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Electron Spin Resonance Study of the Autoxidation of 6-Aminodopamine

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SUMMARY

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Although the mechanism of neurotoxicity of 6-aminodopamine and 6-hydroxydopamine is generally agreed to be initiated by the nonenzymatic oxidation of these compounds by molecular oxygen, most studies have focused on subsequent chemical and biological events. This ESR study indicates that this initial reaction can be described as a one-electron transfer from the neurotoxins to molecular oxygen to form their respective semiquinone (or semiquinone-imine) free radicals. Using a combination of deuterium isotope substitution and a resolution enhancement technique, a nearly complete assignment of the hyperfine splitting constants of the ESR spectrum of the 6-aminodopamine has been made. A relatively large interaction of the unpaired electron with the nitrogen of the amino group attached to the phenyl ring emphasizes the semiquinone-imine character of this free radical. The analysis of the ESR spectrum excludes any possibility that this free radical is a secondary product of autoxidation such as the 5,6-dihydroxyindole semiquinone-imine free radical.

INTRODUCTION

The many studies on the mechanism of the neurotoxicity of 6-hydroxydopamine and 6-aminodopamine have been reviewed (1–3). They appear to agree that the nonenzymatic autoxidation of these compounds leads to the formation of a cytotoxic species. Examples of such species are the quinones (or quinone-imines) which react with sulfhydryl or other nucleophilic groups of critical proteins (4). This autoxidation concomitantly activates oxygen by forming $\dot{O}_2^-$, H_2O_2, and the reactive hydroxyl radical (5, 6). Hydroxyl radical scavengers protect catecholamine nerve terminals against the degenerative action of 6-hydroxy- and 6-aminodopamine (7).

Graham *et al.* recently showed that the quinone oxidation product of 6-hydroxydopamine appeared to be less important in its cytotoxicity to C1300 neuroblastoma cells than the associative activation of oxygen (8). In addition to the formation of superoxide and superoxide-derived species, the autoxidation of 6-hydroxydopamine will form the semiquinone free radical (6, 9, 10). Although oxygen must be the ultimate electron acceptor, contaminating iron may be involved in the initial formation of the semiquinone (6), possibly as a catalyst. Direct one-electron transfer to molecular oxygen is found in many autoxidation reactions and, in general, is thought to be a consequence of molecular oxygen being in a ground state triplet (11).

The analogous free radical derived from 6-aminodopamine can be described as either a catechol semiquinone or a semiquinone-imine as shown in Fig. 1. The possibility that these free radicals react with tissue macromolecules is not usually considered. In any case, the disproportionation of these semiquinone-imines (or semiquinones) will form the corresponding quinone-imines (or quinones) which are thought to be primary sulfhydryl reagents (Fig. 1). Using ESR, both these free radicals have been shown to be reduced by ascorbate, which ultimately leads to the catalysis of ascorbate autoxidation by either 6-hydroxy- or 6-aminodopamine (10).

In the accepted scheme of autoxidation of either dopamine derivative, at least two free radical intermediates should be formed, the initial semiquinone-imine (semiquinone) free radical and a secondary free radical formed by the autoxidation of 5,6-dihydroxyindole (9, 12, 13). The identical secondary free radical would appear in the autoxidation of either 6-hydroxy- or 6-aminodopamine (Fig. 1). ESR spectra of 6-hydroxydopamine (6, 9, 10), 6-aminodopamine (10), and related compounds (10) have been reported. In this study, the earlier assignment of the hyperfine couplings of the free radical formed from 6-hydroxydopamine has been confirmed and is consistent with the structure of a semiquinone (9). Using deuterium isotope substitution and the ESR resolution enhancement technique of 90° out-of-phase detection (14), we

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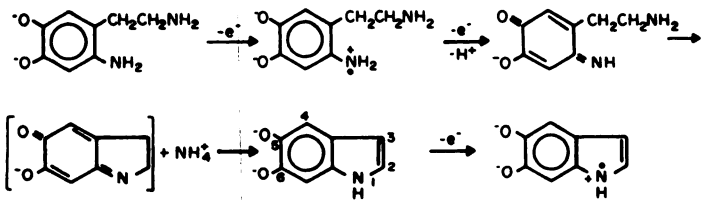

FIG. 1. *Proposed autoxidative pathway for 6-aminodopamine*

have made an assignment of the structure of the free radical formed by the autoxidation of 6-aminodopamine. This assignment is based on an interpretation of the hyperfine pattern which is different from the previous determination (10). Nevertheless, the hyperfine coupling constants are consistent with the structure of the initial semiquinone-imine free radical. By demonstrating that the nitrogen atom in the free radical is attached to two hydrogens, we have excluded the possibility that this free radical is due to the secondary free radical, 5,6-dihydroxyindole semiquinone-imine, whose nitrogen atom would have only one attached hydrogen atom (Fig. 1).

METHODS

6-Hydroxydopamine was obtained from Sigma, and 6-aminodopamine was generously supplied by Dr. E. Engelhardt of Merck, Sharp and Dohme (West Point, Pa.). Both 6-hydroxydopamine and 6-aminodopamine were autoxidized at an elevated pH (1.0 N NaOH), which is a common technique for generating semiquinone anion radicals (9). Both autoxidation and semiquinone stability (10) are diminished at a physiological pH. Otherwise, the autoxidation of those compounds is thought to be qualitatively similar at a pH of 8.3 (10). A major effect of working at the elevated pH of 1.0 N NaOH is the formation of the conjugate bases of the —OH and $—NH_3^+$ groups. Prior to the addition of the neurotoxin, the alkaline solution was saturated with oxygen. Exchangeable protons were replaced with deuterium by using 1.0 N NaOD in D_2O. ESR signals were observed within 30 s and persisted with noticeable decay for about 20 min; thereafter, secondary free radicals became apparent.

ESR spectra were obtained at room temperature with a Varian E-109 spectrometer equipped with a TM_{110} cavity and an aqueous flat cell. Both the in-phase first derivative and the 90° out-of-phase first derivative settings were determined with a Varian strong pitch signal. The amplitude and the resolution of the out-of-phase signal are very dependent upon both microwave power and magnetic field modulation (14). The use of this resolution enhancement technique greatly simplified the determination of hyperfine splittings by decreasing the overlap of spectral lines (compare Fig. 2A with Fig. 2C and Fig. 3A with Fig. 3C). The broader lines to the left of the arrow in Fig. 2B (see expanded spectrum in Fig. 2D) are greatly diminished in the 90° out-of-phase spectrum (Fig. 2C), as is consistent with a shorter relaxation time for the broader lines (15). Both 90° out-of-phase spectra show some asymmetry, as would be characteristic of a contribution of the dispersion signal to the absorption signal, which implies that the 90° out-of-phase setting determined with the Varian strong pitch was not an exactly correct phase setting (15, 16). Ideally, the phase settings should be determined with the signal of the 6-aminodopamine semiquinone-imine free radical, but the decay of that signal made this difficult.

RESULTS AND DISCUSSION

As originally observed by Adams *et al.* (9), the ESR spectrum of 6-hydroxydopamine is the same in either

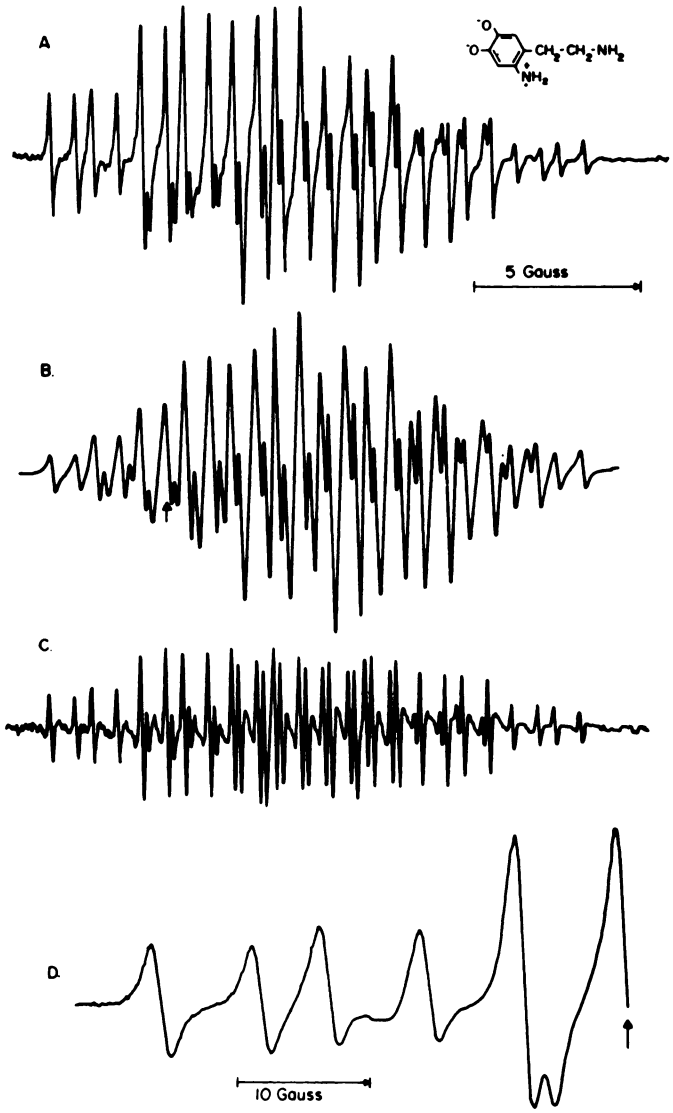

FIG. 2. *(A) The first derivative ESR spectrum of the 6-aminodopamine semiquinone-imine free radical observed on the addition of 6-aminodopamine (200 mM) to alkaline H_2O (1 N NaOH)*

The calibrated modulation amplitude was 0.013 G at 100 kHz and the nominal microwave power was 1 mW. The scan time was 4 min with a 0.128 s time constant.

(B) A computer-simulated ESR spectrum using the hyperfine coupling constants in Table 1.

(C) The 90° out-of-phase ESR spectrum of the 6-aminodopamine semiquinone-imine under the same experimental conditions described in A, except that the microwave power was 0.5 mW, the time constant was 0.064 s, and the scan time was 2 min.

(D) An experimental spectrum of the region to the left of the arrow in B showing the broader lines, which are more apparent in the simulation (B), where a single Lorentzian line width has been used for all the spectral lines.

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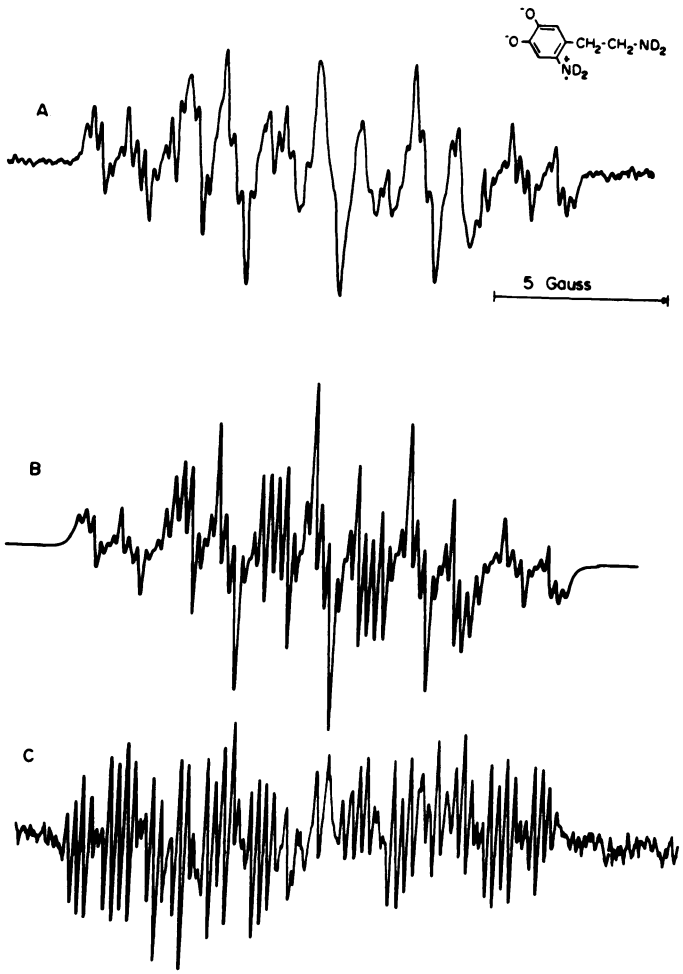

FIG. 3. *(A) The first derivative ESR spectrum of the 6-aminodopamine semiquinone-imine free radical observed on the addition of 6-aminodopamine (20 mM) to alkaline D_2O (1 N NaOD)*

The calibrated modulation amplitude was 0.033 G at 100 kHz and the nominal microwave power was 1.0 mW. The scan time was 4 min with a 0.25 s time constant.

(B) A computer-simulated ESR spectrum using the hyperfine coupling constants in Table 1.

(C) The 90° out-of-phase ESR spectrum of the 6-aminodopamine semiquinone-imine under the same experimental conditions described in A, except the modulation amplitude was 0.10 G.

alkaline H_2O or D_2O. The absence of protons which can be exchanged for deuterium eliminates the possibility that any of the hyperfine couplings could arise from the phenolic hydrogens, the amino hydrogens, or the hydrogen attached to the heterocyclic N of the indole semiquinone-imine (Fig. 1). In special cases involving hydrogen bonding, the protons of aromatic hydroxyl groups do contribute to the ESR spectrum and can be exchanged in alkaline D_2O (17). Since two of the hydrogens have the same hyperfine coupling, these can be assigned to the methylene group (9). The remaining hyperfine couplings are presumably due to the two-ring hydrogens, which are inequivalent. The absence of an effect of D_2O enabled the assignment of the hyperfine couplings that is consistent with a semiquinone dianion (9). This nearly complete assignment is not possible on the basis of the ESR spectrum in H_2O alone (6). We have observed that the larger of the two-ring hydrogen hyperfine couplings increase significantly as the pH is lowered, apparently due to the protonation of the dianion.

The hyperfine splitting constants of 6-aminodopamine were previously determined, but not assigned, as $a^N = 1.32$ G, $4a^H = 2.77$ G, $a^H = 1.32$ G, and $a^H = 0.79$ G. During an investigation of the semiquinone-imine free radical of serotonin and related compounds, we decided to use deuterium substitution with alkaline D_2O to assign these hyperfine splitting constants. The ESR spectrum seen with 6-aminodopamine in D_2O (Fig. 3A) is very different from that seen in H_2O (Fig. 2A), apparently due to the substitution of deuterium for the protons in the 6-amino group. All other possibilities are eliminated by the fact that the 6-hydroxydopamine spectrum is unchanged in D_2O. The spectrum in D_2O was found to be consistent with the magnitude, but not the spin (i.e., $I = ½$ for H; $I = 1$ for N), of the earlier assigned hyperfine splitting constants (Table 1). Although the spectrum in H_2O could be reasonably well simulated by using the previously determined hyperfine coupling constants, a new assignment of the nuclei was required to simulate both the spectrum in D_2O and the spectrum in H_2O simultaneously.

The hyperfine splitting constants for the protons and deuteriums (in D_2O) of the 6-amino group were determined by subtracting the total width of 6-aminodopamine ESR spectra in D_2O from the total width in H_2O (18). If the total width in gauss between the outer lines in H_2O and D_2O is $W(H_2O)$ and $W(D_2O)$, respectively, then

$$W(H_2O) - W(D_2O) = 2a^H_{NH} - 4a^D_{ND_2}, \qquad [1]$$

where $a^H_{NH_2}$ and $a^D_{ND_2}$ are the hyperfine splittings of the proton and the deuterium of the 6-amino group, respectively. From the ratio of the gyromagnetic ratio of H to D,

$$a^D_{ND_2} = 0.1535\, a^H_{NH_2}, \qquad [2]$$

the following relationship is obtained:

$$a^H_{NH_2} = 0.7215[W(H_2O) - W(D_2O)]. \qquad [3]$$

The use of Eq. [3] gave $a^H_{NH_2} = 1.34$ G, then Eq. [2] gave $a^D_{ND_2} = 0.21$ G. The quintet due to this small hyperfine coupling can clearly be seen in the wings of Fig. 3A. It should be noted that enolization can cause substitution of deuterium for ring hydrogens, but this reaction is

TABLE 1

Hyperfine splitting constants in gauss of 6-hydroxydopamine and 6-aminodopamine semiquinone-imine free radicals

	6-Hydroxydopamine	6-Aminodopamine	Partially deuterated 6-aminodopamine
H (methylene)	3.36[a]	2.75	2.75
H (ring)	0.85	1.60	1.28
	0.57	0.77	0.77
H (amino)	—	1.34	0.21
N (amino)	—	2.75	2.75

[a] Our hyperfine splitting constants were not significantly different from those reported by Adams *et al.* (9).

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apparently too slow to cause replacement of the benzene hydrogens in this case (19).

Using arguments identical to that used in the assignment of the hyperfine coupling constants of the 6-hydroxydopamine semiquinone, the other hyperfine constants were assigned as shown in Table 1. The only significant difference between the hyperfine coupling constants in H_2O and D_2O is in the larger of the ring hydrogens. This small difference is probably related to the pH dependence of the larger ring hydrogen hyperfine splitting noted for the 6-hydroxydopamine semiquinone, and may imply an inequivalence of pH and pD, either inherent or in the preparation of the solutions. Alternately, the spin density at this position may have been affected by the partial deuteration of the free radical. The presence of two exchangeable hydrogens is inconsistent with the ESR spectrum being that of the 5,6-dihydroxyindole semiquinone-imine which has only one exchangeable hydrogen attached to the N of the heterocyclic ring.

Both the quinone-imine structure of the two-electron oxidation product of 6-aminodopamine and the relatively large spin density on nitrogen in the free radical emphasize the semiquinone-imine structure of the free radical as shown in Figs. 1 and 2. Although alternate valence bond structures of the radical with the electron localized on oxygen, as in catechol semiquinones, are also important, the one-electron oxidation product of 6-aminodopamine is probably best described as a semiquinone-imine.

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