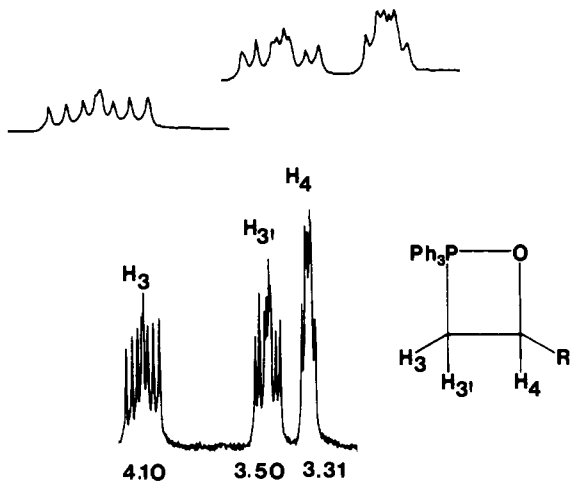


**Figure 1.** A 270 MHz proton NMR spectrum of the pivalaldehyde–methylidenetriphenylphosphorane adduct (toluene-$d_8$, R = $C(CH_3)_3$, methine region): lower plot, 3012-Hz sweep width; upper plot, arbitrary expansion.

(aspirator). The residue was taken up in hexane and filtered through a short column of silica gel (40 g) with more hexane to remove triphenylphosphine oxide. The eluant was concentrated, and the olefin mixture was separated by preparative GLPC (15% 20 M carbowax, 210 °C). Four peaks were observed in order of increasing retention time, (*Z*)-1-phenylpropene, (*E*)-1-phenylpropene, (*Z*)-1-(chlorophenyl)-propene, and (*E*)-1-(chlorophenyl)propene. The relative ratio of *Z*:*E* phenylpropenes was 13.5:1 (93% *Z*) and the *Z*:*E* ratio of chlorophenylpropenes was 6:1 (86% *Z*). No attempt was made to establish response factors for phenyl- vs. chlorophenyl-substituted alkenes, so their relative amounts are not known precisely. The (*Z*)-1-phenylpropene peak was collected and examined by 270-MHz NMR. By integral ratio, $H_1$:$H_2$ was found to be 1:0.66. Thus, 34% of the (*Z*)-phenylpropene has experienced crossover with deuterio ylide.

To establish the % *d* in (deuterioethylidene)triphenylphosphorane, we repeated the experiment with *p*-chlorobenzaldehyde as the reacting partner. The 1-(*p*-chlorophenyl)propene was collected and examined by 270-MHz NMR; intergration showed $H_1$:$H_2$ = 1:0.075, 93% $d_1$ in both cis and trans isomers.

The extent of crossover in the double label experiment is *not* a measure of the extent of oxaphosphetane dissociation since it cannot measure dissociation–recombination of ArCHO + $CH_3CH{=}PPh_3$. A more informative estimate of extent of dissociation is provided by an experiment by using excess labeled aldehyde, assuming the latter does not perturb the system under study. A solution of the adduct from $C_6H_5CHO$ + $CH_3CH{=}PPh_3$ was prepared by careful color end point titration at −78 °C (0.4-mmol scale). After 15 min at −78 °C, 1.1 equiv of *p*-chlorobenzaldehyde was added and the mixture was allowed to reach 20 °C. By NMR analysis of the combined alkene product mixture, the ratio of (*Z*)-(*p*-chlorophenyl)propene:(*Z*)-phenylpropene was 1.4:1. Thus, dissociation under these conditions is more extensive than is crossover determined by the double-label method. The latter requires dissociation of *both* labeled oxaphosphetane and determines only a lower limit for the extent of dissociation.

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# Electron Spin Resonance Characterization of Radicals from 3,4-Dihydroxyphenylalanine: Semiquinone Anions and Their Metal Chelates

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**Abstract:** Semiquinone radicals generated by photolysis of aqueous solutions of 3,4-dihydroxyphenylalanine have been studied over a pH range from 5–11. The semiquinones have distinctive ESR spectra with five major proton couplings all of which have been measured and assigned. The spectra are strongly influenced by the amino acid side chain, reflecting (i) the presence of the chiral carbon center and (ii) restricted rotation of the methylene protons. The major species detected at neutral pH is suggested to be the semiquinone anion with the amino acid group in the zwitterionic form. Above pH 9 the radical with the amino group deprotonated is found to predominate. It is confirmed that these species also are formed by other oxidative procedures, including autoxidation and enzymic oxidation with tyrosinase. Diamagnetic metal ions ($Mg^{2+}$, $Zn^{2+}$, $Cd^{2+}$, $Ca^{2+}$, and $Sr^{2+}$) complex with the semiquinones to give chelate complexes which have been characterized by ESR. With $Cd^{2+}$ and $Zn^{2+}$ steady state concentrations of the metal chelates are very much higher than those of the uncomplexed radicals, allowing hyperfine couplings to $^{111}Cd$, $^{113}Cd$, and $^{67}Zn$ to be measured. It is suggested that this ability to chelate metal ions and associated signal enhancement can be useful in the identification of *o*-semiquinone and related species in complex systems.

Catechols and related compounds are widely distributed in nature. There is current interest in the oxidative degradation of these materials, with clear evidence for the toxicity of their degradation products. Examples of phototoxicity,[1] cytotoxicity,[2,3] and antitumor activity[4] have been reported for a number of these compounds. The cytotoxicity is felt[2,3] to reflect (i) production of superoxide and other damaging oxygen radicals and (ii) the ability of product *o*-quinones to react with sulfhydryl groups of sensitive enzymes. Semiquinones derived from the catechols are almost certainly important intermediates; they are readily formed from most catechols during autoxidation[5] and can participate in the formation of both superoxide and *o*-quinone. Enzymic oxi-

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(3) M. M. Wick, *J. Natl. Cancer Inst.* (*U.S.*), **63**, 1465–1467 (1979), and references therein.
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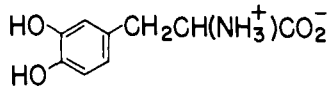
dation[6] and photooxidation[7] similarly often lead to production of semiquinones.

The compound 3,4-dihydroxyphenylalanine (Dopa, **1**) has marked neurologic activity, and is also a key intermediate in the biosynthesis of the photoprotective pigment melanin. It is of some interest to determine the role that semiquinone free radicals play in the metabolic and biosynthetic processes involving this material. Some form of characterization of the radicals is a prerequisite for such a study. One possible way of characterizing and identifying the radicals is by electron spin resonance (ESR) spectroscopy, but while several attempts have been made, none has been completely successful. For example, in a recent study Yoshioka et al.[8] reported on transient radicals from UV photolysis of aqueous solutions of Dopa at pH 7. They indicated that a mixture of radicals was present, but that this mixture did not include the expected semiquinone; hyperfine couplings were not measured and no assignments of spectra were made. Most other studies have been carried out at extremes of pH. For example, radicals were detected under autoxidative conditions by Adams et al.[5] and by Wertz et al.[9] and later by Borg,[10] using cerium(IV) and ferricyanide oxidation, but again their ESR spectra could not be completely interpreted. However, Tomkiewicz et al.,[11] who also obtained spectra of radicals from Dopa at high pH, have assigned their spectra to a mixture of neutral and anionic semiquinones, with a suggested p$K_a$ for the neutral semiquinone of ca. 12.4. This value seems much too high since values of ca. 4 have been measured for related semiquinone radicals by optical spectroscopy.[12] The current situation is therefore one of some confusion.



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One would expect ESR spectra of Dopa semiquinones to show interaction of the unpaired electron with three aromatic protons and the two methylene protons of the side chain. All previous groups have encountered some difficulty in assigning their experimental spectra to Dopa semiquinones since they were unable to detect a clear triplet splitting assignable to two equivalent methylene protons. The situation is further complicated by reports of additional spectral lines, apparently from other radicals. However, as we have pointed out,[13] the methylene protons in Dopa are adjacent to a chiral carbon center, which renders them inequivalent. A triplet splitting from the methylene protons should not, therefore, be expected, raising the possibility that the additional spectral lines observed belong to the same radical species.

We here describe the results of an investigation of Dopa semiquinones and our interpretation of their spectra. Radicals were routinely generated, in the absence of detectable secondary species, by photolysis of aqueous solutions of Dopa at pH 7. Our experiments demonstrate that the radicals formed in this way are the expected semiquinones and confirm the inequivalence of the methylene protons. All observed spectral lines are accounted for in terms of a single species with one-half of the theoretical 32 lines broadened owing to restricted rotation of the methylene protons. The assignment is supported by additional chemical experiments: changes with pH are consistent with expected p$K_a$'s of semiquinone and amino acid moieties, while complexation with diamagnetic metal ions supports the *o*-semiquinone nature of the radical. We have used the results obtained from the photolysis study to confirm that Dopa semiquinones are formed during autoxidation and enzymic oxidation of the parent compound.

## Experimental Section

ESR experiments were carried out with the use of a Varian E-109 spectrometer, and spectra were recorded with the use of 100-kHz field modulation. Hyperfine couplings were measured with a Radiopan gaussmeter, which was also used, in conjunction with a DANA Model 331 microwave counter, in the estimation of *g* values. Hyperfine couplings were measured to ±0.03 G, *g* values to ±0.0001. Spectra were ordinarily recorded with a microwave power of 0.8 to 1.6 mW.

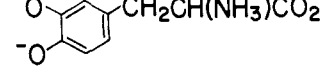
DL-3,4-Dihydroxyphenylalanine (Sigma) and other chemicals employed, glycine (Sigma), magnesium chloride (Fisher), calcium chloride (Fisher), zinc sulfate (Fisher), strontium chloride (Mallinckrodt), and cadmium chloride (Alfa), were of reagent grade or better and were used without further purification. Solutions of Dopa (25 mM) were ordinarily made up in nitrogen-saturated deionized water or glycine buffer (0.1 M) and the pH adjusted to the desired value by the addition of sodium hydroxide solution. In the cases where metal ions were added, this addition was made prior to adjusting the pH to the final value. Unless otherwise stated the final concentration of metal ions was 25 mM. In a few cases the nitrogen-saturated solutions were subsequently saturated with nitrous oxide.

For photolysis experiments, solutions of Dopa were flowed through an aqueous flat cell contained in the ESR cavity in order to avoid depletion of Dopa and excessive buildup of products. The flow rate was usually 1–2 mL/min. The path length of the flat cell (Scanlon Model S-812) was 0.25 mm. Irradiation was with the focused light from an Eimac VIX-300UV 300W xenon arc, filtered through 5 cm of distilled water to remove infrared radiation. Residual heating of the sample was minimized by a purge of gaseous nitrogen through the cavity. For some experiments, the reservoir containing the solution of Dopa was heated or cooled prior to the flow of the solution through the flat cell. In this way sample temperatures in the range 20–55 °C could be obtained during photolysis. Temperatures were measured with the use of a copper–constantan thermocouple in association with a Fluke 2100A digital thermometer. The thermocouple was attached to the outside of the flat cell, just above the irradiation area.

In other experiments radicals were generated by autoxidation and enzymic oxidation of Dopa solutions. In the autoxidation experiments Dopa was dissolved in an oxygen-saturated solution of sodium hydroxide (1 M) and immediately transferred to an aqueous flat cell for ESR measurements. Enzymic oxidation employed buffered (0.06 M phosphate, pH 7.6) solutions of Dopa (20 mM) and mushroom tyrosinase (100 units/mL). The Dopa solution was saturated with nitrogen and the tyrosinase solution saturated with air. The solutions were mixed in a simple two-jet mixing device shortly before entry into a 50 μL capillary tube held in the ESR cavity. The time between mixing and observation was ca. 2 s. No radicals were detected in the absence of the enzyme.

## Results and Discussion

**Radicals from Photolysis. 1. Neutral and Acid Solutions.** At neutral pH, Dopa exists predominantly as the zwitterion (**1**). This material absorbs ultraviolet light of wavelengths <300 nm (Dopa has $\lambda_{max}$ 280 nm, log ($\epsilon_{280}$/$M^{-1}$ $cm^{-1}$) = 3.43)[14] and, by analogy with the monophenolic material tyrosine,[15] one would expect photoejection of an electron to occur, followed by rapid deprotonation to yield the corresponding semiquinone (**2**), reaction 1.



2

$$\mathbf{1} \xrightarrow{h\nu < 300\ nm} \mathbf{2} + e_{aq}^- + 2\ H^+ \qquad (1)$$

One would not expect the p$K_a$ of the amino group in **2** to differ greatly from that of the parent molecule (8.8).[16]

(6) H. S. Mason, E. Spencer, and I. Yamazaki, *Biochem. Biophys. Res. Commun.*, **4**, 236–238 (1961).
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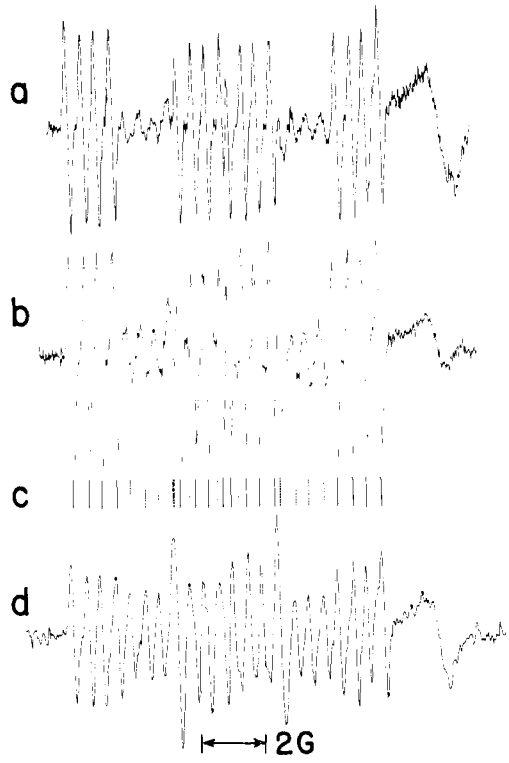
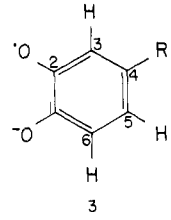



**Figure 1.** ESR spectra of Dopa semiquinone **2** as a function of temperature: (a) 20 °C; (b) 28 °C; (c) stick spectrum for the 28 °C spectrum; (d) 55 °C. The additional broad resonance to high field is an irradiation signal in the flat cell. Conditions: microwave power 1.6 mW, field scan 20 G/30 min (time constant 1 s), modulation amplitude 0.16 G. In (a) and (d) the receiver gain is $1.6 \times 10^5$ and in (b) it is $1.25 \times 10^5$.

Photolysis of Dopa solutions at pH 7 and 20 °C gave the steady state ESR spectrum shown in Figure 1a, which is quite similar to that previously obtained by Yoshioka et al. under similar conditions.[8] The $g$ value is 2.0045, consistent with an anionic *o*-semiquinone structure,[17] and 16 intense hyperfine lines are present, with apparent couplings to *four* inequivalent protons of 0.51, 0.93, 3.58, and 5.23 G.

Hyperfine couplings in *o*-semiquinones (**3**) differ from those




**3**

in *p*-semiquinones, often having one or more couplings to aromatic protons that are >3 G. By comparison of *o*-benzosemiquinone **3** (R = H) with specifically alkylated compounds, it has been shown[18] that these high values are associated with aromatic protons at positions 4 (if the radical is unsubstituted) and 5. Also, in 4-alkyl-substituted *o*-semiquinones **3** (R = alkyl),[19] $a_6^H$, the coupling to the proton at position 6, is typically close to 1.0 G, while $a_3^H$ is generally reported to fall within the range 0.25–0.5 G in protic solvents (this value can be higher in aprotic solvents[19,20]).

Inspection of the experimental data shows that the three smaller couplings are close to the values expected for aromatic protons at the unsubstituted ring positions. If the observed spectrum is indeed that of **2**, one must account for the apparent major coupling of 5.23 G. The side chain of Dopa has a methylene group attached to the ring, and a coupling to a single proton appears anomalous. Furthermore this coupling, if correct, would be unusually large, larger than that for the methyl protons in 4-methyl-*o*-benzosemiquinone for which we find $a^H(CH_3) = 4.82$ G.[21]

However, it should be recognized that the methylene protons are magnetically inequivalent because of their being adjacent to a chiral center[22] and will therefore have different hyperfine couplings, $a_{\beta 1}^H$ and $a_{\beta 2}^H$. Because of this, a 1:2:1 triplet pattern is not expected from these protons, but rather a 1:1:1:1 pattern. Moreover, the inner lines of the 1:1:1:1 multiplet are frequently subject to broadening because of restricted rotation of the methylene protons. This can sometimes make the inner resonances very difficult to detect, in which case[22] an apparent coupling equal to the sum of the two couplings associated with the methylene protons, i.e., $(a_{\beta 1}^H + a_{\beta 2}^H)$, is observed. Now, if this applies in the Dopa system, 5.23 G will be the sum of the methylene proton couplings, and it should be possible to resolve the required additional spectral lines by increasing the temperature, thereby selectively narrowing those lines broadened because of restricted rotation. It can be seen that some weak broad resonances are apparent in Figure 1a, which in other work[8] appear to have been attributed to a second spectral species.

Figures 1b and 1d show the effect that increasing temperature has on the spectrum. As predicted, the broad resonances present in Figure 1a have sharpened considerably and many of the required additional 16 lines can now be observed. The ratio of the signal amplitude of the broad lines to the narrow lines increases from 0.14 at 20 °C to 0.29 at 28 °C and to 0.62 at 55 °C. A stick spectrum (Figure 1c) is shown for the 28 °C spectrum, where the selectively broadened resonances are indicated by the broken lines. All five major couplings can now be distinguished with $a_{\beta 1}^H = 1.89$ G and $a_{\beta 2}^H = 3.33$ G at this temperature. Although the main effect of increasing temperature is to narrow the broader lines, small changes in hyperfine couplings also occur (Table I). The sum of the couplings associated with the methylene protons seems consistent with those obtained in related systems,[19] if one takes into account the restricted rotation[23] of these protons. Thus, if one assumes that the coupling follows the usual $B \cos^2 \theta$ relationship where $\theta$ is the dihedral angle between the orbital of the unpaired electron and the C–H bond, so that a freely rotating alkyl

Table I. ESR Parameters of Dopa Semiquinones[a,b]

| | | hyperfine couplings, G | | | | |
|---|---|---|---|---|---|---|
| pH | $t$, °C | $a_3$ | $a_5$ | $a_6$ | $a_{\beta 1}$ | $a_{\beta 2}$ |
| 7 | 20 | 0.51 | 3.58 | 0.93 | 1.87 | 3.36 |
| | 28 | 0.51 | 3.55 | 0.93 | 1.89 | 3.33 |
| | 32 | 0.51 | 3.57 | 0.93 | 1.89 | 3.36 |
| | 54 | 0.53 | 3.44 | 0.94 | 1.93 | 3.36 |
| 7.5 | 32 | 0.51 | 3.58 | 0.94 | 1.94 | 3.34 |
| 7.6[c] | 23 | 0.49 | 3.59 | 0.94 | 1.95 | 3.35 |
| 8 | 32 | 0.49 | 3.60 | 0.93 | 1.97 | 3.34 |
| 8.5 | 32 | 0.47 | 3.62 | 0.93 | 2.01 | 3.32 |
| 9 | 32 | 0.43 | 3.64 | 0.92 | 2.09 | 3.29 |
| 9.5 | 32 | 0.39 | 3.67 | 0.89 | 2.17 | 3.24 |
| 10 | 32 | 0.37 | 3.69 | 0.89 | 2.22 | 3.26 |
| 11 | 32 | 0.35 | 3.70 | 0.88 | 2.26 | 3.23 |
| *d* | 23 | 0.35 | 3.71 | 0.88 | 2.33 | 3.15 |

[a] Unless otherwise indicated, radicals are from UV photolysis of deoxygenated 0.025 M solutions of Dopa. Solutions above pH 7 also contained 0.1 M glycine (buffer range 8.2–10). [b] All $g$ values 2.0045 ± 0.0001. [c] Radical from oxidation of 0.01 M Dopa with tyrosinase (50 units/mL). [d] Radical from autoxidation of Dopa in 1 M NaOH.

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(20) J. A. Pedersen and J. Spanget-Larsen, *Chem. Phys. Lett.*, **35**, 41–44 (1975).
(21) Other couplings are: $a_3^H$ ca. 0.1 G (poorly resolved); $a_6^H = 3.78$ G; $a_6^H = 0.92$ G. These values are similar to those reported in ref 8.
(22) B. C. Gilbert, J. P. Larkin, and R. O. C. Norman, *J. Chem. Soc., Perkin Trans. 2*, 1272–1279 (1972); B. C. Gilbert and M. Trenwith, *ibid.*, 1834–1839 (1973).
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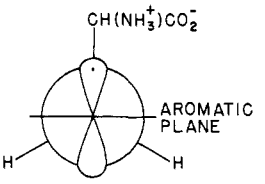



**Figure 2.** Suggested minimum energy conformation for Dopa semiquinones.

substituent (e.g., the methyl group in 4-methyl-*o*-benzosemiquinone) has a coupling of $B/2$, then $B$ = 9.64 G and the mean coupling for the methylene protons is $0.27B$. This value is somewhat smaller than that found for some other 4-alkyl-*o*-semiquinones (e.g., 4-propyl-*o*-benzosemiquinone has a methylene coupling of $0.34B$)[19] but the substituent attached to the methylene carbon is bulkier in this case. The data also suggest that the average value of $\theta$ is close to 60°, i.e., the favored conformation is that shown in Figure 2 with the bulky substituent out of the aromatic plane.

Spectra obtained at lower pHs were less intense. This, at least in part, is likely to reflect a reduced quantum yield for photoionization (cf. tyrosine[24]). It is also possible that the rate of radical termination increases as one approaches the p$K_a$ of the semiquinone group, which should be ca. 4.[12] Radicals could not be detected much below pH 5. At this pH no significant differences in hyperfine couplings from those obtained at pH 7 were found, consistent with a p$K_a$ for the semiquinone moiety of less than 5.

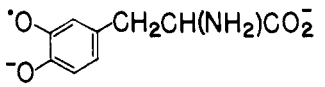
The effect of saturating solutions with $N_2O$ was to increase the intensity of all spectral lines by a factor of 1.5, which presumably reflects partial scavenging of the hydrated electron followed by reaction of hydroxyl with Dopa, reactions 2a and 2b. In the latter

$$e_{aq}^- + N_2O \xrightarrow{H^+} N_2 + \cdot OH \qquad (2a)$$

$$\cdot OH + \mathbf{1} \rightarrow [\text{adduct}] \rightarrow \mathbf{2} + H_2O + H^+ \qquad (2b)$$

reaction, formation of semiquinone probably proceeds via an adduct which rapidly loses water (cf. reactions of hydroxyl with related compounds[25]). A sequence analogous to 2a,b is believed to occur following photoionization of tyrosine.[11]

**2. Alkaline Solutions.** Photolysis at pH 11 gave an ESR spectrum (Figure 3a) somewhat different from that observed at pH 7, but which is quite close to the pH 11 spectrum reported by Tomkiewicz et al.,[11] except that their reported $g$ value is significantly lower. Whereas they interpreted this spectrum in terms of two species, it can be interpreted in a manner similar to that adopted for the spectrum at pH 7, i.e., a single species with coupling to three aromatic protons and to two inequivalent methylene protons that are subject to restricted rotation. The parameters for the pH 11 spectrum are given in Table I. The major differences from the pH 7 spectrum are a decrease in $a_3^H$ and a decrease in the difference between the two methylene proton couplings. We attribute this spectrum to radical 4 where the amino group, which should have a p$K_a$ of ca. 9, is deprotonated.

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Spectra obtained at intermediate pHs are consistent with this assignment. While individual spectra could not be resolved at the intermediate pHs, the difference in couplings between the two species is sufficiently small that apparent couplings can be measured for the mixture of radicals that appear to reflect the weighted average of the individual couplings at that pH.[26] These

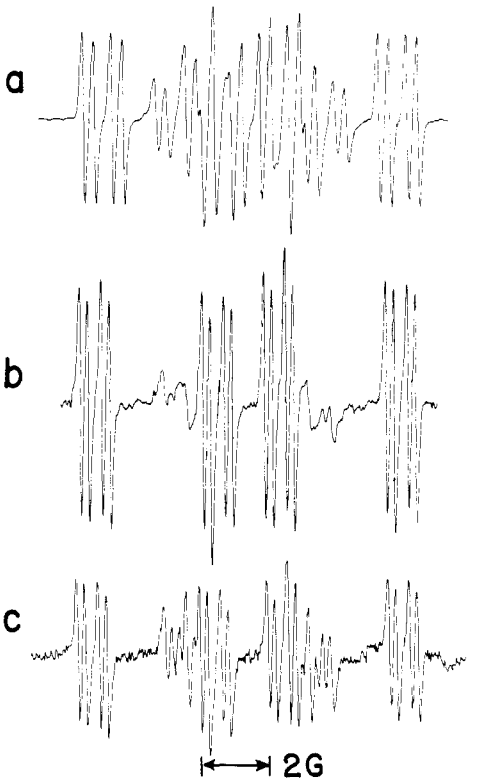


**Figure 3.** ESR spectra of Dopa semiquinones: (a) **4** at 28 °C; (b) and (c) $Zn^{2+}$ complex **5** ($M^{n+}$ = $Zn^{2+}$) at 28 and 55 °C, respectively. Conditions: microwave power 0.8 mW, field scan 20 G/8 min (time constant 0.25 s), modulation amplitude 0.063 G. In (a) the receiver gain is 1 × $10^4$ and in (b) and (c) it is 5 × $10^4$.

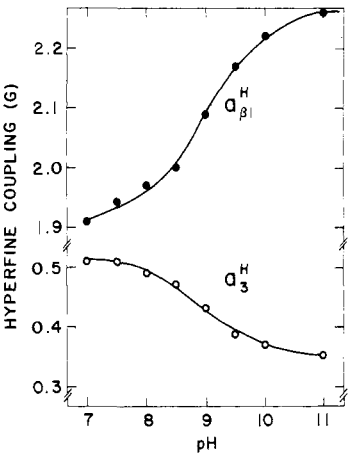


**Figure 4.** Changes in apparent hyperfine couplings $a_3^H$ and $a_{\beta 1}^H$ of Dopa semiquinones as a function of pH.

apparent couplings are reported in Table I, and $a_3^H$ and $a_{\beta 1}^H$ are plotted against pH in Figure 4. One can estimate from Figure 4 that at pH 9 species **2** and **4** are present in approximately equal concentrations. However, this does not necessarily imply that the p$K_a$ for deprotonation of **2** is 9. This would require that (i) the acid–base equilibrium be very much faster than radical termination under these conditions and (ii) the rates of termination of **2** and **4** are identical. Nevertheless, as indicated above, the p$K_a$ of the radical probably is close to that of the parent molecule, i.e., ca. 9.

**3. Solutions Containing Metal Ions.** *o*-Semiquinones are known to form chelate complexes with many complexing metal ions[27]

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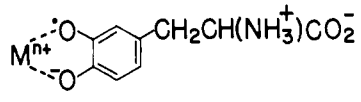
Table II. ESR Parameters of Metal-Chelated Dopa Semiquinones from Photolysis of 0.025 M Deoxygenated Solutions of Dopa Containing 0.025 M Metal Ion at pH 5.5

| metal ion (ionic radius) | $t$, °C | hyperfine couplings, G $a_3$ | $a_5$ | $a_6$ | $a_{\beta_1}$ | $a_{\beta_2}$ | $g$ |
|---|---|---|---|---|---|---|---|
| $Mg^{2+}$ (0.65) | 28 | 0.28 | 3.84 | 0.70 | 2.68 | 3.08 | 2.0042 |
| $Zn^{2+}$ (0.74)[a] | 28 | 0.25 | 3.83 | 0.67 | 2.65 | 3.11 | 2.0039 |
| | 54 | 0.24 | 3.80 | 0.67 | 2.74 | 3.14 | 2.0039 |
| $Cd^{2+}$ (0.97)[b] | 30 | 0.29 | 3.74 | 0.69 | 2.46 | 3.14 | 2.0035 |
| $Ca^{2+}$ (0.99) | 28 | 0.43 | 3.71 | 0.83 | 2.38 | 3.14 | 2.0043 |
| $Sr^{2+}$ (1.13) | 28 | 0.48 | 3.65 | 0.89 | 2.27 | 3.08 | 2.0042 |

[a] Additional coupling to $^{67}Zn$ of 1.40 G. [b] Additional couplings to $^{111}Cd$ and $^{113}Cd$ of 6.81 and 7.13 G, respectively.

and organometallics,[28,29] a property which we have used to identify *o*-semiquinone sites in melanin polymers.[30] Almost the only data available for aqueous solutions are those obtained by Eaton,[27] who generated complexes of *o*-benzosemiquinone by air oxidation of alkaline solutions of catechol containing complexing metal ions. However, under these conditions more than one species was often formed and spectra could not be obtained in aqueous solution for all the metal ions studied. We have observed[31] that metal-complexed radicals are very easily formed by photolysis of aqueous solutions of catechols containing metal ions at pH ca. 5. Dopa reacts similarly to other catechols, giving strong ESR signals consistent with formation of the corresponding chelates, **5**. The reaction can formally be represented as shown in reaction 3.



**5**

$$\mathbf{1} \xrightarrow{h\nu,\ M^{n+}} \mathbf{5} + 2H^+ + e_{aq}^- \qquad (3)$$

Radical concentrations were invariably higher than those obtained in the absence of metal ions at this pH, especially with $Zn^{2+}$ and $Cd^{2+}$ ions for which increases by factors of 15–20 were observed. This probably reflects increased kinetic stability of the complexed radical rather than an increased quantum yield for photoionization, since zinc (and probably the other metal ions studied) does not significantly complex to Dopa at this pH.[32] We have previously shown[30] that reverse disproportionation (comproportionation) of *o*-semiquinones can become significant in the presence of complexing metal ions.

The ESR spectrum obtained in the presence of $Zn^{2+}$ ions at 28 °C (Figure 3b) again shows 16 intense lines together with a number of broad components. The temperature dependence of this spectrum, like that of the spectrum of the uncomplexed radical, is quite marked (Figure 3c). Very similar results were obtained from all other diamagnetic metal ions investigated. Magnetic parameters for the complexes formed are given in Table II. It is notable that $g$ values are lower for the complexes than for the uncomplexed radical, no doubt reflecting spin density in a vacant metal outer orbital.[33]

The effect of complexation on *o*-benzosemiquinone is[27] to decrease spin density at positions 3 and 6, while increasing spin density at positions 4 and 5. Dopa semiquinones exhibit related changes in hyperfine coupling upon complexation in that $a_3^H$ and $a_6^H$ decrease while $a_5^H$ and the mean methylene proton coupling, $^1/_2(a_{\beta 1}^H + a_{\beta 2}^H)$ (which will reflect the spin density at position 4), both increase by about the same amount—ca. 0.25 G for the $Mg^{2+}$ complex. Although the total number of complexes studied is quite small, there does appear to be a correlation between the hyperfine couplings in the complex and the ionic radius of the metal ion (Table II), indicating that the size of the metal ion is an important factor. The difference in hyperfine couplings between the two inequivalent methylene protons decreases with complexation and decreasing ionic radius.

With $Cd^{2+}$ ions, couplings to $^{111}Cd$ and $^{113}Cd$ present in natural abundance (12.86%, $I = ^1/_2$, and 12.34%, $I = ^1/_2$) could easily be resolved. Similarly with $Zn^{2+}$ ions, weak signals showing coupling to $^{67}Zn$ (4% natural abundance, $I = ^5/_2$) were also detected. The couplings to these metal ions (ca. 7 and 1.4 G, respectively) are very similar to those found[34] for the corresponding chelate complexes of *o*-benzosemiquinone, confirming that complexation is indeed occurring at the semiquinone moiety. As with the corresponding semidiones,[35] these semiquinone complexes are evidently largely ionic, with very little spin density residing on the metal ion. Thus the calculated hyperfine coupling for an unpaired electron in the outermost populated s orbital of zinc is 376 G.[22] The s-orbital spin population in **5** ($M^{n+} = Zn^{2+}$) is therefore only about 0.4%.

**Radicals from Autoxidation and Enzymic Oxidation.** Autoxidation of Dopa in 1 M NaOH gave an ESR spectrum with $g = 2.0045$ and hyperfine data (Table I) which compare extremely well with those for the radical obtained from photolysis of Dopa at pH 11 at a slightly higher temperature. There seems little doubt that the radical detected during autoxidation is the same as that from photolysis, i.e., semiquinone **4**.

Oxidation of Dopa with tyrosinase at pH 7.6 gave a radical whose ESR spectrum was essentially the same as that obtained by photolysis at this pH, confirming that the Dopa semiquinone **2** also is produced during the enzymic oxidation. It is believed that tyrosinase acts as a two-electron oxidant,[6] implying that the radicals detected in this system arise from reverse disproportionation involving parent Dopa and oxidized product, for example, small amounts of uncyclized dopaquinone.

We infer from these preliminary experiments that the primary radical species from each of these oxidative processes is the Dopa semiquinone appropriate for the pH employed.

## Conclusions

The primary radical from the photolysis of aqueous solutions of Dopa is the corresponding anionic semiquinone, presumably formed by photoejection of an electron. Semiquinones are also formed during autoxidation and enzymic oxidation of Dopa.

The ESR spectra of the species detected are readily interpreted if one takes into account effects of chirality and restricted rotation of the methylene protons. These effects should be apparent in the spectra of a number of related aromatic radicals. It is also necessary to take into account the different species that can be formed through acid–base equilibria associated with the side chain.

Photolysis provides a direct and convenient method for the generation of specific semiquinones and their metal complexes in aqueous solution. Chelation of *o*-semiquinones by complexing metal ions appears to be a quite general phenomenon. In particular, the hyperfine coupling to cadmium isotopes of ca. 7 G seems to be characteristic of a simple *o*-semiquinone with binding through two oxygens and suggests that measurement of this coupling may be used to help determine ligands in species of this kind. Thus, as we pointed out previously,[30] complex formation in aqueous systems is generally indicative of an *o*-semiquinone structure, while the magnitude of the coupling to certain metal ions can be an important indicator of the identity of the liganding atoms.

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# Nuclear Magnetic Resonance Studies of the Complexation of Trimethyllead by Glutathione in Aqueous Solution and in Intact Human Erythrocytes

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**Abstract:** The complexation of trimethyllead (TML), $(CH_3)_3Pb^{IV}$, by glutathione (γ-L-glutamyl-L-cysteinylglycine (GSH)) has been studied in aqueous solution and in intact human erythrocytes by nuclear magnetic resonance spectroscopy. The deprotonated sulfhydryl group is shown to be the strongest binding site for TML. Formation constants, including microscopic formation constants for sulfhydryl complexes in which the amino group is protonated and deprotonated, have been determined from the dependence of the chemical shift of the exchange-averaged $^1H$ resonance for the methyl protons of TML on solution conditions. To determine if TML is also complexed by GSH in a more complex biological system, we have measured $^1H$ spin–echo Fourier transform (SEFT) NMR spectra for intact erythrocytes to which TML has been added. Resonances are observed for the naturally occurring GSH and several other small molecules, including glycine, alanine, ergothionine, creatine, and lactic acid. Of these potential ligands, TML is found to be complexed only by the intracellular GSH, and the complex is identical with that which forms in much simpler aqueous solutions. The NMR results on the erythrocytes also indicate that TML, added as trimethyllead acetate, rapidly crosses the erythrocyte membrane and that the TML-poisoned erythrocytes continue to metabolize glucose. Measurements have also been made on the competitive complexation of TML in intact erythrocytes by the naturally present GSH and added penicillamine, a molecule which has been tried as a treatment for alkyllead poisoning. The results obtained in this study demonstrate that detailed information about the complexation of metals in intact cellular systems such as erythrocytes can be obtained by $^1H$ NMR spectroscopy.

There is presently considerable interest in the aqueous chemistry of organometallic forms of the metals, particularly as it relates to their environmental and toxicological behavior.[1] Of the various organometallic forms of lead, the trialkyllead(IV) species are of particular interest; inhalation or absorption of tetraalkyllead compounds results in lead in the fluids and tissues of the body, primarily as trialkyllead(IV)salts.[2,3]

In previous studies, we have shown that trimethyllead (TML) reacts as a one-coordinate species in aqueous solution and that, of the potential binding sites in amino acids, the deprotonated sulfhydryl group binds TML most strongly at physiological pH.[4] Of the various sulfhydryl-containing biological molecules, glutathione (GSH, γ-L-glutamyl-L-cysteinylglycine) is among the most abundant. For example, in human erythrocytes, GSH is typically present at the 2 mM level.[5,6] Because of the high affinity of TML for sulfhydryl groups and the abundance of GSH in nature, we have studied the complexation of TML by GSH. The complexation in aqueous solutions has been characterized by $^1H$ and $^{13}C$ NMR spectroscopy. Formation constants, including microscopic formation constants for the binding of TML to the deprotonated sulfhydryl group of the amino protonated and deprotonated forms of GSH,[7] have been derived from the dependence of the exchange-averaged chemical shifts of the $(CH_3)_3Pb^{IV}$ protons on solution composition and pH. To determine if TML is also complexed by GSH in a more complicated biological system, we have made $^1H$ spin–echo Fourier transform NMR measurements on the GSH in intact human erythrocytes to which TML has been added. We also have studied the competitive complexation of TML by GSH and penicillamine in intact human erythrocytes. Penicillamine is a ligand which has been found to be effective in the treatment of several forms of heavy-metal poisoning.[8,9] These studies on intact human erythrocytes clearly demonstrate the power of NMR methods for the elucidation of the coordination chemistry of metal ions in complex biological systems.

## Experimental Section

**Chemicals.** Trimethyllead acetate (Alfa Inorganics) was used as the source of TML. Because acetate forms a complex with $(CH_3)_3Pb^+$ in aqueous solution, it was converted to a stock solution of trimethyllead perchlorate by an ion-exchange procedure described previously.[10] The stock solution was standardized by potentiometric titration. A pH 7.0 solution of trimethyllead acetate was used in the studies of intact erythrocytes.

The glutathione (Sigma) was used as received. The purity of the GSH in terms of the sulfhydryl group was checked by titration with coulometrically generated iodine in acetate buffer using biamperometric endpoint detection.[11]

**Sample Preparation.** All solutions in the formation constant studies were prepared with doubly distilled $H_2O$ which had been freshly boiled and cooled under a stream of argon. The solutions were prepared from the stock trimethyllead perchlorate solution and solid ligand. The ionic strength was adjusted to 0.3 M with $NaClO_4$ for the $^1H$ NMR studies and 0.4 M for the $^{13}C$ NMR studies. *tert*-butyl alcohol was added as a chemical shift reference for the $^1H$ measurements and 1,4-dioxane for the $^{13}C$ measurements. The solutions were usually made acidic (pH ~2.5) with $HClO_4$, and then NaOH was added and NMR samples withdrawn at pH intervals of ~0.4 pH unit up to pH ~12.5. Samples

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