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EFFECTS OF SUPEROXIDE DISMUTASE AND CATALASE ON CATALYSIS OF 6-HYDROXYDOPAMINE AND 6-AMINODOPAMINE AUTOXIDATION BY IRON AND ASCORBATE

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Abstract—The effects of superoxide dismutase and catalase on autoxidation of 6-hydroxydopamine and 6-aminodopamine in several chemical environments were studied. Inhibition by superoxide dismutase of autoxidation of 6-hydroxydopamine to its *p*-quinone required the presence of metal chelators, EDTA or diethylenetriamine pentaacetic acid (DETAPAC). A "lag" period in 6-hydroxydopamine autoxidation in the presence of superoxide dismutase could be prevented by carrying out autoxidation in a mixture of 6-hydroxydopamine and its *p*-quinone, conditions in which adequate levels of the semiquinone are available for reaction with O_2. Catalase potentiated the inhibitory effect of superoxide dismutase in the presence of EDTA but had no effect in the presence of DETAPAC. Superoxide dismutase and catalase had no effect on the initial rate of 6-aminodopamine autoxidation to the *p*-quinone imine or on later intracyclization and polymer formation. Iron chelated by EDTA functioned as a catalyst in 6-hydroxydopamine autoxidation, making the reaction independent of superoxide as shown by the lack of effect of superoxide dismutase. The presence of iron-EDTA resulted in bleaching of the *p*-quinone product and the consumption of the H_2O_2 formed during autoxidation. Catalase had no effect on the rate of 6-hydroxydopamine autoxidation but completely prevented bleaching of the *p*-quinone, probably by preventing formation of hydroxyl radical by a Fenton reaction between iron-EDTA and H_2O_2. Ethanol, which scavenges hydroxyl radical but not superoxide of H_2O_2, similarly prevented bleaching of the *p*-quinone with no other effects on autoxidation. Iron-EDTA also catalyzed 6-aminodopamine autoxidation with associated consumption of H_2O_2. Superoxide dismutase, catalase and ethanol had no effect on 6-aminodopamine autoxidation in the presence or absence of iron-EDTA, showing the independence of the kinetics of 6-aminodopamine autoxidation and polymerization from its products, superoxide, H_2O_2 and hydroxyl radical. Chelation by DETAPAC prevented the effects of iron on 6-hydroxydopamine and 6-aminodopamine autoxidation. Autoxidation of 6-hydroxydopamine in the presence of ascorbate exhibited a lag phase followed by a linear phase of autoxidation. Superoxide dismutase, catalase and ethanol had no effect on 6-hydroxydopamine or 6-aminodopamine autoxidation in the presence of ascorbate. Autoxidation of 6-hydroxydopamine or 6-aminodopamine in the combined presence of iron-EDTA and ascorbate showed the full effects of both additions, except that 6-hydroxydopamine autoxidation exhibited no lag phase and the iron-catalyzed autoxidation of ascorbate occurred simultaneously with the 6-hydroxydopamine or 6-aminodopamine reactions.

6-Hydroxydopamine (6-OHDA) and 6-aminodopamine (6-ADA) are catecholamine analogs that accumulate in catecholamine-containing neurons and cause degeneration of nerve terminals when injected into animals [1–4]. 6-OHDA and 6-ADA autoxidize at neutral pH, generating H_2O_2, superoxide, and hydroxyl radical [5]. The initial events in autoxidation of 6-OHDA or 6-ADA (QH_2) lead to stoichiometric formation of H_2O_2 and the *p*-quinone of 6-OHDA or the *p*-quinone imine of 6-ADA (Q) [6, 7]:

$$QH_2 + O_2 \rightarrow Q + H_2O_2 \qquad (1)$$

The *p*-quinone or *p*-quinone imine may undergo intracyclization [6] followed by polymerization to brown and black pigments related to neuromelanins [7]. Heikkila and Cohen [8] have shown that superoxide has the special property of catalyzing 6-OHDA autoxidation, whereas 6-ADA autoxidation is not catalyzed by superoxide. Thus, superoxide dismutase inhibits 6-OHDA autoxidation but has no effect on 6-ADA autoxidation [5]. Liang *et al.* [9] have shown that 6-OHDA oxidation to the *p*-quinone may be driven by either O_2, as in reaction 1, or by H_2O_2, as in reaction 2:

$$QH_2 + H_2O_2 \rightarrow Q + 2\ H_2O \qquad (2)$$

Catalase might be expected to partially inhibit 6-OHDA autoxidation by removing H_2O_2 that would otherwise be available for 6-OHDA oxidation. We have reported that catalase has a limited inhibitory effect on 6-OHDA autoxidation and that catalase greatly potentiates the inhibitory effect of superoxide dismutase on 6-OHDA autoxidation [10]. Possible protective effects of superoxide dismutase and catalase against 6-OHDA and 6-ADA toxicity might be

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affected by the presence of metals [11, 12] or reactive reducing substances, such as ascorbate, in biological systems. Ascorbate is known to cause cycling between 6-OHDA or 6-ADA and their *p*-quinone or *p*-quinone imine derivatives resulting in increased O_2 consumption and H_2O_2 formation [12–14]. In this paper we study the effects of superoxide dismutase and catalase on 6-OHDA and 6-ADA autoxidation in the presence of chelated iron and ascorbate.*

MATERIALS AND METHODS

Enzymes and chemicals. Catalase (88,823 units/mg) was obtained from CalBiochém-Behring (La Jolla, CA), superoxide dismutase (3,000 units/mg) from Truett Laboratories (Dallas, TX) and 6-hydroxydopamine (6-OHDA) from the Aldrich Chemical Co. Inc. (Milwaukee, WI). 6-Aminodopamine (6-ADA) was a gift from Dr. Edward L. Engelhardt of Merck, Sharp & Dohme (West Point, PA). Ethylenediamine tetraacetic acid (EDTA), $FeCl_2$ and $FeCl_3$ were purchased from the Fisher Scientific Co. (Fair Lawn, NJ) and diethylenetriamine pentaacetic acid (DETAPAC), *L*-ascorbic acid and ADP from the Sigma Chemical Co. (St. Louis, MO).

Spectrophotometric studies. Autoxidation of 6-OHDA and 6-ADA at pH 7.4 and 25° was followed by observing formation of the *p*-quinone of 6-OHDA [15] and of the *p*-quinone imine of 6-ADA [15] in a Cary 14 spectrophotometer. The absorption of visible light by the *p*-quinone or *p*-quinone imine was followed at 490 nm or by scanning the visible spectra. Stock solutions of 11.2 mM 6-OHDA or 6-ADA were prepared in 1.0 mM KCl, pH 2.0. At pH 2.0, autoxidation of 6-OHDA and 6-ADA proceeds very slowly. Autoxidation was initiated by injecting (with a Hamilton CR-700 constant rate syringe) 40 μl of stock solution into a sample cuvette containing 1.5 ml of 0.1 M sodium phosphate, pH 7.4, and various other additions. The solution in the sample cuvette was mixed continually by a small magnetic stirrer. The absorbance at 490 nm was measured against a reference cuvette containing all additions except 6-OHDA or 6-ADA. Mixing was complete, and a clear absorbance recording obtainable, in about 2 sec. The final concentration of 6-OHDA or 6-ADA in the sample cuvette was 0.3 mM.

O_2 consumption and H_2O_2 formation. O_2 consumption during 6-OHDA and 6-ADA autoxidation was monitored using a YSI model 53 Biological Oxygen Monitor with a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH). Autoxidation was initiated by injecting 80 μl of stock solution (11.2 mM 6-OHDA or 6-ADA) into a sample chamber containing 3.0 ml of 0.1 M sodium phosphate, pH 7.4, and various other additions. The temperature of the sample chamber was controlled at 25°. The final 6-OHDA or 6-ADA concentration was 0.3 mM. The solution in the sample chamber was mixed continually by a magnetic stirrer. Additions of other reagents were sometimes made by injection of small volumes before or after the addition of 6-OHDA or 6-ADA. The accumulation of H_2O_2 was quantitated by observing the restoration of O_2 concentration after injection of excess catalase (10 μg/ml).

RESULTS AND DISCUSSION

Autoxidation in the presence of metal chelators. Figure 1a shows the effects of the metal cation chelators, EDTA and DETAPAC, on 6-OHDA autoxidation. EDTA had a 2-fold stimulatory effect on the initial rate while DETAPAC had no apparent effect. The effects of catalase and superoxide dismutase on 6-OHDA autoxidation were studied. In the absence of chelators, 20 μg/ml catalase and 0.7 μg/ml superoxide dismutase had no effect on autoxidation. In the presence of EDTA (Fig. 1b), catalase had an inhibitory effect which was saturable at 10 μg/ml catalase. Higher catalase concentrations had no greater effect. Superoxide dismutase gave rise to sigmoidal kinetics in formation of the *p*-quinone. An initial lag period was followed by a linear phase of *p*-quinone formation and then by a plateau. The higher concentration of superoxide dismutase, the longer the lag phase and the lower the rate of *p*-quinone formation in the linear phase. In the presence of EDTA, catalase potentiated the inhibitory effect of superoxide dismutase on 6-OHDA autoxidation (Fig. 1b). In the presence of DETAPAC (Fig. 1c), catalase had no effect on the rate of 6-OHDA autoxidation. Superoxide dismutase had a much greater inhibitory effect in the presence of DETAPAC than with EDTA (compare Fig. 1c with Fig. 1b), causing a longer lag phase and greater inhibition of the linear phase. In the presence of DETAPAC, catalase has no potentiating effect on the action of superoxide dismutase (Fig. 1c). Figure 2 shows the consumption of O_2 associated with 6-OHDA autoxidation in the presence of EDTA.

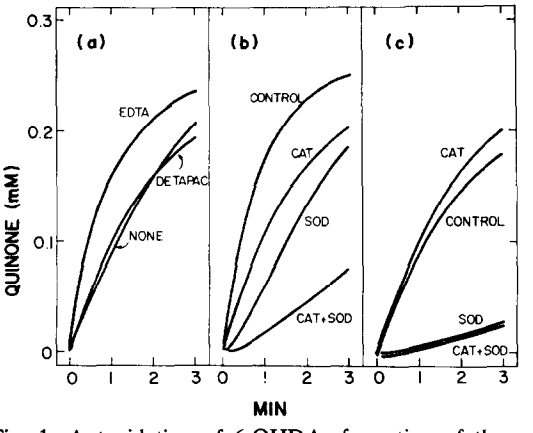

Fig. 1. Autoxidation of 6-OHDA: formation of the *p*-quinone and the effects of catalase and superoxide dismutase in the presence of metal chelators. Autoxidation of 0.3 mM 6-OHDA was carried out in the presence of various chelators (a), 0.3 mM EDTA (b), or 0.3 mM DETAPAC (c). Catalase (CAT) (10 μg/ml) and superoxide dismutase (SOD) (0.2 μg/ml) were present as indicated in the figure.

* A preliminary report of some of this work has been published: S. G. Sullivan and A. Stern, in *Proceedings of the International Conference on Oxygen and Oxy-radicals in Chemistry ad Biology*, (Eds. M. A. J. Rodgers and E. L. Powers). Academic Press, New York, In press.

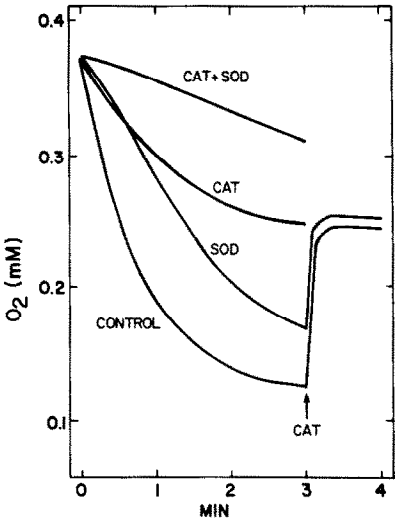

Fig. 2. Autoxidation of 6-OHDA in the presence of EDTA: O_2 consumption and effects of catalase and superoxide dismutase. Reaction mixtures contained 0.3 mM 6-OHDA and 0.3 mM EDTA with 10 μg/ml catalase (CAT) and 0.2 μg/ml superoxide dismutase (SOD) as indicated in the figure. In two of the experiments, catalase was injected at the time indicated by the arrow.

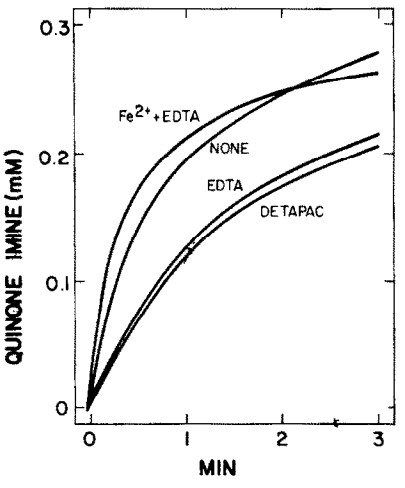

Fig. 3. Autoxidation of 6-ADA: formation of the *p*-quinone imine. Autoxidation of 0.3 mM 6-ADA was carried out in the presence of various additions as shown in the figure. Additions: EDTA, 0.3 mM; DETAPAC, 0.3 mM; and $FeCl_2$, 0.1 mM.

Addition of catalase to the reaction mixture at 3 min showed the typical 1:1 ratio of *p*-quinone formed to H_2O_2 produced that has been reported for 6-OHDA autoxidation. The inhibitory effects of catalase and superoxide dismutase on *p*-quinone formation were reflected in inhibition of O_2 consumption. Catalase also caused apparent inhibition of O_2 consumption by generating O_2 via catalytic decomposition of H_2O_2.

The finding that superoxide dismutase is an effective inhibitor of 6-OHDA autoxidation in the presence of EDTA and DETAPAC but has no effect in the absence of chelators suggests that chelation of trace contaminants such as Cu^{2+} and Mn^{2+} might make 6-OHDA autoxidation dependent on superoxide. Chelation with EDTA blocks the catalytic effect of Cu^{2+} on 6-OHDA and 6-ADA autoxidation [11]. Besides acting as possible catalysts of 6-OHDA autoxidation, Cu^{2+} and Mn^{2+} are efficient scavengers of superoxide, whose effects can be blocked by chelation [16]. Chelation of trace contaminants with EDTA has been shown to make pyrogallol autoxidation dependent on superoxide and inhibitable by superoxide dismutase [17]. The differences between 6-OHDA autoxidation in the presence of EDTA or DETAPAC are probably explained by trace contamination with Fe^{3+}. EDTA enhances the reactivity of Fe^{3+}, whereas DETAPAC blocks Fe^{3+} reactivity [18]. Thus, EDTA has a stimulatory effect on 6-OHDA autoxidation whereas DETAPAC has no effect. We observed that superoxide dismutase had a greater inhibitory effect in the presence of DETAPAC than with EDTA, suggesting that DETAPAC blocked nearly all Fe^{3+} reactivity and made 6-OHDA autoxidation nearly fully dependent on superoxide.

Catalase inhibited 6-OHDA autoxidation in the presence of EDTA but had no effect in the absence of chelators or in the presence of DETAPAC. This result implies that H_2O_2 may have a catalytic role in conjunction with a trace amount of Fe^{3+}-EDTA or other chelated metal. In the particular conditions of our experiments, H_2O_2 was not consumed by reaction 2 [compare Ref. 9] but was accumulated according to reaction 1. Inhibition by catalase, therefore, indicates a catalytic rather than a stoichiometric role for H_2O_2 in the presence of EDTA.

Figure 3 shows the effects of EDTA and DETAPAC on autoxidation of 6-ADA. The initial rate of 6-ADA autoxidation was inhibited by both EDTA and DETAPAC. Unlike the *p*-quinone of 6-OHDA, the *p*-quinone imine of 6-ADA is not stable and

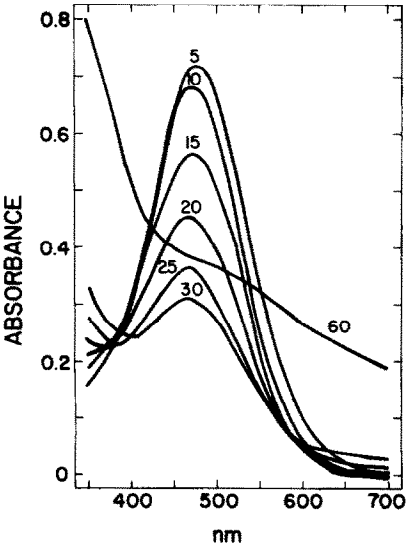

Fig. 4. Autoxidation of 6-ADA: visible spectra of autoxidation products. Spectra were observed at various times (shown in min) during autoxidation. The reaction mixture contained 0.3 mM 6-ADA and 0.3 mM EDTA.

quickly undergoes intracyclization to form other products [6]. Figure 4 shows the visible spectra of the products of 6-ADA autoxidation up to 1 hr. At 5 min the visible spectrum was dominated by the *p*-quinone imine with a broad absorption band around 475 nm. The *p*-quinone imine disappeared and was eventually replaced by brown and black pigments by 60 min. Superoxide dismutase (up to 2 μg/ml) and catalase (up to 20 μg/ml) had no effect either alone or together on the initial rate of 6-ADA autoxidation (as in Fig. 3) or on the rate of later changes leading to polymer formation (as in Fig. 4).

Nature of the "lag" period of 6-OHDA autoxidation caused by superoxide dismutase. Inhibition of 6-OHDA autoxidation by superoxide dismutase in the presence of EDTA or DETAPAC was characterized by an initial "lag" period followed by a linear phase of autoxidation. In the absence of superoxide as catalyst, it is possible that significant levels of some other catalytic intermediate will accumulate before a steady state of 6-OHDA autoxidation is achieved. One possible catalytic intermediate is the semiquinone of 6-OHDA (QH·) which can be formed by the slow initiation reaction,

$$QH_2 + O_2 \rightarrow QH\cdot + H^+ + O_2^- \qquad (3)$$

or, once significant amounts of *p*-quinone (Q) have formed, by reaction 4 [12],

$$QH_2 + Q \rightarrow 2QH\cdot \qquad (4)$$

To test this possibility, the experiment shown in Fig. 5 was carried out. Early formation of the semiquinone by reaction 4 was made possible by observing autoxidation of 6-OHDA in a mixture of 0.15 mM 6-OHDA and 0.15 mM *p*-quinone (Fig. 5, curve b). Superoxide dismutase was added just before the addition of 6-OHDA. No lag period was observed and the initial rate was between the rates of the linear phase achieved with 0.15 mM 6-OHDA (Fig. 5, curve d) and 0.3 mM 6-OHDA (Fig. 5, curve c) in control autoxidations with superoxide dismutase. The results were similar whether EDTA or DETAPAC was present. Studies of O_2 consumption showed the same results. The possibility that H_2O_2 accumulation was necessary for establishment of steady state autoxidation was tested. H_2O_2 (0.15 mM) had no effect on the kinetics of 6-OHDA autoxidation in the presence or absence of superoxide dismutase. The possibility that the transition from the lag phase to the linear phase represented inactivation of superoxide dismutase by H_2O_2 was tested by repeating the experiment of Fig. 5 (curve b) with the dose of superoxide dismutase present during the initial autoxidation of 0.15 mM 6-OHDA to prepare the *p*-quinone. Superoxide dismutase would be inactivated by this procedure if the latter hypothesis were true. The addition of the second dose of 0.15 mM 6-OHDA resulted in formation of *p*-quinone by kinetics identical to that shown in Fig. 5 (curve b), showing that superoxide dismutase was fully active. The results indicate that the early presence of the semiquinone of 6-OHDA prevents the lag period of 6-OHDA autoxidation and are consistent with the following simplified schemes for 6-OHDA autoxidation (compare with Refs. 12 and 14):

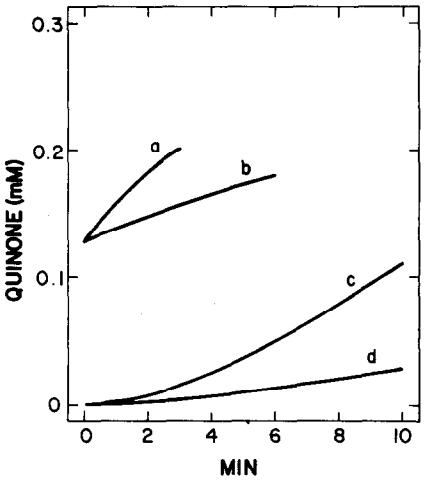

Fig. 5. Kinetics of the inhibition of 6-OHDA autoxidation by superoxide dismutase. Experiments were carried out to determine the mechanism of the "lag" phase of 6-OHDA autoxidation caused by superoxide dismutase. Autoxidation of 0.15 mM 6-OHDA in a reaction mixture containing 0.3 mM DETAPAC was allowed to go to completion. Water (a) or 0.2 μg/ml superoxide dismutase (b) was then added. A second dose of 0.15 mM 6-OHDA was then injected (at zero time on the graph for a and b) and formation of the *p*-quinone was observed. Curves c and d show 6-OHDA autoxidation in reaction mixtures containing 0.3 mM 6-OHDA, 0.3 mM DETAPAC, and 0.2 μg/ml superoxide dismutase (c) and 0.15 mM 6-OHDA, 0.3 mM DETAPAC, and 0.2 μg/ml superoxide dismutase (d).

$$QH_2 + H^+ + O_2^- \rightarrow QH\cdot + H_2O_2 \qquad (5)$$

$$QH\cdot + O_2 \rightarrow Q + H^+ + O_2^- \qquad (6)$$

$$2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2 \qquad (7)$$

The slow initiation reaction 3 provides the initial source of superoxide for catalysis of autoxidation [12]. Reactions 5 and 6 together equal reaction 1 and are probably the main propagation reactions for 6-OHDA autoxidation. In the presence of superoxide dismutase, reaction 7 predominates such that superoxide is less available for reaction 5 and similar propagation reactions. In the presence of superoxide dismutase, the *p*-quinone (Q) would slowly accumulate via reactions 3 and 6 until a significant level of semiquinone (QH·) could be maintained by reaction 4. The sum of reactions 4 plus 6 plus 6 (add reaction 6 twice) plus 7 equals reaction 1 and probably represents the main route of reaction in the presence of superoxide dismutase. The time taken for accumulation of catalytic amounts of QH· in the absence of superoxide is observable as the lag time in 6-OHDA autoxidation in the presence of superoxide dismutase.

Iron-catalyzed autoxidation. Autoxidation experiments were carried out using either 0.1 mM $FeCl_2$ or 0.1 mM $FeCl_3$. The results were essentially identical with either ferrous or ferric iron so that only experiments using ferrous iron are described. As shown in Fig. 6, Fe^{2+}-EDTA greatly accelerated the rate of 6-OHDA autoxidation and caused bleaching of the *p*-quinone. In the presence of Fe^{2+}-EDTA, catalase (up to 20 μg/ml) had no effect on the rate

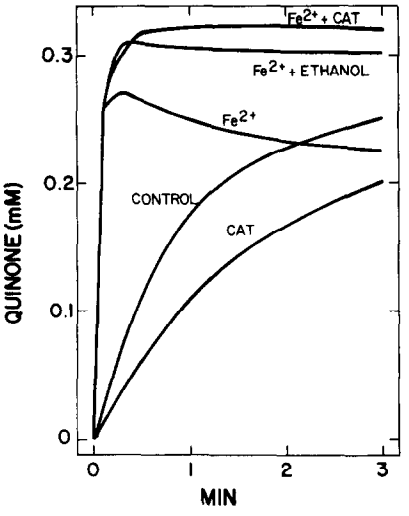

Fig. 6. Effects of iron-EDTA on 6-OHDA autoxidation: formation of the *p*-quinone. All reaction mixtures contained 0.3 mM 6-OHDA and 0.3 mM EDTA with other additions as indicated in the figure. Additions: $FeCl_2$, 0.1 mM; catalase (CAT), 10 μg/ml; and ethanol, 0.5 mM.

of 6-OHDA autoxidation but completely prevented bleaching of the *p*-quinone. Ethanol also prevented bleaching of the *p*-quinone in the presence of Fe^{2+}-EDTA (Fig. 6), whereas ethanol had no effect on rates of autoxidation with any combination of Fe^{2+}-EDTA, catalase and superoxide dismutase. Superoxide dismutase (up to 2 μg/ml) had no effect on Fe^{2+}-EDTA-catalyzed 6-OHDA autoxidation. Figure 7 shows O_2 consumption during Fe^{2+}-EDTA-catalyzed 6-OHDA autoxidation. The addition of catalase after completion of *p*-quinone formation suggests that H_2O_2 had been consumed by reaction with Fe^{2+}-EDTA. Superoxide dismutase had no effect on O_2 consumption in the presence of Fe^{2+}-EDTA. Ethanol had no effect on O_2 consumption with any combination of Fe^2-EDTA, catalase and superoxide dismutase.

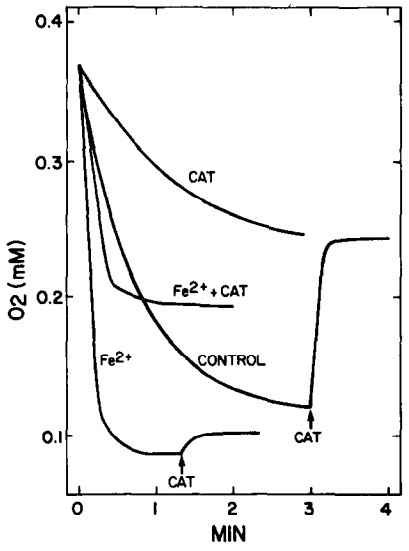

Fig. 7. Effects of iron-EDTA on 6-OHDA autoxidation: O_2 consumption. All reaction mixtures contained 0.3 mM 6-OHDA and 0.3 mM EDTA with other additions as indicated in the figure. Additions: $FeCl_2$, 0.1 mM; and catalase (CAT), 10 μg/ml. In two of the experiments, catalase was injected at the time indicated by the arrow. No measurable quantity of O_2 was consumed in the time period of this experiment by Fe^{2+}-EDTA in the absence of 6-OHDA.

Fe^{2+}-EDTA or Fe^{3+}-EDTA greatly accelerated the rate of 6-OHDA autoxidation and resulted in consumption of H_2O_2 and bleaching of the *p*-quinone. Iron functioned as a catalyst in 6-OHDA autoxidation, making the reaction independent of superoxide as shown by the lack of effect of superoxide dismutase in the presence of iron. In the presence of iron-EDTA, catalase had no effect on the rate of autoxidation but completely prevented bleaching of the *p*-quinone. By removing H_2O_2, catalase may prevent the Fenton reaction between Fe^{2+}-EDTA and H_2O_2 which results in formation of the hydroxyl radical (OH·). Reaction with hydroxyl radical may cause bleaching of the *p*-quinone since ethanol, which scavenges hydroxyl radical but not superoxide or H_2O_2, prevents bleaching of *p*-quinone without having any effect on the rate of 6-OHDA autoxidation. Chelation with DETAPAC blocked most of the effects of iron on 6-OHDA autoxidation (data not shown). Fe^{2+}-DETAPAC had only about 20 per cent of the accelerating effect of Fe^{2+}-EDTA on 6-OHDA autoxidation, with no bleaching of *p*-quinone and very little H_2O_2 consumption. Free iron cation showed some catalysis but with initial sigmoidal kinetics and inhibition by catalase (Fig. 8), while superoxide dismutase had no effect. O_2 consumption studies showed that only about 30 per cent of the H_2O_2 formed in a given time was consumed by Fe^{2+} compared to Fe^{2+}-EDTA (data not shown). The effects of Fe^{2+}-ADP were identical in all respects to those of Fe^{2+} alone.

Iron-EDTA also catalyzed 6-ADA autoxidation (Fig. 3) with consumption of H_2O_2 (not shown).

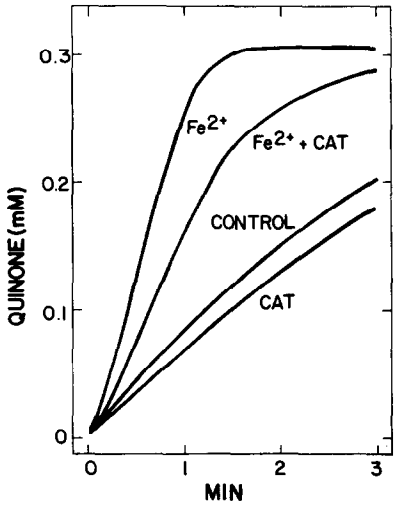

Fig. 8. Effects of free iron cation on 6-OHDA autoxidation: formation of the *p*-quinone. All reaction mixtures contained 0.3 mM 6-OHDA with other additions as indicated in the figure. Additions: $FeCl_2$, 0.1 mM; and catalase (CAT), 20 μg/ml.

These effects were blocked by chelation of iron with DETAPAC. Fe^{2+}-EDTA caused no qualitative changes in the visible spectra of the products of 6-ADA autoxidation (see Fig. 4). Catalase (20 μg/ml), superoxide dismutase (2 μg/ml) and ethanol (0.5 mM) had no effect on the initial rate of autoxidation, O_2 consumption, or on the rate of spectral changes in the presence or absence of iron-EDTA, showing the independence of the kinetics of 6-ADA autoxidation and polymerization from its products, superoxide, H_2O_2 and hydroxyl radical. Reaction 3 has been shown to proceed rapidly with 6-ADA in contrast to the sluggishness of this reaction with 6-OHDA [12].

Autoxidation in the presence of ascorbate. Ascorbate is known to provide an electron source for recycling of 6-OHDA and 6-ADA between 6-OHDA and its *p*-quinone and between 6-ADA and its *p*-quinone imine [12–14]. Recycling of 6-OHDA and 6-ADA by ascorbate (AH_2) results in net formation of dehydroascorbate (A) and H_2O_2:

$$AH_2 + O_2 \rightarrow A + H_2O_2 \qquad (8)$$

Figure 9 shows *p*-quinone formation and O_2 consumption during 6-OHDA autoxidation in the presence of EDTA and ascorbate. 6-OHDA autoxidation exhibited sigmoidal kinetics in the presence of ascorbate. *p*-Quinone formation did not reach the expected end point of 0.3 mM but approached a steady-state ratio of 6-OHDA to *p*-quinone due to recycling by ascorbate. The addition of catalase at 2 min produced evidence of H_2O_2 decomposition, but the generated O_2 was rapidly consumed by net ascorbate oxidation. The total O_2 of the sample chamber was consumed due to the presence of excess ascorbate (10 mM). 6-OHDA autoxidation in the combined presence of Fe^{2+}-EDTA and ascorbate (Fig. 9) showed the full effects of both Fe^{2+}-EDTA and ascorbate (compare with Figs. 6 and 7) except

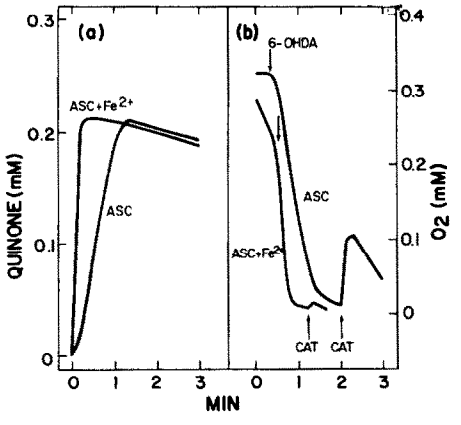

Fig. 9. Effects of ascorbate on 6-OHDA autoxidation: formation of the *p*-quinone (a) and O_2 consumption (b). (a) Each reaction mixture contained 0.3 mM 6-OHDA and 0.3 mM EDTA with other additions as indicated in the figure. Additions: ascorbate (ASC), 10 mM; and $FeCl_2$, 0.1 mM. (b) Each reaction mixture contained (at zero time) 0.3 mM EDTA, 10 mM ascorbate (ASC) and in one of the two experiments, 0.1 mM $FeCl_2$. 6-OHDA (0.3 mM) and catalase (CAT) (10 μg/ml) were injected at the times indicated by the arrows.

that 6-OHDA autoxidation exhibited no lag phase or sigmoidal kinetics and the Fe^{2+}-EDTA-catalyzed autoxidation of ascorbate [19] occurred simultaneously with 6-OHDA reactions. Catalase, superoxide dismutase, and ethanol had no effect, singly or in combination, on the kinetics of *p*-quinone formation in a mixture of ascorbate, 6-OHDA, and EDTA (with or without Fe^{2+}). Superoxide dismutase had no effect on H_2O_2 consumption in mixtures of 6-OHDA, Fe^{2+}-EDTA, and ascorbate. When added to a mixture of ascorbate, 6-OHDA and Fe^{2+}, DETAPAC blocked most of the acceleratory effect of Fe^{2+} on *p*-quinone formation, O_2 consumption, and H_2O_2 consumption (data not shown).

Ascorbate (in the presence of either EDTA or DETAPAC) acted like superoxide dismutase by causing a lag phase before the linear phase of autoxidation was reached. This superoxide dismutase-like effect of ascorbate is probably related to the ability of reduced ascorbate to scavenge superoxide [20, 21]. 6-OHDA autoxidation in the presence of ascorbate was independent of the products, superoxide, H_2O_2 and hydroxyl radical, since superoxide dismutase, catalase and ethanol had no effect on the reaction.

Recycling of 6-ADA by ascorbate was so efficient that 10 mM ascorbate prevented any accumulation of *p*-quinone imine from 0.3 mM 6-ADA (data not shown). Ascorbate prevented *p*-quinone imine accumulation in the presence of EDTA or DETAPAC and in the presence or absence of Fe^{2+}, catalase, superoxide dismutase, and ethanol. In spite of the effective recycling of the *p*-quinone imine by ascorbate, addition of ascorbate at later than 5 min to solutions of autoxidizing 6-ADA did not reverse the spectral changes observed in Fig. 4, showing that recycling was not possible after intracyclization and polymerization. Studies of oxygen consumption by mixtures of 6-ADA, EDTA, and ascorbate showed results similar to those with 6-OHDA (see Fig. 9b) except that the accelerating effect of Fe^{2+}-EDTA was not as pronounced with 6-ADA. As with 6-OHDA, DETAPAC blocked most of the H_2O_2 consumption by Fe^{2+} during autoxidation of 6-ADA.

Conclusions. Sachs *et al.* [15] have shown that intracellular catecholamines such as norepinephrine may scavenge free radicals produced during 6-OHDA autoxidation and protect against 6-OHDA toxicity. Norepinephrine inhibited 6-OHDA autoxidation *in vitro* and conferred protection against 6-OHDA neurotoxicity *in vivo* while having little effect on 6-ADA autoxidation or toxicity. They suggest that norepinephrine may function by scavenging superoxide, which is a catalyst in 6-OHDA autoxidation but not in 6-ADA autoxidation. Other experiments indicate that ascorbate may accelerate 6-OHDA and 6-ADA toxicity [12–14], whereas *in vivo* experiments indicate that scavengers of hydroxyl radical provide protection against toxicity [22]. Superoxide dismutase and catalase may protect against 6-OHDA and 6-ADA toxicity either by inhibiting autoxidation or by removing the toxic oxygen intermediates formed during autoxidation. In this paper we have studied the effects of superoxide dismutase and catalase on 6-OHDA and 6-ADA autoxidation in a few well-characterized chemical environments. We observed inhibitory effects

of superoxide dismutase and catalase in several conditions with 6-OHDA but never with 6-ADA. Superoxide dismutase had no effect on 6-OHDA autoxidation in the presence of chelated iron or ascorbate. Superoxide catalysis of 6-OHDA autoxidation may be of minor importance in biological systems containing metals or reactive reducing substances like ascorbate. The most important role of catalase and peroxidative enzymes in protection against 6-OHDA and 6-ADA toxicity may be to prevent hydroxyl radical formation by preventing the Fenton reaction between iron and H_2O_2.

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