J. Cosmet. Sci., 51, 169–182 (May/June 2000)

Oxygen radicals from photoirradiated human hair: An ESR and fluorescence study

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Accepted for publication March 15, 2000.

Synopsis

Exposure of hair to light causes degradation, the precise mechanism of which is not completely understood. Oxygen free radicals (superoxide and hydroxyl) are believed to be involved, and a variety of indirect evidence has supported this. However, to date, direct observation of oxyradicals from photoirradiated hair has been lacking.

We have used complementary electron spin resonance and fluorescence techniques to assess oxyradical production when human hair is irradiated under UVA and visible light ($\lambda \geq 320$ nm). ESR studies include direct observation of intrinsic melanin and keratin radicals as well as spin trapping with DMPO. The fluorescence technique is based on terephthalic acid dianion (TA) as a hydroxyl radical probe. Radical scavengers are used to compete with the probe (TA or DMPO). Our results indicate that (a) oxyradicals are produced during photoirradiation, (b) terephthalate is a convenient method to study hydroxyl production in hair, (c) radical production upon irradiation varies with hair type, and (d) the effects of radical scavengers suggest potential implications for hair care product development.

INTRODUCTION

Hair fibers consist primarily of fibrous proteins belonging to the keratin family. Morphologically, hair structure has three distinct components: the cuticle, the cortex, and the medulla. The shingle-like cuticles form the hair exterior and enclose the corticular mass. The cortex constitutes the bulk of the hair and contains tightly packed elongated cortical cells oriented parallel to the fiber axis. These cells contain alpha-helical microfibrils embedded in a cystine-rich amorphous protein matrix. The protein components comprising the hair fiber have differing amino acid compositions. The function of the medulla is not clear. It is composed of loosely attached spongy cells. The keratin in hair is accompanied by minor quantities of lipids. Pigmented hair contains melanin granules located within the cortex and medulla (1–3).

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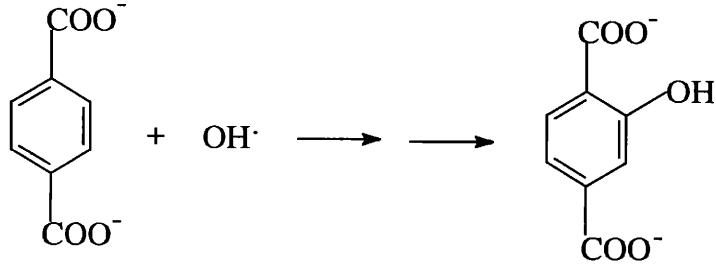
Exposure of hair to light causes chemical and physical degradation of the fiber (4,5). Recent reports on the harmful effects of sunlight on human skin have raised awareness of the deleterious effects of sunlight on biological tissues in general. Because hair does not have the repair mechanism of skin (6), the effects of photodegradation must be dealt with through cosmetic means. The way that light affects hair is still not completely understood. The solar spectrum's UV range is known to lead to photooxidation of the protein structure through the oxidation of specific amino acids, in particular the oxidation of cystine to cysteic acid (7–9). The deleterious effects of solar irradiation are perceived by consumers as changes in texture and color, dryness, etc, and can be evaluated in terms of reduced elasticity, increased porosity or swelling properties, altered dye sorption characteristics, and photofading of natural or artificial hair color (10–12).

The essential first event in hair photodamage, as in all photoprocesses, is light absorption by the fiber. Free radicals have been observed by electron spin resonance (ESR) spectrometry during the light-induced degradation of keratin (13–15). Carbon-centered radicals are formed with an action spectrum maximum of 285 nm, suggesting that they are primary photoproducts of aromatic amino acids (16). Kinetic measurements indicate that there are at least three types of radicals produced at this wavelength (17). Stable sulfur-centered radicals are formed from irradiation of dry keratin in the absence of oxygen, but their ESR signals disappear in the presence of moist air (18). The presence of small amounts of metal ion (trivalent iron or divalent copper) is especially conducive to the production of free radicals from irradiated keratin at wavelengths above 320 nm, apparently due to the formation of metal–protein complexes that absorb at higher wavelengths (19). Smith *et al.* (20) have suggested the presence of tetrahedral iron-sulfur centers that are easily oxidized upon irradiation, giving rise to carbon-centered radicals on neighboring groups. The most significant chromophores in proteins that absorb in the UVB region are the amino acids, tyrosine (Tyr, λ_{max} = 275 nm) and tryptophan (trp, λ_{max} = 280 nm). The irradiation of wool keratin under UVA and visible light does not cause the formation of tryptamine, which is the main photoproduct formed during irradiation of tryptophan itself in aqueous solution (21,22). Like wool photoyellowing and photodamage, a free-radical mechanism may be involved in the photodamage of hair at wavelengths above 320 nm.

Melanin has an intrinsic ESR signal that increases significantly when irradiated with UV-visible light. In the presence of oxygen, superoxide is produced, which dismutates to hydrogen peroxide (23,24). This leads to the formation of hydroxyl radical in the presence of trace amounts of metal ions. We have recently extended the observations of earlier workers to the study of oxygen radical production in suspensions of melanin extracted from red and brown hair (25). By employing terephthalic acid dianion (TA) as a hydroxyl radical trap, we have introduced a convenient method for estimating relative amounts of oxyradicals formed under varying conditions. In the terephthalate analysis, TA reacts with hydroxyl radicals to form hydroxyterephthalate (HTA), as shown in Scheme I (26). HTA is highly fluorescent, with an excitation wavelength sufficiently high that there is little or no interference from tryptophan fluorescence.

Our results on the melanin suspensions show that while red hair melanin (largely pheomelanin) has a smaller intrinsic ESR signal, and a smaller increase upon irradiation, compared to brown hair melanin (largely eumelanin), irradiation of red hair melanin produces a larger yield of oxygen radicals than either brown hair melanin or sepia, which is pure eumelanin. In this paper, we report on the combined use of ESR spectroscopy



Terephthalate (TA) 2-hydroxyterephthalate (HTA)

(Non-fluorescent) (Fluorescent: $\lambda_{ex} = 315$ nm, $\lambda_{em} = 425$ nm)

Scheme 1

and reaction with TA to demonstrate and assess the involvement of oxygen radicals when hair is irradiated at wavelengths above 320 nm.

EXPERIMENTAL

MATERIALS

All chemicals were reagent grade quality and were used without further purification. Terephthalic acid, sodium hydroxide, and potassium phosphate monobasic (KH_2PO_4) were purchased from Fisher Scientific Company (Fair Lawn, NJ). DMPO (5,5-dimethyl-1-pyrroline N-oxide), 2-bromoterephthalate, and sodium azide were purchased from Sigma (St. Louis, MO). Sodium benzoate, and sodium phosphate dibasic ($Na_2HPO_4 \cdot 7H_2O$) were from Allied Chemical (New York). Ethanol (200-proof) was from Quantum Chemical (Tuscola, IL).

Standard 2-hydroxyterephthalate (HTA) was synthesized from 2-bromoterephthalate by the method of Mason *et al.* (27). The crude precipitate was purified using liquid column chromatography until the fluorescence spectrum matched that from the literature (28). The melting point of the purified crystals is 321–325°C (lit. m.p. 320–322°C). This authentic HTA was used for the standard curve.

For the HTA calibration curve, a 2.0 mM HTA (pH 7.6) stock solution was diluted with 50 mM phosphate buffer (pH 7.6). The standard curve was obtained by plotting fluorescence intensity at 425 nm against HTA concentration (25); it is linear up to at least 10 μM.

The milled keratin powder was provided by Zotos Corporation. Hair tresses were purchased from DeMeo Brothers. Before each experiment, the hair samples were washed with 4.5% sodium lauryl sulfate and doubly distilled water, and left at room temperature to dry. The clean and dry hair sample was cut into 1-mm-long pieces. The cut hair or wool powder was added to a 1- or 2-ml buffered solution of ESR spin trap (DMPO) or fluorescence probe (TA) in a 1-cm square Pyrex spectrometer cuvette for irradiation. Samples were normally stirred to maintain air saturation.

All irradiation was done with a 150-W xenon lamp through glass filters transmitting UVA and visible light (≥320 nm). The measured light flux falling on 50% of a 2-ml sample was typically 0.35 mw/cm^2 (±15%). After irradiation, each sample was filtered through a 0.22-μm filter, and the filtrate was transferred to an ESR quartz flat cell for ESR measurements or to a fluorescence cell for fluorescence measurements. To minimize the effects of varying lamp intensity and other factors, series of experiments comparing different samples were always done on the same day. Replicate experiments performed on different days were generally within ±10% for HTA or ±20% for ESR experiments, but *relative* trends for different types of hair were always the same.

INSTRUMENTAL

Electron spin resonance (ESR) measurements were performed at room temperature on a Bruker ER200 spectrometer operating at 9.76 GHZ with 100 KHZ field modulation and microwave power of 20 mW. Direct ESR spectra of hair were taken in 3-mm quartz tubes. For the spin trapping experiments, irradiation (in 1-cm cuvettes) was done outside of the ESR cavity, and samples were transferred to the flat cell after irradiation. While irradiation of melanin within the ESR cavity permitted detection of the superoxide radical (25), the instability of DMPO-O_2^- allows only DMPO-OH to be observed in the present experiments.

Fluorescence spectra were taken with a Spex Fluorolog-1680 spectrometer in 1-cm disposable plastic cells. HTA signals (λ_{ex} = 315 nm, λ_{em} = 425 nm) were identified and quantified by comparison with synthetic HTA. All fluorescence measurements reported here are on the same arbitrary scale, with a value of 10 corresponding to 0.1 μM of HTA. Because of absorption, fluorescence, and/or fluorescence quenching, individual reference solutions were prepared for each experiment containing the same concentrations of all components except hair (or keratin). Irradiated solutions of HA in the absence of hair or other additives did not give significant production of HTA.

RESULTS AND DISCUSSION

The intrinsic ESR signal for a variety of hair types, as well as for milled wool keratin, is shown in Figure 1. The pigmented black, brown, and red hair exhibits comparatively large signals (g = 2.004), due essentially to the presence of melanin. The broader and much weaker signals from blond, white, and bleached hair have a slightly lower g value and are similar to those of milled keratin. Figure 2 shows the buildup and decay of melanin signals from brown and red hair. The increase of the intrinsic melanin signal under irradiation for the red hair is smaller than that for the brown hair, a difference which we also noted with suspensions of extracted melanins (25).

Figure 3 demonstrates that when wool keratin is irradiated with UVA and visible light in the presence of terephthalate ion, HTA is formed, indicating that hydroxyl radicals are produced during this process. The production of HTA is quenched when ethanol or sodium azide is added to the reaction mixture. Both ethanol and sodium azide are hydroxyl radical scavengers, and thus compete with TA for the hydroxyl radicals produced when the keratin is irradiated. Human hair has a structure similar to that of wool (5).

When hair is irradiated in the presence of TA, HTA is also produced. Figure 4 compares the yield of HTA from different varieties of hair after irradiation for 90 minutes. As in our previous study (25), we observe a steady increase in HTA with increasing irradiation

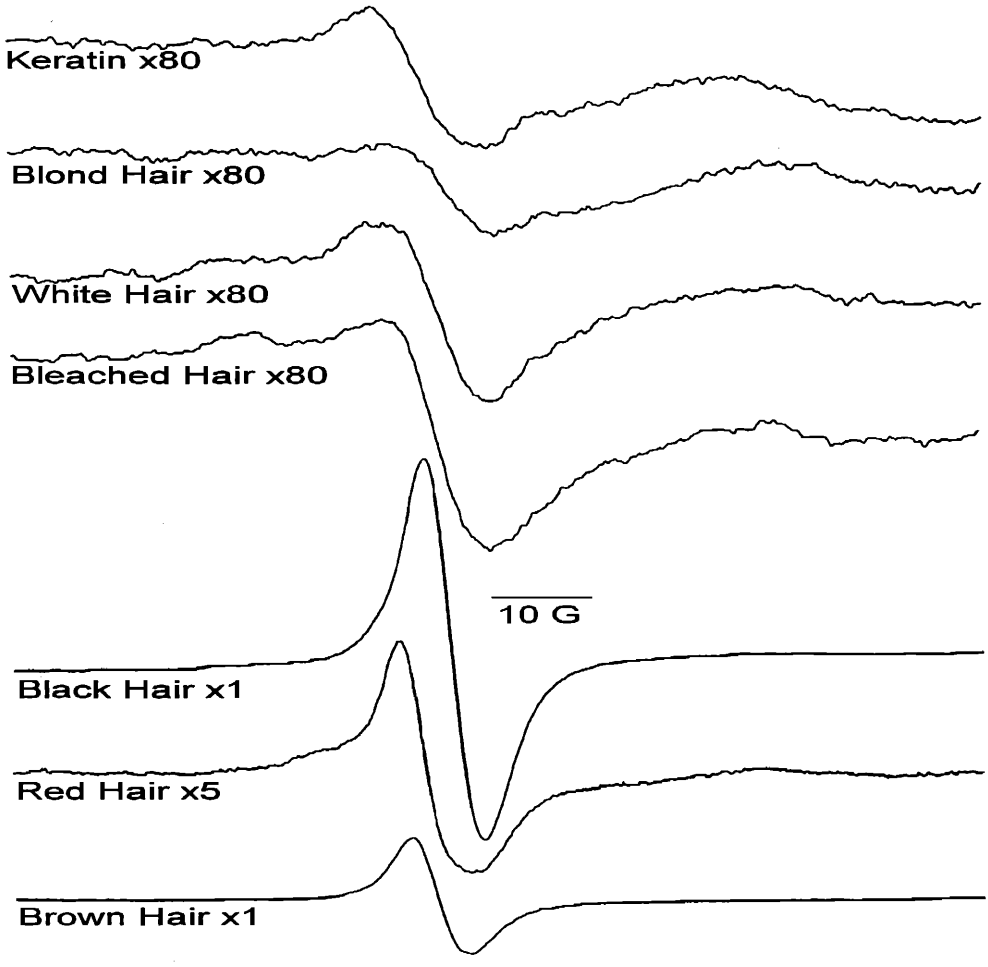


Figure 1. Intrinsic ESR signals for milled wool keratin and human hair. Spectrometer settings: modulation amplitude, 4G; receiver gain, 1.25×10^4; microwave power, 20 mw. Each was 0.03 g in a 2-mm I.D. quartz tube.

time. Figure 5 shows the effects of azide and ethanol on HTA production from irradiated bleached hair. ESR spectra are displayed in Figure 6 for the trapped radical adduct from bleached, red, and brown hair irradiated in the presence of DMPO. The spectra are characterized by hyperfine coupling constants of a_N = 14.9 G, a_H^γ = 14.9 G, showing that the radical is the DMPO-OH adduct (29,30), and confirming that oxyradicals are produced during irradiation. For both the fluorescence and the ESR experiments, bleached hair and red hair give much larger signals than brown hair.

When cinnamic acid is present, no DMPO-OH adduct is observed. Figure 7 gives the result from red hair; other hair types show similar results. Since at the wavelengths ($\lambda \geq 320$ nm) and concentrations of our experiments, cinnamic acid does not have a significant absorption, it seems also to be competing for oxyradicals. This is not unexpected given its aromatic ring and unsaturated side chain. (We note that parallel fluorescence measurements could not be performed because of high background fluorescence of cinnamic acid.)

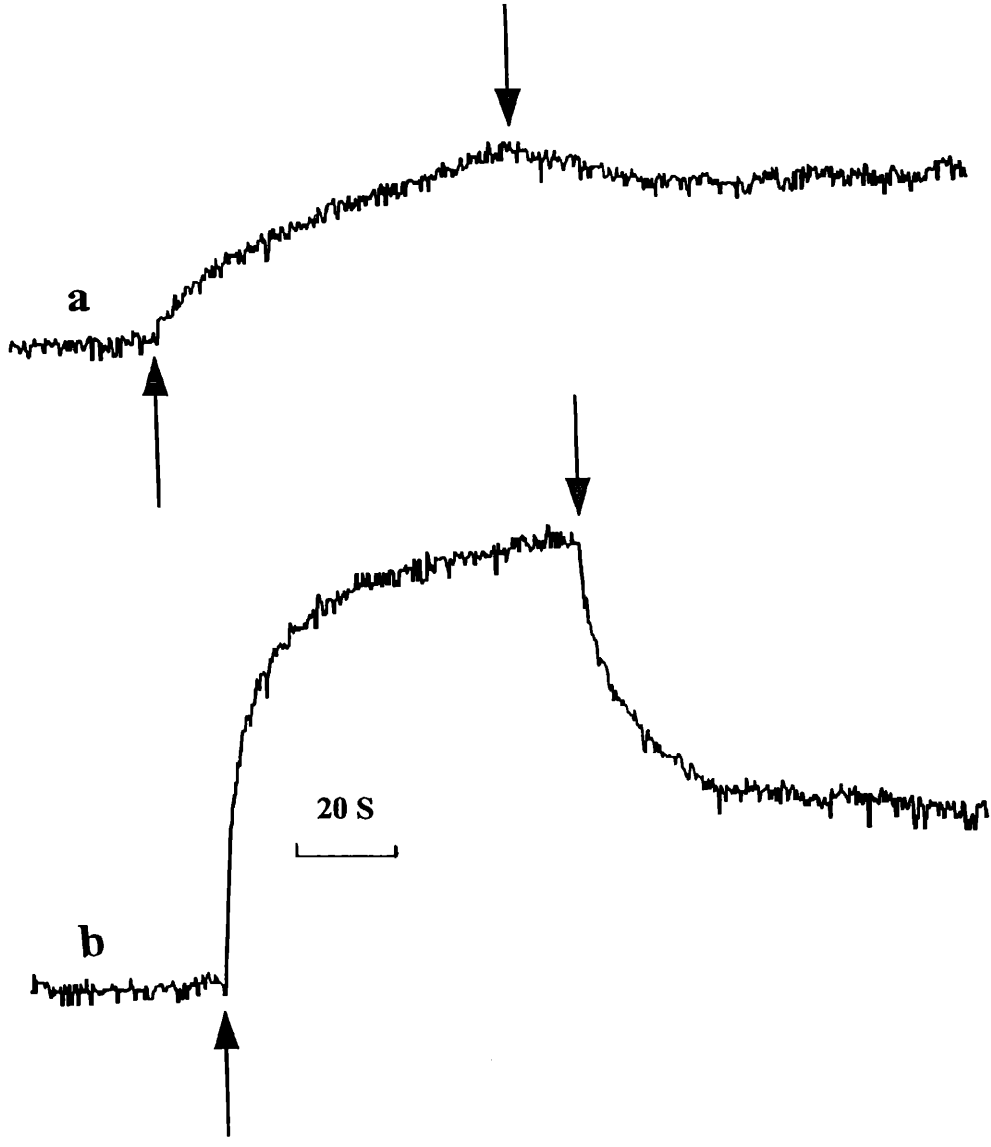


Figure 2. Kinetics of the buildup and decay of hair melanin radicals from human hair. a. Red hair. b. Brown hair. Arrows ↑ and ↓ indicate light on and light off. Other conditions as in Figure 1.

Eumelanin and pheomelanin respond differently to photoirradiation, and this difference is reflected in the melanins extracted from hair. Conclusions of earlier workers (31) that irradiated red hair melanin produces more oxyradicals than black hair melanin are consistent with our results using ESR spin trapping and HTA fluorescence on sepia and on melanin isolated from red and brown hair (25). These results parallel those of the present study for red and brown hair. As with oxyradical production, we also found that the intrinsic ESR signals of red and brown hair melanin respond differently to irradiation above 320 nm. However, in contrast to oxyradical production, stimulation of the intrinsic ESR signal for red hair or melanin isolated from red hair is *less* than that for brown

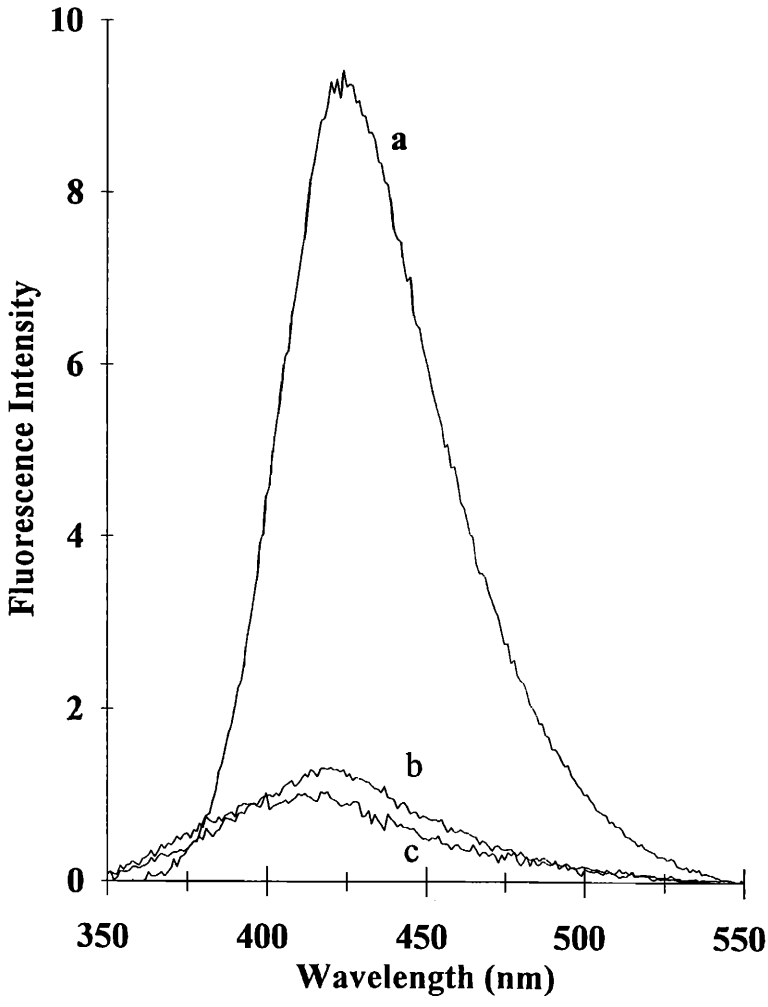


Figure 3. HTA produced from irradiated wool keratin: 0.1 g wool keratin powder in 2 mM TA phosphate buffer, pH 7.6 (phosphate buffer), irradiated for 30 min. a. No additives. b. Sodium azide added (10 mM). c. Ethanol added (1 M).

hair or melanin isolated from brown hair. The difference is illustrated in Figure 2 for the two types of hair.

Results of previous studies indicate that oxygen consumption rates are similar for pheo- and eumelanin (32,33). This result, coupled with the results of the intrinsic ESR signals, would seem to predict greater photoinduced oxyradical production from eumelanin than from pheomelanin, in contrast to the above observation that red hair and red hair melanin give a *higher* overall yield of oxyradicals. Thus, while the initial rate of *production* of oxyradicals may be similar, or even higher, for eumelanin, the fate of these radicals must be different for the two types of melanin/hair. These apparent differences in reactivity may be explained in terms of the different structures of the two types of melanin. Brown hair contains eumelanin that is oxidized enzymatically from tyrosine. Pheomelanin formation includes the cyclization and oxidative polymerization of 5-S-

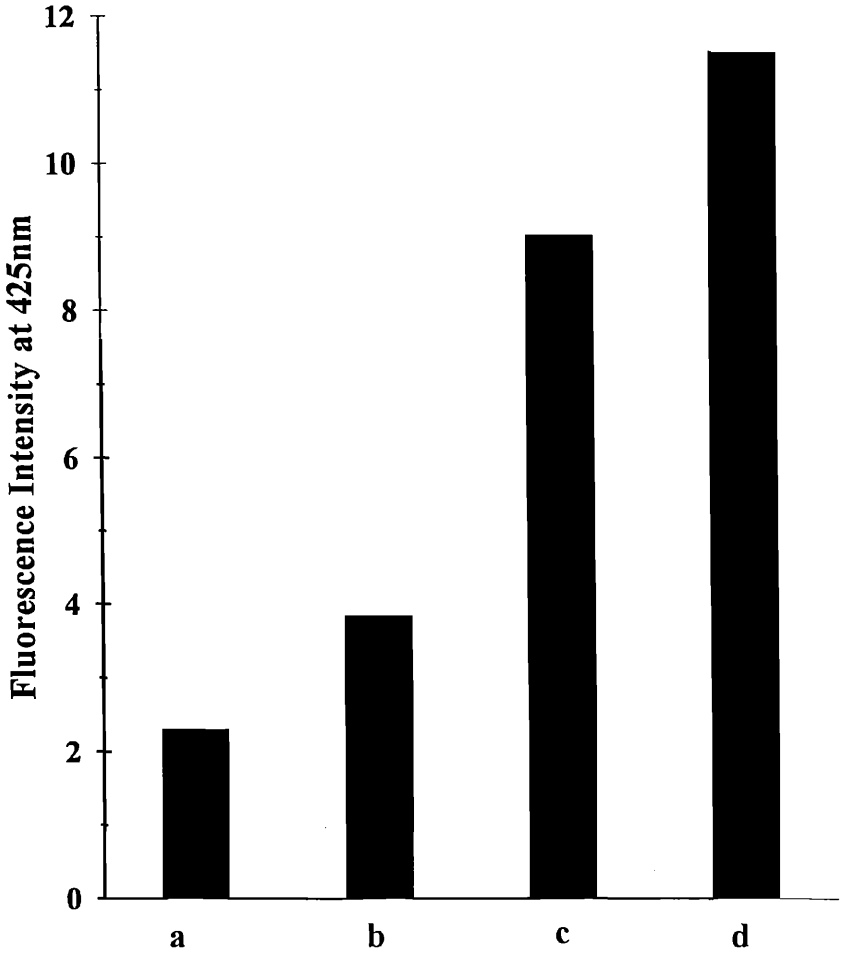


Figure 4. HTA production from human hair: 0.2 g hair (1-mm long) in 2 ml of 2 mM TA at pH 7.6, irradiated for 90 min. a. Brown hair. b. Blond hair. c. Red hair. d. Bleached hair.

cysteinyldopa and its isomers (present in smaller quantities) (34). It would appear from photobleaching studies that the sulfur-containing benzothiazine unit of pheomelanin is more resistant to oxidation than the indolic units of eumelanin (35). Therefore, eumelanin seems to be more efficient than pheomelanin as a scavenger of reduced oxygen species. Hence the amount of $O_2 \cdot^-$/OH· that is available to reach the spin traps (DMPO or TA) (Figures 4, 6; reference 25) is greater for red hair and red hair melanin. The greater ability to scavenge free radicals for eumelanin suggests that the eumelanin has a better photoprotective effect with regard to hair structure than pheomelanin. This is consistent with the fact that brown hair is more easily photobleached than either red (35) or black hair (36).

As shown from the experiments with wool keratin (Figure 3), the structural component of hair produces oxyradicals even in the absence of melanin. In addition to carbon- and oxygen-centered radicals, it has been reported that sulfur-centered radicals were also produced from irradiated wool keratin (17). This involves thiyl radicals, generated via

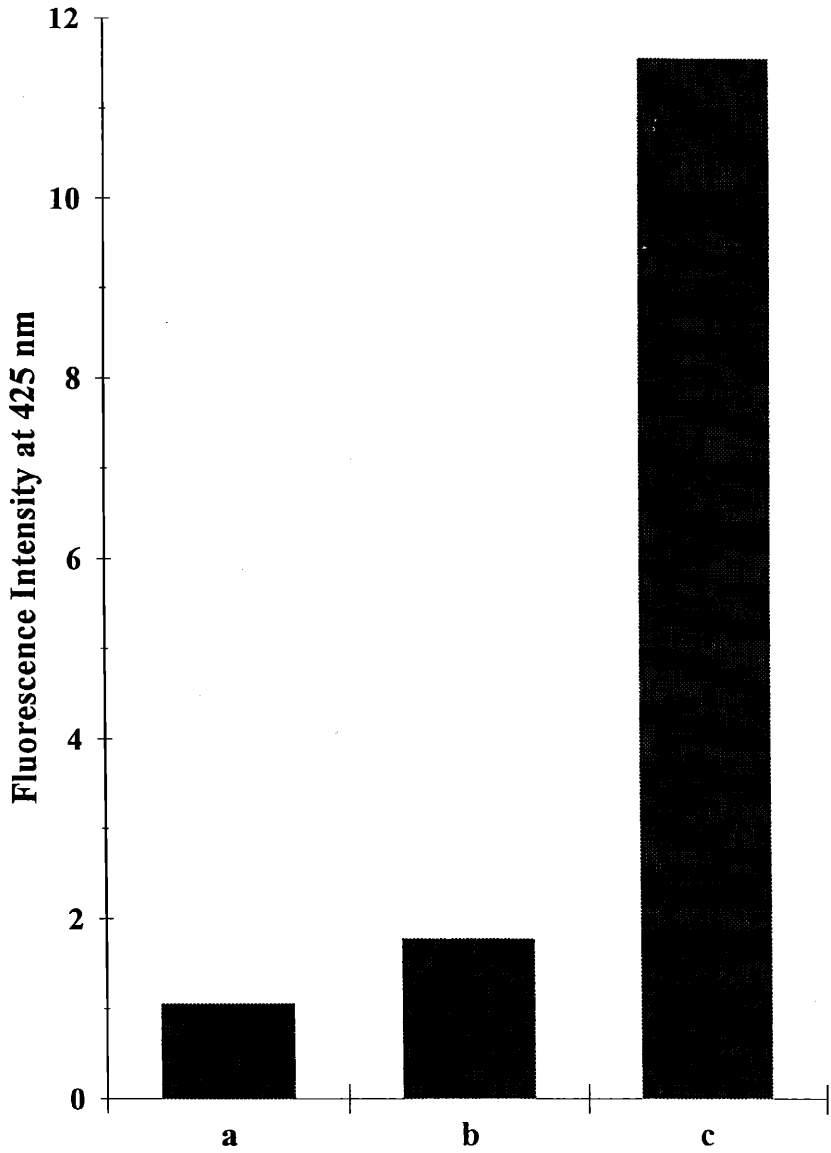


Figure 5. Effects of additives on HTA production from irradiated bleached hair: 0.2 g bleached hair (1-mm long) in 2 mM TA at pH 7.6, irradiated for 90 min. a. Ethanol added (1 M). b. Azide added (10 mM). c. No additives.

metal–sulfur complexes, which have considerable reactivity, and thus provide another pathway for oxyradical production (20,37). The sequence given in Scheme 2 has been suggested to explain the fate of thiyl radicals produced during the autoxidation of cysteine (38,39). In our spin trapping studies, irradiation was not carried out in the ESR cavity, and we find no evidence for the presence of transient sulfur radicals.

Among the free radicals involved in these reactions, superoxide, hydroxyl, and hydrogen peroxide are most cytotoxic species induced by ionizing radiation and photosensitized

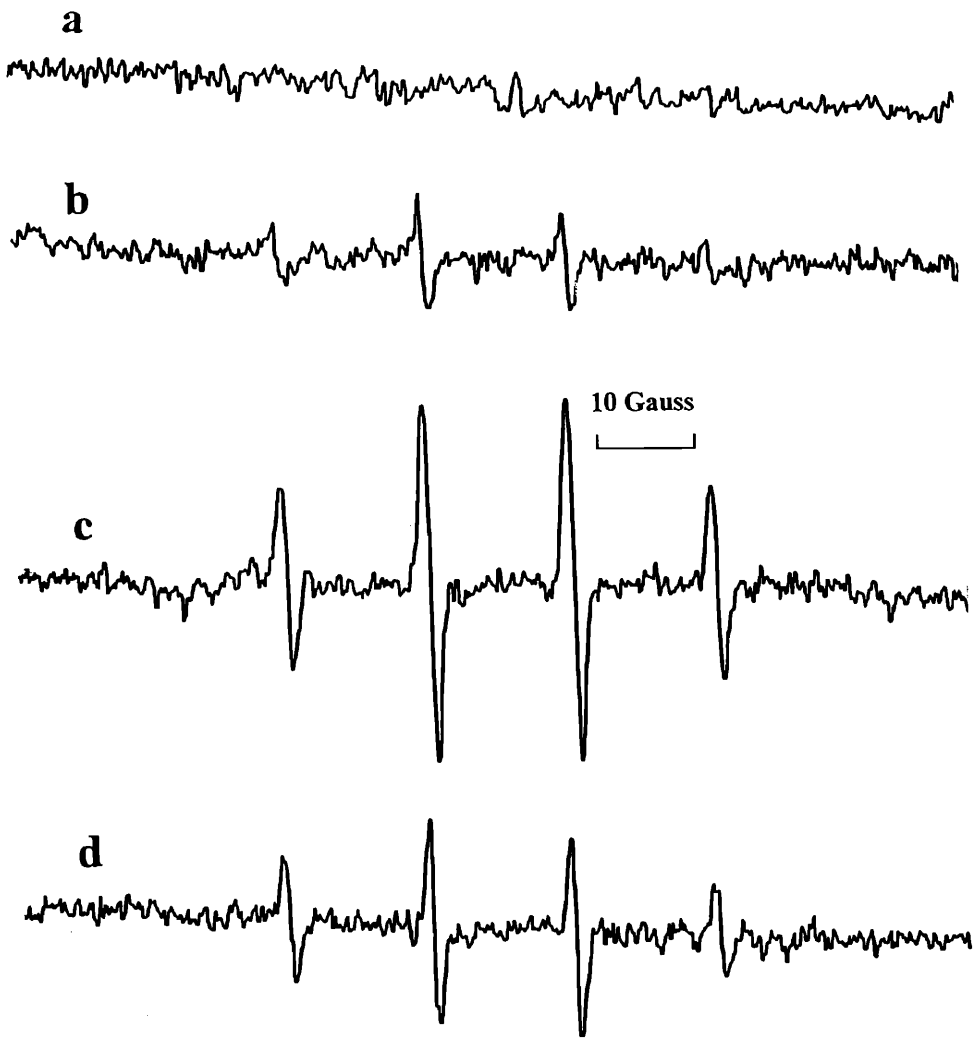


Figure 6. ESR spectrum of the DMPO-OH adduct from irradiated hair. A 0.015-g sample of hair was irradiated for 10 min in 1 ml 20 mM DMPO, after which the sample solution phase was transferred to the quartz flat cell. Spectrometer settings: microwave power, 20 mw; sweep width, 100 G; modulation amplitude, 1 G; receiver gain, 1.25×10^4; number of scans, 5. a. DMPO blank (no hair). b. Brown hair. c. Red hair. d. Bleached hair.

oxidations. Additional reactions result in the oxidation of amino acids, most notably tryptophan, within the hair matrix. Thus, photodamaged hair (and wool) is characterized by reduced tryptophan and increased cysteic acid (5,6,50,41). The reduced fiber strength of weathered hair appears to be the consequence of the scission of disulfide bonds and their conversion to cysteic acid residues. Tryptophan photodamage appears to precede disulfide photooxidation. In cysteine-containing protein such as keratin, the disulfide bond contributes strongly to the overall stability and integrity of the structure. With the destruction of disulfide bonds, the hair may lose its natural elasticity, become more porous, and, hence, become more sensitive to changes in humidity. Degradation of

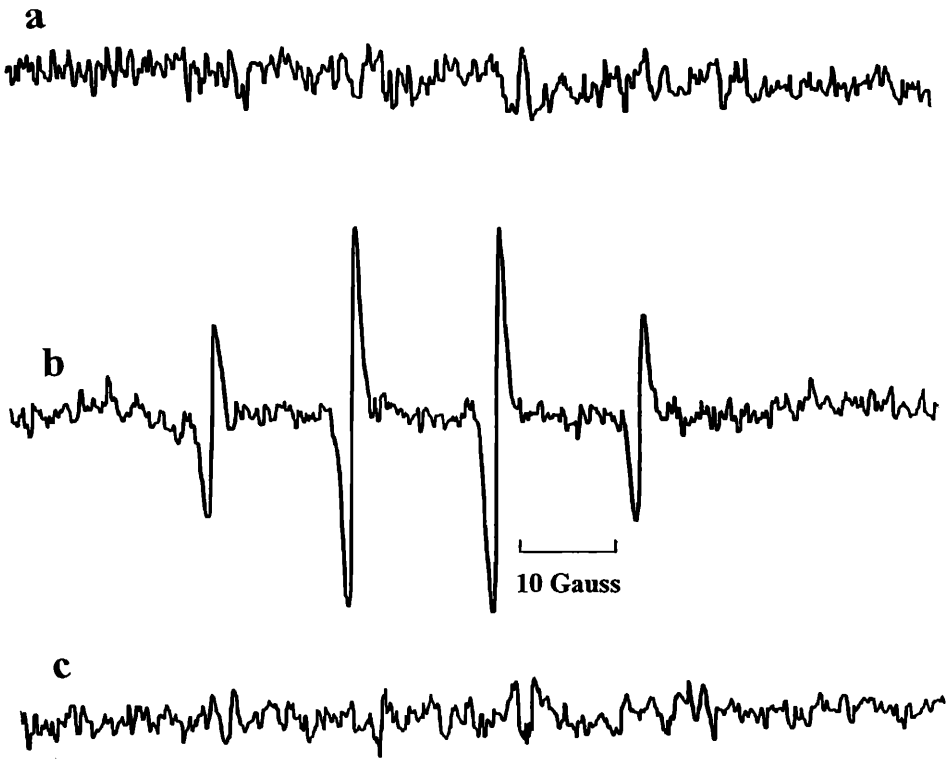


Figure 7. Effect of cinnamic acid on the DMPO adduct from irradiated red hair: 0.02 g red hair in 1 ml of 20 mM DMPO solution. The spectrometer settings are the same as in Figure 6. a. No irradiation. b. Irradiated for 10 min. c. 10 mM cinnamic acid added, irradiated for 10 min.

Propagation:

$$RS\cdot + O_2 \rightarrow RSOO\cdot$$

$$RSOO\cdot + RSH \rightarrow RSOOH + RS\cdot$$

$$RS\cdot + RSH \rightarrow RSSR\cdot^- + H^+$$

$$RSSR\cdot^- + O_2 \rightarrow RSSR + O_2\cdot^-$$

$$O_2 + RSH \rightarrow H_2O_2 + RS\cdot^-$$

Reduction of hydrogen peroxide:

$$RSSR\cdot^- + H_2O_2 \rightarrow RSSR + OH^- + OH$$

Termination:

$$2RS\cdot \rightarrow RSSR$$

$$RS\cdot + RSSR\cdot^- \rightarrow RSSR + RS^-$$

$$2O_2\cdot^- + 2H^+ \rightarrow H_2O_2 + O_2$$

Scheme 2

cystine residues also may cause the hair to lose some of its natural ability to absorb UV light.

Exposure to light induces the oxidative destruction of disulfide and phenolic side chains of the polypeptide chains. The light quanta induce the formation of free radicals that attack the disulfide bonds by chain reactions. Lengthy exposure to sunlight, especially in the summer or in southern regions where the exposure to sunlight is stronger and longer, can bring 20–40% weakening of the hair structure. Air- and water-borne pollution can also contribute to the oxidative damage of hair. In urban areas a great deal of polynuclear hydrocarbons and heavy metal oxide-containing pollutants are airborne and adsorb onto the hair surface. Many of these materials are light sensitizers capable of converting oxygen molecules into oxygen species. These attack and oxidize hair and degrade it (2).

A particularly interesting aspect of the present study is the effect of cinnamic acid. Cinnamates are an important class of sunscreen, perhaps most notably as octylmethoxycinnamate. Since our studies are carried out in aqueous media where octylmethoxycinnamate would not be soluble, we used cinnamic acid as a functional model for cinnamate sunscreens. The absence of a DMPO-OH signal for hair irradiated in the presence of buffered 10 mM cinnamic acid (Figure 7) is an indication that the additive either prevents the formation of oxyradicals ($O^{\bullet}_2{}^-$ or $OH^{\bullet}$) or competes effectively for them with the spin trap. Since, at the concentrations and wavelengths used here, cinnamic acid has no significant absorbance, the mode of action in quenching the oxyradical signal is not simply by blocking or absorbing light. An alternative mode of action for cinnamic acid is scavenging $HO^{\bullet}$. Other aromatic hydrocarbons such as benzoic acid are used as $HO^{\bullet}$ scavengers due to their ability to compete for $HO^{\bullet}$ via aromatic hydroxylation. This observation may have significant implications for the use and development of sunscreens. Sunscreens used in skin care are not always desirable in hair care since they tend to be lipophilic and, as such, impart an undesirable oiliness to the hair. Furthermore, strategies in developing sunscreens for skin care may not yield materials optimal for hair care since different mechanisms may be operative and/or have different levels of importance in one versus the other. For example, the reaction of skin to UV-Vis radiation involves immunological responses not possible for hair.

CONCLUSION

Reduced oxygen radicals have been postulated during photoirradiation of hair, and the results of our studies provide direct evidence for the production of oxyradicals from human hair under UVA-visible irradiation. In addition, we have shown the value of combining direct ESR measurements and ESR spin trapping with a novel fluorescent probe for hydroxyl radical production. Terephthalate ion proves to be a convenient probe for the study of the production of hydroxyl radicals from irradiated samples of melanin and human hair, with potential utility in other areas. While we must emphasize the need for carefully prepared blank experiments, the relatively high excitation wavelength for HTA allows the presence of a variety of additives, including the radical scavengers used in this study and in our experiments with melanin (25).

Our results indicate that bleached and red hair give a greater net yield of hydroxyl radical than brown hair under irradiation, reflecting the role of eumelanin in darker hair

as a more effective radical scavenger. Various other factors, including porosity, metal ions and melanin content, can influence the extent of oxygen radical production and photodegradation of hair. Eumelanin and pheomelanin differ in composition, and regardless of type, the amount varies between individuals in the range of 1–4%. Small amounts of metals are endogenous to hair but are also impacted by exogenous factors such as water hardness. Porosity is mainly a function of environmental stress, such as combing, light, chemical treatment, etc. Our studies demonstrate convenient methods for assessing oxygen radical production in hair and, in combination with other methods, provide the potential to correlate hair damage with oxygen radical production under the influence of various factors.

ACKNOWLEDGMENTS

Financial support from Zotos Corporation is acknowledged. The authors thank Drs. P. and A. Rieger of Brown University for providing the ESR spectrometer and for their helpful suggestions.

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