Studies on Nitrate Reductase. III.[1][2]

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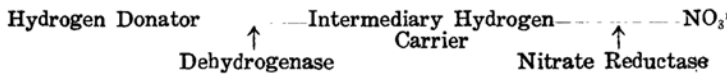
Introduction. Many species of bacteria are able to reduce nitrate to nitrite. The actual agency of this reduction, however, remained obscure until Quastel, Stephenson and Whetham[3] pointed out the existence of a specific enzyme acting on nitrate in Bac. coli and some other facultative anaerobes. In 1938–39 Yamagata[4] obtained a cell-free preparation of this enzyme and proposed the name "nitrate reductase" instead of "nitratase" which had been previously used. Recently we have succeeded in obtaining a cell-free preparation of nitrate reductase from Bac. coli by ultrasonic destruction of the cells and studied several properties and the mode of action of this enzyme.[1][2]

By these foregoing investigations it has been firmly established that nitrate reductase is an enzyme which catalyzes the oxidation of the reduced form of an intermediary hydrogen carrier by nitrate and therefore the biological reduction of nitrate consists of two steps even in the simplest case. This case may be shown schematically as follows:



(I)

As the intermediary hydrogen carrier in this scheme, methylene blue or

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

(2) F, Egami and R. Sato, *J. Chem. Soc. Japan*, **69** (1948), 20; cf. F. Egami, *Chemical Researches*, **1** (1948), 117.

(3) J. H. Quastel, M. Stephenson and M. D. Whetham, *Biochem. J.*, **19** (1925), 304; cf. L. H. Stickland, *Biochem. J.*, **29** (1934), 1543; E. Aubel, *Enzymologia*, **4** (1937), 51, etc.

(4) S. Yamagata, *Acta Phytochimica*, **10** (1938), 283; **11** (1939), 145.

any other suitable oxidation-reduction indicator can be used *in vitro*. For the purpose of the study of nitrate reductase apart from dehydrogenase system, we have constructed the following system.

$$\text{Leucomethylene blue} \longrightarrow \underset{\text{Nitrate Reductase}}{\uparrow} \longrightarrow NO_3' \qquad \text{(II)}$$

Here the leuco form of methylene blue is prepared chemically by catalytic hydrogenation of methylene blue using Pd-$BaSO_4$ as a catalyst.

Further we have shown that flavin-adenine-dinucleotide, and presumably also several flavoprotein enzymes, can act as intermediary hydrogen carriers. However we found an important exception to scheme (I) when succinate was supplied as a hydrogen donator. In this case the succinate-succinic dehydrogenase system can react directly with the nitrate-nitrate reductase system without auy intermediary hydrogen carrier.

$$\text{Succinate} \longrightarrow \underset{\text{Succinic Dehydrogenase}}{\uparrow} \longrightarrow \underset{\text{Nitrate Reductase}}{\uparrow} \longrightarrow NO_3' \qquad \text{(III)}$$

It must be mentioned here that succinic dehydrogenase is known as a so-called "cytochrome reducing dehydrogenase".

All these findings provide an interesting information about the position of nitrate reductase in the reaction chains of biological oxidations. That is, nitrate reductase may perhaps play a similar rôle to that of the Warburg-Keilin system when nitrate is used as an ultimate hydrogen acceptor instead of molecular oxygen.

From these considerations we have been led to an assumption that the chemical nature of nitrate reductase might be similar to that of cytochrome or cytochrome oxidase. In the present paper studies on this line will be reported.

Experimental. Preparation of enzyme: Bac. coli was grown on peptone-bouillon-agar medium containing 0.1 % KNO_3 for 24 hours at 37°. Cells were harvested and washed several times with 0.8 % NaCl solution and suspended thickly in phosphate buffer (*p*H 7.4, $\frac{1}{10}$ mol). The suspension was then exposed to ultrasonic waves (500 KC., anode voltage 1200 volts) for 30 minutes and was centrifuged (10,000 r.p.m.) to remove cell debris completely. The supernatant fluid was dialysed overnight against streaming water. To this solution ammonium sulfate was added to an extent of 0.2 saturation and the precipitate was centrifuged off. Ammonium sulfase was again added to half saturation, and then centrifuged. The precipitate thus obtained was dried in a vacuum desiccator containing concentrated sulfuric acid. Thus we could obtain from ca. 50 g. wet cells ca. 1 g. of dried powder mixed with crystalls of ammonium sulfate.

This preparation can be kept for several months without inactivation in a desiccator at 0°. 300 mg. of this powder was dissolved in 10 c.c. of distilled water and used as a nitrate reductase solution. Besides nitrate reductase our preparation shows a powerful activity of formic dehydrogenase when tested with methylene blue technique. For the purpose of spectroscopic observations solutions containing 100 mg. of powder per c.c. were used.

Measurement of enzyme activity: Thunberg tubes were employed. Reaction mixture was usually as follows: 0.5 c.c. of enzyme solution, 1 c.c. of phosphate buffer (*p*H 7.4, $\frac{1}{10}$ mol), 1 c.c. of KNO_3 solution ($\frac{1}{100}$ mol), 2 c. c. of leucomethylene blue ($\frac{1}{750}$ mol), and 1 c.c. of inhibitor solution or distilled water. The tube was incubated for 1 hour at 37° and the nitrite thus formed was determined.

Determination of nitrite: The reaction mixture was decolorized by activated charcoal and proteins were removed. Then Griess-Ilosvay's reagent was added and the pink color thus developed was determined colorimetrically with Pulfrich's stufenphotometer.

Effects of Inhibitors. In order to elucidate the chemical nature of nitrate reductase, we have studied the effects of some typical respiratory inhibitors on the action of this enzyme. The results of this experiment are summarized in Table 1.

Table 1.

Inhibitors.....	KCN			NaN_3		CO		monoiodo-acetic acid		cysteine	cystine
Concentrations.... (mol/l.)	10^{-4}	10^{-3}	10^{-2}	10^{-3}	10^{-2}	1 atm. (dark)	1 atm.* (light)	10^{-3}	10^{-2}	2×10^{-2}	2×10^{-2}
Degrees of in-.... hibition (%)	7	97	100	56	100	42	0	52	93	92	7

* The tube was illuminated by 100 watt lamp from a distance of 30 cm.

As is shown in Table 1, nitrate reductase is markedly inhibited by so-called "heavy metal reagents" such as KCN, NaN_3 and CO. This fact strongly suggests that a certain heavy metal is related to the activity of this enzyme. In fact Quastel[5] already assumed the participation of iron in the bacterial reduction of nitrate on the basis of his observation with resting bacteria. Our experiment with cell-free enzyme also indicates in accordance with his assumption that the heavy metal contained in nitrate reductase must be iron, because we have shown, as is recorded in Table 1, that CO-inhibition of nitrate reductase completely disappears by light and it is well known that among CO-compounds of various heavy metals and their complexes, so far as examined, only those of iron and its complexes are photodissociable and therefore their catalytic activities, if any,

(5) J. H. Quastel, *Nature*, **130** (1932), 207; cf. H. Burström, *Planta*, **29** (1939), 292.

will be restored by light.

Although the above experiment is not sufficient to acquire an accurate knowledge about the rôle of iron in the process of enzymic reduction, it seems very attractive for us to consider that the essential function of the iron consists in an alternative cycle of valency between ferric and ferrous states, since nitrate reductase is inhibited both by KCN and CO; the former combines with ferric iron in almost all cases and the latter solely with ferrous iron.

In addition it must be noticed that this enzyme is intensively inhibited by a low concentration of monoiodoacetic acid, a specific inhibitor for sulfhydryl enzyme. Cysteine is also poisonous, but cystine is almost without effect. These facts feem to show that the sulfhydryl (—SH) or disufide (—S—S—) group may be connected with the activity in some manner.

Spectroscopic Observations. The crude preparation of nitrate reductase obtained by our method shows a brownish yellow color in a concentrated aqueous solution. This solution, when reduced by $Na_2S_2O_4$, exhibits three absorption bands in the visible region under Zeiss microspectrometer. The non-reduced solution shows only very weak and diffused absorptions (oxidized bands). The positions of the three reduced bands are as follows:

(1) 585 mμ, very weak and diffused.
(2) 560 mμ, intense and sharp.
(3) 530 mμ, weak and sharp.

Among these bands, (2) and (3) are apparently identical with the α- and β-bands of cytochrome b which were observed by Keilin[6] with Bac. coli suspension. In contrast with (2) band (3), band (1) is very weak and diffused, the origin of which is yet unknown.

In order to decide whether there exists any relationship between nitrate reductase and these bands, the following experiments have been carried out. The enzyme solution was precipitated fractionally into five portions by acetone (below 0°) and the activity of nitrate reductase and intensities of the reduced bands were determined. The results are shown in Table 2.

Table 2.

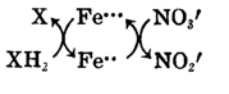
Fractions	Volume % of acetone when precipitated	Relative activities of nitrate reductase	Intensities of the band at 585 mμ	Intensities of the bands at 560 and 530 mμ
I	0 —33.3	100	+	+++
II	33.3—50	15	+	+
III	50 —66.6	4	++	±
IV	66.6—75	0	—	—
V	75 —88.3	0	—	—

Further addition of acetone produced no precipitate.

(6) D. Keilin, *Ergeb. Enzymforsch.*, 2 (1933), 239.

The following facts have been made clear by these results: (1) the band at 585 mμ has no relation to nitrate reductase, and (2) the intensities of the bands at 560 mμ and 530 mμ, which are regarded as the reduced bands of cytochrome b, are apparently parallel with the activity of this enzyme. This parallelism seems to be an evidence, though not decisive, of the identity of cytochrome b with nitrate reductase. That nitrate reductase may contain iron as an essential component (as postulated by our inhibition studies) also agrees with this assumption, for cytochrome b is an iron-porphyrin enzyme.

We have further attempted some experiments to ascertain the identity of these two substances. If our assumption is correct and nitrate reductase (= cytochrome b) undergoes cyclic oxidation and reduction, the process of enzymic reduction of nitrate may be shown by the following scheme.



X, XH_2....oxidized and reduced forms of intermediary hydrogen carrier.
$Fe^{\cdots}$, $Fe^{\cdot\cdot}$....oxidized and reduced forms of nitrate reductase (cytochrome b)

From this scheme it is expected that the reduced bands of cytochrome b will shift to the oxized bands when nitrate is added. We found, contrary to our expectation, that no change occurs in the absorption bands of cytochrome b, previously reduced by $Na_2S_2O_4$, even when a large amount of nitrate was added. This fact is not, however, contradictory with our assumption, since we later found that the enzyme treated with $Na_2S_2O_4$ loses its activity irreversibly. To avoid the use of $Na_2S_2O_4$ we then adopted the following method. In the main room of a Thunberg tube 3 c.c. of enzyme solution, 0.5 c.c. of sodium formate (1 mol), and 0.5 c.c. of methylene blue (1:10,000) were put in. In the side chamber of the tube was placed 0.5 c.c. of KNO_3 (2 mol). Then the tube was evacuated and incubated at 37°. As our enzyme preparation contains powerful formic dehydrogenase as well as nitrate reductase the methylene blue was soon decolorized and simultaneously the reduced bands of cytochrome b appeared. After complete decolorization of methylene blue KNO_3 was mixed with the enzyme solution. Then immediately decrease of the intensities of the bands of reduced cytochrome b was observed and they approached those of oxidized form. But this conversion ceased incompletely probably due to an establishment of an equilibrium between the oxidizing power of nitrate and the reducing tendency of formic dehydrogenase system. This result seems to support our assumption firmly.

It is quite natural to expect then that some changes will take place by KCN and NaN_3 in the absorption bands of cytochrome b, since these reagents inhibit nitrate reductase markedly. But we could not observe any change of absorption spectrum by KCN and NaN_3 in either the

oxidized or reduced enzyme solution. Reduced cytochrome b has not only α- and β-bands, but also γ-band at about 430 mμ which can not be observed by this method. As the possibility still remains that γ-band will be affected by these inhibitors, we will study this point in future.

Discussion. Although many problems are still left unsolved about the chemical nature of nitrate reductase, the identity of this enzyme with cytochrome b becomes quite probable by our present investigations.

Cytochrome b is widely distributed in aerobic cells and is considered as an important factor of aerobic cell respiration, but studies on this substance have been neglected compared with other components of the cytochrome system. Up to the present it has been investigated mainly by spectroscopic method and it is now confirmed that there are two kinds of cytochrome b whose absorption bands lie at somewhat different positions. As one of them is present in the heart muscle of animals etc. and the other in Bac. coli, etc., we will distinguish them as the heart muscle type and Bac. coli types respectively. The positions of the reduced bands are as follows:

	α-band	β-band
Heart muscle type	564 mμ	534 mμ
Bac. coli type	460 mμ	630 mμ

To which component of cytochrome b must be attributed the activity of nitrate reductase? We assume that cytochrome b of Bac. coli type is the very nature of nitrate reductase and that of the heart muscle type is unconcerned with this enzyme. This assumption also answers the question why there are tissues and cells which can not reduce nitrate in spite of a high content of cytochrome b.

We have further clarified by consulting "Bergey's Manual of Determinative Bacteriology[7] and Tamiya and Yamagutchi's[8] list on cytochrome types of microorganisms that (1) bacteria without cytochromes can not reduce nitrate at all, (2) almost all organisms containing cytochrome b of Bac. coli type can reduce nitrate, but those of the heart muscle type are not able to reduce nitrate except in a few cases. Moreover Bernheim and Dixon[9] reported that among various tissues of many animals only the liver (and also the muscle of a few species) can reduce nitrate, while according to Yoshikawa[10], cytochrome b of the liver is Bac. coli type. This parallelism between the ability of nitrate reduction and the existence

(7) D. H. Bergey, "Bergey's Manual of Determinative Bacteriology", 4th Ed., Baltimore (1934).

(8) H. Tamiya and S. Yamagutchi, *Acta Phytochimica*, **6** (1934), 105.

(9) F. Bernheim and M. Dixon, *Biochem. J.* **22** (1928), 125.

(10) H. Yoshikawa, from discussions at the 1st "Symposium on Enzyme Chemistry' held at Tokyo in October, 1948.

of Bac. coli type cytochrome b also seems to be favourable for our assumption.

Summary.

The effects of various inhibitors on nitrate reductase have been investigated and some spectroscopic observations have been made to elucidate the chemical nature of nitrate reductase. From these studies it becomes very probable that cytochrome b of Bac. coli type is identical with nitrate reductase.

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