Inhibition of Biogenic Amine Uptake by Hydrogen Peroxide: A Mechanism for Toxic Effects of 6-Hydroxydopamine

Abstract. *Hydrogen peroxide, dialuric acid, or 6-hydroxydopamine inhibited the uptake of dopamine, norepinephrine, and serotonin into rat brain synaptosomal preparations. The addition of catalase protected all systems, but catalase was only partially protective for 6-hydroxydopamine acting upon catecholamine uptake. The data show that 6-hydroxydopamine generates hydrogen peroxide and that hydrogen peroxide can damage the biogenic amine uptake systems. Part of the damage caused by the 6-hydroxydopamine that accumulates in the catecholamine nerve terminals in vivo may be attributed to the hydrogen peroxide.*

The compound 6-hydroxydopamine (6-OHDA; 2,4,5-trihydroxyphenylethylamine) causes depletion of catecholamine in the brain and in the periphery (*1*), inhibition of catecholamine uptake mechanisms (*2*), and degeneration of nerve terminals (*3*). Although this compound has been widely used, the mechanism of nerve degeneration has not been elucidated. We now present evidence that hydrogen peroxide (H_2O_2) is a toxic intermediate generated from 6-OHDA by its reaction with molecular oxygen (Fig. 1). Hydrogen peroxide damages the uptake mechanisms for the biogenic amines. We believe that H_2O_2 may also be responsible for the destruction of nerve terminals.

Benzenoid compounds with either *o*- or *p*-dihydroxy structures can be oxidized by molecular oxygen with resultant formation of a quinone and H_2O_2. Both *o*- and *p*-dihydroxy groups are present in 6-OHDA; when 6-OHDA is dissolved in aqueous solution at 37°C, there develops within minutes a red color, signifying rapid oxidation. Since 6-OHDA appears to autoxidize rapidly, we set out to determine whether H_2O_2 could be implicated as a toxic intermediate in the damage to nerve terminals. We also studied dialuric acid (Fig. 1) which generates H_2O_2 (*4*) and may be considered a structural analog of 6-OHDA.

The uptake of [^{3}H]dopamine (DA; 8.3 c/mmole), DL-[^{3}H]norepinephrine (NE; 7.5 c/mmole) and [^{3}H]serotonin (5-HT, 5-hydroxytryptamine; 6.0 c/mmole) into rat brain homogenates was studied by a modification of the method of Snyder and Coyle (*5*). The [^{3}H]-catecholamines were purchased from New England Nuclear and the [^{3}H]-serotonin from Amersham/Searle. Nonradioactive stressor compounds, namely 6-OHDA (Regis), dialuric acid (Calbiochem), or H_2O_2 (Fisher) were added to 10-ml samples which were incubated for 30 minutes at 37°C. Then the ^{3}H-labeled biogenic amines were added to 0.4 to 1.1 $\times 10^{-8}M$, and uptake was measured after 15 minutes. In some samples, catalase, approximately 9000 units (Worthington, twice crystallized, 36,000 unit/mg) (*6*), was present throughout the incubation period; in the other samples, the catalase was added immediately prior to the addition of ^{3}H-labeled biogenic amine. Catalase alone had no effect on uptake. Uptake was expressed as the ratio of labeled biogenic amine in the tissue to that in the medium (T/M ratio); that is, the number of counts per minute per gram of tissue divided by the number of counts per minute per milliliter of medium. Final results were calculated as the percentage of control uptake.

Uptake of [^{3}H]DA, [^{3}H]NE, or [^{3}H]-5-HT was inhibited by 5 $\times 10^{-4}M$ 6-OHDA, $10^{-3}M$ dialuric acid, or $10^{-3}M$ H_2O_2 (Table 1). The T/M ratios of the unprotected samples ranged from 1.8 to 32.2 percent of control values. In other experiments, we found that the inhibition of uptake was dependent on concentration. Inhibition was observed for concentrations of stressor compound down to $1 \times 10^{-5}M$. To our knowledge, this is the first report that H_2O_2 or H_2O_2-generating agents can inhibit the uptake of biogenic amines. An initial concentration of at least $10^{-3}M$ 6-OHDA can be calculated to be present in vivo when 200 μg of 6-OHDA is injected into the rat brain (*1*); similarly, an initial circulating level of $10^{-3}M$ can be calculated for a dose of 50 mg/kg administered intravenously (*2*).

Table 1. Inhibition of biogenic amine uptake into rat brain homogenates and protection by catalase. Samples were incubated with stressor agents for 30 minutes at 37°C and then the uptake of biogenic amines was measured. In the unprotected samples, the catalase was added immediately before the addition of biogenic amine. Duplicate specimens agreed to within ± 5 percent of their mean. Each experiment was repeated several times and gave similar results. Mean T/M ratios in the control vessels were 172 : 1 (serotonin), 426 : 1 (dopamine), and 93 : 1 (norepinephrine).

Amine	Uptake (percent of control)	
	Catalase protected	Unprotected
6-Hydroxydopamine (5×10^{-4}M)		
Serotonin	98.0	23.4
Dopamine	8.7	1.8
Norepinephrine	18.9	9.4
Hydrogen peroxide (10^{-3}M)		
Serotonin	101.0	20.4
Dopamine	102.6	22.3
Norepinephrine	102.0	23.0
Dialuric acid (10^{-3}M)		
Serotonin	104.0	32.2
Dopamine	92.0	10.1
Norepinephrine	92.0	24.9

Protection by catalase was evident for each of the stressor compounds. In other experiments, the catalase was no longer effective after it had been inhibited by treatment with 3-amino-1,2,4-triazole (Mann) (*7*); this indicated that protection by catalase protein was due specifically to the removal of H_2O_2.

In the experiments with dialuric acid, full protection of the uptake systems by catalase showed that it was neither dialuric acid nor its oxidation product alloxan which caused the inhibition since both substances were still present. Inhibition in the unprotected samples must be attributed to the H_2O_2 formed prior to the addition of catalase. Likewise in the 6-OHDA experiments, the full protection of the 5-HT uptake system by catalase showed that H_2O_2 was the inhibitory agent rather than 6-OHDA or its corresponding quinone (or other oxidation products).

The interpretation of the effects of 6-OHDA and catalase on the catecholamine uptake systems is more complex. 6-Hydroxydopamine can block the uptake of catecholamines not only indirectly by generating H_2O_2, but also by competing directly with catecholamines for the uptake systems (*2*). The latter action tended to obscure the protective role of catalase in the experiments shown in Table 1. In order to demonstrate more clearly the protective role of catalase, we performed experiments with whole brain tissue slices (1.0 by 0.2 by 0.2 mm, prepared on a McIlwain-Mickle tissue chopper, Brinkmann Instruments). After the 30-minute exposure period, the extracellular 6-OHDA was removed by decantation, and the slices were rinsed twice by resuspension in fresh medium and

redecantation. The slices were then resuspended, and uptake of DA was measured. In two experiments, the unprotected samples gave T/M ratios of only 6 and 8 percent of control values, while the catalase-protected samples gave ratios of 45 and 52 percent of control values. Triplicate samples agreed to within ± 5 percent of their mean; the T/M ratio for control tissue was 46 : 1. Since 6-OHDA accumulates in catecholamine nerve terminals (*2*), the inhibitory effects, not protected by catalase, can be attributed in part to the action of H_2O_2 generated within the tissue and in part to the direct action of residual 6-OHDA. Tissue slice experiments performed with [^{3}H]-5-HT produced results similar to those in Table 1, that is, the system was fully protected by catalase in the medium.

We considered the possibility that oxidation of [^{3}H]catecholamines by the quinone or other oxidation products of 6-OHDA, or by H_2O_2 generated within the tissue, might account for the lowered T/M ratios. In experiments with homogenates, we analyzed both the tissue and the supernatant by thin-layer chromatography (*8*) and found that more than 90 percent of the radioactivity was present as unchanged [^{3}H]catecholamine. Similar observations were made for uptake measurements in the presence of $10^{-3}M$ H_2O_2. Therefore, diminished uptake cannot be attributed to destruction of [^{3}H]catecholamines.

Certain enzymes such as monoamine oxidase generate small amounts of H_2O_2 within neural tissue. However, the mechanisms for detoxifying H_2O_2 are unclear. Protective enzymes, such as glutathione peroxidase or catalase, which act in other tissues to destroy relatively low or high concentrations of H_2O_2, respectively (*9*), appear to be very low or absent in brain (*10*). An enzyme that utilizes H_2O_2 to convert tyrosine to dopa has been found in human brain (*11*); perhaps this enzyme serves in a protective role. However, brain tissue is uniquely sensitive to oxidative inhibition of glycolysis when H_2O_2-generating agents such as the *o*- or *p*-dihydroxy phenols are added in vitro (*10, 12*). This sensitivity may contribute to the susceptibility of catecholamine nerve terminals to degeneration when 6-OHDA is injected in experimental animals.

We have shown that 6-OHDA generates H_2O_2 and that H_2O_2 can damage the uptake systems for the biogenic

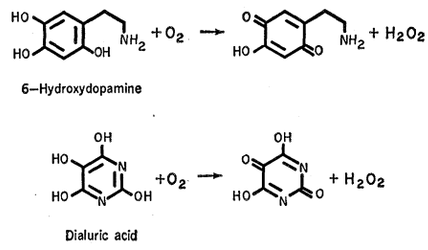

Fig. 1. Formation of hydrogen peroxide from 6-hydroxydopamine or dialuric acid by autoxidation.

amines. The formation of H_2O_2 from 6-OHDA has been reported by others (*13*). While release of catecholamines by 6-OHDA or competitive inhibition of uptake may be the mechanisms for the reversible loss of catecholamines in vivo, damage by H_2O_2 generated from the 6-OHDA that accumulates in catecholamine terminals may be the cause of the long-lasting catecholamine depletion that accompanies the destruction of nerve terminals.

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5. S. H. Snyder and J. T. Coyle, *J. Pharmacol. Exp. Ther.* **165**, 78 (1969). A crude pellet of synaptosomes and mitochondria was isolated by centrifugation at 27,500*g* for 30 minutes. The pellet was suspended in 10 ml of Krebs-Ringer phosphate containing glucose (1 mg/ml), Pargyline (0.016 mg/ml), and ethylenediaminetetraacetic acid (0.05 mg/ml), but no ascorbate. Portions (0.5 ml) were added to 9.5 ml of the same medium for uptake studies. Ascorbate was not added to the medium lest it interfere with the experiments since it is itself an H_2O_2-generating agent [B. Chance, *Biochem. J.* **46**, 387 (1950)], and a catalyst for the conversion of catalase to the inactive complex II [D. Keilin and P. Nicholls, *Biochim. Biophys. Acta* **29**, 302 (1958)]. In other experiments, ascorbate potentiated the inhibition of uptake caused by dialuric acid; this may have been due either to reduction of alloxan to dialuric acid or to the H_2O_2 generated by the ascorbate.
6. The crystalline catalase suspension (1 ml) was centrifuged at 700*g* for 10 minutes. The supernatant was discarded, and the crystals were dissolved in 10 ml of Krebs-Ringer phosphate solution. Samples of 0.5 ml were used.
7. Aminotriazole was added to 4 ml of the catalase solution (*6*) to give a concentration of 0.1*M*, and the catalase was inhibited by exposing the samples to H_2O_2 vapor for 2 hours at 37°C with a gaseous diffusion method in Warburg vessels [G. Cohen and P. Hochstein, *Science* **134**, 1756 (1961); *Biochemistry* **3**, 895 (1964)]. Aminotriazole by itself had no effect in the uptake system.
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Immunocompetent Cells among Mouse Thymocytes: A Minor Population

Abstract. *Suspensions prepared from thymuses of TL+ mice contain a majority of TL+ cells and a minority of TL− cells. The graft versus host reactivity of the TL− population is much greater than that of the whole population, as judged by the numbers of cells required to give splenic enlargements in Simonsen's assay. It is proposed that the TL− thymocyte represents a stage in the differentiation of TL+ thymocytes into immunocompetent lymphocytes.*

Thymocytes can cause graft versus host reactions (GVHR) but their capacity to do so is greatly inferior to that of lymph node cells, many more thymocytes than lymph node cells being required to produce GVHR (*1*). Indeed it is necessary to consider the possibility that the low GVH activity of suspensions of thymocytes may in fact be due to contamination with immunocompetent cells from the blood or from lymph nodes unintentionally included when thymocyte suspensions are prepared (see below). If such contamination can be ruled out, then two explanations may be suggested for the low GVH activity of thymocyte suspensions—either the activity of individual thymocytes is lower than that of individual lymph node cells, or the