The Pigmentary System: Physiology and Pathophysiology, Second Edition
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16 The Physical Properties of Melanins

Tadeusz Sarna and Harold A. Swartz

Summary

1 The complexities of melanins make it difficult to reach very specific conclusions as to the properties and structure of these intractable pigments.
2 Different melanins can have different physicochemical properties, with the most important variables being the nature of the monomeric units.
3 Consequently, there are at least three different types of naturally occurring melanins: eumelanin, pheomelanin, and neuromelanin.
4 Although melanins have been viewed as large heterogeneous polymers and amorphous substances, there appears to be a short-range order within the melanin nanostructure, consisting of several layers of stacking 0.34 nm apart. A growing body of experimental evidence indicates that the assembly of melanin oligomers into nanoparticles, and their aggregation into larger particles, is a key process that determines basic physicochemical properties of melanin pigments.
5 The most important functional groups of the melanin oligomeric units are: aryl and α-amino acid carboxylic groups, fully oxidized, semi-reduced, and fully reduced *o*-quinone groups in eumelanins, and the corresponding *o*-quinonimine, *o*-semiquinonimine, and *o*-aminophenols in pheomelanins.
6 The observable optical properties of natural melanins are a complex function of different melanin monomers and oligomers to absorb light, and ability of melanin particles to scatter and reflect light at different wavelengths.
7 Melanins are poor fluorophores; however, the intensity of fluorescence increases upon their oxidative degradation.
8 Although several band models for melanin have been proposed to explain its optical absorption and electrical properties, there is at present no fully satisfactory theory for melanin to have amorphous semiconductor and related properties.
9 Melanins are the only known biopolymers that both *in vivo* and *in vitro* contain a significant amount of persistent free radical centers that are easily detectable by electron paramagnetic resonance (EPR) spectroscopy.
10 Many of the EPR characteristics and the associated free radical and redox reactions of melanin can be explained on the basis of a comproportionation equilibrium involving key redox groups of the melanin oligomers.
11 The ability of melanin to form complexes with multivalent metal ions is one of its basic physicochemical properties that affects the biological effects of this pigment.
12 Binding of transition metal ions by melanin may result in two opposite effects on its free radical EPR signal: while paramagnetic ions induce magnetic quenching of the intensity of the observable EPR signal of the melanin, diamagnetic ions, such as zinc(II), enhance the EPR signal intensity by shifting the comproportionation equilibrium of the melanin subunits.
13 Melanin polymers are complex redox systems, the resultant properties of which are modified by pH, temperature, illumination with ultraviolet and visible light, and storage conditions.
14 Prolonged illumination of melanin with intense light, in the presence of oxygen, leads to irreversible bleaching of the pigment and its oxidative degradation.
15 Antioxidant properties of melanin, shown in model systems, can be explained by melanin's ability to sequester redox-active metal ions, scavenge oxidizing free radicals, and quench electronically excited states of molecular oxygen and photosensitizing dye molecules.
16 Much is known about melanin as a result of the use of sophisticated physical and chemical approaches, and it is likely that continued progress will be made.

Historical Background

Melanins are a complex group of pigments of different origins, the composition and structure of which depends very much on local conditions, including the type of synthesis (e.g. within specific organelles such as melanosomes vs. polymerization of naturally occurring high concentrations of monomers as in some regions of the brain), the monomeric units that are polymerized (e.g. whether or not cysteine derivatives are involved), the presence of other types of molecules at the time of formation (e.g. tyrosinase, other proteins, lipids, metal ions), and the subsequent history of the usually long-lived polymers (e.g. oxidation, complexing of metal ions). The resulting melanins are usually heterogeneous with very intractable physical properties, which resist attempts to characterize them by simple physical–chemical approaches. Consequently, attempts to characterize melanins have involved the use of a large number of different and often complex techniques. These have led to large amounts of data, but the nature of the data obtained from many different techniques, combined with the heterogeneity of the melanins that have been studied, has resulted in incomplete and sometimes apparently contradictory conclusions on the composition and structure of melanins. Since publication of the first edition of *The Pigmentary System*, significant advances in biophysical studies of melanin have been made. Particularly impressive are the results of recent studies, in which the ultrastructure of melanin

was examined by powerful imaging techniques such as scanning tunneling and atomic force microscopies, and the photodynamics of melanin, after excitation with light, were analyzed by ultrafast emission and absorption spectroscopies. The aim of this chapter is to summarize representative data on the biophysics of melanin that have been published recently and to provide an overview of the existing data on melanins and, where possible, to indicate generally accepted conclusions on their significance. The reader will find, however, that the current state of knowledge, in spite of the recent advances, often does not lead to such conclusions and, therefore, only a summary of experimental findings is provided without fitting them into a comfortably unified picture of melanin. This chapter represents an updated progress report and a source of information that may facilitate understanding and experimental progress on this intriguing and important natural polymer.

Current Concepts

Structure and Composition of Melanin

The usual melanin pigments are amorphous substances with a distinct particulate character. In biological material, melanin is usually present in the form of discernible units such as pigment granules. The size and shape of the mature melanin granules are, to a significant degree, determined by the process that forms them. In most cells, melanin is synthesized in a specific organelle, the melanosome, the phenotype of which determines the geometry of the melanin particles (Seiji, 1981) (see Chapter 15). For example, melanosomes in human retinal pigment epithelium are typically elongated and relatively large (2–3 μm long and 1 μm wide; Feeney-Burns, 1980), while melanin granules in Harding–Passey melanoma cells are almost spherical in shape and much smaller in size (0.40 μm diameter) (Hach *et al*., 1977).

In normal skin, the ultrastructure of melanosomes usually relates to the type of melanin they produce (Hearing *et al*., 1973; Jimbow *et al*., 1979). Typical eumelanosomes have an ellipsoidal–lamellar structure with melanin being deposited in a uniform pattern. Pheomelanosomes, on the other hand, are round and granular with uneven deposition of pigment within the melanosome. It should be noted, however, that another spherical melanosome with a distinct granular ultrastructure, which occurs in the Harding–Passey mouse melanoma, does not produce pheomelanin but mostly eumelanin, as determined by complex physicochemical analysis (Ito and Jimbow, 1983; Jimbow *et al*., 1984).

In human epidermis, melanin granules are transferred to keratinocytes where they appear as single, nonaggregated pigment granules or as aggregates, often termed complex melanosomes (see Chapters 7 and 9). The size and distribution of melanosomes in epidermal keratinocytes depends predominantly on the type of human skin (Toda *et al*., 1973). Eventually, after fusion with lysosomes, the epidermal melanosomes are degraded and broken up into "melanin dust" (Wolf, 1973). It remains to be determined to what extent the physicochemical properties of the melanin are modified by such a disassembly of the melanin granules.

Very significant changes in melanin can occur with age; it has been shown by electron microscopy that, in human retinal pigment epithelium from donors over 90 years old, virtually all the melanin granules are enclosed in other material, thereby creating "melanolipofuscin" or "melanolysosomes" (Feeney-Burns *et al*., 1990).

Neuromelanin has a distinctively different type of pigment granule. Neuromelanin is found in certain dopaminergic neurons of the substantia nigra and locus coeruleus of primate brains (Bazelon and Fenichel, 1967; Marsden, 1969; Van Woert and Ambani, 1974). These neuromelanin granules are irregular in shape and vary in size and, unlike melanin arising from melanosomes, they do not have distinct well-organized limiting membranes. Neuromelanin *in situ* is a material granule consisting of three different components—an electron-dense melanin core, electron-translucent lipid vacuolae, and lipofuscin (Barden and Brizzee, 1987). It has been reported that neuromelanin accumulates with age in the substantia nigra of human brains until it reaches a maximum level at the age of 50–60 years, and then it gradually decreases (Mann and Yates, 1974).

Based on scanning electron microscopy studies, it was proposed that the "primary particles" of natural melanin are spherical, nonporous particles with a diameter in the order of 30 nm, with an inherent specific surface area in the order of 160 m^2/g (Kollias *et al*., 1991; Zeise *et al*., 1992). According to this view, a "melanin aggregate" is composed of strongly associated primary melanin particles placed together in such a fashion that the measured surface area is significantly less than the sum of the specific surface areas of the primary particles of which it is composed. Such aggregates may be similar to, if not identical with, melanin granules derived directly from *Sepia officinalis* melanosomes, which are spherical in shape, have an average diameter about 160 nm, and a specific surface area 29.31 m^2/g (Kollias *et al*., 1991). *Sepia* melanin granules are uniformly electron dense and, under electron microscopy, exhibit no detectable ultrastructure. In this respect, it is an open question whether typical elongated eumelanosomes and round pheomelanosomes with distinct ultrastructure should be classified, according to this scheme, as "agglomerates," i.e. loosely associated clusters of melanin aggregates.

More recent scanning electron microscopy (SEM) study of *Sepia* melanin indicated the existence of much smaller particles with lateral dimensions of about 15 nm that adhered to larger melanin subunits (Nofsinger *et al*., 2000). This study also suggested significant structural differences between *Sepia* melanin and a synthetic melanin obtained by enzymatic oxidation of dopa. The presence of such small particles in purified melanin, isolated from the ink of cuttlefish, has been confirmed by another imaging technique. Taking advantage of the three-dimensional spatial resolution of atomic force microscopy (AFM) and the ability of the technique to cut the

imaged object or perform with it other mechanical manipulations, the Simon group demonstrated that, although the spherical particles 100–200 nm in diameter were stable structures, they were composed of tiny particles with lateral dimensions of 15–25 nm (Clancy *et al*., 2000). In a recent study, images of melanosomes from bovine retinal pigment epithelium and from human hair clearly indicate that these pigment granules are also aggregated assemblies of substructures about 20 nm in diameter (Liu and Simon, 2003a). In a related study, the authors demonstrated the importance of the isolation and purification procedure on the structure and chemical composition of a natural melanin (Liu and Simon, 2003b). Thus, while two different acid/base procedures, used for the isolation of melanin from human hair, yielded amorphous material without any apparent structure, only the enzymatic extraction preserved the normal morphology of the melanosomes.

Even though synthetic melanins, from different substrates, often exist in aggregated forms, it has been concluded that neither chemically nor enzymatically prepared tyrosine-melanin is composed of discrete particles (Zeise *et al*., 1992). Indeed, in a comparative study, Nofsinger *et al*. (2000), using SEM, showed that, unlike *Sepia* melanin, a synthetic melanin, obtained by enzymatic oxidation of DOPA, was an amorphous material without any distinct substructure. On the other hand, light scattering experiments on colloidal aqueous suspensions of synthetic auto-oxidized dopa-melanin revealed a particulate character of this melanin preparation, with a particle size of less than 10 nm (Huang *et al*., 1989). Characterization of the structural units (nodules) of synthetic dopa-melanin was also carried out by the X-ray small-angle scattering technique (XSAS) (Miyake and Izumi, 1984; Miyake *et al*., 1985, 1987). The radius of gyration of melanin in its aqueous solution, calculated from an initial slope of the Guinier's plot, suggests that the elemental molecular unit of this synthetic melanin may be rod-like in shape, 4.8–8.5 nm long, and 0.6–0.8 nm in diameter. Qualitatively similar conclusions about the submolecular structure of melanin have been reached on the basis of ultrasonic measurements of synthetic diethylamine melanin and natural melanin from *Sepia* (*Aconthosepian*), and B16 and Harding–Passey melanomas (Kono, 1984; Kono and Jimbow, 1985; Kono and Yoshizaki, 1987). A pronounced particle wave resonance observed for all studied melanins at 220 MHz was related to a stiff-chain unit that could be approximated by a rod-like rigid molecule.

Perhaps the most detailed structure of the synthetic tyrosine-melanin "protomolecule" has been proposed by Zajac *et al*. (1994). Based on wide-angle X-ray diffraction analysis of dried melanin and scanning tunneling microscopy measurements of monomolecular layers of the melanin deposited on highly oriented pyrolytic graphite, the authors constructed a model of the fundamental unit of synthetic melanin consisting of three stacked sheets with five to eight 5,6-indolequinone residues in each sheet. The spacing between stacks was assumed to be 0.34 nm in order to account for the prominent features observed by X-ray diffraction and scanning tunneling microscopy. Therefore, the overall dimensions of the melanin protomolecule were calculated to be roughly 2.0 nm in lateral extent and 0.76 nm in height. The X-ray and STM results were verified by structure minimization and molecular orbital techniques.

Systematic modeling of the data from X-ray diffraction studies of melanin performed by Cheng *et al*. (1994a, b) can be summarized as follows. The derived structure factor, S(q), typically shows six diffused peaks within the q-range 0.3/Å to 16/Å in reciprocal space (Fig. 16.1). Although some differences are apparent in the magnitude of S(q) oscillations for DOPA-melanin, tyrosine-melanin, and *Sepia* melanin, all these melanins are rather similar in the arrangements of their neighbors. The first peak in S(q) (at q = 1.74/Å) reflects mainly the number and spacing of the melanin monomer layers, suggesting that, in real space, the interlayer spacing is 3.4 Å. The plane-polymerized monomeric units produce the second peak at q = 3.0/Å, while the third peak at q = 5.6/Å refers to the number (four or five) of connected monomers in a layer. The last three peaks in the higher q-region are mainly produced by the single monomer structure, the average band length of which determines their locations. A 1.42-Å distance obtained in real space can be attributed to the average bond length of C–C, C–O, and C–N. The four-layer stacking of four to eight 5,6-dihydroxyindole (or 5,6-indolequinone) units gives a dimension of the fundamental melanin unit of 15 Å. Interestingly, a prepeak at q = 0.45/Å (which corresponds to the length 13–20 Å in real space) has also been observed in some melanin samples (Bridelli *et al*., 1990; Cheng *et al*., 1994b; Thathachari and Blois, 1969). The X-ray diffraction data revealed that melanin has a relatively low X-ray absorption coefficient ($1 < \mu \leq 2$/cm), which is consistent with its elemental composition (Cheng *et al*., 1994b).

The collective results of SEM and AFM studies of *Sepia* melanin, carried out by the Simon group, and of independent structural studies of synthetic melanins, in which the researchers used STM and AFM (Gallas *et al*., 2000), small-angle neutron scattering (Gallas *et al*., 1999), or synchrotron small-angle X-ray scattering (Littrell *et al*., 2003), as well as the results of matrix-assisted desorption ionization (MALDI) mass spectroscopy measurements, are consistent with the proposed model of ultrastructural organization of eumelanins, in which the major building block of eumelanin pigments is a small planar oligomer, probably highly cross-linked, with maximum dimensions 0.4 × 1.0 nm, that is preferentially aggregated into fundamental aggregates of 3–4 π-stocked oligomers (Cheng *et al*., 1994a, b; Clancy and Simon, 2001; Gallas *et al*., 2000; Zajac *et al*., 1994). The macroscopic morphology of eumelanin pigment granules is a result of hierarchical self-assembly, in which the building blocks of eumelanin assemble into hundred-nanometer structures, which then aggregate to form the final pigment granules (Clancy *et al*., 2000). This model is a radical deviation from the existing model, in which melanin was pictured as a huge heteropolymer, consisting of a large number of different monomers

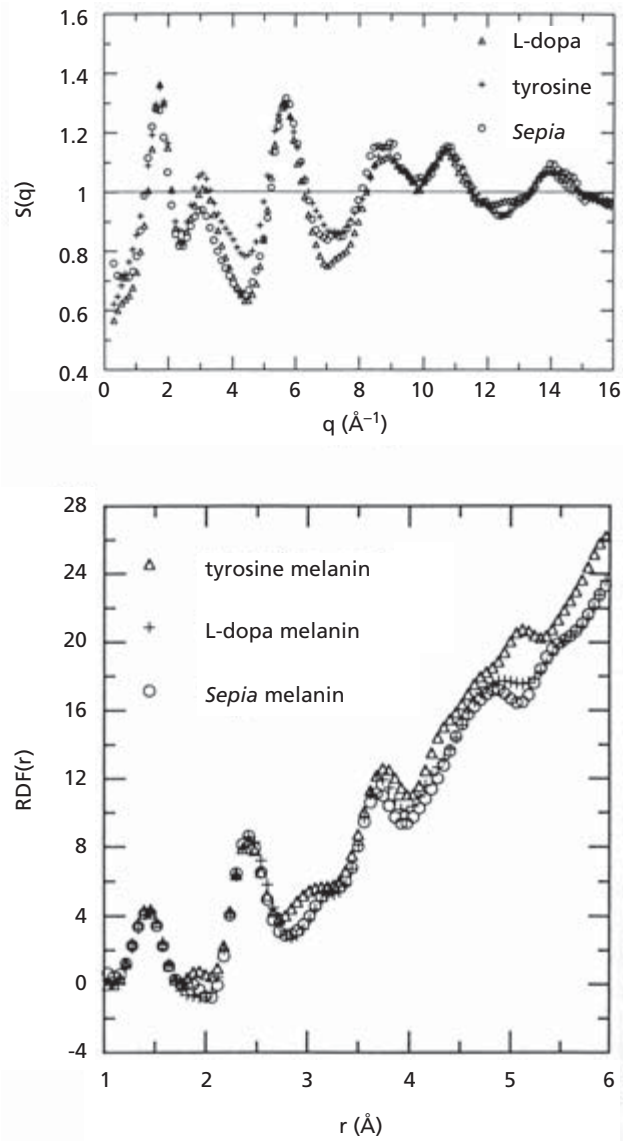

Fig. 16.1. Structure factors S(q) (A) and the radial distribution functions (RDF) (B) of two synthetic melanins and *Sepia* melanin. Reproduced from Cheng *et al.* (1994b), with permission.

linked covalently by a variety of bonds (Nicolaus, 1968). According to the new model, melanin is built of very small building blocks—planar oligomers—consisting of as few as five to eight monomers, which assemble into nanoaggregates that form characteristic stocks. Such nanoaggregates assemble into larger structures, which then aggregate to macroscopic pigment granules. Although the exact nature of the forces that are involved in the assembly of nanoaggregates and of hundred-nanometer structures, as well as in their aggregation, remains unknown, it can be speculated that Van der Waals', π–π, and hydrophobic interactions play a key role. Unfortunately, until now, no comparable structural studies of pheomelanin have been carried out.

Much of our current knowledge about the chemical structure of melanins comes from chromatographic identification and quantitation of the characteristic products arising from chemical degradation of the pigments (Ito, 1986, 1998; Ito and Fujita, 1985; Ito and Jimbow, 1983; Jimbow *et al.*, 1984; Ozeki *et al.*, 1995; Prota *et al.*, 1998a, b). This approach has been applied to human neuromelanin to try to unravel its chemical structure (Carstam *et al.*, 1991; Odh *et al.*, 1994; Wakamatsu *et al.*, 1991). Unfortunately, the conclusions reached by the authors have significant areas of apparent disagreement. The Swedish researchers found large quantities of 4-amino-3-hydroxyphenyl-ethylamine (AHPEA) in neuromelanin samples hydrolyzed with hydriodic acid, whereas the Japanese researchers were unable to detect any significant amount of AHPEA after they analyzed neuromelanins by degradation by hydriodic acid (HI). This discrepancy leaves open whether cysteinyldopamine is incorporated into neuromelanin.

While detectable levels of pheomelanin are found in human skin, regardless of race, color, and skin type, eumelanin is always the major constituent of epidermal melanin (Ito and Wakamatsu, 2003). It appears that high levels of pheomelanin are found only in yellow and red hair of mammals and in red feathers of birds (Ito and Wakamatsu, 2003).

It is believed that the key intermediates in the biosynthetic pathway for eumelanin are 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA), as well as their fully oxidized forms that determine the nature of the fully formed melanin polymer (reviewed by Hearing, 1993; Hearing and Tsukamoto, 1991; Prota, 1992). In the biosynthetic pathway for pheomelanins, a similar role may be played by 1,4-benzothiazynylalanine, derived from cysteinyldopas (Deibel and Chedekel, 1984; Ito and Wakamatsu, 1989; Prota, 1988; Rorsman *et al.*, 1979).

The distributions of the functional groups (CHx, C–O, C=O, O–C=O, $–NH_2$, and R_2NH) of synthetic and natural eumelanins have been studied in solid-state samples by electron spectroscopy for chemical analysis (ESCA or X-ray photoelectron spectroscopy) (Clark *et al.*, 1990). Qualitative and quantitative analysis, calibrated with model compounds (various precursors of eumelanin), revealed interesting differences between the synthetic polymers obtained from DOPA and DHI and *Sepia* melanin. Thus, although none of the synthetic melanins had elemental compositions identical to *Sepia* melanin, DOPA melanins (both auto-oxidized and enzymatically oxidized) were roughly comparable with the *Sepia* eumelanin in elemental composition and distribution of functional groups. The high content of nitrogen observed in *Sepia* melanin was attributed to proteinaceous material still associated with the natural eumelanin. Enzymatically produced DOPA melanin had a higher percentage of carbonyl-type functionalities than auto-oxidized DOPA melanin. As judged by their functional group distribution, the enzymatic DOPA melanin was a better model of natural eumelanin than auto-oxidized DOPA melanin.

Infrared (IR) spectroscopy also has been used in the chemical analysis of melanin (Blois *et al.*, 1964; Bridelli *et al.*, 1980; Garcia-Borrón *et al.*, 1985; Jimbow *et al.*, 1984; Wilczok

et al., 1984; Zecca *et al*., 1992). Using solid pellets of melanin dispersed in KBr, broad absorption bands with varying intensities were observed at 3400/cm, 3200/cm, 3000–2800/cm, 2700–2500/cm, 1700–1650/cm, 1600–1400/cm, and 1045/cm. The bands could arise from symmetric and asymmetric stretching and bending of bonds in a variety of functional groups such as amine, imine, carboxylic, carboxylate, phenolic, aliphatic CH_3, CH_2, C–H, aromatic C–H, etc.; however, precise assignment of these bands is still difficult.

Quantum mechanical calculations of the vibrational structure of the key melanin monomers, carried out by Powell *et al*. (2004), showed that the three main redox forms of 5,6-dihydroxyindole had significantly different infrared and Raman signatures, suggesting that these spectra could be used *in situ* to identify nondestructively the monomeric content of various melanins.

Melanin–protein complexes have been investigated by IR using absorption bands of amide groups. Based on detection of the distinct amide I and amide II bands in natural melanins and in synthetic melanin obtained by auto-oxidation of DOPA in the presence of bovine serum albumin, it was concluded that melanins *in vivo* exist as true melano-protein complexes with the protein moiety covalently bound to melanin (Bilinska *et al*., 1987; Garcia-Borrón *et al*., 1985). It should be noted, however, that no independent unequivocal evidence for covalent binding between melanin and protein has been obtained.

IR also has been used to study the effect of potential degradative treatments on melanin. Characteristic changes in the IR spectra of DOPA melanin were observed upon treatment with HCl. The appearance of sharp methyl and methylene bands at 3000/cm and 2800/cm upon acid hydrolysis of this synthetic melanin was attributed to acid-induced decomposition of some indolic monomers, yielding noncyclic units with aliphatic side-chains (Garcia-Borrón *et al*., 1985). This is consistent with evidence from analysis by degradative chemistry. Ito (1986) found that acid-treated melanins gave much lower yields of pyrrol-2,3,5-tricarboxylic acid (after oxidation of melanin with permanganate) than the corresponding native melanins.

The number of accessible aryl carboxylic acid and α-amino acid carboxylic groups in synthetic tyrosine melanins and natural *Sepia* melanin was estimated by titrimetric analysis using nonaqueous media—2-propanol and acetic acid (Zeise and Chedekel, 1992). Titratable acidic groups were measured to be 180 μEq/q for *Sepia* melanin, 490 μEq/q for melanin prepared by oxidation of tyrosine with persulfate, and only 68 μEq/q for enzymatic tyrosine-melanin. It was concluded that, of the two synthetic melanins, only the enzymatic tyrosine-melanin was an adequate functional group model for the surface structure of eumelanin.

Elemental analysis and quantitative amino acid analysis were employed for estimation of the elemental composition of the melanin backbone in *Sepia* melanin and two synthetic melanins (Chedekel *et al*., 1992a, b). Assuming only one nitrogen atom per monomeric unit of eumelanin, the authors were able to determine the average monomeric backbone chromophore of *Sepia* melanin. The elemental composition of the melanin chromophore backbone is: C, 45.91%; H, 2.66%; N, 6.98%; and O, 29.32%. Accordingly, the C/N and empirical formula for the *Sepia* melanin were 7.67 and $C_{7.67}H_{5.33}NO_{3.68}$ respectively. Of course, the derived formula of any natural melanin will critically depend on accurate estimates of the contribution of any amino acids associated with the melanin, which can obscure the determination of the actual elemental composition of the melanin.

Taking advantage of the enhanced spectral resolution offered by the NMR techniques of cross-polarization, magic angle-spinning (CP/MAS), and high-power proton decoupling, high-resolution ^{13}C and ^{15}N solid-state NMR have been used for the assignment of functional groups of various eumelanins (Aime and Crippa, 1988; Aime *et al*., 1991; Duff *et al*., 1988; Peter and Förster, 1989). Briefly, at a ^{13}C Larmor frequency of 75.7 MHz, the main features of the ^{13}C CPMAS spectrum of *Sepia* melanin consisted of an intense carboxylate resonance centered at 173 ppm as well as broad and strong absorptions in the aromatic and olephenic region (90–120 ppm). In natural melanins, but not in synthetic melanins, a number of variously substituted aliphatic regions (15–75 ppm) was observed, consistent with the presence of unreacted DOPA and a proteinaceous component (Fig. 16.2) (Herve *et al*., 1994). Although solid-state CP/MAS and ^{15}N NMR were used recently for characterization of *Sepia* melanin and human melanin (Adhyaru *et al*., 2003), and high-resolution ^{1}H NMR was employed for quantification of the aromatic protons of the polymer chain in *Sepia* melanin and human hair melanin (Katritzky *et al*., 2002), the application of these advanced analytical methods has not yet yielded any truly unique information about the chemical structure of melanin.

Much of the information on the structure and composition of melanin derived by chemical and structural analysis needs to be considered critically and considered tentative because of the experimental complexities involved in such determinations. This is because of the heterogeneous nature of melanins and their difficult physical–chemical properties, and the possible presence of nonintrinsic proteinaceous material. The procedures required to make it feasible to analyze melanins can readily lead to artifacts. The analyses of its chemical composition often depend on the derivatization of relatively small parts of the macromolecule and so, even if the derivatization procedures are entirely valid from a chemical point of view, the portions of the molecule from which they are obtained may not be fully representative of the entire molecule; for example, they may be derived primarily from parts that are on the surface. Analogous considerations apply to many of the structural studies, especially when these require that the melanin be dried or otherwise altered. This can lead to very distinct changes in its physical properties [e.g. as monitored by electron paramagnetic resonance (EPR) or, equivalently, ESR], which may not reflect the fully hydrated state, etc. (Sealy *et al*., 1980).

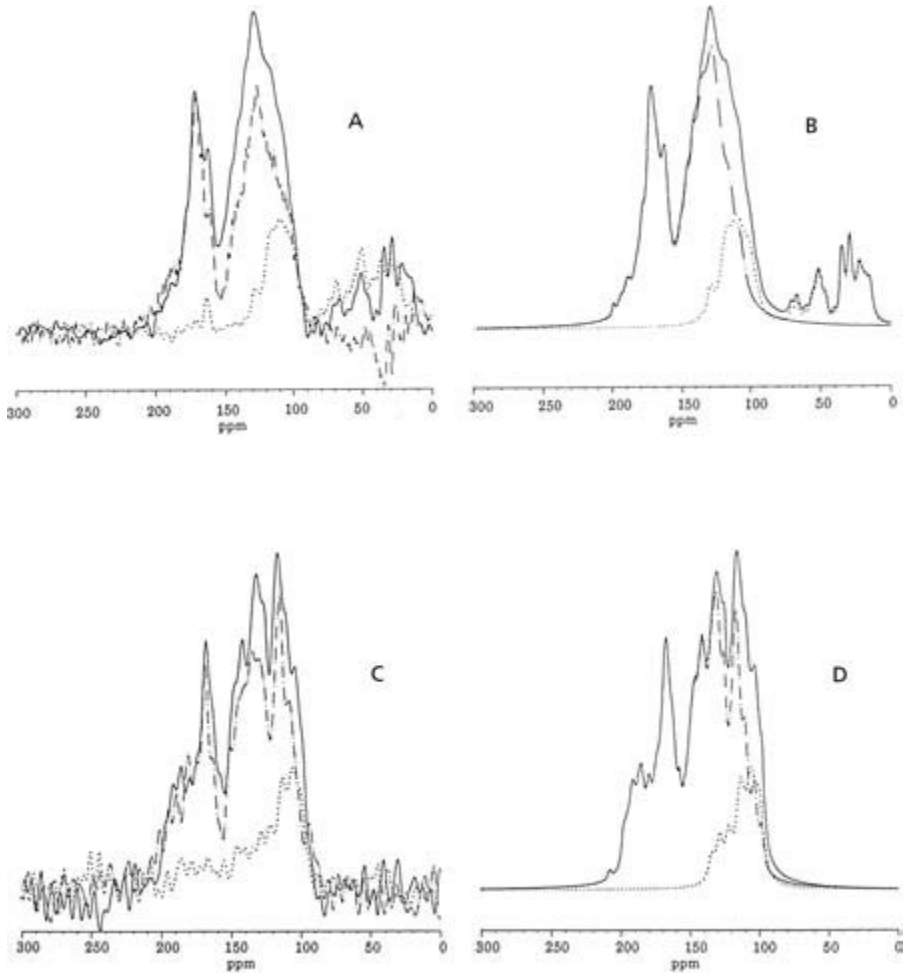

Fig. 16.2. ^{13}C solid-state NMR spectra of *Sepia* melanin (A) and a synthetic melanin obtained by auto-oxidation of 5,6-dihydroxyindole (C), and their Lorentzian peak fitting spectra (B) and (D) respectively; spectra obtained by conventional cross-polarization/magic-angle spinning (CP/MAS) technique are shown by solid lines, while those obtained by short-contact-time CP/MAS (protonated carbons) are shown by dotted lines and those obtained by dipolar dephasing (quaternary carbons) by broken lines. Reprinted from Hervé *et al.* (1994), with kind permission of Elsevier Science-NL.

Optical Properties

The absorption of light is one of the most obvious and important properties of melanins. This has proven to be an immensely complex topic, in terms of both understanding the mechanisms and consequences of optical absorption by melanin and exploiting these properties to enhance an understanding of melanins. In this section, we attempt to provide representative highlights of what appears to be a productive and growing area of study.

The absorption spectrum of human skin melanin *in vivo* is a linear function of the wavelength in the range of 500–750 nm (Kollias and Baqer, 1985). Based on diffused reflectance spectra obtained from patients with vitiligo and normal volunteers, it has been proposed that human melanin absorbs visible radiation through two distinct mechanisms: one that is in effect over the entire visible range and is linear in wavelength, and a second one that is evident at wavelengths in the range 400–500 nm and is exponential in frequency (Kollias and Baqer, 1987; Kollias *et al.*, 1991). As natural melanin *in situ* is in particulate form (Barden and Brizzee, 1987; Feeney-Burns, 1980; Kollias *et al.*, 1991), its observable optical properties are a complex function of melanin's ability to absorb, scatter, and reflect light at different wavelengths. Therefore, it has been very useful to carry out optical studies on synthetic and isolated natural melanins as well as *in situ*.

The optical density of soluble synthetic melanin, such as auto-oxidized DOPA melanin, increases almost monotonically with decreasing wavelength (Crippa *et al.*, 1978; Sarna and Sealy, 1984a). The apparent absorption coefficient of DOPA melanin increases from about 4/mg/cm^2 at 600 nm to over 30/mg/cm^2 at 200 nm (Fig. 16.3). It is important to stress that these are only representative values because the detectable absorption spectra of melanin depend, among other factors, on the conditions used for its synthesis, the redox state of the polymer, the pH and the ionic strength of the aqueous media, temperature, and the handling of the sample (Sarna, 1992). Although the molecular origin of melanin determines many of its physicochemical properties, optical absorption of the polymers synthesized from dopa and cysteinyldopas is quite similar (Fig. 16.3).

The smooth absorption curve of a solution of synthetic auto-oxidized DOPA melanin, with almost monotonic increase in the absorbance with decreasing wavelength, viewed as a characteristic feature of melanin, has been an enigma for years and is still not fully understood. Although different models have been considered to explain the unusual optical

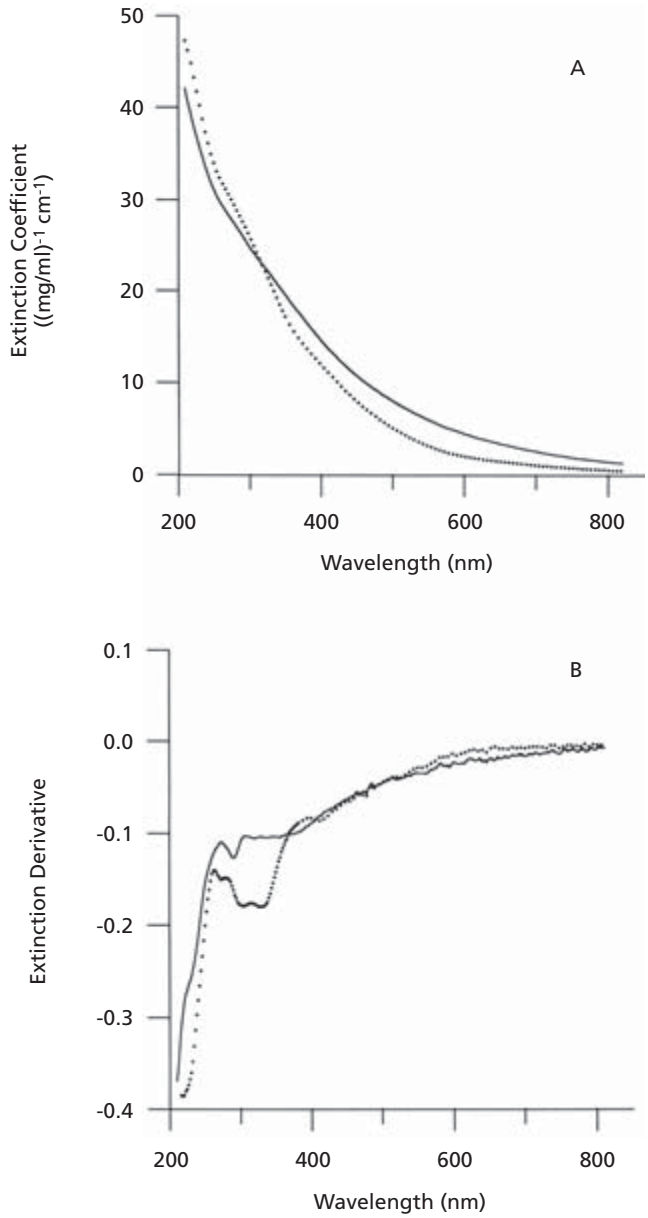

Fig. 16.3. Apparent absorption coefficients (A) and first derivatives of extinction (B) for auto-oxidized dopa melanin (solid lines) and cysteinyldopa melanin (dotted lines). Experimental conditions: melanins dissolved in 50 mM phosphate buffer, pH 7.4, run in 0.1 cm quartz cells.

properties of melanin, including the solid-state model, in which melanin is treated as an amorphous semiconductor with distinct energy bands (Crippa *et al*., 1978; Galvao and Caldas, 1988; Jastrzebska *et al*., 1990; Longnet-Higgins, 1960; McGinness *et al*., 1974), none of the models proved to be satisfactory. One of the most interesting results of the theoretical studies by Galvao and Caldas (1990a, b) was the finding that key physicochemical properties of melanin start to emerge in systems consisting of a small number of monomeric units. Indeed, a remarkably smooth absorption spectrum in the region 300–800 nm for a random composition of basic monomer units such as 5,6-dihydroxyindole, indole-5,6-quinone, and their semiquinone form has been obtained by Bochenek and Gudowska-Nowak (2003a, b), using the intermediate neglect of differential overlap (INDO) and other semi-empirical methods. A similar trend, with significant absorption in the visible spectral region, has also been observed by Stark *et al*. (2003), who carried out much more advanced density functional theory (DFT) calculations for simple melanin oligomers. The relative stability of nine tautomers of 5,6-dihydroxyindole and 5,6-indolequinone and their excitation energies in both gas phase and solution were examined by Il'ichev and Simon (2003), who used DFT, time-dependent DFT, and self-consistent reaction field calculations. The results of their calculations indicated that the indole-quinone units could be responsible for a relatively strong absorption in near infrared. Finally, a first principle density functional theory calculation of the electronic and vibrational structure of key melanin monomers has been reported by Powell *et al.* (2004). The authors postulated that the difference in energies of the highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) of the different monomers could lead to a large range of HOMO–LUMO gaps in eumelanin molecules and thus be related to the observed broadband optical absorption of melanin. In other words, it is the monomer diversity and chemical disorder that are responsible for the optical properties of melanin.

Although the existing theoretical studies of melanin have not yet resulted in a satisfactory description of its optical properties, there is little doubt that advanced quantum mechanical calculations of various melanin oligomers should yield important new results and, ultimately, will lead to better understanding of its structure and physical properties.

The degree of aggregation of the melanin is an especially important factor that will significantly modify the ability of melanin to transmit light at different wavelengths (Huang *et al*., 1989; Pilas and Sarna, 1985). This effect can also be exploited to study the aggregation of melanins. Using static and dynamic light scattering emitted by a NeHe laser, the dynamics of aggregation of a synthetic DOPA melanin in acidic aqueous solution was studied (Huang *et al*., 1989). It was found that, depending on the final pH of the solutions, slow and fast regimes of the kinetics of aggregation could be identified. The precipitates formed in these two regimes could be characterized by fractal structures. It was estimated that, in the fast, diffusion-limited aggregation regime, the aggregate was a fractal with dimension of 1.8, whereas the dimension of the fractal was 2.23 in the slow, reaction-limited aggregation regime. The fractal nature of melanin aggregates has been established over scales ranging from about 0.08 to 2.0 μm (Eisner, 1992). The slow regime of melanin aggregation, with the fractal dimension 2.2, was also observed in the presence of critical amounts of transition metal ions such as copper, nickel, and zinc. That aggregation of melanin affects its optical properties has also been found for very small melanin particles. Thus, Nofsinger *et al*. (1999) reported distinct differences

in the optical absorption of *Sepia* melanin obtained by ultrafiltration of the bulk melanin using a series of different ultrafiltration disk membranes. The results of this study, perhaps predictably, showed a substantial reduction in melanin absorbance in the visible and near-UV spectral region when very low-molecular fractions of this eumelanin were examined. In a related study, the authors demonstrated that differences in the absorption bands were due to varying levels of melanin aggregation (Nofsinger and Simon, 2001).

The optical absorption and scattering properties, and thermal diffusivity of melanin particles from *Sepia officinalis* were recently determined at 580 nm and 633 nm, using photometric and photothermal techniques (Vitkin *et al*., 1994). The absorption coefficient (μ_a) and the transport scattering coefficient (μ_s^1) of *Sepia* melanin were determined from data of diffuse reflectance and transmittance. The scattering anisotropy was obtained from an additional measurement of the total attenuation coefficient and, independently, by goniometry. Pulsed photothermal radiometry was used to deduce the absorption and transport scattering coefficients by a model based on optical diffusion theory, and to provide the thermal diffusivity of solid melanin. At 633 nm, μ_a was found to be 127–157/cm/% and 162–176/cm/% at 580 nm. The corresponding transport scattering coefficient values were 24–28 and 28–30/cm/% at 633 nm and 580 nm respectively. The photometrically measured optical absorption and scattering properties of *Sepia* melanin were consistent with the Mie theory predictions, which indicated strong dependence on the size of melanin particles. The larger the size of melanin particles (melanosomes), the higher their albedo (and more forward-peaked scattering phase functions). These effects are likely to be important for epidermal melanosomes and for retinal pigment epithelial granules, the size of which is significantly larger than that of *Sepia* melanin. Data from both optical and photothermal studies suggest that *Sepia* melanin is not a perfect black body absorber; the melanin significantly scatters light in the yellow–red region.

The internal absorption coefficient of melanosomes *in situ* in human skin was determined, based on measurements of the threshold radiant exposure from a pulsed ruby laser that was necessary to achieve explosive vaporization of the melanosomes (Jacques and McAuliffe, 1991). This phenomenon is observed when the rate of radiant energy deposition within the melanin granule is substantially higher than the thermal relaxation rate of the pigment granule. As the thermal relaxation of melanosomes is estimated to be between 0.5 and 1 μs, threshold radiant exposures for melanosomal injury could easily be obtained using nanosecond Q-switched Nd:YAG or ruby lasers when the energy density exceeds 1 J/cm^2 (Anderson *et al*., 1989; Watanabe *et al*., 1991). Based on their own data and the literature they provided, a summary of the absorption coefficient (μ_a) of the melanosome interior vs. wavelength was presented. The μ_a parameter was found to vary significantly with the wavelength; thus, the average μ_a values were around 80/cm at 1064 nm, 200/cm at 694 nm, 350/cm at 630 nm, 700/cm at 532 nm, and 1500–2000/cm at 355 nm. Dry melanosomes required lower threshold radiant exposure to achieve their explosive vaporization than wet melanosomes, indicating that the effective μ_a for dry melanosomes is higher (202/cm) than that for wet melanosomes (120/cm). The data have been interpreted in terms of swelling, with wet melanosomes having a 70% larger volume than dry melanosomes. The absorption spectrum for human epidermis *in vivo*, measured by an optical fiber spectrometer, matched quite well the relative changes with wavelength of the absorption coefficient of the interior of the melanosome.

The solid-state appearance of typical melanin and its very efficient nonradiative de-excitation, following the absorption of ultraviolet or visible photons, would suggest photo-acoustic spectroscopy to be a method of choice for studying the optical properties of natural melanins. This spectroscopic technique has been applied, with great success, to many biological systems (Balasubramanian and Mohan Raa, 1986; Braslavsky, 1986; Fork and Herbert, 1993; Moore, 1983) but, surprisingly, has been used sparsely to study melanins. The only photo-acoustic spectrum of melanin reported to date that the authors are aware of is a spectrum of a synthetic DOPA melanin obtained in a limited spectral range (Wróbel *et al*., 1997). The PAS spectrum of DOPA melanin is similar to typical optical absorption spectra of solubilized eumelanins. Photo-acoustic techniques were used to determine the thermal properties of melanin and to study nonradiative relaxation of excited states in melanin (Crippa and Viappiani, 1990; Gallas *et al*., 1988). It was found that the acoustic signal induced in synthetic DOPA melanin by chopped light from a krypton laser was dependent on the chopping frequency ($\sim\omega^{-1}$), as predicted by the Rosencwaig–Gersho theory for optically dense samples with a length of thermal diffusion that is greater than the optical extinction length (Rosencwaig and Gersho, 1976). The data suggested, rather unexpectedly, that the optical absorption coefficient in acid- or acetone-precipitated melanin was about 30 times smaller than that of "standard melanin." Photo-acoustic measurements of dense aqueous melanin suspension made at various chopping frequencies with an argon-ion laser as the excitation source revealed strong dependence on pH and the redox state of melanin (Crippa and Viappiani, 1990). Control experiments, performed on natural and synthetic melanins in the form of powders or pastes, confirmed the results of Gallas *et al*. (1988) only to some extent; the photo-acoustic signal intensity varied with the chopping frequency as $\omega^{-0.9}$–$\omega^{-1.1}$.

A very efficient electron–photon coupling, consistent with efficient energy transfer toward the internal degrees of freedom of the melanin macromolecule, was inferred from the effects of phonon amplification observed in a synthetic melanin upon the application of a pulsed electric field with increasing gradient (Crippa *et al*., 1991). This explains why melanin is a very poor fluorescence emitter. The first unambiguous report of melanin-specific fluorescence was reported only in 1984, using natural melanins, both intact and solubi-

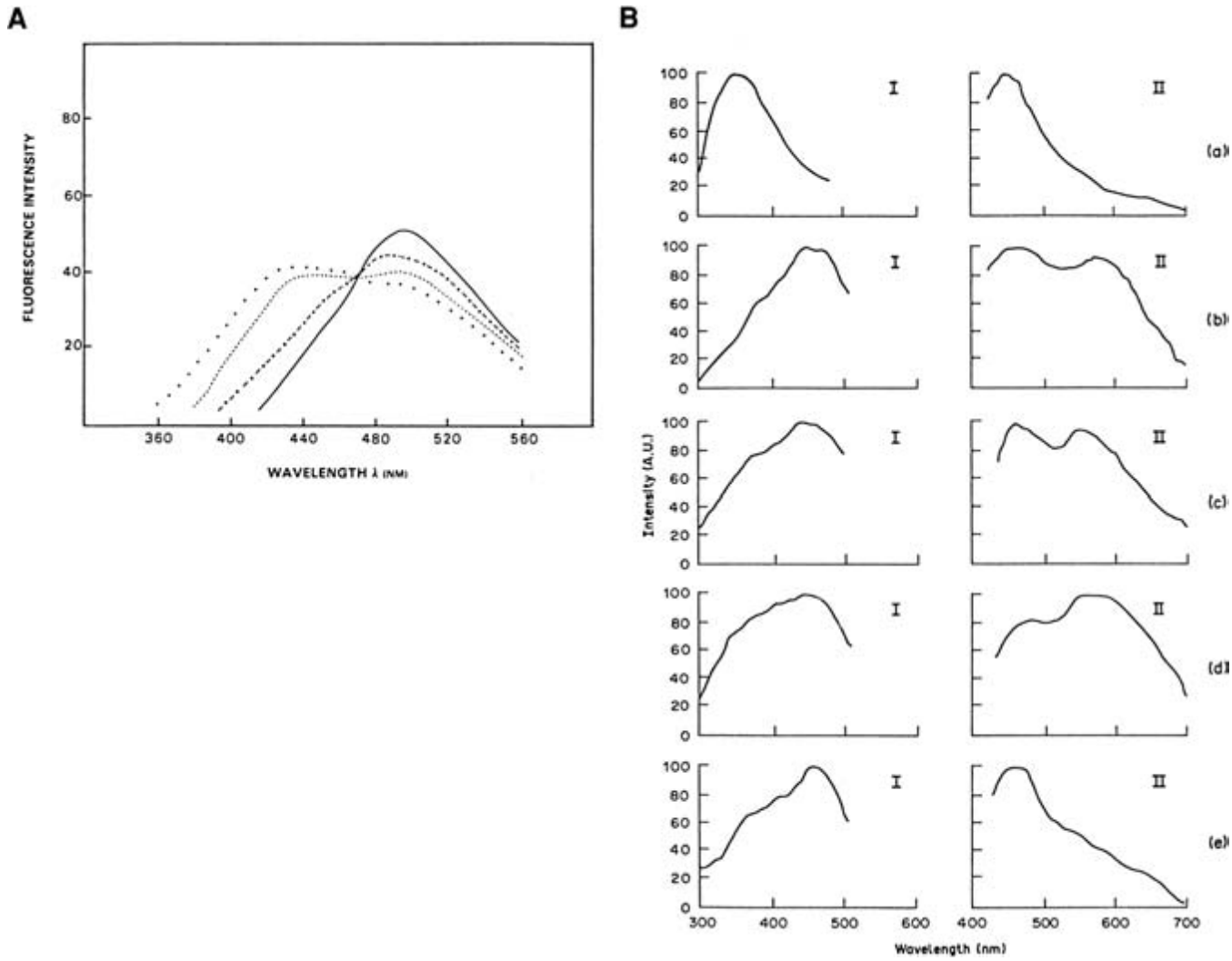

Fig. 16.4. Fluorescence spectra of auto-oxidized dopa melanin (A) and of intact melanin granules from human RPEs of different age groups (B). (A) Fluorescence of melanin was observed as a function of its excitation wavelength: 340 nm (dotted line extending to the shortest wavelengths) and 400 nm (solid line in the longest wavelength region), with fluorescence induced by excitation at 360 nm and 380 nm being characterized by dotted lines in between. (B) Left curves (I) are excitation spectra with emission monitored at 570 nm, and right curves (II) are emission spectra with excitation at 364 nm. (a) Fetal, (b) 5–29 years, (c) 30–40 years, (d) > 50 years and (e) 1 year bovine melanin. Reprinted from Gallas and Eisner (1987), with kind permission; and from Boulton *et al.* (1990), with kind permission from Elsevier Science.

lized, after drying, in solid-state KBr (Kozikowski *et al.*, 1984). The authors observed a weak broad luminescence, centered at about 540 nm, when natural melanins from human hair and *Sepia* ink were excited by an argon-ion laser emitting at 488 nm. A dramatic increase in the fluorescence emission intensity was brought about by the solubilization of the melanin, achieved by treatment with H_2O_2 at high pH. [Similar increases in the detectable fluorescence of synthetic DOPA melanin and melanoproteins, after early oxidative degradation of the melanin by treatment with H_2O_2, were also reported by Soviet workers (Korzhova *et al.*, 1989).] An induction of a distinct fluorescence of melanin *in situ* in tissue sections was observed upon irradiation of the sections with UV light (Elleder and Borovansky, 2001). The phenomenon was explained as being due to an oxidative breakdown of melanin induced by simultaneous action of UV and hydrogen peroxide (Elleder and Borovansky, 2001).

Fluorescence of synthetic DOPA melanin was studied by Gallas and Eisner (1987) as a function of excitation wavelength and melanin concentration. Fluorescence of melanin, excited at 340 nm, corrected for attenuation of excitation and emission beams and removal of background Raman and impurity fluorescence signals, exhibited a broad signal. Upon deconvolution, the data indicated the presence of two emission bands, one with a maximum at about 430 nm and the other with a maximum at 510 nm (Fig. 16.4). When melanin

was excited at longer wavelengths, such as 400 nm, one emission band (at 500 nm) was observed predominantly. Assuming two interacting species (quinone and hydroquinone moieties of melanin) that can form a complex, quinhydrone, the fluorescence data of melanin were interpreted by a simple model, in which excitation occurs mainly through the broad absorption band of the complex. The complex can then dissociate, leaving the quinone or hydroquinone groups in the excited state, or can decay by radiative processes after a series of vibronic transitions. The latter would lead to one band of fluorescence emission, while the radiative decay of the excited quinone (or hydroquinone) units would constitute another band of fluorescence emission. However, the validity of the conclusions discussed above has been questioned by Nofsinger and Simon (2001), who in a more recent study analyzed spectral and kinetic parameters of radiative relaxation of a natural eumelanin. Using different molecular-weight fractions of melanin, extracted from the ink sacs of *Sepia officinalis*, these authors measured excitation and emission spectra of the melanins. They found no structure for the corrected emission spectra in the wavelength range 400–550 nm for excitation wavelengths above 325 nm. On the other hand, the authors demonstrated that emission properties of *Sepia* melanin varied with the aggregation state of the melanin. Although different melanins were examined in these two studies, it seems unlikely that melanin origin was responsible for the observed differences in melanin fluorescence. Indeed, in a recent independent study, Meredith and Riesz (2004) reported on radiative relaxation quantum yields for a synthetic dopa-melanin. Using a strict renormalization procedure to correct for pump beam attenuation and heavy reabsorption of the emission, the authors found no evidence of a double-peaked feature in the emission spectrum of the melanin that was reported by Gallas and Eisner (1987). Meredith and Riesz (2004) observed that the position, width, and intensity of the emission maxima, as well as the quantum yield, varied as a function of the excitation wavelength. The emission quantum yield was calculated to be in the range 0.0005–0.0007, almost an order of magnitude lower than that determined by Nofsinger and Simon (2001) for *Sepia* melanin. The data may indicate that fluorescence emission in a synthetic eumelanin is derived from ensembles of small chemically distinct oligomeric units that can be selectively pumped.

A detailed analysis of the photodynamics of melanin was carried out by the Simon group, using a number of complementary time-resolved techniques, such as femtosecond transient absorption spectroscopy, picosecond fluorescence spectroscopy, and nanosecond photo-acoustic calorimetry (Forrest *et al*., 2000; Nofsinger and Simon, 2001; Nofsinger *et al*., 1999, 2001; Ye and Simon, 2002, 2003). The main results could be summarized as follows. The emission dynamics of melanin are nonexponential and require a sum of exponentials to generate functional forms that provide a fit for experimental data. Thus, for *Sepia* melanin, four exponentials are required with the following lifetimes (and amplitudes): 56 ps (0.54), 0.51 ns (0.22), 2.9 ns (0.16), and 7.0 ns (0.08). Emission decay of a synthetic pheomelanin is also nonexponential and can be fitted by three exponentials: 46 ps (0.66), 1.2 ns (0.166), and 6.2 ns (0.18). When different molecular-weight fractions of *Sepia* melanin were examined, the authors found that, although large size fractions were the source of short emission decay (lifetimes less than 1 ns), small melanin fractions were responsible for long-lived emission dynamics (lifetimes greater than 1 ns). Nonexponential character of transient absorption decay dynamics has also been observed for eumelanin and pheomelanin following photoexcitation with an ultrashort laser pulse at 303 nm. Global fitting parameters, obtained from transient absorption data for *Sepia* melanin, give the following characteristic lifetimes: 0.56 ps, 3.2 ps, and 31 ps. Corresponding parameters for a synthetic pheomelanin are 0.46 ps, 2.9 ps, and 27 ps. The data clearly show that melanin is a system in which a very efficient thermal relaxation occurs. This is to say that energy absorbed by melanin photons is rapidly converted into heat via very fast internal conversion.

Excitation and emission spectra of intact melanin granules of the retinal pigment epithelium (RPE) from human donors of different age groups were studied by continuous wave (CW) and time-resolved spectrofluorometry (Boulton *et al*., 1990; Docchio *et al*., 1991). Fluorescence spectra of RPE melanin exhibited distinct age-related changes; the excitation maximum shifted from 350 to 450 nm and became broader with increasing age of the RPE, and the emission spectrum developed a second peak (in addition to the main intensity at 440 nm) at about 560 nm, which grew in intensity with age. The overall fluorescence intensity of RPE melanin increased with increasing age of the donor. Using time-resolved fluorescence spectroscopy, four decay components in the fluorescence spectrum of RPE melanin have been identified (0.14–0.19 ns, 0.43–0.58 ns, 1.98–2.30 ns, and 5.49–7.90 ns). The resultant time-integrated and time-gated fluorescence spectra of RPE melanin also exhibited marked variations with age. Although the results of these impressive investigations are not completely understood, some of the observed age-related changes could be explained by partial oxidative degradation of the melanin and formation of complex pigment granules such as melanolipofuscin (Rózanowska *et al*., 1995).

Melanin as an Amorphous Semiconductor

The remarkable ability of melanin to absorb near infrared, visible, and ultraviolet radiation almost indiscriminately has not been explained satisfactorily. Even though it cannot be ruled out that the observable optical properties of melanin result from the presence of many chromophores, the absorption bands of which overlap to the extent that an absorption continuum is formed in the entire UV-vis range, no evidence has been provided to prove this view. In fact, the existing relevant data are rather ambiguous in this respect. Thus, irreversible bleaching of melanin, induced by oxidative degradation, is at first accompanied by a gradual decrease in the absorbance by melanin in both the visible and the ultra-

violet regions (Korytowski and Sarna, 1990; Wolfram and Albrecht, 1987). This could be explained by assuming that all the chromophores in melanin are equally susceptible to oxidative degradation induced by light plus oxygen or by hydrogen peroxide. Synthetic melanin, which is extensively bleached, has a severely modified absorption spectrum compared with that of native melanin; the absorption in the visible region, particularly the red, is significantly reduced, whereas it increases in the UV. The absorption changes in bleached melanin seem to be correlated with changes in an important intrinsic molecular probe of melanin—its free radical centers (Sarna *et al*., 2003).

In an attempt to explain the unusual optical behavior of melanin (as well as its electrical properties), a band model, viewing the melanin as an amorphous semiconductor, has been proposed (Crippa *et al*., 1978; Kurtz *et al*., 1987; McGinness and Proctor, 1973; Strzelecka, 1982a, b). An optical band gap value of 3.4 eV, reported for synthetic DOPA melanin, was based on optical absorption and photoconductivity measurements (Crippa *et al*., 1978). On the other hand, significantly lower values for the optical gap (1.4–1.73 eV) of several natural melanins and synthetic DOPA melanin were determined by Strzelecka (1982a, b). Band gaps in the range of 1.0–1.4 eV were also found by Kurtz *et al*. (1987) for various melanins. Finally, an optical gap value of 1.45 eV could be estimated from the photoconductivity edge of about 850 nm reported by Trukhan *et al*. (1973). The reason for significant discrepancies in the reported band gap values is not clear. It can be speculated that some of the differences may arise from variations in the types of melanin and/or the preparation of the samples that were used. A mechanism for band gaps in melanins, based on mobility gaps, typical for amorphous semiconductors, has been proposed by McGinness (1972), and unusual current–voltage (I–V) characteristics for a wet melanin were explained by amorphous semiconductor switching in the melanin (McGinness *et al*., 1974). This explanation was later questioned (Chio, 1977), but the hypothesis about melanin being an amorphous semiconductor has led to additional speculations and experiments.

Measurements of electrical photoconductivity of melanin were reported in 1968 (Potts and Au, 1968), and photoconductivity of natural melanin from frog RPE was studied by the microwave dispersion technique (Trukhan *et al*., 1970, 1973). In the latter works, the current carrier was found to be of hole character, and its mobility was estimated to be 15 cm^2/Vs.

Measuring dark, DC, steady-state conductivity as a function of the applied voltage, and plotting the specific conductivity vs. inverse absolute temperature and optical absorption of melanin vs. the photon energy, basic semiconductor characteristics of synthetic DOPA melanin and several natural melanins were obtained (Strzelecka, 1982a, b, c). Specific conductivity of the natural melanins was in the range 10^{-11}–10^{-10}/Ω/cm, whereas it was almost 10^{-7}/Ω/cm for the synthetic melanin. Thermal activation energies for natural melanins were found to be 0.93–1.04 eV at 298–333 K. Interestingly, the synthetic melanin appeared to have two values of activation energies: below 311 K it was 0.1 eV and above 313 K it increased to 0.78 eV. The energy of 0.1 eV was taken as evidence for a band of states (0.2 eV wide) of the Fermi level. Unfortunately, it does not appear that these results, obtained with DOPA melanin, have been reproduced by an independent study.

Dark and photoinduced, DC, steady-state conductivity measurements were also carried out on synthetic melanins prepared from dopamine, epinephrine (adrenaline), adrenochrome, and adrenolutin (Jastrzebska *et al*., 1990). Specific conductivities of the melanins were in the range 1.3×10^{-12}–1.5×10^{-10}/Ω/cm, which is significantly lower than that reported for DOPA melanin (Strzelecka, 1982a, b). Thermal activation energies determined for the catecholamine melanins, on the other hand, were rather similar to that of DOPA melanin: 0.62–0.73 eV. Except for adrenolutin melanin, no photocurrent was observed for other melanins that were tested.

The importance of absorbed water on melanin conductivity has been demonstrated in a more recent study (Jastrzebska *et al*., 1995). The authors reported that thermal activation energy of dark conductivity varied in the range 47–73 kJ/mol, depending on the hydration state of melanin. Indeed, in a more recent study, Meredith *et al*. (2004) showed that the electrical conductivity of a synthetic eumelanin varied by five orders of magnitude if the melanin was exposed to relative humidity in the range 10–80%. The authors of the latter study concluded that the electronic contribution to the conductivity of melanin was very small and eumelanin should essentially be considered as an insulator. A polaron and hopping model for melanin conductivity, based on the results of dielectric spectroscopy and photoconductivity studies of synthetic DOPA melanin, has been proposed by Jastrzebska *et al*. (2002a, b).

Charge transport in synthetic catechol melanin was studied by analyzing current–voltage characteristics and temperature dependence of DC steady-state conductivity (Osak *et al*., 1989a), and polarization and depolarization currents at different electric fields and temperatures (Osak *et al*., 1989b). The data indicated a deviation from Ohm's law for voltages higher than several hundred volts. The temperature dependence of DC steady-state conductivity at an applied voltage of 85 V (for which Ohm's law was obeyed in the entire temperature range studied) suggested two thermal activation energies: below 3°C, the activation energy was 0.76 eV and, at higher temperatures, it was 1.58 eV. Long-lasting polarizing currents, detected in the melanin, were described in terms of the movement of charges trapped in deep states of the polymer. Polarization of the melanin samples had an activation character, with the thermal activation energy of 0.67 eV. According to the authors, the current carrier in the catechol melanin was predominantly of electron character. The authors further concluded that transport of charges involved both charges generated in the sample and charges injected from electrodes.

The results of the studies on semiconductor properties of melanin, briefly reviewed in this section, are difficult to interpret. The observable DC dark and photoconduction of melanin are very low and depend critically on the water

content in the tested samples. This is a serious consideration in strongly hygroscopic samples such as melanin. Thus, unless all experimental parameters are strictly controlled, including the preparation of melanin and its physicochemical state, DC conductivity measurements on melanin samples may lead to random results and erroneous conclusions. It therefore appears that, at this time, the semiconducting properties of melanin have not been rigorously determined. Even if melanin is an amorphous semiconductor, it remains to be established whether this description of melanin offers any new insight into our understanding of the structure and properties of melanin, and its biological functions.

A band model for melanin that could explain some of its optical absorption, electron exchange, and paramagnetic properties has been suggested following theoretical calculations of the electronic structure of the melanin monomer and dimer units (Longnet-Higgins, 1960; Pullman and Pullman, 1961). Extrapolating the bonding character of the lowest unoccupied orbital (LUMO) of one particular dimer of 5,6-indolequinone to the lowest conduction band of the infinite polymer, the authors pointed out the tendency of such a melanin model to be an electron acceptor and that this would explain the trapping of free radicals. The semiconducting polymer model, however, was found to be inconsistent with EPR data that apparently ruled out the occurrence of any extensive π-electron delocalization in melanin (Blois *et al*., 1964).

More recent theoretical investigations of model polymers for eumelanins have been carried out by Galvao and Caldas (1990a). Using the Hückel π-electron approximation, and the same parametrization used by Pullman and Pullman (1963), the authors studied the electronic structure of a family of ideal ordered polymers arising from 5,6-indolequinone in different redox states. The authors found that structural effects, such as direction of polymerization, began to emerge as the length of the polymer increased. The redox state of the melanin units seemingly played an important role in its band structure, e.g. a polymer built from 5,6-dihydroxyindole units consistently showed larger gaps and narrower bands, whereas finite chains of semiquinone units exhibited bonding character of their lowest unoccupied molecular orbital. An important conclusion, reached by the authors, was the inevitable occurrence of end-type defects in any finite melanin polymer. The existence of defects with deep gaps is consistent with the hypothetical electron acceptor properties of melanin. According to the authors, an electron injected at the surface of the pigment (by a donor molecule) could be trapped at an end-type defect state producing the observable electron paramagnetic resonance (EPR) signal. Furthermore, they argued that capture of a second electron at the same defect would not be favored because of electron–electron repulsion effects and, as a result, such an electron would be easily transferable to another empty defect center. In this model, unpaired electrons are likely to have rather uniform distribution among defects, and the spin concentration is expected to be roughly independent of temperature. The authors speculated that, for samples in solution, the chains of the polymer would behave more like isolated molecules so that double occupation of defects was more probable and, therefore, an enhanced temperature dependence of the spin concentration was predicted for samples in solution.

In an extension of their theoretical study of model polymers for eumelanins, Galvao and Caldas (1990b) investigated the effects of different kind of defects, such as the aggregation of carboxyl radicals into one skeleton monomer, aggregation of a host monomer in a lateral misplaced position, and faults in the polymerizing sequencing. The data indicated that, although the end-type defect is not deactivated by the introduction of other defects, new capture centers might be formed, which could enhance the electron-accepting properties of the melanin.

Free Radicals in Melanin

Melanin is the only known biopolymer that both *in vivo* and *in vitro* contains relatively high concentrations of persistent free radical centers that can easily be detected by EPR spectroscopy (Blois *et al*., 1964; Enochs *et al*., 1993a; Mason *et al*., 1960; Sarna and Lukiewicz, 1971; Sarna and Swartz, 1978; Sealy *et al*., 1980). These free radicals have turned out to be very important experimental parameters both to explain the properties of melanin and to serve as analytical probes for investigating the structure and properties of melanins.

The EPR signals of melanin are specific for the two main types of melanin pigments (Fig. 16.5). At X-band (~9.5 GHz), eumelanins have a single slightly asymmetric line 4–6 G wide with a g-factor close to 2.004. The EPR spectrum of pheomelanin typically consists of three spectral features with an overall width of about 30 G, and g = 2.005.

It is important to stress that, even though the EPR signal of melanin is very persistent, and no physicochemical procedures are known to quench it irreversibly without decomposition of the material, the free radicals in melanin are by no means "stable." In fact, it has been demonstrated that the concentration of free radicals can be changed reversibly by almost two orders of magnitude (Sarna *et al*., 1981). Several physicochemical agents have been shown to modify the amount and/or type of free radicals in melanin: ultraviolet and visible radiation (Cope *et al*., 1963; Felix *et al*., 1979; Ostrovsky and Kayushin, 1963; Sarna and Sealy, 1984b; Sarna *et al*., 1985a, b), pH (Chio *et al*., 1982; Grady and Borg, 1968), temperature (Arnaud *et al*., 1983; Chio *et al*., 1980), complexing of diamagnetic multivalent metal ions (Felix *et al*., 1978a), redox reactions of the melanin polymer (Dunford *et al*., 1995; Korytowski *et al*., 1986; Reszka and Chignell, 1993; Sarna and Swartz, 1993), and the degree of hydration of the melanin (Sealy *et al*., 1980).

As discussed in a later section, it is believed that most of the changes in the free radicals induced by these agents are due to changes in the so-called comproportionation equilibrium, i.e. the equilibrium between fully reduced and oxidized subunits, and the intermediate semi-reduced (semi-oxidized) states that

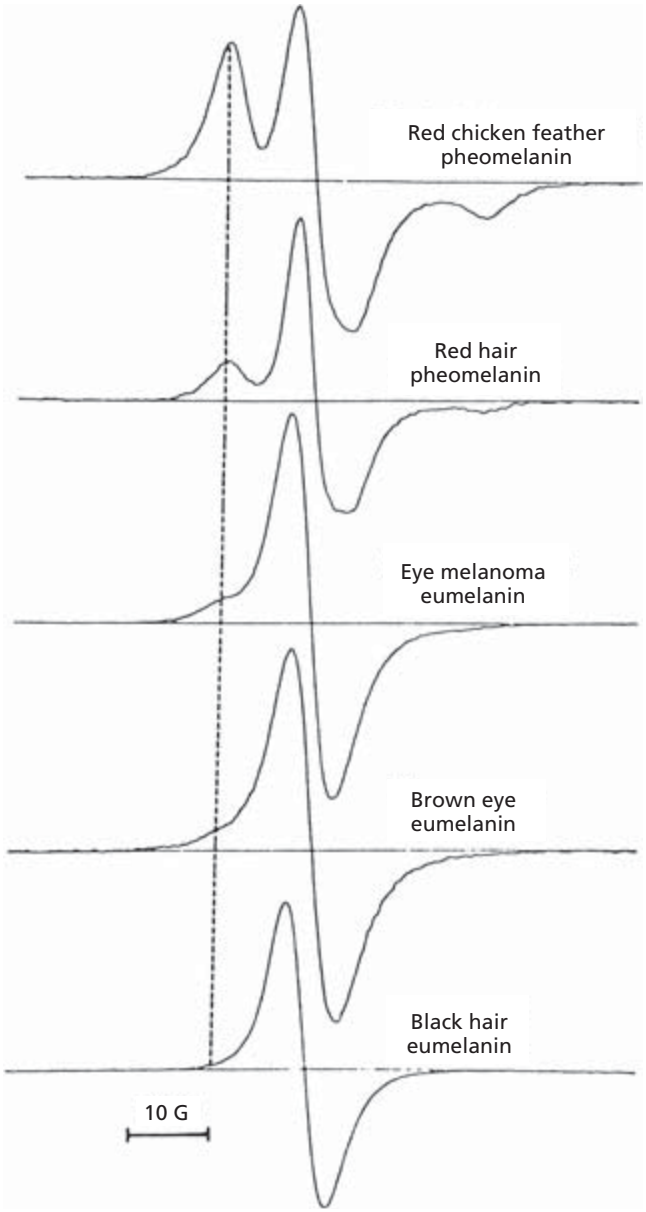

Fig. 16.5. EPR spectra from frozen suspensions of natural melanins (1 mg/ml) containing 3 mM Zn^{2+} (pH 4.5) at –196°C. Spectra were recorded at X-band. Reproduced from Sealy *et al.* (1982b), with permission.

are free radicals (Felix *et al.*, 1978a; Sealy, 1984). The equilibrium is significantly shifted toward the diamagnetic form of the melanin subunits, and the free radical content of synthetic DOPA melanin is around 2×10^{18} spins/g (referred to mass of the dried melanin) under typical experimental conditions: pH 7, ambient temperature, no irradiation with ultraviolet or visible light, no metal ions, and fully hydrated samples. This corresponds to about one free radical per 1500 polymer units (assuming a molecular weight of 200 for the melanin subunit). The free radical content of purified melanin from the choroid of bovine eyes was reported to be about half that of DOPA melanin (Chio *et al.*, 1982). Although the number of melanin free radicals detected under typical experimental conditions is rather low because of the equilibria, the total number of participating units is likely to be substantially higher, similar to the maximum spin concentration obtained under any experimental conditions (Sarna *et al.*, 1981; Sealy *et al.*, 1980). It therefore appears that the comproportionation equilibrium, monitored by EPR in melanin, reflects a significant percentage of total monomer units (Chio *et al.*, 1980).

In addition to changes in the intensity of the EPR signal of melanin detected, there can be small, but distinct, changes in other spectral characteristics. For example, the g-factor of DOPA melanin is 2.0034 at pH 1, 2.0036 at pH 7, and 2.0042 at pH 12 (Chio *et al.*, 1982). The corresponding changes in the spin concentration are 2×10^{18}–1.2×10^{19} spins/g. The EPR signal of eumelanins (particularly synthetic DOPA melanin) at high pH becomes narrower and more asymmetric than at low pH. Dramatically different EPR spectra have been observed for pheomelanins (Sealy *et al.*, 1982a). The spectra, showing distinct changes with varying pH, were interpreted as being due to the presence of a different type of melanin free radical. It has been proposed that, unlike eumelanin, pheomelanin contains *ortho*-semiquinonimine radicals, in which the unpaired electron is delocalized on both oxygen and nitrogen atoms. As a result, an immobilized, nitroxide-like EPR spectrum is observed with the parallel component of the hyperfine coupling ($2\,A_{\parallel}$) being about 30 G. The *ortho*-semiquinonimine radical is in equilibrium with the pheomelanin subunits—fully reduced *o*-aminophenols and oxidized *o*-quinonimines. The free radical can predominantly be observed at low pH or, in the presence of complexing zinc(II) ions, at neutral and slightly acid pH.

The effect of complexing of diamagnetic multivalent metal ions on the melanin EPR signal is an important diagnostic test that can be used to determine the molecular nature of the subunits. Changes in the EPR spectra are consistent with complex formation between the metal ion and chelating polymer radicals (Felix *et al.*, 1978a). The structure of the chelating radicals can be inferred from line width changes that reflect hyperfine splittings associated with the metal ions. The magnitude of the hyperfine splitting from association with a particular metal ion is sensitive to the detailed structure of the free radicals in the melanin (Felix and Sealy, 1981), e.g. radicals complexed with the ^{113}Cd(II) isotope in melanins derived from DOPA, catechol, and cysteinyldopa have splittings of about 3.5 G, 7 G, and 15 G respectively (Sealy, 1984).

The established relationship between the amount of inhomogeneous broadening of the EPR signal of melanin induced by complexation of ^{113}Cd(II) with melanin and the origin of the melanin also has an important practical implication because it can be used for unambiguous differentiation of natural melanins (Jimbow *et al.*, 1984; Sealy *et al.*, 1982a; Vsevolodov *et al.*, 1991). Thus, EPR spectroscopy is a unique

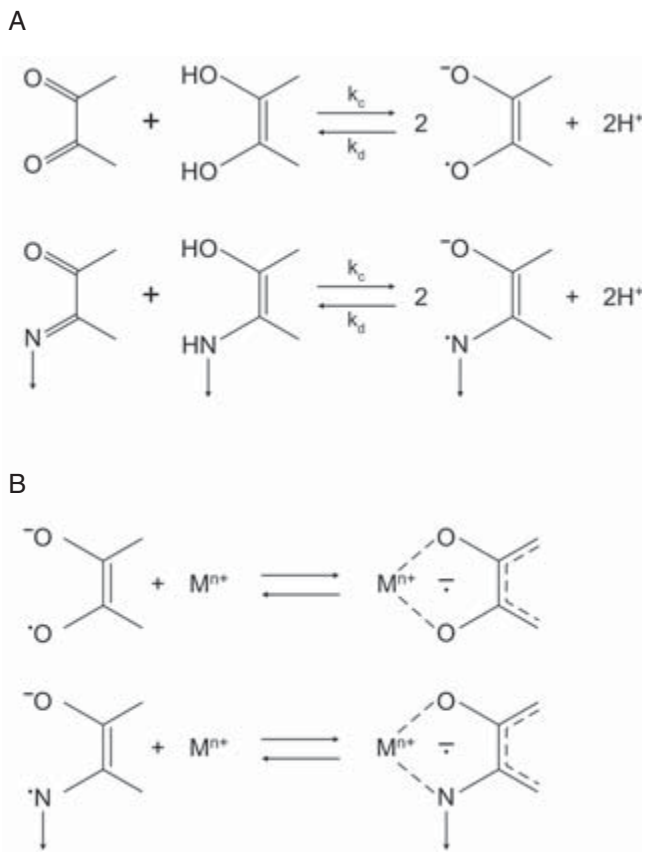

Fig. 16.6. The chemical structure of the monomer units of the melanin oligomers.

physical method that enables nondestructive analysis and characterization of melanins with high sensitivity and accuracy.

The molecular nature of inducible melanin radicals (extrinsic free radical centers) appears to be well understood. It is most likely determined by the chemical structure of the monomer units of the melanin oligomers that can engage in redox equilibria (Fig. 16.6A):

$$Q + QH_2 \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} 2SQ + 2H^+$$

These monomers are *o*-quinones, *o*-hydroquinones, and *o*-semiquinones in the case of eumelanin. Corresponding units for pheomelanin are *o*-quinonimines, *o*-aminophenols, and *o*-semiquinonimines respectively.

Any agent that can influence the equilibrium constant, k_c/k_d, may modify the detectable concentration of free radicals in melanin. A key aspect of the comproportionation equilibrium is stabilization of the radicals (Fig. 16.6B). Thus, diamagnetic metal ions that are able to form chelate complexes with the free radicals in the melanin shift the equilibrium toward the semiquinone free radicals. A similar phenomenon is observed at high pH: the induced deprotonization of the radicals results in their enhanced stabilization. This, in turn, increases the observable concentration of free radicals.

A careful analysis of the results of numerous EPR studies of melanin free radicals leads to the conclusion that the above may not be the only free radical centers present in the melanin polymer (Sarna, 1992; Sealy *et al*., 1980). In the authors' experience, regardless of the experimental conditions under which melanin is examined, its free radical content does not decrease below a certain level (providing the melanin is not decomposed). This seems to indicate the existence of two independent pools of melanin free radicals: extrinsic and intrinsic radical centers. The extrinsic radicals can be viewed as a convenient molecular probe, reporting on the molecular nature of the melanin monomer units and the redox state of its functional groups.

The intrinsic radicals, on the other hand, are somewhat less understood. They are probably paramagnetic centers that were generated during the formation of the melanin and trapped within the growing oligomers and aggregates of basic subunits. Because of severely restricted accessibility to any reactive extraneous agents and low chemical reactivity, these radicals are essentially "stable." The intrinsic radicals being associated with the melanin core can be viewed as a unique endogenous spin label reporting on the molecular state of the melanin and its integrity. For example, it has been demonstrated that the magnitude of the low-pH EPR signal of DOPA melanin (which is a measure of the intrinsic free radicals in melanin), subjected to oxidative degradation, corresponds to the degree of bleaching of the melanin in a reproducible and consistent way (Sarna *et al*., 2003).

Thus, the EPR spectrum of melanin, examined under non-extreme experimental conditions, is usually a superposition of two or more EPR signals arising from the corresponding free radical centers. Detailed analysis of EPR spectra of melanins of various origins, recorded at 35 GHz (Q-band) to increase spectral resolution, over a range of pH, led to a conclusion that the EPR spectrum of melanin at intermediate pH was a composite of two spectra arising from anionic and neutral radicals with different g-factors (Grady and Borg, 1968). Such an analysis has later been refined by recording second derivative Q-band EPR spectra of DOPA melanin at various pH and the use of more advanced computer simulations of the EPR spectra (Pasenkiewicz-Gierula and Sealy, 1986). The authors interpreted the EPR spectra of frozen aqueous dopa-melanin in terms of four different spectral species related to anion *o*-semiquinone radicals with relatively localized unpaired electrons and cation radicals with extended delocalization of their unpaired spins. Recent advances in EPR spectroscopy made possible the examination and analysis of EPR spectra of various melanins at very high frequency (W-band, 95 GHz; Nilges, 1998). It is expected that such high-resolution EPR measurements and sophisticated computer simulations will make possible unambiguous identification of the molecular nature of all radicals in melanin, and determination of their role in the physicochemical activity of melanin.

As noted previously, dramatic changes in the intensity of the EPR spectrum of melanin free radicals can be induced by paramagnetic metal ions (Blois *et al*., 1964; Sarna *et al*., 1976). Even though the quenching effect of copper(II) ions on melanin EPR signal was originally interpreted as a chemical reaction between copper and free radicals (Blois *et al*., 1964), it has later been shown that the effect is purely magnetic in nature (Sarna *et al*., 1976). Using lanthanide ions, which have similar chemical properties but quite different magnetic properties, it was possible to observe consistent changes in the amplitude and microwave power saturation of the EPR signal of the radicals in melanin as a function of the type and concentration of the added metal ion. Lanthanides with a very short spin-lattice relaxation time had a strong effect on microwave power saturability of the melanin EPR signal, but only weakly quenched the signal amplitude. The effect can be understood by viewing the interacting melanin radical with neighboring metal ions as magnetic dipoles fixed in space. As a result of such an interaction, dipolar broadening of the narrow EPR signal of melanin occurs. As the magnitude of the broadening depends inversely on the cube of the distance between the melanin free radical and the metal ions, quenching of the melanin EPR signal is a distinct function of the concentration of paramagnetic metal ions in the melanin environment. This static dipolar interaction is modulated by spin-lattice relaxation of the metal ion, which efficiently decreases the amount of the dipolar broadening of the EPR signal of melanin. The faster the rate of relaxation of the metal ion, the weaker the dipolar broadening observed as the metal ion-induced quenching of the melanin EPR signal. The theory of such an unusual dipolar broadening has been described by Leigh (1970).

For very rapidly relaxing paramagnetic metals, the fluctuating magnetic dipole field sensed by the melanin free radicals provides a powerful spin-lattice relaxation mechanism. Maximum relaxing efficiency of metal ions occurs when the rate of their spin-lattice relaxation approximates the microwave frequency.

The quenching of the EPR signal of melanin is much more pronounced when relatively slowly relaxing metal ions are used, such as copper(II), iron(III), manganese(II) and, among lanthanides, gadolinium(III). It is important to emphasize that, in melanins, the dipolar broadening effect decreases the melanin EPR signal intensity without any apparent broadening of its line width. Thus, the effect can easily be misinterpreted by an inexperienced researcher as a real reduction in the free radical content of the examined melanin. This becomes a serious consideration when analyzing natural melanins, which can, both *in vivo* and *in vitro*, accumulate substantial amounts of transition metal ions, including paramagnetic metal ions (Enochs *et al*., 1993b; Zecca and Swartz, 1993; Zecca *et al*., 1994). One way to deal with such a problem is carefully to determine the microwave power saturability of the melanin samples and their signal amplitude *before* and *after* subjecting the samples to procedures that may stimulate the release of the metal ions that are bound to the melanin. In the authors' experience, washing the melanin samples in an aqueous solution of high concentrations of powerful metal ion chelators such as EDTA or DTPA and desferal for several hours is usually quite effective in this respect. Incubating melanin samples in solutions of hydrochloric or sulfuric acid (0.1–1.0 M) is also effective; however, the latter procedure may be unacceptable because of possible modifications of the melanin chemical structure induced by acids (Liu *et al*., 2003; Prota, 1988).

Ion Exchange Properties

The ability of melanin to bind metal ions is one of its basic physicochemical properties that affects the biological effects of this pigment (reviewed by Enochs *et al*., 1994; Sarna, 1992; Sarna and Rozanowska, 1994; Swartz *et al*., 1992). As is the situation for other properties of melanins, the study of ion exchange properties of melanin has been valuable both to understand the biological effects of these interactions and as a tool to investigate the structure and properties of melanins. It is well known that melanin, both *in vivo* and *in vitro*, can accumulate substantial amounts of multivalent metal ions (Bruenger *et al*., 1967; Cotzias *et al*., 1964; Larsson and Tjälve, 1978; Lydén *et al*., 1984; Okazaki *et al*., 1985; Simonovic and Pirie, 1963; Valkovic *et al*., 1973; Zecca and Swartz, 1993). It has been estimated that the number of metal ion binding sites in eumelanins is about 20% of the number of monomeric units in the polymer (Potts and Au, 1976). Similar estimates were made after titrating the EPR signal of melanin with paramagnetic metal ions; plots of microwave power saturability vs. concentration of metal ions were sigmoidal and yielded a value of 6×10^{20} total metal binding sites per gram of dried melanin (Sarna *et al*., 1976).

Binding of multivalent metal ions by melanin is a pH-dependent phenomenon; the amount of metal ions bound to melanin usually increases with pH in the pH range 1–7. This indicates that melanin behaves as a weak acid ion exchange resin. This is expected because the melanin polymer contains a number of functional groups (Larsson, 1998) that can serve as potential ligands for the interacting metal ion. Thorough analysis of complexing of metal ions with melanin requires precise control of the sample pH. This is because the pH of the sample (which is likely to change after adding substantial amounts of metal ions to an aqueous suspension of melanin) may determine not only the amount of metal ions that bind to melanin, but also the type of complexes that are formed.

The radioactive isotope ^{54}Mn(II) was used to determine binding parameters for interactions with bovine eye melanin (melanoprotein), human hair melanin, and synthetic dopamine melanin, and the binding was analyzed by the method of Scatchard (Lydén *et al*., 1984). Four classes of binding sites were found in bovine eye melanin, with the corresponding number of sites (in μmol/mg melanin) and the apparent association constants: $n_1 = 0.072$, $K_1 = 5.2 \times 10^7$; $n_2 = 0.195$, $K_2 = 1.8 \times 10^6$; $n_3 = 0.661$, $K_3 = 2.0 \times 10^4$; $n_4 = 0.398$, $K_4 = 1.1 \times 10^3$. The total binding capacity was 1.33 μmol/mg melanin, which is equivalent to about 20% of the number of all melanin units, assuming 200 as the molecular weight for an average melanin monomer unit. The

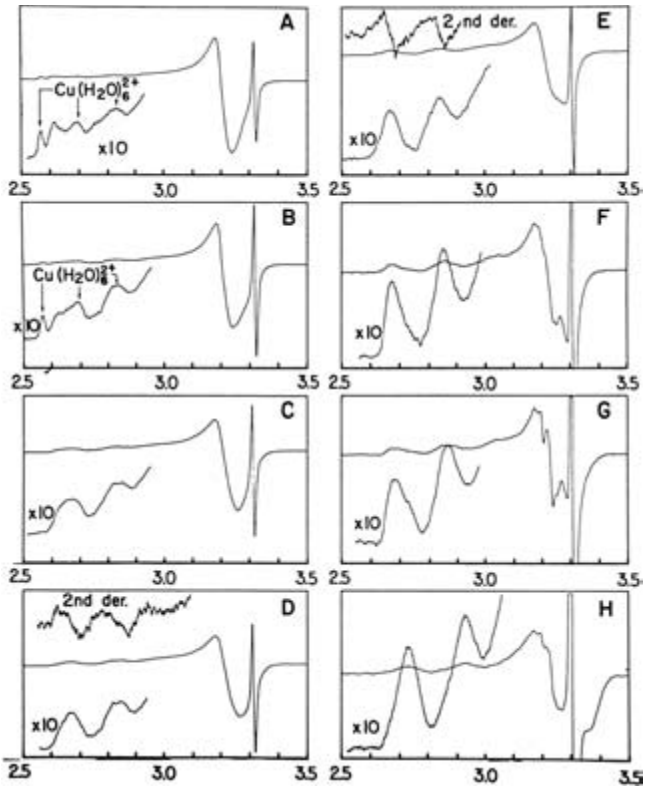

Fig. 16.7. EPR spectra of copper ($^{63}Cu^{2+}$) bound to melanin from bovine eye choroid at: (A) pH 1.6, (B) pH 1.9, (C) pH 2.9, (D) pH 4.3, (E) pH 5.8, (F) pH 8.7, (G) pH 11, (H) pH 12.6. Spectra were recorded at –196°C using an X-band EPR spectrometer equipped with second derivative capabilities. The abscissa indicates magnetic field strength in kilograms. The ratio of melanin isonomers to copper ions was 100:1 (assuming a monomeric molecular weight of 200). Reproduced from Sarna *et al.* (1980a), with permission.

authors indicated, however, that the binding capacity of the bovine eye melanin was rather high, as the total binding capacity was 0.23 µmol/mg melanin for human hair melanin and 0.15 µmol/mg melanin for dopamine melanin.

Using X-band EPR spectroscopy and the isotope $^{63}Cu(II)$ as a molecular probe, the molecular nature of the main binding sites in synthetic DOPA and catechol melanins, and natural melanin from bovine eye choroid have been studied (Froncisz *et al.*, 1980; Sarna *et al.*, 1980a). The results can be summarized as follows. Depending on the pH of the system, copper(II) can form a number of complexes with melanin that involve different functional groups of the polymer and exhibit different stabilities (Fig. 16.7). Nearly all complexes involve just one or two ligands from melanin; the others are presumably H_2O or OH^- groups. At pH < 7, binding is predominantly to monodentate carboxyl complexes and, in eumelanin, also to bidentate nitrogen–carboxyl complexes. The corresponding EPR spectral parameters are within the range: $g_{\|} = 2.26–2.34$, $A_{\|} = 460–560$ MHz, and $g_{\perp} = 2.066–2.076$. The complexing of copper ions by melanin can be observed at low pH (below pH 3) in the EPR spectra of frozen suspensions of melanin in solutions of copper(II) with a ratio of melanin monomers to copper ions of 100:1 (assuming a monomeric molecular weight of 200). At pH 7 and above, binding is to phenolic hydroxyl groups, but the number of such sites is much less in natural melanin than in synthetic DOPA melanin. The corresponding magnetic parameters are: $g_{\|} = 2.24–2.26$, $A_{\|} = 560–579$ MHz, $g_{\perp} = 2.054–2.064$. At very high pH (above pH 11), the EPR signal of natural melanin with Cu(II) is unlike any found in synthetic melanins. Its spectroscopic parameters are: $g_{\|} = 2.18$, $A_{\|} = 610–620$ MHz, $g_{\perp} = 2.050$.

The assignment of complexes of Cu(II) to functional groups of melanin has been further supported by EPR and elemental analysis of chemically modified synthetic DOPA melanin (Sarna *et al.*, 1981). Blocking the melanin phenolic hydroxyl and carboxylic groups by methylation, ethylation, or acetylation was accompanied by consistent changes in the EPR spectra of melanin–Cu(II) complexes and reduction in the amount of Cu(II) that was bound to melanin.

Using a potentiometric method, in combination with mathematical fitting and correlative spectroscopies, Szpoganicz *et al.* (2002) quantitated the binding sites of a colloidal suspension of synthetic melanin, and compared the metal-binding affinities of the melanin functionalities. One of the most interesting conclusions reached by the authors was the apparent role of a quinonimine functionality in metal binding by DHI-melanins. On the other hand, recent quantum mechanical calculations of selected melanin monomer units indicate that the quinonimine tautomer of 5,6-indolequinone should not be present in melanin to any appreciable extent (Il'ichev and Simon, 2003).

As virtually identical EPR spectra of melanin–copper(II) complexes have been observed for choroidal melanoprotein and for protein-free melanin (obtained from the melanoprotein by hydrolysis with cold hydrochloric acid), it has been concluded that the protein component does not play a significant role in metal ion binding. This conclusion was supported by results of the titration of the free radical signal of melanin with Cu(II) in melanoprotein and purified melanin; the corresponding titration curves were identical.

Binding of copper ions to melanin is a time-dependent process. At moderately acidic pH, after addition to suspensions of melanin, cupric ions rapidly form an initial complex, which rearranges within hours to a more stable monodentate or bidentate complex. At pH 3.0, the complex is predominantly the monodentate complex with the carboxyl groups of melanin. Similar intramolecular rearrangements of iron(III) have been observed recently for synthetic neuromelanins (Shima *et al.*, 1997).

The binding of metal ions to melanin was also studied by the utilization of ferric ions as molecular probes (Sarna *et al.*, 1981). The EPR spectra of Fe(III)–melanin complexes at neutral and weakly acidic pH (pH 3–7) were found to be almost indistinguishable (apart from signal intensity). The most prominent feature of the spectra was a single asymmetric line at g = 4.3. Similar EPR spectra of iron were detected in natural melanin from bovine eye choroid (Sarna *et al.*, 1980a), in melanosomes from human and bovine retinal pigment epithelium (Zareba *et al.*, 2005), and in neuro-

melanin (Enochs *et al*., 1993a; Zecca and Swartz, 1993). The EPR data of iron–melanin complexes can be compared with results obtained by another powerful spectroscopic technique—Mössbauer spectroscopy (Bardani *et al*., 1982; Gerlach *et al*., 1995; Kochanska-Dziurowicz *et al*., 1985; Sarna *et al*., 1981). For fully hydrated DOPA melanin samples incubated with the ^{57}Fe(III) isotope at pH 3 and pH 7, the Mössbauer spectra at 77 K were essentially identical, with quadrupole splitting 0.33 ± 0.005 mm/s and isotopic shift 0.17 ± 0.04 mm/s (Sarna *et al*., 1981). The data from EPR and Mössbauer studies suggest that ferric ions bind to melanin predominantly via phenolic hydroxyl groups and form high-spin complexes with distorted octahedral or rhombic symmetry, with a coordination number of 4–6. It can be speculated that the functional groups of melanin provide the four planar ligands for Fe(III) complexes with melanin, while the two remaining ligation sites are occupied by OH^- or H_2O. A significant modification of melanin–iron complexes due to drying may be inferred from the fact that substantially different Mössbauer parameters were observed when examining fully hydrated (Sarna *et al*., 1981) and dried melanin samples (Bardani *et al*., 1982; Kochanska-Dziurowicz *et al*., 1985). Drying of neuromelanin, isolated from human substantia nigra seems to modify the state of iron ions bound to melanin, as their Mössbauer parameters become similar to those of human hemosiderin or ferritin (Galazka-Friedman *et al*., 1996; Gerlach *et al*., 1995).

In a recent study of ion exchange properties of *Sepia* melanin, Liu *et al*. (2004) compared the ability of EDTA to remove Mg(II), Ca(II), Sr(II), and Cu(II) bound to the melanin granules. The authors found that the binding constants of *Sepia* melanin at pH 5.8 for all these ions were higher than that of EDTA. The binding of Fe(III) was concluded to involve coordination to *o*-dihydroxyl groups. The authors also found evidence that Ca(II) and Mg(II) were bound to melanin via coordination to carboxylic groups.

It is worthwhile emphasizing that ionic binding to melanin is by no means restricted to multivalent metal ions. Strong complexes of melanin with organic cations, both *in vivo* and *in vitro*, have been observed (Bielec *et al*., 1986; D'Amato *et al*., 1986; Larsson and Tjalve, 1979; Larsson *et al*., 1977; Lindquist, 1973; Lindquist and Ullberg, 1974; Lindquist *et al*., 1988; Link *et al*., 1989; Lydén *et al*., 1983; Stepien and Wilczok, 1982). Among the molecules that exhibit very high binding affinity with melanin are cationic forms of porphyrin derivatives, phenothiazine derivatives, chloroquine and its derivatives, and quaternary bipyrridyllium salts (paraquat and diquat). Even though different types of interaction can be involved, electrostatic attraction is the dominant force that determines complexing of these organic cations with melanin. Accumulation of drug molecules by melanin was reviewed in depth by Larsson (1998).

Melanin as a Redox System

The redox properties of melanin have been recognized for a long time (Figge, 1939). In fact, one of the principal histological tests used to detect melanin *in situ* is based on its reducing power; the presence of melanin in biological samples is deduced from the ability of the specimen to reduce Ag^+ to metallic silver (Lillie and Fullmer, 1976). The redox properties of melanin have been investigated extensively because of their potential biological roles, especially with regard to oxidative damage. The redox properties have also served as very valuable probes to elucidate the properties and structure of melanins.

The redox properties of melanin are, to a large extent, determined by the redox properties of its monomer units (reviewed by Sarna and Swartz, 1993). The relevant functional groups in eumelanin are most likely 5,6-dihydroxyindole, 5,6-dihydroxyindole-2-carboxylic acid, and their fully oxidized (quinone) and semi-oxidized (semiquinone) forms. In pheomelanins, the relevant monomer units are probably *o*-aminophenols, such as 1,4-benzothiazine and the corresponding fully oxidized *o*-quinonimine and semi-oxidized *o*-semiquinonimine. Although the basic redox features of melanin can be described in terms of the chemical properties of its monomers, significant differences exist between the properties of the free monomers and when they are subunits in melanin. One of the most apparent differences is their chemical reactivity; while free *o*-quinones such as dopaquinone, cysteinyldopaquinone, 5,6-indolequinone, and dopaminequinone are very reactive and extremely unstable (Graham, 1978; Monks *et al*., 1992; Thompson *et al*., 1985), the related subunits are quite stable in melanin. The relative stability and moderate reactivity of melanin subunits are probably due to modifications of their redox potential, electron affinity, and restricted accessibility of these functional groups within the pigment granule as a result of intramolecular interactions and steric hindrance.

Using bipyridinium quaternary salts as a redox probe, the one-electron reduction potential E^{01} (corresponding to the fully oxidized/semi-reduced couples of the melanin subunits) of synthetic DOPA and cysteinyldopa melanins was studied by the pulse radiolysis method (Rozanowska *et al*., 1999). It was found that a synthetic model of pheomelanin could be reduced by a milder reducing free radical than was required for the synthetic model of eumelanin. Even though quantitative determination of the one-electron reduction potential for the melanins studied was not possible (the melanins interacted with all redox probes without establishing an apparent equilibrium), a very approximate estimation of E^{01} suggested that the one-electron reduction potential of cysteinyldopa melanin was more positive than –350 mV, while the E^{01} for the major reactive sites of DOPA melanin was between –450 and –550 mV. Owing to the intrinsic heterogeneity of melanins, a moderate dispersion of the redox properties of the melanin functional group is to be expected and is probably more realistic than a single value.

These preliminary data need to be verified by the use of other methods suitable for the determination of redox properties of other aggregated polymeric materials. It remains to be determined what is the second one-electron reduction

potential (E^{01}) of the semi-reduced/fully reduced couple of the melanin moieties, and it is necessary to show that data obtained with synthetic melanins can be extrapolated to natural melanins.

An interesting attempt to study redox properties of several natural and synthetic melanins by the use of simultaneous electrochemical and EPR measurements has been described by Lukiewicz *et al*. (1980). Distinct changes in the EPR signal of melanin radicals, observed during electrochemical treatment of the sample induced by the applied voltage, were explained by direct interactions of the melanin polymer with the electrodes. The data were interpreted in terms of the melanin being electroreduced and electro-oxidized via discrete one-electron steps. Unfortunately, no quantitative data were provided that could be used for estimation of the melanin redox potential or electron exchange capacity, and these very promising investigations have not been followed up by any systematic studies that could provide much needed reference data on the redox properties of various melanins.

One-electron reduction (and oxidation) reactions, induced by synthetic DOPA melanins, have been unambiguously shown by EPR spectroscopy using several different nitroxide radicals as redox probes (Sarna *et al*., 1985a). The interaction between melanin and nitroxide probes was found to be strongly pH dependent, with the rate of reduction of nitroxides at pH 10 being about 20 times faster than that at pH 5 (Sarna *et al*., 1985a). The data indicate that hydroquinone groups of melanin may be involved in the reduction of the nitroxides. The reduction of the nitroxide radicals was reversible, indicating that a redox equilibrium was established. From equilibrium concentrations of nitroxides and the product of the one-electron reduction of nitroxides, hydroxylamines, the equilibrium constant can be estimated for the reaction between nitroxide and melanin, assuming reasonable values for the concentrations of oxidizing and reducing groups on the polymer. The values that were obtained were in good agreement for a range of nitroxide concentrations, suggesting that the assumptions inherent in the calculations were broadly correct. Thus, for DOPA melanin formed by auto-oxidation, the number of electron-donating groups was found to be 20–30 times higher than that of the electron-accepting groups (the total number of active redox sites on the polymer was assumed to be about 25% of all monomer units). The results are rather surprising; however, if verified, they would suggest that synthetic DOPA melanin occurs predominantly in the reduced state. On the other hand, an estimate, based on available data in the literature, suggested that the reducing and oxidizing capacities of dopa-melanin were about 5 and 3 mEq/g respectively (Sealy *et al*., 1980). Of course, the storage conditions of the sample, including age and exposure to light, high pH, and oxygen, could significantly modify the resultant redox state of melanins.

Molecular oxygen is one of the most common, biologically important, electron acceptors. Although thermodynamically, this species is very reactive with many electron-donating molecules, as a result of spin restriction (its ground state is a triplet), molecular oxygen is kinetically quite unreactive with typical diamagnetic molecules (Koppenol and Butler, 1985). Melanin is no exception in this respect, although its reactivity with O_2 may vary significantly, depending on the experimental conditions.

The effect of pH, temperature, and chemical modification of synthetic DOPA melanin on melanin-induced oxygen consumption has been studied using EPR oximetry (Sarna *et al*., 1980b). EPR oximetry is an indirect, physical method that has proved to be a very convenient tool for measuring the concentration of oxygen and its changes, in a variety of biological samples (reviewed by Hyde and Subczynski, 1989; Swartz *et al*., 1994). The investigation clearly demonstrated that pH is the most effective experimental parameter for enhancing the rate of melanin-induced oxygen consumption: at pH 5.5, the auto-oxidation rate was slow—10^{-6} g of O_2 per minute, per 1 g of DOPA melanin dissolved in 1 l; the rate increased several thousand times at pH 11. The rapid increase in the rate of consumption of oxygen, particularly evident between pH 9 and 10.5, was attributed to ionization of the phenolic hydroxyl groups of the polymer. The process was found to be thermally activated, with the thermal activation energy of the order 10 Kcal/mol. The inhibitory effect of catalase on the consumption of oxygen suggests the formation of hydrogen peroxide as a product of the interaction of melanin with molecular oxygen. This conclusion has been supported by the results of studies in which the formation of H_2O_2 during auto-oxidation of melanin pigment was shown, using an oxidase electrode (Hintz and Kalyanaraman, 1986; Korytowski *et al*., 1985).

The amount of hydrogen peroxide produced upon auto-oxidation of melanin was found to be dependent on the type of melanin, with the melanin obtained by auto-oxidation of DOPA being five times more efficient than DOPA melanin synthesized in the presence of tyrosinase, and 10 times more efficient than purified melanin from bovine eye choroid. Superoxide dismutase accelerated the rate of production of H_2O_2, indicating the involvement of superoxide as an intermediate. The proposed overall scheme for auto-oxidation of melanin consists of one-electron reduction of molecular oxygen to superoxide anion, followed by reduction of superoxide to H_2O_2 and oxidation of superoxide to O_2, and spontaneous dismutation of superoxide to equimolar H_2O_2 and O_2. The data indicate that DOPA melanin can act as a pseudo-dismutase; it is able to oxidize and reduce superoxide anion, albeit the oxidation of superoxide to O_2 seems to be the dominant process, accounting for approximately 80% of the reaction between superoxide anion and DOPA melanin.

Auto-oxidation of melanin may be an important, rate-limiting process in coupled reactions in which melanin acts as an electron transfer agent. An example is the melanin-catalyzed oxidation of NADH and *p*-phenyldiamine (Van Woert, 1968). Oxidation of NADH by melanin was later confirmed in an independent study (Gan *et al*., 1974), which also showed that proteins associated with the melanin polymer (either in natural melanoproteins or in synthetic model systems) efficiently inhibited electron transfer reactions of

melanin. An inhibition of the electron transfer ability of DOPA melanin has also been observed for several different drugs upon binding to melanin (Debing *et al.*, 1988). As the degree of inhibition correlated with the extent of binding and did not show any consistent dependence on the chemical nature of the drugs, the inhibitory effect of the drugs was explained by simple shielding of the redox active sites in melanin. It should be emphasized that the oxidizing equivalents for regeneration of melanin, in its electron transfer reactions, can be provided not only by oxygen, but also by other electron-accepting molecules such as ferricyanide (Gan *et al.*, 1976).

Another oxidation reaction catalyzed by melanin has been reported by Baich and Schloz (1989): in the presence of synthetic and natural melanins, glycine was oxidized to glyoxylic acid and formic acid. It was not clear from this study, however, what exactly was the role of melanin and whether possible products of reduction of oxygen, such as superoxide and H_2O_2, were also involved in the oxidation of glycine.

Photoreactivity of Melanin

Ultraviolet and visible light can significantly modify the physicochemical properties of melanin. The most noticeable examples of the effects of light in melanin are photoinduced free radicals and photomodification of the redox properties of melanin.

Illumination of melanin samples in the EPR spectrometer resonant cavity at room temperature causes a reversible enhancement of the free radical signal of melanin (Cope *et al.*, 1963; Ostrovsky and Kayushin, 1963; Stratton and Pathak, 1968). Steady-state spectra for light-induced radicals observed during continuous photolysis, obtained by subtracting the intrinsic (dark) spectrum from the composite spectrum, revealed that the intrinsic and light-induced species differed in several EPR parameters: line width, g-factor, and microwave saturation (Felix *et al.*, 1979). After termination of the irradiation, the signal decayed with second-order kinetics, suggesting that recombination was the primary process responsible for the termination of the photoinduced radical (Fig. 16.8). As second-order kinetics are typically observed for free radicals in solutions, the data seem to indicate that the free radicals in melanin at ambient temperature have a substantial degree of molecular mobility.

Time-resolved EPR measurements also revealed a new transient species formed in photoirradiated melanin samples (Felix *et al.*, 1979). This species, unlike the "usual" photoinduced radicals, decayed rapidly (in milliseconds) at both ambient and cryogenic temperature, and had an EPR spectrum with a time profile that indicated the occurrence of chemically induced dynamic electron polarization. The data were interpreted in terms of the involvement of a triplet state intermediate. Such interpretation, however, seems inconsistent with the results of recent studies of melanin photodynamics using femtosecond laser flash photolysis and nanosecond photo-acoustic calorimetry (Forrest and Simon, 1998; Nofsinger *et al.*, 2001). The authors failed to detect any long-lived transients with triplet-like properties. On the other hand, the observed very fast repopulation of the melanin ground state was a clear indication of a very efficient mechanism of energy dissipation due to internal conversion.

The increased efficiency of melanin for absorbing light at shorter wavelengths suggests that photoformation of melanin free radicals may be a wavelength-dependent phenomenon. Action spectra for photogeneration of free radicals from bovine eye melanin and synthetic DOPA melanin showed a strong wavelength dependence in the 230–600 nm wavelength range (Sarna and Sealy, 1984b). Quantum yields for the steady-state formation of radicals were generally low; for DOPA melanin, the quantum yield reached the value 0.01 only at the shortest wavelengths studied, and it was well below 0.001 in the visible range. The efficiency of production of radicals from natural eumelanin was about three times greater than for synthetic melanin. As action spectra for the production of radicals differed from the optical absorption spectra, it was concluded that the chromophore(s) most active in free radical production were not the major chromophore(s) that absorb light, particularly in the visible range. A new intriguing interpretation of the spectral dependence of the photoinduced formation of melanin radicals was proposed by

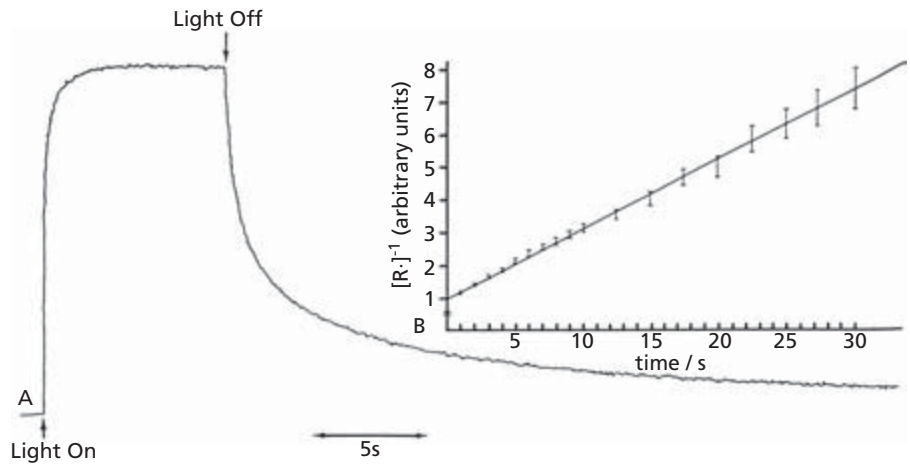

Fig. 16.8. Time-resolved changes in the intensity of EPR spectrum of photoinduced free radicals in melanin from bovine eye choroid (A), and second-order plot of the data (B); [R·] denotes transient free radical concentration. Reproduced from Felix *et al.* (1979), with permission.

Nofsinger *et al.* (1999). Comparing optical properties of different particle-size fractions of *Sepia* melanin with the reported spectral dependence of photoformation of melanin radicals, Nofsinger *et al.* (1999) found striking similarities between the absorption spectrum of the lowest molecular size fraction examined (MW < 1000) and the action spectrum for the photogeneration of melanin radicals. The role of small melanin particles in photogeneration of radicals was further substantiated by photo-acoustic data for different size fractions using an excitation wavelength of 351 nm. Thus, the data revealed that, although the percentage of the absorbed energy that was released as heat by the MW > 10 000, 10 000 > MW > 3000, 3000 > MW > 1000, and MW < 1000 eumelanin fractions was above 90 for the first two fractions, it became about 80 for the third fraction and only about 40 for the smallest molecular weight fraction. The authors concluded that the photochemical properties of melanin are determined by the presence of small molecular size constituents that, unlike the larger melanin particles, retain high photoreactivity. Although the interpretation proposed by Nofsinger *et al.* (1999) is very attractive and seems probable considering the role of different size aggregates in the structure of melanin (Clancy and Simon, 2001), it remains to be determined how representative the smallest particles are among the building blocks of natural melanin.

Photoionization and photohomolysis of melanins occur in the wavelength range 240–300 nm (Kalyanaraman *et al.*, 1984). This was inferred from spin trapping experiments, in which melanin was irradiated in the EPR resonant cavity in the presence of the spin trap 5,5-dimethyl-1-pyrroline-1-oxide (DMPO). In the absence of oxygen, irradiation of the melanin samples resulted in the formation of characteristic spin adducts, DMPO–H·, product of the interaction of DMPO with either H· or e_{aq}^-.

Photoirradiation of melanin leads to the production of both reducing and oxidizing equivalents on the polymer, which below 400 nm seem to originate from a common precursor (or precursors) (Sarna *et al.*, 1985a). The data indicate that irradiation of melanin with light enhances melanin's reducing and oxidizing power.

In aerated aqueous samples, photoexcitation of melanin leads to an enhanced consumption of molecular oxygen and the formation of superoxide anion and hydrogen peroxide (Felix *et al.*, 1978b; Korytowski *et al.*, 1987; Rózanowska *et al.*, 1995; Sarna *et al.*, 1980b; Tomita *et al.*, 1984). Action spectra and quantum yields for photoinduced consumption of oxygen have been determined for eumelanins and pheomelanins using EPR oximetry (Sarna and Sealy, 1984a; Sarna *et al.*, 1984). The results of these studies indicate that the reduction of oxygen induced in melanin by visible light has a low yield: the quantum yield in the visible range is below 0.001 and reaches 0.01 only at 230–220 nm. Action spectra for photoconsumption of oxygen by eumelanin and pheomelanin are comparable with each other and exhibit similarities to the action spectra for anaerobic photogeneration of free radicals in melanin (Fig. 16.9). Using EPR spin trapping, Rózanowska

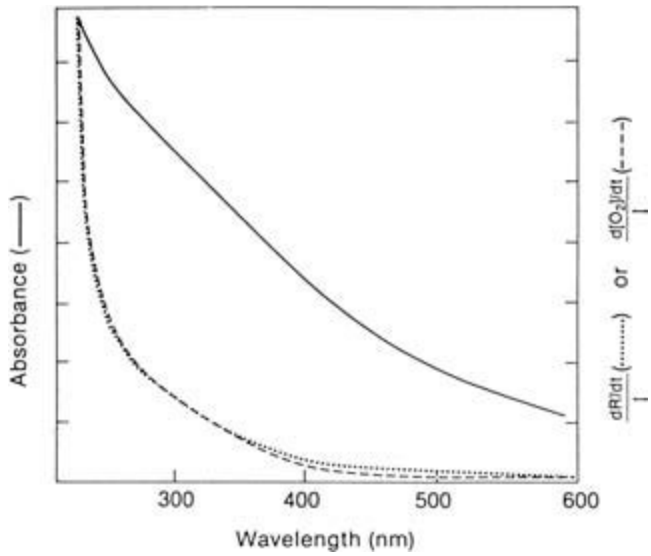

Fig. 16.9. Action spectra for melanin free radical photoproduction (dotted line) and for oxygen photoconsumption (broken line) by eumelanins. For comparison, an apparent optical absorption spectrum of dopa melanin (solid line) is also shown. Reproduced from Sarna and Sealy (1984b), with permission.

et al. (2002) demonstrated a significant generation of superoxide anion when melanosomes, isolated from human retinal pigment epithelium, were irradiated with blue light. The authors also showed that such an aerobic photoreactivity of the human RPE melanosomes increased with the age of the donors. Although the data suggest an increased pro-oxidizing activity of RPE melanin in RPE from older individuals, at this point it is unclear whether this intriguing observation has any biological implications.

A very interesting study regarding the ability of eumelanin to photogenerate reactive oxygen species was recently published by Nofsinger *et al.* (2002). Using a simple spectrophotometric technique, the authors observed that the efficiency of cytochrome *c* reduction, used to monitor the photogeneration of superoxide by different molecular size fractions of *Sepia* melanin, was an order of magnitude higher for the smallest unaggregated oligomers than that characteristic of the bulk pigment. The reduced efficiency of aggregated melanin to photogenerate superoxide anion and hydrogen peroxide was attributed to the decrease in surface concentration of melanin redox active sites upon aggregation.

Using the nitroblue tetrazolium–superoxide dismutase assay, the action spectrum for photoproduction of superoxide from aerated aqueous solutions of pheomelanin was determined (Chedekel *et al.*, 1980). In contrast to the results cited above, these results indicated higher quantum yields for the photogeneration of superoxide even in the visible range. The quantitative aspects of this work should be considered with significant caution, however; it is important to realize that nitroblue tetrazolium can be reduced by many electron donors (including melanin itself), and even the inhibitory effects of

superoxide dismutase may not be used as a specific indicator of primary formation of superoxide anion (Aulair and Voisin, 1985). A model for interfacial photoinduced electron transfer between melanin and oxygen molecules has been proposed by Crippa (2001). It involves adsorption of dioxygen on melanin solid surface and light-induced carriers. The process depends on the surface fractal characteristics and is described by the Marcus theory for electron transfer reactions.

Prolonged aerobic photolysis of pheomelanin is accompanied by loss of a major melanin chromophore (Chedekel *et al*., 1977). Although a role for superoxide anion, hydrogen peroxide, and hydroxyl radicals in the photodestruction of pheomelanin was postulated (Chedekel *et al*., 1978), the mechanism of the observed phenomena has not been established. Although pheomelanin was viewed as being a particularly photolabile type of melanin (Chedekel *et al*., 1977), a comparative study of photobleaching of eumelanin and pheomelanin suggested that eumelanin was actually more susceptible to aerobic photodegradation (Wolfram and Albrecht, 1987).

Bleaching of DOPA melanin induced by its aerobic illumination from near ultraviolet and visible radiation has been studied as a function of pH, concentration of exogenous hydrogen peroxide, oxidation state of melanin, and the presence of copper ions (Korytowski and Sarna, 1990). Based on detection of characteristic products of salicylate hydroxylation (2,3- and 2,5-dihydroxybenzoic acids), the formation of hydroxyl radicals in photolyzed melanin samples has been demonstrated. It was suggested that redox active metal ions that are bound to melanin might be involved in the generation of hydroxyl radicals via Fenton-type processes (Korytowski *et al*., 1987). Indeed, using direct EPR measurements of copper(II) complexes with melanin, it has been shown that copper(I) bound to melanin was rapidly oxidized by either H_2O_2 or oxygen (Korytowski and Sarna, 1990). It has been concluded that photobleaching of melanin involves two distinct stages: reversible oxidation of the hydroquinone moieties of melanin followed by irreversible reactions of the monomers that lead to degradation of the melanin polymer.

An interesting melanin-mediated photo-oxidation of ascorbate has been reported (Glickman and Lam, 1992; Glickman *et al*., 1993). It was demonstrated that melanin granules that were isolated from retinal pigment epithelium of bovine eyes induced nonenzymatic oxidation of ascorbate when illuminated with a continuous-wave argon-ion laser. The photooxidation of ascorbate was explained in terms of two processes—one that might be mediated by melanin-induced oxygen radicals and another that was due to interaction of ascorbate with the free radicals of melanin. However, in a more recent study, it has been demonstrated that melanin acts predominantly as an electron transfer agent in the photooxidation of ascorbate, while the ultimate electron acceptor is molecular oxygen (Rózanowska *et al*., 1997). The results of the latter study also suggested that the primary electron transfer between ascorbate and melanin involved photoinduced melanin radicals.

Antioxidant Properties

The discovery of free radical properties of melanin led to a hypothesis that melanin might act as a free radical trap, thereby protecting cells from the effects of free radicals formed in biological oxidation–reduction reactions (Mason *et al*., 1960). A growing body of experimental evidence suggests that the cellular melanin may be an important antioxidant system (Bustamante *et al*., 1993; Ostrovsky *et al*., 1987; Porebska-Budny *et al*., 1992; Reszka *et al*., 1998; Scalia *et al*., 1990; Slawinska *et al*., 1983; Stepien *et al*., 2000; also reviewed by Sarna, 1992). As the term "antioxidant" is not always used very rigorously in biomedical literature, it may be useful to define it. Following the definition given by Halliwell and Gutteridge (1989), we will consider an antioxidant as "any substance that, when present at low concentration compared to those of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate." Thus, melanin may act as an antioxidant by:

1 scavenging initiating radicals;
2 deactivating electronically excited oxidizing species such as singlet molecular oxygen ($^1O_2$);
3 sequestering redox active metal ions such as iron and copper (metal ions that are bound to melanin may be less efficient in generating diffusible damaging free radicals and/or decomposing lipid peroxides to form propagating radicals);
4 chain breaking, i.e. scavenging intermediate radicals such as peroxyl and alkoxyl.

There is a fairly extensive but amorphous literature on potential antioxidant properties of melanin. The following is aimed at being an indicative, rather than a comprehensive, review of this subject. The antioxidant properties of melanin have been examined by studies of the abilities of melanin to quench electronically excited dye molecules, scavenge reactive free radicals, and sequester redox active metal ions.

Using laser flash photolysis and EPR oximetry as well as conventional absorption and fluorescence spectroscopy, it has been shown that binding of two cationic dye molecules, tetra(*N*-methyl-4-pyrridyl)porphyrin and tetra(4-*N*,*N*,*N*,*N*-trimethyl-anilinium)porphyrin, was accompanied by a broadening of their absorption band, fluorescence quenching, triplet state quenching, and reduction in the dye-photosensitized oxygen consumption (Bielec *et al*., 1986). For anionic dyes such as tetra(4-sulfonatophenyl)porphyrin, there was no binding and, consequently, melanin had little or no effect on the absorption, fluorescence, and triplet states of the dyes and on their photosensitizing abilities (Fig. 16.10). Thus, complexing of positively charged dye molecules via ionic interaction can lead to a very efficient deactivation of their electronically excited states and, as a result, to complete loss of the dye-photosensitizing activity. If not quenched, the dye molecules, via type I or type II photosensitized oxidation reactions, could oxidize many substrate molecules and generate potentially cytotoxic species such as singlet molecular oxygen, superoxide anion, hydrogen peroxide, and hydroxyl radicals (Bensasson *et al*., 1993; Nonell, 1994). An interaction of melanin with potentially photosensitizing molecules was later confirmed for

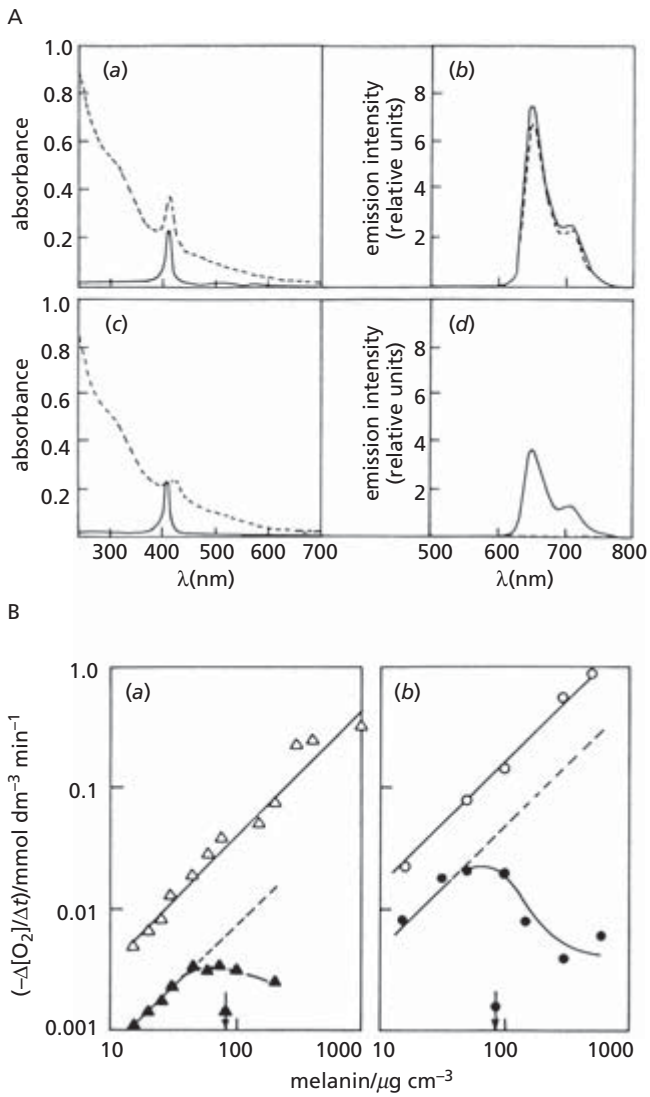

Fig. 16.10. The effects of complexation of a positively charged porphyrin dye by synthetic melanin on the optical properties of the dye (top) and its photosensitizing ability (bottom). (A) Optical absorption (a and c) and fluorescence emission (b and d) spectra of an anionic (a and b) and a cationic (c and d) porphyrin in the presence of cysteinyldopa melanin (broken lines) and its absence (solid lines). (B) Oxygen consumption rates photosensitized by the anionic porphyrin (open symbols) and cationic porphyrin (solid symbols) are plotted as a function of concentration of dopa melanin (a) and cysteinyldopa melanin (b). Reprinted from Bielec *et al.* (1986), with kind permission from the Royal Society of Chemistry, UK.

ground-state complexes with cationic porphyrins (Ito *et al.*, 1992) and demonstrated for the singlet excited state of 8-methoxypsoralen (Losi *et al.*, 1993). The mechanism of the quenching of excited states of melanin bound to tetra(4-*N*,*N*,*N*,*N*-trimethylanilinium)porphyrin was studied recently by femtosecond absorption and picosecond emission spectroscopies (Ye *et al.*, 2003). It has been concluded that such a binding facilitates an ultrafast energy transfer from the excited porphyrin molecule to melanin. The excited energy is then rapidly converted into heat. Because of its speed, the process involves only singlet excited states with no triplet state formation.

Melanin can also be an efficient quencher of singlet oxygen (Sarna *et al.*, 1985b; Sealy *et al.*, 1984). The rate constants of the interaction of $^1O_2$ (generated by a Rose Bengal photosensitized reaction) with synthetic DOPA and cysteinyldopa melanins and natural melanins have been determined under steady-state conditions, using EPR oximetry. The data indicate that chemical quenching of $^1O_2$ by melanin is a rapid reaction, with the corresponding rate constants of 1.3×10^5 mg/ml/s and 6×10^5 mg/ml/s for DOPA melanin and cysteinyldopa melanin respectively. Natural melanins interacted significantly slower with $^1O_2$. This is not unexpected, considering the particulate form of such melanins; as singlet oxygen was generated uniformly in the sample volume, the aggregated melanin had little chance to interact with most molecules of $^1O_2$ before they deactivated via competing processes. Similar values for the apparent rate constants for the interaction of synthetic melanins with $^1O_2$ have been obtained most recently by direct time-resolved detection of the singlet oxygen after pulse laser-induced generation of $^1O_2$ (Wielgus *et al.*, unpublished).

Superoxide anion is another "reactive oxygen species" that may be involved, directly or indirectly, in damage of key cellular constituents (Fridovich, 1983). Superoxide is a product of one-electron reduction of molecular oxygen and can be generated by the interaction of oxygen with suitable electron donors with one-electron reduction potential that is more negative than –0.16 V (Koppenol and Butler, 1985). One of the first reports indicating that melanin can scavenge superoxide anion appeared some 20 years ago (Goodchild *et al.*, 1984). Unfortunately, the experimental details of this study were very sparse. The superoxide scavenging ability of eumelanins was subsequently demonstrated by direct EPR measurements of the characteristic EPR signal of frozen alkaline solutions of H_2O_2 and $NaIO_4$ in the absence and presence of increasing amounts of melanin (Geremia *et al.*, 1984). This method has been refined by the addition of acetone to the reacting mixture (Sichel *et al.*, 1991). It must be stressed, however, that the free radical scavenging properties of melanin were tested by this method under extreme conditions (pH 13), which are not directly relevant in biological systems.

Using stopped-flow EPR measurements of the induced melanin free radical signal and dimethyl sulfoxide (DMSO)/KO_2 as the source of superoxide anion, its interaction with DOPA melanin and melanin from bovine eye choroid has been observed, and the rate constant of the interaction has been calculated (Korytowski *et al.*, 1986). The superoxide-induced melanin radicals decayed with an effective bimolecular rate constant of $0.8–5.1 \times 10^4$ mg/ml/s. The apparent rate of the interaction of melanin with superoxide has been determined from the observed inhibition of the DMPO–O_2H radical adduct formed in the superoxide-generating system in the presence of superoxide dismutase (SOD) and/or melanin. The rate constant was calculated to be

Table 16.1. Apparent second-order rate constants of interaction of melanin with singlet oxygen and free radicals mg/ml/s.

Species	Melanin				
	DM	DMT	CDM	BEM	RHM
$O_2(^1\Delta_g)$	1.3×10^5*		6.0×10^5*	0.06×10^5*	0.3×10^5*
$O_2^{\bullet}$	$(2.0$†$–3.3$‡$) \times 10^3$	10^3†		10^3†	
$^{\bullet}OH$	10^7‡		0.7×10^7‡		
$^{\bullet}OOCH_2OH$	0.018×10^4‡		1.3×10^4‡		
$^{\bullet}CH_2OH$	7.3×10^4‡		1.1×10^6‡		
$CH_3\ ^{\bullet}CHOH$	6.7×10^4‡		0.8×10^6‡		
e_{aq}^-	1.7×10^6‡	0.4×10^6‡	2.4×10^6‡		
$CO_2^{\bullet}$	10^4–10^5‡				
$N_3^{\bullet}$	1.2×10^6‡		1.5×10^6‡		

*Data obtained by EPR oximetry under steady-state illumination in the presence of Rose Bengal as the sensitizer.
†Data obtained by direct EPR measurements and EPR spin trapping using DMSO/KO_2 as the source of O_2.
‡Data obtained by pulse radiolysis.
DM, auto-oxidative DOPA melanin; DMT, DOPA melanin synthesized in the presence of tyrosinase; CDM, cysteinyldopa melanin; BEM, melanin from bovine eye choroid; RHM, red hair melanin.

4×10^5/M/s, assuming a molecular weight of 200 for the DOPA melanin monomer unit. A similar value for the rate constant of DOPA melanin interaction with superoxide anion in fully aqueous media has been obtained by independent measurements using the pulse radiolysis method (6.5×10^5/M/s) (Sarna *et al*., 1986). This powerful method has also been used for the determination of rate constants of the interaction of several different melanins with radicals from water radiolysis (Table 16.1). In addition, it has been shown that superoxide anion interacts with DOPA melanin predominantly by reducing it. Perhaps the most comprehensive study to date of the interaction of DOPA and cysteinyldopa melanins with a variety of oxidizing and reducing radicals has been published by Rózanowska *et al.* (1999). The authors, using pulse radiolysis to generate specifically selected free radicals, observed either directly or indirectly their interaction with both synthetic melanins. The results showed that the efficiency of the interaction depended, in a complex way, on the redox potential, the electric charge, and the lifetime of the radicals. Repetitive pulsing experiments, in which the free radicals, probing the melanin redox sites, were generated from four different viologens, indicated that the eumelanin model had more reduced than oxidized groups accessible to reaction with the radicals. Oxidation of DOPA melanin by oxidizing radicals appeared to be easier than that of cysteinyldopa melanin. Although with many radicals studied, melanin interacted via a simple one-electron process, the reaction of both melanins with the strongly oxidizing peroxyl radical from carbon tetrachloride involved radical addition.

A very efficient scavenging by cysteinyldopa melanin of peroxyl radicals generated from hydroxyethyl and hydroxymethyl radicals with the corresponding rate constant 2.6×10^6/M/s (3.5×10^4/M/s for DOPA melanin) suggests that melanin *in vivo* might be able to participate in chain breaking by scavenging intermediate radicals that are involved in the propagation of the peroxidation process (Dunford *et al*., 1995). Both synthetic melanins were good scavengers of carbon-centered radicals with corresponding rate constants in the range 10^7–10^8/M/s.

The results of the reviewed investigations are consistent with melanin being an efficient scavenger of strongly oxidizing (and reducing) free radicals and a quencher of singlet molecular oxygen. It is rather unlikely, however, that these properties of melanin play a key role in the antioxidant action of the pigment. Unless "site-specific" formation of reactive free radicals or singlet oxygen is considered, natural melanin is a very inefficient scavenger and quencher of such short-lived species. This is because of the limited lifetime of randomly generated reactive species. These species would interact with many constituents of the pigmented cell before having a chance to diffuse to the proximity of the melanosomes (or pigment granules), where they would then need to penetrate the melanosome (pigment granule) surface and interact with the active groups in the polymer. A special case, in which the free radical scavenging and singlet oxygen quenching abilities of melanin might be of importance, is "site-specific" formation of such species, i.e. if the generation of reactive, short-lived species is predominantly within the melanin granule (melanosome) or in its proximity. This could happen if copper, iron, or manganese ions bound to melanin were redox activated and exposed to oxygen or H_2O_2, or when photosensitizing molecules, associated with the melanin granule (melanosome), were activated by light in the presence of oxygen. Under such circumstances, melanin would act as a very powerful antioxidant, and very little reactive species would escape the scavenging and quenching action of the melanin.

Melanin principally exerts its antioxidant activity by binding redox active metal ions. This has been demonstrated in model studies with DOPA melanin (Pilas *et al*., 1988) and dopamine melanin (Zareba *et al*., 1995). The data indicate that iron ions that are bound to melanin are inefficient catalysts for H_2O_2-dependent generation of free hydroxyl radicals (Fig. 16.11). Even though melanin complexes with ferrous ions are readily oxidized by H_2O_2 and oxygen, very few OH radicals leak out of the melanin polymer, as shown by EPR spin trapping and HPLC electrochemical detection of salicylate hydroxylation products. Ferric ions, on the other hand, after binding to melanin, become significantly more difficult to reduce by mild reductants. Sequestration of iron ions has been identified as a major mechanism for the inhibitory effects of melanin on lipid peroxidation (Korytowski *et al*., 1995), and an important role of the degree of iron binding to melanin on the antioxidant/pro-oxidant actions of melanin in protection against chemically and photochemically induced peroxidation of lipids has been considered in an independent study (Krol and Liebler, 1998).

Melanin may lose part of its antioxidant activity, or even become pro-oxidant, when its metal ion-binding capacity is exceeded, or if redox active metal ions, present in melanotic systems, are bound to strong chelators (such as EDTA). The basal rate of lipid peroxidation induced by iron, which was enhanced by dopamine melanin (Ben-Shachar *et al*., 1991), may be explained by the inability of the melanin to complex all ferric ions. Under the conditions employed by the authors of the cited paper, it seems that the dopamine melanin was saturated with iron ions. Similar phenomena are probably involved in a recently reported pro-oxidant action of DOPA melanin in lipid peroxidation (Sotomatsu *et al*., 1994). An enhanced formation of hydroxyl radicals, observed in model systems containing ferric ions complexed with EDTA, induced by DOPA melanin (Pilas *et al*., 1988) and dopamine melanin (Zareba *et al*., 1995), can be explained by the reducing power of melanin, which is able to reduce and thereby activate ferric ions chelated by EDTA and, hence, drive a Fenton reaction (Fig. 16.11).

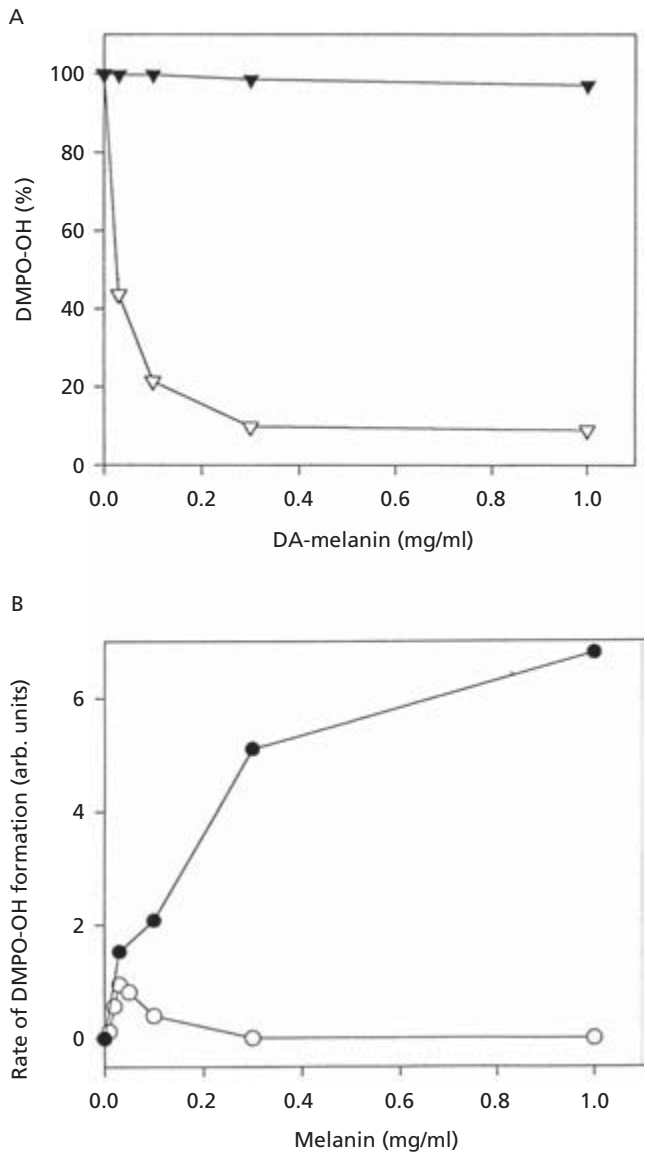

Fig. 16.11. The effects of synthetic dopamine melanin on the generation of free hydroxyl radicals by a Fenton system. (A) shows the inhibitory effect of melanin when ferrous ions were complexed by a weak chelator such as citrate (open triangles); on the other hand, melanin exhibited a negligible effect if Fe(II) was chelated by a strong chelator such as DTPA (solid triangles). (B) illustrates the activating role of melanin, when ferric ions were present in the form of complexes with DTPA (solid circles) and with citrate (open circles). The inhibitory effect of melanin is expressed as normalized maximum levels of the DMPO–OH spin adduct formed (A) or its rate of formation (B). Reprinted from Zareba *et al*. (1995), with kind permission of Elsevier Science-NL.

Perspectives

The complexities of melanins, due to their physical–chemical properties and inherent heterogeneity, make it difficult, at least at this time, to reach very specific conclusions as to the properties and structure of melanins. In spite of these limitations, some very useful generalizations can be drawn. It is necessary, however, to use a variety of different methods to try to characterize melanins and to recognize that, inevitably, the conclusions reached by the use of a single method need to be confirmed by other, independent methods.

The physical state and experimental conditions of melanins can have profound effects on the apparent properties of the melanin. The most important variables of this type include the state of hydration of the molecule (it might be argued successfully that studies on dried melanins will usually have limited validity), the pH, and the present and past history of redox conditions for the sample. Another important consideration is the method of purification of the melanin. It is now recognized that treatment of melanin with strong acids or

alkali may irreversibly modify the physicochemical properties of the melanin polymer.

Different melanins can have very different properties. The most important variables are the nature of the monomeric units, especially whether these include cysteine-containing subunits. As a consequence, there are at least three quite different types of naturally occurring melanins: eumelanin, pheomelanin, and neuromelanin. The biological effects and/or roles of melanin are significantly affected by interactions with metal ions as a result of the binding of metal ions by the melanin; the interactions affect the properties of both the metal ions and the melanin. Even though melanin can bind a variety of different types of molecules (with different forces being involved), the most common and important is binding of metal ions and organic cations via electrostatic interaction.

In vivo it is likely that some melanins, perhaps most, have proteins associated with them and that these affect the properties of the melanin. The proteins probably include those associated with the synthesis of melanin (tyrosine and related enzymes, except for neuromelanin) and proteins derived from the immediate environment, especially from the melanosomes. The redox character of melanin is very complex and is greatly affected by the nature of the melanin and its environment. In order to understand the extent and even the direction of redox reactions that will occur, it is essential to specify the conditions, including the type of melanin, metal ions that are present, proteins associated with the melanin, the redox state of the melanin, pH, and the presence of other redox active species such as molecular oxygen.

The ability of melanin to quench electronically excited states of certain photosensitizing dye molecules, scavenge reactive free radicals, and sequester redox active metal ions makes the pigment an efficient antioxidant. The occurrence of lipids with melanins is very incompletely understood.

Melanins are unique biopolymers with persistent free radicals and a distinct EPR signal. Many of the EPR characteristics and the associated free radical and redox reactions occurring in the presence of melanins can be explained on the basis of a dynamic redox equilibrium involving quinones, hydroquinones, and semiquinones in the monomeric units of the melanin.

The optical properties of melanin are very incompletely understood in spite of the extensive and often productive studies that have been carried out. In particular, we do not yet have a satisfactory and full explanation for the absorption spectrum of melanins and the biological significance of the photochemistry associated with melanins. Irradiation of melanin with ultraviolet or visible light generates transient free radicals and enhances redox reactivity of the electron exchange groups of melanin. Although intriguing, there is at present no fully satisfactory theory or evidence for melanin to have amorphous semiconducting and related properties. Much is known about melanin through the use of sophisticated physical and chemical approaches, and it is likely that continued progress will be made, eventually resulting in a thorough understanding of this important class of biopolymers.

Acknowledgments

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