NITRATE REDUCTASE OF NITRATE RESPIRATION TYPE FROM *E. COLI*

I. SOLUBILIZATION AND PURIFICATION FROM THE PARTICULATE SYSTEM WITH MOLECULAR CHARACTERIZATION AS A METALLOPROTEIN*

S. TANIGUCHI AND E. ITAGAKI

Department of Chemistry, Faculty of Science, Nagoya University, Chikusa, Nagoya (Japan)

(Received February 11th, 1960)

SUMMARY

A particulate nitrate reductase system which included cyt. b_1 as an intermediary carrier from formate or DPNH to nitrate was prepared from *E. coli* cells grown anaerobically in the presence of nitrate; this system possessed remarkably high activity of nitrate reductase of the nitrate respiration type. The particulate preparation, devoid of activities suggestive of not only reduced pyridine nucleotides oxidases but also dehydrogenases except for formate was, as far as has been tested, functionally characterized by its highly active formate–nitrate reductase system. Nitrate reductase, the terminal enzyme of the particulate system, was solubilized by cold alkaline incubation after heat treatment and then purified to a homogeneous state. The enzyme was shown to have a molecular weight of a million and to contain one atom of bound molybdenum and about 40 atoms of bound iron per molecule but no bound flavin. A difference spectrum (oxidized minus reduced) having a broad peak at 445–450 mμ disappeared rapidly on the addition of nitrate with simultaneous production of nitrite. The turnover number of the enzyme was estimated to be about $7.0 \cdot 10^3$ moles/sec from reduced methylviologen, the most effective electron donor. Examination of the physiological donors for the purified enzyme showed that reduced forms of pyridine nucleotides were never effective and flavin derivatives were little effective. Data which suggested that reduced cyt. b_1 would be a more favorable donor for nitrate reductase were presented. The homogeneously purified enzyme seems to function as a terminal enzyme in a nitrate-respiring system *in vivo* and may be regarded as an anaerobic variant of terminal oxidases. Biochemical comparison between *E. coli* enzyme (nitrate respiration type) and *Neurospora* enzyme (nitrate assimilation type) as well as their electron-transferring mechanisms have been discussed.

Abbreviations: nitrate reductase, NaR; cytochrome, cyt.; formate dehydrogenase, FDH; reduced methyl or benzyl viologen, MVH or BVH; methylene blue, Mb; reduced di- or triphosphopyridine nucleotide, DPNH or TPNH; flavinadenine dinucleotide, FAD; flavin mononucleotide, FMN; free riboflavin, FR; 2-heptyl-4-OH-quinoline-N-oxide, HOQNO; *p*-chloromercuribenzoate, PCMB.

* A part of this study was reported in the 11th Symposium on Enzyme Chemistry held at Kyoto, Japan and in the Symposium on Haematin Enzymes held at Canberra in 1959[1].

INTRODUCTION

Recent studies on the enzymic mechanism of microbial nitrate reduction have shown that the reduction does not have a single mechanism and is of no common metabolic significance. The multifunctional nature of nitrate reduction can be now understood by classifying it into two types[2-4].

The first type, "nitrate respiration type", has been found in the group of facultative anaerobes such as *E. coli*. *E. coli* NaR which has been studied in details in this laboratory was, one decade ago, suggested by SATO *et al.*[5] to be an iron enzyme. Then, the enzyme from aerobically grown *E. coli* cells was confirmed by TANIGUCHI *et al.*[6] and IIDA *et al.*[7] to be a terminal enzyme in a particulate electron-transferring system with cyt. b_1 functioning as the indispensable electron carrier. The particulate NaR together with cyt. b_1 has been solubilized by deoxycholate or iso-butanol treatment[7] and the properties of the soluble enzyme systems have been also described[7-9]. Recently, highly active NaR of anaerobically grown *E. coli* cells solubilized by steapsin or chymotrypsin digestion and partially purified was tentatively suggested by TANIGUCHI *et al.*[9] as a metalloflavoprotein. However, any definite conclusions on the chemical nature of the enzyme have never been drawn.

On the other hand, the second type, "nitrate assimilation type", has been found in the group of aerobes such as *Neurospora*. *Neurospora* enzyme, extensively studied by NASON's group in the McCollum–Pratt Institute, has been proved to be a soluble molybdoflavoprotein[10] which does not include any participation of *Neurospora* cytochromes[3].

The present paper describes the solubilization and purification of NaR to a homogeneous state from a particulate electron-transferring system of nitrate-respiring *E. coli* cells together with the physical and chemical characterization of the homogeneous enzyme as not a metalloflavoprotein but as a metalloprotein.

MATERIALS AND METHODS

Micro-organism

E. coli, Yamagutchi strain, was anaerobically grown in 70 l of a medium of the following composition in a 100 l stainless steel tank at 30° with occasional N_2 gas bubbling: 1 % polypeptone (Wako Chemicals), and meat extract paste (Kyokuto Chemicals), 0.1 % KNO_3, casamino acid (Difco Chemicals), and yeast extract powder (Wako Chemicals), 0.2 % K_2HPO_4 and 2.0 % glucose, pH 7.2. The liquor of young cells preliminary grown under the same conditions was inoculated to produce the concentration of 5 % (v/v). As soon as the logarithmic growth phase was accomplished (about 5.5–7.0 h after the inoculation), the liquor was immediately cooled on ice and any solid formed was separated and washed with distilled water; nitrite was removed by a Sharples ultracentrifuge in the cold. The cell paste obtained can be stored in a frozen state at −15° for about 2 weeks without any noticeable loss of MVH-NaR activity.

Cell disruption and preparation of particulate NaR

All the procedures were carried out below 4° unless otherwise described. The frozen cells were ground in a mortar for 30 min with twice their weight of alumina

powder (Wako No. 800) and for another 10 min with five times their weight of cold 0.1 *M* phosphate buffer, pH 7.1 and then centrifuged at 2000 × *g* for 20 min to obtain the cell-free extract. By centrifuging the cell-free extract at 15,000 × *g* for 30 min, the particulate preparation was obtained as a precipitate; this was washed twice with cold distilled water. The particulate preparation of thick paste can be stored in a frozen state at −15° for about 2 weeks without any noticeable loss of MVH-NaR activity.

Other materials

Calcium phosphate gel was prepared according to the method of KEILIN *et al.*[11] and was aged before use for at least a week. The gel was adjusted to contain about 30 mg dry weight per ml of gel. DPNH and TPNH were prepared by the enzymic reduction of the respective nucleotides of 70% or more purity by the methods of PULLMAN *et al.*[12] and by EVANS *et al.*[13], respectively. Authentic samples of FAD and FMN were kindly donated by Dr. J. OKUDA. MV and BV were purchased from British Drug Houses, Ltd., and were used after recrystallization. HOQNO was kindly donated by Dr. R. SATO and used according to the method described by LIGHTBOWN *et al.*[14]. Mammalian cyt. *c* was purchased from L. Light and Co. Ltd.

Solubilization and purification of the particulate NaR preparation

All procedures were carried out at below 4° unless otherwise described. The concentration of the particulate preparation was adjusted to an appropriate value (0.2–0.6 mgN/ml) in 5 m*M* Na_2HPO_4 (pH 8.3) containing 0.1 m*M* of KNO_3 and heated at 60° for 5 min. The heated suspension was immediately cooled to 4° by ice-water and kept for about 15–20 h. The soluble NaR was obtained as the supernatant after Sharples centrifugation at 50,000 × *g* for 10 min. To this supernatant, was added 1 *M* acetate buffer, pH 5.0 to produce a final concentration of 0.02 *M* and calcium phosphate gel of 70–150 mg dry weight per 100 ml of supernatant; the solution was then stirred for 10 min and allowed to stand for 10 min. The gel was collected and twice washed with distilled water and once with 2–10 m*M* phosphate buffer, pH 7.1. A large proportion of the adsorbed enzyme could be eluted from the gel by a phosphate buffer of 0.1 *M*, pH 7.1 containing 0.1 m*M* glutathione as an enzyme stabilizer. The combined eluates (about 80 ml from 100 g cell paste) were fractionated with $(NH_4)_2SO_4$ with standing for 20 min after the slow addition of the solid salt to obtain the fraction at between about 38–53% saturation. The precipitate was dissolved with about half the volume of the same medium and the fraction between about 30–40% saturation was again obtained in the same manner. The resulting precipitate was dissolved in 0.05 *M* phosphate buffer pH 7.1 containing 0.1 m*M* of glutathione of volume one quarter that of the original eluate and was subjected to ultracentrifugation in a Spinco Model L at 110,000 × *g* for 3–4 h. Owing to the large molecular weight of NaR described later, the active material was precipitated and washed three times with the same medium in order to remove the inactive lighter components. Typical results of the solubilization and purification together with the ultracentrifugal patterns of the intermediate and final fractions are summarized in Table I.

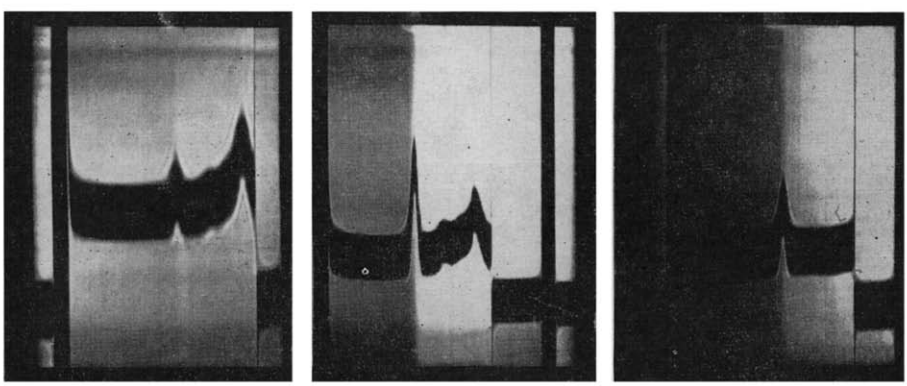
In steps 3, 4 and 5 as designated in Table I, preliminary small scale tests must

always be conducted to determine the most favorable conditions for each purification. The final preparation can be stored for one week in the same phosphate buffer containing glutathione in a frozen state at —15° with less than 30–40 % loss of MVH-NaR activity.

TABLE I

SOLUBILIZATION AND PURIFICATION OF *E. coli* NITRATE REDUCTASE

Step No.	*Fraction*	*Total N (mg)*	*Units (× 10⁻⁴)*	*Specific activity (× 10⁻³)*
1	Cell paste	(270 g wet weight)		0.18
2	Particulate preparation	2500	135	0.54
3	Supernatant solubilized after heat treatment	580	100	1.72
4	Calcium phosphate gel eluate	227	90	3.95
5	$(NH_4)_2SO_4$ 1st ppt. (38–53 % sat.)	55.2	80	14.5
6	$(NH_4)_2SO_4$ 2nd ppt. (30–40 % sat.)	11.2	50	56.2
7	Spinco L 1st pellet	3.04	40	132
9	Spinco L 3rd pellet	1.50	28	186



Step 5 Step 7 Step 9

Sedimentation patterns of nitrate reductase preparations in phosphate buffer, pH 7.1, ionic strength, 0.1. The direction of sedimentation is from right to left and the photographs were taken 24 min after the rotor attained full speed (59,780 r.p.m.). In these experiments, average rotor temperature was 13.5°, protein concentration approximately 0.5 %, and bar angle 55°. The activity was always shown to be associated with the fastest peak in each pattern.

Solubilization of the particulate NaR preparation by dodecylsulfate treatment

To the particulate preparation suspended in 0.05 *M* phosphate buffer, pH 7.1 in 3–5 mg N/ml concentration, was added a 10 % solution of dodecylsulfate to give a final concentration of 0.5–1.0 %. After standing for 2 h at 30°, a reddish brown supernatant was obtained after centrifugation at 20,000 × *g* for 40 min and this was used as the dodecylsulfate solubilized preparation.

Solubilization of the particulate NaR preparation by steapsin or chymotrypsin treatment

The method of solubilization by means of steapsin (Nutritional Biochemical Corp. from pig pancreas) was previously described by the authors[9]. Using the same conditions as for the steapsin method, chymotrypsin (Nutritional Biochemical Corp.)

could be applied in place of steapsin. Pepsin, trypsin, lipase (from *Sclerotinia libertiana*), and crystalline proteases (from *B. subtilis* and *Streptomyces griseus*) were shown to be without effect under the above conditions.

Electrophoretic, ultracentrifugal and spectrophotometric analyses

Criteria of purity for the enzyme preparations were examined with a Spinco Model H electrophoretic apparatus and a Spinco Model E ultracentrifuge. Spectrophotometric analysis was carried out with a Beckman spectrophotometer DK-2 or a Hitachi electrospectrophotometer Model ERB-U.

Determination of enzyme-bound Mo by spectrographic analysis

To determine the Mo content in the homogeneous preparation, an enzyme sample and, as a blank test substance, the almost inactive protein which could be separated at step 7 in Table I as an ultracentrifugal supernatant were simultaneously dialyzed for 20–50 h at 4° in the same bottle against deionized distilled water with stirring and one exchange of the outer solution after several hours from the start of the dialysis. After dialysis, equal volumes of aliquots of the enzyme sample, inactive protein and the outer solution were placed in clean glass tubes and lyophilized. After drying the lyophilized samples for 3 h in an Abderhalden apparatus at 100°, the weights of the three samples were determined. Usually 3–7 mg of the protein samples were oxidatively decomposed by 1.0 ml of fuming nitric acid and 0.5 ml of 30% hydrogen peroxide. After adding the spectroscopically pure graphite powder in approximately equal amounts to those of the protein samples, the mixtures were evaporated to dryness under an infra-red lamp at 110° for 4 h. Definite amounts of the dried materials were then well mixed in an agate mortar with approximately equal amounts of spectroscopically pure sodium chloride which was found to enhance the emission lines, and then the mixtures were subjected to emission spectrography using the cathode layer method. The Mo content actually found in the enzyme sample should be corrected for the minute contents of Mo (about 10–15% of that of the enzyme sample) found in the inactive protein and the outer solution (in both samples the contents were approximately equal) probably resulting from the phosphate salt reagent used as the medium in the enzyme preparation. The determination was carried out by Drs. C. IIDA AND K. YAMASAKI in the Laboratory of Inorganic Chemistry of our Department. For the details of the procedures employed, their papers[15,16] should be consulted.

Determination of enzyme-bound total iron

As already described in the procedure for Mo determination, 0.5 ml of 1 *N* HCl was added to the appropriate volume of the dialyzed enzyme sample and inactive protein or the outer solution employed as the blank material, and decomposed by heating. After the addition of 2.0 ml of 2 *M* acetate buffer, pH 4.0, 5.0 ml of 10% aqueous solution of NH_2OH–HCl and a suitable amount of ammoniacal aqueous solution (1:3) to maintain the final pH at 4.0 correctly, the mixture was allowed to stand for 5 min at room temperature to completely reduce the ferric iron. After developing the green color by the addition of *o*-nitrosoresorcinol monomethyl ether reagent described by TORII[17], the colored solution was washed three times with CCl_4 and centrifuged to remove the turbidity due to any remaining CCl_4. Then, the total

iron was determined colorimetrically (at a wave length of 700 mμ) for the clear supernatant.

Determination of enzyme-bound flavins

The determination was carried out fluorometrically by the lumiflavin method[18] and D-amino acid oxidase assay[19]. In the latter assay, the homogeneous preparation which was boiled for 10 min in a neutral pH condition was treated.

Enzyme assay

(a) *MVH method*: This assay was carried out on a mixture containing 0.05 μmole of MV, 50 μmoles of KNO_3, 250 μmoles of phosphate buffer, pH 7.1 and the enzyme solution with a total volume of 0.5 ml in an open tube at 30°. After 5 min preincubation, the reaction was started by the addition of small amounts (less than 1 mg) of dithionite. After the reaction had proceeded for 5 min, the tube was immediately shaken to oxidize the remaining MVH by the air. The amount of nitrite formed was colorimetrically determined by Griess-Ilosvay reagent. This activity is referred to MVH–NaR in this paper. In testing the other donors, MVH was replaced by others. In the assay of the activities of the particulate preparation, the reaction was carried out in a Thunberg tube in order to restrain the particulate oxidase activity.

(b) *Spectrophotometric method*: Sometimes, the activity was spectrophotometrically assayed utilizing a reduced dye as the electron donor by measuring the change in the characteristic absorption of each reduced dye (*i.e.*, in the case of MVH, by measuring the decrease of the optical density at 590 mμ and using the corresponding molecular extinction coefficient of MVH which has been determined to be $7.65 \cdot 10^6$ cm^2/mol at pH 7.1) using a Thunberg tube-type cuvette. In these cases, a stoichiometrical relationship between the amounts of the reoxidized dye and the nitrite formed was established.

Definition of NaR units and the specific activity

One unit of MVH–NaR was defined as the amount of the enzyme which produced 1 μmole of nitrite in 1 h under the above conditions. The specific activity was defined as units per mg Kjeldahl nitrogen.

Other assay methods

The assay method with Mb and the expression of the units and the specific activity for FDH and other dehydrogenases was as previously described[7]. The protoheme content in the particulate preparation was determined by the method of MOSS[20].

RESULTS

Nitrate-respiring E. coli *cells*

The multi-functional character of the nitrate reduction of *E. coli* has already been reported by TANIGUCHI *et al.*[2] who demonstrated that this microbe can perform simultaneously two different types of nitrate metabolism: nitrate respiration (anaerobiosis) and apparent nitrate assimilation (aerobiosis).

The former activity can be shown by measuring the anaerobically reduced

amount of nitrate in the presence of a suitable electron donor such as formate. The anaerobic reduction of nitrate cannot proceed further than the nitrite stage and is profoundly retarded by low oxygen pressure, reflecting the character of this nitrate respiration[2].

On the other hand, the latter activity can be detected by measuring the aerobically reduced amount of nitrate in the presence of a suitable respiratory substrate such as formate[21]. Under the aerobic conditions, accompanying the simultaneous oxidation of formate, nitrate can be reduced to nitrite and to ammonia which is also rapidly metabolized suggesting an apparently assimilative character of the aerobic reduction[2,21].

As shown in Table II, *E. coli* cells grown aerobically in the standard medium with shaking possess much higher activity of aerobic reduction than that of anaerobic reduction. In the anaerobically grown cells, on the contrary, much lowered activity of aerobic reduction and about 20-fold enhanced activity of anaerobic reduction can be found. The addition of nitrate to the medium which supports anaerobic growth was found to stimulate not only the bacterial growth and formate–NaR activity but also to enhance the cyt. b_1 content of the cells. Thus, *E. coli* cells anaerobically grown by apparently respiring nitrate and used in the present study were shown to possess an active mechanism for nitrate respiration.

TABLE II

NITRATE-REDUCING ACTIVITIES OF *E. coli* CELLS

Reaction mixture: KNO_3, 100 μmoles; formate, 1 μmole; phosphate buffer pH 7.1, 0.08 *M*; cell suspension. Total volume, 3.0 ml at 30°. Figures represent the reduced NO_3^- (μmoles) per h and mg N of the cells.

Reaction condition	*Growth condition*	
	Aerobic	*Anaerobic*
Aerobic* ($\longrightarrow NH_3 \dashrightarrow$)	30–50	0–5
Anaerobic** ($\longrightarrow NO_2^-$)	10–20	200–500

* The reaction was carried out in a Warburg vessel with vigorous shaking under air and the nitrate reduced was measured according to the procedure previously described[2,21].

** The reaction was carried out in a Thunberg tube under anaerobic conditions and the nitrite produced was measured according to the procedure previously described[7].

Particulate NaR system and the solubilized NaR systems

Examination of localization patterns of a NaR system by centrifugal fractionation of the cell-free extract revealed that about 70% of the units of MVH–NaR, formate–NaR and protoheme contents together with about twice* the number of FDH units which were initially found in the cell-free extract were concentrated in the particulate preparation. The specific activities of the MVH–NaR and FDH of the particulate preparation have the values of 200–1000 and 300–1000, respectively. The present localization patterns are essentially the same as those previously reported in the case of aerobically grown *E. coli* cells[2,7] indicating that these enzymes are bound to cellular structures.

* This suggests the presence in the cell-free extract of some uncharacterized factor inhibiting FDH activity.

For the enzymic reduction of nitrate by the particulate preparation, as shown in Table III, natural electron donors such as $FMNH_2$, formate and DPNH (effective in this order) but not TPNH could be utilized. Among the artificial donors, MVH and BVH, both one-electron donors with very low redox potentials, were the most effective.

TABLE III

COMPARISON OF ELECTRON DONORS FOR PARTICULATE *E. coli* NITRATE REDUCTASE ACTIVITY

Electron donors except formate, DPNH and TPNH were reduced by dithionite as described in MVH method. Concentrations of these electron donors were all in sufficiently saturating levels for the particulate NaR activity.

Electron donor (2 mM in final concentration)	*Units (relative value)*
Methylviologen-H	100
Benzylviologen-H	100
Phenosafranine-H_2	58
Pyocyanine-H	55
Methylene blue-H_2	5.3
Phenazine methosulfate-H	2.7
FMN-H_2*	31
FAD-H_2*	29
Formate**	16
DPNH*	6.8
TPNH*	0–2

* 0.2 m*M*.
** 50 m*M*.

The difference spectrum (reduced minus oxidized) of the particulate preparation of which the reduced state was anaerobically maintained by the FDH activity is shown in Fig. 1.

The peaks of the difference spectrum at 432, 532, and 560 mμ clearly indicate the abundant presence of cyt. b_1 in the particulate preparation. As seen from the

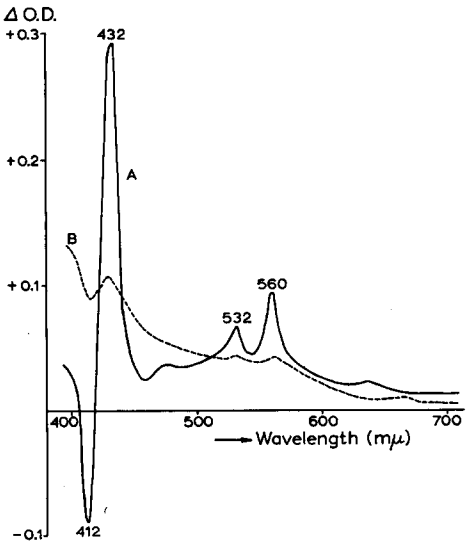


Fig. 1. Difference spectra of the *E. coli* particulate preparation. When the particulate preparation of 1.8 mg N was anaerobically incubated in the presence of 50 μmoles of phosphate buffer, pH 7.1 and sodium formate in a Thunberg tube-type cuvette for 15 min, the difference spectrum (reduced minus oxidized) was observed (Curve A). The spectrum obtained by reducing with formate was very similar to that reduced with dithionite except that partial reduction (about 80%) was observed with formate. After adding 10 μmoles of solid KNO_3 from the side tube into the main tube, the difference spectrum (Curve A) changed to Curve B within 1–2 min.

same figure, cyt. b_1 enzymically reduced by formate was instantaneously re-oxidized by the addition of nitrate together with the simultaneous production of nitrite.

As shown in Table IV, formate–NaR activity is strongly inhibited by HOQNO, the specific inhibitor for cyt. b_1. The same extent of inhibition of DPNH–NaR activity by quinoline oxide was also found whereas no inhibition of the MVH–NaR

TABLE IV

EFFECT OF INHIBITORS AND ACTIVATORS ON THE PARTICULATE *E. coli* NITRATE REDUCTASE ACTIVITY

Compound	*Final concentration (M)*	*Inhibition (%)*	
		from formate	*from MVH*
Amytal	$2 \cdot 10^{-3}$	75	20*
Menadione**	$5 \cdot 10^{-3}$	40*	0
Dicumarol	$3 \cdot 10^{-5}$	70	25
Dicumarol + Menadione	$(3 \cdot 10^{-5}) + (5 \cdot 10^{-3})$	70	—
HOQNO	10^{-5}	85	0
PCMB	$5 \cdot 10^{-4}$	60	20
Phenazine methosulfate	$3.3 \cdot 10^{-4}$	160*	5

* Activation per cent.
** Ethanol solution was used with the appropriate control.

activity by the compound could be confirmed. These observations led the authors to the conclusion that cyt. b_1 participated in the reaction as an indispensable intermediary carrier for the formate– and DPNH–NaR but not for the MVH–NaR. Table IV also shows that formate–NaR activity differs remarkably from MVH–NaR activity in its sensitivity towards amytal, dicumarol (never reversed by menadione) and PCMB suggesting the participation in the former system of flavoprotein, a dicumarol-sensitive site and an –SH group, respectively. Among the redox dyes tested, only phenazine methosulfate caused noticeable activation probably owing to its functioning as an effective intermediary carrier in place of cyt. b_1. Cyanide and azide at 0.1 m*M* final concentrations resulted in an appreciable inhibition (65 and 80%, respectively) of both formate– and MVH–NaR activities, indicating a heavy metal component probably in the common terminal enzyme of the particulate system. Various chelating reagents tested* caused no remarkable inhibition.

Michaelis constants (K_m) of the particulate formate–NaR for nitrate and formate were estimated to be $1.3 \cdot 10^{-4}$ *M* and $7.0 \cdot 10^{-4}$ *M*, respectively.

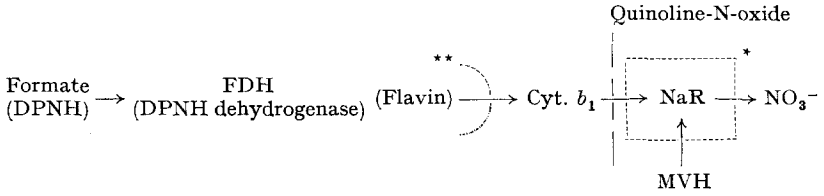
Regardless of the kind of buffers tested (glycyl–glycine, Tris and phosphate), the pH optimum for the particulate formate–NaR activity was 7.0–7.2 and the activity was not specifically affected.

The particulate preparation was found to be devoid of not only dehydrogenase activity (Mb assay) for lactate, succinate, β-OH-butyrate and glucose, but hydrogenase (H_2 production from MVH), DPNH and TPNH oxidase activities. Besides the very strong activity of FDH, only a weak activity of formate oxidase was found.

Summarizing the results described above, the particulate electron-transferring system to the nitrate of *E. coli* of nitrate respiration type can be illustrated by the following scheme.

* Chelators tested were the same as those described in the footnote on p. 277.

PARTICULATE *E. coli* NITRATE REDUCTASE SYSTEM



* Only the terminal moiety is solubilized by either alkaline cold incubation, or steapsin or chymotrypsin digestion after heat treatment.

** This functional connection can be split by dodecylsulfate treatment which simultaneously solubilizes all the entities FDH, cyt. b_1 and NaR bound in the particulate preparation.

The recovery of the activities of particulate enzymes in the solubilized preparations which can be brought about by three different procedures—alkaline cold incubation after heat treatment, steapsin or chymotrypsin treatment and dodecylsulfate treatment is summarized in Table V. The former two treatments can specifically solubilize only MVH–NaR activity. On the other hand, dodecylsulfate treatment can solubilize a large proportion of the FDH activity and cyt. b_1 whereas formate– and DPNH–NaR activities have never been detected in the solubilized preparation. However, as in the case of the original particulate preparation, it was found that a reduced form (with dithionite) of cyt. b_1, present in the dodecylsulfate-solubilized preparation, was quite an effective electron donor for nitrate reduction by the preparation. As shown in the previous scheme, it appears that a factor functionally connecting DPNH and formate with cyt. b_1 was removed by the dodecylsulfate treatment while alkaline incubation more specifically cleaved only the terminal entity, MVH–NaR. A similar mode of cleavage was found also in the case of solubilization by means of steapsin or chymotrypsin. The highly active preparations were purified chromatographically (Dowex-2) after solubilization by hydrolyzing enzymes[9] and were found to exhibit several diffuse peaks with a slow rate of

TABLE V

RECOVERY OF ACTIVITIES OF PARTICULATE ENZYMES IN THEIR SOLUBILIZED PREPARATIONS

Fraction	*Relative units (particulate preparation taken as 100)*				*Relative content of cyt. b_1***
	MVH → NaR	*FDH (→ Mb)*	*formate → NaR*	*DPNH → NaR*	
Particulate preparation	100	100	100	100	+++
Solubilized supernatant prepared by:					
Alkaline cold incubation after heat treatment	70*	0	0	0	±
Steapsin or chymotrypsin digestion after heat treatment	85*	0	0	0	±
Dodecylsulfate treatment	95*	75*	0	0	++

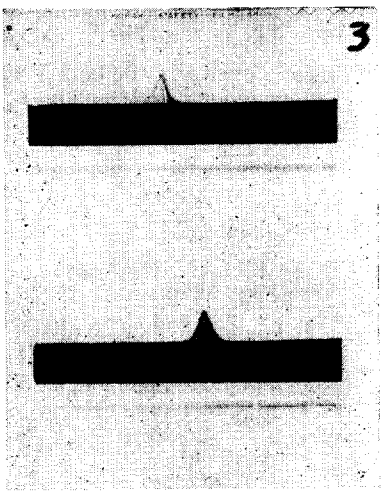
* These activities were found to remain in the high speed supernatant (110,000 × *g* for 1 h) of each solubilized preparation.

** Intensity of α-band (560–565 mμ) of cyt. b_1 reduced with dithionite was spectroscopically observed.

sedimentation in the patterns from the ultracentrifugal analysis which indicated heterogeneity probably caused by enzymic splitting of the NaR molecule*. For the purpose of enzyme purification the milder procedure of solubilization by cold alkaline incubation after heat treatment was advantageously employed. The effect of dodecylsulfate treatment is similar to that of isobutanol treatment employed for the particulate preparation from aerobically grown *E. coli* cells[7].

Physical and chemical characterizations of homogeneous NaR

Molecular constants and turnover number of NaR: The sedimentation and electrophoretic patterns of the final preparation are shown in Table I and Fig. 2, respectively. A single component with a symmetrical pattern associated with the brown–yellow



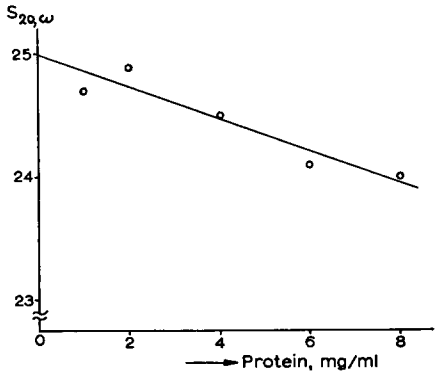


Fig. 3. Plot of $S_{20,w}$ of *E. coli* nitrate reductase against protein concentration.

Fig. 2. Electrophoretic patterns of nitrate reductase (specific activity, 180,000) in phosphate buffer, pH 7.1, ionic strength, 0.1. In this experiment, the time of electrophoresis was 120 min, protein concentration, 0.5%, current, 2.5 mA and temperature, 2.2°. Upper diagram: ascending pattern. Lower diagram: descending pattern.

color of the enzyme was found in each analysis. The electrophoretic mobility of the enzyme at pH 7.1 was estimated under the conditions stated in Fig. 2 to be $-9.55 \cdot 10^{-5}$ cm^2 $volt^{-1}$ sec^{-1}.

The measurement of the molecular weight of the enzyme was carried out by Dr. N. Ui of Gunma University. From the data presented in Fig. 3, the sedimentation coefficient ($S_{20,w}$) of nitrate reductase was estimated by extrapolation to zero concentration to be 25.0 Svedberg units. The measurement of the diffusion constant in the same buffer and at 0.4% protein concentration showed that the value of $D_{20,w}$ was $2.27 \cdot 10^{-7}$ cm^2 sec^{-1}. Assuming the partial specific volume of the protein to be 0.75 ml g^{-1}, the molecular weight of nitrate reductase was calculated to be a million. By assuming that the nitrate reductase molecule is a prolate ellipsoid without hydration, the frictional ratio (f/f_0) estimated to be 1.38 gives an axial ratio of 7–8 suggesting a considerable deviation of the molecular shape from a spherical one. On the basis of this established molecular weight, the specific activity of MVH–NaR of the final

* For the experimental evidence, see the legend for Table VII.

preparation of $13–22 \cdot 10^4$ gives the turnover number of the nitrate reductase as $5.2–8.8 \cdot 10^3$ moles/sec.

Enzyme-bound metals: The results of spectrographic analysis revealed the characteristic presence of Mo and Fe in the homogeneous enzyme samples. Besides these metals, small but significant amounts of Mg and trace amounts of Mn were detected. However, the presence of almost equal amounts of these metals were confirmed in the blank materials.

As shown in Table VI, on the basis of a molecular weight of a million and the dry weight of the enzyme protein, the Mo content determined indicates that the nitrate reductase has one atom of Mo per molecule. On the other hand, the Fe content determined colorimetrically indicates the presence of about 40 non-heme Fe atoms per molecule. Prolonged dialysis of the enzyme protein against deionized distilled water seemed to cause no significant change in the contents of these enzyme-bound metals.

Flavin detection: Data from the analysis of flavin components in the homogeneous preparation, as shown in Table VII, indicate that the detectable flavin components in the enzyme are almost near the limit of error of the determination. It is concluded

TABLE VI

METALLIC CONTENTS IN *E. coli* NITRATE REDUCTASE

Expt. No.	*Specific activity* ($\times 10^{-4}$)	*Dialysis time against deionized water (h)*	*Number of atoms per molecule*	
			Mo	*Fe*
1	16	20	1.2–0.8*	45
2	18	15	—	43
		50	1.1–0.7**	36
3	18	36	—	42
4	14	30	—	25

* Determined by the internal standard method with Pd and V[15,16].
** Determined by the visual method[15,16].

TABLE VII

FLAVIN DETECTION

Expt. No.	*Specific activity of nitrate reductase* ($\times 10^{-4}$)	*Detected as free riboflavin (μg/g dry weight)*	*Number of flavin molecules per molecule of nitrate reductase*
1	22	2.8	0.009
2	22*	0.0	0.000
3	18	0.8	0.002
4	15	0.2	0.0005
5	20	—	0.02**

* The enzyme protein used in expt. 1 was digested by chymotrypsin of weight 8% that of NaR at pH 8.15 and 37° for 3 h. By ultracentrifugal analysis the digested mixture was shown to consist of almost a single component having a smaller value of sedimentation coefficient in spite of no remarkable loss of activity during the digestion. For this digested mixture, fluorometric assay was carried out.
** This was determined by D-amino acid oxidase assay.

therefore, that the enzyme-bound flavin component is, if any, a trace and not significant in its amount which indicates that the present final product is a metalloprotein but not a metalloflavoprotein. From the authors' previous data including the absorption spectra of the partially purified preparation, NaR was temporarily regarded as a metalloflavoprotein[9]. In fact, the partially purified preparation (*i.e.*, preparation at step 5) showed a spectrum similar to that previously presented[9]. However, large portions of the contaminated inactive flavoproteins were shown to be effectively eliminated in the initial $(NH_4)_2SO_4$ precipitate in steps 5–6 and also in the ultracentrifugal supernatant in steps 7–9.

Absorption spectra: As seen from Fig. 4, the homogeneous preparation had no specific absorption peak except that of protein in the vicinity of 277 mμ and the absorbance decreased gradually over the entire near u.v. and visible region with

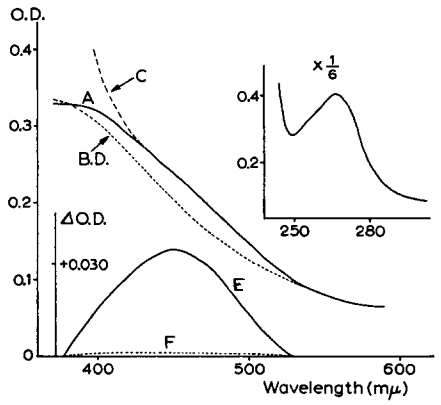


Fig. 4. Absorption spectra of *E. coli* nitrate reductase. The homogeneous enzyme preparation of specific activity, 150,000 and of 1.9 mg protein/ml concentration in 0.02 *M* phosphate buffer, pH 7.1 was used. Temperature, 25°: A, oxidized form (without additions); B, reduced with $NaBH_4$ (less than 1 mg) (after 5 min); C, A + DPNH (3 μmoles) (after 30 min); D, B + CO (1 atm.) (after 30 min incubation in the dark); E, A (oxidized form) – B (reduced with $NaBH_4$); F, A (oxidized form) – B (reduced with $NaBH_4$ plus 10 μmoles solid KNO_3) (after 5 min). The deviation of curve C from curve A in the short wave length region was due to the absorption caused by the DPNH added.

increasing wave length, indicating none of the characteristics of a heme enzyme. The difference spectrum (oxidized minus reduced) showed a broad peak at 445–450 mμ which instantly disappeared under anaerobic conditions on the addition of nitrate with the simultaneous production of nitrite. However, the oxidized spectrum could not be transformed by means of DPNH to a reduced one such as that formed with $NaBH_4$. The reduced spectrum was not affected by darkness and anaerobic incubation with 1 atm. of CO. A deproteinization procedure for the homogeneous preparation which consisted of treatment with cold trichloroacetic acid (5% of final concentration), yielded a completely colorless supernatant and a yellow precipitate of the denatured enzyme protein. Though the nature of these spectra cannot yet be fully understood, the brown–yellow color may presumably be due to combination between the enzyme protein and heavy metals.

Other properties: Table VIII shows that MVH and BVH are still the most effective electron donors for the activity of the homogeneous preparation in view of their values of both V_{max} and K_m. There seems to be no essential difference in the reactivity with the electron donors listed in Table VIII from those exhibited by the particulate enzyme (Table III) except that the reactivities for reduced flavin derivatives exhibited by the purified enzyme are relatively much reduced. Ferricyanide, $FeSO_4$, ascorbate, reduced mammalian cyt. *c*, reduced cyt. c_3*, hypoxanthine and DPNH were all inactive as electron donors for nitrate reduction. No detectable

* The purified sample of cyt. c_3 was kindly donated by Dr. M. ISHIMOTO.

activity of MVH–, DPNH– and TPNH–cyt. *c* (mammalian) reductase, aldehyde or xanthine dehydrogenase (from acetaldehyde or hypoxanthine to indophenol dye), even in the presence of externally added FAD, was confirmed in the homogeneous preparation. Remarkable sensitivities for cyanide and azide but not for various chelators tested* were seen for the MVH–NaR activity representing essentially

TABLE VIII

COMPARISON OF ELECTRON DONORS FOR *E. coli* NITRATE REDUCTASE

The spectrophotometric assay method was employed.

Electron donor (reduced with dithionite)	*Vmax. (relative value)*	K_m *for the donor* ($\times 10^{-5}$ *M*)
Methylviologen-H	100	>0.1*
Benzylviologen-H	100	>0.1*
Phenosafranine-H_2	84	>0.1
Methylene blue-H_2	2.8	4.5
FAD-H_2	1.2	1.3 — 2.0
FMN-H_2	6.0	2.6
FR-H_2	3.2	1.6

* These figures, though they could not be determined quantitatively, were much less than those for phenosafranine-H_2.

TABLE IX

COMPARISON BETWEEN PROPERTIES OF *E. coli* ENZYME (NITRATE RESPIRATION TYPE) AND *Neurospora* ENZYME (NITRATE ASSIMILATION TYPE)

	E. coli enzyme	*Neurospora enzyme*
Enzyme-bound components:		
Mo	1 atom /molecule	Present
Fe	~40 atoms/molecule	Not reported
flavin	Not detected	FAD
Preferable electron donor	Reduced methylviologen	Reduced indophenol
Physiologically required electron donor	Reduced cyt. b_1	TPNH
K_m for NO^-_3	$5.1 \cdot 10^{-4}$ *M* (from reduced methylviologen)	$1.4 \cdot 10^{-3}$ *M* (from TPNH)
Cyt. *c* reductase activity	Not detected	Actively associated
Cytochrome participation	Cyt. b_1	None
Original form	Particulate	Soluble
Physiological function	Nitrate respiration	Nitrate assimilation

* 8-OH-quinoline, *o*-phenanthroline, α,α′-dipyridyl, diethyl dithiocarbamate (these in 1 m*M* final concentration), *o*-nitrosoresorcinol monomethyl ether (0.24 m*M*), thioglycolate (10 m*M*) and allyl thiourea (0.5 m*M*) produced only about 10–30% inhibition. Neither NaF, ethylenediamine tetraacetic acid (these in 10 m*M*), salycylaldoxime (1 m*M*) nor dithizon (80 μ*M*) exhibited any inhibitory effect.

similar sensitivities to those in the case of particulate MVH–NaR activity. Moreover, the same sensitivities as described above were also found for $FADH_2$–NaR activity. No significant sensitivity for CO in the dark was found for not only the activities of MVH– and $FADH_2$–NaR of the homogeneous preparation but also those of formate–, DPNH–, and MVH–NaR of the particulate preparation.

The optimum pH for MVH–NaR activity was 7.1 in phosphate buffer, which was found to be a more effective buffer for restoring MVH–NaR activity than Tris, citrate and pyrophosphate buffers. The Michaelis constant (K_m) for the MVH–NaR of the homogeneous preparation for nitrate was estimated to be $5.1 \cdot 10^{-4}$ *M*. Heat treatment at 60° for 5 min resulted in no inactivation while treatment at 70° caused 70 % inactivation.

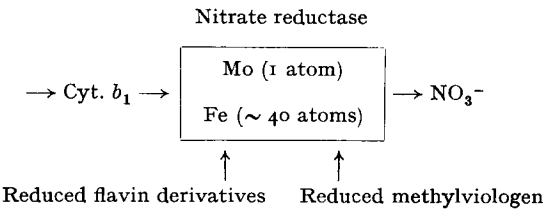
DISCUSSION

Summarizing the results described above, a comparison of properties between *E. coli* enzyme and *Neurospora* enzyme is presented in Table IX.

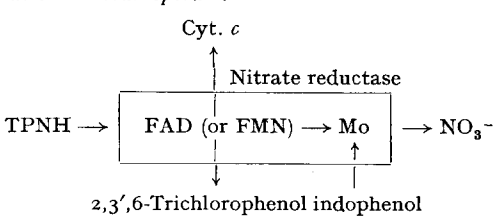
The electron-transferring sequences of these two NaR systems are also schematized as follows. In the light of the general functioning of Mo recently established

ELECTRON-TRANSFERRING SEQUENCES OF *E. coli* NITRATE REDUCTASE AND OF *Neurospora* NITRATE REDUCTASE

(a) Nitrate respiration in *E. coli*:



(b) Nitrate assimilation in *Neurospora*[10]:



in the NaR of *Neurospora*[10,22] and higher plants[23] or in the action of xanthine dehydrogenase from milk and liver[24–26], Mo found in the enzyme protein seems to take part in the terminal electron transfer of the nitrate reduction. On the other hand, the presence of bound iron atoms has not been reported for the *Neurospora* enzyme. The function and significance of the iron atoms still remain to be elucidated. It is of some interest, however, that the large molecule, succinate dehydrogenase, a flavoprotein of molecular weight of about several millions, was recently isolated from *M. lactilyticus* and was shown to bind multiple non-heme iron atoms of which the

function is also not yet clarified[28]. The other most noticeable difference from the *Neurospora* enzyme is that the *E. coli* enzyme has no significant level of bound flavin component and neither DPNH nor TPNH, but possibly reduced cyt. b_1 can specifically function as a physiological and direct electron donor. *E. coli* enzyme has been purified by the milder procedure in which, generally speaking, the possibility that a flavin moiety would be released from an enzyme or would be decomposed seems improbable.

The present enzyme cleaved from the terminal moiety of the particulate system of *E. coli* grown anaerobically in the presence of nitrate may be regarded as an anaerobic variant of the terminal oxidases and the functional system of nitrate respiration *in vivo*.

Evidence for the functioning of Mo by valency change in enzymic reactions as well as data indicative of the nature of the bound iron atoms and the acceptor specificity of the enzyme will be presented in a second paper[29].

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to Professor F. EGAMI for his valuable and encouraging suggestions, and to Mr. K. OHMACHI for his kind collaboration and discussions throughout this work. The authors are deeply indebted also to Drs. N. UI and C. IIDA for their co-operation. Thanks are also due to Drs. J. OKUDA, K. TAKATA and Mr. S. TSUKAMOTO of Nagoya University for making facilities available for carrying out the fluorometric flavin assay, for carrying out the electrophoretic and ultracentrifugal analyses, respectively.

This research was supported in part by a grant from the Scientific Research Fund of the Japanese Ministry of Education.

REFERENCES

1 F. EGAMI, M. ISHIMOTO AND S. TANIGUCHI, *Proceedings of the Symposium on Haematin Enzymes*, Canberra, 1959, in the press.
2 S. TANIGUCHI, R. SATO AND F. EGAMI, in W. D. MCELROY AND B. GLASS, *Inorganic Nitrogen Metabolism*, The Johns Hopkins Press, Baltimore, U.S.A., 1956, p. 87.
3 R. SATO, in W. D. MCELROY AND B. GLASS, *Inorganic Nitrogen Metabolism*, The Johns Hopkins Press, Baltimore, U.S.A., 1956, p. 163.
4 A. NASON, *Ann. Rev. Microbiol.*, 12 (1958) 203.
5 R. SATO AND F. EGAMI, *Bull. Chem. Soc. Japan*, 22 (1949) 137.
6 S. TANIGUCHI, A. ASANO, K. IIDA, M. KONO, K. OHMACHI AND F. EGAMI, *International Symposium on Enzyme Chemistry, Tokyo and Kyoto, 1957*, Maruzen Co., Ltd., Tokyo, 1958, p. 238.
7 K. IIDA AND S. TANIGUCHI, *J. Biochem.*, 46 (1959) 1041.
8 E. ITAGAKI AND S. TANIGUCHI, *J. Biochem.*, 46 (1959) 1419.
9 S. TANIGUCHI AND E. ITAGAKI, *Biochim. Biophys. Acta*, 31 (1959) 294.
10 A. NASON, in W. D. MCELROY AND B. GLASS, *Inorganic Nitrogen Metabolism*, The Johns Hopkins Press, Baltimore, U.S.A., 1956, p. 109.
11 D. KEILIN AND E. F. HARTREE, *Proc. Roy. Soc. London*, B124 (1938) 397.
12 M. E. PULLMAN, A. SAN PIETRO AND S. P. COLOWICK, *J. Biol. Chem.*, 206 (1954) 129
13 H. J. EVANS AND A. NASON, *Plant Physiol.*, 28 (1954) 233.
14 W. J. LIGHTBOWN AND F. L. JACKSON, *Biochem. J.*, 58 (1954) xlix.
15 C. IIDA AND K. YAMASAKI, *Biochim. Biophys. Acta*, 44 (1960) 352.
16 C. IIDA, *J. Spectroscopical Soc. Japan*, 8 (1960) 141.
17 T. TORII, *Bull. Chem. Soc. Japan*, 76 (1955) 328, 333.
18 K. YAGI, *Biochemistry of Flavins*, Kyoritsu Co., Ltd., Tokyo, 1957.
19 O. WARBURG AND W. CHRISTIAN, *Biochem. Z.*, 298 (1939) 150.
20 F. MOSS, *Australian J. Exptl. Biol. Med. Sci.*, 32 (1954) 571.
21 S. TANIGUCHI AND M. KONO, *J. Biochem.*, (1960) in the press.

[22] D. J. D. NICHOLAS AND H. M. STEVENS, in W. D. MCELROY AND B. GLASS, *Inorganic Nitrogen Metabolism*, The Johns Hopkins Press, Baltimore, U.S.A., 1956, p. 178.
[23] D. J. D. NICHOLAS AND A. NASON, *Plant Physiol.*, 30 (1955) 135.
[24] W. D. MCELROY AND B. GLASS, *Inorganic Nitrogen Metabolism*, The Johns Hopkins Press, Baltimore, U.S.A., 1956, chapter VI.
[25] W. W. WESTERFELD, D. A. RICHERT AND R. J. BLOOM, *J. Biol. Chem.*, 234 (1959) 1889.
[26] W. W. WESTERFELD, D. A. RICHERT AND E. S. HIGGINS, *J. Biol. Chem.*, 234 (1959) 1897.
[27] M. G. P. J. WARRINGA, O.H. SMITH, A. GIUDITTA AND T. P. SINGER, *J. Biol. Chem.*, 230 (1958) 97.
[28] M. G. P. J. WARRINGA and A. GIUDITTA, *J. Biol. Chem.*, 230 (1958) 111.
[29] E. ITAGAKI AND S. TANIGUCHI, manuscript in preparation.