ELECTRON SPIN RESONANCE STUDY OF MELANIN TREATED WITH REDUCING AGENTS

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ABSTRACT The electron spin resonances (ESR) of several native and modified melanins have been determined. Melanins isolated from black wool and synthesized from 3,4-dihydroxy-L-phenylalanine (L-DOPA) and tyrosine all show similar ESR signals. Modification of the isolated melanins by treatment with reducing agents causes some lightening in color and slight changes in the ESR spectra. Lithium and liquid ammonia (Birch) reduction applied to melanins from wool and L-DOPA gave very different results, as reflected by ESR spectra, but in both cases the changes were much greater than those produced by other treatments. In general, reductive treatments in nonaqueous media in the presence of metals increase the free radical content and line width, whereas treatment in aqueous media resulted in decreased free radical content. These observations are consistent with a melanin pigment which is an irregular polymer and has unpaired electrons localized on different but similar monomer units.

INTRODUCTION

The black and brown colorations found so widespread in nature are attributable to a class of pigments known as melanins. Our interest in melanins derives from work with wool. Even sheep bred to give white fleeces often have small amounts of pigmented hairs that decrease the fleece value. Removal of the black hair by most bleaching techniques is often only partly successful and is likely to damage the wool severely. Better understanding of the chemical properties of melanins should lead to better methods of removing the color. However, characterization of melanins has been exceptionally difficult because of the intractability of the pigments towards usual physical and chemical probes. No melanin, natural or formed in vitro, has yet been isolated and fully characterized.

One promising clue to the nature of melanins is the presence of an endogenous free radical, which can be detected by electron spin resonance (1). ESR spectra of several natural and synthetic melanins have been shown to be strikingly similar with respect to line width, line shape, and g-value (2). The source of these signals is inferred to be free radicals trapped within the matrix of the melanin. However, the signals characteristically lack hyperfine structure, so that details of composition, structure,

and environment of the radicals cannot be determined precisely. This lack of detail, coupled with the dark color of the pigment suggests that the signal might result from delocalization of the unpaired electron throughout a conjugated polymer system.

We have subjected the melanin pigment obtained from black wool to several homogenous reducing systems in an attempt to disrupt its conjugation and render the ESR signal more informative.

MATERIALS AND METHODS

Isolation of Melanins from Wool

By Hydrolysis. Melanin was isolated from black wool fibers by digesting the keratin either with 6 N HCl or with a solution of phenol and thioglycollic acid (PHT) according to the method of Green and Happey (3). A typical run in which 13 g wool in 500 ml 6 N HCl was refluxed for 4 h yielded 0.52 g pigment. The highly hygroscopic black pigment was dried over phosphorus pentoxide under vacuum for 72 h. (Found: C, 56.40; H, 3.60; N, 10.30; S, 1.65; no ash.)

By Enzyme Digestion. The enzymatic solution was prepared by dissolving 2 ml mercaptoethanol, 36 g urea, and 31.6 mg papain in 200 ml distilled water in a glass stoppered flask (4). Black wool (2 g) was added to the solution and the stoppered flask placed into a 50°C oven. In 4 h, the wool was completely solubilized. Another 15.8 mg of the enzyme was added and the stoppered solution allowed to stand another 48 h. The pigment obtained included a small amount of fibrous material that was removed by hand.

Synthesis of Melanin

Melanin was formed by autoxidation of L-DOPA and by enzymatic action on L-DOPA and L-tyrosine according to the procedures described by Blois et al. (2). The enzyme was a commercial preparation of mushroom tyrosinase (Worthington Biochemical Corp., Freehold, N.J.). The enzymatic solution contained 30 mg enzyme in 100 ml of Sorensen's buffer pH 7. This solution (15 ml) was added to 150 mg L-DOPA in 500 ml pH 7 buffer or to 500 ml buffer saturation with tyrosine. After 2 wk, the black pigment which precipitated was removed by filtration, washed with distilled water, and dried over phosphorus pentoxide.

Physical Properties

The isolated pigments were tested for solubility in the following solvents: dimethyl formamide (DMF), formic acid, dimethyl sulfoxide (DMSO), 2,2,2-trifluoroethanol, nitrobenzene, hexafluoroacetone, hexamethylphosphotriamide, 6 M sodium hydroxide, acetic acid, concentrated sulfuric acid, and pyridine. These liquids, which range from slightly polar to extremely polar and from non-hydrogen bonding to strongly hydrogen bonding, did not dissolve the pigments. It can be noted that solvents such as DMSO and DMF which are good swelling agents for wool keratin had no effect on the isolated melanin pigment. However, the pigment could be suspended in aqueous acid solvents such as formic and sulfuric acids. In no case did chemical treatment increase the solubility of the pigment.

The pigments exhibited generalized absorption in both the ultraviolet (dispersed in formic acid) and in the infrared (KBr pellet). Raman spectra, too, showed no useful absorption peaks.

Chemical Modifications

Reduction of the pigment obtained from wool by hydrolysis in 6 N HCl was attempted in several homogenous reducing systems. Each reducing agent used in this study has been demon-

strated to be capable of reducing the indole nucleus which is purported to be a major constituent of the melanin pigment. A sample of wool melanin was oxidized by hydrogen peroxide for comparison of the ESR signal.

Birch Reduction (5). Melanin (50 mg), 2 g lithium metal, and 150 ml of liquid ammonia were stirred for 2 h, then allowed to evaporate overnight. The isolated "reduced" pigment was light brown in color compared with the original black.

Borohydride/Aluminum Chloride Reduction (6). Anhydrous aluminum chloride (6 g) in 100 ml of diglyme was added dropwise with stirring to a mixture of melanin (25 mg) and sodium borohydride (5 g) in 200 ml diglyme. The mixture was heated to 90° and maintained at that temperature for 1 h. After cooling, the "reduced" melanin was isolated by filtration, washed with water, and dried as before. Though still quite dark, the pigment had a brownish tinge.

Borohydride/Ferric Chloride Reduction. The reaction was run as above but with ferric chloride substituted for aluminum chloride. The "reduced" product appeared identical to that obtained with aluminum chloride.

Ascorbic Acid (7). Melanin (50 mg) was refluxed in 25 ml 2 M ascorbic acid overnight. The product was isolated by filtration, washed and dried. Its color was dark brown.

Tris(Triphenylphosphine)chlororhodium (I) Hydrogenation Catalyst. Melanin (25 mg) was added to 10 mg of catalyst (prepared by the procedure of Osburn and Wilkerson as described by Monson [8]) dissolved in chloroform in a steel shaker bomb. The system was subjected to a hydrogen pressure of 2,000 lb/in^2 and allowed to shake overnight. The solvent was decanted and the pigment washed and dried as before. The isolated "reduced" pigment showed no evidence of color change from the original sample.

Oxidation with Hydrogen Peroxide. Melanin (20 mg) was placed in 50 ml of 3% hydrogen peroxide solution at 50° for 2 wk. During this time approximately 70% of the melanin dissolved. The particulate pigment that remained was recovered. It showed little color change from the original sample.

ESR Methods

Melanin samples in the form of nominally dry powders were weighed and placed in 3 mm ID quartz tubes, stoppered and examined at room temperature with a Varian E-3 EPR spectrometer (Varian Associates, Palo Alto, Calif.).

For purposes of comparison, a normalized intensity (NI) was calculated using the expression (9)

$$\frac{h(\mathrm{LW})^2}{(\mathrm{mod})(\mathrm{gain})(\mathrm{wt})},$$

where: h is peak to peak height of derivative spectrum; LW is line width measured between points of maximum slope; wt is sample weight; and mod, gain are the spectrometer modulation amplitude and gain settings. It was assumed that the line shape for all of the melanin spectra were the same. Microwave power levels of 2 mW or lower were used to minimize saturation effects.

The electron g-values were determined by direct measurement of the magnetic field with the Fieldial (Varian Associates, Palo Alto, Calif.) and of the microwave frequency with a Hewlett-Packard X532 direct-reading frequency meter (Hewlett-Packard Co., Palo Alto, Calif.). The magnetic field at the sample was determined by calculating a correction term to the Fieldial reading using a series of solid substances for (diphenylpicrylhydrazyl, charred dextrose, and Varian's strong pitch) which g-values were accurately known. Values of g were routinely reproducible to $\pm 2 \times 10^{-4}$.

TABLE I

ESR DATA FOR MELANINS SEPARATED BY VARIOUS METHODS

Source	*g*-values ±0.0002	Line width, *G* ±0.2	Normalized intensity (NI)
Wool/HCl hydrolysis	2.0036	5.4	8.0
Wool/PHT hydrolysis	2.0038	5.5	6.4
Wool/papain digestion	2.0040	8.0	2.8
L-DOPA/autoxidation	2.0037	5.0	10.0
L-DOPA/tyrosinase	2.0038	5.8	18.8
L-Tyrosine/tyrosinase	2.0040	6.4	1.2
Black oriental hair*	2.0037	5.2	—

*Included as an example of intact melanin in hair.

RESULTS

The native and modified melanins all gave essentially the same type of ESR signal, a slightly asymmetrical derivative curve devoid of any hyperfine structure. The line shape was intermediate between Lorentzian and Gaussian. Saturation behavior indicated that the lines were inhomogenously broadened. Tables I and II show *g*-values, line widths, and normalized free radical intensities for the native melanins and melanins subjected to various reductive procedures and to hydrogen peroxide.

The data in Table I indicate that the endogenous free radical of melanin is not appreciably affected by the method of isolation. Melanins from several sources exhibit very similar spectra and *g*-values, although the concentrations of free radicals indicated by normalized intensity (NI) values vary somewhat more. The exception is melanin obtained by enzyme (papain) treatment. Its *g*-value and line width are larger, while its NI is definitely smaller than the corresponding values for the other melanins from wool. The melanin samples obtained synthetically from L-DOPA by enzyme

TABLE II

ESR DATA FOR MODIFIED MELANINS

Source	Treatment	*g*-values ±0.002	Line width, *G* ±0.2	Normalized intensity (NI)	Color
Wool/HCl hydrolysis	$NaBH_4/AlCl_3$	2.0037	6.1	15.8	DBrn
Wool/HCl hydrolysis	$NaBH_4/FeCl_3$	2.0036	5.9	11.2	DBrn
Wool/HCl hydrolysis	Catalytic reduction	2.0035	6.0	12.9	BLK
Wool/HCl hydrolysis	Ascorbic acid	2.0037	5.7	2.9	DBrn
Wool/HCl hydrolysis	Birch reduction	2.0043	7.4	0.13	LBrn
Wool/HCl hydrolysis	H_2O_2	2.0036	5.0	0.09	BLK
L-DOPA/autoxidation	Birch reduction	2.0038	6.6	30.0	BLK

treatment or by autoxidation are quite similar to those from wool by HCl or PHT hydrolysis. On the other hand, melanin synthesized from tyrosine appears more like that obtained from wool by papain digestion.

The modified melanin data (Table II) can be grouped into two categories. The first encompasses cases in which treatment resulted mainly in a change in the number of free radicals present, with very little effect upon the *g*-values and only a small effect on line widths. In the second category, the normalized intensity, line widths and *g*-values have been altered significantly. The first category includes treatments with sodium borohydride, reduction catalysts, ascorbic acid and hydrogen peroxide. Interestingly, the reductive treatments in nonaqueous media in the presence of metals increased the melanin free radical content and line width, whereas in aqueous media, ascorbic acid and hydrogen peroxide treatment resulted in decreased free radical content. In the second category are the Birch reductions, which gave very different results when applied to melanins from wool and DOPA, but in both cases the changes were much greater than those produced by other treatments.

DISCUSSION

Previous reports of the effects of reducing agents on melanin have been limited mainly to the action of ascorbic acid (1, 2), but have led to opposite conclusions.

According to Blois et al. (2), reduction of squid melanin with ascorbic acid results in a color change but no reduction in free radical concentration, whereas excess Cu^{2+} ion destroyed the ESR signal without altering the absorption spectrum. He therefore concluded that the melanin color and paramagnetism are independent of each other. The results and conclusions reported by Mason et al. (1), are in direct contrast. Mason found that both color and free radical content were decreased by ascorbic acid reduction. From this observation, he concludes that there is a direct correlation between color and free radical content.

Our results for the ascorbic acid reduction of wool melanin are in accord with those of Mason. However, our other reductive treatments produced the opposite result. The free radical content increased and the color remained the same or turned from black to dark brown. A remarkable result of this study is the large, opposite difference in the effects of Birch reduction on the normalized intensity of the ESR signals for the two melanins derived from wool and L-DOPA (Table II). The wool melanin lost most of its ESR signal on reduction while the corresponding signal for the L-DOPA melanin product increased three times. A plausible explanation for this result is that structural details in the L-DOPA melanin induce disproportionation of a two-electron reduction product to one-electron derivatives that are relatively stable, while in the natural melanin, possibly because of different aggregate size or compactness, this process does not take place. Thus, an initial product with decreased radical content could disproportionate to one with increased content. This conjecture is illustrated in Fig. 1. Treatment with hydrogen peroxide had no effect on the color but resulted in a large decrease in free radical content.

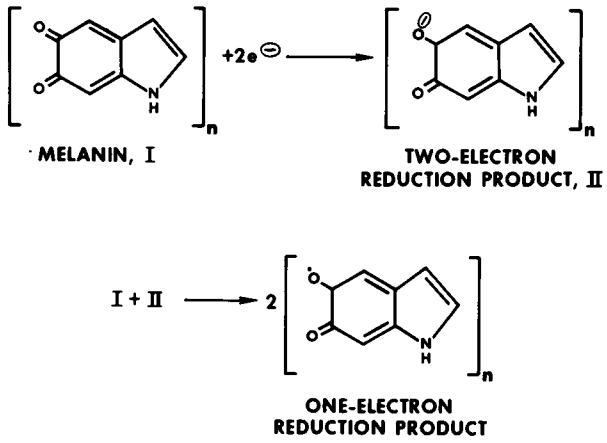

FIGURE 1 Diagram depicting disproportionation of a two-electron melanin reduction product to a one-electron reduction product.

These results support Blois's contention that the melanin color and free radical content are independent, even though our results are at variance with his for ascorbic acid. Blois also cites the quenching of the melanin signal in the presence of an 80-fold excess of Cu^{++}, without any corresponding change in the visible spectrum, as evidence for the independence of melanin color and free radical content. The disappearance of the signal was attributed to destruction of the unpaired spins. However, in view of the great excess of Cu^{++} required and concomitant decrease of the melanin T_1 (2), the disappearance of the signal could be due to copper-melanin spin-spin interactions giving a line too broad to detect. In this case, no conclusion about radical content and color can be inferred.

Mason et al. (1) have concluded from their observations on hair containing various degrees of melanin and melanizing *Calliphora puparia* that there is a correlation between pigmentation and free radical content. However, since the free radical content in melanin derived from different sources is the same to within an order of magnitude (1, 2), then for an intact sample, such as hair or puparium cuticle, a change in the degree of pigmentation and a corresponding change in ESR signal probably measure the change in the amount of melanin present rather than a relationship between color and free radical content in the melanin itself.

Mason also has reported that the melanin signal in black hair is increased by ultraviolet irradiation. We have confirmed this observation with black oriental hair illuminated with a high pressure mercury lamp. However, such illumination has no effect on the ESR signal from isolated melanin pigments. This is in accord with Zahlan et al. (11) who found no effect of UV irradiation on the ESR signal from melanin powders at room temperature and who attributed increases in signal amplitude with irradiation at 77°K to an increase in the spin lattice relaxation time. Cope et al. (10) also have found that illumination by visible light of dried beef eye melanin has no ef-

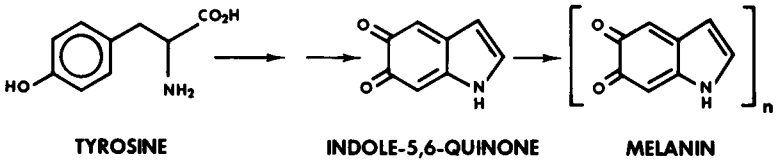

FIGURE 2 Schematic diagram depicting melanin biosynthesis.

fect on its ESR signal. This suggests that for hair, the free radicals are not being produced directly in the melanin, but that the melanin is acting as a trap for the free radicals known to be produced by ultraviolet light; in much the same way that the cysteine residues act as traps in irradiated nonpigmented wool (12).

It is becoming increasingly apparent that the structure of black melanin may differ significantly depending upon its origin. However, the generally accepted concept of melanin formation is that suggested by Raper (13) and later expanded by Cromartie and Harley-Mason (14). Their picture of melanin considers it to be a highly conjugated polymer formed from the tyrosine-derived monomer, indole-5,6-quinone (Fig. 2). Later, Nicolaus and co-workers (15, 16) suggested that melanin is a highly irregular three-dimensional polymer not only in the way the units are linked together, but also in the units themselves.

Our results are most in accord with the Nicolaus picture. The *g*-values suggest that the unpaired electrons are centered on N or O atoms and are not extensively delocalized. In such a model, no correlation between color and free radical content would be expected. The superposition of spectra from several types of trapped radical and anisotropic effects in the solid would tend to smear out any hyperfine structure, giving a single asymmetric line that will be inhomogenously broadened.

In conclusion, the different reductive treatments have had varied effects on the melanin pigments. These variations are manifest by differences in color and free radical content. We attribute these differences to the changes in nature of the persistent free radical species and in the molecular structure of the pigments. These changes may perhaps include disruption of a conjugated aromatic system.

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