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Nitrate Reductase in Cell-Free Extracts of a Haemin-Requiring Strain of *Staphylococcus aureus*

By JOAN P. CHANG and JUNE LASCELLES
Microbiology Unit, Department of Biochemistry, University of Oxford

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Staphylococcus aureus strain SG 511 Var differs from the parent organism, SG 511 A, in being streptomycin-resistant and in requiring haemin for growth in certain media (Jensen & Thofern, 1953). There is, however, no haemin requirement if the medium is supplemented with acetate plus purines and uracil (Lascelles, 1956). Organisms grown in the absence of haemin neither respire nor exhibit catalase activity, but addition of haemin to the suspensions activates them in both respects (Jensen & Thofern, 1953; Jensen, 1957; Gardner & Lascelles, 1962). The fact that the suspensions respond to haemin suggests that organisms grown without this factor nevertheless contain proteins that can combine with haemin to give functional haemoproteins.

Anaerobic growth of the mutant strain with nitrate as final electron acceptor is dependent upon haemin, suggesting that the staphylococcal enzyme system for reduction of nitrate to nitrite (nitrate reductase) has a haemin component. This is supported by the finding that suspensions of organisms grown without haemin reduce nitrate but only provided that haemin is added; this forms the basis for a sensitive assay for haemin (Lascelles, 1956).

Enzyme systems that catalyse the reduction of nitrate to nitrite with reduced dyes as electron donor have been isolated from other bacteria capable of anaerobic growth with nitrate as terminal acceptor (Taniguchi & Itagaki, 1960; Fewson & Nicholas, 1961). These studies have provided evidence that cytochromes participate in electron transfer to nitrate with donors such as $NADH_2$ but that the terminal enzyme itself is not a haemoprotein. It seems likely therefore that the effect of haemin in nitrate reduction by the mutant strain of *Staphylococcus* is due to its conversion into a cytochrome that mediates in electron transfer to nitrate.

In the present work some of the properties of the staphylococcal nitrate-reductase system have been studied in cell-free preparations with particular reference to the action of haemin. Conditions affecting the formation of the enzyme system and its localization within the cell have been examined. Evidence was sought for the presence of a protein component in cell-free preparations that could combine with haemin to give a haemoprotein active in the nitrate-reductase system.

MATERIALS AND METHODS

Organisms. The parent and mutant strains of *Staphylococcus aureus* (SG 511 A and SG 511 Var) were those isolated and described by Jensen & Thofern (1953). Maintenance of stock cultures and other details have been previously given (Lascelles, 1956; Gardner & Lascelles, 1962).

Medium. The basal medium described by Gardner & Lascelles (1962) was supplemented with sodium acetate (10 mM) and adenine, xanthine and uracil (each 0·1 mM). In the present work this supplemented medium is referred to as 'basal medium' and additions of nitrate and haemin were made as noted in the text.

Growth of organisms and preparation of extracts. Bulk cultures were sown with organisms that had been sub-cultured once through the basal medium; about 5 ml. of such a culture was used to sow 1 l. of medium. Organisms were normally grown in static culture in Erlenmeyer flasks filled to the neck, and incubation was for 18–24 hr. at 37°. Aerobic cultures (200 ml. in 2 l. Erlenmeyer flasks) were incubated on a reciprocating shaker operating at about 110 cyc./min.

The harvesting of cultures and preparation of cell-free extracts by shaking with glass beads have been described by Gardner & Lascelles (1962).

Fractionation of extracts. The crude extracts (Gardner & Lascelles, 1962) were spun for 60 min. at 100 000 *g* in a Spinco ultracentrifuge model L. After removal of the supernatant ('soluble fraction') the gelatinous pellet was rinsed twice with 40 mM-potassium phosphate

buffer, pH 7·5 (3 ml.), and resuspended in a volume of the same buffer equal to one-quarter of that of the original extract. This fraction is referred to as the 'particulate fraction'.

Assay of nitrate-reductase activity. Two incubation systems were used. (*a*) The pyocyanine system contained, in 1 ml.: extract, 0·5–1 mg. of protein (0·2–0·3 mg. with particulate fractions); potassium phosphate buffer, pH 7·5, 50 μmoles; sodium lactate, 50 μmoles; potassium nitrate, 100 μmoles; L-cysteine, 3 μmoles; NAD, 0·5 μmole; pyocyanine perchlorate, 0·25 μmole; lactic dehydrogenase (type 1; Sigma Chemical Co., St Louis, Mo., U.S.A.), 50 μg. (*b*) The haemin system was as (*a*) except that haemin (0·4 μm-mole) and FMN (0·1 μmole) were used instead of pyocyanine.

The tubes (10 mm. × 150 mm.) were filled with nitrogen, stoppered and incubated for 30 min. at 37°. The reaction was terminated by addition of 2 ml. of the zinc acetate–ethanol reagent of Fewson & Nicholas (1961). After centrifuging of the reaction mixture the nitrite was estimated in the supernatant fluid as described by Lascelles (1956). Enzyme activity is expressed as μmoles of nitrite formed/mg. of protein in 30 min.

Estimation of protein. The method of Lowry, Rosebrough, Farr & Randall (1951) was used; the standard was crystalline bovine serum albumin (Armour Laboratories Ltd., Eastbourne, Sussex).

Measurement of absorption spectra. Micro-cells of 0·5 ml. volume and 1·0 cm. light-path were used. The reference cell contained particulate fraction (from the mutant strain grown without haemin) adjusted to give about the same extinction at 500 mμ as the experimental material. The contents of each cuvette were treated with one drop of saturated dithionite before recording the spectrum with an Optica recording spectrophotometer, model CF4DR (Optica Ltd., Gateshead, Co. Durham).

Special chemicals. Stock solutions of haemin were made in 50% (v/v) ethanol containing 2 mN-NaOH; these were stored at −20° and dilutions made in water just before use. Haemin was added aseptically after autoclaving of the medium. Other porphyrin–metal complexes have been described by Burnham & Lascelles (1963). The haemoproteins used were crystalline products; haemoglobin and cytochrome *c* were purchased from the Sigma Chemical Co., and myoglobin and catalase were obtained as described by Burnham & Lascelles (1963). Pyocyanine perchlorate was purchased from Mann Research Laboratories, New York 6, N.Y., U.S.A., and NAD and FMN from the Sigma Chemical Co. Chelex X-100 analytical-grade chelating resin was obtained from the Bio-Rad Laboratories, Richmond, Calif., U.S.A. The following were gifts: Amytal and antimycin A (Professor Sir Hans Krebs, F.R.S.), 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (Dr J. Lightbown), chlorpromazine, 4,7-dimethyl-1,10-phenanthroline, 2,4,6-tripyrridyl-*s*-triazine and neocuproine (Dr D. Griffiths).

RESULTS

Properties of nitrate reductase. Crude extracts of both mutant and parent strains reduced nitrate to nitrite in the reaction mixtures containing either haemin or pyocyanine (Table 1). Extracts of the mutant strain grown in the absence of haemin formed little or no nitrite unless either haemin or pyocyanine was present in the assay mixture; nitrite formation with pyocyanine was greater than with haemin (Table 1). Addition of haemin to the pyocyanine assay system did not increase the activity. Extracts of the parent strain reduced nitrate to nitrite in the absence of either haemin or pyocyanine but pyocyanine, though not haemin, considerably increased nitrate reduction (Table 1).

Nitrate reduction by extracts of the mutant increased with haemin concentration over the range 0·02–0·2 μM (Fig. 1). Other iron porphyrins and protoporphyrin did not replace haemin, nor did they affect the action of haemin (Table 2). Cytochrome *c* did not replace haemin; haemoglobin had considerable activity though myoglobin and catalase had little effect (Table 2).

Nitrite was not formed in the absence of lactate and omission of either NAD or cysteine diminished the yield by about 30%. Nitrate reduction was

Table 1. *Effect of pyocyanine and haemin on nitrate reduction by extracts of mutant and parent organisms*

Organisms were grown on the basal medium supplemented with 10 mM-KNO_3 and, where shown, with μM-haemin. Crude extracts were assayed for activity (see the Materials and Methods section) in the haemin test system, without and with 0·4 μM-haemin, or in the pyocyanine test system with 0·25 mM-pyocyanine. Nitrite formation is expressed as μmoles/mg. of protein/30 min.

Organism	Haemin in growth medium (μM)	Nitrite formation		
		Without addition	With haemin	With pyocyanine
Mutant	−	0·16	0·64	0·98
	+	0·39	0·39	0·49
Parent	−	0·61	0·59	1·13

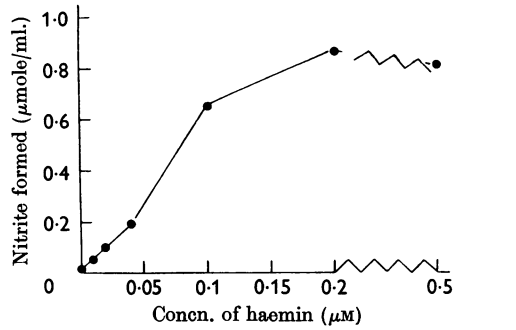
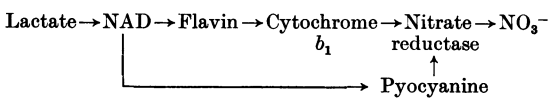


Fig. 1. Effect of haemin concentration on nitrate reduction. A particulate preparation from the mutant strain, grown on the basal medium with 10 mM-KNO_3, was used. Incubation was in the haemin assay system for 30 min. Protein concn., 0·25 mg./ml. of reaction mixture.

increased to a variable extent (10–50 %) by FMN or FAD in the test system containing haemin, though not in that containing pyocyanine; FMN was therefore always added to the systems containing haemin. Omission of lactic dehydrogenase did not affect nitrite formation but the enzyme was always included in the reaction mixtures.

These experiments, together with those described in the next sections, suggested that the path of the electron transfer to nitrate was as follows:



$NADH_2$ generated from NAD by the lactic dehydrogenase could presumably be coupled to the nitrate reductase either directly by the artificial carrier pyocyanine or by the mediation of components of the staphylococcal electron-transfer chain.

Effect of inhibitors. A number of inhibitors were examined for their effects on nitrate reduction in the presence of either haemin or pyocyanine with extracts of the mutant strain (Table 3). In both test systems there was considerable inhibition by cyanide, azide and chlorpromazine. Compounds that inhibited activity with haemin, but had little or no effect in the presence of pyocyanine, included 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide, Amytal and the metal chelators EDTA, neocuproine, diphenylphenanthroline and tripyrridyltriazine. The action of 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide suggested the participation of a *b*-type cytochrome in electron transfer to nitrate and evidence for the formation of a haemoprotein of this type is given below.

Effect of iron. Inhibition by the metal chelators suggested that a metal was also a component of the electron-transfer system via the pathway requiring haemin. This was supported by the fact that nitrite formation was increased by the addition of iron citrate to crude extracts (Table 4, Expt. 1). The effect of iron was more pronounced with particle preparations (see below) that had been treated with the ion-exchange resin Chelex (Table 4, Expt. 2); addition of molybdate with or without iron had no effect.

Nitrate reduction in the pyocyanine test system was also affected by Chelex treatment, but to a smaller extent; iron completely restored the activity (Table 4, Expt. 3).

Fractionation of crude extracts. Crude extracts of the mutant strain were divided into the soluble and particulate fractions by centrifuging for 60 min. at 100 000 *g*. Both fractions reduced nitrate in the presence of pyocyanine and the major proportion of the activity originally present in the crude extracts was recovered in the soluble fraction (Table 5). The system responding to haemin was, however, located in the particulate fraction (Table 5). Total recoveries of this activity were low, amounting usually to only 30–40 % of that in the original extract.

Table 2. *Effect of iron porphyrins and haemoproteins on nitrate reduction by extracts of mutant strain*

The mutant organism was grown on the basal medium supplemented with 10 mM-KNO_3. Crude extracts were assayed in the haemin-containing test mixture without and with 0·2 μM-haemin, together with the additions shown. The concentration of the haemoproteins is expressed as haemin equivalents. –, Not tested.

Additions	Concn. (μM)	Nitrite formed (μmoles/mg. of protein/30 min.) Without haemin	With haemin
None	0	0·19	1·46
Protoporphyrin	0·2	0·34	1·13
Haematohaemin	0·6	0·25	1·18
Deuterohaemin	0·5	0·28	1·39
Coprohaemin	0·5	0·17	1·05
Haemoglobin	0·6	0·83	–
Myoglobin	0·3	0·30	–
Catalase	0·8	0·36	–
Cytochrome *c*	0·2	0·15	–

Table 3. *Effect of inhibitors on nitrate reduction by extracts of mutant strain*

Organisms were grown on the basal medium supplemented with 10 mM-KNO_3. The inhibitors were examined with crude extracts in the pyocyanine or haemin test systems and results are expressed as percentages of the activity without inhibitor. The results are from two experiments in which the control activities (μmoles of nitrite/mg. of protein/30 min.) were 1·33 and 1·74 with pyocyanine and 1·12 and 0·98 with haemin.

Compound	Concn. (mM)	Percentage of control activity With pyocyanine	With haemin
2-*n*-Heptyl-4-hydroxyquinoline-*N*-oxide	0·07	88	23
Amytal	0·5	84	49
Antimycin A	0·04	115	87
Potassium cyanide	0·5	21	11
Sodium azide	1·0	48	29
Sodium sulphite	0·5	100	69
Thiourea	0·5	100	75
Chlorpromazine	0·3	33	25
EDTA	0·5	110	27
4,7-Diphenyl-1,10-phenanthroline	0·2	78	19
2,4,6-Tripyrridyl-*s*-triazine	0·5	130	56
Neocuproine	0·3	93	63

The distribution of enzyme activity in extracts of the parent strain showed a similar pattern, most of the activity with pyocyanine again being found in the soluble fraction (Table 5). The particulate fraction exhibited activity in the absence of haemin and was not stimulated by its addition.

These results suggested that part of the staphylococcal nitrate reductase was associated with the particulate electron-transfer chain ('particulate enzyme') but that most of it (60% or more) remained in the supernatant fraction after removal of the particles by centrifuging ('soluble enzyme'). The enzyme in both fractions could be revealed by assay with pyocyanine whereas only the particulate enzyme showed activity in the haemin test system. The latter assay presumably measured nitrate reduction mediated by a particulate electron-transfer chain which included a cytochrome; the pyocyanine test system by-passed this chain.

Activity of organisms grown with haemin. Crude extracts of the mutant strain grown with nitrate and haemin (μM) reduced nitrate in the absence of both haemin and pyocyanine. Addition of haemin had no effect though pyocyanine slightly increased the activity (see Tables 1 and 6). When assayed with pyocyanine extracts from haemin-grown organisms had only about 50% of the activity of those from organisms grown with nitrate alone. The distribution of activity between the soluble and particulate fractions also differed. At least 70% of the nitrate-reductase activity measured with pyocyanine was found in the particulate fraction of

Table 4. *Effect of iron and molybdenum on nitrate reduction by extracts of mutant strain*

Organisms were grown on the basal medium with 10 mM-KNO_3. In Expt. 1 a crude extract was used. In Expts. 2 and 3 particulate fractions (1·6 and 2·1 mg. of protein/ml. respectively) were first treated at 0° for 15 min. with Chelex resin (4 parts of extract to 1 part of resin, by vol.) and then decanted. Enzyme activities were determined in the haemin (Expts. 1 and 2) or pyocyanine (Expt. 3) test systems with the additions shown.

Expt.	Chelex treatment	Additions (0·01 mM)	Nitrite formation (μmoles/mg. of protein/30 min.)
1 (haemin system)	−	Nil	1·11
	−	Iron citrate	1·43
2 (haemin system)	−	Nil	2·16
	+	Nil	0·94
	+	Iron citrate	1·74
	+	Sodium molybdate	1·08
	+	Iron citrate + sodium molybdate	1·56
3 (pyocyanine system)	−	Nil	1·17
	+	Nil	0·83
	+	Iron citrate	1·37
	+	Sodium molybdate	0·71
	+	Iron citrate + sodium molybdate	1·20

Table 5. *Distribution of nitrate-reductase activity in extracts of parent and mutant strains*

Organisms were grown in the basal medium supplemented with 10 mM-KNO_3 and, where shown, with μM-haemin. The crude extracts were separated into the soluble and particulate fractions and assayed with pyocyanine or haemin as described in the Materials and Methods section.

Organism	Concn. of haemin in growth medium (μM)	Fraction	Nitrite formed (μmoles/mg. of protein/30 min.)		Total activity recovered (%)†	
			With pyocyanine	With haemin	With pyocyanine	With haemin
Mutant	−	Crude extract	1·08	0·96	.	.
		Soluble	0·90	0·16	88	13
		Particulate	1·23	2·80	17	43
	+	Crude extract	0·33	0·28	.	.
		Soluble	0·07	< 0·02	18	< 5
		Particulate	1·39	1·32	73	59
Parent	−	Crude extract	0·74	0·44*	.	.
		Soluble	0·56	< 0·02*	67	< 3
		Particulate	1·50	0·83*	25	33

* Haemin was not added to the test mixture.

† Percentage of the total activity in the original crude extract assayed with pyocyanine or haemin.

Table 6. *Effect of growth conditions on nitrate-reductase activity in extracts of mutant strain*

Organisms were grown on the basal medium supplemented where shown with 10 mM-KNO_3 and μM-haemin. Incubation under semi-anaerobic and aerobic conditions is described in the Materials and Methods section. Nitrate-reductase activity was measured in crude extracts using both the pyocyanine and haemin test systems.

Additions to growth medium	Incubation conditions	Nitrite formation (μmoles/mg. of protein/30 min.) With pyocyanine	With haemin
Nil	Semi-anaerobic	0·08	—
KNO_3		1·11	0·55
KNO_3 + haemin		0·49	0·39
KNO_3	Aerobic	< 0·015	< 0·015
KNO_3 + haemin		< 0·015	< 0·015

extracts from haemin-grown organisms, whereas in extracts from organisms grown without haemin the enzyme activity was predominantly in the soluble fraction (Table 5). It is notable, however, that the specific activity of the particulate fractions from organisms grown either with or without haemin was of the same order. This suggested that the higher total activity found in organisms grown without haemin was due to increased formation of the soluble enzyme.

Induction and repression of nitrate reductase. Formation of nitrate reductase by the mutant strain depended on the presence of nitrate in the growth medium (Table 6); induced enzyme formation was, however, completely prevented by growth under aerobic conditions (Table 6). Such repression occurred both in the presence and absence of haemin, showing that a functional cytochrome system was not necessary for the repressing mechanism to act.

Formation of a b*-type cytochrome in extracts.* Jensen & Thofern (1953) detected cytochrome b_1 spectroscopically in intact organisms of both the parent strain and of the mutant grown with haemin. The action of haemin in promoting nitrate reduction by either crude extracts or particulate fractions from the mutant strain grown without haemin suggested that the cell-free preparations contained a protein that combined with haemin to give cytochrome b_1.

Direct evidence for cytochrome b_1 formation was sought by observation of the spectra of particulate preparations reduced with dithionite.

Particulate fractions from the parent organism exhibited a three-banded spectrum with maxima at 428, 559–560 and 525–534 mμ (Fig. 2). This spectrum resembled that designated as cytochrome b_1 in other bacteria (Morton, 1958). The spectrum of particles from the mutant strain grown with haemin was identical. No absorption maxima were detected in preparations from organisms grown without haemin and these ('apoenzyme particles') were consequently used in the reference cell of the spectrophotometer to balance the light-scattering effects of the turbid particulate preparations.

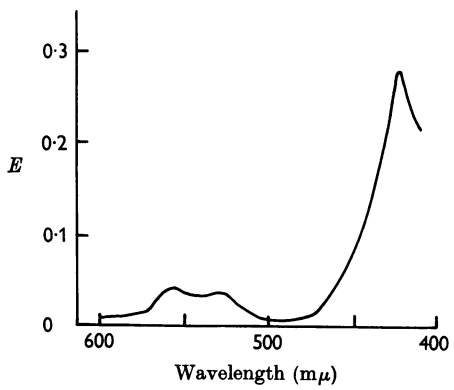


Fig. 2. Absorption spectrum of particulate fraction from the parent strain, grown on the basal medium supplemented with 10 mM-KNO_3. The preparation (2·65 mg. of protein/ml.) was reduced with dithionite and read against a reference cell containing mutant 'apoenzyme' particles plus dithionite.

Crude extracts of the mutant strain (grown without haemin) were incubated in buffered cysteine with or without haemin for 30 min. The particulate fractions were prepared by centrifuging; the washed particles were tested for nitrate-reductase activity and their spectra measured. The particulate fraction from extracts incubated with haemin reduced nitrate in the absence of added haemin (Table 7) and exhibited a spectrum typical of cytochrome b_1 (Fig. 3). The supernatant after removal of the particulate fraction contained no detectable haemin (estimated as the pyridine haemochrome), showing that the haemin added to the crude extract had become completely bound to the particles; haem-dependent nitrate-reductase activity was also absent.

The spectrum of cytochrome b_1 was observed when particles alone were incubated with haemin

Table 7. *Nitrate reduction by fractions from extracts of mutant strain pretreated with haemin*

Organisms were grown in the basal medium supplemented with 10 mM-KNO_3. Crude extract (10 ml., 4·6 mg. of protein/ml.) was incubated for 30 min. at 37° with 40 mM-potassium phosphate buffer, pH 7·5, and 4 mM-L-cysteine with addition of haemin as shown. Extracts were then separated by centrifuging into the soluble and particulate fractions (see Materials and Methods section). Nitrate-reductase activity was measured in the haemin test system without and with 0·4 μM-haemin.

Addition to crude extract	Fraction	Nitrite formed (μmoles/mg. of protein/30 min.) Without haemin	With haemin
None	Soluble	< 0·035	< 0·035
	Particulate	< 0·035	3·00
Haemin (2 μM)	Soluble	< 0·035	< 0·035
	Particulate	2·48	2·67
Haemin (10 μM)	Soluble	< 0·035	< 0·035
	Particulate	3·64	3·25

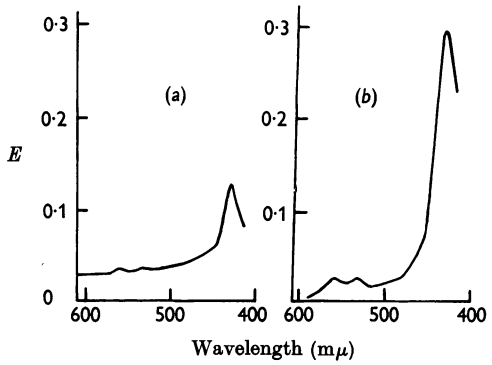


Fig. 3. Absorption spectrum of particulate fraction after incubation of crude extracts from mutant strain with 2 μM-haemin (*a*) and 10 μM-haemin (*b*) (see Table 7). The preparations were reduced with dithionite and read with the reference cell containing mutant 'apoenzyme' particles plus dithionite. Protein concn.: (*a*) 2·1 mg./ml.; (*b*) 2·2 mg./ml.

and cysteine (Fig. 4*a*, *b*). Heated particles in the same system showed only the more diffuse spectrum of haemin alone (Fig. 4*c*). The cytochrome b_1 spectrum was also shown by measuring the difference spectrum with heated particles plus haemin in the reference cell and unheated particles plus haemin in the experimental cell (Fig. 4*d*).

Extracts from the mutant strain grown with aeration had no nitrate-reductase activity (see Table 6). Particulate fractions from such extracts gave the cytochrome b_1 spectrum when incubated with haemin and cysteine, showing that formation of the protein component was not prevented by aeration.

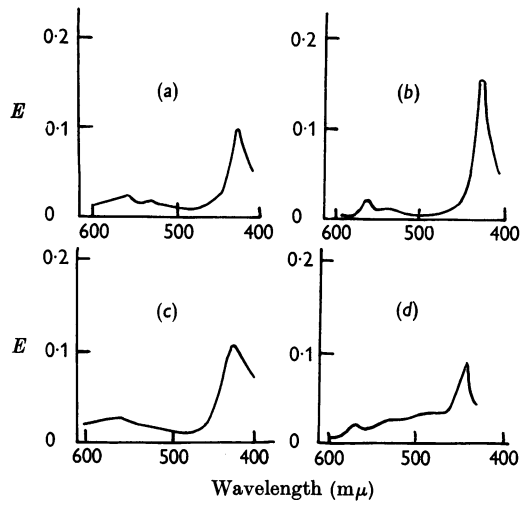
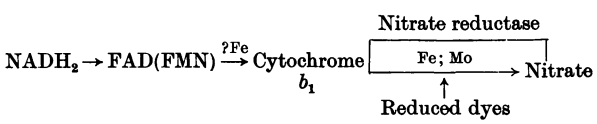


Fig. 4. Absorption spectrum of particles incubated with haemin. The particles (1·9 mg. of protein/ml.) were prepared from an extract of the mutant strain grown with 10 mM-KNO_3. They were incubated for 30 min. at 37° with 40 mM-potassium phosphate buffer, pH 7·5, 6 mM-cysteine and 2 μM-haemin, in a final volume of 0·5 ml. under N_2. (*a*) and (*b*) contained 0·15 and 0·4 ml. of particles respectively; (*c*) contained 0·15 ml. of heated (2 min. at 100°) particles. After addition of dithionite, (*a*), (*b*) and (*c*) were read against a reference cell containing mutant 'apoenzyme' particles plus dithionite; in (*d*) preparation (*a*) was read with preparation (*c*) in the reference cuvette.

DISCUSSION

In micro-organisms the reduction of nitrate to nitrite is the first step in the utilization of nitrate as an ultimate electron acceptor in place of oxygen (dissimilatory nitrate reduction). The staphylococcal enzyme system serves in a dissimilatory capacity since both parent and mutant strains use nitrate as terminal acceptor for anaerobic growth under defined conditions (Lascelles, 1956).

The dissimilatory system has been studied in several bacteria including *Escherichia coli*, *Pseudomonas aeruginosa* and *Aerobacter aerogenes* (Nason, 1962). In *E. coli* the dissimilatory nitrate reductase is largely associated with the particulate electron-transport chain but may be solubilized by various treatments (Taniguchi & Itagaki, 1960). The evidence suggests that the path of electron transport to nitrate in the particulate system from *E. coli* is shown as follows:



The present work has demonstrated that *Staphylococcus aureus* has two forms of nitrate reductase, a soluble and a particulate one. After the particulate fraction has been removed by centrifuging the soluble enzyme can only be revealed by assay with pyocyanine and is therefore not firmly linked to components of the electron-transport chain. The soluble enzyme probably does interact with this system in unfractionated extracts as suggested by the higher haemin-dependent activity in such extracts compared with either fraction alone. The dissimilatory nitrate reductase in *E. coli* and *P. aeruginosa* is also found in the soluble and particulate portions of the cell-free extracts and is predominant in the particulate fraction (Taniguchi & Itagaki, 1960; Fewson & Nicholas, 1961). In the extracts from the *S. aureus* mutant, grown without haemin, and from the parent organism, the nitrate reductase is predominantly in the soluble fraction. The enzyme in this fraction may have originated from the particulate fraction of the cell, being released by disintegration of the particles during the preparation of the extracts. Alternatively, it may exist in soluble form in the intact organism. The latter possibility is favoured by the constancy of the specific activity of the particulate fractions compared with the variations found in the soluble fractions according to whether the organisms were grown with or without haemin.

For the particulate enzyme the evidence accords with the pathway of electron transfer suggested for *E. coli*. (1) Added haemin (or pyocyanine) is needed for nitrate reduction by particulate preparations from the mutant strain grown without haemin. (2) Cytochrome b_1 can be detected spectroscopically in such preparations upon addition of haemin. (3) Haemin-dependent nitrate reduction is inhibited by 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide which is known to inhibit reactions involving *b*-type cytochromes (Lightbown & Jackson, 1956). (4) Chelating agents, azide and cyanide inhibit nitrate reduction whereas iron increases it; this indicates that a heavy metal (or metals) is a component of the enzyme system. (5) Flavins increase haemin-dependent nitrate reduction.

The nitrate reductase of *S. aureus* is inducible, in that it is formed only in the presence of nitrate; enzyme formation is repressed by aeration. It therefore resembles the enzyme in the denitrifier *Micrococcus denitrificans* (Chang & Morris, 1962) and in other bacteria (Nason, 1962). How oxygen exerts this effect is unknown. It is clear, however, that the action of oxygen is specifically concerned with the formation of nitrate reductase and is not a general effect on the electron-transport system. Particles prepared from organisms grown with aeration formed cytochrome b_1 when incubated with haemin, showing that the protein moiety had been formed aerobically. Also organisms grown aerobically respire at the same rate in the presence of haemin as those grown anaerobically (J. P. Chang & J. Lascelles, unpublished work).

Evidence that the protein component of cytochrome b_1 is formed by the *S. aureus* mutant when grown without haemin and that this protein is capable of combining *in vitro* with haemin to give the functional haemoprotein of the nitrate-reductase system is based on the following observations. (1) Haemin-dependent nitrate reduction by the cell-free extracts is inhibited by 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide. (2) Particulate preparations incubated with haemin show absorption maxima (reduced form) at 428, 559–560 and about 529 mμ, which are typical of cytochrome b_1 and are identical with those found in the parent strain. (3) Cytochrome b_1 formation is exhibited only in the particulate fraction where the haemin-linked nitrate reductase is also located. The reconstitution of haemoprotein enzymes *in vitro* has been observed with catalase in a haemin auxotroph of *E. coli* (Beljanski & Beljanski, 1957) and with tryptophan pyrrolase from liver (Greengard & Feigelson, 1962). To our knowledge this is the first demonstration of reconstitution of a cytochrome in cell-free extracts.

Cytochrome b_1 has been identified spectroscopically in several bacteria (Smith, 1961), and soluble forms of *b*-type cytochromes have been isolated from denitrifying bacteria (Vernon, 1956). In other bacteria such as *E. coli* and *Corynebacterium diphtheriae* partially purified preparations of cytochrome b_1 have been described (Pappenheimer, 1955; Itagaki, Fugita & Sato, 1962). The way in which the protohaem prosthetic group is attached to the protein component is unknown. The reconstitution of the haemoprotein in cell-free extracts raises the question of its biosynthesis. Formation of the protein component, at least in the *S. aureus* mutant, is independent of the presence of haemin, and the final attachment of the prosthetic group occurs in the absence of the requirements for protein synthesis. It is possible that the combination of protein and haemin may be mediated by an enzyme required to activate either the haemin or protein moiety. The present experiments do not exclude this possibility.

SUMMARY

1. Nitrate-reductase activity has been demonstrated in cell-free extracts of a mutant strain of *Staphylococcus aureus* requiring either haemin or acetate plus purines and uracil for growth.

2. Extracts from the mutant grown without haemin require either haemin or pyocyanine for nitrate reduction in a system containing phosphate buffer, lactate, lactic dehydrogenase, cysteine and

NAD. FMN or FAD stimulate the reduction in the presence of haemin but not with pyocyanine.

3. Extracts from the parent organism, which does not require haemin for growth, or from the mutant grown with haemin, reduce nitrate in the absence of haemin or pyocyanine, though the latter stimulates.

4. Haemin-dependent nitrate reductase is inhibited by 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide. The inhibitory effects of metal chelators cyanide and azide and the stimulation by iron suggest the participation of this metal in nitrate reduction.

5. Nitrate reductase is induced by growth in the presence of nitrate and is repressed by aeration. The repression occurs irrespective of the presence of haemin in the medium.

6. Fractionation of crude extracts of the mutant organism into soluble and particulate portions by centrifuging shows that nitrate reductase is present in both fractions. Both the soluble and particulate forms of the enzyme are revealed by assay with pyocyanine, but the haemin-dependent activity is located in the particles. In organisms grown without haemin there is more soluble enzyme than in organisms grown with this factor.

7. Particles from the mutant strain, grown without haemin, form a haemoprotein when incubated with haemin and cysteine; the reduced absorption maxima are similar to those of cytochrome b_1 and to those in particles from the parent organism.

8. It is concluded that the *S. aureus* mutant forms the protein component of cytochrome b_1 when grown without haemin; this combines with haemin to give cytochrome b_1, which participates in electron transfer to nitrate, mediated by nitrate reductase.

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Intracellular Distribution of Isocitrate–Nicotinamide–Adenine Dinucleotide Phosphate - Oxidoreductase Activity during Development of the Chick Embryo

BY W. W. BAKER AND R. W. NEWBURGH
Science Research Institute and Department of Chemistry, Oregon State University, Corvallis, Oregon, U.S.A.

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The unique characteristic of the embryonic system is the development of a heterogeneous cell population from a homogeneous one. An approach towards an understanding of this process is to attempt to correlate biochemical and morphological changes occurring in the developing embryo. Certainly a biochemical parameter involved is the activity of individual enzymes. Not only does this include activity per cellular unit but also intracellular site of a particular enzyme activity. Bio-