

analyzed by a computer program<sup>21</sup> using equations we have derived for complexation of tribasic species which are identical with the equations of Albert.<sup>25</sup> Our program was able to reproduce literature results from published data.<sup>7,25,26</sup> Stability constant calculation involves a determination of  $\bar{n}$ , the average number of ligands bound per metal. We used from 7 to 11 values of  $\bar{n}$  between 0.3 and 0.7 to determine  $K_1$ , and an equivalent number between 1.3 and 1.7 to determine  $K_2$ .<sup>33</sup> The results are listed in Table II.

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## Interactions of Melanin with Metal Ions. Electron Spin Resonance Evidence for Chelate Complexes of Metal Ions with Free Radicals

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**Abstract:** ESR experiments reveal, for the first time, that di- and tripositive metal ions bind to *o*-semiquinone radical centers within melanin polymers to yield chelate complexes. The binding is often accompanied by large increases in total radical concentration, which are considered to arise via metal ion induced shifts in a comproportionation equilibrium. The effects observed are likely to be general for radicals with chelating ability, and may find use in the separation and identification of composite ESR signals in other biological systems.

There have been several reports that naturally occurring melanin from various sources will incorporate a variety of metal ions, and that synthetic melanin has a similar affinity for these same ions.<sup>2,3</sup> This similarity has led to the suggestion<sup>2</sup> that the protein associated with natural melanins is not of major importance in metal ion binding.

The melanin polymer is heterogeneous<sup>4</sup> and contains carboxyl, quinol, and amine groups which have been suggested

to be the binding sites. From similarities in metal ion affinity between melanins and carboxylic ion exchange resins it has been proposed<sup>2,3</sup> that at both pH 5.0<sup>3</sup> and 7.6<sup>2</sup> melanin metal binding is a function of free carboxyl groups within the polymer. However, it seems likely that other groups also can participate in binding. Thus, dependent upon pH, L-Dopa, a melanin precursor with many of the structural features attributed to the polymer, appears<sup>5,6</sup> to form at least three kinds

of copper(II) complexes with carboxyl, quinol, or mixed carboxyl/quinol binding. (Several other metal ions are also known<sup>7</sup> to form chelates with Dopa and other derivatives of catechol.) A distribution of melanin binding sites was also inferred from experiments<sup>8</sup> involving competition between metal ions for binding sites on the polymer.

It has been known for some time<sup>9</sup> that both natural and synthetic melanins exhibit single-line ESR spectra attributable to randomly oriented semiquinone-type free-radical species. However, binding of metal ions to free radicals does not seem to have been specifically considered, despite the fact that strong interactions of melanin radicals with paramagnetic ions have been demonstrated.<sup>8,10</sup> Blois, Zahlan, and Maling observed<sup>10</sup> that the free-radical signal disappeared when  $\text{Cu}^{2+}$  was added to melanin granules in a ratio of ca. 80  $\text{Cu}^{2+}$  ions to one free radical. This effect was confirmed by Sarna and Żukiewicz<sup>11</sup> and has now been satisfactorily explained<sup>8</sup> as a consequence of magnetic interaction between  $\text{Cu}^{2+}$  and the free radical, rather than of a chemical oxidation by the metal ion.

Sarna and Żukiewicz made the additional observation<sup>11,12</sup> that the integrated intensity of the free-radical signal of melanin increased in the presence of  $\text{Zn}^{2+}$ ,  $\text{Cd}^{2+}$ , or  $\text{Mg}^{2+}$  ions. Their discovery prompted further effort to understand this effect of diamagnetic ions, and we have now established that there occurs a chemically induced increase in the number of free-radical centers. In this paper we investigate the effect, show that it occurs with a number of different diamagnetic metal ions, and propose a hypothesis based on chemical models to explain it. This hypothesis involves the chelation of free radicals by metal ions.

The additional signals induced by metal ions are referred to here as extrinsic. The present work also indicates that at least part of the intrinsic free-radical signal (that present in aerated solutions at around neutral pH) is modified in the presence of metal ions. Free-radical signals in melanin can also be induced by light,<sup>13</sup> by raising the pH,<sup>14</sup> and by drying.<sup>10</sup> The present work is restricted, however, to a consideration of signals induced by diamagnetic metal ions.

## Experimental Section

ESR experiments were carried out using a Varian E-9 spectrometer equipped with 100-kHz and 35-Hz field modulation. First-derivative spectra were recorded using 100-kHz modulation; second derivatives were obtained using both.  $g$  values were measured to  $\pm 0.0001$  relative to a pitch standard ( $g = 2.0028^{15}$ ) contained in the rear port of a dual sample cavity. Microwave power saturation data were expressed in terms of  $P_{1/2}$ , the attenuation of the klystron microwave power (200 mW) at which the ESR signal is half as large as it would be in the absence of saturation. Estimated errors are  $\pm 1$  dB. Spectra displayed in Figures 1–3 were recorded with a microwave power of 20  $\mu\text{W}$ , at which saturation was negligible.

The data reported herein refer to experiments on melanin samples maintained at 77 K. This temperature was selected in order to better study saturation of the radical system, which is more pronounced at low temperatures. Apart from the difference in saturation, results obtained at ambient temperature were similar in all respects.

Chemicals employed were of reagent grade or better and were used as purchased. Samples of metals enriched in magnetic isotopes were obtained in the form of the oxides from Oak Ridge National Laboratory. The oxides were dissolved in acid and the pH of the solution was adjusted to the desired value by the addition of sodium hydroxide solution.

Natural melanin was extracted from bovine eyes according to the method<sup>16</sup> of Plumer and Kopac, while synthetic melanin was synthesized from DL-Dopa as follows. A solution of DL- $\beta$ -hydroxyphenylalanine (10 g; 0.051 mol) in 2 L of deionized water was adjusted to pH 8 by the addition of concentrated ammonia solution. Air was bubbled through the stirred solution for 3 days. Concentrated hydrochloric acid was then used to bring the pH to 2, and the resulting black precipitate was washed several times with 0.01 M hydrochloric acid and then with deionized water. The total yield was 3.5 g.

**Table I.** Changes in Melanin Free-Radical ESR Spectrum after Incubation of Melanin Suspensions with 2.1 mM Metal Ions at pH 5.5

Melanin	Metal ion [magnetic isotope, $\mu$ , $I$ ]	$\Delta H_{pp}^a /$		$P_{1/2}^b /$ dB <sup>c</sup>	
		G	$g$		
Natural, A type	None	4.9	2.0040	29	
	$\text{Mg}^{2+}$ [ <sup>25</sup> Mg (10%), 0.85, <sup>5/2</sup> ]	4.8	2.0041	29	
	$\text{Zn}^{2+}$ [ <sup>67</sup> Zn (4%), 0.87, <sup>5/2</sup> ]	4.9	2.0041	25	
	$\text{Zn}^{2+}$ [ <sup>67</sup> Zn (90%), 0.87, <sup>5/2</sup> ]	6.0	2.0040	23.5	
	$\text{Cd}^{2+}$ <sup>d</sup>	5.0	2.0041	26	
	$\text{Cd}^{2+}$ [ <sup>113</sup> Cd (96%), 0.62, <sup>1/2</sup> ]	6.3	2.0040	26	
	$\text{Al}^{3+}$ [ <sup>27</sup> Al (100%), 3.64, <sup>5/2</sup> ]	6.3	2.0040	28	
	$\text{Sc}^{3+}$ [ <sup>45</sup> Sc (100%), 4.75, <sup>7/2</sup> ]	7.3	2.0039	24	
	$\text{La}^{3+}$ [ <sup>139</sup> La (100%), 2.76, <sup>7/2</sup> ]	8.0	2.0036	19	
	$\text{In}^{3+}$ [ <sup>113</sup> In (4%), 5.50, <sup>9/2</sup> ] [ <sup>115</sup> In (96%), 5.51, <sup>9/2</sup> ]	5.5 <sup>e</sup>	2.0039 <sup>e</sup>	26 <sup>e</sup>	
	Synthetic, from Dopa	None	3.9	2.0034	
		$\text{Ca}^{2+}$ <sup>f</sup>	4.4	2.0038	28
$\text{Sr}^{2+}$ [ <sup>87</sup> Sr (7%), 1.09, <sup>9/2</sup> ]		4.5	2.0038	28	
$\text{Zn}^{2+}$ [ <sup>67</sup> Zn (4%), 0.87, <sup>5/2</sup> ]		4.5	2.0040	28	
$\text{Zn}^{2+}$ [ <sup>67</sup> Zn (90%), 0.87, <sup>5/2</sup> ]		5.0	2.0038	26	
$\text{Cd}^{2+}$ <sup>d</sup>		4.3	2.0039	28	
$\text{Cd}^{2+}$ [ <sup>113</sup> Cd (96%), 0.62, <sup>1/2</sup> ]		5.5	2.0037	27	
$\text{Al}^{3+}$ [ <sup>27</sup> Al (100%), 3.64, <sup>5/2</sup> ]		6.2	2.0037	22	
$\text{Sc}^{3+}$ [ <sup>45</sup> Sc (100%), 4.75, <sup>7/2</sup> ]		7.2	2.0037	19	
$\text{La}^{3+}$ [ <sup>139</sup> La (100%), 2.76, <sup>7/2</sup> ]		7.0	2.0036	20	
$\text{In}^{3+}$ [ <sup>113</sup> In (4%), 5.50, <sup>9/2</sup> ] [ <sup>115</sup> In (96%), 5.51, <sup>9/2</sup> ]		5.6 <sup>e</sup>	2.0036	24 <sup>e</sup>	

<sup>a</sup> Peak to peak line width. <sup>b</sup> Saturation parameter, microwave power required to reduce signal to half what it would be in the absence of saturation. <sup>c</sup> dB below 200 mW. Dual cavity configuration, sample contained in quartz insert. <sup>d</sup> 99% <sup>114</sup>Cd ( $I = 0$ ). <sup>e</sup> Approximate data for the narrower spectral component. <sup>f</sup> >99% isotopes with  $I = 0$ .

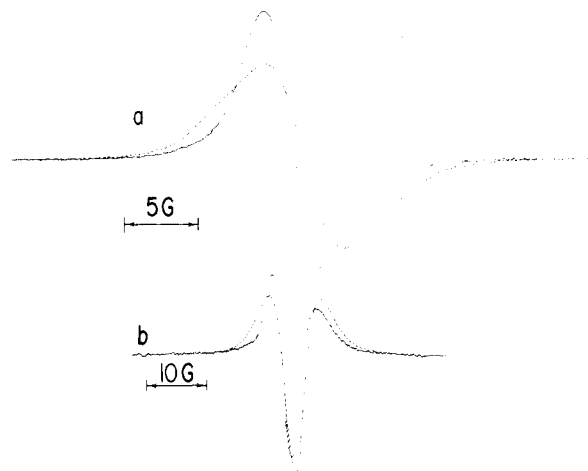
Samples for ESR investigation were prepared in the following manner. To 0.5 mL of melanin suspension (containing approximately 10 mg of melanin/mL) at pH ca. 5 was added 5 mL of 0.22 M sodium chloride solution and 5 mL of 4.4 mM metal ion solution. The mixture was brought to pH 5.5 by the addition of sodium hydroxide solution and then allowed to stand for 30 min before the melanin was isolated by centrifugation. Samples were stored at 77 K.

## Results and Discussion

Spectral changes and free-radical concentration changes from the melanin intrinsic ESR signal upon incubation with metal ions are presented and discussed sequentially.

(1) **Spectral Changes.** Unipositive alkali metal ions bind very poorly to melanin.<sup>2,3</sup> Accordingly, we did not anticipate and were indeed unable to find any changes in the melanin free-radical ESR spectrum in the presence of  $\text{Na}^+$ ,  $\text{K}^+$ , or  $\text{Cs}^+$  ions. Since no interaction was evident with these ions, an alkali metal salt (sodium chloride) was used to minimize differences in ionic strength between solutions containing other metal ions.

Several di- and tripositive ions were next studied. The effects of incubation with solutions of these metal ions on ESR spectral parameters are shown in Table I and in Figures 1 and 2. The



**Figure 1.** (a) First-derivative ESR spectra for synthetic melanin after incubation with solutions enriched in: (—)  $^{114}\text{Cd}^{2+}$ ; (---)  $^{113}\text{Cd}^{2+}$ . (b) Second-derivative spectra after incubation with solutions of  $\text{Zn}^{2+}$  ions containing (—) 4%  $^{67}\text{Zn}$ ; (---) 90%  $^{67}\text{Zn}$  (gain  $\times 1.6$ ).

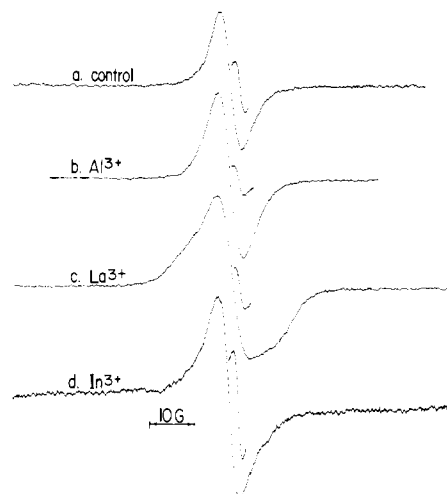
data in Table I, with one exception, are the parameters for the composite line, although it seems probable that the spectra obtained under our conditions contain components from both intrinsic and extrinsic chemically induced radicals. In at least the case of  $\text{In}^{3+}$ , where the lines from induced radicals are particularly broad (see below), there is some evidence for residual intrinsic radicals with relatively narrow lines. Synthetic melanin spectra are displayed in Figure 1, natural melanin spectra in Figure 2. As the data in Table I indicate, results for synthetic and natural melanin were generally similar, except that the line width of the intrinsic signal in natural melanin is somewhat broader than that in the synthetic preparation.

Significant changes in line width were found with these ions. Most importantly, the effect is largely dependent upon possession by the metal ion of a nuclear moment, since isotopic substitution in appropriate cases produced marked differences. For instance, a large difference in line width of first-derivative spectra is seen (Figure 1a) between samples incubated in solutions containing 2+ ions of  $^{113}\text{Cd}$  ( $I = 1/2$ ) and  $^{114}\text{Cd}$  ( $I = 0$ ), the former being much broader. A similar marked difference was observed between 2+ ions of naturally occurring zinc, which contains just 4% of the magnetic isotope  $^{67}\text{Zn}$  and zinc enriched to 90% in this isotope. Second-derivative spectra (Figure 1b) reveal the differences better in this instance.

Equally dramatic changes in line width from the intrinsic melanin signal after incubation with magnetic isotopes of tripositive metal ions are shown in Figure 2. The induced resonance appears as an extremely broad background signal in the case of  $\text{In}^{3+}$ , which has the highest nuclear moment and the highest nuclear spin of the ions investigated.

The magnitude of the line broadening in the above examples is evidence for very strong interaction between melanin free radicals and the metal ion. We believe that this interaction results from the formation of radical-metal ion complexes.

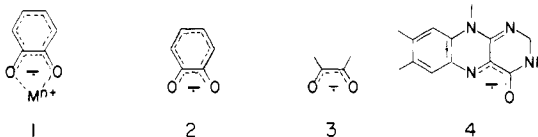
The magnetic interaction between free radical and metal ion must be some combination of dipole-dipole and contact. As an example, for  $^{113}\text{Cd}$  the order of magnitude of the incremental broadening of the free-radical line width because of dipole-dipole interaction with the cadmium nuclear spin is  $\gamma\hbar/r^3$  (where  $\gamma$  is the gyromagnetic ratio of the cadmium nucleus and  $r$  is the electron-nuclear separation) = 6 G at 1 Å. Thus, if the interaction were purely dipolar in character, the separation would be between 1 and 2 Å. The contact interaction is generally considered to be a measure of covalent bonding. It can only arise through an overlap of wave functions of the metal ion and free radical. One angstrom is roughly the



**Figure 2.** First-derivative ESR spectra for natural melanin before and after incubation with tripositive metal ions: (a) control, no added metal ions; (b)  $^{27}\text{Al}$ ; (c)  $^{139}\text{La}$ ; (d)  $^{113}\text{In}$  (4%) plus  $^{115}\text{In}$  (96%). Additional maxima shown are those of the pitch standard used for calibrating the spectra. Relative gains are (a)  $\times 1.6$ ; (b)  $\times 1$ ; (c)  $\times 1.25$ ; (d)  $\times 3$ .

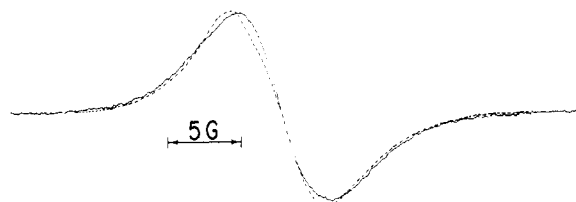
separation expected for a largely ionic metal ion complex. So, while we do not know the relative magnitudes of the dipolar and contact interactions, any assumed combination of these two interactions leads to the same conclusion: viz.,  $r$  lies between 1 and 2 Å. The observed broadening using nuclear isotopes is the key experimental result of this paper leading to the hypothesis of free radical-metal ion complex formation.

Experiments involving radicals which we consider to be suitable models have demonstrated their potential for complexation in solution. Some chelate complexes (**1**) of metal ions with *o*-benzosemiquinone have been characterized;<sup>17</sup> hyperfine splittings from metal ions were observed in several instances where the metal ion possessed nuclear spin. The requirements for effective chelation of this kind have been summarized<sup>18</sup> (with particular reference to semidiones): viz., the metal ions should be di- or tripositive while the radicals should be negatively charged and possess a *cis* stereochemistry. Of course, the metal ions studied here satisfy the former condition, while *o*-semiquinones (**2**), *cis*-semidiones (**3**), and flavin semiqui-



ones (**4**) satisfy the latter. In this respect we note that chelates of flavin semiquinones (with zinc and cadmium ions) have also been characterized, both optically<sup>19</sup> and with ESR.<sup>20</sup> It is concluded from this brief survey of model systems that complex formation between di- and trivalent metal ions and the *o*-semiquinone moieties considered to be present within the melanin polymer is indeed to be expected.

The marked dependence of the melanin free-radical line-width parameter on the nature of the added metal correlates well with data reported for the above models. Data for *o*-semiquinone complexes in aqueous solution are limited, but *cis*-semidiones should be very good models for *o*-semiquinones and, in fact, the magnitude of the hyperfine coupling to  $\text{Al}^{3+}$  in semidiones<sup>18</sup> (1.6 G) is close to that in *o*-benzosemiquinone<sup>17</sup> (1.96 G). Where comparison with the semidiones is possible, the broadening of the melanin signal parallels increases in total spectral width arising from isotropic metal ion splittings in *cis*-semidiones. Thus, for  $\text{Zn}^{2+}$ ,  $\text{Al}^{3+}$ , and  $\text{La}^{3+}$ , increases in the natural melanin radical line-width parameter (from that of the intrinsic signal) are 1.1, 1.4, and 3.1 G, respectively,



**Figure 3.** First-derivative ESR spectra for natural melanin in the presence of  $^{113}\text{Cd}$ : (—) experimental; (---) calculated from the spectrum in the presence of  $^{114}\text{Cd}$  assuming a further isotropic coupling to  $^{113}\text{Cd}$  ( $I = 1/2$ ) of 3.2 G.

while increases in total spectral width calculated from the data for semidiones are 4.5, 7.8, and 12.6 G. (For zinc and cadmium ions, a similar correlation exists with hyperfine coupling data for flavin semiquinones.<sup>20</sup>) Furthermore, it was possible to simulate the natural melanin spectrum in the presence of  $^{113}\text{Cd}^{2+}$ , where the line broadening is ca. 1.4 G, reasonably well by taking the spectrum in the presence of  $^{114}\text{Cd}^{2+}$  and introducing an isotropic hyperfine coupling of 3.2 G; see Figure 3. The closeness of the fit in the wings of the spectrum is particularly encouraging, while discrepancies in the center probably result from neglect of hyperfine anisotropy and the admixture of a proportion of intrinsic radical. We emphasize again that the data of Table I almost certainly refer to such a mixture of radicals; they should not be taken to characterize the complexed free radical for which line widths in particular are likely to be greater than the parameters reported.

Changes in  $g$  value and saturation characteristics (Table I, columns 4 and 5) of the melanin signal in the presence of metal ions are in the direction expected for complex formation. Thus, for ions of the same charge (the effect is most pronounced for the 3+ ions) there is a negative contribution to the  $g$  value that increases with increasing atomic number (again cf. semidiones<sup>18</sup>), probably owing to spin density in a vacant metal outer orbital,<sup>21</sup> while saturation becomes progressively more difficult to achieve.

It may appear surprising that the equilibrium for complex formation is favorable for the di- and tripositive metal ions employed in this study, the majority of which are generally considered to be weakly complexing species. Significantly, however, many of the known complexes<sup>22</sup> of the 3+ ions are chelates and include those with catechols, dicarboxylic acids, and 8-hydroxyquinoline, which can chelate either through two oxygen atoms (cf. *o*-semiquinones, *cis*-semidiones), or through oxygen and nitrogen (cf. flavin semiquinones).

The poorest chelators are expected to be the alkaline earth metal ions, which have lowest charge and highest ionic radii. With these ions, which contain only low percentages of isotopes possessing nuclear spin, evidence for complexation is less clear, but there are still indications of interaction with the melanin radical, viz. changes in  $g$  similar to those described above were found for synthetic melanin and small increases in line width were also apparent. This increase in line width (also found for nonmagnetic isotopes of zinc and cadmium) is likely to arise from small changes in proton hyperfine couplings or  $g$  anisotropy upon complexation, similar to those described<sup>17,18</sup> for model radicals. It is probable, however, that the complexation equilibrium is much less favorable with these ions.

**(2) Concentration Changes.** Several of the metal ions induced large increases (up to a factor of nine) in integrated signal intensity, while others gave small or zero increases. It is noteworthy that the ions which gave the smallest effects ( $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ , and  $\text{Sr}^{2+}$ ) are the alkaline earth metal ions, which we expect to form the least stable complexes. The data are shown in Table II. Natural melanin samples gave consistently smaller increases than synthetic melanin under the same conditions.

In an earlier paper<sup>11</sup> it was speculated that the enhancement

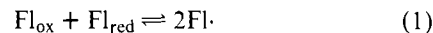
**Table II.** Free-Radical Concentration Changes after Incubation of Melanin Suspensions with 2.1 mM Metal Ions at pH 5.5

Metal ion	Enhancement <sup>a</sup>	
	Natural melanin	Synthetic melanin
$\text{Mg}^{2+}$	1.0	
$\text{Ca}^{2+}$		1.5
$\text{Sr}^{2+}$		1.2
$\text{Zn}^{2+}$	3.1 <sup>b</sup>	6.6 <sup>b</sup>
$\text{Cd}^{2+}$	1.6 <sup>b</sup>	3.8 <sup>b</sup>
$\text{Al}^{3+}$	3.0	6.7
$\text{Sc}^{3+}$	4.1	9.0
$\text{La}^{3+}$	3.9	7.8
$\text{In}^{3+}$	3.3	7.3

<sup>a</sup> Defined as integrated signal intensity after incubation with metal ions relative to the "intrinsic" signal. <sup>b</sup> Average of values obtained with different isotopes (see text).

found for the ESR signal of natural melanin after incubation with zinc ions could perhaps arise from the displacement of intrinsic paramagnetic ions in natural melanin. However, since similar results are obtained with synthetic melanin, where concentrations of bound metal ions are very low, we are able to preclude this possibility.

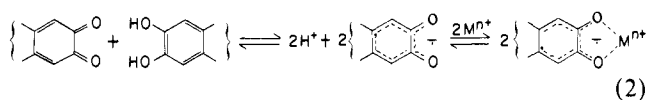
A second possibility<sup>12</sup> is that there exists an equilibrium between free radicals and their oxidized and reduced counterparts (or, in principle, any species having a formal oxidation-reduction state equivalent to a pair of semiquinones) that can be disturbed by metal ions. An example of such a system which has been studied in some detail is that involving flavin semiquinones, reaction 1 (where Fl  $\equiv$  flavin).



For model flavins in solution Hemmerich et al.<sup>23</sup> have observed a 20-fold increase in radical concentration in the presence of  $\text{Zn}^{2+}$ . Since Hemmerich was also able to demonstrate<sup>19</sup> that interaction of the metal ion with radical (equilibrium constant  $K \approx 10^4$ ) was very much stronger than with either oxidized or reduced flavin, it was concluded that metal ion chelation serves to remove uncomplexed flavin radicals from the system, shifting the equilibrium expressed in reaction 1 to the right.

Melanin is known<sup>4</sup> to contain a number of quinone and quinol moieties which are the oxidized and reduced forms required to establish an equilibrium analogous to reaction 1. Furthermore, it is likely that a number of these units will be present within the polymer as quinhydrone or other quinone-quinol complexes. Such a situation with quinone and quinol groups in close proximity would seem ideal for such an equilibrium, removing any requirement for diffusion together of quinone and quinol groups, although the latter possibility cannot be ruled out.

A problem with such a model is that it would appear to give, at least initially, a pair of radicals in extremely close proximity. Our failure to observe strong radical-radical interaction would require either a measure of spatial diffusion or conceivably electron tunneling. Furthermore, whether the metal ions react initially with a small proportion of radicals present (as depicted in reaction 2) or with a quinhydrone or similar complex having some radical character is not yet clear. Equilibrium 2 should therefore be regarded as depicting an overall reaction for which mechanistic details remain to be established.



We have conducted model experiments in solution with partially oxidized catechol that lend support to our view that

both quinone and quinol are required for radical induction. We find that whereas an excess of zinc ions does not yield ESR-detectable concentrations of free radical from a solution of catechol at pH 5, prior treatment of the catechol solution with a deficiency of potassium ferricyanide as oxidant with subsequent addition of zinc ions yields high concentrations of the zinc-chelated catechol semiquinone with  $a^H(2H) = 3.9$  G,  $a^H(2H) = 0.5$  G (cf.<sup>17</sup> 3.95 and 0.45 G).

As indicated above, the alkaline earth ions do not induce significant amounts of additional free radicals, while they do affect at least part of the intrinsic signal. The reason for this is probably (vide supra) that complexation of these ions with melanin radicals is not strong enough to affect the disproportionation equilibrium to a marked degree, i.e. complexation may well be significantly reversible under our conditions.

### Conclusions

We conclude that reversible complexation of metal ions with the melanin free radical is a general phenomenon. Many of the ions studied here are likely to interact strongly with other free-radical species which possess the structural and electronic requirements for chelation, e.g., flavin semiquinones, for which studies of metal ion interaction have been largely restricted to d-electron ions.

A comproportionation equilibrium within the melanin polymer between quinone-quinol units and free radicals seems to best explain the induction of free radicals by complexing metal ions and it may well be that other reversible effects (with light, base, etc.) can be explained in a similar manner. For instance, many disproportionation reactions become reversible at high pH, resulting in increased free-radical concentrations.<sup>24</sup>

The effects of metal ions described here may aid in the identification of free-radical signals in other biological systems. A positive test of course requires the presence of a chelating radical, so that broadening of the free-radical signal found in fulvic or humic acids, with, e.g., <sup>67</sup>Zn would be evidence for the detectable radicals being *o*- rather than *p*-semiquinones.<sup>25</sup> (*p*-Semiquinones are structurally unable to chelate.)<sup>17</sup> Also, quantitative changes in line width may, in favorable cases, serve to distinguish between components of the free-radical signal found in tissue. Thus, complexation of a flavinoid semiquinone component with a metal ion possessing a magnetic nucleus should result in radical induction and extensive broadening (based on the large hyperfine couplings to metal ions in flavin semiquinone chelates<sup>20</sup>), while melanin or melanin-like components are expected to show an increase in radical concentration but with less broadening. (An increase in the concentration of free radicals in tissue after incubation with zinc ions has been observed in this laboratory.)<sup>26</sup> Also, since neutral radicals appear not to form chelates of comparable stability, one may be able to estimate  $pK_a$  of chelating

radicals, which should also assist in the separation and characterization of contributing resonances.

Finally, the several points of similarity between melanin and flavins evident here and in other studies<sup>27</sup> are notable. Part of the biological function of melanins may similarly be<sup>27</sup> to participate in one- and two-electron oxidation-reduction systems.

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