MOLECULAR PHARMACOLOGY, 8, 241–248

Further Studies on the Generation of Hydrogen Peroxide by 6-Hydroxydopamine

Potentiation by Ascorbic Acid

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(Received June 30, 1971)

SUMMARY

We studied the inhibitory action of various compounds, including hydrogen peroxide, on the uptake of [^{3}H]dopamine by rat brain slices. Dialuric acid, 6-hydroxydopamine, and 5-hydroxydopamine consumed oxygen and generated hydrogen peroxide in solution as a result of aerobic oxidation, as measured with an oxygen electrode. The regeneration by catalase of half the oxygen consumed by 6-hydroxydopamine confirmed that oxygen consumption was equal to H_2O_2 production. The rate of oxygen uptake (H_2O_2 production) by dialuric acid or 6-hydroxydopamine was augmented by the addition of ascorbic acid. In addition, alloxan, which is the oxidized form of dialuric acid, consumed oxygen when ascorbate was added. The mechanism for this can be envisaged as reduction of the oxidized compounds by ascorbate, followed by reoxidation to form more H_2O_2, with continuous recycling. Concomitant with increased production of H_2O_2, there was increased inhibition of [^{3}H]dopamine uptake. Ascorbate by itself did not inhibit the uptake of [^{3}H]dopamine and did not produce measurable quantities of H_2O_2. 6-Hydroxydopamine, a compound that causes nerve terminal degeneration *in vivo*, was compared with 5-hydroxydopamine, which does not. As both compounds are structural analogues of dopamine, they can inhibit the uptake of [^{3}H]dopamine into brain slices by competing for the uptake mechanisms. Additionally, both may inhibit uptake irreversibly by generating H_2O_2, which causes oxidative damage. 6-Hydroxydopamine produced H_2O_2 at about 12 times the rate yielded by 5-hydroxydopamine. Ascorbate potentiated H_2O_2 production by 6-hydroxydopamine but suppressed that from 5-hydroxydopamine. These findings are consistent with an important role for H_2O_2 in the 6-hydroxydopamine-induced degeneration of nerve terminals and may explain why 5-hydroxydopamine does not produce degenerative changes.

INTRODUCTION

6-Hydroxydopamine (2,4,5-trihydroxyphenylethylamine) produces a number of changes in both the sympathetic and central nervous systems. These include inhibition of catecholamine uptake mechanisms (1–3), depletion of catecholamine levels (4–7), lowering of catecholamine-biosynthetic enzymes (5, 8, 9), and degeneration of adrenergic nerve terminals (6, 10). Two mechanisms for nerve degeneration have been set forth. Both involve the autoxidation of 6-hydroxydopamine to hydrogen peroxide and

This work was supported by Grant NS-05184 from the Clinical Research Center for Parkinson's and Allied Diseases. NS-05184

a quinone. Previously, we had shown that H_2O_2 generated by 6-hydroxydopamine caused inhibition of biogenic amine uptake systems *in vitro* (11); we proposed that H_2O_2 was responsible for nerve degeneration *in vivo*. Saner and Thoenen (12), on the other hand, reported that the quinone of 6-hydroxydopamine binds *in vitro* to proteins such as albumin. They postulated that a similar reaction involving tissue protein nucleophilic groups might be the cause of nerve degeneration. Since the quinone and H_2O_2 are formed simultaneously during the autoxidation of 6-hydroxydopamine, it is quite possible that both mechanisms play a role.

In the present study we found that ascorbic acid, which reduces the quinone of 6-hydroxydopamine, actually potentiates the damage to the catecholamine uptake systems *in vitro*. The augmentation of H_2O_2 production by ascorbate was measured with an oxygen electrode. A suggested reaction scheme for ascorbate potentiation of H_2O_2 production is shown in Fig. 1. Our results show that H_2O_2 generated by autoxidation, rather than the quinone of 6-hydroxydopamine, is the agent responsible for damage to the catecholamine uptake systems *in vitro*. In contrast, 5- hydroxydopamine (3,4, 5-trihydroxyphenylethylamine), an agent that is taken up and accumulated by sympathetic nerve terminals but does not cause nerve degeneration, was a very much weaker H_2O_2-generating agent *in vitro*. These results are consistent with the point of view that H_2O_2 may be the agent responsible for the nerve degeneration *in vivo*.

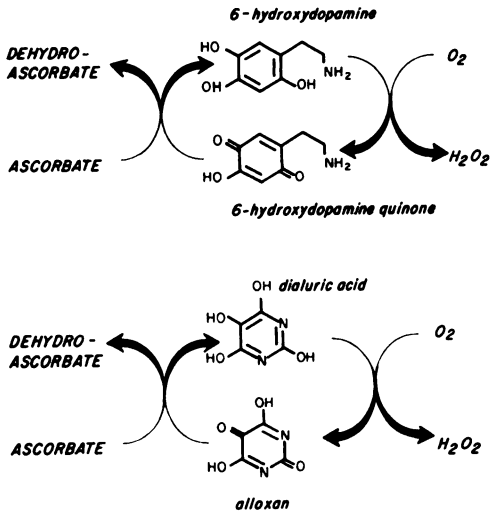

FIG. 1. *Suggested reaction sequence for production of hydrogen peroxide by 6-hydroxydopamine or dialuric acid, with recycling of the corresponding quinones by ascorbic acid*

In aqueous solution at pH 7.4, the 6-hydroxydopamine quinone is rapidly transformed to 6-hydroxyindoline *p*-quinone and other products (12, 13). We did not attempt to distinguish among the several oxidized forms of 6-hydroxydopamine in our studies. The reduction of quinones by ascorbate is a 2-electron transfer process, and intermediate free radicals are generated. We did not attempt to distinguish any intermediary radical species of the organic compounds or of oxygen in our studies, or evaluate their role in controlling the kinetics of appearance of H_2O_2.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing approximately 100 g were used. After decapitation, the brains were removed, rinsed in cold 0.9% NaCl, weighed, and cut with a razor blade into slices approximately 1 mm thick. These were then cross-chopped at 0.2 mm on a McIlwain-Mickle tissue chopper (Brinkmann Instruments). The slices (1.0 × 0.2 × 0.2 mm) were distributed homogeneously by stirring with a magnetic stirrer for approximately 10 min with 100 volumes of ice-cold Krebs-Ringer-phosphate buffer containing glucose (1 mg/ml), pargyline (0.016 mg/ml), and ethylenediaminetetraacetic acid (0.05 mg/ml). Triplicate aliquots (2 ml) containing 20 mg of brain tissue were then pipetted with an Aupette (Clay-Adams) into 25-ml Erlenmeyer flasks containing various amounts of ascorbic acid (Fisher) in 8 ml of the same buffer. The nonradioactive stressor compounds, namely, 6-hydroxydopamine (Regis), 5-hydroxydopamine (Regis), dialuric acid (Calbiochem), alloxan (Calbiochem), or H_2O_2 (Fisher), were added, and samples were incubated with shaking at 37° in an air atmosphere for 30 min. Then the [^{3}H]dopamine (8.3 Ci/mmole, New England Nuclear Corporation) was added to a final concentration of 5 nM, and the incubation was continued for another 15 min. The tissue was assayed for radioactivity according to Shaskan and Snyder (14). Data were expressed as a tissue to medium ratio (counts per minute per gram of tissue divided by counts per minute per milliliter of medium).

Oxygen uptake was measured on a biological oxygen monitor (Yellow Springs Instruments) connected to a Honeywell Electronik 19 recorder. The temperature of the system was kept at 37° by a circulating water pump. The rate of O_2 uptake was measured in 1.0 ml of the phosphate-buffered medium described above. Various compounds, including dopamine (Winthrop), were added with an Oxford automatic pipette from solutions prepared with deoxygenated water; mixing was achieved with a small magnetic stirrer in the electrode chamber. Full scale of the recorder corresponded to 204 nmoles of O_2 per milliliter. Data were calculated as the rate of O_2 disappearance per minute per milliliter.

RESULTS

Experiments with the oxygen electrode. We used an oxygen electrode to study the reaction between molecular oxygen and various compounds. Oxygen consumption was taken as a measure of the generation of H_2O_2 within the solution; this assumption was verified, as discussed below.

A number of compounds are compared in Table 1. Dopamine was used as a control. Although dopamine is recognized as being readily oxidized by molecular oxygen, the rate of reaction for concentrations up to 1 mM was too slow to be detected under our experimental conditions. On the other hand, oxygen consumption by 0.1 mM 6-hydroxydopamine was about 3 times greater than by 0.1 mM dialurate, while the rate for 0.1 mM 5-hydroxydopamine was barely detectable. At 0.5 mM concentrations of compound, oxygen consumption by 6-hydroxydopamine was very vigorous (sufficient to remove all oxygen within 1 min) and about 4 times greater than by dialurate; oxygen consumption by 5-hydroxydopamine was only 6.2% of that by 6-hydroxydopamine.

The augmentation of oxygen consumption by ascorbate is illustrated in Fig. 2. Addition of 0.1 mM 6-hydroxydopamine to the buffered medium (Fig. 2, curve *A*) resulted in oxygen consumption at a rate of 24 nmoles/min. Subsequent addition of ascorbate to 10 mM accelerated the rate to 54 nmoles/min. The reaction continued until the solution was totally deoxygenated. At completion of the reaction, 100 nmoles of 6-hydroxydopamine had consumed 200 nmoles of oxygen. Ascorbate by itself did not provoke measurable consumption of oxygen (data not shown, but compare Fig. 2, curve *D*). With a lower concentration (0.01 mM) of 6-hydroxydopamine in the presence of ascorbate (Fig. 2, curve *E*), the reaction proceeded at a slower rate, but by 20 min 150 nmoles of oxygen had been consumed by only 10 nmoles of 6-hydroxydopamine.

Catalase caused the regeneration of approximately half the oxygen consumed by 6-hydroxydopamine (Fig. 2, curve *B*). This shows that oxygen consumption is a measure of H_2O_2 production within solution. Other investigators[1] have used catalase in conjunction with the oxygen electrode to measure the H_2O_2 produced by dialuric acid. In Fig. 2, curve *C*, 0.1 mM alloxan by itself did not provoke oxygen consumption, but a vigorous reaction did ensue when 10 mM ascorbate was added. In the presence of 10 mM ascorbic acid, the rates of oxygen consumption for

TABLE 1

Rate of disappearance of oxygen from solution in the presence of various autoxidizable substances

Oxygen consumption from 1 ml of solution was measured with an oxygen electrode. The same medium was used as for catecholamine uptake studies. Concentrated stock solutions of the various compounds were prepared in deoxygenated water, and these were added to the medium in the electrode chamber by micropipette (10 μl). Rate data are from the linear portion of the curve, and represent the mean and standard deviation of three separate experiments.

Compound	Rate
	nmoles O_2/min
0.1 mM dopamine	<2.0
0.1 mM 5-hydroxydopamine	2.0 ± 1.0
0.1 mM dialuric acid	8.8 ± 3.1
0.1 mM 6-hydroxydopamine	28.6 ± 10.8
0.5 mM 5-hydroxydopamine	14.6 ± 3.5
0.5 mM dialuric acid	57.1 ± 20.4
0.5 mM 6-hydroxydopamine	237.3 ± 35.0
1 mM dopamine	<2.0

[1] D. G. Cornwell and D. W. Deamer, unpublished observations.

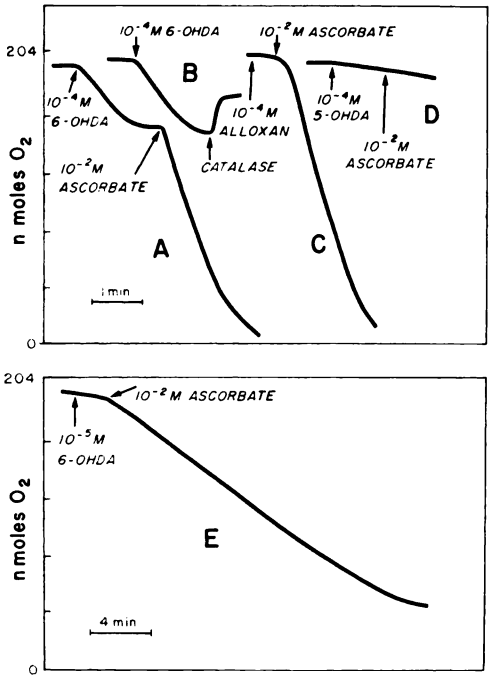

FIG. 2. *Oxygen consumption by various compounds*
The rate of oxygen consumption was measured with an oxygen electrode. The experimental conditions were the same as in Table 1. Points of addition of the various compounds and their final concentration in solution are indicated by arrows. Full scale of the recorder represented 204 nmoles of oxygen in the 1-ml sample. 6-OHDA, 6-hydroxydopamine. 5-OHDA, 5-hydroxydopamine.

alloxan, dialurate, and 6-hydroxydopamine were similar. A similar study with 0.1 mM 5-hydroxydopamine (Fig. 2, curve *D*) revealed very little oxygen consumption either alone or with added 10 mM ascorbate. At higher concentrations of 5-hydroxydopamine (up to 1 mM) no acceleration by ascorbate was observed. In fact, a diminution was evident. For example, at 0.5 mM 5-hydroxydopamine, oxygen consumption was 14.6 nmoles/min without ascorbate and 4.9 nmoles/min with ascorbate. The data show that 6-hydroxydopamine is a potent H_2O_2-generating agent while 5-hydroxydopamine is much weaker. Dialuric acid and alloxan were also potent H_2O_2-generating agents.

Uptake experiments with [^{3}H]catecholamines. We studied the uptake of [^{3}H]dopamine into slices of whole rat brain and the inhibition of uptake by 6-hydroxydopamine, dialuric acid, or alloxan. Previously, an H_2O_2-mediated inhibition in synaptosomes had been observed for 6-hydroxydopamine or dialurate acting upon the uptake mechanisms for dopamine, norepinephrine, or serotonin (11). Since ascorbate augmented H_2O_2 production (Fig. 2), concomitant potentiation of inhibition of amine uptake was expected. The data of Table 2 show potentiation of the inhibition of [^{3}H]dopamine uptake by increasing concentrations of ascorbic acid. Similar results were obtained when the uptake of [^{3}H]norepinephrine was studied. Ascorbate alone was not inhibitory. While 0.1 mM dialuric acid by itself was without effect, the addition of 1 mM ascorbate produced 48% inhibition, and this rose to 90% with 10 mM ascorbate. Ascorbate similarly potentiated the inhibition by 0.1 mM alloxan. It had previously been established with the use of the enzyme catalase that dialuric acid itself was not inhibitory, but that the damage to the uptake systems was caused by H_2O_2 (11). At 0.01 mM 6-hydroxydopamine, 52% inhibition of [^{3}H]-dopamine uptake was evident. This can be attributed in part to H_2O_2, since a portion of the inhibition is prevented by catalase (11), and in part to competition between [H]^3-dopamine and 6-hydroxydopamine for the catecholamine transport systems (3). Once again there was potentiation of the inhibition, which rose from 52% without ascorbate to 87% with 10 mM ascorbate.

The data of Table 3 show dose-response relationships for the inhibitory effects of 6-hydroxydopamine, dialuric acid, or alloxan on the uptake of [^{3}H]dopamine by rat brain slices. Ascorbate (10 mM) potentiated the inhibition over the entire concentration range of stressor agents. The results with alloxan were particularly striking. Alloxan alone was not inhibitory up to a concentration of 1 mM; however, when ascorbate was present, as little as 0.01 mM alloxan caused 74% inhibition. Dialuric acid at 1 mM was strongly inhibitory. While 0.1 mM dialuric acid by itself was not inhibitory, the addition of 10 mM ascorbate produced 96% inhibition, and even 0.01 mM dialurate gave 43% inhibition with ascorbate. Results with 6-hydroxydopamine were once again complicated by

TABLE 2

Inhibition of [^{3}H]dopamine uptake into rat brain slices by 6-hydroxydopamine, dialuric acid or alloxan: potentiation by ascorbic acid

Rat brain tissue slices (20 mg) were incubated for 30 min at 37° in the presence of inhibitory agents and with various amounts of ascorbic acid. [^{3}H]Dopamine was added to a concentration of 5 nM, and uptake was measured after 15 min. Data are expressed as the tissue to medium ratio (T/M) ± the standard deviation for triplicate samples.

Inhibitor	Ascorbate	T/M	T/M with inhibitor	Inhibition
	mM			%
0.01 mM 6-hydroxydopamine	0	34.3 ± 3.1	16.6 ± 1.5	52
	1	40.1 ± 2.4	16.4 ± 0.7	59
	5	35.1 ± 2.3	6.1 ± 0.6	83
	10	39.3 ± 1.9	5.1 ± 0.1	87
0.1 mM dialuric acid	0	34.4 ± 3.0	33.3 ± 1.2	3
	1	31.6 ± 2.4	16.4 ± 1.0	48
	5	29.7 ± 2.3	5.2 ± 0.9	83
	10	29.2 ± 1.4	3.0 ± 0.6	90
0.1 mM alloxan	0	32.0 ± 2.8	32.6 ± 4.3	+2
	1	30.1 ± 1.6	15.6 ± 2.4	48
	5	34.6 ± 2.0	4.2 ± 0.4	88
	10	33.5 ± 3.0	1.4 ± 0.2	96

TABLE 3

Effect of ascorbic acid on dose-response relationship for inhibition of [^{3}H]dopamine uptake into rat brain slices by 6-hydroxydopamine, dialuric acid, or alloxan

Experimental conditions were the same as in Table 2, except that the concentrations of inhibitory agents were varied and only a single concentration of ascorbate (10 mM) was used. Data are expressed as the tissue to medium ratio (T/M) ± the standard deviation of triplicate samples.

Inhibitor	Concentration	T/M	Change	T/M with 10 mM ascorbate	Change
	mM		%		%
6-Hydroxydopamine	0	36.0 ± 2.4		31.7 ± 3.3	
	0.001	26.2 ± 3.8	−27	16.2 ± 1.4	−49
	0.01	13.7 ± 2.5	−62	3.7 ± 0.6	−88
	0.1	4.9 ± 0.6	−86	0.9 ± 0.1	−97
	1	1.4 ± 0.4	−96		
Dialuric acid	0	28.6 ± 3.4		33.9 ± 3.5	
	0.01	31.5 ± 1.2	+10	19.3 ± 0.9	−43
	0.1	30.6 ± 1.0	+7	1.5 ± 0.2	−96
	1	4.1 ± 0.8	−86		
Alloxan	0	28.6 ± 3.4		33.9 ± 3.5	
	0.01	30.3 ± 0.3	+6	8.8 ± 1.9	−74
	0.1	34.8 ± 1.4	+18	1.2 ± 0.1	−97
	1	36.1 ± 2.9	+26		

the capacity of this agent both to generate H_2O_2 and to block competitively the transport system for [^{3}H]dopamine. However, potentiation by 10 mM ascorbate was evident at all concentrations; the increase was about one order of magnitude (compare 0.1 mM without ascorbate to 0.01 mM with ascorbate). In these experiments, the incubation medium was bright red and the isolated tissues were gray to black in appearance after incubation with 6-hydroxydopamine in the absence of ascorbate. With ascorbate, the color of the medium was diminished and the color of the tissue was virtually eliminated. This indi-

TABLE 4

Inhibition of [^{3}H]dopamine uptake into rat brain slices by H_2O_2 or 5-hydroxydopamine: lack of potentiation by ascorbic acid

The experimental conditions are the same as in Table 2. Data are expressed as the tissue/medium ratio (T/M) ± the standard deviation of triplicate samples.

Inhibitor	Ascorbate	T/M	T/M with inhibitor	Inhibition
	mM			%
0.1 mM H_2O_2	0	33.8 ± 4.9	28.1 ± 0.2	17
	1	34.7 ± 2.9	27.9 ± 1.0	20
	5	36.6 ± 2.1	36.2 ± 1.8	1
	10	35.7 ± 2.0	35.4 ± 2.5	1
0.001 mM 5-hydroxydopamine	0	34.4 ± 3.0	11.4 ± 0.4	67
	1	31.6 ± 2.4	11.6 ± 0.6	63
	5	29.7 ± 2.3	11.2 ± 1.2	62
	10	29.2 ± 1.4	11.6 ± 1.0	60

cated reduction of the extracellular and intracellular oxidation products of 6-hydroxydopamine.

The uptake of [^{3}H]dopamine into rat brain slices was inhibited by H_2O_2 and by 5-hydroxydopamine (Table 4). Previously we had observed that H_2O_2 inhibited the uptake of [^{3}H]dopamine, [^{3}H]norepinephrine, and [^{3}H]serotonin into rat brain synaptosomes (11). Ascorbate did not potentiate the inhibition by H_2O_2 (Table 4). Indeed, the higher concentrations of ascorbate exerted a protective action; this may have been due to some destruction of the H_2O_2. 5-hydroxydopamine, like the 6-hydroxy analogue, is taken up and accumulated by adrenergic nerve endings (15, 16); hence 5-hydroxydopamine undoubtedly exerted some inhibitory action by competing for the transport system for [^{3}H]dopamine. However, 0.001 mM 5-hydroxydopamine was not potentiated by ascorbate (Table 4), whereas the same concentration of 6-hydroxydopamine was potentiated (Table 3).

DISCUSSION

In a previous study (11) we showed that catalase, an enzyme specific for H_2O_2, prevented a portion of the inhibition of [^{3}H]-catecholamine uptake caused by the action of 6-hydroxydopamine on rat brain synaptosomes. In addition, catalase completely prevented the inhibition of amine uptake caused by H_2O_2 itself. These data were interpreted to indicate that 6-hydroxydopamine generates H_2O_2 in aqueous solution and that the H_2O_2 can inhibit the uptake systems for the catecholamines. A complication in this study arose from the fact that 6-hydroxydopamine can inhibit uptake by competitively blocking the transport systems for the catecholamines. Thus, two factors were operative simultaneously when 6-hydroxydopamine was present: the cytotoxicity of H_2O_2 and the competitive blockade of transport. To clarify the role of H_2O_2, we performed three different types of studies. First, the effect of 6-hydroxydopamine was studied on the serotonin uptake system, which was not inhibited competitively by this agent. Catalase fully protected the serotonin uptake system. Second, we washed out the excess 6-hydroxydopamine from tissue slices before studying the uptake of [^{3}H]catecholamines. By reversing the competitive blockade of transport, the cytotoxic action of H_2O_2 (prevented by catalase) was more clearly evident. Last, we studied dialuric acid, which is not a competitive inhibitor of catecholamine uptake, but which generates H_2O_2. Dialuric acid inhibited the uptake systems for dopamine, norepinephrine, and serotonin, and each was fully protected by catalase. These data clearly showed that H_2O_2 generated from 6-hydroxydopamine was toxic to the uptake systems for the biogenic amines. In view of the well-known general toxicity of H_2O_2, we suggested (11) that the selective degeneration of sympathetic neurons by 6-hydroxydopamine might be brought about by the intraneuronal generation of relatively large amounts of H_2O_2 by the 6-hydroxydopamine which accumulates at these sites.

In the current experiments, the rates of generation of H_2O_2 (oxygen consumption) by 6-hydroxydopamine and by dialuric acid in aqueous solution were measured with an oxygen electrode (Fig. 2 and Table 1). Ascorbate augmented the rates[2] for both agents.

[2] It should be noted that ascorbate increased the initial rate of reaction as well as the over-all amount of oxygen consumed (Fig. 2, curves *A* and *E*). The increased rate may be the result of complex interactions between the ascorbate and the multiple oxidation products (13) of 6-hydroxydopamine, or perhaps it is due to faster generation

The amount of H_2O_2 generated in the presence of excess ascorbate was many times greater than the amount of autoxidizable agent, and appeared to be limited mainly by the availability of oxygen in the system. For example, with 10 nmoles of 6-hydroxydopamine (Fig. 2, curve *E*), the oxygen consumption (i.e., H_2O_2 production) was over 150 nmoles in 20 min in the 1-ml reaction system (which contained 10,000 nmoles of ascorbate). Since ascorbate stimulates the continuous generation of H_2O_2, it seems likely that tissue ascorbate may be involved in potentiating the toxic actions of H_2O_2 generated *in vivo*. The concentration of ascorbate in the brain of rats is approximately 2 mM (17), which is in the range of our experiments. Additionally, 6-hydroxydopamine is often administered in a reducing vehicle (such as ascorbic acid) because most workers recognize the rapidity with which this agent is oxidized (3, 9, 18). The apparent protection of 6-hydroxydopamine by ascorbate may actually increase the cellular damage by increasing the amounts of H_2O_2 formed.

Ascorbate potentiated the inhibition of catecholamine uptake by 6-hydroxydopamine, dialuric acid, and alloxan (Tables 2 and 3). This is consistent with the increased production of H_2O_2. With 6-hydroxydopamine, part of the increment in inhibition may have been due to increased competitive inhibition of transport from increased amounts of 6-hydroxydopamine formed by reduction of its quinone. With dialuric acid and alloxan, on the other hand, there is no competition for transport systems (11), and the increased inhibition is clearly due to the increased formation of H_2O_2.

Saner and Thoenen (12) had suggested that 6-hydroxydopamine quinone might be the agent responsible for destruction of nerve terminals *in vivo*. They found that radioactive 6-hydroxydopamine remained bound irreversibly to tissue protein *in vivo* for long periods of time; this may indicate damage to enzymes or structural proteins by cross-linking with 6-hydroxydopamine quinone. In our studies with the uptake systems for catecholamines in brain slices *in vitro*, the quinone did not appear to play any role. Ascorbate, which reduced the 6-hydroxydopamine quinone or alloxan, actually potentiated the damage by both these agents. We observed the reduction of extracellular 6-hydroxydopamine quinone visually as a diminution in red color in the presence of ascorbate. Additionally, when the tissue slices were rinsed free of the suspension medium, the ascorbate-treated slices were relatively colorless compared to 6-hydroxydopamine samples without ascorbate, which were gray or black in color. These data showed that ascorbate diminished the intracellular as well as the extracellular quantities of the quinone. These experiments rule out 6-hydroxydopamine quinone as a toxic factor in the studies *in vitro*. However, it may be that the quinone contributes to nerve terminal degeneration *in vivo*. This possibility is not ruled out by our studies *in vitro*. However, the recycling of the quinone by tissue ascorbate to yield much larger amounts of H_2O_2 appears to be an attractive alternative.

Another possibility for a contributory mechanism for nerve terminal destruction emerges from the observation of an extremely rapid depletion of oxygen from solution by 0.1 mM 6-hydroxydopamine in the presence of ascorbate (Fig. 2, curve A). Even higher concentrations of 6-hydroxydopamine are injected experimentally, and accumulation of this compound in nerve terminals might yield intraneuronal concentrations in the range of 1 mM, which is the estimated level of catecholamine neurotransmitters (19). One wonders whether or not some degree of neuronal hypoxia over the period of time during which 6-hydroxydopamine persists in high concentration within nerve terminals may contribute to the eventual degenerative process.

A comparison of 6- and 5-hydroxydopamine may provide some insight into the mechanism of nerve terminal degeneration. Both compounds are actively accumulated in catecholamine nerve terminals; however, 5-hydroxydopamine does not appear to cause permanent neural damage (15, 16). One difference uncovered by this investigation is the relatively greater ease of oxidation of 6-hydroxydopamine (Fig. 2 and Table 1).

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of a rapidly reacting, intermediate, free radical species (semiquinone), or simply to the reduction of quinones which may have formed during handling.

This points to an oxidation product of 6-hydroxydopamine as the toxic agent. As discussed earlier, H_2O_2 is the agent which causes inhibition of uptake mechanisms; it is a likely candidate for participating in the degenerative process as well. Ascorbate did not potentiate H_2O_2 formation from 5-hydroxydopamine but it did with 6-hydroxydopamine (Fig. 2). At higher concentrations of 5-hydroxydopamine (up to 1 mM), ascorbate actually diminished the rate of oxygen consumption by this compound. The reason for this was unclear; however, it may involve an interaction between ascorbate and the semiquinone intermediate in the oxidation of 5-hydroxydopamine. Tissue ascorbate may participate in creating the great pathological distinction between 5- and 6-hydroxydopamine.

In conclusion, we have shown that 6-hydroxydopamine generates H_2O_2 and that ascorbate potentiates the generation of H_2O_2. We have shown that H_2O_2 inhibits catecholamine uptake. It seems likely that H_2O_2 may play a causative role in nerve degeneration as well. This might follow from oxidative inhibition of sulfhydryl-dependent enzymes concerned with intermediary metabolism, oxidation of crucial membrane sulfhydryl groups, or peroxidation of structural lipids.

It has been shown that agents such as desmethylimipramine, which inhibit 6-hydroxydopamine uptake, also decrease the extent of damage caused by this agent (9). We postulate that the catecholamine nerve terminals are destroyed because they are the sites of high concentrations of H_2O_2 emanating from the 6-hydroxydopamine which accumulates there. The mechanisms for protecting brain and peripheral nerve tissue against peroxidative damage are as yet unclear (11). Brain in particular appears to be extremely vulnerable to damage by H_2O_2-generating agents *in vitro* (20). Further work will be required in order to test whether H_2O_2 generated intraneuronally from 6-hydroxydopamine is in fact the causative agent for nerve terminal degeneration.

ACKNOWLEDGMENT

We thank Felicitas Cabbat for her expert technical assistance.

REFERENCES

1. G. Jonsson and C. Sachs, *Eur. J. Pharmacol.* **9**, 141 (1970).
2. G. Hellman, G. Hertting and B. Peskar, *Brit. J. Pharmacol. Chemother.* **41**, 270 (1971).
3. L. L. Iversen, *Eur. J. Pharmacol.* **10**, 408 (1970).
4. K. D. Evetts, N. J. Uretsky, L. L. Iversen, and S. D. Iversen, *Nature* **225**, 961 (1970).
5. N. J. Uretsky and L. L. Iversen, *J. Neurochem.* **17**, 269 (1970).
6. H. Thoenen and J. P. Tranzer, *Arch. Exp. Pathol. Pharmakol. (Naunyn-Schmiedebergs)* **261**, 271 (1968).
7. H. Goldman and D. Jacobowitz, *J. Pharmacol. Exp. Ther.* **176**, 119 (1971).
8. G. R. Breese and T. D. Traylor, *J. Pharmacol. Exp. Ther.* **174**, 413 (1970).
9. G. R. Breese and T. D. Traylor, *Brit. J. Pharmacol. Chemother.* **42**, 88 (1971).
10. F. E. Bloom, S. Algeri, A. Groppetti, A. Revuelta and E. Costa, *Science* **166**, 1284 (1969).
11. R. Heikkila and G. Cohen, *Science* **172**, 1257 (1971).
12. A. Saner and H. Thoenen, *Mol. Pharmacol.* **7**, 147 (1971).
13. S. Senoh and B. Witkop, *J. Amer. Chem. Soc.* **81**, 6231 (1959).
14. E. G. Shaskan and S. H. Snyder, *J. Pharmacol. Exp. Ther.* **175**, 404 (1970).
15. J. P. Tranzer and H. Thoenen, *Experientia* **23**, 743 (1967).
16. H. Thoenen, W. Haefely, K. Gey and A. Hurlimann, *Arch. Exp. Pathol. Pharmakol. (Naunyn-Schmiedebergs)* **259**, 17 (1967).
17. R. Rajalakshmi and A. J. Patel, *J. Neurochem.* **15**, 195 (1968).
18. J. de Champlain and R. Nadeau, *Fed. Proc.* **30**, 877 (1971).
19. H. Corrodi and G. Jonsson, *J. Histochem. Cytochem.* **15**, 65 (1967).
20. G. Cohen and P. Hochstein, *Dis. Nerv. Syst.* **24**, Suppl. 44 (1963).