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6-Hydroxydopamine: Evidence for Superoxide Radical as an Oxidative Intermediate

Abstract. *Superoxide dismutase inhibited the autoxidation of 6-hydroxydopamine as measured by the rate of formation of a quinone and the rate of oxygen consumption. These observations demonstrate the formation of the superoxide radical during the autoxidation process. This finding may be relevant to the mechanism of adrenergic nerve terminal degeneration caused by 6-hydroxydopamine.*

Superoxide dismutase (*1*) is the enzyme that catalyzes the decay of the superoxide anion ($O_2^{\cdot-}$, a free radical) according to the reaction

$$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (1)$$

In a recent review, Fridovich (*2*) discussed how the presence of $O_2^{\cdot-}$ in biological systems could be ascertained through the use of superoxide dismutase. It has been shown, for example, that $O_2^{\cdot-}$ was involved in oxygen-dependent hydroxylations (*3*), the mechanism of xanthine oxidase (*4*), the mechanism of tryptophan pyrrolase (*5*), hemolysis induced by dialuric acid (*6*), and the autoxidation of epinephrine at elevated *p*H (*7*).

6-Hydroxydopamine (6-OHDA) accumulates within catecholamine nerve terminals after injection into experimental animals and subsequently causes degeneration of these nerve terminals (*8*). It spontaneously reacts with oxygen to form a quinone (*9*) and H_2O_2 (*10*). We used superoxide dismutase to look for $O_2^{\cdot-}$ during the autoxidation of 6-OHDA. We found that $O_2^{\cdot-}$ is formed and that it catalyzes the autoxidation of 6-OHDA.

The rate of reaction between 6-OHDA and molecular oxygen was studied in two ways. First, the rate of formation of the quinone of 6-OHDA [or other oxidation products (*11*)] was measured by the increase in absorbancy at 490 nm on a Gilford model 300 spectrophotometer. This was done at 37°C with constant agitation in 0.05*M* potassium phosphate buffer at *p*H 7.4. Second, a Clark oxygen electrode (Yellow Springs Instruments) connected to a Honeywell Electronik 19 recorder was used to monitor O_2 consumption by 6-OHDA. The reaction was carried out in a closed chamber with continuous stirring at 37°C in 1 ml of the potassium phosphate buffer. Superoxide dismutase (100 μg/ml, 300 units per milliliter, Truett Laboratories) was added in some experiments. In both systems, the initial rates were calculated and the data analyzed statistically by using Student's *t*-test.

Table 1. Effect of superoxide dismutase on the initial rate of O_2 consumption by 6-OHDA ($10^{-4}M$). The experimental details are the same as for Fig. 1B. The data are given as mean ± S.D., with the number of experiments in parentheses. The buffers used were: *p*H 7.4 and 8.0, 0.05*M* potassium phosphate; *p*H 8.5, 0.05*M* tris(hydroxymethyl)aminomethane hydrochloride; and *p*H 9.0, 0.05*M* sodium carbonate.

*p*H	O_2 consumed (nmole/min)		Inhibition (%)
	Without enzyme	With enzyme	
7.4	31 ± 11 (6)	8 ± 4 (6)	74
8.0	45 ± 11 (7)	8 ± 5 (6)	82
8.5	173 ± 15 (4)	45 ± 6 (4)	74
9.0	203 ± 29 (4)	42 ± 5 (4)	79

The rate of formation of the quinone from $2.5 \times 10^{-4}M$ 6-OHDA was inhibited by superoxide dismutase (Fig. 1A). The initial rate in the absence of enzyme was 0.415 ± 0.148 absorbancy unit per minute [mean ± standard deviation (S.D.); $N = 5$], while in the presence of superoxide dismutase the initial rate was 0.027 ± 0.010 unit per minute ($N = 5$). Superoxide dismutase inhibited the rate of formation of the quinone by 93 percent ($P < .001$).

6-Hydroxydopamine spontaneously reacted with O_2 at a rapid rate (Fig. 1B). We showed previously with the use of the enzyme catalase (*12*) that O_2 consumption could be equated with H_2O_2 formation according to the reaction scheme

$$\text{6-OHDA} + O_2 \rightarrow Q + H_2O_2 \qquad (2)$$

where Q is the quinone of 6-OHDA. The initial rate of O_2 consumption by $2.5 \times 10^{-4}M$ 6-OHDA was 75 ± 11 nmole of O_2 per minute (mean ± S.D.; $N = 9$) in the absence of superoxide dismutase and 20 ± 1 ($N = 4$) in the presence of superoxide dismutase. Superoxide dismutase inhibited the rate by 73 percent ($P < .001$).

We also studied the reaction between 6-OHDA ($10^{-4}M$) and O_2 over the range of *p*H 7.4 to 9.0 (Table 1). The rate of O_2 consumption increased with increasing *p*H, but the effect of superoxide dismutase was similar over the *p*H range.

The data showed that $O_2^{\cdot-}$ was formed during the autoxidation of 6-OHDA. They also indicated that $O_2^{\cdot-}$ formed from 6-OHDA catalyzed the oxidation of 6-OHDA. For a decrease in the rate of reaction to have occurred after the addition of superoxide dismutase (Fig. 1, A and B), $O_2^{\cdot-}$ must have played a catalytic role in the overall reaction. This was the means by which Misra and Fridovich (*7*) showed that $O_2^{\cdot-}$ was an intermediate in the autoxidation of epinephrine at elevated *p*H and catalyzed the reaction. However, in the epinephrine system $O_2^{\cdot-}$ was detected only in a reaction catalyzed by ferrous ion at *p*H 9.0 or higher, or in a reaction catalyzed by base at *p*H 10.2. In contrast, the generation of $O_2^{\cdot-}$ at physiologic *p*H was

readily detectable in the 6-OHDA system.

A theoretical reaction sequence in which $O_2^{\cdot-}$ can be formed from 6-OHDA and then further catalyze the autoxidation of 6-OHDA has been adapted from the reaction scheme proposed by Misra and Fridovich (*7*) for the autoxidation of epinephrine:

$$6\text{-OHDA} + O_2 \rightarrow SQ\cdot + O_2^{\cdot-} + H^+ \quad (3)$$

$$H^+ + 6\text{-OHDA} + O_2^{\cdot-} \rightarrow SQ\cdot + H_2O_2 \quad (4)$$

$$SQ\cdot + O_2 \rightarrow Q + O_2^{\cdot-} + H^+ \quad (5)$$

$$SQ\cdot + O_2^{\cdot-} + H^+ \rightarrow Q + H_2O_2 \quad (6)$$

where SQ· is the semiquinone free radical of 6-OHDA and Q is the quinone of 6-OHDA. The sum of this reaction sequence is

$$2(6\text{-OHDA}) + 2O_2 \rightarrow 2Q + 2H_2O_2 \quad (7)$$

Thus, the autoxidation of 2 moles of 6-OHDA leads to the formation of 2 moles of the quinone of 6-OHDA and 2 moles of H_2O_2. Reactions 4 and 5 constitute a self-propagating chain leading to the formation of the quinone of 6-OHDA and H_2O_2. The $O_2^{\cdot-}$ consumed in reaction 4 is regenerated in reaction 5. Superoxide dismutase would decompose $O_2^{\cdot-}$, and reactions 4 and 6 would be eliminated. If the reaction between 6-OHDA and O_2 (reaction 3) were the rate-limiting step, the addition of superoxide dismutase would lead to an inhibition in the overall rate of autoxidation. Since the rates we observed (Fig. 1, A and B) were considerably slower in the presence of superoxide dismutase, this reaction sequence appears reasonable. A catalytic role for H_2O_2 in the reaction sequence is untenable since the addition of catalase in place of superoxide dismutase was without effect on the rate of formation of the quinone (*13*).

The superoxide anion is a very reactive free radical whose capacity to initiate cytotoxic effects has not been fully ascertained. On the basis of the distribution of superoxide dismutase in aerobic microorganisms, strict anaerobes, and aerotolerant anaerobes, McCord *et al.* (*14*) have argued that this enzyme is vital to the existence of organisms that metabolize oxygen. Additionally, there is evidence that $O_2^{\cdot-}$ may play a role in the dialuric acid–induced hemolysis of erythrocytes of rats deficient in vitamin E (*6*) and in the lethal action of oxygen at elevated pressures on certain microorganisms (*15*).

The superoxide anion can also generate another very reactive species, the OH· radical, through a reaction with H_2O_2, as proposed by Haber and Weiss (*16*):

$$O_2^{\cdot-} + H_2O_2 \rightarrow OH\cdot + OH^- + O_2 \quad (8)$$

Since the autoxidation of 6-OHDA leads to formation of both $O_2^{\cdot-}$ and H_2O_2, it seems likely that OH· is also generated. The existence of still another free radical species, the semiquinone of 6-OHDA, is also suggested by our results. This would arise during the one-electron transfer in the reaction of O_2 with 6-OHDA (reaction 3) and also in the chain-propagating step (reaction 4).

Our data show the formation of $O_2^{\cdot-}$ and indicate the importance of this free radical in controlling the overall rate of oxidation of 6-OHDA. It had been postulated that either H_2O_2 (*10*) or the quinone of 6-OHDA (*9*) might be the toxic product responsible for the degeneration of adrenergic nerve terminals caused by 6-OHDA. Our results indicate one new candidate for the chemical species responsible for initiating the neuronal damage, namely $O_2^{\cdot-}$. Additionally, OH· and the semiquinone of 6-OHDA may also be involved, although their presence in the system has not been established. If the autoxidation of 6-OHDA is intimately involved with its destructive properties, and this is likely, local concentrations of superoxide dismutase may be important in regulating the degree of susceptibility of various tissues to this compound. This would follow from the removal of $O_2^{\cdot-}$ and from the simultaneous slowing of the rates of formation of H_2O_2 and the quinone of 6-OHDA.

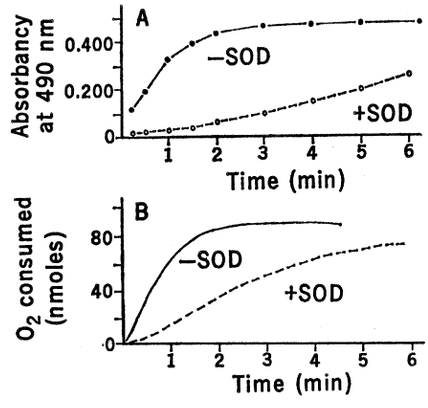

Fig. 1. Representative experiments showing the effect of superoxide dismutase (SOD, 100 μg/ml) on (A) the formation of a quinone and (B) oxygen consumption by $2.5 \times 10^{-4}M$ 6-OHDA. The reactions took place at 37°C in 0.05*M* potassium phosphate buffer at *p*H 7.4. The SOD was added just before the 6-OHDA. In (A) colorimetry was used; the sample was constantly agitated in air. In (B) oxygen consumption was measured; the reaction was carried out in a closed system which contained 1 ml of the medium (204 nmole of O_2 per milliliter). Boiled SOD was without effect in either system. The effect of SOD on the change in absorbancy (A) was more pronounced than the effect on O_2 consumption (B). The reason for this was not clear, but it may have involved either the different conditions used for (A) and (B) or the complexities in the identification of the substances absorbing light at 490 nm [see (*11*)].

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