Enzymatic Reduction of Aromatic Nitro, Nitroso and Hydroxyl amino Compounds

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In the previous paper[1], the nature of the enzyme system participating in the reduction of aromatic nitro-compounds was described.

This paper describes the route of the enzymatic reduction of nitro-compounds to amino-compounds. It is well known that when aromatic nitro-compounds such as trinitrotoluene or dinitrophenol are administrated to animals, the corresponding amino, hydroxylamino, and azoxy compounds are excreted in the urine[2][3]. These products are considered as intermediate or by-products in the biological reduction of nitro-compounds to amino-compounds[4][5][6][7][8]. Concerning the route of the reduction, we assumed at first the following successive reduction of the nitro to the amino, taking into consideration that biological oxido-reductions generally take place by the two-electron steps:

Nitro→Nitroso→Hydroxylamino→Amino

However, we paid attention also to such substances as azoxy, azo, and hydrazo compounds, which are known to be intermediates in organo-chemical reduction of aromatic nitro-compounds.

Experimental Methods

We used as substrates nitroso[9], hydroxylamino[9], azoxy[10], azo[11], and hydrazo[12] compounds in the series of *o*-benzoic acid, because they are all sufficiently water-soluble and stable for the enzymatic study. The preparation of the enzymes from a halotolerant bacterium, the experimental conditions, and the methods of estimation of the reaction velocity were the same as for those described in the preceding report.

Results and Discussion

Enzymatic Reduction of *o*-Nitroso- and *o*-Hydroxylamino Benzoic Acid:

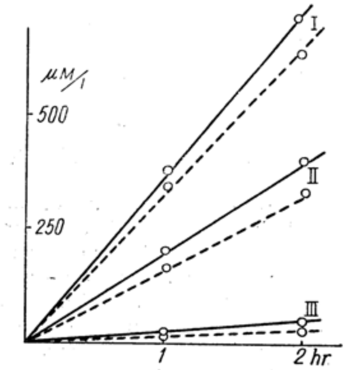

Fig. 1. The formation of anthranilic acid from *o*-nitroso, *o*-hydroxylamino benzoic acids in the presence of hydrogen carriers, –○– nitroso, – –○– – hydroxylamino. System: Substrate (6×10^{-3}M) 1 cc., Reductase 1 cc., H. Carrier (10^{-3}M) 0.4 cc., Dehydrogenase (5 mg./cc.) 1 cc., Buffer (pH 6.8, 10^{-1}M, phosphate) 1 cc., HCOONa (10^{-1}M) 1 cc., H_2O 1.6 cc., I: Nile Blue, II: Methylene Blue, III: no carrier.

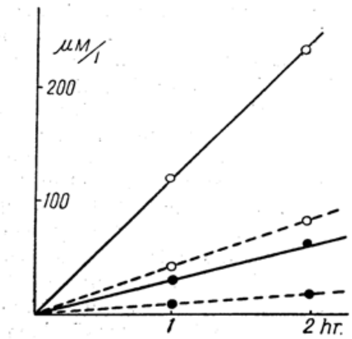

Fig. 2. The formation of anthranilic acid with leuco M.B. as hydrogen donator, –○– nitroso, –●– hydroxylamino, ----- no enzyme. System: Substrate (6×10^{-3}M) 1 cc., Leuco M.B. (10^{-2}M) 2 cc., Buffer 1 cc., Reductase 1 cc..

The measurements of the reaction velocity were carried out by the determination of the anthranilic acid produced. As shown in Fig. 1 and Fig. 2, they were reduced enzymatically by the hydrogen donator system such as the

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system consisting of hydrogen donator, dehydrogenase and an appropriate intermediary hydrogen carrier or by the leuco-form of appropriate redox dyes.

It is noteworthy that methylene blue is effective as a hydrogen carrier in these cases, contrary to the case of the reduction of the nitro group. From these results it may be concluded that the water-extract of the freeze-dried cells of the halotolerant bacterium contains the enzymes which accelerate the reduction of aromatic nitroso and hydroxylamino groups. We should like to call them "nitroso-reductase" and "hydroxylamino-reductase" respectively. The effects of various inhibitors on nitroso- and hydroxylamino- reductases are shown in TAB. I. The inhibiting action of cyanide and azide are so strong that the participation of some heavy metal to the enzyme reaction may be supposed. The inhibition is even greater than that on nitro-reductase.

TABLE I

THE EFFECTS OF SEVERAL INHIBITORS

	Nitroso	Hydroxylamino
KCN 10^{-3} M	80%	70%
NaN_3 10^{-2} M	40	30
NaF 10^{-2} M	10	10
Mn^{++} 10^{-4} M	—	0

As shown in Fig. 1 and Fig. 2, the velocity* of the enzymatic reduction of nitro is larger than that of hydroxylamino. It is not due to the inhibition by the excess of substrate in the latter case, because the velocity of the formation of amino from hydroxylamino is quite proportional to the concentration of the substrate (Fig. 3).

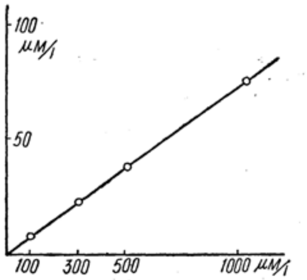

Fig. 3. The relation of the formation of anthranilic acid and the conc. of substrate (*o*-hydroxylamino benzoic acid. System: Substrate 1 cc., Reductase 1 cc., Leuco M.B. (10^{-2}M) 2 cc. Buffer 1 cc. Time: 2 hr.

These facts suggest that the hydroxylamino compound may not be a normal intermediate in the enzymatic reduction of nitroso compounds.

Experiments with *o, o′*-Azoxy, *o, o′*-Azo, and *o, o′*-Hydrazo Benzoic Acids:

They were not reduced in the presence of our enzyme preparation by the hydrogen donator-dehydrogenase system (nile blue or methylene blue as hydrogen carriers) nor by leuco methylene blue. So these substances can be excluded from the possible intermediates of the reduction from the nitro compound to the amino compound. However *o, o′*-azoxy benzoic acid was formed as a by-product in the reduction of *o*-nitrobenzoic acid as will be shown later (Fig. 5).

Route of the Enzymatic Reduction:

As the velocity of the enzymatic formation of amino group from nitroso group is larger than that from hydroxylamino group as mentioned above, the main route of nitroso to amino cannot be

nitroso→hydroxylamino→amino,

but it must be

nitroso→X→amino

nitroso→amino.

As azoxy, azo and hydrazo compounds, which had been supposed to be X in the reaction scheme, were not reduced enzymatically, we have been led to the conclusion that the direct four-electron reduction from nitroso to amino may be the main route of the enzymatic reduction.

In order to further ascertain the conclusion, we tried to detect the intermediates and the by-products of the enzymatic reaction by paper-chromatography. The reaction mixture (Each of the above-mentioned compounds and dehydrogenase system) was extracted with other, dried in the refrigerator and the residue was dissolved in a small quantity of ether and tested by paper chromatography. The composition of the developing solvents and *Rf* values of various compounds are shown in TAB. II.

TABLE II. THE DEVELOPING SOLVENTS AND *Rf* VALUES

Solvents; (1)

Ethanol	40%	Butanol	33%
Butanol	20	Pyridine	33
Ammonia (s.g. = 0.90)	20	sat. NaCl aq.	33
Benzene	20	The organic layer of above mixture	

(2) *Rf* Values:

Benzoic acids	Solvent (1)	Solvent (2)
o–nitro	0.63	0.68
o–amino	0.51	0.91
o–nitroso	0.20	0.30
o–hydroxyl-amino	0 02	—
o, o′–azoxy	0.24	0.65
o, o′–azo	0.28	—
o, o′–hydrazo	0.33	—

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*) Measured by the formation of amino group.

For the detection of each spots, Ehrlich's reaction for amino compounds was carried out directly or after the reduction by $SnCl_2$-HCl. Axoxy, azo and hydrazo compounds appeared as red spots and others as yellow spots.

The enzymatic reduction of nitroso was followed by paper chromatography as shown in Fig. 4. At the first stage of the reaction the spot of hydroxylamino appeared intensely; it then gradually became feeble and afterwards disappeared. From these results it must be considered that besides the direct route there is also a route by which the nitroso is reduced enzymatically to amino via hydroxylamino.

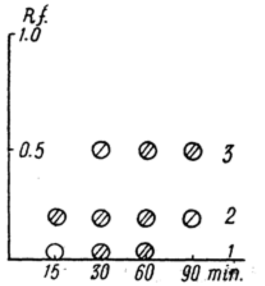

Fig. 4. Solvent (1) (Table II)
1: o-hydroxylamino benzoic acid
2: o-nitroso " "
3: o-amino " "

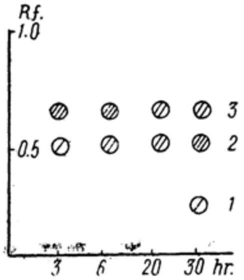

Fig. 5. Solvent (1) (Table II)
1: o,o'-azoxy benzoic acid
2: o-amino " "
3: o-nitro " "

On the paper chromatogram of the reduction products of the nitro to the amino, as shown in Fig. 5, the spot of azoxy compound was detected, besides the spots of decreasing nitro and of increasing amino. Taking into consideration the fact that the azoxy compound cannot be reduced by our enzyme preparation, it must be regarded as a by-product. The reason why any substance other than nitro, amino and azoxy benzoic acids was not detected may be explained by assuming that the possible intermediates, nitroso and hydroxylamino benzoic acids, are so rapidly reduced that they do not accumulate appreciably. This consideration is also supported by the fact that the quantity of the amino compound formed was equivalent to that of the nitro compound reduced, which in turn was estimated by the polarography.

TABLE III.

	0 hr.	9 hr.	Degree of diminution or formation
nitro	100%	68%	32%
amino	0	33	33
nitro	100	76	20
(in the presence of 10^{-3} M KCN)			(degree of inhibition is 25%)

Moreover, the paper chromatography disclosed the existence of the nitroso compound as a reduction product in the enzymatic reduction of the nitro compound under the presence of 10^{-3} M KCN. In this condition, the enzymatic formation of amino from nitro was reduced to 30%, that from nitroso to 20% and that from hydroxylamino to 30%, while the rate of the enzymatic reduction of nitro measured by polarography was reduced only to 75%. These facts are easily explained by considering the that primary product of the enzymatic reduction of the nitro compound is the nitroso compound and that this stage of successive reduction is less inhibited by KCN than the latter stages.

All of the results concerning the reduction process of nitro to amino may be summarized as follows:

$$\begin{tikzcd}
\text{Nitro} \arrow[r, "\text{Nitro-reductase}"'] & \text{Nitroso} \arrow[r, "\text{Nitroso-reductase}"'] \arrow[dr] & \text{Amino,} \\
& & \text{Hydroxylamino} \arrow[u, "\text{Hydroxylamino-reductase}"']
\end{tikzcd}$$

although it must be mentioned that we have as yet not been able to obtain experimental evidence that nitroso-reductase and hydroxylamino-reductase are different. The accumulation of azoxy compound, a by-product, is probably due to the condensation of nitroso and hydroxylamino compound.

Summary

1) A cell-free enzyme preparation of a halotolerant bacterium activates aromatic nitroso and hydroxylamino compounds as well as nitro compounds and accelerates their reduction by hydrogen donator systems.
2) The enzymatic reduction of nitroso and hydroxylamino was more strongly inhibited by cyanide and azide than that of nitro.
3) Methylene blue, which can not be used as an intermediary hydrogen carrier in the case of nitro reduction, can be used in the

cases of nitroso and hydroxylamino reduction.

4) From these facts, we have been led to the consideration that there exist special enzymes such as nitroso-reductase and hydroxylamino-reductase besides nitro-reductase.

5) Azoxy, azo, and hydrazo compound cannot be reduced by the enzyme preparation.

6) From the studies of reaction velocity and paper chromatography of the intermediates and the end products, we proposed a scheme concerning the enzymatic reduction of nitro to amino, namely:

$$\begin{tikzcd}
\text{Nitro} \arrow[r] & \text{Nitroso} \arrow[rr] \arrow[dr] & & \text{Amino} \\
& & \text{Hydroxylamino} \arrow[ur] &
\end{tikzcd}$$

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Science, Nagoya University