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Photodestruction of pheomelanin: Role of oxygen

(superoxide/skin cancer)

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ABSTRACT **Pheomelanin, the red-brown polymeric pigment in the skin and hair of red-headed humans, is composed of a protein fraction covalently bound to a colored chromophore. Photolysis of aerated aqueous pheomelanin solutions, isolated from human red hair, results in destruction of the chromophore and liberation of the peptide fraction. The rate of photolysis depends on the pH and the concentration of both pigment and oxygen and is slightly inhibited by the enzyme superoxide dismutase (superoxide:superoxide oxidoreductase EC 1.15.1.1). Pheomelanin photolyzed in the presence of nitroblue tetrazolium results in the formation of a blue diformazan, whether or not oxygen is present. Superoxide dismutase inhibits the aerobic photoreduction of nitroblue tetrazolium whereas, in the absence of oxygen, no inhibition is observed. These experiments strongly suggest the involvement of superoxide in the aerobic photolysis of pheomelanin and point out a possible mechanism for ultraviolet-induced cell damage in redheads.**

The black or brown eumelanins of human skin afford protection against the damaging effects of the ultraviolet component in sunlight (1–4). Light of these wavelengths, 280–380 nm, is nondestructive to eumelanin and produces reversible changes that are believed to function as mechanisms in this pigment's photoprotective ability. Two such reversible reactions are (*i*) immediate pigment darkening (3, 5, 6), an apparent oxidation-reduction reaction resulting in the darkening of preformed pigment, and (*ii*) an increase in the number and a change in the nature of the unpaired electrons in the pigment (7–9).

Fair-skinned humans exhibit a number of abnormal reactions to sunlight, including freckling (10) and a high susceptibility to skin cancer (11–14). These have usually been attributed to the fact that the skin of these people has a poor tanning capacity, sunburns readily, and contains little pigment. Although pheomelanin, the red-brown or yellow pigment found in the hair of fair-skinned humans (15, 16), has yet to be isolated from human skin, there is an increasing amount of indirect evidence that it occurs in melanosomes found in various parts of the body, including the skin (17–22). Pheomelanin is readily photodegraded under physiologically relevant conditions (23, 24), an observation that has led us to suggest the following four mechanisms by which ultraviolet light may deleteriously affect cells containing this pigment: (*i*) loss of a pigment purportedly responsible for photoprotection; (*ii*) formation of dermatitic or carcinogenic photoproducts; (*iii*) formation of dermatitic or carcinogenic compounds by reactions of photochemically produced intermediates with normal cell constituents; and (*iv*) formation of photoproducts that inhibit the enzymatic systems responsible for repair of ultraviolet-induced damage. In this paper we support the viability of mechanism *iii* by presenting evidence that a highly reactive intermediate is formed during the course of the photolysis.

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Oxygen has been shown to affect the course and rate of the photolysis of pheomelanin (23, 24). In this work we focus on the role and biological significance of oxygen in the photodegradation of this pigment. Our results support formation of the superoxide anion, O_2^-, as the reactive oxygen species initially produced during the photolysis.

MATERIALS AND METHODS

Chemicals. Human hair, judged to be red by at least four individuals, was collected from beauty salons, barber shops, and individuals in the Columbus, OH, area. Superoxide dismutase (superoxide:superoxide oxidoreductase, EC 1.15.1.1) from bovine blood (activity, 2500 units/mg of solid: lot 107C-8100), protease from *Streptomyces griseus* (type VI, activity 4 units/mg of solid; lot 114C-0168), and 3,3′-(3,3′-dimethoxy[1,1′-biphenyl]-4,4′-diyl)bis[2,5-diphenyl-2H-tetrazolium] dichloride (nitroblue tetrazolium), grade III, were purchased from Sigma. Rose Bengal (C.I. no. 45440) was purchased from Matheson, Coleman and Bell. Hydrogen peroxide (30%, lot 753872) was purchased from Fischer. All other chemicals used were reagent grade.

Pheomelanin. Human red hair was digested in 1 M NaOH (600 g/4 liters) for 24 hr at room temperature. The digest was centrifuged and filtered thru diatomaceous earth to remove particulate matter. The pH of the filtrate was adjusted to 1 by addition of concentrated HCl, and the dark-brown precipitate was collected by centrifugation (Sorvall model GLC-1, 6000 rpm, 10 min). This crude pigment was dissolved in the minimal amount of 0.17 M Tris buffer (pH 8.8) and subjected to gel filtration (4 ml at a time on 100 × 2.5 cm columns packed with Bio-Gel P-6 hydrated in 0.17 M Tris buffer at pH 8.8). The first dark-brown fraction to be eluted from each run was pooled and concentrated, and the pigment was precipitated by adjusting the pH to 1 with HCl. Tris was removed from the precipitate by washing several times with 1 M HCl. The precipitate was stored at 10°C as a slurry in 1 mM HCl.

A stock solution of this pigment was prepared by dissolving the solid in 0.2 M phosphate buffer (pH 7.5). Aliquots of this solution were taken and adjusted to the desired concentrations and pH (Corning model 7 pH meter). Concentrations normally used in the photolyses, unless otherwise specified, averaged between 0.40 and 0.50 mg (dry weight) per ml of buffer.

Amino Acid Analyses. Amino acid composition of pheomelanin was determined by using a Beckman model 116 amino acid analyzer [modified to use a Durrum DC-6A resin (Pierce)] after a 24-hr hydrolysis in 6 M HCl (25). The amino acid composition of the photolysate was determined by lyophilization of the photolysate and hydrolysis with acid as above.

Photolyses. A Hanovia 450-W medium-pressure mercury lamp placed in a water-cooled quartz immersion well (Ace Glass) was used for all photolyses. Aqueous solutions of

pheomelanin in Pyrex test tubes were irradiated in a "merry-go-round" photochemical reactor (Rayonet model MGR 500) and monitored with a spectrophotometer (Perkin–Elmer Hitachi model 200). Test tubes were standarized by placing pigment in Pyrex tubes, photolyzing for 80 min, averaging the net change in absorbance at 400 nm, and eliminating all tubes that varied from the average by more than 0.003 absorbance unit. A Spectronic 20 (Bausch and Lomb) was used to follow the nitroblue tetrazolium experiments.

For most photolyses, air-equilibrated solutions in open test tubes were used. The oxygen-free system was obtained by the cyclic freeze/thaw method (six or more cycles). The test tubes were attached to cuvettes and sealed after the final degassing. Oxygen-saturated solutions were prepared by degassing as above, followed by the addition of oxygen at 1 (1 atm = 1.013 $\times 10^5$ Pa) to each test tube and sealing.

Unless otherwise noted, all photolyses were performed in 0.2 M phosphate buffer. The reference test tubes contained air-equilibrated pheomelanin and 0.2 M phosphate buffer (and nitroblue tetrazolium when applicable) of the same concentrations and pHs as used in the experimental test tubes and were photolyzed along with the experimental test tubes. The following experiments were performed.

(*i*) Air-equilibrated, phosphate-buffered pheomelanin solutions with pHs of 6.5, 7.5, 8.5, 9.0, and 10.0 were photolyzed in open test tubes.

(*ii*) Air-equilibrated, phosphate-buffered (pH 8.0) solutions containing 0.77, 0.49, 0.20, 0.10, or 0.06 mg of pheomelanin per ml of buffer were prepared by diluting aliquots of the stock solution and photolyzed in open test tubes.

(*iii*) Phosphate-buffered (pH 7.5) solutions of pheomelanin were photolyzed in the absence of oxygen, in the presence of air at 1 atm and in the presence of oxygen at 1 atm.

(*iv*) Fresh solutions of Rose Bengal were prepared in buffer (pH 8.0). Aliquots of these and pheomelanin were placed in volumetric flasks and diluted with buffer (pH 8.0) to give solutions that were 0.1, 1.0, and 10 μM in Rose Bengal. These were placed in open test tubes and photolyzed.

(*v*) Superoxide dismutase [activity checked by the standard assay of Beauchamp and Fridovich (26)] was mixed with aliquots of pigment and diluted with pH 7.6 buffer to give a final concentration of 0.62 mg of enzyme per ml of buffer. These solutions were aerated, transferred to open test tubes, and photolyzed together with a reference.

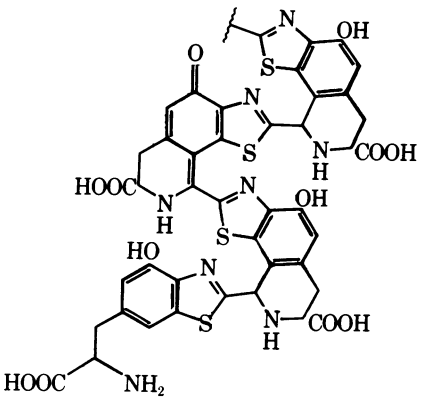
(*vi*) A buffered (pH 7.3) solution containing pigment, nitroblue tetrazolium (64 μM) and superoxide dismutase (1.6 μg/ml) was photolyzed in test tubes under an atmosphere of oxygen along with a reference.

(*vii*) Solutions of hydrogen peroxide were mixed with aliquots of pigment and diluted with pH 7.3 buffer to give solutions 0.08 M in peroxide. Half of these solutions were aerated and then photolyzed in open test tubes together with a reference; the other half were stored in the dark at room temperature and monitored at the same time as the photolyzed solutions.

(*viii*) Solutions of various pheomelanin concentrations (pH 7.3) were prepared. A 3-ml sample of each of these solutions was placed in the electrode chamber of a Yellow Springs Instrument model 5331 oxygen probe, and a Clark type polarographic electrode was inserted. The solutions were equilibrated for 3 min and the probe drift was recorded on a Sargent Welch recorder (model SRG) for 5 min prior to photolysis. Oxygen uptake was likewise recorded during photolysis (a Pyrex-filtered, 100-W high-pressure xenon arc, placed 11 cm from the electrode chamber, was used).

RESULTS AND DISCUSSION

Pheomelanin, as isolated, is a dark-brown solid soluble in aqueous alkali, trifluoroacetic acid, formic acid, and several highly polar organic solvents. This pigment is composed of a polymeric backbone covalently bound to a protein fraction. Structure 1 has been proposed for the polymeric chromophore of pheomelanin (15). The Lowry method (27) was used to demonstrate that the pigment is 87% protein by weight. The protein-free chromophore is easily prepared by treatment of the pigment with protease followed by chromatography on Bio-Gel P-30. This procedure affords a dark-brown alkali-soluble pigment that is more highly colored than the pheomelanin itself ($E^{1\,cm}_{1\%} = 2.9 \times 10^{-3}$ vs. $E^{1\,cm}_{1\%} = 5.5\ 10^{-4}$ at 400 nm). All photolyses reported in this paper were performed on the protein–chromophore complex herein referred to as pheomelanin.



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Aerated aqueous solutions of pheomelanin rapidly lost their color when irradiated by ultraviolet light of wavelengths longer than 300 nm (Fig. 1) but were stable to the action of visible light. Amino acid analyses of irradiated pheomelanin solutions indicated little change in the relative abundances of the various amino acids compared to unphotolyzed pheomelanin (Table 1). This indicates that most of the photochemistry apparently involves destruction of the chromophore. Pheomelanin solutions exhibited a photoreaction even in the absence of oxygen; however, the rate of pheomelanin destruction was slower than

Table 1. Amino acid composition of pheomelanin

Amino acid	Relative abundance, % Prior to photolysis	After photolysis
Asp + Asn	11.82	11.20
Thr	3.79	3.96
Ser	5.44	5.19
Glu + Gln	19.77	17.89
Pro	6.68	5.60
Gly	3.41	4.12
Ala	7.40	7.79
Val	7.01	7.90
Met	0.31	0.59
Ile	4.57	4.91
Leu	13.97	14.39
Tyr	1.97	1.89
Phe	4.14	6.44
Lys	2.68	2.25
His	0.98	0.37
Arg	6.03	5.49
Total	99.97	99.98

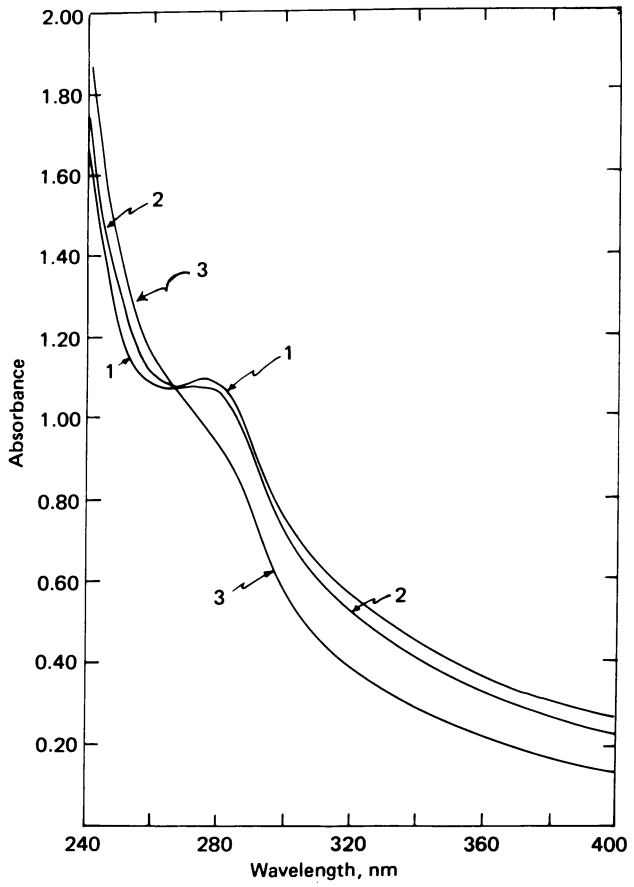


FIG. 1. Photolysis of pheomelanin (0.43 mg/ml in pH 8.0 phosphate buffer; air-equilibrated in Pyrex test tubes). Spectra 1–3 correspond to photolysis times of 0, 158, and 1113 min, respectively.

in the case of aerated photolyses. In the absence of oxygen, no isobestic point was observed at 265 nm during the photolysis; instead, a uniform decrease in absorbance at all wavelengths was observed. The rate of photolysis, conveniently monitored by the loss of absorbance at 400 nm, was found to be dependent on pH and the initial concentration of both pigment and oxygen.

In order to ascertain the role of oxygen in the photoreaction, we arbitrarily chose to run the photolyses at pHs between 7.3 and 8.0 (Fig. 2). At these pHs, and with pigment concentrations in excess of 2.0 mg/ml, the loss of oxygen was observed to be pseudo-first order (Fig. 3). We believe that this result, along with the pigment concentration dependence, points to a type II photooxidation (scheme 1) involving either transfer of excitation from the pigment to oxygen, producing an electronically excited singlet state of oxygen, $^1O_2$, or transfer of an electron from the excited pigment to oxygen to give the superoxide anion, $O_2^{\cdot-}$ (28). The possibility of oxygen addition to an electronically excited state of the pigment (e.g., Pig* + O_2 → $PigO_2$*) was considered a viable alternative but was discarded when the facile photoreduction of oxygen was demonstrated (see below).

$$\text{Pig} \xrightarrow{h\nu} {}^1\text{Pig}$$

$${}^1\text{Pig} \longrightarrow {}^3\text{Pig}$$

$${}^3\text{Pig} + O_2 \longrightarrow {}^1O_2 + \text{Pig}$$

$${}^3\text{Pig} + O_2 \longrightarrow O_2^{\cdot-} + \text{Pig}^{\cdot+}$$

Scheme 1

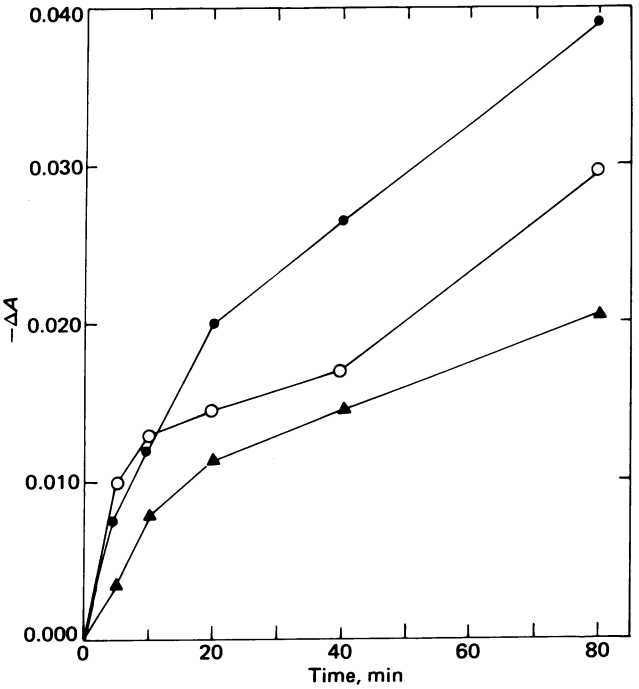
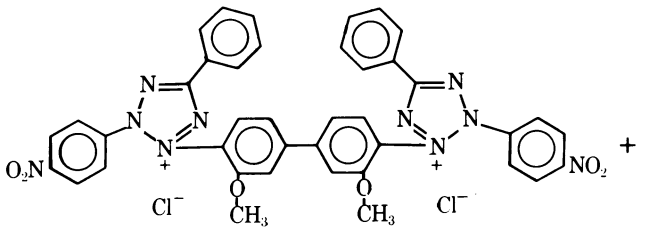


FIG. 2. Photolysis of pheomelanin (0.49 mg/ml in 0.2 M phosphate buffer) at pH 6.2 (▲), pH 8.0 (○), and pH 10.0 (●). The rate of photolysis is measured as $-\Delta A$ at 400 nm.

Although we have been able to demonstrate that known singlet oxygen sensitizers such as Rose Bengal (29) accelerate the photodestruction, numerous other mechanistic tests for the involvement of singlet oxygen have been inconclusive.

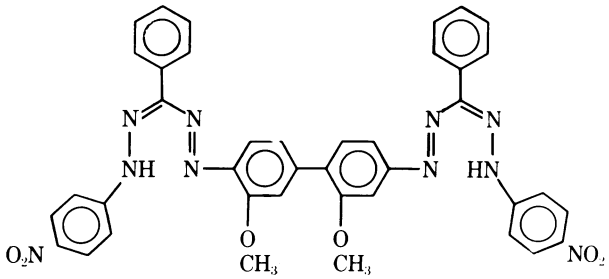
The reaction of superoxide with nitroblue tetrazolium, **2**, results in the formation of a highly colored diformazan, **3** (26).



nitroblue tetrazolium

2

$$4O_2^{\cdot-} + 2H_2O \longrightarrow 2HO^- + 4O_2 + 2Cl^-$$



Diformazan

3

This reaction is conveniently monitored spectrophotometrically by the increase in absorbance at 560 nm. Photolysis of oxygenated solutions of pheomelanin and **2** resulted in the rapid formation of **3**. This reaction was quenched by superoxide dismutase [maximum of 71% inhibition by 16 μg of enzyme per ml of buffer (Fig. 4)].

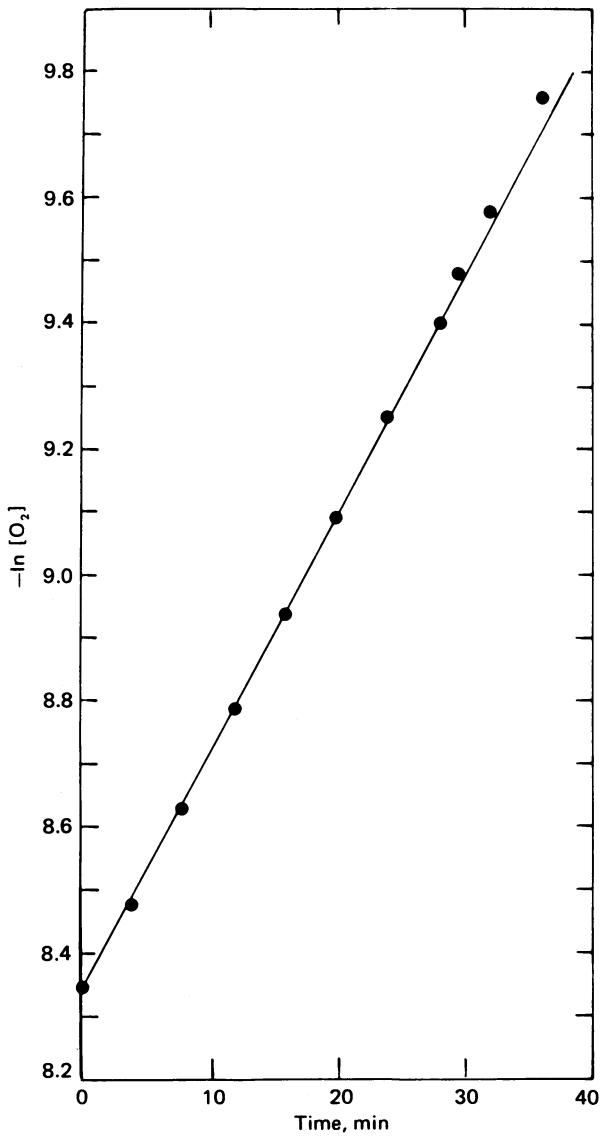


FIG. 3. Oxygen uptake during photolysis of pheomelanin (2.80 mg/ml in pH 7.3 phosphate buffer).

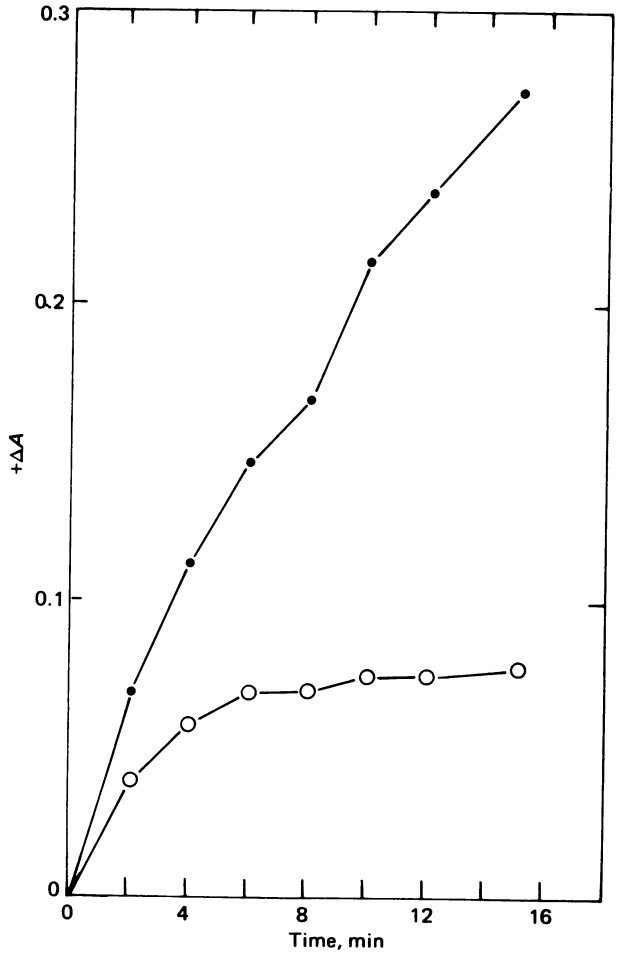


FIG. 4. Nitroblue tetrazolium reduction in the presence (○) and absence (●) of superoxide dismutase. Conditions: pheomelanin, 0.50 mg/ml; nitroblue tetrazolium, 64 μM; superoxide dismutase, 1.6 μg/ml. Both solutions were air-equilibrated and phosphate-buffered (pH 7.3).

The photolysis of degassed solutions of pheomelanin and **2** also resulted in the production of **3**; however, this reaction was not quenched by superoxide dismutase. We suggest that the initial steps in the photodegradation of pheomelanin involve the photochemically mediated transfer of an electron from the pigment to oxygen as outlined in scheme 1.

Production of superoxide *per se* does not account for the destruction of the pheomelanin chromophore. Superoxide has been reported to exhibit nucleophilic reactivity (30), to behave as a one-electron reducing agent (31), and to behave as a one-electron oxidizing agent (32). Serious questions have been raised as to the ability of superoxide to behave as an oxidizing agent (33), and it is unlikely that a process that results in the overall reversible transfer of an electron would cause the destruction of the pigment's chromophore. Furthermore, this type of process would be catalytic in oxygen, a situation contrary to our studies on the effect of oxygen concentration on the rate of photolysis. Thus, of the three types of direct superoxide–pigment interaction, the most likely is direct nucleophilic attack of the quinoid units, ultimately leading to hydroperoxides. The reaction of superoxide with pheomelanin does not effectively compete with the reaction of superoxide with nitroblue tetrazolium. Thus, if there is a direct reaction of pheomelanin with superoxide it is not fast enough to prevent superoxide from escaping to the free solution. Because the photolysis is only minimally inhibited by superoxide dismutase, direct nucleophilic attack of superoxide on pheomelanin cannot be a major pathway leading to pigment destruction.

The dismutation of superoxide, both catalyzed and uncatalyzed, results in the formation of hydrogen peroxide via the following reaction:

$$2O_2^{\cdot-} + 2H_2O \rightarrow O_2 + H_2O_2 + 2HO^-$$

Our results indicate that there is both a dark and a light reaction between hydrogen peroxide and pheomelanin. Irradiation of solutions of peroxide and pigment resulted in the rapid loss of the chromophore. Under the continuous photolytic conditions of our experiments, it seems unlikely that substantial quantities of hydrogen peroxide survive long enough to attack the pigment in a dark reaction. We suggest that the hydroxyl radical, HO·, produced by

$$Fe^{3+} + O_2^{\cdot-} \rightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + HO\cdot$$

is the species responsible for the initial attack on the chromophore. The reagent grade chemicals used to prepare the buffers would afford sufficient concentration of iron(III), typically between 0.1 and 1 μM, for this sequence.

In conclusion, evidence has been presented that oxygen reacts with photochemically excited pheomelanin by means of an electron transfer affording the superoxide anion. Although many other aspects of the photolysis are still unknown (e.g., involvement of singlet oxygen, the mechanism of pigment destruction and the possible involvement of hydroxyl radicals), the identification of superoxide as a primary photoproduct has serious implications. Superoxide has been suggested to cause cleavage of DNA (34, 35), depolymerization of acid polysaccharides (36, 37), and peroxidation of unsaturated lipids (38–40). The conclusion from our experiments, that superoxide is produced in a primary photochemical event during the irradiation of pheomelanin, raises the question of the possible role of superoxide in ultraviolet-induced skin cancer and points out the importance of understanding its reactions with biological molecules.

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