CHAPTER 7

Structure and Reactivity of Melanins: Influence of Free Radicals and Metal Ions

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I. INTRODUCTION

Melanins are biological polymers that are largely responsible for pigmentation in animals. Substantial amounts are present in the hair, skin, and in the choroid and iris of the eye, while smaller quantities have been found in the substantia nigra of primates and in the inner ear. They are intriguing materials for study in that, with the possible exception of photoprotection, their function remains unknown.

The untoward physical and chemical properties of melanin render its study especially challenging. The material is insoluble and opaque, has little order and few characteristic spectroscopic absorption bands, and is without well-defined molecular weight. Improvement in our understanding of its structure has been largely based on chemical degradation studies which have demonstrated not only that melanin is a heterogeneous polymer but that it contains many quinone and hydroquinone units; i.e., melanins are redox polymers.

Further progress has been possible by virtue of another unusual property that in part characterizes melanins: they contain free radicals under all known conditions. This property makes them accessible for study by electron spin resonance spectroscopy, which selectively monitors species with unpaired electrons. This technique has provided information on both intrinsic free radicals and those generated by light and other treatments.

Another esr approach that has proved useful in investigating melanin structure utilizes paramagnetic metal ions as probes of the nature of metal-binding sites available on the polymer. Metal binding is of some importance, for natural melanins generally contain high concentrations of metal ions.

Several reviews [*1–3*] are available on the use of chemical modification and degradation techniques in the elucidation of melanin structure. In this chapter we instead choose to emphasize the chemical reactivity of melanins and in particular their free-radical properties as revealed by esr measurements. The aim of the chapter is threefold: to provide an introduction to the study of melanins by esr spectroscopy; to review the (rapidly accumulating) published data; and to indicate the present status of research pertaining to melanin structure and reactivity.

II. GENERAL PROPERTIES OF MELANINS

A. Classification and Melanogenesis

The original classification of melanins was based on color [*1*]. The *eumelanin* class includes the brown to black pigments found in skin and

hair and is usually extended to include those of the eye and sometimes the substantia nigra ("neuromelanin"). These are the melanins which have received the greatest amount of attention and with which we shall largely be concerned. Other classes are *phaeomelanins* (yellow to red animal pigments) and *allomelanins* (brown to black plant and fungal pigments).

An alternative classification [*1*] (Table I) is according to known or presumed chemical precursors: hydroxyaromatics and other small incorporated molecules such as cysteine. Thus, it is believed that eumelanins are derived from tyrosine or dihydroxyphenylalanine (dopa), phaeomelanins from dopa in the presence of cysteine, and allomelanins from catechol or other simple polyhydroxyaromatics. (Humic acids are generally included in the allomelanin class; these have free-radical properties closely related to those of eumelanins.) Some believe [*4*] that neuromelanin may be derived from dopamine and perhaps other neuroregulators.

"Synthetic melanins" are prepared *in vitro* by oxidation of hydroxyaromatics, and are named according to their precursors: thus "dopa-melanin" and "catechol-melanin" are the synthetic polymers derived from dopa and catechol, respectively. The primary advantages of synthetic melanins are that (a) they are readily and reproducibly obtained from melanin precursors, and (b) their behavior is qualitatively (and often quantitatively) similar to that of natural preparations. They are extremely convenient to use in preliminary experiments prior to using scarce and more complex natural melanin samples. One disadvantage in a sense is allied to their close similarity with natural materials—they also are difficult to characterize in terms of chemical structure and organization.

The prevailing view [*2*, *5*, *6*] of the melanogenesis of eumelanins is represented in Fig. 1, a modification of the classic Raper–Mason scheme [*7*, *8*]. The initial oxidative step(s) from dopa (tyrosine) to dopaquinone is

TABLE I Classes of Melanin

Class	Source	Colors	Precursor	Synthetic model
Eumelanins	Hair Skin Choroid Pigment epithelium	Brown, black	Tyrosine/dopa	Dopa-melanin
Phaeomelanins	Hair Feathers Skin	Yellow, red, brown	Dopa + cysteine	Dopa/cysteine melanin
Allomelanins	Seeds Spores Humic acids	Brown, black	Catechol, other polyhydroxyaromatics	Catechol melanin

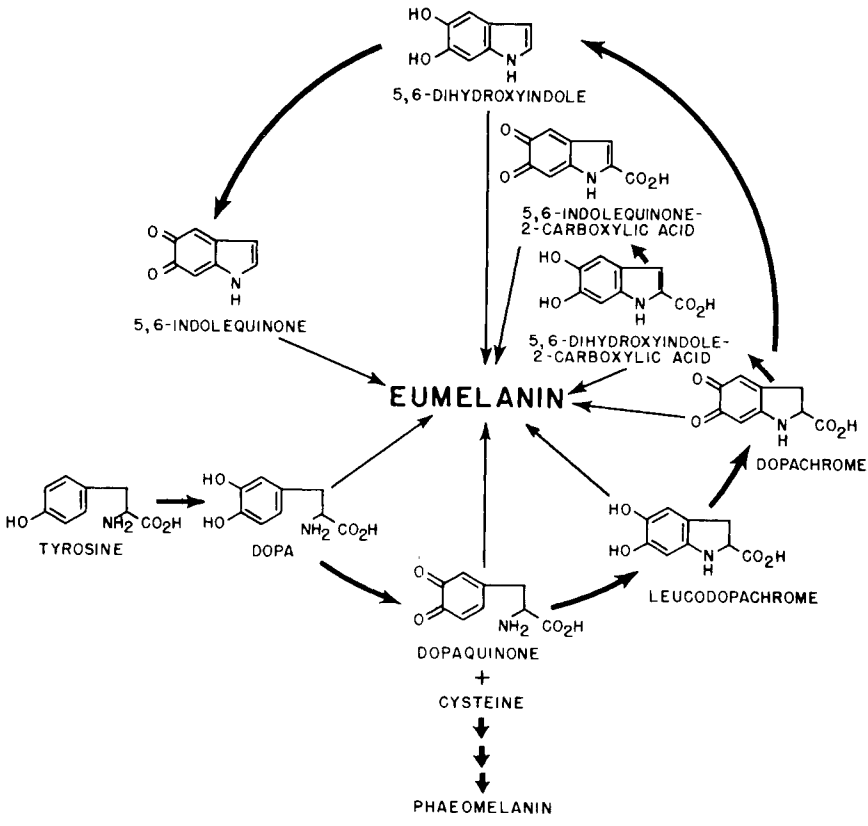


Fig. 1. Melanogenesis of eumelanins from tyrosine.

generally considered to be mediated by the copper-containing enzyme tyrosinase, although an alternative mechanism in which oxidation of tyrosine is catalyzed by hydrogen peroxide-peroxidase has been postulated [*9*, *10*]. Dopa itself has been shown to accelerate the enzymatic oxidation of tyrosine [*11*]. The succeeding sequence of steps does not have an enzyme requirement and appears to occur spontaneously, at least at physiological pH. Zinc ions may be involved in the decarboxylation of 5,6-dihydroxyindole-2-carboxylic acid to 5,6-dihydroxyindole [*12*]. It should be noted that although the original Raper–Mason scheme indicated that melanin is essentially an indolequinone polymer, if polymerization is essentially random, as many now believe, then some or all of the intermediates between dopa and indolequinone are likely to be incorporated into the final polymeric product. This indeed seems to be the case (see below). There is generally protein associated with natural melanins, some of which may be the enzyme tyrosinase.

B. Structure

Information on melanin structure has largely been derived from models of synthetic pathways and from chemical degradation experiments carried out by the research groups of Nicolaus, Swan, and Hempel. This work has served as a basis for the interpretation of essentially all other data. Since melanins are such inert materials, methods required to break down the structure are severe (fusion with alkali or permanganate oxidation), and yields of products are small (<1%). A nondegradative analytical technique would obviously be preferred: more recent approaches [^{13}C nuclear magnetic resonance (nmr) [*13*, *14*] and spin-probe electron spin resonance (esr) [*15*] methods] have this advantage, but share the disadvantage of selectivity (the nmr method monitors only those portions of the polymer that retain some mobility, whereas the esr method can provide information only on accessible metal binding sites). The consensus from the degradation studies [*2*] (Table II) is that the polymer is indeed complex: it is heterogeneous in its monomer content, and it appears to be cross-linked in a random manner. Some of the moieties that have been shown to be present are given in Table II and grouped together in Fig. 2 (a representation due to Nicolaus). While there may be some differences between melanins from different sources (one must remember that the ratios expressed in Table II are extrapolated from very low yields of degradation products and errors are probably high), several general points can be made [*2*]. First, cyclized units based on indole and indole carboxylic acid are always present. Second, uncyclized units based on dopa are also possible. Third, there does appear to be a very large proportion of units in either quinone or hydroquinone oxidation states; i.e., melanin may be considered [*16*, *17*] to be a naturally occurring redox polymer [*18*], a property which may largely determine its chemical behavior. (Nicolaus's model [*21*] does not distinguish among quinone, hydroquinone, or semiquinone oxidation states of the units.)

It is convenient here to introduce the notation $\sim\!\!\sim Q$, $\sim\!\!\sim QH_2$, and $\sim\!\!\sim Q^{\cdot}$ to represent *o*-quinone, *o*-hydroquinone, and *o*-semiquinone groups on the polymer, respectively. We shall use $\sim\!\!\sim Q^{\cdot}$ for semiquinone radicals without regard to their state of protonation.

Several authors [*22–24*] have considered the likelihood of quinone–hydroquinone association to form quinhydrone electron donor–acceptor complexes, i.e., $\sim\!\!\sim Q...H_2Q\sim\!\!\sim$. Here the evidence is circumstantial but chemically reasonable. Thathachari and Blois [*25–27*] carried out x-ray experiments on samples of both natural and synthetic melanins from which they concluded that there exists a degree of short-range order in each. This short-range order is characterized in the natural material from animal sources and in synthetic melanins by an interlayer spacing of 3.4

TABLE II Structural Components of Melanin[a]

Melanin	5,6-Dihydroxyindole + 5,6-indolequinone (%)	Leucodopachrome + dopachrome (%)	5,6-Dihydroxyindole-2-carboxylic acid + 5,6-indolequinone-2-carboxylic acid (%)	Dopa + dopaquinone (%)
Sepia	50–80	20–50[b]		0
Mouse melanoma[c] (Harding–Passey)	36	—	17	47
Dopa[d]	65	10	—	10

[a] From Nicolaus [*1*], Swan [*2*], and Hempel and Männl [*19*].

[b] Sum of leucodopachrome and 5,6-dihydroxyindole-2-carboxylic acid (both oxidation states).

[c] Based on incorporation of ^{14}C-labeled dopa into mouse melanoma. The high proportion of dopa units has been questioned [*2*, *20*].

[d] Substantial amounts (15%) of pyrrolecarboxylic acid units also found [*2*].

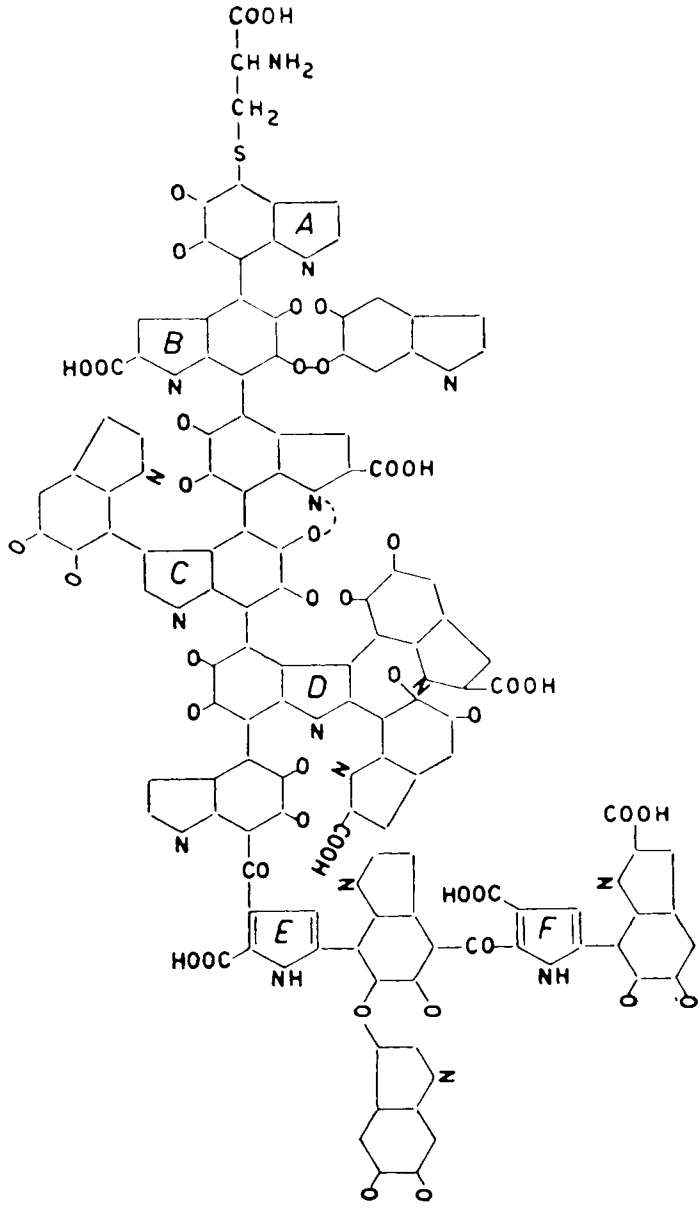


Fig. 2. Proposed structural components of sepiomelanin. (From Nicolaus [21].)

Å, which is just that expected for an electron donor–acceptor complex [*28*]. The outward similarities of melanin and quinhydrone (i.e., the complex between *p*-benzoquinone and *p*-hydroquinone) have been commented on [*22*]. Furthermore, if the random oxidative polymerization model of melanogenesis is correct, then one would anticipate that quinhydrones (much less soluble than hydroquinones) would precipitate from solution to act as nuclei for the continuing polymerization.

There is certainly evidence that such complexes are formed in synthetic redox polymers. Optical absorption measurements made on partially oxidized polyvinylhydroquinones are consistent with the presence of both intra- and intermolecular quinhydrones [*18*].

As indicated earlier, free radicals are also present under all experimental conditions, in concentrations generally reported to vary between 10^{17} and 10^{19} spins per gram of dried material (i.e., up to 1 free radical per 400 monomers).* The evidence for this is entirely based on esr data which we describe in the following pages.

Natural melanins always contain protein, metal ions, and water. Protein incorporated into natural melanin is reported [*29–31*] to account for perhaps 20–50% of its weight. Electron microscopy studies of melanosomes [*5, 32*] suggest a certain regularity of structure of the complexed melanin. Any tyrosinase in the protein will contain the paramagnetic copper(II) ion. However, this is difficult to detect by esr methods owing to [*33, 34*] the proximity of copper ions within the enzyme. It is clear that metal ions are also readily bound to the polymer [*35, 36*]. Ions found in natural melanins include Cu, Fe, and Zn in concentrations ranging from 25–950 μg/g in melanin from bovine eyes [*31, 37–39*]. One can estimate from published data [*36, 40*] that at pH 5 up to 15% of monomer units in natural (bovine eye) and synthetic (dopa) melanin are available for metal ion binding. Values may be even higher at physiological pH. (Many metal-binding experiments are carried out in weakly acid solution in order to avoid the problem of precipitation of metal hydroxides.) Finally, one cannot dismiss the role played by water in the melanin structure. This is discussed in Section VII.

C. Melanin Preparations

Melanins have been isolated for chemical study from all kinds of animal and plant systems. Although some work has been carried out on intact tissue, most melanin preparations have been isolated from tissue before use. We feel that the melanin should be in as close a state as possible to that in the tissue; i.e., the protein normally associated with the polymer

* This assumes a monomer molecular weight of 150.

should be left attached and unmodified, and the polymer should be in its naturally hydrated state. Melanins isolated with protein attached are sometimes termed A-type [*41*]. Pure pigment granules can be prepared by gentle homogenization of tissue followed by filtering, layering over sucrose, and centrifugation (see, e.g. [*42*]). Protein and metal ions attached to the polymer can be largely removed by treatment with concentrated (6 *M*) hydrochloric acid at 105°C in a sealed tube for 100 hr [*41*] (giving B-type melanin).

Synthetic melanins may be obtained by oxidation of dopa, catechol, and related hydroxyaromatics. Thus, a eumelaninlike material is obtained from dopa by simple autoxidation [*1*, *41*], by enzymatic oxidation with mushroom tyrosinase [*1*, *41*], or by oxidation with mitochondrial incubations [*43*]. The melanin is precipitated by lowering the pH of the solution and should then be washed and dialyzed prior to use. As far as one can judge, the structure and properties of these model compounds are closely related to the natural materials, with the exception that they are protein-free. Synthetic polymers of restricted molecular weight (8000–45,000) have been made by terminating autoxidative polymerization of α-methyldopa with borate [*44*].

III. INTRINSIC FREE RADICALS

The observation of esr signals from melanin preparations has engendered a field of research that has maintained its share of interest and controversy. There has been much discussion as to whether the *intrinsic* free radicals (those ordinarily present at neutral pH) are the inert by-products of a polymerization process [*45*] or whether they are involved in some aspect of melanin function.

In this, as in subsequent sections, it must be stressed that data reported from different laboratories have frequently been obtained under very different experimental conditions. In particular, many groups have used dried melanin preparations. It seems that measurements should be made on hydrated melanin suspensions wherever possible—the physical properties of melanin are substantially changed [*17*, *46*, *47*] on drying, which can result in suppressed chemical reactivity.

A. Electron Spin Resonance Parameters

The use of the esr technique in biology has been covered in detail in an earlier volume in this series [*48*]. An esr spectrum may be characterized by such parameters as g-value, line width, and hyperfine couplings to nuclei. However, for melanin the information content of the free-radical

spectrum is limited by the inhomogeneous broadening typical of slowly tumbling macromolecules in solution. No hyperfine coupling is generally detected, and line widths are relatively broad.

The original observations of signals with g ca. 2.004 and a line width of 4–10 G, broadly consistent with those found for immobilized semiquinones [*49*] have since been confirmed in many experiments.* There is agreement that the esr signal is that of an organic free radical. The possibility that the signal might arise from paramagnetic metal ions incorporated into the polymer is ruled out by the finding [*16*] that a very similar signal is found in synthetic materials generated in the absence of metal ions. Semiconductor models have been proposed [*51–53*], but the argument by Blois [*54*] and co-workers, based on the g-value of the observed signal, that the unpaired electron is largely restricted to one or at most two monomer units, is generally accepted. The possibility that the paramagnetic species are thermally accessible triplets derived from electron donor–acceptor complexes has been considered and rejected [*54*]: data for dried samples indicate [*41*] that the free-radical concentration is invariant over the temperature range 4.2°–500°K. However, whether free-radical concentrations vary with temperature in hydrated samples has not yet been tested.† While we feel that a triplet model cannot therefore be completely discounted at this time, we shall assume for the remainder of this review that the species detected are in fact simple radicals ($S = \frac{1}{2}$).

Data for natural melanins from many sources and for synthetic melanins from dopa, catechol, and other hydroxyaromatics are contained in Tables III and IV. Parameters reported are generally g and line width and, in some instances, spin concentration. While careful measurements show reproducible differences in g and line width between melanins from different sources (e.g., between synthetic dopa melanin and natural melanin from the choroid of bovine eyes), these differences are generally small. The relatively large differences existing between some of the entries in Tables III and IV may in part result from differences in isolation procedure (e.g., drying, differences in pH, concentrations of metal ions) and from simple differences in spectrometer conditions (e.g., modulation amplitude, microwave power) under which spectra were recorded, all of which may modify the esr response, as discussed in later sections. Where more than one value exists for the free-radical line width in a particular melanin preparation, the lowest value is likely to be the most reliable.

The reproducibility of spin concentration measurements in particular

* There has appeared a recent report [*50*] of a very different esr signal with $g = 2.013$ and hyperfine splitting from chicken feather phaeomelanin obtained by alkali extraction.

† Note added in proof: Chio, Hyde, and Sealy (*Arch. Biochem. Biophys.*, in press) have observed temperature-dependent paramagnetism in hydrated melanin suspensions.

TABLE III Electron Spin Resonance Data for Natural Melanins

Melanin	Sample form[a]	g-Value	Line width (G)	Spins (g)	Reference
Sepia ink	Suspension	2.003 ± 0.001	~8[b]	6×10^{17}	*16*
Sepia ink	Dried	2.003 ± 0.001		5.4×10^{18}	*16*
Squid ink, A-type	Dried	2.0048 ± 0.0001	4.8 ± 0.3	10^{19}	*41*
Squid ink, B-type	Dried	—	—	5×10^{18}	*41*
Squid ink, HCl extraction	Suspension	2.0045	6		*55*
Squid, A-type	Dried	—	—	1.9×10^{18}	*56*
Squid, A-type	Oven dried	—	—	2.1×10^{19}	*56*
Amphiuma liver	Suspension	—	~7.5[b]	—	*43*
Amphibian eggs	Suspension	—	~5–6[b]	—	*57*
Amphibian eggs	Suspension	2.0050 ± 0.0005	4.5	—	*58*
Calliphora puparia	Suspension	2.003 ± 0.001	~9[b]	—	*16*
Frog eye pigment epithelium	Suspension	[c]	~6	—	*59*
Frog eye stratum pigmenti	Suspension	—	~7.5[b]	—	*60*
Frog eye pigment epithelium	Suspension	2.003	—	5×10^{17}	*61*
Bovine eye	Suspension	2.001 ± 0.006	5.0 ± 0.4	—	*62*
Bovine eye, choroid	Suspension	2.0039	4.8	8×10^{17}	*63*
Bovine eye	Dried	2.001 ± 0.006	5.0 ± 0.4	—	*62*
Bovine eye, choroid	Dried	2.0043	4.9	3.6×10^{18}	*63*
Bovine eye, choroid	Suspension	2.0040 ± 0.0001	4.9 ± 0.1	—	*24*
Bovine eye, iris	Dried	2.0048 ± 0.0005	6.2 ± 0.2	10^{16}	*64*
Bovine eye, pigment epithelium	Dried	2.0046 ± 0.0005	6.2 ± 0.2	10^{16}	*64*
Pig eye, iris	Dried	2.0044 ± 0.0005	6.2 ± 0.2	10^{16}	*64*
Pig eye, pigment epithelium	Dried	2.0044 ± 0.0005	6.0 ± 0.2	10^{16}	*64*

(*continued*)

TABLE III *(Continued)*

Melanin	Sample form[a]	g-Value	Line width (G)	Spins (g)	Reference
Guinea pig eye, iris	Dried	2.0040 ± 0.0005	7.3 ± 0.2	10^{16}	*64*
Hen eye, iris	Dried	2.0042 ± 0.0005	6.3 ± 0.2	10^{16}	*64*
S-91 mouse melanoma	Suspension	2.0039 ± 0.0006	4–5	7×10^{16}[d]	*65*
S-91 mouse melanoma	Intact	[c]	—	—	*66*
Harding–Passey mouse melanoma	Dried	2.0031 ± 0.0005	4–8	—	*41*
Harding–Passey mouse melanoma	Intact	[c]	—	—	*66*
B16 mouse melanoma	Intact	[c]	—	—	*66*
Wool, HCl extraction	Dried	2.0036 ± 0.0002	5.4 ± 0.2	—	*67*
Wool, phenol and thioglycollic acid extraction	Dried	2.0038 ± 0.0002	5.5 ± 0.2	—	*67*
Wool, enzyme digestion	Dried	2.0040 ± 0.0002	8.0 ± 0.2	—	*67*
Chicken feathers	Dried	—	8.6	—	*68*
Human hair	Intact	2.0034 ± 0.0006	5.1 ± 0.4	—	*69*
Human hair, black	Intact	2.0037 ± 0.0002	5.2 ± 0.2	—	*67*
Human hair, black	Dried	2.0043 ± 0.0001	4–8	—	*41*
Human hair, black	Intact	2.003	—	4.7×10^{16}	*16*
Human hair, black	Intact	2.004	—	$4.5–36 \times 10^{16}$	*70*
Human hair, black	Intact	2.003–2.004	4.8 ± 0.2	—	*71*
Human hair, black	Intact	[c]	6.8	—	*72*

Human hair, dark brown	Intact	2.003	—	2.8×10^{16}	*16*
Human hair, medium brown	Intact	2.003	—	4.6×10^{15}	*16*
Human hair, gray	Intact	2.003	—	3.1×10^{15}	*16*
Human hair, dark red	Intact	2.003	—	1.8×10^{15}	*16*
Human hair, fair	Intact	2.003	—	1.1×10^{15}	*16*
Human hair, blond	Intact	2.003	—	4.3×10^{14}	*16*
Rabbit hair	Dried	—	8.2	—	*68*
Human skin, Negro	Intact	2.003–2.004	5.6 ± 0.2	—	*71*
Human skin	Intact	2.004 ± 0.001	6 ± 1	—	*73*
Human comedone	Intact	2.0034 ± 0.0006	5.2–6.6	—	*69*
Human Substantia Nigra	Suspension	2.005	11.7	—	*43*
Human Substantia Nigra	Dried	~2.003	5.9 ± 0.2	—	*64*
Ustilago Maydis	Dried	—	6.0	—	*68*
Nadsoniella Nigra	Dried	~2.003	5–6	$3–16 \times 10^{18}$	*74*
Sunflower seeds	Dried	—	8.8	—	*68*
Luffa Cylindrica seeds	Dried	—	8.6	—	*68*
Potato	Dried	2.0040 ± 0.0001	4–8	—	*41*
Sheep liver pigment[e]	Dried	—	8.26	—	*75*

[a] Neutral pH assumed unless otherwise stated; measurements usually made either at ambient or at −196°C.

[b] Calculated from data in reference cited.

[c] "Close to free electron value" (2.0023).

[d] Spins per gram of homogenate.

[e] Suggested to be melanin.

TABLE IV Electron Spin Resonance Data for Synthetic Melanins

Melanin	Sample form[a]	g-Value	Line width (G)	Spins (g)	Reference
L-Dopa, autoxidation	Dried	2.0037 ± 0.0002	5.0 ± 0.2		*67*
L-Dopa, autoxidation	Dried	2.0038	4–8		*41*
L-Dopa, persulfate oxidation	Suspension	—	—	6.2×10^{17} [b]	*76*
L-Dopa, persulfate oxidation	Dried	—	—	6×10^{17}	*77*
L-Dopa, enzymatic oxidation	Dried	2.0038 ± 0.0002	5.8 ± 0.2	—	*67*
L-Dopa, enzymatic oxidation	Suspension	—	~6[b]	—	*43*
D-Dopa, autoxidation	Dried	2.0038	4–8	—	*41*
D-Dopa, enzymatic oxidation	Dried	2.0038	4–8	—	*41*
DL-Dopa, autoxidation	Suspension pH 5.5	2.0034 ± 0.0001	3.9 ± 0.1	—	*24*
DL-Dopa, autoxidation	Suspension pH 1–2	2.0035	4.6	6.6×10^{17}	*15*
DL-Dopa, autoxidation	Suspension	2.003–2.004	4.8 ± 0.2	—	*71*
DL-Dopa, enzymatic oxidation	Suspension	2.003 ± 0.001	~6.5[b]	4×10^{17}	*16*
DL-Dopa, enzymatic oxidation	Dried	—	—	2×10^{18}	*56*
L-Tyrosine, enzymatic oxidation	Dried	2.0040 ± 0.0002	6.4 ± 0.2	—	*67*
Dopamine, rat liver mitochondrial oxidation	Suspension	—	~6[b]	—	*43*
L-Epinephrine, enzymatic oxidation	Dried	2.004	4–8	—	*41*
L-Epinephrine, autoxidation	Dried	2.0038	4–8	—	*42*

L-Epinephrine, rat liver mitochondrial oxidation	Suspension	[c]	[c]	—	*43*
L-Norepinephrine, rat liver mitochondrial oxidation	Suspension	—	~9[b]	—	*43*
Catechol, autoxidation	Dried	2.0038	4–8	—	*41*
Catechol, autoxidation	Suspension pH 1–2	2.0031	3.4	1×10^{18}	*15*
Catechol, autoxidation[d]	Dried	—		2.3×10^{18}	*56*
Catechol, enzymatic oxidation	Dried	2.0038	4–8	—	*41*
Resorcinol, autoxidation	Dried	2.0036	4–8	—	*41*
p-Hydroquinone, autoxidation	Dried	2.0040	4–8	—	*41*
p-Hydroquinone, autoxidation[e]	Suspension pH 9	2.0040 ± 0.0001	2.9 ± 0.1[f] 3.1 ± 0.1[g]	—	*78*
p-Hydroquinone, enzymatic oxidation	Dried	2.0037	4–8	—	*41*
Adrenochrome, autoxidation[e]	Suspension pH 9	2.0040 ± 0.0001	3.3 ± 0.1[f] 3.6 ± 0.1[g]	—	*78*
	Suspension pH 9	2.0037 ± 0.0001 2.0038 ± 0.0001	2.0 ± 0.1[f] 2.3 ± 0.1[g]	—	*78*
Tyramine, rat liver mitochondrial oxidation	Suspension	[c]	[c]	—	*43*
Serotonin, rat liver mitochondrial oxidation	Suspension	[c]	[c]	—	*43*
Tryptamine, rat liver mitochondrial oxidation	Suspension	[c]	[c]	—	*43*

[a] Neutral pH assumed unless otherwise stated; measurements usually made either at ambient or at −196°C.
[b] Calculated from data in reference cited.
[c] Spectra "similar to other melanins"
[d] Autoxidation in the presence of amino acids.
[e] Simultaneous irradiation with light.
[f] N_2 atmosphere.
[g] O_2 atmosphere.

appears to suffer from their having been carried out under very different experimental conditions. Although one must not overlook the possibility of changes with pH (Section III,C) and perhaps with temperature, several measurements on hydrated suspensions of both natural and synthetic melanins fall within the range $4–10 \times 10^{17}$ spins/g and appear to be fairly reliable. Recent data [*15*] tend to support the hypothesis that under a given set of experimental conditions, the spin concentrations of hydrated melanins from bovine eyes, from dopa, and from catechol are quite similar. Drying is reported [*16, 63*] to increase the spin concentration by factors of 5–9.

B. Relaxation Data

Knowledge of the spin-lattice relaxation time T_1 of free radicals in melanin is expected to be useful in several ways: it may further assist in characterizing melanins and perhaps distinguishing between them; it may provide evidence for existence of different radical types or pools within a particular sample; it would aid in the design and interpretation of various more sophisticated experiments, including endor and eldor; and, finally, there is an interest in relaxation mechanisms in such heterogeneous systems with both liquid- and solidlike properties. It should be noted that measurements of the intrinsic T_1 should be made in the absence of paramagnetic metal ions and molecular oxygen, both of which can induce free-radical spin-lattice relaxation.

Knowledge of the transverse relaxation time T_2 would permit inferences to be drawn concerning aggregation or nonuniform distribution of spins and possibly concerning restricted rotational motions of the free radicals.

However, direct measurements of these parameters are rarely attempted. Instead, progressive saturation experiments are often carried out on melanin samples. The experimental parameter $P_{\frac{1}{2}}$ is determined, which is the microwave power at which the signal is half as great as it would be in the absence of saturation. Such experiments yield information on the product T_1T_2. It is not straightforward, and indeed it may not be possible, to relate $P_{\frac{1}{2}}$ to this product T_1T_2 in a rigorous theoretical manner, nor can T_1 and T_2 be separated. This is because there are simply too many uncertainties including: motional effects, nonuniform radical distribution, cross-relaxation, anisotropic magnetic interactions, the structure of the inhomogeneous line, and so-called "spin-flip" lines involving simultaneous change of electron and solvent proton quantum numbers.

Sarna and Hyde [*79*] have reported saturation recovery measurements on bovine eye melanin preparations in frozen media and in dried samples. They obtained a value of about 10 μsec for T_1. In these same samples, the

parameter $P_{\frac{1}{2}}$ was found to vary by an order of magnitude, while T_1 varied by a factor of 2. It was therefore argued that the combination of saturation-recovery and progressive-saturation experiments gave good evidence for a wide variation of transverse relaxation times, presumably because of variations in average and local radical concentrations. Unfortunately, it was not possible in these studies with the available equipment to make measurements on fully hydrated samples in the liquid phase. Thus, to this date no firmly based experimental information exists on T_1 and T_2 in melanin under normal physiological conditions.

It is found that oxygen-saturated melanin suspensions show microwave power saturation of the free-radical signal at higher incident power than do air-saturated or deoxygenated samples, and that differences in this oxygen-induced relaxation exist between samples of different types. No changes in free-radical line shape or total free-radical content because of the presence of oxygen have been observed. It is not clear whether the magnetic interactions giving rise to the effects are Heisenberg exchange or dipole–dipole, nor is it clear whether oxygen is in solution or adsorbed in some way on the melanin surface.

Despite these uncertainties, microwave power-saturation measurements provide a convenient qualitative indication of oxygen concen-

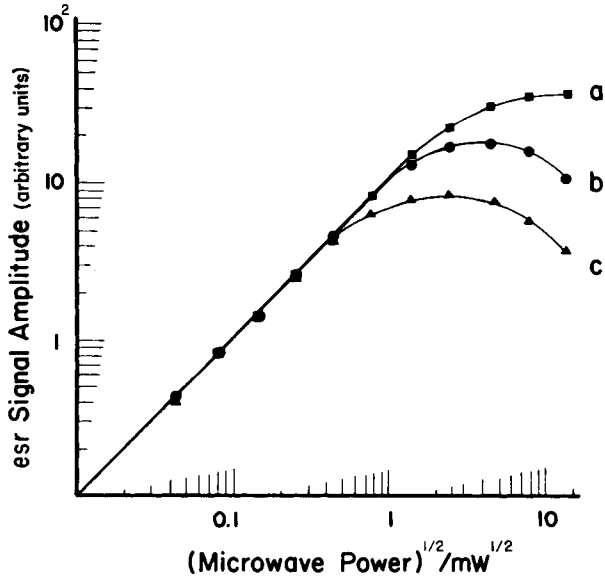


Fig. 3. The esr signal amplitude of the intrinsic free radical in: (a) oxygen saturated (■); (b) air saturated (●); and (c) deoxygenated (▲) aqueous suspensions of dopa-melanin as a function of microwave power. (From [*80*].)

tration in melanin. An illustration of the effect of oxygen on the saturation of radicals in synthetic dopa-melanin is given in Fig. 3 [*80*]. In this plot of esr signal amplitude versus (microwave power)$^{\frac{1}{2}}$, deviation from linearity indicates the occurrence of microwave power saturation. For synthetic melanin at 25 °C it has been found [*80*] that the following relationship holds: $P_{\frac{1}{2}}$ (mW) = 2.5 + 0.57 (%O_2), where 100% O_2 corresponds to 1 atmosphere of pure oxygen over the aqueous melanin suspension. Thus one has a means of monitoring changes in oxygen concentration directly in the sample as it is situated in the cavity of the esr spectrometer. This technique has been used [*80*] to follow depletion of oxygen in photoirradiated systems.

C. Effects of pH

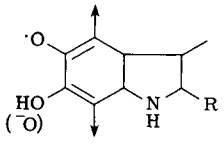
It was the consensus from early studies that the melanin free-radical population is chemically inert and/or is inaccessible to a wide variety of chemical reagents [*41*]. This view no longer seems wholly tenable, in that at least part of the free-radical content of melanin suspensions evidently results from an equilibrium that can be altered by various chemical treatments. The first evidence for this was the reported increase in free-radical concentration with pH [*46*, *81*], which was attributed [*56*] to the operation of an equilibrium as in (1).

$$\sim\!\!Q + H_2Q\sim\!\!\sim \rightleftharpoons 2\sim\!\!Q^{\cdot -} + 2H^+ \qquad (1)$$

Very similar effects were observed in humic acids [*56*] which, as indicated above, fall within the allomelanin class. While the possibility of an alternative interpretation in terms of a chemical reaction at high pH involving oxidation by molecular oxygen (cf. *p*-hydroquinone and related systems [*82*]) was not considered in these early experiments, later work has ruled out this possibility: the increase in radical concentration does not have an oxygen requirement [*47*]. It should be noted that acid–base equilibria of the hydroquinone will be important in determining the increase in radical concentration (again cf. humic acids [*83*]). Very recent work [*63*] on natural melanins has confirmed the above findings: ratios of radical concentrations in aqueous suspensions at neutral pH, in 1 *M* HCl, and in 1 *M* NaOH are 1:0.5:7, respectively.

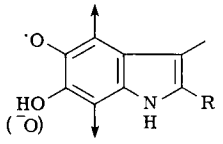
This work also established [*63*] that small spectral changes occur as the pH is varied. Careful measurements of *g*-value and line width of free radicals in natural melanin preparations from bovine eyes indicated differences among acid, neutral, and alkaline media. It seems likely that different acid–base forms of the intrinsic free radicals exist at these different pH's, although assignments to specific species were not at-

tempted. Readily ionizable groups of melanin radicals should be semiquinone and (if present) carboxylate functions. Values of pK_a between 3.7 and 4.8 have been reported [*84*] for several *o*-semiquinones from pulse radiolysis measurements employing optical detection of transients. These values show the expected [*85*] correlation with the redox potential of the parent carbonyl compound. Accessible melanin semiquinones will probably behave in a similar fashion, ionizing around pH 4–5. Some of the free-radical species that may be present over a range of pH are shown in structures **1–3**, where arrows denote possible sites of attachment to other monomer units within the polymer.



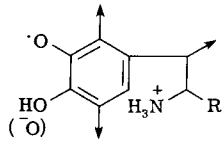
1

R = H, CO_2H, CO_2^-



2

R = H, CO_2H, CO_2^-



3

Radical heterogeneity may be chemical, as expressed in the above structures, or physical, e.g., otherwise identical free radicals having different accessibility to solvent, chemical reagents, etc., or a combination of the two. This is a problem which remains to be satisfactorily resolved. There are certainly indications that more than one species is present. For example, Grady and Borg [*86*] have argued from data on dopa-melanin obtained at Q-band (35 GHz) frequencies (it is often advantageous to undertake esr measurements at more than one microwave frequency in order to resolve spectral features) that the spectrum is a combination of overlapping spectra from two species, only one of which is affected by changes in the pH. It seems that at least two pools of $\sim\!\!\sim\dot{Q}^-$ free radical may be present within the polymer, some of which are largely inaccessible unreactive species, while others are in equilibrium with diamagnetic precursors.

Experiments as a function of temperature and melanin dilution may assist in the differentiation between different types of radical and may improve our understanding of the nature of the interactions (intra- or intermolecular) that give rise to radicals.

IV. INTERACTION WITH METAL IONS

The information that one can obtain using diamagnetic and paramagnetic metal ions is to some extent complementary. With diamagnetic ions one

can study the interaction of metals with paramagnetic (i.e., free-radical) sites within the polymer, whereas paramagnetic ions largely report on their interactions with diamagnetic (nonfree-radical) sites. In each case information comes from the metal ion complexes that are formed. The interaction of paramagnetic ions with free-radical sites is a more complex problem but does provide an approach to investigate the distribution of metal ions with respect to the free radicals.

A. Interaction of Free Radicals with Diamagnetic Ions

The interaction of free radicals with diamagnetic ions has been the subject of some confusion. The first report of an effect of diamagnetic metal ions on melanin free-radical spectra was made by Sarna and Łukiewicz [*87*], who worked with denatured amphibian eggs. They found a significant increase in esr signal intensity in the presence of zinc and cadmium ions. Although subsequent investigation [*88*] of the effect of zinc on radicals in dried dopa-melanin suggested that the esr signal was unchanged, further work on hydrated suspensions has demonstrated [*24*] that there are indeed increases in esr signal intensity in both dopa-melanin and bovine eye melanin, and that these increases do reflect increases in radical concentration. Table V shows data obtained from hydrated aqueous suspensions at pH 5.5 in the presence of a variety of di- and tripositive metal ions. Increases in concentration in the synthetic polymer are seen to be paralleled by those in the natural material.

Concomitant with the changes in concentration were striking changes in

TABLE V Induction of Free Radicals by Diamagnetic Metal Ions[a]

	Enhancement[b]	
Metal ion	Bovine eye melanin	Dopa-melanin
Mg^{2+}	1.0	
Ca^{2+}		1.5
Sr^{2+}		1.2
Zn^{2+}	3.1[c]	6.6[c]
Cd^{2+}	1.6[c]	3.8[c]
Al^{3+}	3.0	6.7
Sc^{3+}	4.1	9.0
La^{3+}	3.9	7.8
In^{3+}	3.3	7.3

[a] From Felix *et al.* [24].

[b] Integrated signal intensity after incubation with 2.1 m*M* metal ions relative to that in the absence of metal ions. Measurements made on aqueous suspensions at pH 5.5 and −196°C.

[c] Average of values obtained with different isotopes.

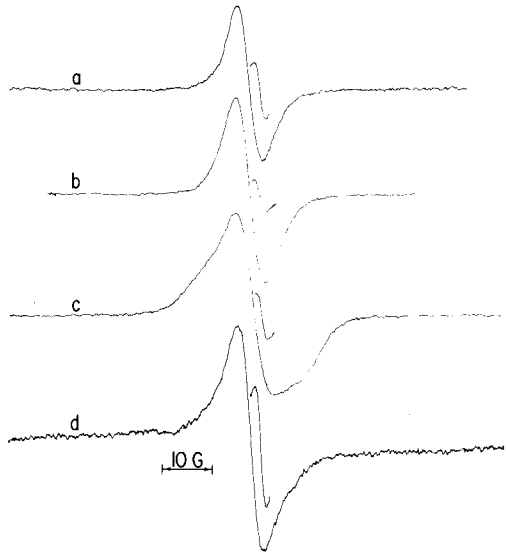


Fig. 4. The esr spectra for bovine eye melanin before and after incubation with 2 m*M* metal ions at pH 5.5: (a) control; (b) Al^{3+}; (c) La^{3+}; (d) In^{3+}. Additional traces shown are those of the pitch standard used for calibrating the spectra. Relative gains are (a) × 1.6; (b) × 1; (c) × 1.25; (d) × 3. (From [*24*].)

free-radical line shape (Fig. 4) which varied with metal ions. The intrinsic free-radical signal was observed to decrease, and additional, broader spectra appeared with the ions Al^{3+}, La^{3+}, and In^{3+}, each of which possesses a nuclear moment. The extent of broadening increased along the series. Confirmation that the effect arises from interaction of the radical with the nuclear moment of the metal ion came from experiments with magnetic and nonmagnetic isotopes of cadmium and zinc: spectra were significantly broader with the magnetic isotopes (Table VI). Calculations based on the extent of broadening indicated a metal ion–free radical separation of about 1–2 Å, sufficiently short for the complex formation expected by analogy with model compounds (e.g., *o*-benzosemiquinone) in solution, for which chelate formation is highly favored [*89*]. Moreover, the spectrum in the presence of the magnetic isotope ^{113}Cd ($I = \frac{1}{2}$) could be simulated from that in the presence of ^{114}Cd ($I = 0$) taking an isotropic coupling to the metal ion of 3.2 G. Formation of complexes of type **4** was therefore proposed [*24*].

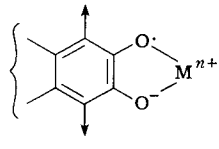


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TABLE VI Effect of Diamagnetic Metal Ions on Electron Spin Resonance Parameters of Melanin Free Radicals[a]

Melanin	Metal ion [magnetic isotope, μ, I]	Line width[b] (G)	g^b
Bovine eye	None	4.9	2.0040
	Zn^{2+} [^{67}Zn (4%), 0.87, $\frac{5}{2}$]	4.9	2.0041
	Zn^{2+} [^{67}Zn (90%), 0.87, $\frac{5}{2}$]	6.0	2.0040
	Cd^{2+}[c]	5.0	2.0041
	Cd^{2+} [^{113}Cd (96%), 0.62, $\frac{1}{2}$]	6.3	2.0040
Dopa	None	3.9	2.0034
	Zn^{2+} [^{67}Zn (4%), 0.87, $\frac{5}{2}$]	4.5	2.0040
	Zn^{2+} [^{67}Zn (90%), 0.87, $\frac{5}{2}$]	5.0	2.0038
	Cd^{2+}[c]	4.3	2.0039
	Cd^{2+} [^{113}Cd (96%), 0.62, $\frac{1}{2}$]	5.5	2.0037

[a] From Felix *et al.* [24].
[b] Data are for frozen aqueous suspensions at −196°C.
[c] Isotopes >99% with $I = 0$.

Complex formation indicates that some of the melanin radicals are accessible to chemical reagents, while radical induction shows that they are capable of interacting with each other in an equilibrium with other monomer units that can be modified, in this case by complexing metal ions as in (2). The equilibrium concept is an important one that is essential to explain radical induction by metal ions with little or no ability to engage in one-electron-transfer reactions. While the oxidized and reduced counterparts to the semiquinone (i.e., ~~Q and ~~QH_2 units) seem by far the most likely species to be involved in the equilibrium (cf. the analogous equilibrium between flavin semiquinones and oxidized and reduced flavins [*90*]), in principle any species having a formal oxidation–reduction state equivalent to a pair of free radicals (e.g., a radical dimer) could account for these observations.

$$\sim\!\!\sim Q + H_2Q\sim\!\!\sim \rightleftharpoons 2H^+ + 2\sim\!\!\sim Q^{\cdot} \overset{2M^{n+}}{\rightleftharpoons} 2\sim\!\!\sim Q^{\cdot}\,M^{n+} \qquad (2)$$

The extent of radical induction with a particular metal ion was found to depend on the propensity of that ion for complexation. Large increases in radical concentration were found for Zn^{2+}, Al^{3+}, Sc^{3+}, La^{3+}, and In^{3+}, while small or zero increases were found for Ca^{2+} and Sr^{2+}. However, the esr line shape of radicals in dopa-melanin was found to change slightly in the presence of these latter ions to approach that found for the complexes. It was proposed that the change in line shape could still arise from complexation if reactions with Ca^{2+} and Sr^{2+} are significantly reversible. Another possibility is that attachment of metal ions to nonradical sites on

the polymer lowers the effective pK_a of the free radicals. This might account for the observation that the esr spectrum in the presence of these ions resembles that found at higher pH values where one presumes that the semiquinone anion dominates.

The demonstration of complex formation is perhaps the best evidence to date that the free radicals are indeed *o*-semiquinone species. *p*-Semiquinones are structurally unable to chelate [*89*] and do not form stable complexes in aqueous media [*91*].

B. Interaction of Free Radicals with Paramagnetic Ions

While addition of diamagnetic metal ions to melanin preparations *increases* the number of free radicals by an equilibrium shift as expressed in eq (2), addition of paramagnetic metal ions is observed to *decrease* the apparent free-radical signal intensity almost to zero without noticeable change in the free-radical line shape.

It seems very likely that paramagnetic metal ions will also induce free radicals, but that the extremely close proximity (1–2 Å) of the free radical and paramagnetic metal ion in the complex will result in such strong magnetic interaction that the esr signal from the induced free radicals will be undetectable. In addition, equilibrium (2) may lead to a *reduction* of the signal intensity of *uncomplexed* free radicals, but decreases observed for diamagnetic metal ions are small [*24*]. Thus one must look for another explanation for the observed decrease.

Blois *et al.* [*41*] first observed this effect when adding copper to melanin, and suggested that a chemical reaction occurred between free radical and copper. Sarna *et al.* [*40*], however, have established that this observed decrease in signal intensity was largely magnetic in character. A key experiment in this paper was the comparison of effects of La^{3+} (diamagnemetic and Gd^{3+} (paramagnetic), which have similar chemical properties. At concentrations similar to those employed by Blois, addition of Gd^{3+} nearly eliminated the free-radical signal, while no decrease was found for La^{3+}.

Leigh [*92*], using Redfield theory, calculated the effect of the A term of the dipolar Hamiltonian for paramagnetic metal–free radical interaction on the free-radical line shape, where the correlation time was T_{1m}, the spin-lattice relaxation time of the metal ion. His calculation was for a single ion–radical pair at a fixed distance. He showed that under an appropriate range of conditions the free-radical signal could be observed to appear to decrease in intensity with little *apparent* change in line shape. The word apparent is important: theoretically, there is no change in the

total number of free radicals. Sarna *et al.* [*40*] suggested that the same argument must also be valid in melanin, although the circumstances are somewhat different: more than one metal ion can interact with each free radical, there is no single radial distance, and there is no fixed orientation of the radial vector with respect to the free-radical molecular coordinate system. Hyde and Rao [*93*], using the Van Vleck "method of moments" on the *B*, *C*, *D*, *E* and *F* terms of the dipolar Hamiltonian, calculated both $P_{\frac{1}{2}}$ and saturation recovery parameters when more than one metal interacts with a particular free radical at varying radial distances. The conclusion that the effects are always dominated by the "distance of closest permissible approach" seems quite general. Dipolar-induced changes in $P_{\frac{1}{2}}$ were found to depend linearly on the concentration of metal ions and on the third power of the minimum possible distance between ion and radical. While the application of the method to the *A* term of the dipolar Hamiltonian has not been attempted, it seems likely that the Leigh-type apparent loss of free-radical signal intensity will also be dominated by the "distance of closest approach."

Leigh's theory predicts that the apparent free-radical signal intensity should increase as T_{1m} decreases, which was experimentally observed in the melanin system [*40*]. It was found that as the Leigh-type effect decreases because of short T_{1m}'s, there was a corresponding increase in $P_{\frac{1}{2}}$ of the free radical. The elements of the lanthanide series other than Gd^{3+} were particularly effective in increasing $P_{\frac{1}{2}}$, presumably because of their high magnetic moments and the fact that $\omega T_{1m} \sim 1$, where ω is the microwave frequency. The ions Dy^{3+} and Tm^{3+} were the most effective inducers of spin–lattice relaxation of the metals investigated. Typical data are given in Table VII.

For fast-relaxing metal ions, plots of $P_{\frac{1}{2}}$ versus metal ion concentration showed sigmoidal character, yielding a value of 6×10^{20} metal binding sites per gram of dried material. Similar results were obtained with slow-relaxing metal ions (Cu^{2+}, Gd^{3+}), where free-radical signal height was plotted. Competition experiments between diamagnetic metal ions and paramagnetic metal ions can be carried out [*40*]. One observes either $P_{\frac{1}{2}}$ or the free-radical signal after incubation with one type of ion and then carries out a titration with the other type. Competition for metal ions between melanin and metal ion chelators such as EDTA also can be observed using the free-radical resonance characteristic as a convenient passive indicator. In this way, it was established that melanin is a very effective binder of metal ions, and that competition between melanin and EDTA for metal ions may take several hours to reach equilibrium. Although such competitions afforded some evidence [*40*] for the existence of several different kinds of binding site, the chemical nature of these sites

TABLE VII Effect of Paramagnetic Metal Ions on Free Radical Signal Amplitude and Microwave Saturation Parameters, $P_{\frac{1}{2}}$, for Bovine Eye Melanin[a]

Metal ion	Concentration (mM)	Amplitude[b]	$P_{\frac{1}{2}}$ (mW)
None	—	1.0	1.3
Mn^{2+}	0.5	0.37	22.5
Gd^{3+}	0.5	0.40	16.0
Gd^{3+}	1.5	0.05	—
Cu^{2+}	0.5	0.70	9.0
Cu^{2+}	1.5	0.20	—
Ni^{2+}	5	0.17	11.0
Ho^{3+}	5	0.30	112
Tm^{3+}	5	0.29	400
Dy^{3+}	5	0.26	315
Er^{3+}	5	0.35	200
Co^{2+}	5	0.33	141
Nd^{3+}	5	0.40	56
Sm^{3+}	5	0.38	24
Pr^{3+}	5	0.48	20

[a] From Sarna *et al.* [40].

[b] Measurements made on aerated aqueous suspensions at −150°C.

could only be speculated on. However, rather detailed information on diamagnetic binding sites is possible through use of the spin-probe technique which we next describe.

C. Paramagnetic Ions as Spin Probes

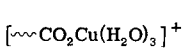
There are four paramagnetic ions that exhibit esr spectra under nearly all conditions of temperature and motion: Cu^{2+}, VO^{2+}, Mn^{2+}, and Gd^{3+}. Of these, the first two have been used as probes of metal-ion binding sites. Each prefers square-planar coordination, with oxygen and nitrogen the common ligands. The g-values and metal hyperfine couplings as revealed in the powder esr spectra provide useful (but not definitive) information on ligands and charge of the complex. Assignments are usually based on $g_{\|}$ and $A_{\|}$ values (H_0 parallel to the normal of the plane). Occasionally, superhyperfine interaction with ^{14}N can be observed in the $g_{\perp}$ region.

The first attempt to use such an approach was made by Blois *et al.* [*41*]. They showed that copper bound to squid melanin displayed a spectrum with $g_{\perp} = 2.073$, $g_{\|} = 2.27$–2.31, and $A_{\|} = 348$–378 MHz, and concluded that the metal was bound to four oxygens, four nitrogens, or two of each, and also that the binding was ionic rather than covalent. Both natural and synthetic melanins (and melanin precursors) have now been studied in some detail [*15, 47*]. Use of a single copper isotope (^{63}Cu) was found to

greatly facilitate spectral analysis. Redox reactions of Cu^{2+} were not of major importance under the conditions of the experiments.

Effective metal-ion binding sites in natural melanin are expected [*24*] to be hydroquinone, amino acid, and carboxylate moieties whose acid–base properties should result in a strong pH dependence of metal binding. The esr spectra of copper complexes with all materials were therefore studied [*15*] as a function of pH. Chemical degradation experiments [*2*] had shown that a major portion of synthetic (dopa, catechol) melanin monomers remains intact and possesses the same potential binding sites as their precursors. Thus the experiments with the precursors provided a basis for analysis of results with synthetic melanins, which in turn served as a basis for studies of natural melanin. Well-resolved spectra in the $g_{\|}$ range permitted resolution of overlapping spectra from as many as three types of complexes.

It was concluded from this work [*15*] that copper can form a number of complexes with melanins, depending on the pH of the system. In general, only one or two ligands to copper originate from melanin—the others are presumably H_2O or OH^- groups. At low pH, binding appears to be mainly to carboxylate groups, while up to pH 7 interaction with amino acid sites predominates. At pH 7 and above, synthetic dopa-melanin shows a resonance attributable to copper bound to hydroquinone, but this resonance is weaker in the natural material. Complexes of types **5–7** were suggested. Reduction of the polymer with sodium dithionite gave esr spectra only of those copper sites that are most resistant to reduction [copper (I) is of course diamagnetic]. Under these conditions, both natural and synthetic materials gave resonances showing superhyperfine coupling to one or more nitrogen ligands. No major effect of associated protein on the copper-binding properties of the melanin was found in these studies.



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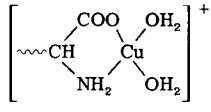

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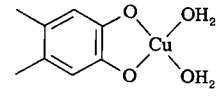

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V. INTERACTION WITH LIGHT

While the concept of an equilibrium between radicals and quinone and hydroquinone moieties was initially invoked to account for the effects of base and diamagnetic metal ions, it is possible to view not only thermal changes but also photolytic changes as being derived from a shift in the position of a fundamental equilibrium. But first one must consider how light is absorbed by melanin.

A. Light Absorption

Melanin absorbs strongly throughout the ultraviolet (uv) and visible (vis) regions of the electromagnetic spectrum. Published spectra [*41*, *68*] show broad structureless absorption bands increasing toward shorter wavelengths. As Blois has pointed out [*22*], organic substances that are black in color can arise where the material has low-lying conduction bands or where charge-transfer interactions predominate, as in quinhydrones. Thus the quinhydrone complex formed between *p*-benzoquinone and *p*-hydroquinone has [*94*] a strong absorption in the visible (λ_{max} = 440 nm; ϵ_{max} = 890 M^{-1} cm^{-1}) that is not present in the isolated molecules. Simple intramolecular complexes [*95*] show similar behavior. Consistent with complex formation is the previously mentioned finding by Thathachari and Blois [*25-27*] of some short-range order, which was taken to indicate stacking of aromatic units to form complexes. Also, by analogy with polyvinyl hydroquinone–quinone polymers [*18*], such interactions are expected to be facile. The fact that free radicals can be generated by light extending from the uv into the visible is consistent with the hypothesis that they are derived from quinhydrone ~~Q...H_2Q~~ or similar complexes.

However, it is clear that additional physical data to confirm the presence of complexes are highly desirable. This appears to be an area in which advances can be made using optical spectroscopy, provided that technical problems with light scattering can be overcome. Certainly, the possibility of detecting intra- and intermolecular complexes similar to those found in synthetic redox polymers has not been explored.

B. Transient Free Radicals

The suggestion that additional free radicals may be induced by light was first made by Mason and co-workers [*16*], who studied the free-radical signal in hair. This was disputed by others who claimed either that the effect was thermal [*96*] or that it could be accounted for by a change in microwave saturation of the free radical [*97*] resulting in an increased esr signal without a change in radical concentration.

There has since appeared an overwhelming number of reports of free-radical induction by light in other melanin preparations (Table VIII). While it must be recognized that the magnitude of the effects observed will depend on such factors as sample geometry and irradiation time, which are not always specified, several conclusions can be drawn from these data. Thus, it is clear that, even after allowing for thermal effects and changes in microwave saturation, radicals are induced in both intact

tissue and in hydrated melanin suspensions by wavelengths throughout the visible and uv. These induced radicals are at least partly stabilized at cryogenic temperatures. With the possible exception of hair, radical induction in melanin preparations can be removed by ceasing irradiation and (for frozen samples) thawing. There is some indication that a change in microwave saturation may occur on irradiation and that radical induction may be greater in natural than in synthetic materials. The effect of light is suppressed in dried samples.

Work carried out in the mid 1960's by Cope and colleagues [*62, 100*] and by Stratton and Pathak [*71, 101, 102*] showed that the free radicals were transient at ambient temperature with a half-life of about 1 sec. However, several puzzling observations remained to be interpreted. Decay of the radicals at ambient temperature was reported [*62*] to be extremely complex, but the data were later [*103–105*] fitted to the Elovich equation. The transient radical concentration was suggested to depend on oxygen concentration, and the mechanism proposed for radical decay had an oxygen dependence. It was not clear how, if at all, the induced radicals differed from the intrinsic ones.

Recent measurements on deoxygenated samples of dopa-melanin and melanin from bovine eyes have shown [*106*] that the induced and intrinsic radicals do not have identical esr spectra. Computer subtraction of the intrinsic radical signal from the spectrum obtained during continuous illumination gave the data tabulated in Table IX. The light-induced radicals (a mixture, see below) have a composite spectrum characterized by a higher g-value and a somewhat broader line width, and they are more difficult to saturate with microwave power.

Time-resolved experiments were also carried out, with time resolution down to 1 msec. It was confirmed that a slowly decaying transient radical species is formed that decays within seconds (Fig. 5). However, measurements of its decay indicated second-order kinetic behavior over a wide range of concentration and temperature. No effect of oxygen on the radical concentration was found. The observation of second-order kinetics suggests a diffusive process, implying a significant degree of radical mobility in the system. [Mobility is also implied by the equilibrium expressed in eq (2).] The effective recombination rate constant is of the order of $10^4\ M^{-1}\ \text{sec}^{-1}$, similar to that obtained for the decay of hindered semiquinones [*107*] and for some other radicals in polymers [*108*]. The questions of polymer surface mobility and site interaction are discussed later. The quantum yield of this radical species is estimated [*106*] to be very low ($< 1\%$), indicating that its generation cannot account for very much of the absorbed light.

However, on the millisecond time scale, a much faster decaying com-

TABLE VIII Induction of Free Radicals in Natural and Synthetic Melanins by Light

Melanin	Sample form[a]	Irradiation	T (°C)	Enhancement[b]	Reference
Squid, A-, B-types	Dried	vis + uv	−196	1.0[c]	*97*
Frog, pigment epithelium	Suspension	vis	Ambient	~2.75[d]	*60*
Frog, pigment epithelium + retina	Intact	vis	−20	~2[c]	*59*
Frog, pigment epithelium	Intact	vis	−196	1.17–1.19	*59*
Frog, pigment epithelium	Suspension	vis	Ambient	1.75	*61*
Bovine eye	Dried	vis	Ambient	1.0	*62*
Bovine eye	Suspension	vis	Ambient	2–4[c]	*62*
S-91 Mouse melanoma	Suspension	vis	Ambient	2.5	*65*
S-91 Mouse melanoma	Suspension	vis	Ambient	1.25[d]	*98*
Human hair, black	Intact	λ = 366 nm	Ambient?	2.1	*16*
Human hair	Intact	vis + uv	Ambient	Increase	*67*
Human hair	Intact, dry	$\lambda \geq$ 320 nm	−196	1.4	*71*
Human hair	Intact, wet	$\lambda \geq$ 320 nm	−196	2.4	*71*
Human hair	Intact	$\lambda \geq$ 366 nm	Ambient	0.96–1.08	*96*
Wool	Dried	vis + uv	Ambient	1.0	*67*
Human skin	Intact	uv	−196	Increase	*99*
Human skin, Negro	Intact	$\lambda \geq$ 320 nm	−196	~2.0[c,d]	*71*
Human skin	Intact	vis	−196	1.5–2.2	*73*
Human substantia nigra	Suspension	vis	Ambient	1.6[d]	*43*
Nadsoniella nigra	Dried	uv	Ambient	1.0	*74*
Dopa, autoxidation	Suspension	$\lambda \geq$ 320 nm	Ambient	Increase	*71*
Dopa, autoxidation	Suspension	$\lambda \geq$ 320 nm	−173	3.5[d]	*71*
Dopa, autoxidation	Suspension	vis	Ambient	1.1–1.15	*62*
Dopa, autoxidation	Dried	$\lambda \geq$ 320 nm	−196	1.1	*71*
Hydroquinone, autoxidation[e]	Suspension pH 9	404–780 nm	Ambient	2.3[d,f] 1.7[d,g]	*78*
Adrenochrome, autoxidation[e]	Suspension pH 9	404–780 nm	Ambient	1.55[f] 1.56[g]	*78*
Purpurogallin, autoxidation[e]	Suspension pH 9	404–780 nm	Ambient	3.0[d,f] 2.7[d,g]	*78*

[a] pH ~7 unless otherwise indicated.
[b] Signal amplitude relative to that in the absence of light.
[c] Change in microwave saturation noted.
[d] Calculated from data in reference cited.
[e] Autoxidation in the presence of light.
[f] Nitrogen atmosphere.
[g] Oxygen atmosphere.

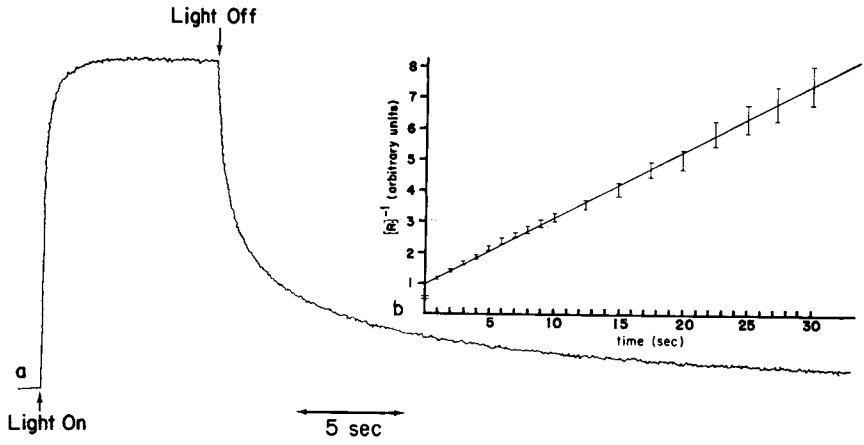


Fig. 5. (a) Slow kinetic behavior of transient free radicals in a neutral aqueous suspension of bovine eye melanin at 298°K from time-resolved esr measurements. Signal average of 28 scans. (b) Second-order plot of the data. $[R\cdot]$ = transient free radical concentration. (From [*106*].)

ponent was detected (Fig. 6). The yield of this component, which corresponds to ca. 50% of the steady-state signal obtained under continuous illumination, is 50–100 times that of the slow component. Thus, after taking account of the fast-decaying component, radical production may not be trivial. Although complex decay kinetics are frequently encountered in systems containing hindered phenoxyls and semiquinones and which can be accounted for [*107, 109*] by the formation of a metastable dimer, this is a rather different phenomenon. Preliminary experiments indicate that the two kinetic components are derived from independently decaying species. In contrast to a large temperature dependence of the slow decay, the fast decay displays little if any temperature dependence

TABLE IX Electron Spin Resonance Parameters for Intrinsic and Light-Induced Free Radicals in Natural and Synthetic Melanins at Ambient Temperature[a]

Melanin	$g \pm 0.0001$	Line width (G)	$P_{\frac{1}{2}}$[b] (mW)
Bovine eye			
Intrinsic radicals	2.0041	4.7 ± 0.1	7 ± 2
Light-induced radicals[c]	2.0045	5.4 ± 0.1	14 ± 2.4
Dopa			
Intrinsic radicals	2.0034	3.8 ± 0.1	2.5 ± 0.1
Light-induced radicals[c]	2.0039	3.9 ± 0.1	11 ± 1.3

[a] From Felix *et al.* [*106*].
[b] Data for deoxygenated aqueous suspensions.
[c] Steady-state mixture of slow- and fast-decaying species.

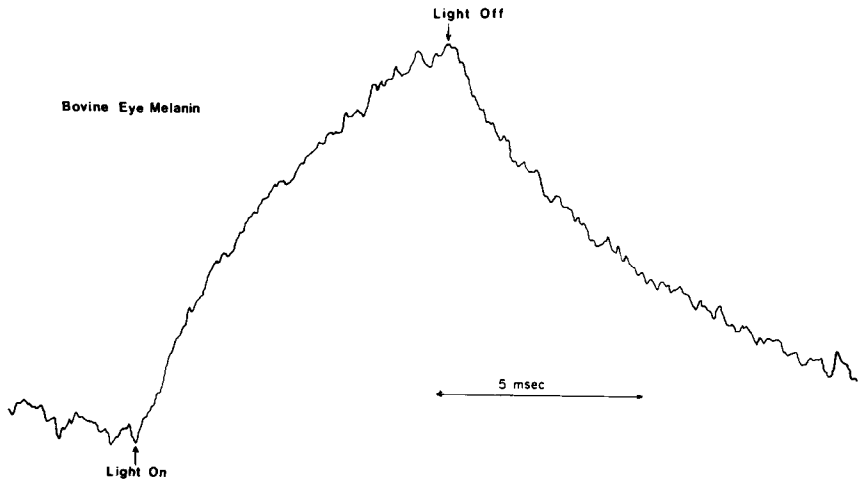


Fig. 6. Fast kinetic behavior of transient free radicals in bovine eye melanin at 298°K. Signal average of 5500 scans. (From [*106*].)

over the temperature range −196° to +80°C. This latter observation could suggest decay by an electron-tunneling mechanism [*110*] rather than by a diffusive process.

Both species are generated by light throughout the visible spectrum. The radical species detected in all previous experiments appears to be the one which decays slowly at ambient temperature and is stabilized at cryogenic temperatures. Further work is necessary to establish the nature of the two radical species. Their dependence on wavelength, pH, etc. may help clarify the situation.

Work with aerated samples has revealed an additional complexity. Changes in microwave saturation in a closed system following irradiation indicate [*80*] that oxygen is being removed, with the result that the mixture eventually becomes deoxygenated. This phenomenon may partly account for the reported [*62*] changes in microwave saturation behavior of irradiated samples. If one assumes that oxygen concentrations in aerated and oxygen-saturated melanin suspensions are those measured for water, then average rates of oxygen uptake are those shown in Table X. The average rate is approximately the same for both air- and oxygen-saturated solutions. It seems likely [*80*] that initial rates of oxygen uptake are higher, since the irradiated part of the sample cell will lose oxygen rapidly and become deoxygenated. Oxygen must then diffuse in from the unirradiated part if it is to react.

The mechanism of oxygen removal was studied by the addition of the enzymes catalase and superoxide dismutase, whose function is to destroy

TABLE X Oxygen Uptake from Dopa Melanin Suspensions during Irradiation with Visible Light[a]

Melanin suspension[b]	Oxygen uptake[c] (μM/sec)
Air saturated	2.2 ± 0.1
Air saturated + superoxide dismutase	2.0 ± 0.1
Air saturated + catalase	1.34 ± 0.05
Air saturated + catalase + superoxide dismutase	1.30 ± 0.05
Oxygen saturated	2.0 ± 0.1

[a] From Felix *et al.* [*80*].

[b] 10 mg/ml melanin at pH 7, ambient temperature. Irradiation with light of 320–600 nm from a 300 W high-pressure xenon lamp.

[c] Average rate of uptake over deoxygenation period.

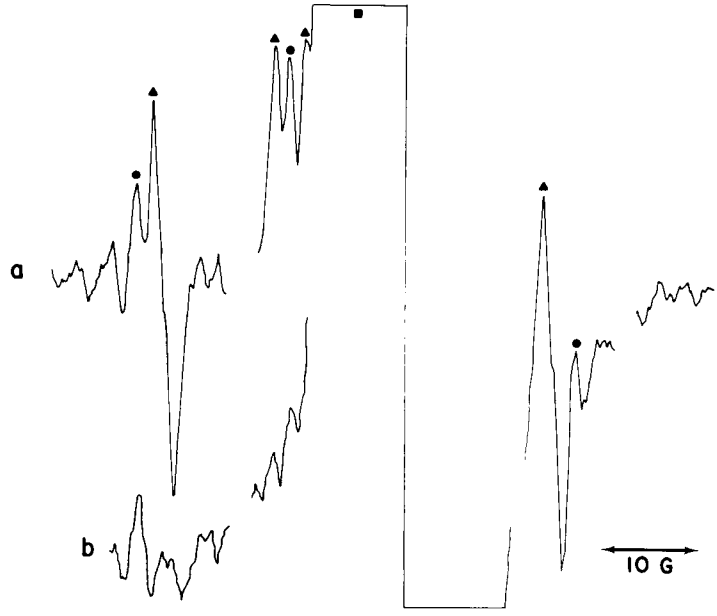
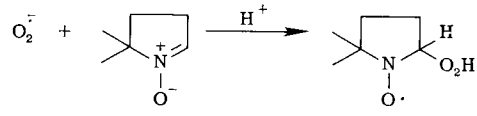


Fig. 7. The esr spectra of free radicals detected during photoirradiation of an oxygen-saturated aqueous suspension of dopa–melanin in the presence of the spin trap 5,5′-dimethylpyrroline-1-oxide. Lines marked ▲ are from the adduct of O_2^- with DMPO. The melanin resonance (■) is off scale in this display. (a) Without superoxide dismutase; (b) + superoxide dismutase. (From [*80*].)

hydrogen peroxide and to accelerate the disproportionation of superoxide as in eqs (3) and (4), respectively. A high conversion of oxygen to hydrogen peroxide was indicated by the finding that catalase reduces oxygen uptake by 40%. Changes in oxygen uptake with superoxide dismutase were much smaller, but are not inconsistent with generation of superoxide provided that production of the latter is the rate-determining step in oxygen removal; i.e., disproportionation of $O_2^{\cdot-}$ (presumably via $O_2^{\cdot-}$ and $\dot{O}_2H$) is relatively fast even in the absence of the enzyme. Supporting evidence for the mediation of superoxide came from spin-trapping experiments [*80*]. In this technique [*111*] a free radical that is difficult to detect directly by esr for magnetic reasons or owing to its low yield or chemical reactivity is added to a spin trap, usually a nitroso compound or nitrone, to give a relatively persistent spin-adduct nitroxide whose esr spectrum is characteristic of the addend. 5,5′-Dimethylpyrroline-1-oxide (DMPO) is a nitrone which is an excellent scavenger for $O_2^{\cdot-}$ (and/or $HO_2^{\cdot}$) [*112, 113*], as in eq (5). Addition of DMPO to a melanin suspension followed by continuous photolysis gave the spectrum shown in Fig. 7(a), where a number of the lines of the superoxide adduct are clearly visible (the others are masked by the melanin resonance). Confirmation of this assignment came from the absence of the spectrum in the presence of superoxide dismutase [Fig. 7(b)].

$$H_2O_2 \xrightarrow{\text{catalase}} H_2O + \tfrac{1}{2}O_2 \tag{3}$$

$$2O_2^{\cdot-} \xrightarrow[\text{dismutase}]{2H^+ \text{ superoxide}} H_2O_2 + O_2 \tag{4}$$



(5)

The *overall* quantum yield for oxygen removal and of the $O_2^{\cdot-}$ adduct is relatively low, probably because of local deoxygenation as outlined above. The spin-adduct concentration is likely to be further depressed owing to its reduction either by superoxide itself or transient melanin free radicals. There is also some evidence [*114*] to show that even in the absence of other radicals the spin-adduct lifetime is relatively short (ca. 30 sec at pH 7). The source of the reducing electron from the photoirradiated melanin could be the fast-decaying transient free radicals, although reaction of oxygen with the transient radicals has not yet been detected directly. The reversible equilibrium between transient semiquinones and oxygen [eq (6)] is known [*115*] from pulse radiolysis experiments employing optical detection.

It should be noted that no effect of oxygen on the concentration of the intrinsic radicals has been reported. In this instance, any equilibrium between the radicals and oxygen of the kind expressed in eq (6) must lie well to the left.

$$Q^{\cdot-} + O_2 \rightleftharpoons Q + O_2^{\cdot-} \tag{6}$$

C. Evidence for a Triplet State Precursor

Until very recently, no information has been available on how free radicals are generated in melanin on photoirradiation, although it seemed likely that photoexcited states were involved. However, information of this kind can often be obtained at short observation times via spin-polarization phenomena (chemically induced dynamic electron polarization, CIDEP) [*116, 117*]. These phenomena have been interpreted [*118*] in terms of two general mechanisms, the radical-pair mechanism (RP) and the triplet mechanism (TM). The latter, encountered in photochemical systems, is sometimes referred to as the photochemical mechanism. In the radical-pair mechanism, polarization arises through spin selection in radical recombination, whereas the triplet mechanism requires [*119*] the rapid chemical reaction of a spin-polarized triplet precursor. Figure 8 indicates the steps typically involved in the production of spin polarization in triplet and radical species. Light absorption promotes acceptor A to an excited singlet state, ^1A. Intersystem crossing to the triplet ^3A manifold results in a nonequilibrium population (i.e., spin polarization) of

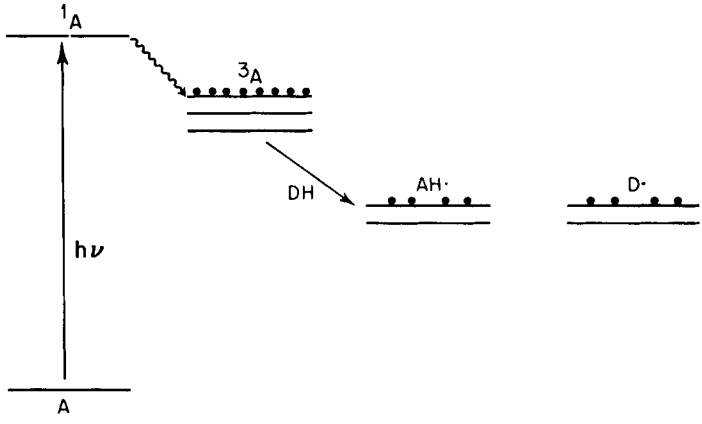


Fig. 8. Production of spin-polarized free radicals via the triplet mechanism. Preferential intersystem crossing from an excited singlet (^1A) to the nondegenerate Zeeman levels of the triplet (^3A) in the external magnetic field is followed by very fast chemical reaction which competes efficiently with spin-lattice relaxation in the triplet.

the triplet sublevels. This spin polarization is normally very rapidly degraded by relaxation processes. However, if chemical reaction with, e.g., a hydrogen atom donor DH is fast enough to compete effectively with this relaxation, the polarization will be carried over into free-radical products AH· and D·.

Experimental observations of spin polarization induced by the TM mechanism have mostly been in systems in which the radical population is allowed to build up to a steady state before its decay is monitored. In such systems, the phenomenon takes the form of an initial emission signal and an emissive contribution to the steady-state signal that is removed when the light is turned off [*120*]. (A different profile results if the polarization is derived from a RP mechanism.)

Such time-resolved spectra are readily obtained [*106*] from melanin preparations provided one uses either synthetic melanin or natural melanins from which protein and metal ions have been removed by treating the melanin with concentrated acid. A typical spectrum is shown in Fig. 9. Observation was on the millisecond time scale. We interpret this as strong evidence that the transient free radicals are derived from a photoexcited triplet. With a knowledge of the free-radical lifetime and its spin-lattice relaxation time T_1, the initial polarization with which the radicals are generated, i.e., before they have time to relax, can be estimated [*121*] (one must also correct for the finite instrument response time). If this is done, taking a reasonable value for the transient radical T_1 of 10 μsec (the value for the intrinsic radical [*79*]) gives an initial polarization of several hundred, around the maximum predicted by theory [*121*]. Although this estimate needs to be confirmed by direct measurements using a microsecond response spectrometer, it appears that essentially all the spin

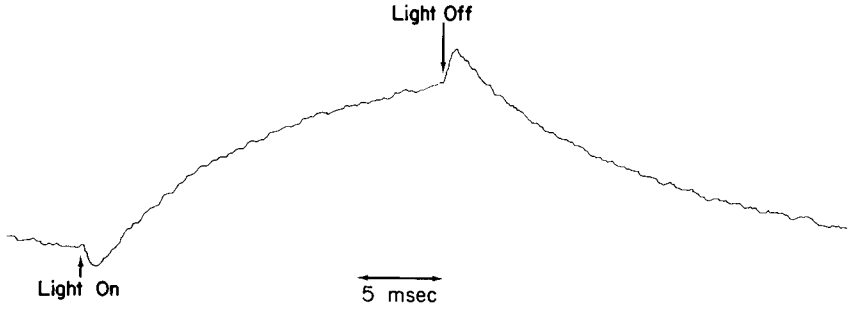


Fig. 9. Spin polarization in the time-resolved esr spectra of transient free radicals from dopa–melanin. The polarization observed is limited by the time response of the spectrometer (ca. 200 μsec). (From [*106*].)

polarization generated in the triplet state is transferred to free-radical products before the triplet has time to relax.

It is thought that polarization is suppressed in A-type natural melanins because they contain paramagnetic metal ions. It has been demonstrated [*106*] that paramagnetic ions quench CIDEP in synthetic melanins. The addition of relatively small amounts of Cu^{2+} ions to synthetic melanin (in the ratio of 1 Cu^{2+} to 200 monomers) removed all detectable polarization, whereas a similar concentration of Zn^{2+} ions was without effect. The paramagnetic ions are likely to operate by facilitating relaxation both in the triplet and in the free radicals.

Direct observation of the triplet state has not yet been accomplished. Detection using the esr technique may be extremely difficult, for, even at −196°C, the triplet lifetime is evidently extremely short.

D. Overall Mechanism

It is suggested that the major pathways for free-radical generation and destruction are those shown below, in which key intermediates are quinones and hydroquinones (or quinhydrone or similar complexes), excited singlet and triplet states, and semiquinone free radicals. These are cycled in the major reaction sequence (route A of Fig. 10). The exact nature of the excited singlet and triplet states is not yet clear. The excitation energy may be localized on the quinone moiety, in which case the sequence would be analogous to that found for photoreaction between quinones and

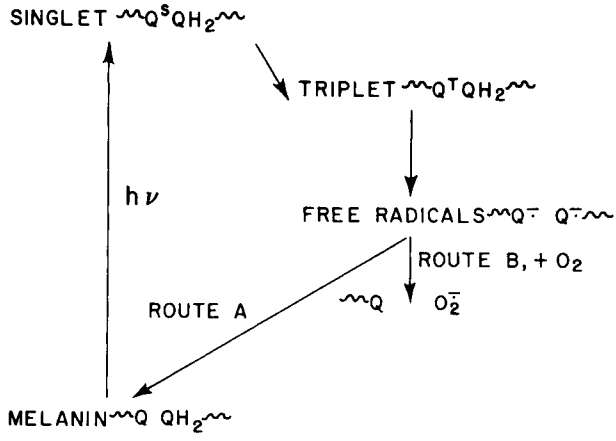


Fig. 10. Possible mechanism for light absorption and degradation in melanins. Route A is thought to predominate in deoxygenated media. Route B becomes important if oxygen is present.

hydroquinones in solution [*122*]. Electron transfer between semiquinones returns the system to its original state.

A minor pathway (route B of Fig. 10) is available in the presence of oxygen. Here chemical reaction occurs with the oxidation of some hydroquinone groups to quinones.

E. Relevance to Photoprotection

Light absorption by melanin is thought [*123*] to lead to both degradation of light to heat and some oxidation (immediate pigment darkening [*124, 125*] appears to have a requirement for oxygen [*101*]). It is possible that the sequences outlined above represent fundamental pathways in the mechanism of photoprotection. Thus, route A is a cyclic mechanism that appears capable of indefinite repetition in the absence of oxygen (the transient free-radical response is unaffected by continuous photolysis for many hours [*106*]). Route B, on the other hand, operates only when oxygen is present and leads to a partial oxidation of the polymer, with some conversion of $\sim\!\!\sim QH_2$ to $\sim\!\!\sim Q$ moieties. Cassidy and Kun [*18*] have described data obtained in their laboratory on synthetic redox polymers (polyvinylhydroquinones) that had been partially oxidized. After addition of oxidant, a slow equilibration was evident, consistent with a regrouping of quinone units within the polymer to form quinhydrone complexes. If melanin quinones can similarly regroup in the presence of excess hydroquinone to yield additional quinhydrone complexes, then the light-absorbing properties of the polymer may be enhanced, as may its capacity to afford photoprotection. However, the products of the oxidation, superoxide and hydrogen peroxide, are potentially damaging species. They may interact, in a reaction presumably catalyzed by traces of metal ion [*126*], to yield hydroxyl radicals [*127*], while hydrogen peroxide itself can react with melanin, ultimately to bleach the polymer [*128*]. It is interesting to note that both superoxide dismutase and catalase activity have been found in eye tissue [*129*].

VI. OTHER CHEMICAL REACTIONS

A. Redox Reagents

The principal effect of oxidizing (Ox) and reducing (Red) agents on melanin evidently is to alter the proportions of $\sim\!\!\sim Q$ and $\sim\!\!\sim QH_2$ groups, as in eq (7). Redox properties of melanin have been known and employed in histochemical analysis for many years. The main histochemical test for

melanins is based on their ability to reduce silver nitrate at pH 4 to metallic silver [*23*]. For a particular melanin, reduction occurs at a rate believed to be related to the relative amounts of $\sim\!\!\sim QH_2$ and $\sim\!\!\sim Q...H_2Q\sim\!\!\sim$ that it contains. Prior reduction with dithionite greatly enhances the rate of blackening [*128*].

$$\sim\!\!\sim QH_2 + \text{ox} \rightleftharpoons \sim\!\!\sim Q + \text{red} \qquad (7)$$

Emphasis in later studies has been placed on effects on free-radical properties [*16, 88*] and the kinetics of the redox reactions [*130–134*]. Despite these investigations, the exact proportions of $\sim\!\!\sim QH_2$ and $\sim\!\!\sim Q$ within the melanin structure are not known with certainty. Attempts have been made to assay accessible groups by titration. Recent optical measurements [*132*] of the reduction of ferricyanide by synthetic dopa-melanin indicate a reducing capacity of 5.29 meq of ferricyanide/g of melanin, equivalent to about 0.4 hydroquinone units/monomer. A reducing capacity equivalent to 0.01 hydroquinone units/monomer has been calculated [*17*] from polarographic data obtained using Fe^{3+} as oxidant, but this value may be low because, although a two-step reduction was observed, it is based solely on the initial rapid step. In systems where Ti^{3+} was employed as a reducing agent, an oxidizing capacity equivalent to 0.24 quinone units/monomer was derived [*17*].

Taking the higher value for the reducing capacity with that for the oxidizing capacity, it appears that for dopa-melanin reducing and oxidizing capacities are ca. 5 and 3 meq/g, respectively, with a $[\sim\!\!\sim QH_2]:[\sim\!\!\sim Q]$ ratio of ca. 1.8. These values are similar to the highest values reported [*135, 136*] for the related humic acid family of redox polymers: namely, hydroquinone-reducing capacities from 2.1 to 5.1 meq/g, quinone-oxidizing capacities from 1.6 to 2.1 meq/g with $[\sim\!\!\sim QH_2]:[\sim\!\!\sim Q] =$ 1.2–2.9. They are also in reasonable accord with an estimate [*2*] of total hydroquinone and quinone in dopa-melanin from chemical degradation experiments (0.47 and 0.37 units/monomer, $[\sim\!\!\sim QH_2]:[\sim\!\!\sim Q] = 1.3$). One would anticipate that natural melanins with their associated protein would be less reactive, and there is evidence [*133, 134, 137*] that this is so. The ability of melanoma-melanin to oxidize NADH and to reduce ferricyanide is much lower than that of dopa-melanin unless its protein is removed by acid hydrolysis. Protein added to dopa-melanin or incorporated into dopa-melanin during synthesis reduced its redox capacity.

A partial list of reagents investigated is shown in the left-hand column of Table XI. (A more complete list may be found in Lillie [*128*].) Melanin behavior is paralleled by that of polyvinylhydroquinone/quinone redox polymers [*18*] (right-hand column, Table XI), which undergo similar reactions. It should be noted that such synthetic redox polymers can also

TABLE XI Redox Reactions of Melanins and Synthetic Redox Polymers[a]

Melanin	Polyvinylhydroquinone/quinone
Oxidants:	
Fe^{3+}	Fe^{3+}, Ce^{4+}
I_2	Cl_2, Br_2, I_3^-
OCl^-, OBr^-	OCl^-, OBr^-
CrO_3, $Cr_2O_7^{2-}$	$Cr_2O_7^{2-}$
$Fe(CN)_6^{3-}$, MnO_4^-	$Fe(CN)_6^{3-}$
Benzoquinone	Benzoquinone
Reductants:	
Sn^{2+}, Ti^{3+}	Sn^{2+}, Ti^{3+}
NADH	NADH
Ascorbate	Ascorbate
$S_2O_4^{2-}$	
Thiols	

[a] From Lillie [*128*] and Cassidy and Kun [*18*].

bind metal ions and that some contain esr signals [*138*]. The relationship between melanins and redox polymers has received little recognition to date, although Finkle [*139*] has pointed out that polyvinyl catechols may be related to humic acids.

Effects on the melanin radical esr signal intensity have been reported (Table XII). These reports are mostly from isolated measurements, and some have been disputed. It is a moot point at present to what extent redox reagents, with the possible exception of hydrogen peroxide which slowly bleaches melanin with large decreases in esr signal intensity, affect the free-radical content. However, we note that reported decreases are mostly from hydrated suspensions, and that the free-radical signal is not completely quenched. This is consistent with the notion that there exist pools of intrinsic free radicals in hydrated melanins with differing chemical reactivity and/or accessibility.

An interesting example of a redox reaction catalyzed by melanin is that between NADH and molecular oxygen to give NAD^+ and hydrogen peroxide [*141*] (synthetic redox polymers have been suggested [*18*] as possible commercial catalysts for the generation of H_2O_2). The reaction may be stimulated to some extent by visible light. During this experiment some decrease in esr signal intensity was detected.

B. Reactions with Free Radicals

Two accounts [*77, 142*] have appeared of the scavenging by melanins of persistent free radicals such as diphenyldipicrylhydrazyl (DPPH) and

TABLE XII Effects of Redox Reagents on ESR Signal Intensity of Free Radicals in Melanins

Melanin	Sample form[a]	Reagent	Enhancement[b]	Reference
Dopa, autoxidation	Dried	Dithionite	1.0	*88*
Sepia ink	Suspension	Ascorbic acid	0.5[c]	*16*
Squid, A-type	Dried	Ascorbic acid	1.0	*41*
Dopa, autoxidation	Dried	Ascorbic acid	1.0	*88*
Wool, HCl extraction	Dried	Ascorbic acid	0.36[d]	*67*
S-91 mouse melanoma	Suspension	Cysteine	0.3–0.37[d]	*65*
S-91 mouse melanoma	Suspension	Penicillamine	0.2	*140*
Sepia ink	Suspension	H_2S	0.5	*16*
Dopa, autoxidation	Dried	$NADH/O_2$	1.0	*88*
Dopa, enzymatic oxidation	Suspension	$NADH/O_2$	0.75	*141*
Frog pigment epithelium	Suspension	2-ethyl 6-methyl-3-hydroxy pyridine	0.4	*61*
Human hair	Intact	H_2O_2	$<<1$	*72*
Wool, HCl extraction	Dried	H_2O_2	0.01	*67*
Dopa, autoxidation	Dried	$Fe(CN)_6^{3-}$	1.0	*88*
Dopa, autoxidation	Dried	$Ce(SO_4)_2$	1.0	*88*
Dopa, autoxidation	Dried	Tetramethyl-phenylenediamine	1.0	*88*

[a] Experiments at ambient temperature; pH usually not stated.
[b] Signal amplitude relative to that of intrinsic radicals.
[c] 3×10^{17} spins/g.
[d] Calculation from data in reference cited.

nitroxides. It is felt that these radicals are reduced by $\sim\!\!\sim QH_2$ units on the surface of the melanin which are ultimately oxidized to $\sim\!\!\sim Q$ groups via transient semiquinones. This seems convincing, given the ability of nitroxides to oxidize phenolic OH groups of exposed tyrosine residues in proteins [*143*].

Reaction with DPPH was monitored by measuring the decrease in DPPH optical absorption with time. Separate esr measurements showed that the concentration of melanin free radicals did not change significantly [*77*]. Thus, no evidence to suggest that external free radicals are scavenged by intrinsic melanin radicals was forthcoming. Experiments with modified melanins established that reduction of DPPH requires hydroquinone groups on the polymer: blocking these groups by methylation or by oxidation suppressed the ability of the melanin to reduce DPPH to diphenyldipicrylhydrazine. The oxidized polymer could be recycled using ascorbate or NADH.

The suggestion [*16, 144*] that part of the function of melanin may be to remove transient external free radicals, e.g., those formed in photolytic or

radiolytic reactions, has not been tested, although as judged by the chemical properties of the groups contained within the polymer, it is reasonable to expect that melanin should be an excellent scavenger for $\cdot OH$, $H\cdot$, e^-_{aq}, and alkyl and other radicals.

C. Drug Binding

A number of studies [*145–160*] have demonstrated strong binding of drugs (cocaine, epinephrine, dopamine, phenothiazines, etc.) and quaternary bipyridylium salts to melanin, and there is evidence that such binding can affect their properties. An improved knowledge of melanin chemistry may enable one to understand why certain drugs bind to melanin and how they function when bound. Although simple ionic binding has been considered [*151*], several authors [*55, 145, 149*] have argued, on the grounds that the majority of these drugs are known for their ready electron donation and ease of formation of free-radical derivatives, that they probably form charge-transfer complexes in which the drug acts as an electron donor, melanin as an electron acceptor. Thus, on mixing melanin with chlorpromazine in dimethylsulphoxide, an increase in conductivity above that of the individual components was found [*55*].

There are conflicting reports of changes in free-radical signal intensity on addition of chlorpromazine to melanin. Two studies [*55, 161*] indicate a decrease, whereas another [*88*] suggests no significant change. However, it should be noted that the sample form and other experimental conditions differed widely between these studies.

D. Modification Procedures

Chemical modification procedures have been extensively used in the investigation of the chemical structure of melanin, but have infrequently been used to modify materials for esr studies. Modifications can provide information on the identity and importance of different functional groups in melanins with regard to their metal binding, redox, and photochemical properties. Many of the modifications reported in the literature are directly applicable. For instance, diazomethane alkylates all the carboxylate, hydroquinone, pyrrole, and indole units, while selective blocking can be achieved by methanol esterification and by reaction with thionyl chloride and dimethyl sulfate. Boyd [*77*] and Rubin and Boyd [*162*] have described experiments in which dried, synthetic melanin samples were acetylated and alkylated. These procedures block the reducing capacity of the polymer, but effects on the free-radical concentration were less marked. Thus, acetylation of dopa-melanin absorbed on cellulose did not

change the radical content significantly, while the action of dimethyl sulfate and diazomethane on dopa-melanin resulted in reported decreases by 10% and 40–70%, respectively.

A number of reductive procedures have been attempted [*67*] with the aim of bleaching melanin from wool by reducing the indole nucleus. The esr properties of the melanins were examined after the modification. Some changes were observed after Birch reduction, borohydride reduction, or treatment with hydrogen in the presence of a Tris(triphenylphosphine) chlororhodium(I) catalyst. Borohydride reduction has been used in other experiments to solubilize synthetic melanin for assay via its optical absorption at 290 nm [*46*]. The major effect of borohydride thus seems to be the reduction of ~~Q to ~~QH_2.

VII. ROLE OF SOLVENT AND MOTIONAL EFFECTS

A. Influence of Solvent

Both natural and synthetic melanins, as isolated, contain enormous quantities of water. Thus, 1 ml of an acid-precipitated dopa-melanin preparation contains only ca. 10 mg of dried material. It seems that water may be essential to preserve the structure of the polymer in a solvent-swollen state.

The role of the solvent may be especially crucial. Many of the chemical properties of melanins are based on equilibria that are probably dependent on motion. One might expect that such equilibria would be affected by drying and that chemical reactivity would be severely curtailed. Consistent with this, free-radical concentrations in dried material have been reported to be not only higher [*16, 63*], but little affected [*41*] by chemical reagents. Experiments in this laboratory [*47*] have confirmed that induction of free radicals by high pH, metal ions, and light can be suppressed in dopa-melanin that has been dried, even after resuspension in water for several days. Horak and Gillette [*17*] and Das *et al.* [*46*] have also reported that melanin, once dried, changes its physical characteristics and loses chemical reactivity. The use of dried samples may thus have contributed to the confusion regarding free-radical induction.

It is possible to investigate water in the vicinity of the intrinsic radicals through the electron nuclear double resonance (endor) technique [*163*]. Besides being useful to measure small hyperfine couplings that may not be resolved in many esr experiments, this technique reports on matrix protons which interact with the free radicals; these give rise to a characteristic line at an nmr frequency corresponding to that of the free proton.

Incubation in D_2O can provide information on the accessibility of water to the free-radical sites. Endor experiments on melanins have not as yet revealed any resolved hyperfine structure, but they have shown [*164*] signals from matrix protons. Only small changes with D_2O were observed, indicating either that solvent accessibility is poor or that the major part of the signal arises from matrix protons in the polymer rather than in the solvent.

The role of the solvent appears in a more subtle form in the analysis of microwave saturation data. Anomalous saturation behavior has been interpreted [*79*] in terms of spin-flip lines appearing in the wings of the spectrum at high microwave power. These lines, which reflect simultaneous electron–proton transitions, are more apparent at low microwave frequencies, as required by theory. Additional data suggest that they are more prominent in hydrogen-bonding solvents. (Hales has demonstrated [*165*] for a model semiquinone system that other anomalous saturation effects can reflect hydrogen-bonding between immobilized semiquinones and solvent.) Exchange with D_2O does not suppress the spin-flip lines entirely, indicating that only part of the intrinsic free-radical population is solvent accessible. This approach promises to be a useful adjunct to endor studies in probing free-radical interactions with solvent.

B. Motion

Where motion in melanin polymers has been implicitly considered, which is seldom, it has usually been concluded that there is none. However, many polymers display a surprising degree of motion, in particular those that are swollen by solvent. Weak ^{13}C nmr spectra have been obtained [*13*] from some fungal melanins, indicating that these materials possess some flexible regions.

An important consideration in all polymer systems is the propensity for site interaction, i.e., the potential of one site on the polymer to interact with any other site. There is now evidence to suggest that site interaction in melanin may be relatively facile. The experiments with diamagnetic metal ions described in Section IV led to the conclusion that there exists an equilibrium within the polymer between free radicals and, e.g., quinone and hydroquinone groups, while the finding [*106*] that part of the transient free-radical population induced by photoirradiation decays with second-order kinetics suggests a diffusive process. Thus it has become important to investigate motion in melanin granules. Essentially, nothing is known.

Anisotropic interactions in immobilized free radicals give rise to an asymmetric line shape. Considerable variation in symmetry of esr lines in melanins has been observed, and it may be that this reflects motion in the

free radical. Saturation-transfer spectroscopy [*166, 167*] is appropriate for study of molecular motion with rotational correlation times in the range 10^{-3}–10^{-7} sec. It is possible that one can gather quantitative motional information from measurements on either the intrinsic free radicals or on nitroxide spin labels bound to hydroquinone units on the polymer surface.

VIII. BIOMEDICAL APPLICATIONS

Excess quantities of melanin are produced in tumors (malignant melanomas), and there are a number of other disorders in which abnormalities of pigmentation occur [*124*]. Speculations are often made about the role of melanin in these disorders either as a cause or as a direct reflection of altered physiology. Our increased knowledge of melanin should eventually enable us systematically and rationally to consider the role of melanin in these disorders.

Melanin also seems to be involved, directly or indirectly, in several accepted therapies: it is the primary light acceptor in laser-induced retinal coagulation [*168*] and in conventional phototherapy; phenylephrine used to effect pupillary dilations binds to the melanin of the iris [*169, 170*]; and patients under phenothiazine treatment often exhibit enhanced photosensitivity of the skin associated with binding of the drugs to melanin [*123, 161, 171*].

A. Electron Spin Resonance Tests for Melanin

The classic histochemical test for melanins [*172*] is the ready reduction of 0.1 *M* silver nitrate at pH 4. This requires that the melanin contain oxidizable *o*-hydroquinone groups (*p*-hydroquinones apparently react much more slowly [*173*]) so that melanins with the greatest degree of quinonization react the least readily. Similarly, esr spectroscopy may be able to assist in distinguishing between melanin and other pigments and even possibly between melanins themselves. This possibility has been investigated by Sarna and Swartz [*63*], who have delineated a series of tests whereby natural melanin can be distinguished from other black pigments via an esr assay of its free-radical properties. These tests were originally proposed on an empirical basis, but the chemistry behind the characteristic responses now appears to be fairly well understood. They rely on the stability of the material and its fundamental internal equilibria. The tests are as follows.

1. Nature of the esr signal: fairly symmetrical, 4–10 G wide, g-value 2.004, $P_{\frac{1}{2}} \sim 0.2$ mW at 77°K.
2. Stability: indefinitely stable at room temperature, minimal effects from boiling in concentrated acid.
3. Photosensitivity: exposure to visible light increases the free-radical content by a factor of 2–3 at cryogenic temperatures.
4. Effect of zinc: incubation in 0.1–1.0 M Zn^{2+} solutions at pH 5.5 increases the signal intensity by a factor of 2–4 and increases its asymmetry.
5. Effect of pH: signal intensity decreases by 50% at pH 1 and increases by a factor of up to 8 in base with an increase in asymmetry.
6. Effect of paramagnetic ions: paramagnetic ions such as Cu^{2+} or Dy^{3+} decrease microwave power saturation (i.e., increase $P_{\frac{1}{2}}$).

By using this series of tests as a criterion for melanin, it has been possible to identify melanin in amelanotic melanomas [*63*] and to show that the pigment in the liver of patients with the Dubin–Johnson syndrome does not have melaninlike properties [*174*]. Distinction between different melanins has not yet been systematically attempted. However, preliminary evidence has been obtained [*73*] that the melanin associated with the pigmented lesions that accompany neurofibromatosis has properties that differ from normal cutaneous melanins. Increases in free-radical content in the presence of zinc or hydroxide ions by factors of up to 25 have been observed.

IX. CONCLUDING REMARKS

The lack of quantitative knowledge concerning the properties of melanins has led to inconclusive results from many experiments designed to study their chemical reactivity. This problem and others remain, and where possible we have attempted to define them. It must be recognized that results may depend strongly on the physical state of the melanin preparation, and that experimental conditions should be carefully controlled. A means of routinely monitoring concentrations both of quinone and hydroquinone groups and of quinhydrone complexes is needed. The development of improved synthetic models of well-defined structure may lead to a better understanding of melanin reaction mechanisms, while the study of intact tissue samples is needed to confirm the generality of these mechanisms. For example, we need to investigate mechanisms of site interaction and to test for the involvement of radical pair states. Further details of the degradation of light by melanins are required. It may be

possible to obtain these by carrying out measurements at shorter time scales.

Nevertheless, progress is being made toward improving our understanding of melanin structure and reactivity. We know that melanins are naturally occurring redox polymers, containing high concentrations of *o*-hydroquinone and *o*-quinone units which appear to largely determine their chemistry. Some of these units are likely to be associated as quinhydrones.

Melanins are excellent ion-exchange materials, containing a number of functional groups capable of binding metal ions. The propensity for binding by these groups, which include carboxyls, amino acids, *o*-hydroquinones, and *o*-semiquinones, is strongly pH dependent.

The intrinsic free-radical population in hydrated melanin suspensions appears to be heterogeneous. Part of the population is in equilibrium with, e.g., *o*-hydroquinone and *o*-quinone moieties, and its concentration is consequently modified by changes in pH and concentration of metal ions. The remainder is much less reactive and seems to be similar to those species present in dried melanin preparations. Despite their lack of chemical reactivity, they can be approached fairly closely by metal ions.

Melanin absorbs light throughout the visible and ultraviolet to generate transient free radicals. A free-radical mechanism may account for part of the photoprotective process believed to be the primary function of melanin in man. However, photoirradiation of melanin can yield the potentially damaging species superoxide and hydrogen peroxide in a reaction leading to partial oxidation of the polymer.

With an improved knowledge of melanin structure and function, it is likely that esr spectroscopy can be a useful tool in both detecting melanin and understanding its role in disease conditions.

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