from a first-order value of 0.60 eV) in comparison with the observed separation of 0.47 eV. The second-order SO coupling involves an admixture of 13% of the $8a_1$ ($e_{1/2}$) orbital with the $e_{1/2}$ component of 10e. This result suggests that Jahn-Teller distortions are not required to explain the observed patterns. The σ_{CI} band is prominent at 9.81 eV and, together with the n_I bands and the second σ_{CI} band at 12.7 eV, is reduced in relative intensity under photoionization by He II radiation.

Inductive electron withdrawal by the bromine and iodine atoms is again responsible for stabilization of the MOs of **3a**. Exceptions arise when the halogen orbitals are of appropriate symmetry and energy for conjugative interaction with the alkane MOs. The resulting destabilization brought about by the resonance electron donation of the halogen atoms offsets their inductive effect, and in the case of the σ_{CI} MOs causes an overall destabilization.

Conclusion

The bromo and iodo derivatives of the polycycloalkyl halides considered in this study are each substituted at a tertiary carbon atom and are of C_{3v} symmetry. Consequently their He I photoelectron spectra show similarities to those of *tert*-butyl bromide and *tert*-butyl iodide. However, the competition between conjugative and spin-orbit coupling effects which influence the ionization energies and the splittings observed for the halogen bands in each spectrum show interesting variations which are difficult to simulate by molecular orbital calculations. An indication of the complexities involved is that while the bromine 4p orbitals lose their localized nonbonding character except for one sharp peak in **1b**, the iodine 5p orbitals exhibit considerable localized character, though in varying amounts. The 6-bromotricyclo[$3.1.1.0^{3,6}$]heptane molecule (**1b**) is unusual in that its first photoelectron band is essentially of hydrocarbon character. In each of **1c**, **2c**, and **3c**, the σ_{CI} band is distinctive in that it is relatively narrow and intense in the He I spectra. The VEOMP calculations accurately simulate the observed splittings in the first PE band of the iodo compounds and indicate that the inclusion of second-order effects is necessary to explain the reduced separations obtained experimentally. In the halo adamantanes **3b** and **3c** the σ_{CX} bonding character is shared between two orbitals of a_1 symmetry. This is supported both by variations in their He I spectra and comparisons made with their He II spectra. The quality of calculations required to explain these observations in more detail is beyond present capabilities for molecules of the size represented here. Electrooxidation studies on these iodopolycycloalkanes[24] give $E_{1/2}$ values which correlate poorly with the first IEs measured here. The $E_{1/2}$ (IE) values for iodoadamantane (**3c**) are 1.72 V (8.79 eV) which show the same correlation noted for the iodobicycloalkanes,[8] that IE − $E_{1/2}$ = 7.09 ± 0.02. By comparison, **1c** with values of 1.52 V (7.27 eV) and **2c** with 1.57 V (8.76 eV) do not fit this pattern.

Acknowledgment. This work was supported by the Australian Research Grants Scheme. We acknowledge the Commonwealth Postgraduate Awards to M.K.L. and P.E.P. and the Flinders University Research Award to R.S.A. We thank G. D. Willett for assistance with the ab initio calculations.

Registry No. 1a, 51273-50-8; **1b**, 78995-60-5; **1c**, 74725-76-1; **2b**, 59346-69-9; **2c**, 74725-77-2; **3b**, 768-90-1; **3c**, 768-93-4.

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Photoionization and Photohomolysis of Melanins: An Electron Spin Resonance-Spin Trapping Study

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Contribution from the National Biomedical ESR Center, Department of Radiology, Medical College of Wisconsin, Milwaukee, Wisconsin 53226. Received April 30, 1984

Abstract: Electron spin resonance studies of the photolysis of synthetic and natural melanins in the presence of the spin trap 5,5-dimethyl-1-pyrroline 1-oxide are reported. Synthetic melanin was from autoxidation of dopa and natural eumelanin was extracted from bovine eyes. Spin adducts from reaction of transient species with the trap were identified and shown to arise from reaction with hydrated electrons and hydrogen atoms. It is inferred from this that e_{aq}^- and H· are products of melanin photolysis. Action spectra and quantum yields for these processes are reported and compared with those obtained for a synthetic melanin derived from catechol, which contains hydroxy aromatic, but no hydroxyindole, units.

Melanins are pigments derived from the enzymic oxidation of 3,4-dihydroxyphenylalanine (dopa) and/or cysteinyldopa.[1] They fall into two main classes: eumelanins, which are derived predominantly from dopa, and pheomelanins, which contain substantial amounts of units derived from cysteinyldopa. Natural melanins also contain protein. Following initial oxidation of the precursor, cyclization occurs, followed by polymerization to give a heterogeneous amorphous pigment. In eumelanins, the major units in the polymer appear to be based on 5,6-dihydroxyindole.[2]

Melanins function to protect tissue against the harmful effects of UV radiation.[3] However, damage to the pigment can occur during this process: photolysis of isolated pigments in air leads to the consumption of oxygen, which is reduced to superoxide and hydrogen peroxide, and associated pigment oxidation.[4] Recent quantitative data[5] have shown that the active chromophore is similar in both eumelanin and pheomelanin systems and that yields of net photooxidation are comparable. Thus photoinduced oxygen consumption is common to both classes of melanin.

The chromophore most active in oxygen consumption appears not to be the major eumelanin visible chromophore since the action spectrum for oxygen consumption[5,6] shows a much greater wavelength dependence than does the melanin optical absorbance

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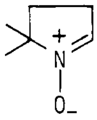
spectrum. The efficiency of oxygen consumption is low with visible light but increases rapidly at shorter wavelengths. For synthetic melanin from dopa (i.e., "dopa melanin", a model compound for natural eumelanin) the quantum yield is about 0.005 at 300 nm, while for most natural melanins the yield is about 3 times higher.[6]

The photochemical mechanism(s) whereby oxygen is reduced to superoxide and hydrogen peroxide is not clear at the present time, except that free-radical pathway(s) appear to dominate: no evidence for a singlet oxygen mechanism has been forthcoming.[6] One possible radical mechanism involves photoionization (to give e_{aq}^-) and/or photohomolysis (to give H·), as occurs with melanin precursors, for example, 3,4-dihydroxyphenylalanine and other catechols and catecholamines.[7] A model compound for pheomelanin, 4-hydroxybenzothiazole, also has been shown to photoionize: e_{aq}^- has been detected optically following flash photolysis of this material.[8] Production of e_{aq}^- also was reported from the laser photolysis of pheomelanin.[8]

We now report a study of the photolysis of eumelanin and its model compound and show that these materials, like their precursors, produce both hydrated electrons and hydrogen atoms upon UV irradiation. We used the spin trap 5,5-dimethyl-1-pyrroline 1-oxide (DMPO, **1**) as a scavenger for hydrated electrons and hydrogen atoms. These species both react to give spin adduct **2**,



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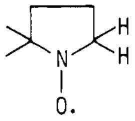

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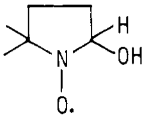

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the electron by attachment followed by protonation of the electron adduct, the hydrogen atom by direct addition (reactions 1 and 2). **2** is readily detected by electron spin resonance (ESR). The

$$e_{aq}^- + \mathbf{1} \xrightarrow{H^+} \mathbf{2} \tag{1}$$

$$H\cdot + \mathbf{1} \rightarrow \mathbf{2} \tag{2}$$

$$e_{aq}^- + N_2O \xrightarrow{H^+} N_2 + \cdot H \tag{3}$$

relative amounts of **2** originating from these two pathways were determined by carrying out experiments on solutions saturated with N_2O, where the hydrated electron is scavenged, as in reaction 3.[9] Action spectra and quantum yields are reported, and the data have been compared with those obtained from catechol melanin, a melanin formed by oxidation of catechol, which contains hydroxy aromatic but no hydroxyindole units.

Experimental Section

Dopa and catechol melanins were prepared by the autoxidation of dopa and catechol as described previously.[12] Like dopa melanin, catechol melanin synthesized in the absence of salt is soluble in neutral and alkaline solutions.[13] Melanosomes were isolated from bovine eyes[14] and protein removed by treatment with 6 N HCl for 72–100 h at ambient temperature. DMPO was obtained from Aldrich Chemical Co. and was redistilled in vacuo prior to use.

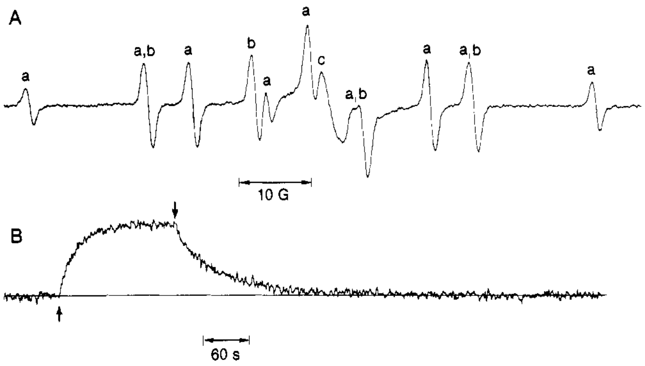

Figure 1. (A) Electron spin resonance spectra of spin adducts produced during the irradiation of a solution of dopa melanin (0.5 mg/mL, pH 8.4) in the presence of 8.7 mM DMPO. Lines marked a and b are derived from spin adducts 2 and 3, respectively. The line marked c is from free radicals in the melanin polymer. (b) Buildup and decay of radical **2** (see text) from irradiatin of 0.5 mg/mL dopa melanin.

Samples typically contained 0.02–0.05 mg/mL melanin and 8.7 mM DMPO in 50 mM phosphate buffer, pH 7.6, and generally were saturated with nitrogen to remove oxygen, which is an excellent scavenger of hydrated electrons and hydrogen atoms.[10,11] The samples were inserted into a quartz flat cell (path length 0.025 cm) for irradiation and ESR observations. For some experiments, samples were saturated with nitrous oxide in order to scavenge the hydrated electron.

Samples were irradiated at ambient temperature (23 °C) in situ in the cavity of the ESR spectrometer. Irradiation was with light from a Bausch and Lomb monochromator (33-86 series) using an Eimac VIX200UV 200-W Xe lamp as light source. The monochromator slit width was set at 3 mm, corresponding to a bandwidth of 10 nm. The light intensity incident on the sample was measured by using a YSI Radiometer (Model 65A) and by hematoporphyrin actinometry.[15] The irradiated area of the flat cell was 225 mm^2.

Electron spin resonance measurements were made using a Varian E-109 spectrometer operating at 9.5 GHz with 100-kHz field modulation. Spectra were obtained with nonsaturating microwave powers (typically 1 mW) and a modulation amplitude of 1 G. The time dependence of radical formation and decay was monitored by setting the magnetic field to the position of one of the hyperfine lines of the spin adduct. The light was then turned on and the buildup of the radical to a steady state recorded; subsequently the light was turned off and the decay of the radical obtained. No light-induced radicals were detected in the absence of the melanin. Radical concentrations were estimated by comparing the double integrals of selected lines from the spectrum of the spin adduct nitroxide and from the spectrum of the spin probe 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrroline-1-yloxy (Aldrich Chemical Co).

Optical spectra were measured on a Perkin-Elmer 320 spectrophotometer.

Results

Dopa melanin and bovine eye eumelanin absorb continuously throughout the visible and ultraviolet. Optical spectra of these materials have previously been reported.[6] The spectrum of catechol melanin was similar to that of dopa melanin except that it showed a steeper wavelength dependence, with $A_{300}/A_{600} = 13$. Estimated values of ϵ (units (mg/mL)$^{-1}$ cm^{-1}) at 300, 400, 500, and 600 nm were 17.5, 5.7, 2.6, and 1.3, respectively.

Figure 1A shows the ESR spectrum obtained during continuous irradiation with light of 250 nm of a solution of dopa melanin in the presence of DMPO. The incident fluence rate was 1.5 W/m^2. The dominant ESR spectrum is that of radical **2** (see above), which has the following magnetic parameters: $a_N = 16.6$ G, $a_{2H} = 22.5$ G, and $g = 2.0054$. Generation of this spin adduct implies that hydrated electrons and/or hydrogen atoms are produced in the system (reactions 1 and 2 above). Smaller amounts of radical **3**, $a_N = 14.9$ G, $a_H = 14.9$ G, $g = 2.0055$, also are present.[16] It is possible that radical **3** is formed by the addition to DMPO of ·OH from the photolysis of H_2O_2. The latter has been shown to

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[9] Reaction of e_{aq}^- with N_2O is rapid ($k = 8.7 \times 10^9$ M^{-1} s^1 at pH 7).[10] Reaction with the hydrogen atom is much slower, at least up to pH 11.[11]

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[13] Melanins are heterogeneous polymers having a range of molecular weights. Molecular weights of dopa and catechol melanins used in this study are > ca. 50 000 based on the molecular weight cutoff of the ultrafiltration membrane employed in their isolation.

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[16] Magnetic parameters reported for **2** and **3** are close to literature values.

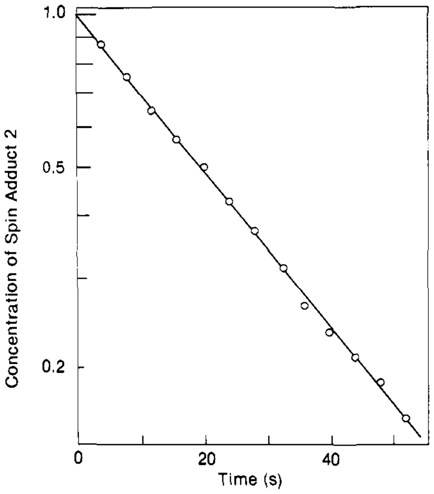

Figure 2. First-order plot of the decay of radical **2**.

be present in small amounts in stored melanin suspensions.[18] Spectra similar to that shown in the figure were obtained from bovine eye melanin and from catechol melanin under these conditions.

The time dependence of the spectrum of radical **2** is shown in Figure 1B. Whereas at low melanin concentrations the decay follows secnd-order kinetics, as found for this radical when generated in solutions of dopa and catechol,[7] moderate melanin concentration were found to enhance the radical decay so that a first-order pathway dominates. This is the case for the conditions under which Figure 1B was obtained. First-order decay is demonstrated by the linear dependence of the logarithm of the radical concentration vs. time (Figure 2). Rate constants determined from Figure 2 and similar plots obtained for a range of melanin concentrations were proportional to the melanin concentration. It can be concluded from this that the decay is pseudo first order at high melanin concentrations, where the spin adduct nitroxide is destroyed by a reaction with the melanin. It is probable that reduction to the corresponding hydroxylamine occurs since melanins are known to contain substantial amounts of reducing groups.[19] To summarize, at low melanin concentrations reaction 4 predominates, whereas at high concentrations reaction 5 represents the major decay pathway.

$$\mathbf{2} + \mathbf{2} \rightarrow \text{products} \qquad (4)$$

$$\mathbf{2} + \text{melanin} \xrightarrow{k} \text{products} \qquad (5)$$

From the pseudo-first-order rate constants obtained as a function of melanin concentration, a second-order rate constant, k, of 2.1×10^{-2} (mg/mL)$^{-1}$ s^{-1} was derived for reaction 5. If we take 1 mg/mL melanin to be approximately 6.7 mM on the basis of monomer units (this assumes a monomer molecular weight equivalent to that of dihydroxyindole, one of the major melanin units,[2] a rate constant of approximately 3 M^{-1} s^{-1} is obtained. A similar rate constant (1 M^{-1} s^{-1}) was obtained for catechol melanin. Melanins are thus weak scavengers for these radicals, although better than either dopa or catechol.[7] To date little information is available on radical scavenging by melanins, although their chemical structure does suggest that they should be effective in this respect.[20]

Under conditions in which radical decay is first order, the rate of spin adduct formation is given by

$$d[\mathbf{2}]/dt = I\phi_2 \qquad (6)$$

Here I is the rate at which photons are absorbed, ϕ_2 is the quantum yield for formation of nitroxide **2**, and k' is a pseudo first-order constant ($=k$[melanin]). At steady state

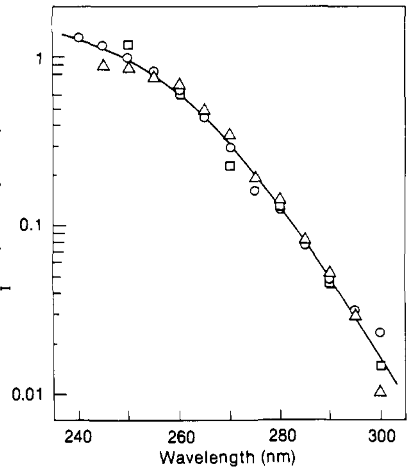

Figure 3. Action spectra for production of radical **2** (formed via addition of e_{aq}^- and H· to DMPO) from the UV photolysis of melanins at pH 8.0: (○) dopa melanin, (△) bovine eye melanin, (□) catechol melanin. Note that the ordinate is on a logarithmic scale.

Table I. Quantum Yields (ϕ) for Spin Adduct Formation from Melanins at pH 8.0

λ, nm	dopa melanin, $10^3\phi$	catechol melanin, $10^3\phi$
240	4.7	
245	4.5	2.5
250	3.9	2.5
255	3.2	2.3
260	2.5	1.9
265	1.7	1.5
270	1.2	1.1
275	0.63	0.60
280	0.50	0.45
285	0.32	0.26
290	0.20	0.18
295	0.13	0.10
300	0.10	0.04

$$d[\mathbf{2}]/dt = 0 = I\phi_2 - k'[\mathbf{2}]_{ss} \qquad (7)$$

where $[\mathbf{2}]_{ss}$ is the steady-state radical concentration, so that

$$(d[\mathbf{2}]/dt)_{\text{formation}} = I\phi_2 = k'[\mathbf{2}]_{ss} \qquad (8)$$

Furthermore, if the spin trap is present in concentrations sufficient to scavenge all the hydrated electrons and hydrogen atoms formed, the nitroxide yield becomes equal to the sum of the yields of the primary species, i.e., $\phi_2 = \phi_{e_{aq}^-} + \phi_{H\cdot}$. Experimentally, we tested this by varying the concentration of the spin trap and found that the steady-state radical concentration reached a plateau at a DMPO concentration of about 8.7 mM for a melanin concentration of 0.5 mg/mL. In the calculations that follow, we assume that this concentration of DMPO traps all the hydrated electrons and hydrogen atoms formed in the system.

Under these conditions (i.e., high DMPO concentration and first-order decay of the spin adduct), we obtained rates of spin adduct formation over a range of wavelengths. Since the radical yield is proportional to the steady-state radical concentration (eq 8), action spectra data could be obtained by dividing the steady-state radical concentration by the number of incident photons and plotting this parameter against wavelength. Data obtained in this way for the three melanins are shown in Figure 3, where the ordinate is on a logarithmic scale. The wavelength profile is essentially the same for all three systems, so that a common action spectrum can be drawn through the data points. The efficiency of radical production increases sharply with decreasing wavelength. No radical production was detected above ca. 300 nm.

Quantum yields at each wavelength were obtained for dopa and catechol melanins from measurements of the steady-state radical

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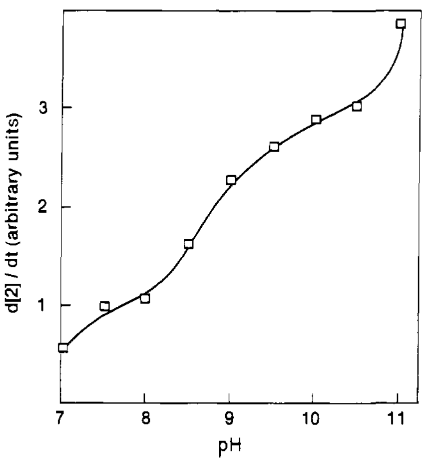

Figure 4. Effect of pH on the yield of radical **2** from dopa melanin.

concentration, the rate constant for termination, and the number of absorbed photons, as described above. These data are given in Table I. The reaction is moderately effective at short wavelengths: for dopa melanin at 240 nm, ϕ_2 was estimated to be 0.005. The yield from bovine eye eumelanin varied somewhat for sample to sample, ranging between 10% and 20% of that measured for dopa melanin under comparable conditions.

As indicated above, the yield of spin adduct should equal the sum of the yields of hydrated electrons and hydrogen atoms generated in each system. Since hydrated electrons are selectively scavenged by nitrous oxide, the relative amounts of e_{aq}^- and H· that contribute to the formation of **2** can be determined from experiments with nitrous oxide saturated solutions. At pH 8.0 in the presence of the electron scavenger, the yield of **2** from dopa melanin was decreased to about 35% of that measured in nitrogen-saturated solutions, implying that roughly 35% of the formation of **2** involves hydrogen atoms, with the remaining 65% involving hydrated electrons, i.e., $\phi_{e_{aq}^-}/\phi_{H\cdot} = 1.8$.

We studied the effect of pH on the yield of radicals from dopa melanin and found a significant increase between pH 7 and 11. Between pH 8 and 10 the yield increased by a factor of about 2.8, with an apparent further increase above about pH 10.5 (Figure 4).

Discussion

It has been demonstrated that melanins produce both hydrated electrons and hydrogen atoms upon photolysis. The reaction occurs at short wavelengths, where it is of moderate efficiency, and is promoted by increasing pH. Photoionization predominates over photohomolysis at neutral pH. In all these aspects the melanins qualitatively resemble their precursors, dopa and catechol.[7] However, unlike the precursors, action spectra for the melanins do not show a maximum in the ultraviolet; instead the efficiency of radical formation continues to increase with decreasing wavelength over the wavelength range investigated (Figure 3).

Since the action spectra are so similar for the three melanins, it appears that a similar chromophore is active in the photoionization/photohomolysis reaction in each system. While the exact nature of this chromophore is not known, it seems not to be dependent on groups in the polymer that are specifically related to dopa. Probably hydroxy aromatic units in the melanin are involved (cf. photolysis of aromatic amino acids in proteins[21]).

The chromophore active in photoionization/photolhomolysis is not the major eumelanin visible chromophore. This is evident from Figure 5, which shows the action spectrum for $e_{aq}^- + H\cdot$ production (on a linear scale) with the optical absorbance spectrum of dopa melanin. Figure 5 also shows action spectra for oxygen consumption[6] and melanin free-radical production.[22] If these latter processes are dependent on photoionization/photohomolysis, then their action spectra (which were obtained[6,22] under conditions of bandwidth and optical density very similar to those used in the present study) should be closely related. Comparison of both action spectra and quantum yields indicates a moderately good correlation at short wavelengths (<250 nm), but both oxygen consumption and melanin free-radical production occur at longer wavelengths where no measurable photoionization or photohomolysis was detected by spin trapping. From this we can conclude that the photoionization/photohomolysis process detected by spin trapping may contribute to oxygen consumption and melanin free-radical production at short wavelengths but does not contribute appreciably to these processes induced by near-UV or visible light.

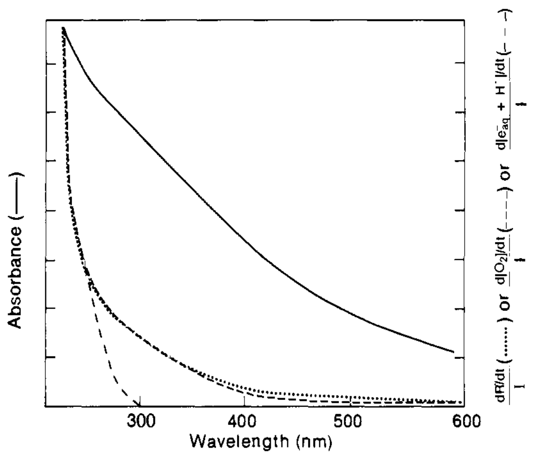

Figure 5. Comparison of melanin action spectra for photoionization and photohomolysis (– –) with those for oxygen consumption (- - -) and free radical production (···). The optical absorbance spectrum for dopa melanin (—) also is shown.

It remains possible, although rather unlikely, that at longer wavelengths a second photoionization/photohomolysis pathway occurs which we were unable to detect by the spin trap method. (It has been reported[8] that hydrated electrons are detectable by optical spectroscopy following laser photolysis of pheomelanin with visible light.) If a second pathway is present in the systems that we have studied, the spin adduct formed must decay much faster than with irradiation at shorter wavelengths. Conceivably this might occur if the spin adduct rapidly reacts with a melanin counter radical formed directly or indirectly from the primary photochemical step at longer wavelengths:

$$\mathbf{2} + \text{melanin radical} \rightarrow \text{products} \qquad (5)$$

However, all melanin radicals detected so far have a strong propensity for reacting with each other: to date no chemical means of intercepting these radicals has been found. (For example, they are not spin trapped by DMPO.)

Acknowledgment. This work was supported by Grants AM-26950 and RR-01008 from the National Institutes of Health.

Registry No. **1**, 3317-61-1; **2**, 40936-29-6; **3**, 55482-03-6; hydrogen atom, 12385-13-6.

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