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The Free Radical Property of Melanins

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The electron spin resonances of *Calliphora* puparia, of *Sepia* ink, of human hair, and of 3,4-dihydroxyphenylalanine melanin have been determined. The pigment in each case displays absorption with $g = 2.003 \pm .001$. The free radical property of these melanins is ascribed to a stabilized semiquinonoid form of the polymeric pigment.

INTRODUCTION

Free radicals have been detected by electron spin resonance spectroscopy in pigmented frog's eggs (1) and in the pigments formed by alkaline autoxidation and enzymic oxidation of 3,4-dihydroxyphenylalanine (2–4). The chemical and biological significance of free radical development during the formation of natural and synthetic melanins is unknown. In the present study we have examined the process of melanization in metamorphosing *Calliphora* puparia with electron spin resonance spectroscopy. We have, in addition, determined the free radical contents of variously colored human hair before and after exposure to ultraviolet radiation, of *Sepia* ink melanin, and of melanins formed enzymically from 3,4-dihydroxyphenylalanine.

EXPERIMENTAL METHODS

Electron Spin Resonance Spectroscopy

A 3-cm. wavelength electron resonance spectrometer with a transmission cavity operating in the H_{014} mode was employed in these investigations. Magnetic field modulation at 100 kc./sec. with phase-sensitive detection (5) were used to overcome crystal noise and obtain high sensitivity. The final signal was therefore plotted by the pen recorder as a first derivative curve, as will be seen in the figures. Measurements were made at both room temperature and at 90°K., the rectangular cavity being immersed in a liquid oxygen bath for the latter measurements, with suitable insulating "heat breaks" incorporated in the waveguide run.

At the level of sensitivity at which this spectrometer operates, it is important to avoid signals from traces of paramagnetic impurities. For this reason each specimen tube was tested before measurements were made on the samples themselves. It was found that glass tubes were usually unsatisfactory, with relatively high impurity content, and silica tubes were therefore used in all these experiments. These were of 6 mm. external and 4 mm. internal diameter and could be inserted directly down the central axis of the narrow side of the cavity, when about 1 cm. length of the specimen was in the region of concentrated microwave magnetic field.

The magnetic field strengths were calibrated by a proton resonance meter, and g values could be determined by direct comparison with the position of the signal for a very small crystal of diphenylpicrylhydrazyl inserted beside the specimen. Quantitative intensity determinations were made from a double integration of the derivative tracing, and comparison of this with the signal from a calibrated free-radical carbon standard was obtained under identical conditions.

Ultraviolet and Visible Spectroscopy. Ultraviolet and visible absorption spectra were measured in a Cary model 14 recording spectrophotometer, using 1 cm. silica cuvettes.

Radiation Experiments. A 500-w. high pressure mercury vapor lamp with maximum spectral density at 3660 A. was employed. The silica specimen

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tubes were held at a distance of 15 cm. from the light source.

Preparation of Melanins

Several hundred white puparia of *Calliphora erythrocephala* were collected and allowed to melanize at room temperature. It was then possible to select 15 or 20 puparia at an identical stage of melanization for any experiment. To obtain isolated cuticles, the puparia were pierced and their contents squeezed out by firm horizontal strokes with a flat steel edge.

Sepia ink was collected from *Sepia officinalis* with the kind help of Dr. J. Gilpin-Brown at the Marine Biological Laboratory, Plymouth. The ink sacs of the urethan-anesthetized *Sepia* were dissected out, and their contents were milked into glass vials. Each sac yielded from 2 to 10 ml. ink, which consisted of a suspension of black granules (0.2 μ diameter) at a concentration of 20% *w/v*. No admixed proteins could be demonstrated in the ink by means of paper electrophoresis at pH 8.4 (Veronal buffer). The absorption spectrum of this substance is discussed below.

In order to prepare melanin from 3,4-dihydroxyphenylalanine, 100 mg. of the DL-form (L. Light and Co.) was dissolved in 30 ml. of distilled water and oxidized in the presence of 4 mg. of purified mushroom tyrosinase. After 4 hr. the melanin was separated by centrifugation and studied as a sludge. The yield, determined by subsequent drying of the product, was 30–32 mg.

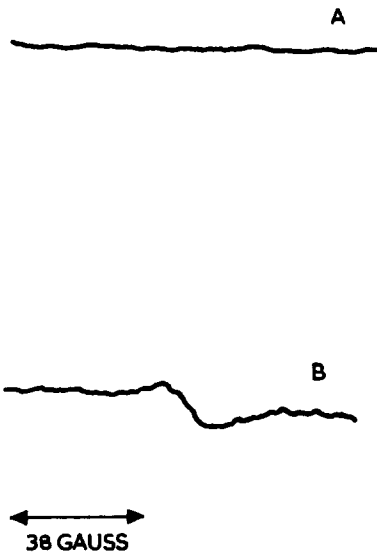


Fig. 1. Electron spin resonance of whole *Calliphora* puparia. The specimen tubes contained 6–8 puparia which had been very rapidly frozen in liquid oxygen: (*a*) unmelanized puparia; (*b*) naturally melanized puparia.

RESULTS

Calliphora Melanin

The whole white puparia displayed no electron spin resonance at 70°K. (Fig. 1*a*), whereas naturally melanized puparia showed a resonance absorption with a g value of 2.003 ± 0.001 (Fig. 1*b*). In order to determine whether or not the electron resonance was associated with the cuticles of the insects, the cuticles were separated from the rest of the puparia and studied in an isolated condition. It was found that the cuticles from white insects showed no absorption, but the the cuticles of naturally melanized puparia displayed an absorption similar in g value; the free radical content was 2.8×10^{17} free spin/g. cuticle.

In order to determine whether the darkening process and free radical formation were associated phenomena, cuticles isolated from white puparia were allowed to darken spontaneously. As these cuticles darkened over a period of 12 hr., electron spin resonance developed, at a g value of 2.003 ± 0.001, as depicted in Fig. 2*b*. After 12 hr., however, the free-radical signal was found to remain more or less constant, whereas the darkening noticeably increased.

Sepia Ink

This intensely black melanin displayed an absorption maximum at 327 mμ and a shoulder at 640 mμ (Fig. 3). Upon reduction with either ascorbic acid or H_2S, the maximum at 640 mμ disappeared while absorption at 327 mμ became greatly intensified (Fig. 3*b*). The difference spectra between the oxidized and reduced forms are shown in Fig. 4. The difference spectrum very clearly demonstrates the strong absorption which was lost at 630 mμ and gained at 340 mμ as a result of reduction, but the differences themselves cannot be ascribed to the oxidized or reduced states of the chromophores because they are superimposed upon the marked scattering caused by the granular nature of the pig-

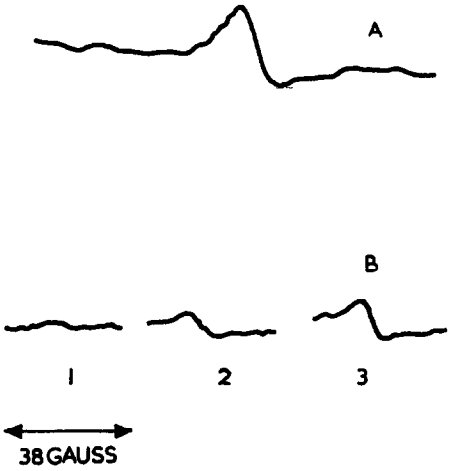


FIG. 2. Electron spin resonance of isolated cuticles of *Calliphora* puparia. (*a*) Naturally melanized cuticles. The specimen tubes contained 16 cuticles weighing approximately 87 mg. in total. (*b*) The development of electron spin resonance in isolated cubicles which had stood for (*1*) no hours, (*2*) 2 hr., and (3) 12 hr., with concomitant darkening.

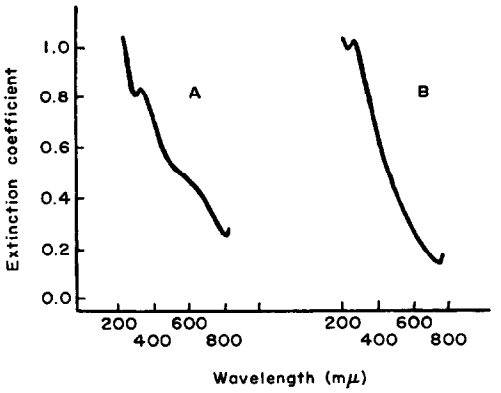


FIG. 3. The absorption spectrum of *Sepia* melanin: the spectrum of a suspension of 0.33 mg./ml. in distilled water, pH 6.0, of the natural melanin (*a*) before, and (*b*) after reduction with H_2S. An identical spectrum was obtained by reducing the melanin with ascorbic acid.

ment. If the melanin was hydrolyzed in 6 *N* HCl for 36 hr. at 125°, at least 12 amino acids could be demonstrated by two-dimensional paper chromatography in the supernatant acid, indicating that at least a part of the absorption of the native *Sepia* melanin in the ultraviolet region was due to protein.

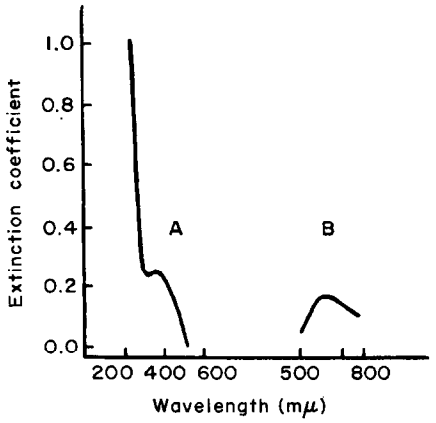


FIG. 4. The absorption spectrum of (*a*) the reduced form minus that of the natural form and (*b*) the spectrum of the natural form minus that of the reduced form of *Sepia* melanin.

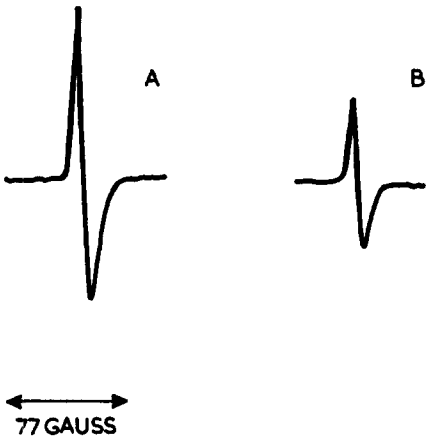


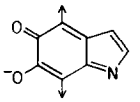
FIG. 5. (*a*) Electron spin resonance of native *Sepia* melanin, and (*b*) of *Sepia* melanin after partial reduction with ascorbic acid.

Lyophilized *Sepia* melanin displayed a very strong electron resonance with $g = 2.003$, and a concentration of unpaired spins of 5.4×10^{18}/g. The original ink gave a value of 6×10^{17} spins/g. (Fig. 6*a*). Upon reduction with excess ascorbic acid, the intensity of absorption decreased to 3×10^{17}/g. but did not vanish (Fig. 5).

DIHYDROXYPHENYLALANINE MELANIN

This substance gave a strong electron resonance signal at a *g* value of 2.0003 (Fig. 5), which corresponded to a free radical content of 1.1×10^{-4} moles/mole of polymer unit,

assumed to be derived from indole-5,6-quinone by loss of two hydrogen atoms:



Melanins of Human Hair

A short survey of the free radical content of human hair was made. Samples of unambiguously black, brown, red, blond, and gray hair were selected from a large number of samples, and all were found to give detectable electron resonance signals at a value of $g = 2.003$. The free radical concentrations are listed in Table I. Of the samples studied, black hair contained a higher free radical content than the rest, up to 4.7×10^{16} spins/g. Upon exposure to ultraviolet light from a mercury arc lamp for 15 min., this free radical concentration increased to 9.9×10^{16} spins/g. (Fig. 7). The curves in Fig. 8 were measured at an amplification of 100× that for Fig. 7. Similar but less marked changes were observed by irradiation with sunlight.

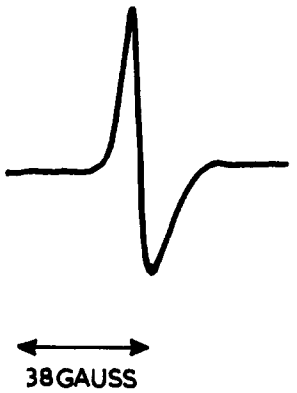


Fig. 6. Electron spin resonance of melanin formed by the enzymic oxidation of 3,4-dihydroxyphenylalanine.

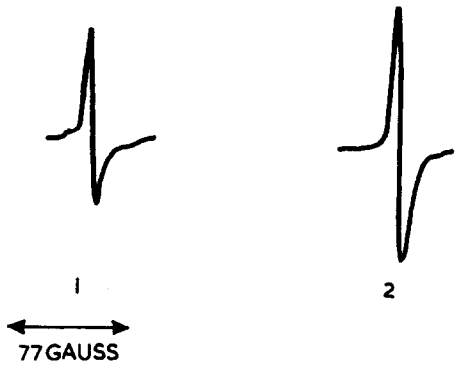


Fig. 7. Electron spin resonance absorption of black human hair (*1*) before, and (*2*) after exposure to ultraviolet light.

Fig. 8. Electron spin resonance absorption of blond human hair (*1*) before, and (*2*) after exposure to ultraviolet light.

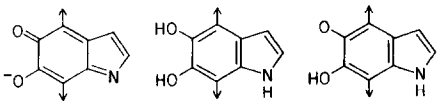
Discussion

Melanins are polymerization products of enzyme-generated quinones, often associated with proteins. A description of melanin structure must include identification of the polymer units, the nature of the inter-unit linkage, the length of the chains, the redox state of the polymer, and the nature of the protein and its linkage to the pigment. Little of this information has been obtained for the intractable natural melanins, although some progress has been made with synthetic melanins derived from catechol and 3,4-dihydroxyphenylalanine, and the free radical property accordingly cannot be correlated with structure in an unequivocal way. Nor can the free radical content be calculated from the melanin content of a tissue because there is no method of determining melanin quantitatively. In the present study, we have tried to establish that the free radical property of melanized tissues is in fact a property of the contained melanin.

There is a correlation between pigmentation and free radical content in *Calliphora* puparia, in human hair, and in *Sepia* ink (Table I). This correlation is shown in three ways. Whole white *Calliphora* puparia have no free radical content, whereas the black puparia give an electron spin resonance signal (Fig. 1), and as isolated cuticles darken, a signal develops (Fig. 2). With human hair samples, selected from a large number of

varied colors to represent clear differences, an increasing order of free radical content is also observed (Table I). When *Sepia* melanin is reduced with ascorbic acid or H_2S, loss of color is observed (Figs. 3 and 4); simultaneously, there is a decrease in free radical content (Fig. 6 and Table I).

The synthetic melanin formed by the enzymic oxidation of 3,4-dihydroxyphenylalanine is a polymer of indole-5,6-quinone (6, 7). The metastable intermediates between 3,4-dihydroxyphenylalanine and indole-5,6-quinone are not free radicals, and there is no evidence that any detectable free radicals form during the enzymic transformations of these melanin precursors. The free radical property of the melanin is therefore undoubtedly a property of the polymeric pigment itself. Indole-5,6-quinone is believed to polymerize by reductive condensations involving, tentatively, the 4- and 7-positions (6), and the unit of polymer may therefore be in a quinonoid (II*a*), a hydroquinonoid (II*b*), or a semiquinonoid (II*c*) form:



IIa IIb IIc

We ascribe the free radical property of this melanin to a semiquinonoid form (II*c*) which must greatly stabilized by the possibility of resonance throughout the highly conjugated polymer, and by steric restrictions on reactivity. The stabilization of such a semiquinonoid form would therefore depend upon the degree of conjugation, upon the chain length of the polyphenylene system, and upon the redox state of the polymer (*cf.* 8).

Sepia melanin loses free radical character upon reduction (Fig. 5). The oxidized and reduced spectra cannot be correlated with known spectra of any synthetic melanins or their precursors, and as no quinone-generating oxidase has been found to be associated with the *Sepia* granules, there is little ground from which to infer the structure of the precursor. There is a general relationship between the infrared spectra of *Sepia* melanin and dihydroxyphenylalanine melanin which will be reported elsewhere,[3] but otherwise, only the redox property, the association with protein, the color, and comparative biology place this pigment within the category of melanins. Nevertheless, it is a reasonable presumption that its free radical character has a source in structural properties similar to those of dihydroxyphenylalanine melanin.

TABLE I

FREE RADICAL CONTENT OF MELANINS

Substance	Free spins/g. dry weight
1. *Sepia* melanin	
Natural ink (wet state)	6.0×10^{17}
Reduced ink	3.0×10^{17}
Lyophilized melanin	5.4×10^{18}
2. *Calliphora* cuticle	
Unmelanized (wet state)	0
Naturally melanized	2.8×10^{17}
3. Human hair	
Very black	4.7×10^{16}
Very black after ultraviolet irradiation	9.9×10^{16}
Dark brown	2.8×10^{16}
Medium brown	4.6×10^{15}
Gray	3.1×10^{15}
Dark red	1.8×10^{15}
Fair	1.1×10^{15}
Blond	4.3×10^{14}
4. Dopa melanins (wet state)	3.9×10^{17}
	4.2×10^{17}

On the other hand, the melanin of human hair is very probably related to dihydroxyphenylalanine melanin because the enzyme system known to oxidize dihydroxyphenylalanine to melanin is present in the hair follicle. Since under the same conditions of irradiation with ultraviolet light, black (melanized) hair gives a strong increase in free radical content, whereas unmelanized hair gives a poor response, melanin may act in *some* organisms as a biological electron exchange polymer able, by means of its capacity for oxidation and reduction, and its stable free radical state, to protect a melanin-containing tissue or associated tis-

[3] Mason, H. S. and Sheppard, H., unpublished results.

sues against reducing or oxidizing conditions which might otherwise set free within living cells reactive free radicals capable of disrupting metabolism. These experiments provide evidence for the electron trap concept of Commoner *et al.* (1), and the proposal by Daniels (9) that melanins may protect against radiation by this mechanism. However, melanin also occurs in forms such as *Sepia* ink in which it is difficult to see how the operation of such a mechanism could come about. It is probably best, at present, to regard the free radical property of melanins as a reflection of the underlying structure of these pigments which may, or may not, serve a metabolic or biological function.

While the specific precursors of melanin in *Calliphora* puparia are not known, several catecholic compounds have been isolated from the cuticles (10), and it is very probable that the free radical character of this melanin has a structural origin very similar to that of dihydroxyphenylalanine melanin. The observation that the free radical signal develops during the initial 12-hr. period of darkening but remains relatively constant thereafter while darkening continues may suggest that there is a maximum possible free radical content for melanins, beyond which the pigment becomes increasingly quinonoid upon oxidation. Alternatively, the cuticulation process may involve more than one process, as has already been suggested by Goodwin (11) and by Malek (12).

ACKNOWLEDGEMENTS

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One of us (H. S. M.) wishes to express his gratitude to Professor Sir Alexander Todd for the hospitality of his Department while this study was being made.

Mr. D. A. T. Carter of Richardson's hairdressing shop, Cambridge, collected a large number of samples of human hair of every color for this study. We are pleased to acknowledge his assistance.

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