Electron Spin Resonance-Spin Stabilization of Semiquinones Produced during Oxidation of Epinephrine and Its Analogues*

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The ESR-spin stabilization approach has been employed to detect and characterize o-semiquinone radicals from the oxidation of epinephrine and related materials (norepinephrine, 3,4-dihydroxynorephedrine, isoproterenol, and adrenaline) in aqueous solutions. Semiquinones were generated by various oxidative procedures—enzymatic oxidation (with horseradish peroxidase/H₂O₂), chemical oxidation (with Ag₂O) and photooxidation—and subsequently kinetically stabilized through complexation with Zn²⁺ ions. This "spin stabilization" affords high radical concentrations, which has allowed unambiguous identification of the radical intermediates. Where appropriate, spectral assignments have been supported by deuterium substitution experiments and computer simulations of spectra. Two types of free radical have been identified: primary "open chain" semiquinones, formed by one-electron oxidation of the parent catecholamines, and secondary semiquinones, formed subsequent to cyclization reactions. The mechanism of formation of the secondary radicals is discussed, and it is concluded that they are derived from product aminochromes. Thus, oxidation of adrenochrome gives a radical identical to the secondary species observed from oxidation of epinephrine.

Oxidation of epinephrine (1) and its analogues is unusually complex because of the many possible free radical and molecular species that can be formed by sequential one-electron oxidations of the parent compound (Table I). Whereas the molecular products shown in the scheme are well established (1), radical intermediates have rarely been detected. Nonetheless, o-semiquinones are postulated as intermediates during the autoxidation (2) and enzymatic (3), superoxide-catalyzed (4), and metal ion-catalyzed oxidation (5, 6) of these materials. The toxicity of catecholamines in fact is generally considered (7–9) to be related to the production during oxidation of damaging free radicals and toxic molecular products such as o-quinones. In addition, there is evidence (10) that microsomal reduction of adrenochrome (2), the four-electron oxidation product of epinephrine, leads to a superoxide-generating cycle in which o-semiquinone free radicals are again intermediates.

Direct detection and identification of these radicals in the above systems should be possible using the ESR technique which, because of its sensitivity and selectivity, is well suited for studies of free radicals in complex systems. However, although several attempts have been made (11–14) using this technique to unambiguously identify semiquinone species from epinephrine in aqueous systems, none has been completely successful. Problems associated with low radical concentrations, poor spectral resolution, and the presence of more than one spectral species owing to secondary radical reactions have not been overcome.

![Diagram](https://example.com/diagram.png)

In this paper we show that such studies can be greatly facilitated though the use of the spin stabilization approach. In chemical systems where o-semiquinones and related species are formed from catechols (3), it has been found (15–18) that significant enhancements of radical concentrations are possible through the inclusion in the system of di- or tripositive metal ions, which form chelate complexes with the free radicals. (In nonaqueous solvents, the diphenylthallium cation has been used in an analogous manner (19).) The enhancements are possible because of scavenging of the semiquinone (4) by the metal ion (M⁴⁺), as in reaction 1, to form a much longer lived complex (5).

![Diagram](https://example.com/diagram.png)

Apart from studies on chemical systems, we recently showed (20) that the method is applicable to enzymatic systems: spin-stabilized radicals were detected in high concentrations from the oxidation of some simple catechols by the horseradish peroxidase-hydrogen peroxide system. The levels of metal ion required to optimize radical concentrations were without marked effect on the enzyme activity. We now report the use of spin stabilization to identify radicals formed from epinephrine and its analogues in a number of oxidative systems. Two

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1. The abbreviations and trivial names used are: epinephrine, 1-(3,4-dihydroxyphenyl)-2-(methylamino)ethanol (DL); isoproterenol, 1-(3,4-dihydroxyphenyl)-2-isopropylaminoethanol (DL); norepinephrine, 1-(3,4-dihydroxyphenyl)-2-aminoethanol (DL); dopamine, 3,4-dihydroxy-2-(methylamino)acetoephone; adrenochrome, 3,4-dihydroxy-1-ethyl-5,6-indolinedione; aminochromes, a family of highly colored 2,3-dihydroxindole-5,6-quinones; leucadrenochrome, 3,5,6-trihydroxy-N-methylhydroindole; adrenolutin, 5,6-dihydroxyl-2-aminodihydroindole; HRP, horseradish peroxidase.
major types of radical are described—primary "open chain" semiquinones, which are intermediates in the production of aminochromes, and secondary semiquinones derived from the aminochrome generation. Production of radicals was by enzymatic or chemical means, using either HRP/H2O2, Ag2O, or UV irradiation. Zn2+ was used as the stabilizing metal ion.

MATERIALS AND METHODS

Adrenalone hydrochloride was obtained from Fluka Chemical Corp., DL-epinephrine-α,β,β-d3 from Merck. All other catecholamines and adrenochrome were from Sigma. These materials were used as received.

Solutions containing 6 mM catecholamine were made up in acetic buffer, prepared by the addition of zinc acetate (2.5 M) to 0.2 M acetic acid followed by dropwise addition of either 1 M NaOH or glacial acetic acid to bring the buffer to the desired pH. Typical Zn2+ concentrations were 0.225–0.45 M. For experiments designed to study the effects of deuterium exchange, acetate buffer in D2O was prepared by adding zinc acetate to acetic acid in D2O (99.8%). pD was estimated taking pD = pH + 0.4. Experiments mostly were carried out over a range of pH (pD) from 4.0 to 5.5. Reduced solubility of Zn2+ at lower hydrogen ion concentrations ruled out its use in neutral and alkaline solutions. In addition, phosphate buffer could not be used because of the low solubility of zinc phosphate.

Enzymatic oxidation of catecholamines and adrenochrome was with HRP and H2O2 as previously described (20). Semiquinone radicals were easily detected in steady state concentrations in a static system. The concentration of HRP in stock solutions was calculated taking into account the effect of the metal ion. The small splitting of 0.23 G that is observed presumably is a γ-coupling to one of the methylene hydrogens in the side chain. (These hydrogens are inequivalent, being adjacent to a chiral center (16).) It cannot arise from the OH proton, since the spectrum was unchanged in D2O solution where this proton would be exchanged.

From isoproterenol under the above conditions we obtained a closely related spectrum (Fig. 2c) which by similar reasoning was assigned to species 5; R = CHOHC6H4NH2CH3, with the magnetic parameters given in Table II.

Primary radicals from the above systems (epinephrine and isoproterenol) were studied most conveniently using UV irradiation to generate them. In the static enzymatic system, buildup of secondary radicals (below) soon was observed. However, spectra attributable to primary o-semiquinones from other catecholamines (norepinephrine, 3,4-dihydroxy-epinephrine) revealed no stable radicals.

Table 1

<table>
<thead>
<tr>
<th>Electron Nature</th>
<th>Oxidation of DOPA</th>
<th>Oxidation of Leuconorechrome</th>
<th>Oxidation of Adrenolutin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Epinephrine</td>
<td>1 e</td>
<td>Adrenolutin</td>
</tr>
<tr>
<td>1</td>
<td>Epinephrine</td>
<td>1 e</td>
<td>Adrenolutin</td>
</tr>
<tr>
<td>2</td>
<td>Epinephrine quinone → leuconorechrome</td>
<td>1 e</td>
<td>Adrenolutin</td>
</tr>
<tr>
<td>3</td>
<td>Leuconorechrome</td>
<td>1 e</td>
<td>Adrenolutin</td>
</tr>
<tr>
<td>4</td>
<td>Adrenochrome → adrenolutin</td>
<td>1 e</td>
<td>Adrenolutin</td>
</tr>
<tr>
<td>5</td>
<td>Adrenolutin</td>
<td>1 e</td>
<td>Adrenolutin</td>
</tr>
</tbody>
</table>

RESULTS

Detection and Identification of Primary o-Semiquinones—Both enzymatic oxidation and photooxidation of solutions of epinephrine in acetic buffer (pH 5.0) containing Zn2+ ions gave the steady state ESR spectrum shown (Fig. 1a). There is considerable overlap of spectral lines, but the major spectral features expected for the primary semiquinone are present. Computer simulation of the spectrum (Fig. 1b) gave the ESR parameters shown in Table II. The measured g value was 2.0039 ± 0.0001. These parameters agree well with data for zinc-complexed primary o-semiquinones from related molecules (16, 18). (The effect of zinc is to modify hyperfine couplings and to decrease the g-value through stabilizing negative charge on the oxygens (17).) The spectrum can therefore be attributed to the chelated primary radical from epinephrine 5; R = CHOHC6H4NH2CH3, where the side chain is protonated at pH 5. Assignments of major hyperfine couplings to specific protons (Table II) is based on arguments developed for other 4-substituted o-semiquinones (23), taking into account the effect of the metal ion. The small splitting of 0.23 G that is observed presumably is a γ-coupling to one of the methylene hydrogens in the side chain. (These hydrogens are inequivalent, being adjacent to a chiral center (16).) It cannot arise from the OH proton, since the spectrum was unchanged in D2O solution where this proton would be exchanged.

From isoproterenol under the above conditions we obtained a closely related spectrum (Fig. 2c) which by similar reasoning was assigned to species 5; R = CHOHC6H4- NH2CH(CH3)2, with the magnetic parameters given in Table II.

Primary radicals from the above systems (epinephrine and isoproterenol) were studied most conveniently using UV irradiation to generate them. In the static enzymatic system, buildup of secondary radicals (below) soon was observed. However, spectra attributable to primary o-semiquinones from other catecholamines (norepinephrine, 3,4-dihydroxy-epinephrine) revealed no stable radicals.

![FIG. 1. ESR spectra of zinc-complexed primary o-semiquinones from epinephrine and related materials. a, radicals from UV irradiation of epinephrine (20 mM); b, computer simulation of the above spectrum with proton hyperfine splittings as 3.62, 3.15, 0.65, 0.38, and 0.23 G and a line width of 0.1 G; c, radicals from UV irradiation of isoproterenol (20 mM); d, radicals from peroxidatic oxidation of 3,4-dihydroxyephedrine (6 mM). Concentrations of HRP and H2O2 were 100 nM and 1.6 mM, respectively. All reactions were carried out in acetic acid/zinc acetate buffer, pH 5.0. The arrow in d indicates the position of an irradiation signal in the ESR cell.]
Electrospin resonance data for Zn²⁺-complexed primary o-semiquinones from epinephrine and its analogues

<table>
<thead>
<tr>
<th>Parent catecholamine</th>
<th>Derived radical</th>
<th>Hyperfine couplingsa¹</th>
<th>g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epinephrine</td>
<td>5, R = CH(OH)CH₃NH₂CH₃</td>
<td>0.38 0.32 0.65 3.15 0.23</td>
<td>2.0039</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>5, R = CH(OH)CH₃NH₃</td>
<td>0.40 0.36 0.70 3.20 0.26</td>
<td>2.0039</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>5, R = CH(OH)CH₃NH(CH₃)₂</td>
<td>0.38 0.37 0.65 3.10 0.26</td>
<td>2.0039</td>
</tr>
<tr>
<td>Dihydroxynorephedrine</td>
<td>5, R = CH(OH)CH(CH₃)₂NH₂</td>
<td>0.36 0.37 0.60 3.70 0.26</td>
<td>2.0039</td>
</tr>
<tr>
<td>Adrenalone</td>
<td>5, R = C(=O)CH₂NH₂CH₃</td>
<td>0.55 2.90 1.65 3.20 0.23</td>
<td>2.0041</td>
</tr>
</tbody>
</table>

*The notation used to designate individual protons is that used previously (16): ring protons are numbered 3, 5, and 6 (the side chain substituent is at position 4) and protons in the side chain are designated β (attached to the carbon atom adjacent to the ring) and γ.

⁺⁺ ±0.03 G.  
* ±0.0001.

Fig. 2. ESR spectra of zinc-complexed secondary o-semiquinones from epinephrine and related materials. a, radicals from autoxidation of adrenochrome (10 mM), b, computer simulation of the above spectrum with hyperfine couplings of 5.10 (3H), 4.44 (1N), and 0.91 (2H) G and a line width of 0.1 G. c, radicals from Ag₂O oxidation of isoproterenol (20 mM). d, radicals from peroxidatic oxidation of 3,4-dihydroxynorephedrine. Concentrations of HRP and H₂O₂ were 250 nM and 6 mM, respectively. All reactions were carried out in acetic acid/zinc acetate buffer, pH 5.0. The arrow to the right of d indicates the position of an irradiation signal in the ESR cell; the arrow in the center indicates the position of the spectrum of another radical species, probably derived from polymerization (see text).

We studied the secondary radical from epinephrine in the greatest detail, since we found that this species was easily generated by other methods, including autoxidation or enzymatic oxidation of adrenochrome in the presence of Zn²⁺ ions and chemical oxidation of epinephrine with Ag₂O followed by treatment with Zn²⁺. The spectrum of the radical is shown in Fig. 2a. A computer simulation is shown in Fig. 2b.

This radical shows a large hyperfine splitting to nitrogen (as do all the other secondary radicals), a large splitting from methyl hydrogens and from two additional hydrogens that are equivalent (or almost equivalent). Individual hyperfine lines are broad suggesting further, unresolved, hyperfine splittings. The g value is 2.0040, similar to that measured for primary o-semiquinones.

These data are consistent with a radical structure where cyclization has occurred so that the electron is now delocalized over a nitrogen atom. Since the fact that the radical can be spin-stabilized implies that a chelating o-semiquinone structure is retained, the probable structure for this secondary radical is 6: R₁ = H, R₂ = CH₃, where the minor proton splittings are from the methylene hydrogens in the ring and splittings from hydrogens attached to the aromatic ring are

| Semiquinones from Oxidation of Epinephrine |

Electron spin resonance data for Zn²⁺-complexed primary o-semiquinones from epinephrine and its analogues.
within the line width. (Small values for the aromatic protons would be consistent with withdrawal of electron density onto nitrogen.) Note that structure 6 is simply the one-electron oxidation product of adrenochrome or adrenolutin 7 (which is derived from adrenochrome by an intramolecular redox reaction and occurs in the keto form shown rather than as a trihydroxyindole (26).)

If structure 6 and the assignments to protons are correct, then oxidation of epinephrine in which the side chain is deuterated (R = CD(OH)CD2NH2CH3) is predicted to yield a radical with an ESR spectrum in which the methylene splitting has collapsed owing to replacement of —CH2— by —CD2—, deuterium having a much lower nuclear moment than hydrogen. This expectation was borne out in practice: oxidation of epinephrine gave a spectrum in which the methylene splitting was absent (other splittings were unaffected), consistent with Structure 6 and the assignments given in Table III.

Secondary radicals detected from other catecholamines had spectra closely related to that from epinephrine, also showing hyperfine splitting to nitrogen and a g value close to 2.040. For example, prolonged enzymatic oxidation of isoproterenol by HRP/H2O2 resulted in an ESR spectrum identical to that shown in Fig. 2d can be assigned to structure 6; R1 = CH3, R2 = H. Norepinephrine under similar conditions gave a short-lived spectrum corresponding to the expected secondary radical which likewise was sensitive to D2O again indicating a hyperfine splitting from an exchangeable proton. With 3,4-dihydroxynorephedrine and norepinephrine, formation of polymeric species was rapid (note the presence of a line attributable to a growing polymeric radical in the center of Fig. 2d).

**DISCUSSION**

We have demonstrated that both primary and secondary radicals from epinephrine and related materials can easily be identified using the spin stabilization approach. The success of the procedure depends on the high radical concentrations that can be generated, even where rates of radical production are very low. This is a result of a greatly decreased termination rate for the complexed semiquinone: for simple o-semiquinones complexed with zinc we find a second order rate constant (2k) for decay of ~104 M^-1 s^-1 at pH 5. This rate constant is extremely low for a semiquinone at this hydrogen ion concentration, which is close to the pK of the neutral semiquinone (27). We foresee no general problem in the extension of the method to identify o-semiquinones and to determine their rates of formation in more complex systems. Other pH values and buffer systems can be accommodated through an appropriate choice of metal ion.

Oxidation of epinephrine and related materials to amino-chromes is known to occur via an intermediate o-quinone which undergoes rapid cyclization followed by further oxidation (24). This is depicted for epinephrine in Table I, in which we also include radical intermediates and adrenolutin, the rearrangement product of adrenochrome.

In our systems, initial one-electron oxidation to the primary o-semiquinone is accomplished either enzymatically or by UV irradiation. Production of o-quinone follows, via disproportionation of primary semiquinones. With deprotonation of the amino group in the side chain, o-quinones undergo 1,4-intramolecular cyclization to the unstable leucoaminochrome, which is rapidly oxidized to the aminochrome (24). In this latter step, the oxidant may be another molecule of o-quinone or perhaps another oxidizing species in the system. At this time we have no direct evidence to determine whether the primary o-semiquinone or the o-quinone is the oxidizing species.

**TABLE III**

<table>
<thead>
<tr>
<th>Parent catecholamine</th>
<th>Solvent</th>
<th>Derived radical</th>
<th>Hyperfine couplings</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epinephrine</td>
<td>H2O</td>
<td>6; R1 = H; R2 = CH3</td>
<td>4.44</td>
<td></td>
</tr>
<tr>
<td>Epinephrine-α,α,β,δ-d3</td>
<td>D2O</td>
<td>6; R1 = CD2; R2 = CH3</td>
<td>4.44</td>
<td></td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>H2O</td>
<td>6; R1 = H; R2 = H</td>
<td>3.40 3.40 (1H)</td>
<td>1.2 (H)</td>
</tr>
<tr>
<td>D2O</td>
<td>6; R1 = H; R2 = D</td>
<td>3.40 0.52 (1D)</td>
<td>1.2 (H)</td>
<td></td>
</tr>
<tr>
<td>Isoproterol</td>
<td>H2O</td>
<td>6; R1 = H; R2 = CH(CH3)2</td>
<td>4.58 1.90 (1H)</td>
<td>0.90 (H)</td>
</tr>
<tr>
<td>Dihydroxynorephedrine</td>
<td>D2O</td>
<td>6; R1 = CH3; R2 = H</td>
<td>3.40 3.38 (1H)</td>
<td>0.90 (1H)</td>
</tr>
<tr>
<td></td>
<td>D2O</td>
<td>6; R1 = CH2; R2 = D</td>
<td>3.40 0.5 (1D)</td>
<td>0.90 (1H)</td>
</tr>
</tbody>
</table>

* a" and a"NH denote couplings from nitrogen and from hydrogen bonded to nitrogen respectively; a"m and a"m denote couplings from hydrogens on carbon atoms attached to nitrogen (R1 and ring hydrogens, respectively).
* ±0.03 G.
* ±0.0001.
* An identical spectrum was obtained from HRP/H2O2 oxidation of adrenochrome.
* In this structure, the other ring hydrogen also is replaced by deuterium.

2 C. C. Felix and R. C. Sealy, unpublished observations.
time we do not know whether a radical intermediate (which would be 3 electrons removed from the parent catecholamine) is involved in this reaction. Aromatic rings are tautomers of semiquinones; rearrangement of aminochromes that possess a 3-hydroxy group to the corresponding aminolutins has been shown to be catalyzed by metal ions such as Zn$^{2+}$ (28).

The secondary radicals we have detected by ESR could arise from oxidation of either aminochromes or aminolutins. In the enzymatic system, HRP/H$_2$O$_2$ is the likely oxidant, identical radical. Note that the electron densities at the various atoms in secondary radicals are, like the primary radicals, sensitive to substituents in the nonaromatic moiety. In particular, for the cyclized radicals electron density in the aromatic ring is rather small. The fairly large hyperfine coupling to nitrogen for radicals of this kind is indicative of resonance stabilization in which unpaired spin density is delocalized onto nitrogen (below). A similar stabilization has been suggested for the related $\alpha$-semiquinone from 6-hydroxydopamine, which also has a nitrogen para to one of the semiquinone oxygens (29).

Although 5,6-dihydroxy-$N$-methyldiindole is reported also to be an oxidation product of leucoadrenochrome (30), we did not observe an ESR spectrum corresponding to that of the corresponding Zn$^{2+}$ complexed $\alpha$-semiquinone. However, further degradation of the materials to melamins or melanin-like products was evident from the observation of ESR signals of polymeric radicals, in particular from norepinephrine and dihydroxynorephedrine. These reactions may be of significance in connection with the formation of neuromelamins (31, 32).

With these radical systems now well characterized, we feel that it should be possible to quantitatively study enzyme kinetics of the peroxidatic oxidation of catecholamines and related reactions using the spin stabilization approach. In addition the stabilization of semiquinones should allow the effects of oxidogenic (e.g. phenols) and redogenic (e.g. ascorbate) materials (33) to be directly studied by ESR.

REFERENCES

B Kalyanaraman, C C Felix and R C Sealy


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