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INHIBITION OF ADRENAL DOPAMINE-β-HYDROXYLASE BY 6-HYDROXY DOPAMINE

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Abstract—Incubation of 6-hydroxydopamine with highly purified bovine adrenal dopamine-β-hydroxylase resulted in a complete inhibition of enzyme activity. When reciprocal rates were plotted vs reciprocal tyramine concentration or reciprocal ascorbate concentration, the inhibition appeared to be of the non-competitive type. The interaction between the inhibitor and the enzyme molecule is strong and irreversible. The addition of catalase protected all systems only when catalase was added during the preincubation time. The data show that the inhibitor is not 6-hydroxydopamine but hydrogen peroxides generated from 6-hydroxydopamine.

ADMINISTRATION of a large dose of 6-hydroxydopamine (6-OHD) causes a long-lasting depletion of norepinephrine from sympathetically innervated organs as a result of an acute and selective degeneration of the sympathetic adrenergic nerves.[1–5] Very little is known about the exact molecular mechanisms for the degenerative effect of 6-OHD. Saner and Thoenen[6] have suggested that the destruction results from covalent binding of oxidation products of 6-OHD to nucleophilic groups of macromolecules of great importance to the adrenergic neuron. This suggestion implies that the reaction is non-specific and that the selectivity of the drug is due to 6-OHD being taken up and accumulated by the adrenergic nerves.

Brimijoin and Molinoff[7] have studied the effect of 6-OHD on the activity of tyrosine hydroxylase (*O*-diphenol:O_2 oxidoreductase, EC 1.10.3.1) and dopamine-β-hydroxylase (DBH) [3,4-dihydroxyphenylethylamine, ascorbate:O_2 oxidoreductase (hydroxylating), EC 1.14.2.1.] in sympathetic ganglia of the rat. They have shown that the drug causes no change in tyrosine hydroxylase but there is a long-lasting 50 per cent decrease in DBH activity. The cause of this decrease could be an inhibition of the synthesis of the enzyme or changes in microenvironment or an inhibitory effect. This was not clarified. Breese and Taylor[8] have shown that tyrosine hydroxylase activity was found to be reduced in brain stem, caudate nucleus and whole brain in 6-OHD treated animals. Conversion of tyrosine to norepinephrine and dopamine was also markedly diminished. But here again several causes could be considered: decrease of the enzyme synthesis, changes in the microenvironment or an inhibitory effect. In both cases, the experiments performed *in vivo* could hardly explain the molecular events.

The present study was undertaken in order to investigate a possible direct interaction between DBH and 6-OHD. A highly purified enzyme from bovine adrenal medulla was used.

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Abbreviations used: 6-OHD: 6-hydroxydopamine; DBH: dopamine-β-hydroxylase.

MATERIALS AND METHODS

Materials. Catalase from beef liver (hydrogen-peroxide: hydrogen peroxide oxidoreductase, EC 1.11.1.6.) with an activity of 39,000 units/mg, was purchased from Boehringer (Mannheim, Germany). Tyramine, octopamine, fumarate and bovine serum albumin were obtained from Calbiochem (Los Angeles, Calif.). 6-Hydroxydopamine was obtained from Ab Biotec (Stockholm, Sweden). All other reagents of analytical grade were obtained from Merck (Darmstadt, Germany).

Measurements of enzyme activity. Tyramine was used as the substrate. The octopamine that was formed, was determined by oxidation with periodate, as described by Pisano, Creveling and Udenfriend.[9] Since addition of the monoamine oxidase inhibitors pargyline (10^{-3} M) and iproniazide (10^{-3} M) did not change the results, they were not routinely added to the incubation mixture. We found under our conditions that ascorbate did not interfere with the formation and the spectrophotometric measurement of *p*-hydroxybenzaldehyde. That allowed us to omit the Dowex-H^+ column step originally included in the method of Pisano *et al.*[9] The reaction was performed in an open test tube and the reaction mixture contained in micromoles: fumarate, 20; potassium phosphate pH 6·5, 100; tyramine, 10; ascorbate, 10 and the enzyme to be assayed. When interactions between 6-OHD and DBH were studied in the presence of catalase or albumin, 200 μg of each of these proteins were added. The final volume was 1 ml. The reaction was started by the addition of substrates as described in the text. The reaction mixtures were incubated for 30 min at 37° and the reaction was stopped with 2 ml of 4 M-ammonia. A mixture where enzyme sample was added after ammonia was used as blank. Mixtures were left until they had reached room temperature. Then 0·1 ml of 6% $NaIO_4$ were added and the test tubes were vigorously shaken. The mixtures were allowed to stand for 4 min, 0·1 ml of 30% $NaHSO_3$ was then added and the test tubes were again vigorously shaken. The *p*-hydroxybenzaldehyde formed from octopamine was measured spectrophotometrically at 333 nm in a Zeiss spectrophotometer. The absorbance obtained was compared with the absorbance of standard octopamine solutions which had been oxidized by periodate in the same way. Absorbance was linear with octopamine concentration from 0 to 0·26 μmole. The latter gave an absorbance of 2·0. The blank absorbance was routinely about 0·06–0·07.

Purification of the enzyme. Having isolated the chromaffin granules from bovine adrenal medulla and solubilized the enzyme with Triton, DBH was purified by affinity chromatography, using a Sepharose coupled with tyramine. This method, which will be published in detail elsewhere,[10] allows a rapid, reproducible and convenient purification of bovine adrenal DBH. The enzyme appeared to be electrophoretically pure. No contaminating monoamine oxidase activity could be detected.

Preparation of 6-OHD solution. 6-OHD is described as being labile.[6] In our experiments, 6-OHD was dissolved in water, at 4° just before incubations.

Proteins. Proteins was determined by the procedure of Lowry *et al.*[11]

RESULTS

Effect of 6-OHD on DHB activity with varying tyramine concentrations. Preliminary incubations of 6-OHD with the purified enzyme resulted in 100 per cent inhibition of DBH activity with a 6-OHD concentration of 35×10^{-6} M. The inhibition occurred at very low levels of the drug compared to the concentration of tyramine (Fig. 1).

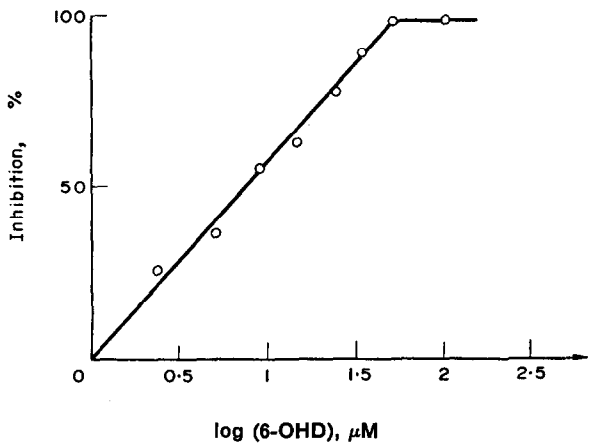

FIG. 1. Effect of 6-OHD on DBH activity. Per cent inhibitions are plotted vs log of 6-OHD concentration. Inhibition is linear until complete inhibition. Tyramine concentration was 2 mM, and ascorbate concentration was 10 mM. Fifty μl of enzyme and 5 μg of highly purified DBH were used at each point. One-hundred per cent activity = 12·5 μmoles octopamine formed 30 min^{-1} mg^{-1} of enzyme, in the conditions described for the assays.

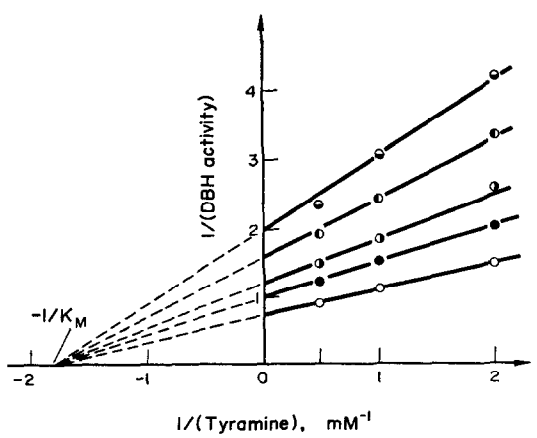

FIG. 2. Effect of 6-OHD on DBH activity with tyramine as varying substrate. The data are plotted by the method of Lineweaver and Burk. Ascorbate concentration: 10 mM. 6-OHD concentration: (○) 0 μM; (●) 2·5 μM; (◑) 5 μM (◐) 10 μM (◒) 15 μM. Assays were initiated by the addition of substrate tyramine and carried out as described. Preincubation and incubation were carried out without catalase. DBH activities are expressed in μmole of octopamine formed 30 min^{-1} 100 μg^{-1} of enzyme. The intersection point is the apparent Michaelis constant for tyramine: K_m = 0·55 mM.

When data are plotted by the method of Lineweaver and Burk[12] (Fig. 2), 6-OHD non-competitively inhibited DBH over the range of $2{\cdot}5 \times 10^{-6}$ to 15×10^{-6} M. The apparent Michaelis* constant for tyramine is $0{\cdot}55 \times 10^{-3}$ M which is in good agreement with our previous results[10] than those given in the literature.[13] However, above an inhibitor concentration of 16×10^{-6} M, the curves seemed to become hyperbolic. As DBH was strongly inhibited in this 6-OHD concentration range measuring

* Since assays were performed only with saturating concentrations of tyramine and ascorbate, all Michaelis parameters are referred to as "apparent".

DBH activities was difficult. Nevertheless, it is certain that inhibition type was modified over the 6-OHD concentration of 16×10^{-6} M. If the data are plotted according to Dixon,[14] i.e. reciprocal velocity vs 6-OHD concentration (Fig. 3), the inhibition is non-competitive and the inhibition constant is 9×10^{-6} M. This value is very low, indicating a very high affinity between 6-OHD and the enzyme.

Each test tube, contained 50 μg of enzyme and since the molecular weight of DBH is 250,000,[10] it corresponds to a concentration of $0{\cdot}2 \times 10^{-6}$ M. Thus, interaction between 6-OHD and DBH could be equimolecular. Further application of the Hill equation afforded a measure of the order of interaction of 6-OHD and the enzyme molecule.

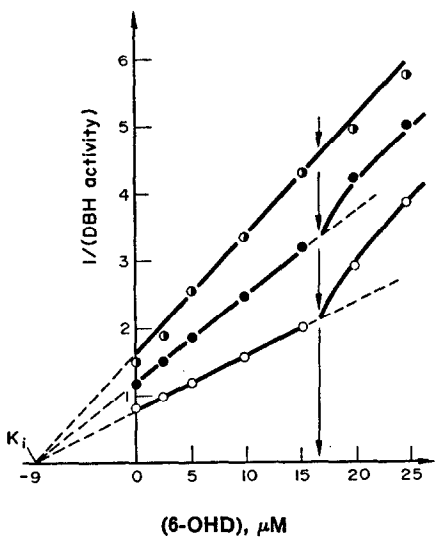

FIG. 3. Effect of 6-OHD on DBH activity, with tyramine as varying substrate. The data are plotted by Dixon's method. Ascorbate concentration: 10 mM. Tyramine concentrations: (○) 2·0 mM; (●) 1·0 mM; (◑) 0·5 mM. DBH activities are expressed in μmoles of octopamine formed 30 min^{-1} 100 μg^{-1} of enzyme. The intersection point is the apparent inhibitory constant for 6-OHD. K_i = 9 μM. Above 6-OHD concentration of 16 μM, note the change of the curves. After being linear, they become hyperbolic. The enzyme used had the same specific activity as in Fig. 1.

In the case of the determination of the non-competitive interaction of inhibitor and an enzyme, the form of the Hill equation used is: $\log [V_i/(V - V_i)] = n \log (I) + \log 1/K_i$ and this form has been considered by Wilson.[15] This modification of the Hill equation is a limiting form, valid only if a saturating concentration of the inhibitor gives a 100 per cent inhibition. In our case, DBH is completely inhibited by saturation levels of 6-OHD, without catalase and with preincubation times of 10 min. In this equation, V is the initial velocity, V_i the initial velocity in the presence of 6-OHD, I the concentration of 6-OHD, and n refers to the order of the interaction of inhibitor with the enzyme. In the case of DBH and 6-OHD, when $\log [V_i/(V - V_i)]$ is plotted against $\log (I)$ the results are shown in Fig. 4. It can be seen that the plots give straight parallel lines over the range of $2{\cdot}5 \times 10^{-6}$ M to 5×10^{-6} M of 6-OHD. However, by increasing the 6-OHD concentration above 15×10^{-6} M, the straight parallel lines are broken. The slopes of the curves pass from $n = 1$ to $n = 2$. The explanation

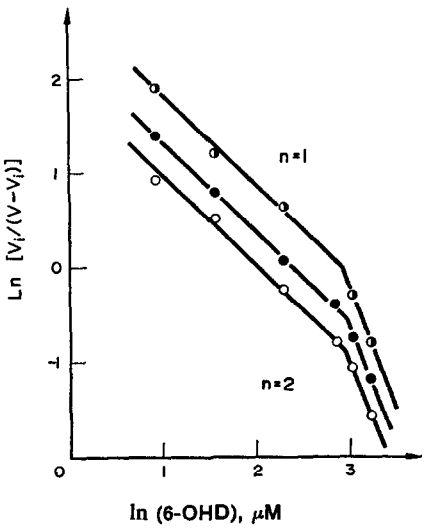

FIG. 4. Application of the Hill equation to the non-competitive inhibition of DBH activity of 6-OHD. The concentration of the inhibitor is expressed in molarity and has a range between 2·5 and 25 μM. Ascorbate concentration: 10 mM. Tyramine concentration: (○) 2 mM; (●) 1 mM; (◑) 0·5 mM.

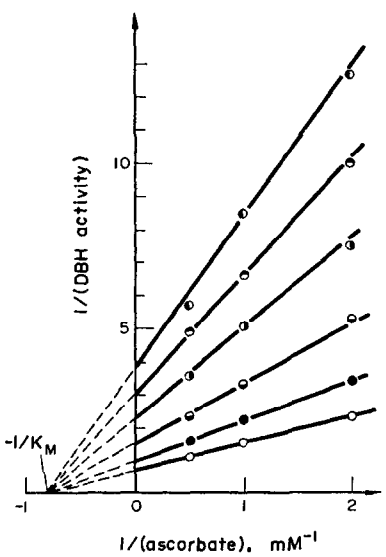

FIG. 5. Effect of 6-OHD on DBH activity with ascorbate as the varying substrate. The data are plotted by the method of Lineweaver and Burk. Tyramine concentrations: 10 mM (saturation levels). 6-OHD concentrations: (○) 0 μM; (●) 2·5 μM; (◒) 5 μM; (◑) 10 μM; (◓) 15 μM; (◐) 20 μM. Assays were initiated by the addition of substrate ascorbate and carried out as described. Preincubation and incubation were carried out without catalase. DBH activities are expressed in μmoles octopamine formed 30 min^{-1} 100 μg^{-1} enzyme. The intersection point is the apparent Michaelis constant for ascorbate: K_m = 1·25 mM.

is that one molecule of 6-OHD causes inhibitions of a non-competitive type when $n = 1$, until a concentration of 15×10^{-5} M. Then 2 molecules of 6-OHD interact with DBH molecule when $n = 2$. The typical feature is that lines are broken for a determined concentration of 6-OHD, whatever the tyramine concentration. That means that there is a direct interaction between enzyme molecule and inhibitor molecule, independently of the substrate, and that throughout the enzymatic reaction time, the inhibitor is tightly bound to the enzyme molecule.

Effect of 6-OHD on DBH activity with varying ascorbate concentrations. It is apparent from Fig. 5 that when reciprocal rates are plotted vs reciprocal ascorbate concentrations, the inhibition is still non-competitive, confirming that the inhibitor is bound to the enzyme molecule throughout the enzymatic reaction time. The intersect point is the apparent Michaelis constant for ascorbate, and is $1{\cdot}25 \times 10^{-3}$ M. As can be seen in Fig. 6, when reciprocal rates are plotted vs 6-OHD concentrations, lines intersect on the abscisse axis. This point is the inhibitor constant and is $4{\cdot}5 \times 10^{-6}$ M, but no hyperbolic sections could be observed.

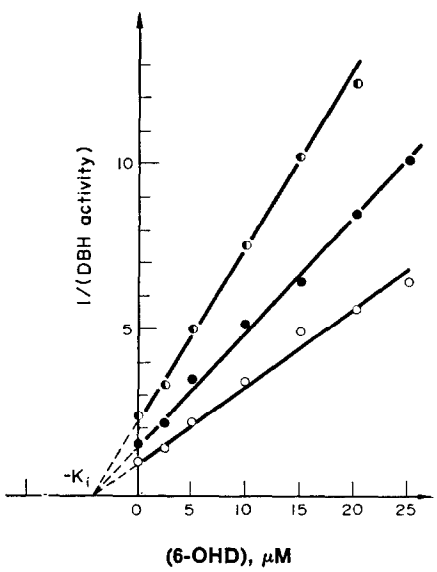

FIG. 6. Effect of 6-OHD on DBH activity with ascorbate as the varying substrate. The data are plotted by Dixon's method. Tyramine concentrations: 10 mM; ascorbate concentrations: (○) 2 nM; (●) 1 mM; (◐) 0·5 mM DBH activities are expressed in μmoles of octopamine 30 min^{-1} 100 μg^{-1} of enzyme. The intersection point is the apparent inhibiting constant for 6-OHD. $K_i = 4{\cdot}25$ μM.

If *EI* is the complex when one molecule of 6-OHD is combined with one DBH molecule, and EI_2 when two molecules of 6-OHD are combined with DBH molecule, the *EI* complex affects the velocity of both reactions, and not the affinity of the enzyme for tyramine and ascorbate, i.e.: ascorbate → dehydroascorbate; tyramine → octopamine. But the EI_2 complex affects, in addition, the second reaction: tyramine → octopamine and not the first reaction: ascorbate → dehydroascorbate. For example, the second inhibitor molecule could bind with an amino-acid which participates in the hydroxylation of tyramine, and not in the reduction of ascorbate, whereas the first inhibitor molecule attacks an amino-acid which participates in both reactions.

Effect of catalase on 6-OHD inhibition. In our laboratory it has been shown that catalase protects DBH from inactivation by H_2O_2-generating agents.[10] When DBH, 6-OHD and catalase were preincubated, no inhibition of the enzyme occurred. The recovered activity is exactly the same as without 6-OHD in the medium (Fig. 7). When DBH and 6-OHD were preincubated, inhibition occurred, even if catalase was added later throughout the enzymatic reaction time. It appeared that the enzyme was degraded even when it was incubated without 6-OHD and without catalase. This is certainly due to peroxides generated by reduction of ascorbate during pre-incubation. When DBH was incubated with 6-OHD, but without catalase, degradation

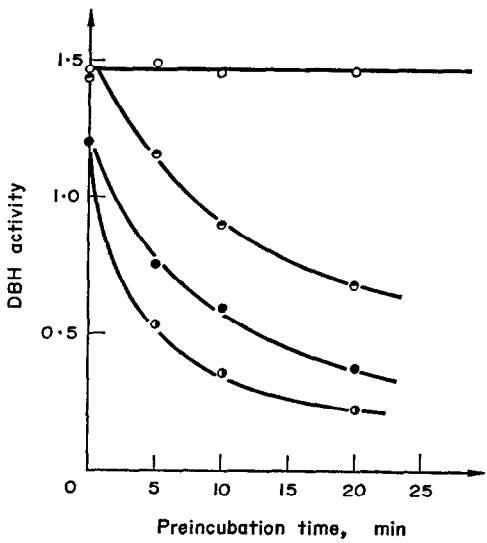

FIG. 7. Effect of preliminary incubation time on enzyme inhibition by 6-OHD. Effect of catalase: (○) enzyme preincubated with catalase, with or without 6-OHD; (◓) enzyme preincubated without catalase and without 6-OHD and assays were carried out with catalase; (●) enzyme preincubated without catalase but with 6-OHD. Assays were carried out with catalase; (◑) enzyme preincubated with 6-OHD. Assays were carried out without catalase. In all cases assays were initiated by the addition of tyramine (10 mM). DBH activities are expressed as μmoles of octopamine formed $30\ min^{-1}$ $50\ \mu g^{-1}$ of enzyme. 6-OHD concentration was 5 μM.

was substantial and appeared to occur very quickly. In 5 min, about 60 per cent of the enzyme was degradated, compared to the activity of DBH when preincubated with catalase. It is relevant to note that benzenoid compounds with either ortho- or para-dihydroxy structures (quinoid) can be oxidized by molecular oxygen with the formation of both quinone and H_2O_2.[16] It is the case with 6-OHD, since this compound appears to oxidize very easily at pH 6·4[16] (Fig. 8). From our results (i.e. DBH fully protected by catalase) it can be suggested that H_2O_2 generated within the medium by oxidation of 6-OHD is implicated in the DBH inhibition. Moreover our results show that the inhibition by H_2O_2 is irreversible since the recovered activities were always weaker when catalase was added after the preincubation time but throughout the enzymatic reaction time, than when catalase was added during preincubation and incubation times.

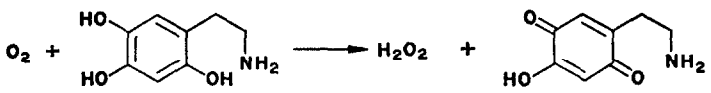

FIG. 8. Oxidation of 6-OHD by molecular oxygen. 6-OHD oxidized to its *p*-quinone derivative, with formation of hydrogen peroxide.

Effect of albumin on 6-OHD inhibition. Instead of catalase, enzyme was preincubated with serum albumin (Fig. 9). When enzyme activities are expressed vs preincubation times the velocities decrease in contrast to incubation with catalase. Albumin does not play such a highly protective role as does catalase, but a slight protection occurred, and was due to an oxidation of albumin instead of DBH. Saner and Thoenen have shown that oxidation products of 6-OHD undergo covalent binding with nucleophilic groups of biological macromolecules.[6] From our results, it appears that quinone products do not affect DBH activity, whereas H_2O_2 generated at the same time as these oxidation products altered the DBH molecule and consequently its activity.

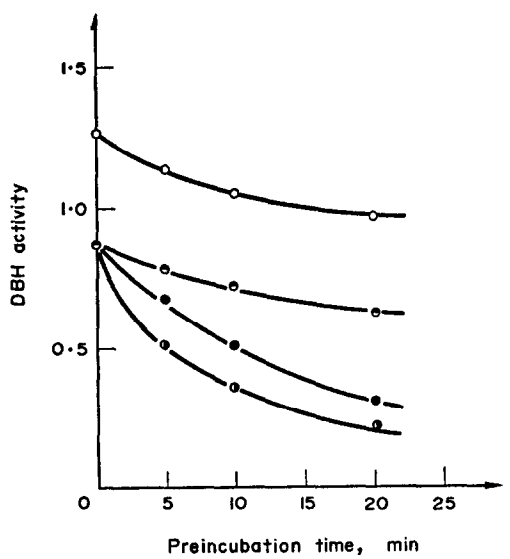

FIG. 9. Effect of preliminary incubation time on enzyme inhibition by 6-OHD. Effect of albumin: (○) enzyme preincubated with albumin and without 6-OHD; (◓) enzyme preincubated with albumin and 6-OHD; (●) enzyme preincubated without albumin but with 6-OHD. Assays were carried out with albumin; (◑) enzyme preincubated with 6-OHD. Assays were carried out without albumin. Assays were initiated by the addition of tyramine (10 mM). DBH activities are expressed as μmoles of octopamine formed 30 min^{-1} 50 μg^{-1} of enzyme. 6-OHD concentration was 5 μM.

DISCUSSION

We have shown that when DBH was incubated with 6-OHD there is a strong inhibition of enzyme activity. The present investigation, at the molecular level, provides good evidence that H_2O_2 formed by oxidation of 6-OHD by molecular oxygen is the real inhibitor of DBH. This is supported by the fact that catalase, which protects against H_2O_2, completely protects DBH from inactivation by 6-OHD. This inactivation is irreversible and non-specific, since it affects biological macromolecules.

Moreover, it appeared that 6-OHD is not a substrate for DBH since when DBH was incubated with tyramine and 6-OHD in the presence of catalase, no competitive inhibition occurred.

Inhibition occurred with very low levels of 6-OHD concentrations ($K_i = 9 \times 10^{-6}$ M when tyramine was the varying substrate, and ascorbate the fixed substrate. $K_i = 4{\cdot}5 \times 10^{-6}$ M, when ascorbate was the varying substrate, and tyramine the fixed substrate), consistent with the administered dose of 6-OHD *in vivo* (10 mg/kg i.v. in mice;[17] 500 μg by ventricular injections to rats;[18] 100 mg/kg s.c. in rats[18]).

Heikkila and Cohen[16] have shown that biogenic amine uptake is inhibited by 6-OHD and hydrogen peroxide. When they studied the amine uptake in the presence of catalase, all systems were fully protected with hydrogen peroxide, but partially protected with 6-OHD. This would mean that catalase does not prevent the 6-OHD uptake.

Certain enzymes such as monoamineoxidase and DBH generate small amounts of H_2O_2 within the neural tissue. However, in brain the mechanisms for detoxifying H_2O_2 are unclear, but they appear to be absent or of very low activity.[19] Thus, it is possible that the lack of peroxidase systems is responsible for the 6-OHD degenerative action in neuronal structures.

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