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Effects of 2- and/or 5-Methylated Analogues of 6-Hydroxydopamine on Norepinephrine- and Dopamine-Containing Neurons

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SUMMARY

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To characterize further the molecular mechanism whereby 6-hydroxydopamine (6-OHDA) exerts its cytotoxic effects, a series of 2- and/or 5-methylated derivatives of 6-OHDA was prepared. These analogs exhibit oxidation-reduction potentials similar to that of 6-OHDA, but the corresponding quinones showed different reactivities toward nucleophiles *in vitro*. The quinone forms of 6-OHDA and 5-methyl-6-OHDA reacted with glutathione, whereas 2-methyl-6-OHDA quinone reacted very slowly, if at all, and 2,5-dimethyl-6-OHDA quinone was unreactive. *In vivo* both 6-OHDA and 5-methyl-6-OHDA were shown to produce long-term depletion of norepinephrine in mouse brain, whereas 2-methyl-6-OHDA and 2,5-dimethyl-6-OHDA were substantially less active. Only 6-OHDA produced long-term depletion of dopamine. These compounds were also tested for their abilities to inhibit uptake or cause release of [^{3}H]norepinephrine or [^{3}H]dopamine, using chopped rat cerebral cortex or corpus striatum. The observed order of potencies for both release and inhibition of uptake was 6-OHDA > 2-methyl-6-OHDA $\cong$ 5-methyl-6-OHDA > 2,5-dimethyl-6-OHDA. The differences in potency between 6-OHDA and 5-methyl-6-OHDA in producing long-term depletion of catecholamines can be explained on the basis of differences in affinities for the uptake systems. However, the differences in potency between 5-methyl-6-OHDA and 2-methyl-6-OHDA in producing long-term norepinephrine depletion cannot be rationalized on a similar basis. A more reasonable explanation for this difference is that 5-methyl-6-OHDA quinone can react with cellular nucleophiles, whereas 2-methyl-6-OHDA quinone cannot. Therefore 5-methyl-6-OHDA produces neuronal destruction through a mechanism similar to that of 6-OHDA, that being generation of a reactive electrophilic species, as well as hydrogen

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peroxide, superoxide anion, and hydroxyl radical. In contrast, the neurotoxicity produced by 2-methyl-6-OHDA probably results only from the effects of hydrogen peroxide, superoxide anion, and hydroxyl radical.

INTRODUCTION

2,4,5-Trihydroxyphenylethylamine (6-hydroxydopamine) has become widely used as a pharmacological tool, since it produces selective destruction of norepinephrine- and dopamine-containing nerve terminals (1, 2). The molecular mechanism whereby 6-hydroxydopamine exerts its cytotoxic effects on catecholaminergic neurons is still in question; however, its specificity apparently results from the fact that it is selectively transported by the neuronal membrane pump. This transport into norepinephrine- and dopamine-containing neurons is a prerequisite to its degenerative effects (1, 2). The chemical events which subsequently occur intraneuronally and eventually produce the destruction of the nerve terminal are apparently initiated by the intraneuronal autoxidation of 6-OHDA.[3] Two molecular theories (2) have been proposed to explain these cytotoxic effects: (a) the quinoid-like compounds generated by nonenzymatic autoxidation of 6-OHDA may act as alkylating agents (3), or (b) the hydrogen peroxide, superoxide anion, and hydroxyl radical also generated upon oxidation of 6-OHDA may function as oxidizing agents (4–8). Both processes could result in alterations in the properties of important cellular proteins and lipids, thereby initiating the neuronal degeneration. The relative importance of these two proposed mechanisms in the cytotoxicity of 6-OHDA has yet to be clarified.

Saner and Thoenen (3) initially proposed the alkylation theory as an explanation for the cytotoxic effects of 6-OHDA, since it was known that strong electrophiles are generated upon autoxidation of this hydroquinone. The electrophilic species which could potentially be generated intraneuronally include 6-OHDA quinone, aminochrome *1*, and aminochrome *2* (Fig. 1), each capable of rapidly reacting with cellular nucleophiles to form covalent linkages. Several studies *in vivo* (4–6) have subsequently confirmed that the covalent interaction of one or more of these oxidation products with intraneuronal proteins can be correlated with the irreversible damage and neurodegeneration produced by this amine.

Several research groups have attempted to characterize the chemical nature of the interaction between the electrophiles produced from autoxidation of 6-OHDA and model nucleophiles *in vitro* (3, 7–16). Saner and Thoenen (3), using bovine serum albumin as a model protein nucleophile, were able to demonstrate the formation of covalent linkages with the oxidation products of 6-OHDA. They proposed that this interaction involved a 1,4-position Michael addition of the protein nucleophile to 6-OHDA quinone as depicted in Fig. 1 (conversion of 6-OHDA quinone to *3*). Subsequently Creveling *et al.* (7) and Rotman *et al.* (8) confirmed this covalent interaction of the oxidation products of 6-OHDA with bovine serum albumin as well as other model proteins. They also showed that these electrophilic agents can serve as cross-linking ligands for proteins (7, 8).

As another protein model system, the interaction of the oxidation products of 6-OHDA, 6-aminodopamine, and structurally related hydroquinones *in vitro* with the enzyme catechol *O*-methyltransferase has been investigated (9–12). These studies have shown that a nucleophilic residue, probably a sulfhydryl group at the active site of this enzyme (13) is specifically modified by these quinoid oxidation products. The exact chemical nature of this adduct has yet to be elucidated.

Using glutathione as a model system, Liang *et al.* (14, 15) observed that 6-OHDA quinone rapidly reacts with this nucleophile *in vitro* to form 2,4,5-trihydroxy-6-*S*-(glutathionyl)phenylethylamine (*3*; R–SH = glutathione), thereby confirming the chemical mechanism proposed earlier by

[3] The abbreviations used are: 6-OHDA, 6-hydroxydopamine; SCE, standard calomel electrode.

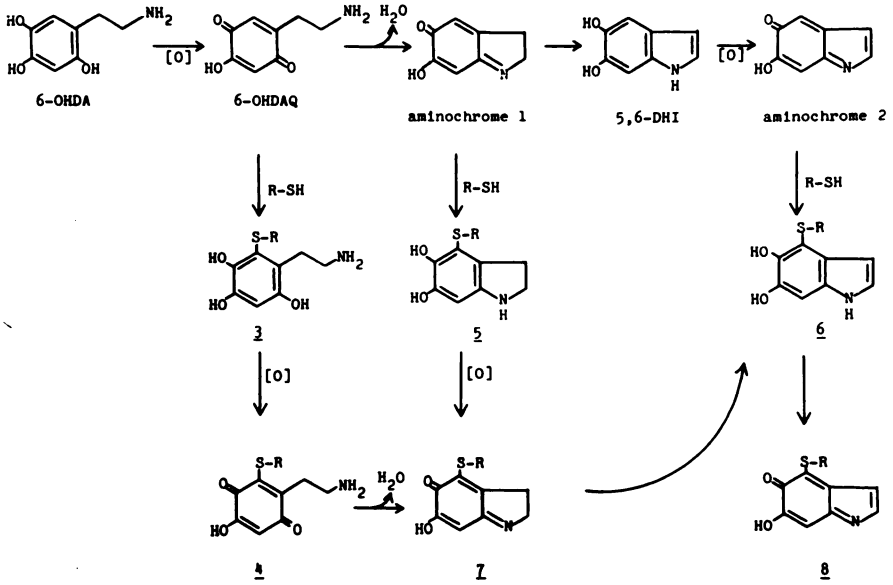
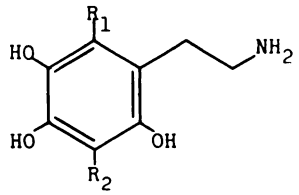


FIG. 1. *Possible modes of reaction between oxidation products of 6-hydroxydopamine and nucleophiles*
For convenience of discussion, several of the intermediates were given trivial names (6-OHDA, 6-hydroxydopamine; 6-OHDAQ, 6-hydroxydopamine quinone; 5,6-DHI, 5,6-dihydroxyindole). [O] refers to an autoxidation process.

Saner and Thoenen (3). In addition, Liang *et al.* (15) isolated the quinone adduct *4* (Fig. 1) from rat brain 1-3 hr after 6-OHDA injections, providing the first chemically identified species resulting from the reaction of 6-OHDA with central nervous system tissue *in vivo*.

Studies of the interaction of aminochromes *1* and *2* with nucleophiles *in vitro* have not been reported; however, data are available on related systems. For example, the reaction of adrenochrome with glutathione, cysteine, and homocysteine was shown to yield 5,6-dihydroxyindole-4-substituted thioethers (16). These results suggest that aminochromes *1* and *2*, upon reaction with a nucleophile, might initially yield addition products *5* and *6* (Fig. 1), respectively, which could undergo further oxidation and rearrangement to yield a wide spectrum of highly reactive quinoids (e.g., *7* and *8*).

The results described above provide strong evidence to support the hypothesis that the oxidation products of 6-OHDA generated *in vivo* could react with intracellular nucleophiles and that this interaction might in part be responsible for the cytotoxic effects of this amine. In addition, these results suggest that the interaction between nucleophiles and the oxidation products of 6-OHDA might, indeed, involve 1,4-position Michael addition reactions as depicted in Fig. 1, consistent with the mechanism originally proposed by Saner and Thoenen (3). In an effort to test this alkylation hypothesis further, we have synthesized 2-methyl-6-OHDA, 5-methyl-6-OHDA, and 2,5-dimethyl-6-OHDA (Fig. 2) and evaluated their effects



	R_1	R_2
6-OHDA	H	H
2-Methyl-6-OHDA	CH_3	H
5-Methyl-6-OHDA	H	CH_3
2,5-Dimethyl-6-OHDA	CH_3	CH_3

FIG. 2. *Analogs of 6-hydroxydopamine synthesized to test the alkylation hypothesis*

in vivo on norepinephrine- and dopamine-containing neurons, as well as their reactivity with glutathione *in vitro*. These analogues of 6-OHDA were chosen since they had been shown earlier (11) to have oxidation-reduction potentials similar to that of 6-OHDA. Therefore they should be equally capable of generating hydrogen peroxide, superoxide anion, and hydroxide radical *in vivo*. However, because of the nature of the methyl substituents, these analogues should exhibit different reactivities toward endogenous nucleophiles. Thus 2-methyl-6-OHDA, 5-methyl-6-OHDA, and/or 2,5-dimethyl-6-OHDA may serve as agents capable of distinguishing the relative importance of the two proposed mechanisms of 6-OHDA action.

MATERIALS AND METHODS

The following compounds were commercially available from the indicated sources: [^{3}H]dopamine hydrochloride (2 Ci/mmole) and DL-[7-^{3}H]norepinephrine hydrochloride (5–15 Ci/mmole), Amersham/Searle or New England Nuclear; 2-(2′,4′,5′-trihydroxyphenyl)ethylamine hydrobromide (6-OHDA), glutathione, and pargyline hydrochloride, Sigma; and 3,4-dihydroxybenzylamine hydrochloride, Aldrich. 2-(6′-Methyl-2′,4′,5′-trihydroxyphenyl)ethylamine hydrobromide (2-methyl-6-OHDA), 2-(3′-methyl-2′,4′,5′-trihydroxyphenyl)-ethylamine hydrobromide (5-methyl-6-OHDA), and 2-(3′,6′-dimethyl-2′,4′,5′-trihydroxyphenyl)ethylamine (2,5-dimethyl-6-OHDA) were synthesized using previously described procedures (11). Sprague-Dawley rats (male, 250–300 g) and ARS(ICR)$_f$ outbred Swiss albino mice (male, 20 g) were purchased from ARS/Sprague-Dawley.

Electroanalytical chemistry. The reduction potentials ($E^{0\prime}$) and the rate constants for cyclization (k_1) were determined in phosphate (0.2 M)–citrate (0.1 M) buffer, pH 7.4, and were measured with a PAR 174 polarograph equipped with a dropping mercury electrode and a standard calomel reference electrode as previously described (11, 17). The rate constants for cyclization (k_1) were calculated by plotting the logarithm of the quinone concentration, measured polarographically, against time, and all were in good accord with first-order kinetic behavior. The reaction of glutathione with the quinones generated from oxidation of 6-OHDA, 2-methyl-6-OHDA, 5-methyl-6-OHDA, and 2,5-dimethyl-6-OHDA were examined in phosphate-citrate buffer, pH 7.4, using the techniques of cyclic voltammetry, ultraviolet spectroscopy, and high-performance liquid chromatography as described in detail earlier (14, 15). The quinones were formed by air oxidation of the corresponding hydroquinones. Following air oxidation the solutions were deoxygenated with a stream of nitrogen to allow polarographic measurement without interference from oxygen polarographic waves. High-performance liquid chromatography was utilized to separate the products generated by reaction of the quinones with glutathione. A Zipax SCX strong cation resin was used as the stationary phase, and 0.1 M $HClO_4$ as the eluent (14).

Long-term effects on mouse brain norepinephrine and dopamine levels. Stock solutions of the drugs (6-OHDA, 2-methyl-6-OHDA, 5-methyl-6-OHDA, and 2,5-dimethyl-6-OHDA) as the hydrobromide salts were prepared in 0.9% NaCl containing 0.1% ascorbic acid and stored frozen in sealed ampoules under nitrogen. Drug treatment groups consisted of 6–12 animals, with the control group receiving injections of 0.9% NaCl containing 0.1% ascorbic acid. Mice were injected intracranially (25 μg/5-μl injection) on days 1, 2, 3, and 4, and on day 7 the animals were killed. The whole brains were immediately removed and frozen individually in a Dry Ice–Acetone bath. The brains were homogenized in 0.1 N $HClO_4$ and centrifuged, and the dopamine and norepinephrine contents in the supernatant were determined by high-performance liquid chromatography using an electrochemical detector (18). The results were expressed as nanograms of catecholamine per gram of tissue, wet weight.

Uptake and release of [^{3}H]norepinephrine in mouse heart in vivo. Inhibition of uptake of [^{3}H]norepinephrine by mouse heart *in vivo* was estimated using a

procedure similar to that described by Creveling *et al.* (19). Inhibition of uptake was estimated by measuring the tritium content of cardiac tissue following intravenous administration of [^{3}H]norepinephrine and comparing it with the amount retained following the simultaneous administration of the same dose of [^{3}H]norepinephrine and varying doses of a test compound. The test compounds and [^{3}H]norepinephrine for both uptake and release experiments were prepared in 0.9% NaCl containing 5% heparin and stored frozen in sealed ampoules under nitrogen. The stock solution of [^{3}H]norepinephrine hydrochloride (0.1 mCi/ml) was diluted with an equal volume of a solution containing the test compound or 0.9% NaCl (control animals), and 0.1 ml of this mixture was administered via the tail vein. Animals were killed by cervical dislocation after 20 min, and their hearts were immediately excised and frozen with Dry Ice. Hearts from animals in each dosage group (four or five mice) were pooled, weighed, and homogenized in 10 volumes of ice-cold 0.5 N $HClO_4$. The homogenates were centrifuged for 20 min at 16,000 × *g*, and an aliquot (0.5 ml) of the supernatant was removed and added to 10 ml of a dioxane-based scintillation fluid. Radioactivity was determined in a scintillation spectrometer. The results were expressed as percentages of control values. ED_{50} values were obtained from the linear portion of the dose-response curve.

Release of [^{3}H]norepinephrine was estimated using a procedure similar to that described by Daly *et al.* (20). A 0.1-ml solution of [^{3}H]norepinephrine (1 nmole, 5 μCi) was injected into the tail veins of mice. After 1 hr the solutions of the test compounds were administered subcutaneously. After 3 hr the mice were killed by cervical dislocation and the hearts were removed, pooled (four or five mice per assay), weighed, and homogenized in 10 volumes of 0.4 N $HClO_4$. After centrifugation, the tritium content of 0.2 ml of the supernatant solution was measured by scintillation spectrometry. Results were expressed as percentages of control values. ED_{50} values were obtained from the linear portion of the dose-response curve.

Release of [^{3}H]norepinephrine or [^{3}H]dopamine from chopped rat brain. The effects of the 6-OHDA analogues on release of [^{3}H]norepinephrine from chopped rat cerebral cortex or of [^{3}H]dopamine from chopped corpus striatum were measured using a modification of the procedure of Ziance and Rutledge (21) and Tessel and Rutledge (22). Chopped rat brain tissue (cerebral cortex or corpus striatum) was incubated in a physiological saline solution (23) containing 0.24 mM pargyline, 1.7 mM ascorbate, and a 1 μM concentration of either [^{3}H]norepinephrine or [^{3}H]dopamine. The [^{3}H]catecholamine was accumulated in the nerve ending by incubation (15 min, 37°); the unbound and nonspecifically bound [^{3}H]catecholamine was removed by washing. The [^{3}H]catecholamine released into the incubation medium upon incubation (30 min, 37°) with the test compound was then measured. [^{3}H]Catecholamines in the tissue and medium were then determined by separating them from the metabolites using cation-exchange chromatography (Dowex 50, Na^+ form), and the results were generally expressed as a percentage of [^{3}H]catecholamine in the incubation medium, calculated as

$$\frac{([^3\text{H}]\text{catecholamine in medium}) \times 100}{([^3\text{H}]\text{catecholamine in medium}) + ([^3\text{H}]\text{catecholamine in tissue})}$$

Inhibition of [^{3}H]norepinephrine or [^{3}H]dopamine uptake into chopped rat brain. The methods used to measure inhibition of [^{3}H]catecholamine uptake were those described earlier by Ziance and Rutledge (21) and Kalisker *et al.* (24). The chopped tissue was suspended and incubated for 10 min in physiological medium (23) containing 0.24 mM pargyline, 1.77 mM ascorbate, and various concentrations of the drug. [^{3}H]Norepinephrine (cerebral cortex) or [^{3}H]dopamine (corpus striatum)

was added to the incubation medium to attain a final concentration of 0.1 μM. The incubation was continued at 37° for 20 min, after which the uptake of [^{3}H]catecholamine was terminated by centrifugation at 4°. The [^{3}H]catecholamines present in the tissue and medium were determined by separating them from the metabolites using cation-exchange chromatography (Dowex 50, Na^+ form) followed by liquid scintillation spectrometry, and the percentage inhibition of uptake was calculated according to the following equation:

$$\frac{T/M_0 - T/M_s}{T/M_0 - T/M_{100}}$$

where T/M_0 is the ratio for the vehicle control, T/M_s is the ratio for sample incubated in the presence of drug, and T/M_{100} is the ratio of the control incubated in the presence of 100 μM unlabeled norepinephrine or dopamine.

RESULTS

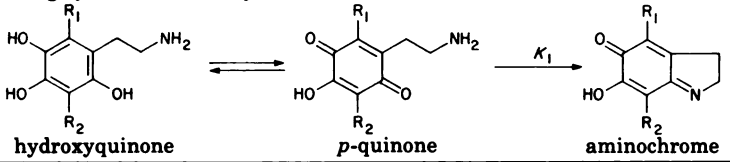
Chemical and electrochemical properties of 6-OHDA analogues. As shown in Table 1, 6-OHDA and its methylated analogues (2-methyl-6-OHDA, 5-methyl-6-OHDA, and 2,5-dimethyl-6-OHDA) have similar oxidation-reduction potentials, suggesting that these compounds would be easily oxidized *in vivo* to the corresponding quinones, hydrogen peroxide, superoxide anion, and hydroxide radical. The *p*-quinones generated from oxidation of 6-OHDA, 2-methyl-6-OHDA, and 5-methyl-6-OHDA all show similar rates of cyclization to the corresponding aminochromes. However, the 2,5-dimethyl-6-OHDA quinone exhibits a substantially faster rate of cyclization, suggesting a shorter half-life *in vivo* for both the hydroquinone and the quinone. The most striking differences in the chemical properties of these quinones are their reactivities *in vitro* with nucleophiles. As a test system, we examined the reactions of these quinones with glutathione using the techniques of cyclic voltammetry, ultraviolet spectroscopy, and high-performance liquid chromatography.

The cyclic voltammograms of the three methylated 6-OHDA analogues are similar to the cyclic voltammogram for 6-OHDA and show a typical hydroquinone-quinone oxidation-reduction couple in pH 7.4 buffer. Peak *A* in Fig. 3 illustrates just the quinone reduction part of the 5-methyl-6-OHDA quinone cyclic voltammogram. Upon addition of GSH, this peak decreases almost immediately (curve *B*), decreases almost immediately (curve *B*), illustrating very rapid reaction with the quinone. The rate of this nucleophilic addition at pH 7.4 is too rapid to be easily

TABLE 1

Electrochemical properties of analogues of 6-OHDA and reactivity of corresponding quinones with glutathione

The reduction potentials ($E^{0\prime}$), the rate constants for cyclization (k_1), and the reactivity with glutathione were measured using cyclic voltammetry as described in MATERIALS AND METHODS (11, 17).



hydroxyquinone *p*-quinone aminochrome

Hydroquinone	R_1	R_2	$E^{0\prime}$ (*p*-quinone)	Cyclization		Reactivity of *p*-quinone with GSH, T_2[a]
				$k_1 \times 10^4$	$T_{\frac{1}{2}}$	
			V vs. SCE	sec^{-1}	*min*	*sec*
6-OHDA	H	H	0.210	3.0[b]	39	31
2-Methyl-6-OHDA	CH_3	H	0.220	6.8[b]	17	—[c]
5-Methyl-6-OHDA	H	CH_3	0.210	4.9[b]	24	23
2,5-Dimethyl-6-OHDA	CH_3	CH_3	0.230	18.3[d]	6	—[c]

[a] The reactions of the *p*-quinones with glutathione were carried out at 25°, and the $T_{\frac{1}{2}}$ values were based on 1 mM glutathione.

[b] Measured at 37°.

[c] No reaction.

[d] Measured at 25°.

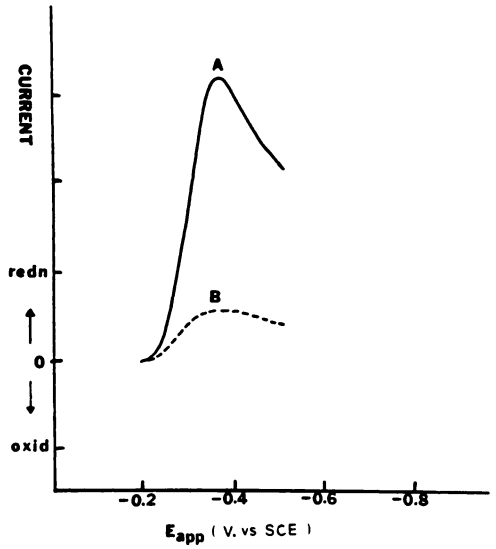


FIG. 3. *Peak polarogram of 5-methyl-6-OHDA alone and in the presence of glutathione*

A, Air-oxidized 5-methyl-6-OHDA at pH 7.4; *B*, immediately after addition of glutathione (1 mM).

studied electrochemically but can be scanned spectroscopically. The ultraviolet spectra showed that at pH 7.4 the air-oxidized 5-methyl-6-OHDA is violet and has λ_{max} at approximately 270 nm; when GSH is added the color fades away and gives λ_{max} near 300 nm. The rate constants for the reaction of GSH and 5-methyl-6-OHDA quinone were determined by following the increase in absorbance at approximately 300 nm. The nucleophilic addition of GSH to 5-methyl-6-OHDA quinone was further confirmed by high-performance liquid chromatography, which showed the appearance of a new peak that had the same retention time as the 2,4,5-trihydroxy-6-*S*-(glutathionyl)phenylethylamine, suggesting that this new peak was a 5-methyl-6-OHDA quinone–GSH adduct.

The rate constants for reactions of GSH with 6-OHDA quinone and 5-methyl-6-OHDA quinone are almost identical (Table 1). In contrast, 2-methyl-6-OHDA quinone reacted extremely slowly with GSH, if at all, and 2,5-dimethyl-6-OHDA quinone was unreactive. The marked differences in these rates of reaction were observed both by cyclic voltammetry and by ultraviolet spectroscopy. Liang *et al.* (14, 15) previously reported the isolation by liquid chromatography and chemical identification of 2,4,5-trihydroxy-6-*S*-(glutathionyl)phenylethylamine as the adduct formed upon reaction of 6-OHDA quinone and GSH. In the case of 5-methyl-6-OHDA quinone, a similar GSH adduct was detected using liquid chromatography. In contrast, the liquid chromatographic analysis of the reaction mixtures of 2-methyl-6-OHDA quinone plus GSH or of 2,5-dimethyl-6-OHDA quinone plus GSH, after even prolonged reaction times, showed no similar GSH adduct product, nor any changes in the cyclic voltammograms or ultraviolet spectra with time. From the intramolecular cyclization rates (k_1, Table 1) of these methylated analogues of 6-OHDA, it is clear that at pH 7.4, GSH addition to 5-methyl-6-OHDA quinone and 6-OHDA quinone occurs much more rapidly than intramolecular cyclization. However, in the case of 2-methyl-6-OHDA quinone and 2,5-dimethyl-6-OHDA quinone, intramolecular cyclization occurs before GSH addition at pH 7.4.

Long-term effects of 6-OHDA analogues on mouse brain norepinephrine and dopamine levels. The methylated analogues of 6-OHDA were tested for their abilities to produce long-term depletion of norepinephrine and dopamine in mouse brain as an index of their neurodegenerative effects. The drugs were administered intracranially, and the catecholamine levels were determined by liquid chromatography using electrochemical detection (18). As shown in Table 2, 6-OHDA depletes norepinephrine to 28% of control values, and dopamine to 55% of control values, under the dosage schedule used in these experiments. 2-Methyl-6-OHDA, 5-methyl-6-OHDA, and 2,5-dimethyl-6-OHDA, in our study, produced no long-term depletion of dopamine; however, interesting differences were observed in their abilities to produce long-term depletion of norepinephrine. 5-Methyl-6-OHDA, which readily reacts with nucleophiles *in vitro*, depleted norepinephrine to 52% of control values, whereas 2-methyl-6-OHDA and 2,5-dimethyl-6-OHDA, which did not react *in vitro* with nucleophiles, were substantially less effective, depleting norepinephrine to 78% and 87% of control levels,

TABLE 2

Long-term depletion of mouse brain norepinephrine and dopamine after intracranial injections of 6-OHDA analogues

Drugs, as the hydrobromide salts, were injected intracranially (25 μg/5-μl injection) in 0.9% NaCl containing 0.1% ascorbic acid. Groups of 6–12 mice were used, with injections being made on days 1, 2, 3, 4, and the animals were killed on day 7. Brains were homogenized in 0.1 N $HClO_4$, and the catecholamine content was determined by high-performance liquid chromatography using electrochemical detection (18). See MATERIALS AND METHODS for details. Values are means ± standard errors.

Drug	Brain catecholamine content			
	Norepinephrine		Dopamine	
	ng/g	*% control*	*ng/g*	*% control*
None[a]	503 ± 14	100	1011 ± 17	100
6-OHDA	143 ± 12	28	555 ± 40	55
2-Methyl-6-OHDA	393 ± 54	78	1155 ± 99	114
5-Methyl-6-OHDA	264 ± 28	52	956 ± 129	95
2,5-Dimethyl-6-OHDA	438 ± 44	87	988 ± 107	98

[a] Control animals were injected with an equal volume of 0.9% NaCl.

TABLE 3

Effects of analogues of 6-OHDA on uptake and release of [³H]norepinephrine in mouse hearts in vivo

To estimate the inhibition of [³H]norepinephrine uptake, varying amounts of the drug were coadministered with a constant amount of [³H]norepinephrine to mice intravenously, and the amount of tritium retained in the heart was determined after 20 min (see MATERIALS AND METHODS for details). Release studies were carried out by intravenous injection of [³H]norepinephrine 60 min prior to the subcutaneous injection of various doses of the drug. Animals were killed after another 2 hr. Mean tritium content per heart was determined according to the procedures described in MATERIALS AND METHODS. ED_{50} values were determined from dose-response curves (percentage of control values vs. log dose).

Compound	ED_{50}	
	Inhibition of [³H]norepinephrine uptake	[³H]Norepinephrine release
	mg/kg	*mg/kg*
6-OHDA	0.3	6
2-Methyl-6-OHDA	1.0	50
5-Methyl-6-OHDA	4.0	65
2,5-Dimethyl-6-OHDA	>10	>100

respectively. The depletion of norepinephrine produced by 2-methyl-6-OHDA was statistically significant ($p < 0.005$) compared with control values. Similarly, the difference in depletion produced by 2-methyl-6-OHDA and 5-methyl-6-OHDA was also significant ($p < 0.005$).

Effects of 6-OHDA anlogues on uptake and release of [³H]norepinephrine in mouse heart in vivo. To evaluate the relative affinities of 6-OHDA, 2-methyl-6-OHDA, 5-methyl-6-OHDA, and 2,5-dimethyl-6-OHDA for the neuronal uptake system, we tested these compounds for their abilities to inhibit [³H]norepinephrine uptake in mouse heart *in vivo*. All the 6-OHDA analogues inhibited the uptake of [³H]norepinephrine; however, 2,5-dimethyl-6-OHDA produced inhibition only at high doses (Table 3). The inhibition was dose-dependent, and ED_{50} values were obtained from the linear portions of the dose-response curves. The relative order of potencies for inhibition of [³H]norepinephrine uptake was 6-OHDA > 2-methyl-6-OHDA ≧ 5-methyl-6-OHDA ≫ 2,5-dimethyl-6-OHDA.

6-OHDA, 2-methyl-6-OHDA, and 5-methyl-6-OHDA also produced dose-dependent release of [³H]norepinephrine from mouse heart (Table 3). The relative order of potencies was 6-OHDA > 2-methyl-6-OHDA = 5-methyl-6-OHDA ≫ 2,5-dimethyl-6-OHDA, which was consistent with the order of potencies observed for inhibition of uptake. However, substantially higher doses were required to cause release than to inhibit uptake of the catecholamine.

Inhibition of [³H]catecholamine uptake into chopped rat brain tissue. All the methylated analogues of 6-OHDA inhibited uptake of [³H]norepinephrine into chopped cerebral cortex (Fig. 4). The ana-

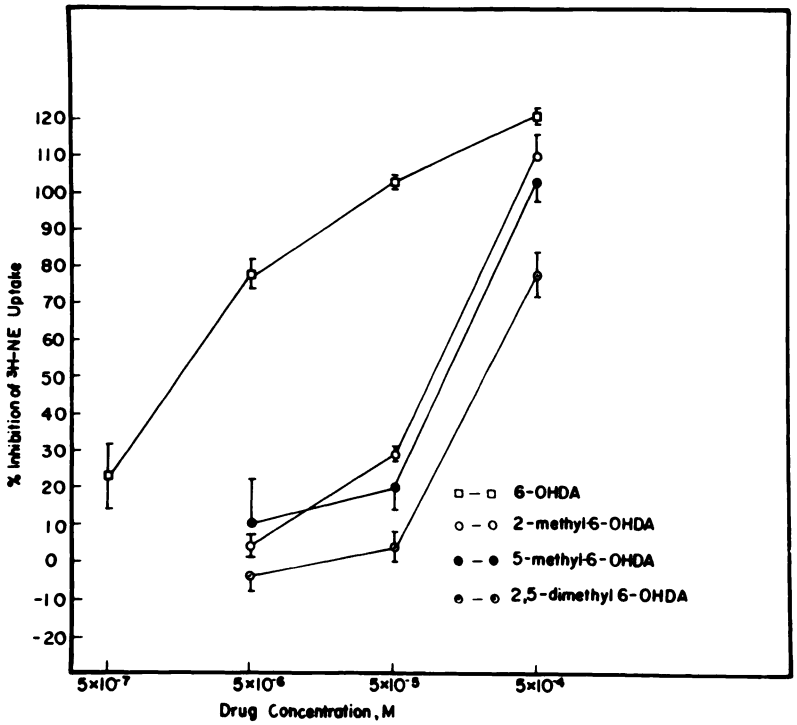


FIG. 4. *Inhibition of [³H]norepinephrine (³H-NE) uptake into chopped rat cerebral cortex by analogues of 6-OHDA*

Chopped rat cerebral cortex was incubated for 10 min with various concentrations of the drug. [³H]Norepinephrine was then added to the incubation medium so as to attain a final concentration of 0.1 μM. Incubation was continued at 37° for 20 min, after which the reaction was stopped by centrifugation at 4°. The radioactivity present in the tissue and medium was determined by liquid scintillation spectrometry, and the percentage inhibition of uptake was calculated as described in MATERIALS AND METHODS. The values shown represent the means ± standard errors of at least three determinations.

logues exhibited dose-dependent inhibition of [³H]norepinephrine uptake, with their potencies decreasing with addition of methyl substituents to the basic 6-OHDA structure. The relative order of potencies was 6-OHDA > 2-methyl-6-OHDA = 5-methyl-6-OHDA > 2,5-dimethyl-6-OHDA. The compounds all showed approximately equal efficacies (maximal responses). This order of potencies was consistent with that observed for inhibition of [³H]norepinephrine uptake into mouse heart *in vivo* (Table 3).

Similar results were observed for inhibition of [³H]dopamine uptake into chopped corpus striatum (Fig. 5). All the compounds showed equal efficacies (maximal responses); however, the potencies decreased with increasing number of methyl substituents (6-OHDA > 2-methyl-6-OHDA = 5-methyl-6-OHDA > 2,5-dimethyl-6-OHDA). The differences in potency between 6-OHDA and 2-methyl-6-OHDA (or 5-methyl-6-OHDA) for inhibition of [³H]dopamine uptake are less dramatic than for inhibition of [³H]norepinephrine uptake. 2-Methyl-6-OHDA and 5-methyl-6-OHDA appear to have a higher affinity for the dopamine uptake system than for the norepinephrine uptake system.

Release of [³H]catecholamine from chopped rat brain. [³H]Norepinephrine was released from chopped cerebral cortex by 6-OHDA and the various methylated analogues (Fig. 6). 6-OHDA, 2-methyl-6-OHDA, and 5-methyl-6-OHDA exhibited dose-dependent release of [³H]norepinephrine, with equal efficacies but varying potencies. 2-Methyl-6-OHDA and 5-methyl-6-OHDA were of about equal potency, but both were substantially less po-

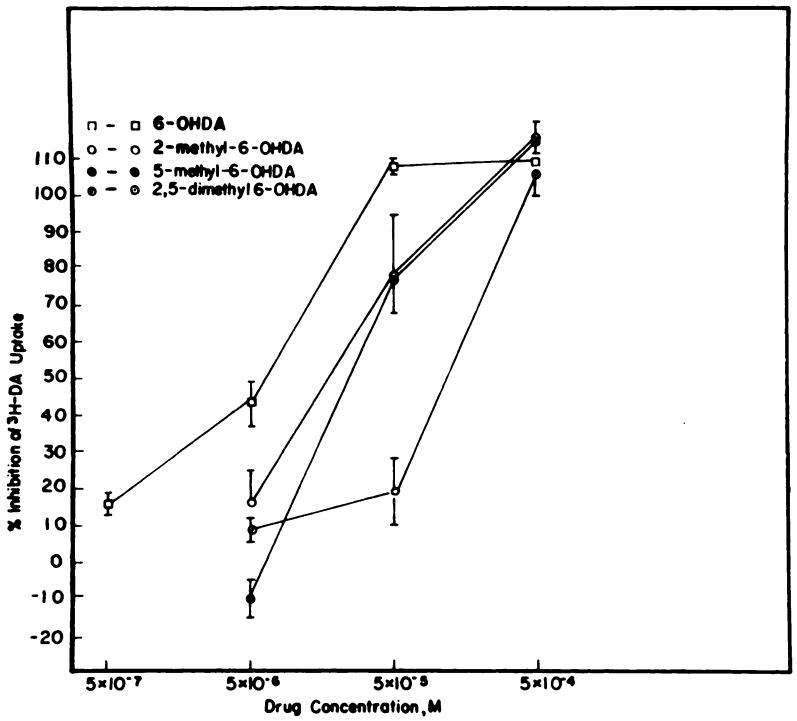


FIG. 5. *Inhibition of [³H]dopamine (³H-DA) uptake into chopped rat corpus striatum by analogues of 6-OHDA*

Chopped rat corpus striatum was incubated with various concentrations of the drug and then with [³H]dopamine as described in Fig. 4. The radioactivity present in the tissue and medium was determined, and the percentage inhibition of uptake was calculated relative to control samples, which contained no inhibitor. The values shown represent the means ± standard errors of at least three determinations.

tent than 6-OHDA. 2,5-Dimethyl-6-OHDA released [³H]norepinephrine, but only at a high concentration (1 mM).

These compounds also produced release of [³H]dopamine from chopped corpus striatum with approximately equal efficacies (maximal responses) (Fig. 7). The order of potencies for release of [³H]dopamine was 6-OHDA ≧ 5-methyl-6-OHDA > 2-methyl-6-OHDA > 2,5-dimethyl-6-OHDA.

DISCUSSION

In an effort to gain some insight into the relative importance of the various oxidation products of 6-OHDA in the neurodegenerative process, we set out to synthesize analogues of 6-OHDA which would exhibit oxidation-reduction chemistry similar to the parent compound but which would generate electrophiles upon oxidation that showed different reactivities with nucleophiles. If these analogues were concentrated in catecholaminergic neurons like 6-OHDA, we should then be able to test the relative importance of the electrophiles (6-OHDA quinone, aminochromes *1* and *2*) vs. the oxygen oxidation products (hydrogen peroxide, superoxide anion, and hydroxyl radical) in the neurotoxicity of this amine.

The oxidation-reduction potentials of the analogues of 6-OHDA synthesized in this study were shown to be identical with that of 6-OHDA. However, as was predicted, the quinones generated from autoxidation of these hydroquinones show different reactivities *in vitro* with the nucleophile glutathione. Liang *et al.* (14, 15) previously studied the reaction of 6-OHDA quinone with glutathione and identified the addition product as 2,4,5-trihydroxy-6-*S*-(glutathionyl)phenylethylamine. In this study we found that 5-methyl-6-OHDA

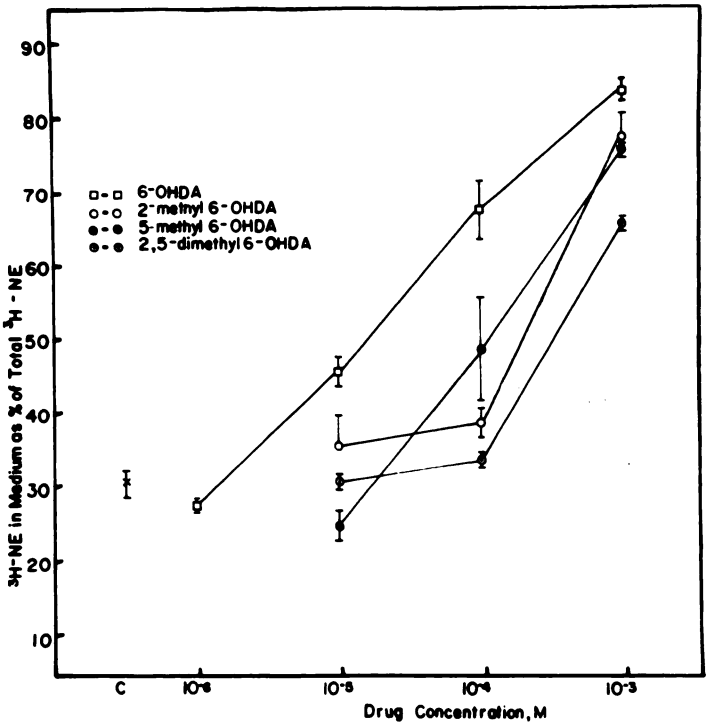


FIG. 6. *Release of [³H]norepinephrine (³H-NE) from chopped rat cerebral cortex by analogues of 6-OHDA*
Neurons were labeled with [³H]norepinephrine in a preliminary incubation. The tissue was washed and then incubated for 30 min in the presence and absence of drug. The proportion of norepinephrine released into the incubation medium was calculated as (norepinephrine in the medium × 100)/(total norepinephrine). The values shown represent the means ± standard errors of at least three determinations. The symbol × above abscissa point *C* represents release from control samples.

quinone reacts rapidly with glutathione (Fig. 3), resulting in the formation of a similar 1,4-position Michael addition adduct. Consistent with our predictions was the observation that 2-methyl-6-OHDA quinone and 2,5-dimethyl-6-OHDA quinone did not yield similar addition products when incubated *in vitro* with glutathione. In fact, these quinones, which have methyl substituents in the chemically active 2-position of 6-OHDA, were completely inert to nucleophilic addition by glutathione (Table 1). Therefore the results of the chemical studies suggest that these 6-OHDA derivatives possess the chemical properties needed to test the relative toxicity of the oxidation products of 6-OHDA.

The results of our studies *in vivo* using 6-OHDA analogues support the idea that many, if not all, of the oxidation products of 6-OHDA are involved in the neurotoxicity of this amine. The order of activity observed for producing long-term depletion of norepinephrine in mouse brain, which was used here as an index of neurotoxicity, was 6-OHDA > 5-methyl-6-OHDA > 2-methyl-6-OHDA > 2,5-dimethyl-6-OHDA (Table 2). The observation that 5-methyl-6-OHDA was less effective than 6-OHDA in producing depletion of norepinephrine was somewhat surprising, since 5-methyl-6-OHDA, like 6-OHDA, should yield both a chemically reactive nucleophile (5-methyl-6-OHDA quinone), as indicated from studies *in vitro*, and the oxygen oxidation products. However, the results obtained from studying the inhibition of uptake and release of norepinephrine both *in vitro*, from chopped cerebral cortex (Figs. 4 and 6), and *in vivo*, from mouse heart (Table 3), indicate that 5-methyl-6-OHDA apparently has a lower affinity than 6-OHDA for the neuronal

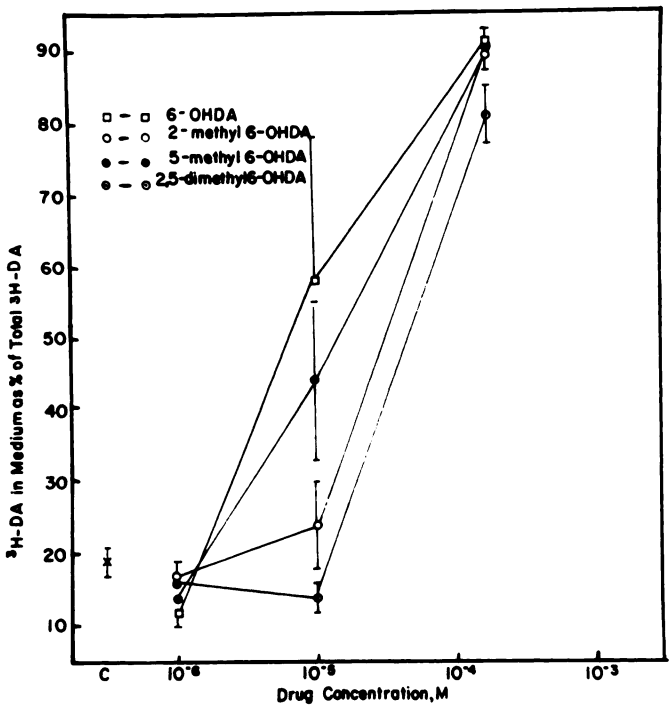


FIG. 7. *Release of [^{3}H]dopamine (^{3}H-DA) from chopped rat corpus striatum by analogues of 6-OHDA*
Neurons were labeled by incubating the tissue with [^{3}H]dopamine and then incubated in the presence or absence of drug as described in Fig. 6. The values shown represent the means ± standard errors of three determinations. The symbol × above abscissa point *C* represents release from control samples.

membrane pump. This difference in affinity for the transport mechanism could account for the differences observed in neurotoxicity. 2-Methyl-6-OHDA and 2,5-dimethyl-6-OHDA, which have oxidation-reduction potentials similar to 6-OHDA (Table 1) but apparently produce a less reactive electrophile, were substantially less effective in producing long-term depletion of norepinephrine in mouse brain than 6-OHDA or 5-methyl-6-OHDA (Table 2). The differences observed in the capability of 5-methyl-6-OHDA and 2-methyl-6-OHDA to produce long-term depletion of norepinephrine cannot be explained on the basis of differences in affinity for the membrane pump, since these amines showed equal potencies in inhibiting uptake or causing release of norepinephrine *in vitro*, from chopped cerebral cortex (Figs. 4 and 6), and *in vivo*, from mouse hearts (Table 3). The differences in the neurotoxicity of 5-methyl-6-OHDA and 2-methyl-6-OHDA (Table 2) can best be explained by the differences in the chemical reactivities of the corresponding quinones (5-methyl-6-OHDA quinone and 2-methyl-6-OHDA quinone) with cellular nucleophiles. The inability of 2,5-dimethyl-6-OHDA to produce any long-term depletion of norepinephrine probably results from its lack of affinity for the neuronal membrane pump (Figs. 4 and 6). The "apparent" differences in affinities of the neuronal membrane pump for 2-methyl-6-OHDA (or 5-methyl-6-OHDA) vs. 2,5-dimethyl-6-OHDA may be due to the more rapid rate of cyclization of 2,5-dimethyl-6-OHDA quinone to the corresponding aminochrome *in situ*. As shown in Table 1, the rate of cyclization of 2,5-dimethyl-6-OHDA quinone was substantially greater than that for 2-methyl-6-OHDA quinone (or 5-methyl-6-OHDA quinone), which would suggest that the apparent half-life *in vivo* for 2,5-dimethyl-6-OHDA would be shorter than that for 2-methyl-6-OHDA (or 5-methyl-6-OHDA). Therefore smaller extraneuronal concen-

trations of 2,5-dimethyl-6-OHDA would be available for selective transport into the neuron. Thus a critical intraneuronal concentration would not be attained. This chemical property of 2,5-dimethyl-6-OHDA is probably also an explanation for its lower potency in causing inhibition of uptake and release of norepinephrine *in vivo* (Table 3) and *in vitro* (Figs. 4 and 6).

The inability of these analogues to produce any long-term depletion of dopamine after intracranial injections in mice (Table 2) probably reflects in part the known resistance of dopaminergic neurons to the cytotoxic effects of 6-OHDA (1, 2). The uptake and release studies in chopped corpus striatum (Figs. 5 and 7) suggest that 2-methyl-6-OHDA, 5-methyl-6-OHDA, and 2,5-dimethyl-6-OHDA may have lower affinity than 6-OHDA for the dopaminergic membrane pump. Therefore the observation that these methylated analogues of 6-OHDA did not have neurotoxic effects on dopaminergic neurons might be attributable to their lower affinity for the membrane pump. This lower affinity would mean that critical intraneuronal concentrations of these amines, which are needed to produce neurodegeneration, could not be achieved. The general resistance of dopaminergic neurons to the neurotoxic effects of 6-OHDA is of interest, since the data shown in Figs. 4–7 would suggest that the dopaminergic and noradrenergic membrane pumps have approximately equal affinities for 6-OHDA. This observation makes the apparent resistance of dopaminergic neurons to 6-OHDA difficult to rationalize simply on the basis of differences in uptake. A possible explanation for this difference in sensitivity to 6-OHDA is that norepinephrine-rich areas are preferentially exposed to higher extraneuronal concentrations of 6-OHDA than are dopamine-rich areas. Alternatively, it might be possible that differences exist in the biochemical environment within dopaminergic neurons as compared with noradrenergic neurons, making dopaminergic neurons inherently more resistant to the oxidation products generated intraneuronally from oxidation of 6-OHDA. This latter possibility is intriguing and perhaps worthy of further experimental investigation.

In conclusion, the results of this study indicate that there exists a role for the electrophiles (6-OHDA quinone, aminochromes *1* and *2*) as well as for the oxygen oxidation products (hydrogen peroxide, superoxide anion, and hydroxyl radical) in a mechanism explaining the neurotoxicity of 6-OHDA. The observed neurotoxic effects of 2-methyl-6-OHDA, which upon oxidation generates an inactive electrophile (2-methyl-6-OHDA quinone), can best be explained on the basis of the toxicity of the oxygen oxidation products (hydrogen peroxide, superoxide anion, and hydroxyl radical). In comparison, the greater neurotoxic effects of 5-methyl-6-OHDA compared with 2-methyl-6-OHDA can best be explained by a combination of the toxic effects of the active electrophile (5-methyl-6-OHDA quinone) and the oxygen oxidation products. Therefore the results of this study make it impossible to rule out a contribution by any of the oxidation products of 6-OHDA to the neurodegeneration process. The observed neurodegeneration produced by 6-OHDA probably results from a series of complex chemical interactions involving both the electrophiles (6-OHDA quinone, aminochromes *1* and *2*) and the oxygen oxidation products.

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