Studies on Nitrate Reductase. VI. Acceptor Specificity of the Enzyme

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Despite a considerable amount of knowledge obtained in our laboratory on nitrate reductase of *Escherichia coli*,[1]~[5] the acceptor specificity of this enzyme remains largely uninvestigated, except a few informations indicating that nitrate reductase of *E. coli* can reduce chlorate as well as nitrate,[1] and that molecular oxygen can not supplant the function of hydrogen acceptor for this enzyme.[6],[7]

Since there are a number of organic nitrogen compounds derived from or structurally related to nitrate, such as nitric acid esters, nitramines, and aliphatic and aromatic nitro compounds, it seems desirable to study whether these compounds can also serve as hydrogen acceptors for nitrate reductase. Among them, nitro compounds are of special interest in view of the long known facts that nitro groups of numerous aromatic compounds are reduced by many microorganisms and animal tissues. Furthermore, in recent years, a considerable attention has been paid to the biochemical significance of nitro groups, since two nitro compounds, chloramphenicol[8] and β-nitropropionic acid[9],[10] have been isolated from biological sources, contrary to the hitherto universally presumed idea that nitro compounds do not occur in nature. It appears, therefore, of interest to find a relation, if exists, between

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(1) F. Egami and R. Sato, *J. Chem. Soc. Japan*, **68**, 39 (1947).

(2) F. Egami and R. Sato, *J. Chem. Soc. Japan*, **69**, 160 (1948).

(3) R. Sato and F. Egami, This Bulletin, **22**, 137 (1949).

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(6) M. Niwa, R. Sato and S. Taniguti, *Symposia on Enzyme Chem.*, **6**, 40 (1951).

(7) L. H. Stickland, *Biochem. J.*, **25**, 605 (1931).

(8) J. Ehrlich, Q. R. Bartz, R. M. Smith, D. A. Joslyn and P. R. Burkholder, *Science*, **106**, 417 (1947).

(9) C. L. Carter and W. J. McChesney, *Nature*, **164**, 575 (1949).

(10) M. T. Bush, O. Touster and J. E. Brookman, *J. Biol. Chem.*, **188**, 685 (1951).

the metabolism of these compounds and nitrate reductase.

The results described below, however, indicate that none of the nitrogen compounds tested can replace nitrate as hydrogen acceptor for nitrate reductase.

Materials and Methods

Dried cells of *E. coli*, which contain nitrate reductase and dehydrogenase systems with their carrier systems, were used as the enzyme preparation. The organism was grown on a peptone-bouillon-agar medium containing 0.1 per cent potassium nitrate for 24 hours at 37°. The cells were then harvested and washed thrice with physiological saline and once with distilled water. The washed cells were dried by lyophilization in the presence of phosphorous pentoxide and stored at 0° in a vacuum desiccator. Dried cell suspension was prepared just before the use by suspending 2—5 mg. of the dried cells in distilled water.

Most experiments were conducted at 37° under an anaerobic condition with the use of Thunberg tubes. Sodium formate was used as the hydrogen donator throughout the investigation. The reaction mixture usually consisted of 1 ml. of each dried cell suspension, $M/10$—$M/25$ sodium formate, substrate solution, $M/5$—$M/15$ phosphate buffer of *p*H 7.4 or 7.6, and distilled water (or inhibitor solution). At the end of incubation periods the reaction mixtures were deproteinized with 2 ml. of saturated uranyl acetate solution or 20 per cent trichloroacetic acid. The reduction of nitrate was followed by determining the formation of nitrite in the deproteinized filtrates. Determination of nitrite was performed by a colorimetric method using Griess-Ilosvay reagent,[11] and analysis for aromatic amines formed from the corresponding nitro compounds was made by the method of Smith and Worrel,[12] N-[1-naphthy]-ethylene diamine being substituted by α-naphthylamine as coupling reagent.

Reoxidation of leuco form of methylene blue by nitrogen compounds was tested as follows. One ml. of each dried cell suspension (3 mg. of the cells), $M/5$ phosphate buffer of *p*H 7.4, $M/25$ sodium formate, and 10^{-4} M methylene blue was pipetted into the main room of a Thunberg tube, and 1 ml. of the solution of the compound to be tested into the side arm. The tube was then incubated at 37° until the dye was completely decolorized. Then the contents of the both parts were mixed. If the compound can reoxidize the leuco dye in the presence of the dried cells, recolorization of the dye takes place immediately after the mixing.

Results

Action of the Dried Cells upon Ethyl Nitrate.

—Ethyl nitrate was used as a representative of nitric acid esters. When the ester is acted by the dried cells of *E. coli*, one may anticipate the following four possibilities: (1) the ester is first hydrolyzed spontaneously or by an esterase to liberate inorganic nitrate, which is in turn reduced to nitrite by nitrate reductase; (2) the ester is enzymatically reduced to the nitrous acid ester; (3) the ester combines with nitrate reductase, but no further change takes place; and (4) no reactions occur at all.

The results of experiments (Curves I and II, in Fig. 1) showed that no nitrite is produced from ethyl nitrate by the action of the dried cells. The hydrolysis of the ester, therefore, seems to be improbable. To test the formation of the nitrous acid ester, the reaction mixture of Curve I was deproteinized after 3 hours incubation and acidified to *p*H 1.0 with hydrochloric acid and then boiled for 30 minutes to hydrolyze the ester. However, no nitrite liberation was detected by this procedure. This seems to exclude the second possibility. Heppel and Hilmoe[13] have reported an enzyme in hog liver which catalyzes the reduction of nitroglycerol and erythritol tetranitrate by glutathione or cysteine in the presence of cyanide. The addition of cysteine (10^{-2} M) and potassium cyanide (4×10^{-3} M), however, did not activate our preparation to reduce ethyl nitrate. It was further found, as is recorded in Fig. 1 (Curves III and IV), that nitrate reduction by the dried cells is entirely

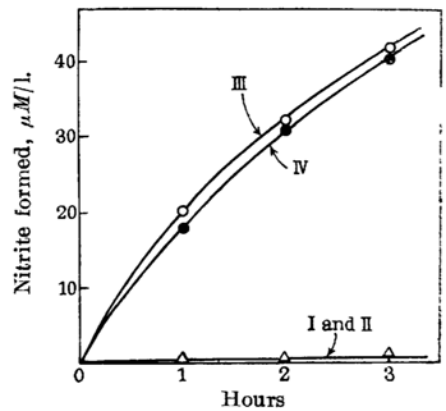

Fig. 1.—Action of the dried cells of *E. coli* on ethyl nitrate (4×10^{-3} M/l.) under aerobic (Curve I) and anaerobic (Curve II) conditions; and reduction of KNO_3 (2×10^{-4} M/l.) by the cells in the presence (Curve III) and absence (Curve IV) of ethyl nitrate (4×10^{-3} M/l.). *p*H = 7.6, and 3 mg. of the dried cells were contained in 5 ml. of medium.

(11) S. Yamagata, *Acta Phytochimica*, **10**, 283 (1938).
(12) G. N. Smith and C. S. Worrel, *Arch. Biochem.*, **24**, 216 (1949).
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unaffected by the presence of ethyl nitrate at a concentration 20-fold as much as that of the inorganic nitrate supplied. This fact appears to show that the third possibility is also unlikely. After all, we are compelled to take the fourth possibility and assume that the ester is indifferent to nitrate reductase at all.

That ethyl nitrate has no affinity to nitrate reductase receives further support from the observation that the ester can not reoxidize leuco methylene blue in the presence of the cells, in contrast to the fact that inorganic nitrate can rapidly recolorize the leuco dye under the influence of nitrate reductase.[11]

Effect of Nitrourea on Nitrate Reduction.—Nitrourea, a nitramine, was found to be a strong inhibitory reagent for nitrate reductase. Nitrate reduction by the dried cells was almost completely inhibited by $4\times10^{-3}\,M$ nitrourea. This compound failed to reoxidize leuco methylene blue.

Effect of Nitromethane on Nitrate Reduction.—As a means of elucidating an interaction between nitromethane and nitrate reductase, the effect of this aliphatic nitro compound on the activity of nitrate reductase was studied (Fig. 2). It was found that $4\times10^{-3}\,M$ nitromethane exhibits no inhibition on nitrate reduction by the dried cells, concentrations of nitrate being varied from $2\times10^{-5}\,M$ to $4\times10^{-3}\,M$. This fact may be thought to indicate that nitromethane has no affinity to nitrate reductase. Furthermore, leuco form of methylene blue was not recolorized by this compound in the presence of the cells.

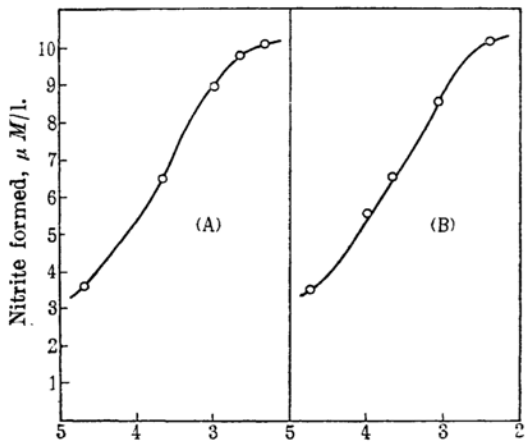

Fig. 2.—Activity-pS-curve of nitrate reduction by the dried cells of *E. coli* in the presence (A) and absence (B) of nitromethane (4×10^{-3} M/l.). pH = 7.6, and 3 mg. of the cells were contained in 5 ml. of medium. Incubation period = 120 minutes.

While rabbit liver homogenates have been reported to decompose nitromethane liberating nitrite ion both under aerobic and anaerobic conditions,[14] the dried cells of *E. coli* produced no nitrite from nitromethane under any conditions.

Reduction of p-Nitrobenzoic Acid to p-Aminobenzoic Acid.—The dried cells of *E. coli* were found to reduce p-nitrobenzoic acid (PNBA) to p-aminobenzoic acid (PABA) in the presence of formate. But the amount of PABA produced was very small (Table 1). This reduction was inhibited by $2\times10^{-3}\,M$ cyanide about 80 per cent. Aerobic condition also retarded the reduction, and in the absence of formate the formation of PABA was decreased to 65 per cent of the complete system.

Table 1

The Reduction of PNBA to PABA by the Dried Cells of *E. coli*

Exp. No.	1	2	3	4	5	6
Dried cell suspension (3 mg. per ml.), ml.	1	1	1	1	1*	1
Phosphate buffer (pH 7.4, M/5), ml.	1	1	1	1	1	1
PNBA ($2\times10^{-3}\,M$), ml.	1	1	1	1	1	—
Sodium formate(M/10), ml.	1	1	—	1	1	—
Distilled water, ml.	1	1	2	1	1	3
Condition	an.	an.	an.	ae.	an.	an.
Incubation period, min.	60	120	120	120	120	120
PABA formed ($10^{-5}\,M$)	0.2	0.3	0.2	0.2	0	0

* The suspension was heated at 100° for 5 minutes. an. = anaerobic; ae. = aerobic.

It was further found that PNBA reduction is also inhibited by nitrate to a small extent. At the first glance this fact was thought to be an evidence indicating that there may be a competitive interaction between PNBA and nitrate. Further study of the effect of PNBA on nitrate reduction, however, provided a strong evidence suggesting that PNBA is indifferent to nitrate reductase. As will be seen in Fig. 3, PNBA ($1.4\times10^{-3}\,M$, a concentration 7-fold as much as the nitrate present) does not inhibit the reduction of nitrate ($2\times10^{-4}\,M$), and, moreover, there is even a slight acceleration in nitrite production. These phenomena can be explained as follows. The

(14) F. Egami and R. Sato, *Nature*, **165**, 365 (1950). F. Egami and M. Itahashi, *J. Biochem.*, **38**, 329 (1951).

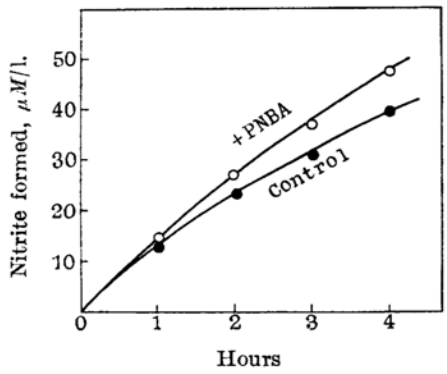

Fig. 3.—Effect of PNBA on nitrate reduction by the dried cells of *E. coli*. pH=7.4, PNBA=1.4×10^{-3} M/l., KNO_3=2×10^{-4} M/l., and 2 mg. of the dried cells were contained in 5 ml. of medium.

enzyme system responsible for PNBA reduction is not identical with that for nitrate reduction, but the observed reduction of PNBA is due to nitrite reductase system contained in the dried cells of *E. coli* at a minute concentration,* and consequently there exists a competitive relation between PNBA reduction and nitrite reduction (but not nitrate reduction). Thus the inhibitory action of nitrate to PNBA reduction mentioned above is not directly caused by nitrate itself, but by nitrite formed from nitrate as the product of its reduction. The slight acceleration of nitrite formation by PNBA is also explicable, since PNBA prevents in a competitive manner the further reduction of nitrite once formed. This competition between nitrite and aromatic nitro groups in their reduction by bacteria has been more clearly demonstrated with a cell-free extract from a bacterial strain, which lacks nitrate reductase, but possesses a much greater activity of nitrite reductase than *E. coli*. We have already published[15] such a competitive interaction between the reduction of the nitro group of chloramphenicol and that of nitrite.

The failure of PNBA to reoxidize leuco methylene blue in the presence of the dried cells of *E. coli* also supports the view that PNBA has no affinity to nitrate reductase.

Several aromatic nitro compounds tested other than PNBA (including chloramphenicol, nitrobenzene, and p-nitrosalicylic acid) were also found to behave similarly.

Discussion

From the evidence presented in this paper it seems very probable that all the nitrogen compounds tested are unable to accept hydrogen from formate by way of nitrate reductase system. Though the compounds tested in the present study are only a few representatives of many related compounds, it might be expected that all the other nitric acid esters, nitramines, and nitro compounds also behave similarly toward nitrate reductase. Thus, based on the results available up to the present, we might be able to state that only two compounds, nitrate and chlorate, can be reduced by this enzyme system. Chlorate has been known to be reduced to chlorite by *E. coli* and some other bacteria.[16] The ability of this compound to accept hydrogen from nitrate reductase was concluded by us[1] from the observations that chlorate, which may be regarded as a chlorine analog of nitrate, exihibts a marked competitive inhibition on nitrate reduction by a cell-free preparation of nitrate reductase from *E. coli*. We have further found, by analyzing the inhibition data, that the apparant Michaelis constant for chlorate reduction by this enzyme is $10^{-2.98}$ M/l., while that for nitrate reduction is $10^{-3.85}$ M/l. Chlorate reduction, however, could not be regarded as a normal function of this enzyme, since its reduction product, chlorite, has been shown to be a very powerful bacterial poison.[16] Quastel, Stephenson, and Whetham[16] claimed that for chlorate reduction is responsible an enzyme which is similar but distinct from nitrate reductase. It might be quite natural that Stephenson[17] was led by this opinion to a somewhat queer conclusion, which she wrote as follows: "Chlorate is a salt which so seldom forms a part of bacterial substrate that it can hardly worth while to possess an enzyme for using it.... the (chlorate reducing) enzyme can hardly be of service to the cell except for purposes of suicidal war". On the other hand, according to our demonstration of the identity of the chlorate reducing enzyme with nitrate reductase, such a queer consideration is not necessary; chlorate reduction is a mere result of a chemical resemblance of chlorate to nitrate, which permits the former to act as hydrogen acceptor for nitrate reductase.

* The content of nitrite reductase in *E. coli* varies from a strain to the other. One strain is completely devoid of it, while the other contains a relatively high activity. But our experiences indicate that the cell-free preparations of nitrate reductase extracted from many strains of *E. coli* by ultrasonic destruction of the cells are free from nitrite reductase activity.

(15) F. Egami, M. Ebata and R. Sato, *Nature*, **167**, 118 (1951).

(16) J. H. Quastel, M. Stephenson and M. D. Whetham, *Biochem. J.*, **19**, 304 (1925).

(17) M. Stephenson, in "Perspectives in Biochemistry", Cambridge, 1938, p. 92.

Summary

Evidence is presented to indicate that ethyl nitrate, nitromethane, and possibly also nitrourea are not able to accept hydrogen from nitrate reductase system of *E. coli*. *p*-Nitrobenzoic acid is reduced to *p*-aminobenzoic acid by the action of the dried cells of *E. coli* to a small extent. This reduction is, however, not achieved by nitrate reductase. Nitrite reductase contained in the organism is responsible for the reduction of aromatic nitro groups.

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