volumes, or errors due to inactivation of diluted extracts had to be avoided. Corrections for the soluble NADH oxidase activity must be made, since this rate becomes comparable to that of nitrite reductase at the lowest

nitrite concentrations. Finally the NADH concentration used must be carefully selected: if too low, only an apparent Michaelis constant (which is NADH as well as nitrite dependent) can be obtained, and if too high, complications due to substrate inhibition are encountered.

Inhibitor sensitivities of nitrite and cytochrome c reductases

The effect of several inhibitors of bacterial and mitochondrial electron transport reactions on the activities of these enzymes was studied. Table V shows that $5 \cdot 10^{-4}$ M Cu^{2+} or CN^- inhibit 82 % and 85 %, respectively, of the NADH oxidation in the presence of nitrite. This amounted to nearly complete inhibition of nitrite reductase, once soluble NADH oxidase had been subtracted.

TABLE V

INHIBITOR SENSITIVITY OF NADH–NITRITE OXIDOREDUCTASE

Aliquots of a high speed supernatant extract from cells grown anaerobically with nitrite (0.1 ml; 2.5 mg protein) were assayed in the presence of various inhibitors of mammalian electron transport enzymes. Inhibitor concentrations refer to the final concentration in the cuvette.

Inhibitor	*Concentration (mM)*	*Inhibition (%)*
None	0	—
Azide	15	<10
Amytal	0.1*	<10
Cyanide	0.5	85
Cu^{2+}	0.5	82
Na_2EDTA	2.7	<10
Thiourea	5	11
Rotenone	8.5**	<10

* mg/ml.
** μg/ml.

Cytochrome *c* reductase was totally inhibited by Cu^{2+}, but was insensitive to cyanide and all other chemicals tested. If cytochrome *c* reductase as measured in this assay is a component of nitrite reductase, it is clear from these inhibition studies that cyanide inhibits the reoxidation by nitrite of reduced cytochrome c_{552}.

Stoichiometry of nitrite reduced to NADH oxidised

The quantity of nitrite reduced for different amounts of NADH oxidised was determined by incubating an enzyme preparation with known amounts of NADH and excess nitrite in Thunberg tubes filled with N_2. Tubes were incubated at 30° until the NADH was completely utilised, and the residual nitrite determined. 0.5, 1.0, and 2.0 μmoles NADH reduced 0.120, 0.255, and 0.515 μmole nitrite, respectively. Thus approx. 4 molecules of NADH were oxidised for each molecule of nitrite reduced.

Fractionation of nitrite reductase with $(NH_4)_2SO_4$

Nitrite reductase and cytochrome *c* reductase activities of high speed supernatant extracts were predominantly insoluble in 40 % satd. soln. of $(NH_4)_2SO_4$. Part of each activity was precipitated by 30 % satd. soln. (Table VI).

Most of the cytochrome c_{552} was soluble in 40 % satd. $(NH_4)_2SO_4$, but proteins precipitated by 50 % or 60 % satd. solns. were rich in this pigment. No stimulations

TABLE VI

AMMONIUM SULPHATE FRACTIONATION OF HIGH SPEED SUPERNATANT EXTRACTS

Satd. $(NH_4)_2SO_4$ soln. was slowly added at 2° to a stirred high speed supernatant extract (HSS) from cells grown anaerobically in the complex medium containing 10 mM nitrate. Proteins which were precipitated when the solution was 30%, 40%, 50% and 60% satd. with the salt were collected by centrifugation and dissolved in 20 mM phosphate buffer (pH 7.9). Their cytochrome content, and nitrite and cytochrome *c* reductase activities were assayed spectrophotometrically. The cytochrome content is expressed as absorbance units per g protein, multiplied by 10.

Fraction	*Nitrite reductase activity* (*μmole/h per mg protein*)	*Cytochrome c reductase* (*μmole/h per mg protein*)	*Cytochrome content* (*Specific units*)
HSS	2.7	9.1	12.0 (b_1) 20.0 (c_{552})
30% ppt.	9.9	26.8	50 (b_1)
40% ppt.	3.2	15.3	9.1 (b_1) 3.6 (a_1) 20.0 (c_{552})
50% ppt.	0.3	1.7	175 (c_{552})
60% ppt.	0.0	0.05	30 (c_{552})

in reductase activities were observed when cytochrome-rich preparations were added back to the 30% or 40% satd. fractions.

When cell extracts prepared in the French press were fractionated with $(NH_4)_2SO_4$, the fraction precipitating at 30% satn. contained most of the cytochrome b_1 initially observed in the high-speed supernatant. Cytochrome b_1 and a_1, and small quantities of cytochrome c_{552} were also detected in the 40% satd. fraction. This cytochrome b_1 was sedimented by centrifugation at $100000 \times g$ for 1 h at 4°, but the cytochrome a_1, cytochrome c_{552}, nitrite reductase activity and cytochrome *c* reductase activity remained soluble.

Fractionation of nitrite reductase on Sephadex gel columns

A high speed supernatant extract was chromatographed on a Sephadex G-100 gel column equilibrated with 20 mM phosphate buffer (pH 7.9). No nitrite reductase activity and very little cytochrome *c* reductase could be detected in the eluate. Since it has been reported that nitrite stabilises nitrite reductase in *E. coli* Bn[9], the column was washed and a fresh enzyme preparation eluted with 20 mM phosphate (pH 7.9) containing 1 mM $NaNO_2$. Once again no nitrite reductase activity was detected in the eluate. Both reductases were eluted in high activity in the first protein-containing fractions when nitrite was omitted, but 2 mM dithiothreitol and 2 mM gluthathione were included in the phosphate buffer. NADH oxidase and traces of cytochrome b_1 were also detected in these early fractions, but cytochrome c_{552} was eluted in later fractions (Fig. 5). Although a clear separation of cytochrome c_{552} from both nitrite and cytochrome *c* reductase was obtained, the cytochrome-rich fractions did not enhance either activity when they were recombined with the early fractions.

The same enzyme sample was chromatographed on the G-200 column equilibrated with phosphate, dithiothreitol and glutathione. Once again cytochrome b_1 and NADH oxidase were detected in the earliest protein fractions to be eluted, but cytochrome *c* reductase and nitrite reductase were retained and subsequently eluted in

cytochrome-free fractions (Fig. 6). Cytochrome c_{552} was detected in the final protein fractions, and again failed to stimulate nitrite or cytochrome *c* reductase when the appropriate fractions were recombined.

Cytochrome a_1 could not be detected in the fractions collected in these experiments, presumably because its concentration in the eluate was too small.

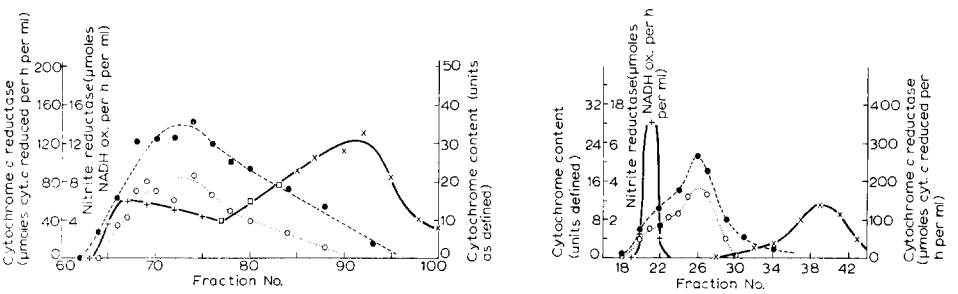


Fig. 5. Fractionation of a high speed supernatant extract on Sephadex G-100. *E. coli* was grown anaerobically in the complex medium containing 10 mM nitrate, harvested, and disintegrated in the French pressure cell. The concentrated high speed supernatant extract (2.0 ml) was loaded onto a 110 cm by 2.2 cm (internal diameter) column which contained Sephadex G-100 gel. The sample was eluted with 20 mM phosphate buffer (pH 7.9) containing 2 mM dithiothreitol and 2 mM glutathione. The eluate was collected in 2.0 ml fractions and assayed for nitrite and cytochrome *c* reductase activities. Cytochromes were determined on the basis of difference spectra of dithionite reduced against untreated samples. One unit of cytochrome b_1 or c_{552} corresponds to a Soret peak of 10^{-3} absorbance units (*i.e.* $A_{428\ m\mu}-A_{470\ m\mu}$ or $A_{420\ m\mu}-A_{470\ m\mu}$, multiplied by 1000). Samples collected between fractions 75 and 85 contained both cytochromes so their relative concentrations could not be determined accurately. ○·····○, nitrite reductase activity (μmole NADH oxidised per h per ml); ●---●, cytochrome *c* reductase (μmole cytochrome *c* reduced per h per ml); +——+, cytochrome b_1 (units defined above); ×——×, cytochrome c_{552} (units defined above); □——□, Soret peaks of cytochrome *b* and cytochrome c_{552} overlap.

Fig. 6. Fractionation of a high speed supernatant extract on Sephadex G-200. The cell-free extract, elution buffer, and assay procedures were the same as those given in the caption to Fig. 5. The length of the G-200 column bed was 75 cm, and the diameter 2.0 cm. A sample volume of 1.0 ml was loaded onto the column, and once again the eluate was collected in 2.0 ml fractions. ○·····○, nitrite reductase activity (μmole NADH oxidised per h per ml); ●---●, cytochrome *c* reductase activity (μmole cytochrome *c* reduced per h per ml); +——+, cytochrome b_1 (units as defined above); ×——×, cytochrome c_{552} (units as defined above).

DISCUSSION

The apparent induction of cytochrome c_{552} synthesis by both nitrite and nitrate suggests that this pigment is an electron carrier for the reduction of one or both of these compounds. The absence of cytochrome c_{552} under conditions which favour maximal nitrate reductase activity, and the fact that membranes alone catalyse this activity suggest that it is involved in the reduction of nitrite rather than nitrate.

When nitrite was added to the growth medium, nitrite reductase activity and the cytochrome c_{552} content increased considerably. No comparable changes in cytochrome b_1 or nitrate reductase were observed. Conversely, high concentrations of nitrate stimulated cytochrome b_1 and nitrate reductase, but inhibited or repressed cytochrome c_{552} synthesis and nitrite reductase activity[1].

The activity of nitrite reductase was not consistently proportional to the amount of cytochrome c_{552} present in cells or extracts. This in itself is neither evidence

for nor against the involvement of cytochrome c_{552} in a particular reaction, though it was initially the reason for proposing a connection between the cytochrome and nitrite reductase. If Scheme 1 (below) is correct, however, it now appears that the rate-limiting step for nitrite reduction involves the enzyme that interacts directly with NADH, namely bacterial cytochrome c_{552} reductase:

$$\text{NADH} \longrightarrow \text{cytochrome } c_{552} \longrightarrow NO_2^-$$

Scheme 1

Cytochrome *c* reductase, as measured in the present experiments using mammalian cytochrome *c* as substrate, was indeed proportional to nitrite reductase in most cases. The noteworthy exception was that this activity was still present at a reduced level in aerobically grown cells which lacked nitrite reductase activity[10]. Cytochrome c_{552} could not, however, be detected in these preparations. The observed substrate inhibitions of both reductases by similar concentrations of NADH, and their similar temperature sensitivities and pH optima provide further evidence that these activities are due to the same enzyme.

An alternative scheme was suggested because of the results of the fractionation experiments on Sephadex gel columns. Although both reductase activities were found in the same fractions at an approximately constant ratio, cytochrome c_{552} could not be detected. The rate of nitrite reduction was not increased when these fractions were recombined with cytochrome-rich fractions. Since cytochrome c_{552} appears to be unnecessary for nitrite reduction by NADH, but reduced cytochrome c_{552} is rapidly oxidised by nitrite, it is unlikely that the enzyme reducing mammalian cytochrome *c* functions physiologically as a cytochrome c_{552} reductase. Rather it appears that mammalian cytochrome *c* can substitute for nitrite as an oxidant for the enzyme that catalyses the reaction shown in Scheme 2.

$$\text{NADH} \longrightarrow NO_2^- \text{ (or mammalian cytochrome } c\text{)}$$

Scheme 2

FUJITA AND SATO[16] reported that whole cells catalyse the reduction of nitrite to ammonia when glucose is the electron donor. This activity could not be detected in broken cells even when gentle methods of disruption were used. They further showed that this activity was proportional to the cytochrome c_{552} content of cells or spheroplasts. It therefore seems probable that a further nitrite reductase remains to be studied and that this enzyme is cytochrome c_{552} linked. This would explain the lack of proportionality between the cytochrome c_{552} content of cells and their NADH–nitrite oxidoreductase activity. As was observed by FUJITA AND SATO, the existence of more than one nitrite reductase could be teleologically advantageous, since nitrite detoxification and nitrite assimilation could be controlled according to different cellular requirements. Experiments are in progress to test these ideas.

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