Effects of Cultural Conditions on Nitrate Reductase in *Photobacterium sepia*

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(Received 11 November 1963)

SUMMARY

The effect of cultural conditions on the growth and nitrate reductase activity of a bacterium, now identified as *Photobacterium sepia*, were examined. The bacterium grew well with ammonium chloride, ammonium nitrate or potassium nitrate as sole nitrogen source. Nitrate reductase activity was markedly decreased in organisms deficient in molybdenum or iron and in organisms grown at high oxygen pressures. The *P. sepia* enzyme is thus similar to nitrate reductases from other bacteria. The *P. sepia* enzyme is constitutive; it is present in organisms grown with ammonium chloride in the absence of nitrate; the ammonium radical did not depress enzyme activity. Under certain conditions (NH_4NO_3 medium, 10%, v/v, O_2 in N_2) molybdenum (10 $\mu\text{g./l.}$) depressed the nitrate reductase activity, but this effect was annulled by repeated subcultivation in molybdenum-deficient medium. Vanadium and especially tungsten, also inhibited the enzyme. The uptake of nitric oxide was decreased in organisms depleted of iron but was increased in organisms deficient in molybdenum. The behaviour of nitrate reductase preparations during fractionation whether from *P. sepia* grown with KNO_3 or with NH_4Cl suggests that the enzyme is the same from both sources.

INTRODUCTION

There are two types of microbial enzymes which reduce nitrate to nitrite. The assimilatory enzyme contains flavin and molybdenum and usually utilizes reduced nicotinamide adenine dinucleotide (NADH_2), or NADPH_2 (Nicholas & Nason, 1954) as a hydrogen donor, whereas the dissimilatory one has an additional iron requirement (Verhoeven, 1952; Sato, 1956; Fewson & Nicholas, 1961; Nicholas, 1963). During assimilation, nitrate is incorporated into cell nitrogen, whereas in the dissimilatory process or 'nitrate respiration' nitrate serves as an alternative hydrogen acceptor to oxygen.

A nitrate reductase characterized in *Achromobacter fischeri* by Sadana & McElroy (1957) was shown to be induced by its substrate only. They claimed that organisms deficient in iron had decreased enzyme activity but that a deficiency of other micro-nutrients, including molybdenum, was without effect. Lindeberg, Lode & Somme (1963) found that nitrate reductase in *Achromobacter* species decreased when the oxygen pressure in the medium was increased and in this respect it resembled the

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dissimilatory enzyme from other bacteria. We have re-examined the cultures used by Sadana & McElroy (1957) and find that the bacterium used by them is in fact *Photobacterium sepia* which can be readily trained to grow with nitrate as sole nitrogen source. By using reduced benzylviologen as hydrogen donor we have found that the enzyme is dependent on both molybdenum and iron for its activity (Nicholas, Redmond & Wright, 1963). This enzyme is constitutive since it is present in *P. sepia* grown with ammonium chloride but no nitrate. The effect of oxygen pressure on enzyme production was also examined.

METHODS

Organism. The bacterium used in these experiments was kindly supplied by Dr W. D. McElroy (McCollum-Pratt Institute, Johns Hopkins University, Baltimore, U.S.A.) as *Achromobacter fischeri*. However, subsequent biochemical tests by Miss Anne Morrison (National Collection Type Cultures, Torrey Research Station, Aberdeen) identified the organism as *Photobacterium sepia*.

Culture media. The organism was grown on the following basal medium: NaCl, 30 g.; Na_2HPO_4 , 5.5 g.; KH_2PO_4 , 2.1 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g.; NH_4NO_3 , 5 g.; glycerol, 3 ml.; Fe, 2 mg. (as FeCl_3); Mo, 0.1 mg. (as $(\text{NH}_4)_2\text{MoO}_4$); water, 1 l. KNO_3 (12.2 g.) and NH_4Cl (6.5 g.) were substituted for NH_4NO_3 in some cultures. Stocks of organism were maintained on nutrient agar slopes of the following composition: NaCl, 30 g.; peptone, 5 g.; beef extract, 3 g.; glycerol, 5 ml.; CaCO_3 , 0.1 g.; agar, 10 g.; water 1 l. The water was deionized in a Permutit Deminrolit Mark IV plant and distilled once in an all-glass still. The media were sterilized at 115° for 15 min.

Purification of culture media. Molybdenum was removed by the copper sulphide co-precipitation method (Nicholas, 1952) and iron by the calcium carbonate adsorption technique of Steinberg (1935). The micronutrient elements Fe, Mo, V and W, spectrographically tested for purity, were supplied by Johnson Matthey (Hatton Garden, London, E.C. 1). Culture solutions purified from Mo were adjusted to pH 7.1 with 5% (w/v) sodium hydroxide which had been passed through a well washed Amberlite (IRA-400) anion-exchange column (24 in. $\times$ 1 in.) to remove molybdate. The Mo content of the sodium hydroxide after passage through the resin was $<0.001 \mu\text{g./ml.}$ as determined by bioassay (Nicholas, 1952). The Fe-deficient cultures were adjusted to pH 7.1 with redistilled 6 N-HCl. It was not found necessary to purify the glycerol.

Cultures grown in 1.5 l. Erlenmeyer flasks containing 400 ml. medium were shaken continuously on a reciprocator (2 in. throw) at 100 strokes/min. Gas mixtures of oxygen in nitrogen, prepared by means of rotameter tubes, were dispersed through the medium by No. 2 porosity glass sinters (250 ml. gas/min./flask). A 3% (v/v) inoculum grown in the appropriate medium was transferred into the large flasks aseptically and the cultures incubated at 24° for 18 hr.

Collection of organisms. Bacteria were harvested in an M.S.E. refrigerated centrifuge at 6,000 g at 4° and washed twice with 3% NaCl to remove nitrite.

Preparation of cell-free extracts. The washed bacteria lysed in distilled water (1 g. wet wt./10 ml.) were centrifuged at 25,400 g for 20 min. at 4° as described by Sadana & McElroy (1957). The sediment was re-extracted with water (1 g. wet

wt./15 ml.) and centrifuged as before. Most of the nitrate reductase activity was in the second water extract.

Nitrate reductase. Method 1. The enzymic reduction of nitrate by whole bacteria was followed anaerobically in Thunberg tubes by using reduced benzylviologen (BVH) as a hydrogen donor. In the side-arm, 0.2 ml. 10^{-3} M-benzylviologen (BV), 1 mg. palladized asbestos and 0.3 ml. 2.5×10^{-1} M-phosphate buffer (pH 7.5); in the tube 0.1 ml. 10^{-1} M-KNO₃, 0.1 ml. bacterial suspension (equiv. 2 mg. N/ml.), 0.3 ml. 2.5×10^{-1} M-phosphate buffer (pH 7.5). The tube was evacuated and flushed with high purity hydrogen passed through a Deoxo-catalytic deoxygenator (Baker Platinum Division, Englehard Industries Ltd., 52 High Holborn Street, London) at which stage the dye was fully reduced. The tubes when finally evacuated were pre-incubated for 5 min. at 30° before tipping in the reduced dye; the reaction was terminated after a further 10 min. by adding 0.1 ml. M-zinc acetate and 1.9 ml. 95 % (v/v) ethanol in water (Medina & Nicholas, 1957). After centrifuging at 4000 g for 5 min., nitrite was determined in a sample of the supernatant solution by the sulphanilamide method (Fewson & Nicholas, 1961).

Method 2. Reduced benzylviologen, prepared by the palladized asbestos-hydrogen method, did not function as a hydrogen donor for nitrate reductase in cell-free extracts. The dye reduced with sodium dithionite was, however, a suitable donor for the extracted enzyme (Sadana & McElroy, 1957). The specific activity of the enzyme is defined as $\mu\text{m-mole NO}_3^-$ formed/10 min./mg./bacterial total-N.

Nitric oxide (NO) reductase. The uptake of NO was measured in a Warburg apparatus. The main compartment contained 0.5 ml. bacterial suspension (equiv. 2 mg. N/ml.), 1.5 ml. 0.1 M-phosphate (pH 7.5); the side-arm contained 0.2 ml. 10^{-2} M-NADH₂, 0.1 mg. crystalline alcohol dehydrogenase, 0.1 ml. 5 % (v/v) ethanol in water, and the centre well 0.2 ml. 20 % (w/v) KOH. The vessels were flushed with oxygen-free nitrogen for 40 min. and then with approximately 20 % (v/v) NO in N₂ until damp litmus paper turned red when held in the exit gas stream from the side-arm stopper. The apparatus was equilibrated at 30° for 15 min. and then the NADH₂-generating system tipped in to start the reaction. The nitric oxide was prepared by the reaction: $3\text{Cu} + 8\text{HNO}_3 = 2\text{NO} + 3\text{Cu}(\text{NO}_3)_2 + 4\text{H}_2\text{O}$. The NO evolved was displaced by a slow stream of oxygen-free nitrogen and after passing through Dreschel bottles containing boiled water the gas mixture was collected over water in a glass aspirator. The NO content of the gas mixture was determined in a gas burette connected to a reservoir containing saturated pyrogallol in 20 % (w/v) KOH. The volume of gas was measured and oxygen introduced from a cylinder. After vigorous shaking the contraction in gas volume, due to the reaction $2\text{NO} + \text{O}_2 \rightarrow 2\text{NO}_2$, was measured. Nitrogen dioxide in the presence of excess O₂ dissolves readily in the alkaline pyrogallol. The gas mixtures prepared in this way contained between 20 and 25 % (v/v) NO in N₂. Oxygen must be rigorously removed from the system since it reacts readily with NO to give NO₂ which dissolves in water to give a mixture of nitrous and nitric acids.

Protein and total-nitrogen determinations. Protein was determined by the Folin method (Lowry, Rosebrough, Farr & Randall, 1951) and the total bacterial-nitrogen by micro-Kjeldahl. The ¹⁵N technique used was that described by San Pietro (1957) and Simms & Cocking (1958).

RESULTS

Identity of the organism used. The organism has the following characteristics: single short rods with rounded ends, motile, non-sporing, 2 mm. convex off-white colonies with shiny surface and an entire edge, self-luminous; is Gram-negative, aerobic, insensitive to 'vibriostat' (Vibriostatic compound 0/129; 2,4-diamino-6-7-di-isopropyl pteridine; Spencer, 1955 made by Difco Laboratories, Michigan, U.S.A.), liquefies gelatin, produces acid from glucose, sucrose, maltose and mannitol, reduces nitrate to nitrite.

These results suggest that this bacterium, which has been used under the name *Achromobacter fischeri* by Sadana & McElroy (1957), is very similar to *Photobacterium sepia* described by Spencer (1955). The genus *Photobacterium* is a member of the Pseudomonadaceae and is no longer referred to as *Achromobacter* (*Bergey's Manual*, 1957). Since *P. fischeri* is a curved rod sensitive to vibriostat and does not liquefy gelatin, it seems likely that the organism used here is *P. sepia*. Spencer (1955) suggested that *P. sepia* be placed in the genus *Aeromonas* and *P. fischeri* in the genus *Vibrio*.

Table 1. *Nitrate reductase activity of Photobacterium sepia whole organisms, in relation to the nitrogen source in the growth medium*

Nitrogen compounds in culture medium	Nitrate reductase specific activity Hydrogen donors for the enzyme	
	Reduced benzylviologen (BVH)	NADH ₂
	(μm-mole NO ₂ ⁻ formed/10 min./mg. bacterial-N)	
KNO ₃	560	0
KNO ₃ + peptone	1090	680
NH ₄ NO ₃	1190	40
NH ₄ NO ₃ + peptone	2860	1300
NH ₄ Cl	3090	—
NH ₄ Cl + peptone	4650	—

Table 2. *The uptake of ¹⁵N by Photobacterium sepia from various forms of nitrogen in relation to molybdenum requirements and nitrate reductase activity of the organisms*

Culture solutions A and B were equivalent in total nitrogen.

Nitrogen source (g./l. medium)		Mo content (μg./l. medium)	Specific activity of enzyme*	Bacterial-N (mg.)	¹⁵ N atom % excess in bacterial-N
A	5 g. ¹⁵ NH ₄ NO ₃ (3.05 atom % excess N)	0	2	672	2.35
		10	1020	546	2.71
B	6.3 g. K ¹⁵ NO ₃ (3.08 atom % excess N) 3.35 g. NH ₄ Cl	0	330	496	0.35
		10	820	504	0.32

* μm-mole NO₂⁻ formed/10 min./mg. bacterial-N.

Effect of various factors on nitrate reductase activity

Nitrogen source. The results in Table 1 show that the nitrate reductase activity was greatest when the ammonium radical was the only nitrogen source. Thus the enzyme is constitutive since it is formed in the absence of its substrate. The inclusion of peptone in the medium not only increased enzyme production but also enhanced its stability in organisms stored at 4° for 24 hr. The results in Table 2 show that molybdenum deficiency restricted nitrate reductase activity in organisms grown with NH_4NO_3 or $\text{KNO}_3/\text{NH}_4\text{Cl}$. The ^{15}N data confirm that ammonia was more readily utilized than was nitrate.

Fig. 1

Fig. 2

Fig. 3

Fig. 1. The effect of oxygen pressure and molybdenum content of the culture medium on nitrate reductase activity and total bacterial-N of *Photobacterium sepia* grown with NH_4Cl (6.5 g./l.). Ordinate left: specific activity of nitrate reductase (μm-mole NO_2^- /10 min./mg. bacterial-N). Ordinate right: mg. bacterial-N/400 ml. culture. Abscissae: 0, 10, 100 μg. Mo/l. culture medium. Top: 1% (v/v) O_2 in N_2 ; middle: 10% (v/v) O_2 in N_2 ; bottom: 20% (v/v) O_2 in N_2 .

Fig. 2. The effect of oxygen pressure and molybdenum content of the culture medium on nitrate reductase activity and total bacterial-N of *Photobacterium sepia* grown with KNO_3 (12.2 g./l.). Ordinate left: specific activity of nitrate reductase (μm-mole NO_2^- /10 min./mg. bacterial-N). Ordinate right: mg. bacterial-N/400 ml. culture. Abscissae: 0, 10, 100 μg. Mo/l. culture medium. Top: 1% (v/v) O_2 in N_2 ; middle: 10% (v/v) O_2 in N_2 ; bottom: 20% (v/v) O_2 in N_2 .

Fig. 3. The effect of oxygen pressure and molybdenum content of the culture medium on nitrate reductase activity and bacterial-N of *Photobacterium sepia* grown with NH_4NO_3 (5 g./l.). Ordinate left: specific activity of nitrate reductase (μm-mole NO_2^- /10 min./mg. bacterial-N). Ordinate right: mg. bacterial-N/400 ml. culture. Abscissae: 0, 10, 100 μg. Mo/l. culture medium. Top: 1% (v/v) O_2 in N_2 ; middle: 10% (v/v) O_2 in N_2 ; bottom: 20% (v/v) O_2 in N_2 .

Aeration of cultures and effects of trace metals. The effects of nitrogen source (NH_4Cl , KNO_3 , NH_4NO_3), oxygen supply (0, 10 and 20 %, v/v, O_2 in N_2) and molybdenum content (0, 10, 100 $\mu\text{g./l.}$) of the culture medium on the nitrate reductase activity of the organisms are illustrated in Figs. 1, 2, 3. Molybdenum was required for nitrate reductase activity when the nitrogen source was either NH_4Cl or KNO_3 irrespective of the gas mixture used. At 20 % (v/v) O_2 level, molybdenum deficiency restricted growth when KNO_3 was sole nitrogen source, but was without effect when NH_4Cl or NH_4NO_3 were used. Total yields of organism were markedly decreased at 1 % (v/v) O_2 when the organism was grown with any of the nitrogen compounds tested.

Table 3. *Nitrate reductase activity and NO uptake of Photobacterium sepia in relation to the molybdenum content of the culture medium*

NH_4NO_3 was the nitrogen source; 10 % (v/v) O_2 in N_2 was sparged through the medium.

Mo content ($\mu\text{g./l.}$ medium)	Nitrate reductase specific activity ($\mu\text{m-mole NO}_2^-$ formed/10 min./mg. bacterial-N)	NO uptake $\mu\text{l. NO/hr/mg. bacterial-N}$
0	4150	20.4
2	3160	16.6
5	1870	11.9
10	1390	10.9
20	1910	12.8

The amount of constitutive nitrate reductase formed with NH_4Cl was markedly decreased by increasing the O_2 pressure in the medium (Fig. 1).

An unusual effect of molybdenum (10, 100 $\mu\text{g. Mo/l.}$) in depressing nitrate reductase activity and yield of organism was observed in the NH_4NO_3 cultures (Fig. 3). This occurred only when 10 % (v/v) O_2 was sparged through the cultures. Both nitrate reductase activity and the uptake of nitric oxide were depressed by increasing the Mo content of the culture media from 2 to 20 $\mu\text{g./l.}$ (Table 3). These toxic effects, however, became less pronounced after repeated subculturing in Mo-deficient medium (Table 4). At the 13th subculture the effects were reversed, since then a deficiency of Mo depressed nitrate reductase activity. The toxic effects did not occur with media which contained KNO_3 or NH_4Cl .

Table 4. *The effect of transferring Photobacterium sepia through several subcultures of medium deficient in molybdenum on the nitrate reductase activity of the organisms*

NH_4NO_3 was the nitrogen source; cultures were aerated with 10 % (v/v) O_2 in N_2 .

Mo content ($\mu\text{g./l.}$ medium)	Number of transfers					
	2		12		13	
	Specific activity* of enzyme	Bacterial-N (mg.)	Specific activity of enzyme	Bacterial-N (mg.)	Specific activity of enzyme	Bacterial-N (mg.)
0	9100	22.4	7950	14.7	790	15.3
10	3550	13.2	5600	10.1	3280	15.3
100	3140	14.5	5360	10.1	3410	13.1

* Specific activity of nitrate reductase $\mu\text{m-mole NO}_2^-$ formed/10 min./mg. bacterial-N.

An inhibition of nitrate reductase activity in organisms grown with NH_4NO_3 was also observed with vanadium and tungsten (Table 5). Tungsten brought about a drastic decrease in enzyme activity. Although nitrate reductase was markedly decreased in amount, the yields of organism were unaffected, since the ammonium radical was being utilized.

Table 5. *Effect of molybdenum, vanadium and tungsten on nitrate reductase activity in Photobacterium sepia*

NH ₄ NO ₃ medium, second subculture, aeration with 10% (v/v) O ₂ in N ₂ .			
	($\mu\text{g. metal/l. medium}$)	Nitrate reductase specific activity*	Bacterial-N (mg.)
Control	0	1840	22.7
Mo (as molybdate)	10	940	26.0
V (as vanadium oxychloride)	10	795	22.8
W (as tungstate)	10	173	22.0

* Nitrate reductase specific activity $\mu\text{mole NO}_2^-/10 \text{ min./mg. bacterial-N}$.

Fig. 4

Fig. 5

Fig. 4. The effect of the iron content of the culture medium on nitrate reductase activity of *Photobacterium sepia*.

Fig. 5. The effect of the iron content of the culture medium on the uptake of nitric oxide by *Photobacterium sepia*.

Nitrate reductase activity and nitric oxide uptake were both dependent on an adequate supply of iron in the culture medium (Figs. 4, 5); about 1.5 mg. Fe/l. produced maximal activity for nitrate and nitric oxide reductases.

Extraction of nitrate reductase

The nitrate reductase activity in cell-free extracts of *Photobacterium sepia* grown in peptone medium with KNO_3 or NH_4Cl , respectively was determined (Table 6). The enzyme appeared to be similar whether extracted from organisms grown with KNO_3 or with NH_4Cl ; in both cases the second water-extract contained most of the activity, and when the extracts were adjusted to pH 4.5 with acetic acid the enzyme was similarly distributed between the precipitate and the supernatant fractions. The extracts from organisms grown with KNO_3 had significantly higher

activities than did whole organisms. The amount of enzyme in whole organisms from these cultures increased markedly after storage overnight at 4°, e.g. from a specific activity of 555 to one of 2510. This suggests that an inhibitor of the enzyme was present in the freshly collected organisms.

Table 6. *The extraction of nitrate reductase from Photobacterium sepia grown in peptone medium with KNO₃ or NH₄Cl*

Nitrogen source	Preparation				
	Intact organisms	1st water extract	2nd water extract	Precipitate* obtained at pH 4.5	Supernatant liquid at pH 4.5
	Nitrate reductase specific activity†				
KNO ₃	380	410	1880	292	740
NH ₄ Cl	4590	340	2540	417	400

* Water extract adjusted to pH 4.5 by adding M-acetic acid and centrifuged for 10 min. at 25,000 g at 4°; precipitate taken up in 0.05 M-phosphate buffer (pH 6.5).

† Nitrate reductase specific activity: $\mu\text{m-mole NO}_3^-/10 \text{ min./mg. bacterial-N.}$

DISCUSSION

Contrary to a previous report, the halophylic bacterium *Photobacterium sepia* (previously assumed to be *Achromobacter fischeri* by Sadana & McElroy, 1957) grows with nitrate as sole nitrogen source. Since it requires molybdenum and iron for growth and for its nitrate reductase activity and since the amount of enzyme was decreased at high oxygen pressures, it resembles the dissimilatory nitrate reductase systems found in other bacteria and fungi (Verhoeven, 1952; Sato, 1956; Fewson & Nicholas, 1961; Walker & Nicholas, 1961; Nicholas, 1961, 1963).

It is of interest that nitrate reductase in *Photobacterium sepia* (*Achromobacter fischeri*) is constitutive; it is formed in this organism with ammonium chloride and without nitrate, its substrate. In other micro-organisms, nitrate or nitrite is required in the medium to produce nitrate reductase activity in the cells. The role of this constitutive enzyme in the organism grown with ammonium chloride is not clear, since under these conditions it has neither an assimilatory nor a dissimilatory function. Ammonia does not depress the formation of nitrate reductase in *P. sepia* as it does in *Neurospora*, where a 'feed back repression' mechanism functions presumably as a physiological control for nitrate utilization *in vivo* (Kinsky, 1961).

Pichinoty & d'Ornano (1961*a, b*) showed that the induction of nitrate reductase in *Aerobacter aerogenes* by nitrate was associated with a *de novo* synthesis of this enzyme from free amino acids and that this process was greatly inhibited in aerobic cultures. They established that oxygen reversibly inhibited the biosynthesis of nitrate reductase. A similar inhibitory effect of oxygen on the production of this enzyme has now been observed in *Photobacterium sepia* grown with either nitrate or ammonia.

The unusual inhibitory effect of molybdenum on growth and on nitrate reductase activity in *Photobacterium sepia* grown with NH₄NO₃ at 10% (v/v) O₂ and the annullment of this effect after 13 subcultures, is difficult to explain. At first sight it might seem that the toxic effect of molybdenum is on the uptake of the ammonium radical, since ¹⁵N experiments showed that about seven times as much nitrogen was

derived from that source. This, however, is not borne out by the fact that no toxic effects from molybdenum were observed in cells grown with ammonium chloride. Vanadium and especially tungsten had an effect like molybdenum. Tungstate was shown by Higgins, Richert & Westerfeld (1956) to be a competitive inhibitor of nitrate reductase in *Neurospora* and Takahashi & Nason (1957) obtained similar results with *Azotobacter*.

The uptake of nitric acid was depressed in *Photobacterium sepia* deficient in iron; similar results were obtained by Fewson & Nicholas (1960) and in *Pseudomonas fluorescens* (unpublished results).

We are grateful to Miss Anne Morrison (National Collection Type Cultures), Torry Research Station, Aberdeen, for carrying out the biochemical tests that identified the organism used here as *Photobacterium sepia* and for establishing the purity of the cultures; to Mr D. J. Fisher for assistance with ^{15}N experiments.

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