Enzymatic Reduction of Aromatic Nitro Compounds

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(Received July 23, 1953)

There have been found only two nitro compounds in nature, e. g. β-nitro-propionic acid and chloramphenicol, but it is well known that many species of micro-organisms have the ability to reduce aromatic nitro compounds. The bacterial reduction of chloramphenicol is considered to be one of the reasons for the tolerance.[1,2] And it is also known that in animals, amino-, hydroxylamino and azoxy compounds are excreted in urine when aromatic nitro compounds are administered[3].

In our laboratory, Egami, Ebata and Sato prepared a cell-free bacterial enzyme preparation which accelerates the reduction of chloramphenicol and other aromatic nitro compounds[2]. However, the present author has found that the cell-free extract[4] of a halotolerant bacterium, *Bacillus pumilus* var., has a stronger activity. Utilizing the enzyme preparation of the bacterium, the author studied the nature of the enzyme system and at the same time reinvestigated the identity of nitrite reductase and the enzyme "nitroreductase" because the identity was suggested by Egami and others in our laboratory. It may be worthy to note here that the bacterium has a series of enzymes participating in the successive reduction of nitrate to ammonia[4] and has a strong activity of denitrification[5].

Experimental

Extraction of Nitro-Reductase.—The bacterium was cultured on peptone-bouillon agar containing 10% NaCl, and 1% KNO_3 at 37°C. After 40 ~50 hours the cultured cells were gathered, washed with 10% NaCl by centrifugation and then lyophilized. A clear enzyme solution was obtained by extracting the lyophilized cells with 10^{-1} M phosphate buffer solution (*p*H 6.8, 50~100 mg. cells/cc.) overnight.

Methods of Measurements.—The enzyme activities were measured under anaerobic condition by using Thunberg tubes at 37°C. Before the estimation, the reaction mixture (6~7 cc.) was deproteinized by $Cd(OH)_2$ gel, which was formed by adding 2 cc. $CdSO_4$ solution ($CdSO_4$ 13 g. in 300 cc. H_2O containing 20 cc. N H_2SO_4) and 0.5 cc. N NaOH to the reaction medium. When the dyes such as methylene blue or nile blue were employed, Japanese acid clay or Kaolin was used as the adsorbing agent. The measurements of the reaction were carried out by the determination of the amino compounds formed, or bypolarography of the remaining nitro compounds[6,7]. The methods of the determination of various amino compounds are as follows:

***p*-, *m*-Aminobenzoic Acids (PABA, MABA) and *p*-Aminosalicylic Acid.**—The solution containing amino compounds (8 cc.) was diazotized by adding 1 cc. 6 N HCl and 0.5 cc. 0.2% $NaNO_2$ at room temperature. After 15 min. excess nitrite was removed by some solid aminosulfonic acid. Then, 0.5 cc. 0.2% β-diethylaminoethyl-α-naphthylamine oxalate was added and the optical density of the red dyes formed was measured by Coleman's spectrophotometer at 650 mμ.

***o*-Aminobenzoic Acid.**—In place of 6N HCl, 2N HCl was employed and diazotization was carried out under cooling in ice water. And the measurement of the optical density was carried out after keeping the solution for 15~20 hours at 37°C. after the addition of the coupling agent.

***m*-Aminophenol**[8].—Diazotization was carried out under cooling in ice water and the optical density was measured at 570 mμ after keeping the coupled solution for 15~20 hours at 37°C.

***p*-Aminophenol.**[8]—To the solution of *p*-aminophenol were added 2 cc. 1N Na_2CO_3, 0.4 cc. 5% phenol, 2 cc. 0.1% $K_3Fe(CN)_6$ and 1.6 cc. dist. water. After 30 min. the measurement of the optical density was carried out at 650 mμ.

Measurement of the Reduction of Chloramphenicol[9].—The solution of the reduction product of chloramphenicol was treated similarly as the solution of PABA. By the measurement of the optical density of the red dye formed, the relative velocity of the reduction of chloramphenicol could be estimated. But, for the determination of the absolute velocity polarography was employed. So, after the removal of most of the dyes by some Japanese acid clay, the test solution was deoxygenated by the stream of H_2 gas for 15 min. and measured by SHIMAZU's polarograph.

1) G. N. Smith and C. S. Worrel, *Arch. Biochem.*, **21**, 216 (1949).
2) F. Egami, M. Ebata and R. Sato, *Nature*, **167**, 118 (1951); *J. Agr. Chem. Soc. Japan*, **25**, 347 (1952).
3) R. T. Williams, G. T. Mille and H. J. Channon, *Biochem. J.*, **38**, 73 (1944).
4) S. Taniguchi, H. Mitsui, J. Toyada, Y. Yamada, and F. Egami, *J. Biochem.*, **40**, 175 (1953).
5) I. Yamashina, unpublished.
6) J. E. Page, J. W. Smith and J. G. Waller, *J. Phys. & Colloid. Chem.*, **53**, 545 (1949).
7) J. W. Smith and J. G. Waller, *Trans. Farad. Soc.*, **46**, 290 (1950).
8) D. Robinson, J. N. Smith and R. T. Williams, *Biochem. J.*, **50**, 221 (1951).
9) G. B. Hess, *Analyt. Chem.*, **22**, 649 (1950).

Results and Discussion

Reaction System[10,11,12,13].—It is known that the enzymatic reductions of nitrate, nitrite and hydroxylamine proceed according to the following reaction system:

Substrate—Reductase—Intermediary—Hydrogen Carrier—Dehydrogenase—Hydrogen Donator.

So, in the case of aromatic nitro compounds the existence of a similar reaction system was expected and it was really ascertained (Table I, Fig. 1).

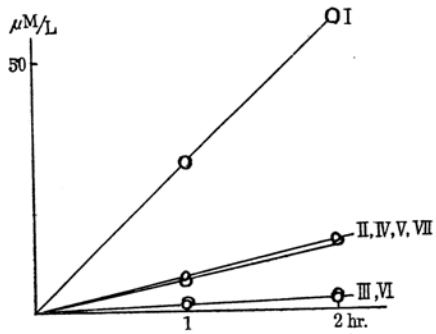


Fig. 1. The formation of PABA at various conditions (Table 1)

TABLE I

	I	II	III	IV	V	VI	VII
Substrate (PNBA 6×10^{-3} M)	1	1	1	1	1	—	1
Reductase*1	1	—	1	1	1*3	1	1
H. Carrier (Nile Blue 2×10^{-3} M)	1	1	1	—	1	1	1
Dehydrogenase*2	1	1	—	1	1	1	1
Buffer (10^{-1} M, *p*H 6.8, phosphate)	1	1	1	1	1	1	1
H. Donator (10^{-1} M, HCOONa)	1	1	1	1	1	1	—
H_2O	—	1	1	1	—	1	1 cc.

*1 The enzyme extracted from halotolerant bacterium
*2 Lyophilized cells of Pseud. fluor. (5 mg/cc.).
*3 heated at 100°C, for 5 min.

Intermediary Hydrogen Carrier.—As intermediary hydrogen carriers, brilliant cresyl blue, thymol blue, *o*-chlorophenolindophenol, methylene blue, K-indigotetrasulfonate, nile blue, neutral red, pyocyanine and several pteridine derivatives were tested. Among these substances only nile blue and brilliant cresyl blue were effective (Fig. 2). The structures and the redox potentials of these two dyes are as follows:

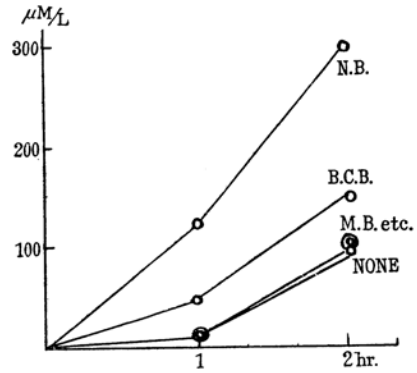
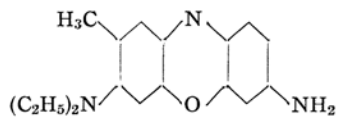
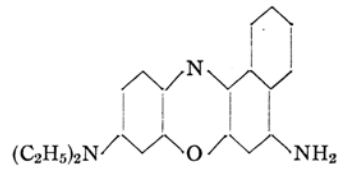


Fig. 2. The formation of PABA in the presence of various hydrogen carriers (3×10^{-5}M)



brilliant cresyl blue

E_0(*p*H 7.0 30°C.) +0.047 volts



nile blue

−0.125 volts

It is interesting to note that the nitroreductase requires the specially shaped molecule, oxazine ring, as the intermediary hydrogen carrier. But, as to the relation between the velocity of the reaction and the concentration of dyes, a marked difference has been observed between two dyes; in the case of nile blue the activity was proportional to the concentration, on the other hand, in the case of brilliant cresyl blue there was an optimum (Fig. 3). The natural hydrogen carrier we have not yet investigated, however, coenzymes such as DPN and FAD may be supposed to function in the reduction of nitro compounds in vivo.

Reaction by the use of leuco dyes.—In order to ascertain the reaction system above mentioned, it was tested whether the reaction proceeds in accordance with the following simple system;

10) B. B. Westfall, *J. Pharmacol.*, **79**, 23 (1944).
11) B. B. Westfall, *J. Pharmacol.*, **78**, 386 (1943).
12) E. Bueding and N. Jolliffe, *J. Pharmacol.*, **88**, 300 (1946).
13) V. H. Parker, *Biochem. J.*, **51**, 363 (1952).

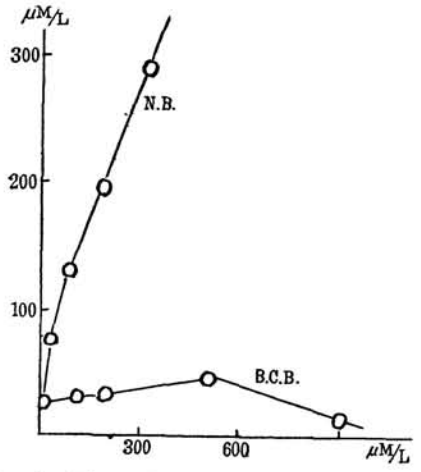


Fig. 3. The relation of the formation of PABA and the conc. of hydrogen carriers

Substrate—Nitro-Reductase—Leuco Dyes. The leuco base of nile blue or brilliant blue was prepared by the catalytic reduction, Pa-$BaSO_4$ being used as the catalyser. In the case of the nile blue, the enzymatic reduction of PNBA was confirmed. An example was

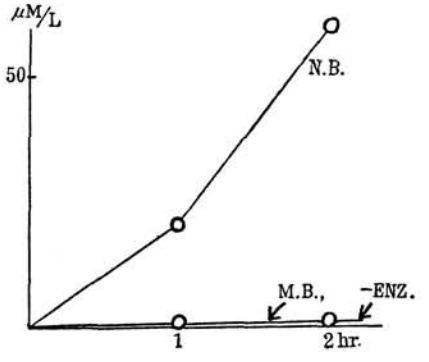


Fig. 4. The formation of PABA with leuco dyes as hydrogen donators System: Substrate (6×10^{-3} M) 1 cc., Reductase 1 cc., Leuco Dye (10^{-2} M) 2 cc., Buffer 1 cc.

shown in Fig. 4, but the curve of the formation of amino compounds plotted against time was not always monotonous. It may be attributed to the low solubility of leuco nile blue in neutral solution, especially in the presence of phosphate. Accordingly, it is unsuitable to use leuco nile blue for the investigation of the reaction velocity. On the other hand, in the case of brilliant cresyl blue, the enzymatic reduction of PNBA was not confirmed. This may be explained by the inhibitory action by the excess of brilliant cresyl blue mentioned above. From these results it was concluded that the enzymatic reduction of aromatic nitro compounds proceeds by the mechanism analogous to that of nitrate, etc.

Michaelis constants were obtained graphically from PS-activity curve in the enzymatic reduction of *o*-, *m*-, and *p*-amino-benzoic acid (Fig. 5).

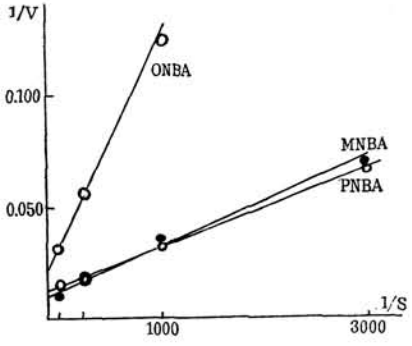


Fig. 5. v=the formation of amino benzoic acids (*o*-, *m*-, *p*-) in 1 hr. in μM/L. s=the conc. of substrates (M/L.)

Specificity, Especially the Relation to Nitrite Reductase.—Various aromatic nitro compounds such as *o*-, *p*-, *m*-nitro benzoic acids (ONBA, PNBA, MNBA), *m*-, *p*-nitrophenols, *p*-nitro salicylic acid. and chloramphenicol were reduced to the respective amino compounds by the enzyme system mentioned above. In the series of nitro benzoic acids the ratio of the reduction velocities at the same concentration of the substrates was *o*-: *m*-: *p*-=0.25 : 0.78 : 1.00.

According to Michaelis equation

$$v=-\frac{kES}{S+K_m}$$

using each K_m values, the ratio of the velocity constants (k) was estimated.

$$o\text{-}:m\text{-}:p\text{-}=0.5:1.2:1.0$$

If K_m values or the ratio of k values were estimated on many nitro compounds, the material for discussing the problems as to the combination of enzyme and its substrate or the influences of the substituents in the reducibility of nitro group could be obtained.

The enzyme solution which was used as nitro-reductase contained also active nitrite reductase. Moreover, as the intermediary hydrogen carrier in nitrite reduction, various substances besides nile blue, brilliant cresyl blue and methylene blue are effective[4]. On the other hand, it was previously shown that methylene blue was not effective in nitro reduction. Now, the competitive inhibition of enzyme action by nitrite reported[2] was reinvestigated and confirmed (Fig. 6). The

effects of aromatic nitro compounds on nitrite reduction were studied as follows. In these experiments, the hydrogen carrier being effective in both cases, nile blue was used and as the carrier effective only in nitrite reduction, methylene blue was used (Table II). As

TABLE II

THE DEGREE OF INHIBITION OF NITRITE REDUCTION BY NITRO COMPOUND

(PNBA)

Conc. of PNBA (M/L.)	Conc. of nitrite (M/L.) 10^{-3}		10^{-2}	
	N.B.	M.B.	N.B.	M.B.
10^{-3}	5%	0%	5%	0%
10^{-2}	40	0	60	0

shown in Table II, only in the presence of the common carrier, nile blue, the competitive inhibition of nitrite reduction by aromatic nitro compounds was observed. These facts show the possibility of the presence of the

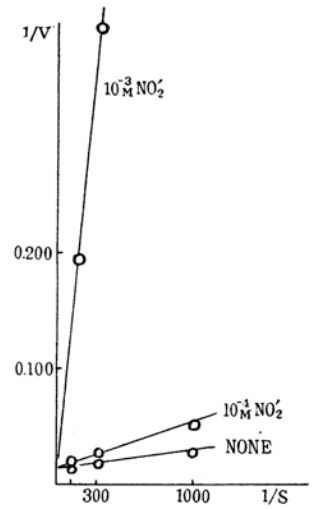
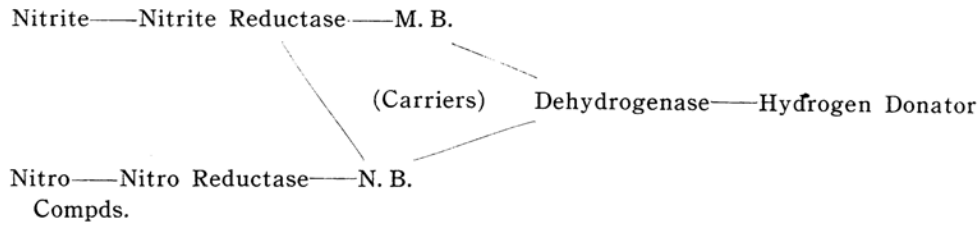


Fig. 6. v=the formation of PABA (μM/L.) in 1 hr.
s=the conc. of PNBA (M/L.)

competition of the two different enzyme systems to the common intermediary hydrogen carrier, as illustrated below:



Then, the effects of several inhibitors on the reduction of nitro compounds compared with that on nitrite reduction are as follows (Table III)[3]. In this case, the inhibition to

TABLE III

THE DEGREES OF INHIBITION BY SEVERAL INHIBITORS

	Nitro	Nitrite
KCN 10^{-3} M	70%	40%
NaN_3 10^{-2} M	5	20
NaF 10^{-2} M	0	0
Mn 10^{-4} M	0	80
Thiourea 10^{-2} M	0	0

formic dehydrogenase was negligibly small. In these data it is shown that the effects of *Mn* are remarkably different in the two cases.

These results seem to be contradictory to the idea that nitrite reductase and nitroreductase may be one and the same enzyme. Anyhow the final solution of the problem will be given by the separation of the enzymes.

Discussion.—The results and the theories described above seem to be consistent with all of the previous reports on the reduction of aromatic nitro compound. E. g. furacin, by bacteria is also considered to take place by a simillar mechanism, taking into consideration the observations that the reduction of furacin by bacteria proceeds only in the presence of oxazine dyes such as nile blue.[14-16]

The author expects that aromatic nitro compounds are not directly but stepwise re-

14) J. S. Gots, V. E. Jordan and A. F. Brodie, *Arch. Biochem.*, **36**, 285 (1952).
15) J.S. Gots, R.E. Asnis and A.F. Brodie, *Arch. Biochem.*, **30**, 25 (1951).
16) J. S. Gots, R. E. Asnis and A. F. Brodie, *Arch. Biochem.*, **30**, 35 (1951).

duced to amino compounds, then there must be many reductases in the enzyme solution which may be named nitro-reductase, nitroso-reductase etc.

Summary

1) The enzyme "nitro-reductase", which activates aromatic nitro-compounds and accelerates their reduction, was extracted from a halotolerant bacterium.

2) The reduction proceeds by the mechanism analogous to that of nitrate reduction, namely, by the system consisting of nitro-reductase, intermediary hydrogen carrier, dehydrogenase and hydrogen donator.

3) Oxazine dyes such as nile blue and brilliant cresyl blue are the only effective hydrogen carriers so far examined for the reaction.

4) Various aromatic nitro compounds can be reduced to amino compounds by the enzyme system. Some kinetic studies were performed.

5) The relation between nitro-reductase and nitrite reductase, the identity of which was suggested formerly, was reinvestigated. The competitive inhibition of nitro reduction by nitrite was confirmed, but taking into consideration various experimental evidences, it may be concluded that the two enzymes are not identical.

The author would like to thank Prof. F. Egami for his kind guidance and encouragement throughout this study. The expence of this study was defrayed in part by a grant from Japanese Biological Research Works, Ltd. and Seikagaku-Kenkyujo Ltd. to which the author's thanks are due.

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