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Evidence for the Involvement of Superoxide Anion in Dopamine-β-Hydroxylase System[1] (38038)

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The enzyme, dopamine-β-hydroxylase (DBH), is a mixed function oxidase that oxidizes dopamine to norepinephrine, an essential step in the biosynthesis of the neurotransmitter (1). This enzyme is known to be a copper-containing protein (2, 3) and ascorbate has been shown to be essential for the optimal enzymatic activity (4). However, the exact role of this cofactor has not been adequately described. In this report, we present data which indicate that the superoxide anion (O_2^-) is involved in the DBH system, and which suggest that the role of ascorbate may be in the generation of O_2^-. Our results also indicate that ascorbate may be replaced as a cofactor in the reaction, without the loss of enzyme activity, by a nonenzymatic system which generates O_2^-.

Materials and Methods. *S*-(methyl-^{14}C) adenosylmethionine (specific radioactivity 53.1 mCi/mmole) was purchased from New England Nuclear Corporation (Boston, Massachusetts). Catalase was obtained from Boehringer Mannheim Corporation (San Francisco, California). Pargyline was a gift from Abbott Laboratory (Chicago, Illinois). Phenazine methosulfate and NADH were products of California Biochemical (Los Angeles, California). Tyramine HCl and nitro blue tetrazolium were purchased from Sigma Corporation (St. Louis, Missouri).

Superoxide dismutase was purified from fresh bovine erythrocytes according to the procedure of McCord and Fridovich (5).

Phenylethanolamine-*N*-methyltransferase (PNMT) was purified from bovine adrenal glands by a modification of the procedure of Molinoff *et al.* (6). Separation of the PNMT fraction from DBH was carried out on Sephadex G-200 column. The fractions which showed the highest DBH activity were pooled and used for the present studies. The DBH fraction prepared by this procedure catalyzed the formation of 53 nmoles of octopamine from tyramine substrate per mg protein per 30 min at 37°C.

DBH activity was measured by two procedures. The two-step procedure of Molinoff *et al.* (6) was used to study the effects of nitro blue tetrazolium (NBT) and phenazine methosulfate-NADH system on DBH activity. This assay was based on the sequential conversion of the product of the DBH reaction to a radioactively labeled *N*-methyl derivative by reaction with partially purified PNMT in the presence of *S*-(methyl-^{14}C) adenosylmethionine. The *N*-methyl derivative was separated by solvent extraction and its radioactivity was used as an index of DBH activity.

The complete incubation system referred to the following components added in two stages: at the first stage, the reaction mixture contained in 325 μl: 0.048 *M* ascorbic acid (pH 5.5), 25 μl; 0.5 *M* sodium fumarate (pH 5.5), 25 μl; 0.0006 *M* pargyline, 20 μl; catalase, 10 μl (1500 units); 1.0 *M* Tris buffer (pH 5.5), 10 μl; 0.03 *M*

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tyramine, 10 μl; 0.1 m*M* $CuSO_4$, 25 μl; and 200 μl of partially purified DBH enzyme solution containing 110–310 μg of protein. An equivalent amount of water was added to substitute the deleted cofactor. The reaction mixture was incubated at 37°C for 30 min. The DBH portion of the assay was stopped, and the PNMT reaction initiated by adding the following components in a volume of 100 μl: *S*-adenoxylmethionine-methyl-^{14}C (2 nmoles), 10 μl; 14.3 m*M* EDTA, 10 μl; 1.0 *M* Tris buffer (pH 8.6), 70 μl; and 10 μl of partially purified PNMT enzyme solution, containing 20 μg of protein. The PNMT reaction was carried out for another 30 min at 37°C and then stopped by the addition of 0.5 ml of 0.2 *M* borate buffer, pH 10.0. ^{14}C-*N*-methyl-octopamine formed in the reaction mixture was extracted into 6 ml of a mixture of toluene and isoamyl alcohol, 3:2 (v/v). After centrifugation, 4 ml of the organic phase was dried in a chromatographic oven at 80°C. The residue was dissolved in 1 ml of absolute alcohol and radioactivity was counted in a liquid scintillation counter.

The effect of superoxide dismutase on DBH activity was carried out by a simple and rapid procedure developed in our laboratory (7).

Results and Discussion. The involvement of ascorbate and molecular oxygen in the hydroxylation reaction catalyzed by the copper-containing DBH suggest a possible O_2^- participation in the reaction. We examined this possibility by using superoxide dismutase (Erythrocuprein) which catalyzed the dismutation of O_2^- to hydrogen peroxide. As seen in Fig. 1, DBH activity was markedly inhibited when various concentrations of superoxide dismutase were included in the reaction mixture. The involvement of O_2^- was further substantiated by the use of nitro blue tetrazolium as a competitor for the free radical (8). As evident from Figure 2, ascorbate-dependent DBH activity was also greatly inhibited by this superoxide scavenger.

The role of O_2^- was further confirmed by examining the reaction in the presence of a nonenzymatic O_2^--generating system consisting of phenazine methosulfate and NADH. This system, as described by Nishikimi *et al.* (9) mimics pyridine-nucleotide-dependent flavoprotein hydroxylase, and it hydroxylates a variety of aromatic substrates presumably through the mediation of O_2^- (10). As indicated in Table I, optimal DBH activity occurs when ascorbate was included in the reaction mixture. Elimination of ascorbate lowered the enzyme activity to 19% of the control. However, when NADH-phenazine methosulfate was added to the reaction mixture in place of ascorbate, DBH activity was comparable to that observed in the controls or even greater. These data strongly suggest that the role of ascorbate in the DBH system is in the generation of O_2^-.

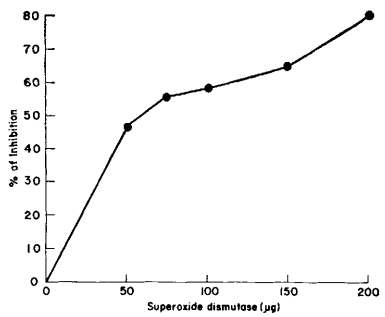

FIG. 1. Inhibition of ascorbate-dependent DBH activity by superoxide dismutase.

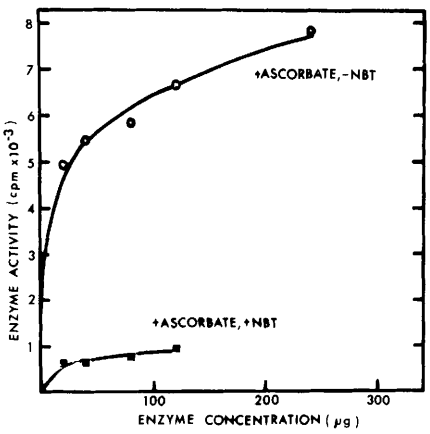

FIG. 2. The effect of nitro blue tetrazolium (NBT) on DBH activity. The incubation mixtures are those described in Table I, ○ - - ○, in the absence of NBT; ■ - - ■, in the presence of NBT (0.3 m*M*).

TABLE I. Effect of NADH-Phenazine Methosulfate (PMS) System on DBH Activity in the Absence of Ascorbate.

Experiment number	Addition or Deletion	DBH Activity[a] (CPM/tube)	% of Control
1	Complete system[b]	5,213	100
	−Ascorbate	983	19
	−Ascorbate, +NADH − PMS[c]	7,348	140
	−Enzyme,[d] −Ascorbate, +NADH − PMS[c]	24	0.5
2	Complete system[b]	5,105	100
	−Ascorbate, +NADH − PMS[c]	5,542	108
3	Complete system[b]	7,052	100
	−Ascorbate, +NADH − PMS[c]	6,489	90

[a] Results indicated were the average value of two determinations.

[b] Ascorbate was present in the reaction mixture.

[c] The final concentrations of NADH and phenazine methosulfate used were 0.15 and 0.30 m*M*, respectively.

[d] Boiled enzyme.

The effect of varying the concentration of phenazine methosulfate on DBH activity in the absence of ascorbate was examined, and these data are presented in Fig. 3. It is evident from this figure that increased DBH activity was observed with increasing concentration of phenazine methosulfate. Near maximum enzyme activity was seen at a concentration of 0.15 m*M*.

It is of interest to ascertain whether the activation of DBH activity by NADH–phenazine methosulfate system in the nonascorbate system was also due to the supply of O_2^-. As indicated in Fig. 4, NADH–phenazine methosulfate-supported DBH activity in the nonascorbate system was progressively inhibited by nitro blue tetrazolium. These results confirm the involvement of O_2^- in the DBH system and suggest that NADH–phenazine methosulfate could replace ascorbate by providing the O_2^- needed for the enzymic action.

Tryptophan-2,3-dioxygenase is another copper-containing enzyme which requires enzyme-bound cuprous ions (Cu^+) for its catalytic activity (11). Oxidative inactivation of this enzyme resulted in the formation of enzyme-bound cupric ions (Cu^{2+}). Reductive activation resulted in the forma-

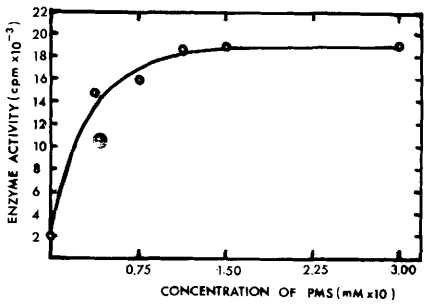

FIG. 3. Effect of phenazine methosulfate (PMS) on DBH activity. Incubations were carried out in the absence of ascorbate. Each incubation contained 0.15 m*M* NADH. Other components were the same as those described in Table I except that the enzyme concentration used in this experiment was 310 μg/tube. Reactions were started by adding various concentrations of PMS to the incubation tubes.

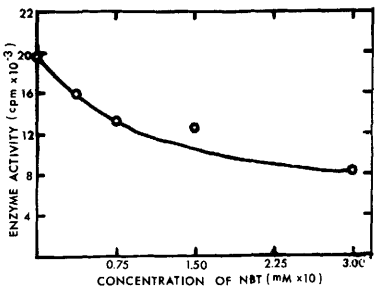

FIG. 4. NBT inhibition of DBH activity supported by NADH-phenazine methosulfate system in the absence of ascorbate. The concentrations of NADH and phenazine methosulfate used for each incubation tube were 0.15 and 0.30 m*M*, respectively.

tion of Cu^{1+}, which was shown to be caused by O_2^-. In analogy to tryptophan-2,3-dioxygenase, Friedman and Kaufman demonstrated that the cupric copper of DBH underwent cyclic reduction and oxidation during catalysis (3). The present studies suggest that superoxide radicals generated during the catalytic process reactivate DBH by converting enzyme-bound cupric form to cuprous form. The function of ascorbate in the reaction mixture is presumable to provide O_2^- for the reductive activation of the enzyme. Similar observations on the reductive activation by O_2^- of certain other metal-requiring enzymes have been reported elsewhere (12, 13).

Summary. Dopamine-β-hydroxylase activity was markedly activated by a NADH-phenazine methosulfate-O_2 model system known to generate superoxide anion (O_2^-) in the absence of ascorbate. This activation of the enzyme activity was shown to be dependent on the concentration of phenazine methosulfate. When superoxide dismutase was added along with ascorbate to the system, DBH activity was markedly inhibited. Furthermore, the addition of the superoxide scavenger, nitro blue tetrazolium, to the enzyme either in the presence of ascorbate or NADH-phenazine methosulfate, resulted in the inhibition of DBH activity. These data suggest that O_2^- is essential for the enzyme activity and that the role of ascorbate is probable in the generation of O_2^-.

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