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MELANIN PHOTOREACTIONS IN AERATED MEDIA: ELECTRON SPIN RESONANCE EVIDENCE FOR PRODUCTION OF SUPEROXIDE AND HYDROGEN PEROXIDE

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Summary. Electron spin resonance measurements on aerated melanin suspensions during photoirradiation show changes in the microwave saturation of melanin free radicals and formation of adducts in the presence of spin traps. These observations indicate that oxygen is reduced to superoxide and hydrogen peroxide.

Introduction. Melanins form a class of biological pigments whose primary function in man is believed to be photoprotection. They are characterized, in part, by their containing persistent free radicals[1] (probably o-semiquinones) which serve as a convenient probe for electron spin resonance (ESR) investigations. Additional, transient free radicals are produced upon irradiation with light; we have characterized the ESR spectra of these radicals in deaerated media and adduced evidence for their having a triplet precursor.[2] However, a sound understanding of the effect of oxygen on irradiated systems is essential if one is to model in vivo systems, and the mode of interaction of oxygen with melanins is poorly understood, both in the dark and in the presence of light. A slow uptake of oxygen by melanin suspensions in the dark has been reported,[3] and Cope et al.[4] have described reversible changes of ESR signal amplitude with oxygen concentration for both intrinsic free radicals and the mixture of radicals (i.e. intrinsic plus light-induced) obtained during irradiation, but the processes involved are unclear. We here provide evidence for (i) rapid scavenging of oxygen by melanin in the presence of light, causing an increase in the micro-

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Abbreviations: ESR - Electron Spin Resonance, Dopa - β-dihydroxyphenyl alanine, DMPO - 5,5'-dimethyl-1-pyrroline-1-oxide, SOD - Superoxide dismutase.

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wave saturation of the ESR resonances of the free radicals and for (ii) reduction of the scavenged oxygen to hydrogen peroxide, at least in part by way of an initial one-electron reduction to yield superoxide. These findings may have important implications in melanin biology: immediate pigment darkening may[5] have an oxygen requirement, and there is a possibility[6] that hydrogen peroxide is involved in melanogenesis.

Methods. Experiments described were carried out with samples of synthetic melanin from DL-β-dihydroxyphenyl alanine (DL-Dopa), prepared as previously described.[7] However, experiments with natural melanin from bovine eyes[8] gave similar results. Melanin aqueous suspensions (ca. 10 mg/ml) at ambient temperature were irradiated with filtered light of 320-600 nm from an Eimac VIX-300UV 300 W Xenon lamp in the cavity of a Varian E-109 spectrometer. Deoxygenation was achieved by freeze-pump-thaw cycles on a vacuum line.

Results and Discussion. It is well known that ESR signal amplitudes depend on the extent of microwave saturation, which is in turn dependent upon the presence of fast-relaxing paramagnetic species such as oxygen, a ground state triplet. This phenomenon is demonstrated for the intrinsic melanin radicals in Fig. 1, where ESR signal amplitudes for deoxygenated, air-saturated and oxygen-saturated suspensions are shown as a function of incident microwave power. (ESR lineshapes were independent of oxygen concentration.) In the absence of saturation, signal amplitudes should be proportional to the square root of the microwave power, and at powers below 0.1 mW, this behavior is found for all samples. At higher powers the deoxygenated sample saturates much more readily than those samples containing dissolved oxygen.

Upon photolysis of oxygen-containing samples, rapid changes in saturation of the melanin ESR signals with time were observed, whereas in the absence of light no changes were observed over the time period of the experiment (up to 1 hour). We believe that these light-induced changes reflect oxygen depletion in the medium, since whereas photolysis of deoxygenated samples gave no change in microwave saturation of the ESR signals from either the intrinsic free radicals or the irradiation mixture, photolysis of aerated samples resulted, within a very few minutes, in an increase in saturation of these signals to the same levels found for deoxygenated samples. (Data

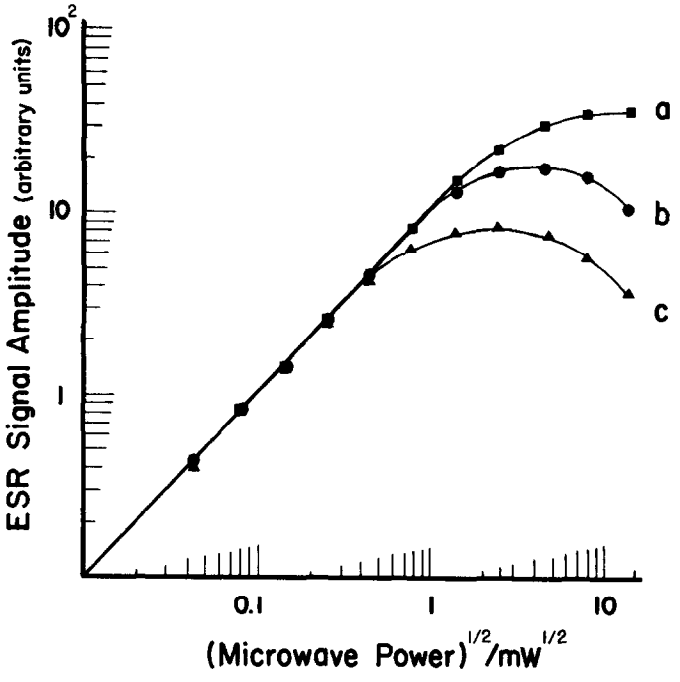


Fig. 1. ESR signal amplitude of the intrinsic free radical in: (a) oxygen-saturated (■); (b) air-saturated (●); and (c) deoxygenated (▲) aqueous suspensions of synthetic melanin as a function of microwave power.

obtained at low microwave power established that no detectable change in radical concentration was occurring.)

By monitoring the change in amplitude of the ESR spectrum of the irradiation mixture recorded under conditions of extreme microwave saturation (200 mW microwave power) as a function of time after the onset of irradiation, we have measured the time required to deoxygenate melanin suspensions and find (see Table) that this time is approximately five times greater for oxygen-saturated solutions than for air-saturated solutions, indicating that the overall rate of oxygen removal, $\Delta[O_2]/\Delta t$, is approximately the same (ca. 2×10^{-6} M s^{-1}). This rate, which may be limited by oxygen diffusion from the unirradiated portion (ca. 50%) of the sample volume, is much lower than the rate of removal of transient radicals by self-reaction (from Ref. 2, $> 2 \times 10^{-4}$ M s^{-1}). The Table also shows the effect of catalase and superoxide dismutase

TABLE

Melanin Suspension	Deoxygenation time/min[a]	$10^6 \frac{\Delta[O_2]}{\Delta t}$/M s^{-1} [b]
Air saturated	1.9 ± 0.1	2.2 ± 0.1
Air saturated + superoxide dismutase	2.1 ± 0.1	2.0 ± 0.1
Air saturated + catalase	3.1 ± 0.1	1.34 ± 0.05
Air saturated + catalase + superoxide dismutase	3.2 ± 0.1	1.30 ± 0.05
Oxygen saturated	11.4 ± 0.5	2.0 ± 0.1

[a]Time required for 95% change of the difference between the original ESR signal amplitude and that for the deoxygenated sample. [b]Average rates of oxygen removal calculated from deoxygenation times assuming that air- and oxygen-saturated suspensions have oxygen concentrations close to those for water, viz. 0.25 and 1.4 mM respectively.

on the rate of oxygen removal. These enzymes catalyze the destruction of hydrogen peroxide and superoxide respectively, reactions (1) and (2). Catalase (360 μg/ml) decreases the rate by 40%, consistent with hydrogen peroxide formation (cf. Van Woert[3]) whereas superoxide dismutase (120 μg/ml) has a much smaller affect. However, the latter is consistent with superoxide production if its predominant mode of disappearance in the absence of enzyme is also disproportionation to oxygen and hydrogen peroxide.

$$H_2O_2 \xrightarrow{\text{catalase}} H_2O + \tfrac{1}{2}O_2 \qquad (1)$$

$$2O_2^{\cdot-} \xrightarrow[\text{dismutase, } 2H^+]{\text{superoxide}} H_2O_2 + O_2 \qquad (2)$$

To demonstrate superoxide production during melanin irradiation, we have used the method of spin trapping[9] which has recently provided[10,11] novel evidence for superoxide generation in other biological systems. The method is based upon the addition of a transient free radical to a radical scavenger such as 5,5'-dimethylpyrroline-1-oxide (DMPO) to give a relatively persistent nitroxide spin-adduct, whose ESR spectrum is characteristic of the addend. The reaction between DMPO and $O_2^{\cdot-}$ (and/or $HO_2^{\cdot}$ [12]) is shown in equation (3).

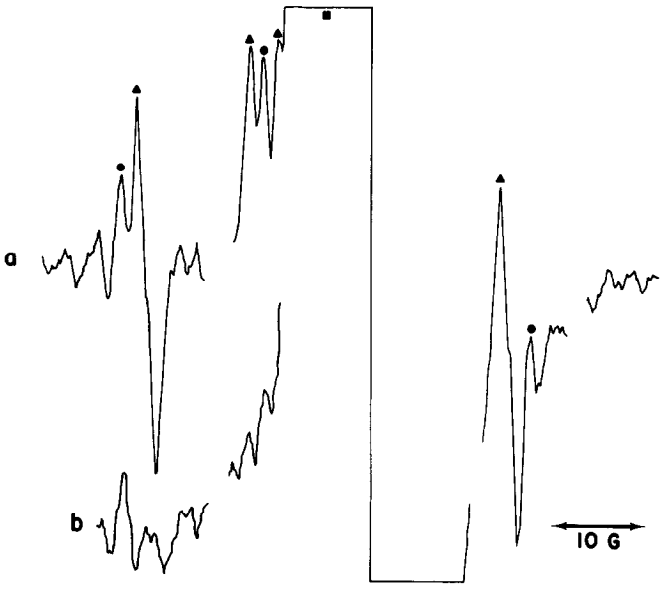
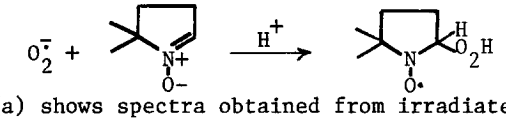


Fig. 2. ESR Spectra of free radicals detected during photoirradiation of an aqueous suspension of synthetic melanin in the presence of the spin trap DMPO. Lines marked ▲ are attributed to the adduct of superoxide with DMPO. The melanin resonance (■) is off-scale in this display. Owing to loss of spin adduct following oxygen depletion (rapid under these conditions) the spectra are composites from several samples scanned over adjoining magnetic field intervals. (a) without superoxide dismutase; (b) with 120 μg/ml superoxide dismutase (only part of this spectrum is shown).



(3)

Fig. 2(a) shows spectra obtained from irradiated oxygen-saturated solutions of melanins containing 0.15 M DMPO. Besides lines from melanin (■) and an impurity in the DMPO (●), other resonances (▲) attributable to the superoxide spin-adduct are clearly visible. We are confident that the lines marked ▲ are indeed from the superoxide adduct since their positions exactly match those of an authentic $O_2^{\cdot-}$ adduct spectrum[11] and they are not present (Fig. 2(b)) when superoxide dismutase is included in the reaction medium.

It is therefore concluded that reduction of oxygen proceeds by a one-electron reduction to superoxide and thence to hydrogen peroxide (reactions (4) - (5)).

$$O_2 \xrightarrow{e} O_2^{\cdot -} \quad (4)$$

$$2O_2^{\cdot -} \xrightarrow{2H^+} O_2 + H_2O_2 \quad (5)$$

The identity of the one-electron reductant has not yet been firmly established. However, although we have not yet found a dependence of the concentration of ESR-detectable free radicals on oxygen concentration, it does seem likely that electron donation is from the transient photoinduced free radicals formed on the polymer surface (cf.[13] the well-documented reversible reaction of semiquinones with oxygen). Our failure to detect such a dependence may well reflect the low overall quantum yield for the reduction.

That irradiated melanins are capable of one-electron reduction of oxygen to give superoxide and hydrogen peroxide raises intriguing possibilities. Despite their low overall yield under our experimental conditions, production of these potentially damaging[14] species may prove to have important consequences in melanin biology although there is evidence for a slow scavenging of hydrogen peroxide by melanin (high concentrations can bleach[15] or otherwise chemically modify[16] the pigment). Other one-electron reductions by irradiated melanin are also to be expected.

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