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15 Chemistry of Melanins

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Summary

1 Melanin pigments can be classified into two major groups: the brown to black insoluble eumelanins and the alkali-soluble yellow to reddish-brown pheomelanins. Both pigments derive from the common precursor dopaquinone formed via the oxidation of L-tyrosine by tyrosinase. Trichochromes are a variety of pheomelanic pigments with structures that are fully elucidated.

2 Dopaquinone, a highly reactive *ortho*-quinone, plays pivotal roles in *chemically* controlling melanogenesis. When sulfhydryl compounds are absent, it undergoes intramolecular cyclization to form cyclodopa, which is rapidly oxidized by redox reaction with dopaquinone to give dopachrome (and dopa). Dopachrome then gradually rearranges to give mostly 5,6-dihydroxyindole (DHI) and a trace of 5,6-dihydroxyindole-2-carboxylic acid (DHICA). Oxidation of these dihydroxyindoles leads to the production of eumelanins. However, intervention of cysteine with this process gives rise preferentially to the production of cysteinyldopa isomers. Cysteinyldopas are then oxidized through redox reaction with dopaquinone to cysteinyldopaquinones that eventually give rise to pheomelanins.

3 Kinetic data, provided by pulse radiolysis studies of the early stages of melanogenesis involving dopaquinone (and cysteine), indicate that the process of mixed melanogenesis proceeds in three distinctive steps: the production of cysteinyldopas, the oxidation of cysteinyldopas to give pheomelanins, followed finally by the production of eumelanins. The switching from pheomelanogenesis to eumelanogenesis is *chemically* controlled by the cysteine concentration.

4 Isolation and properties of natural and synthetic melanin pigments are discussed. Artificial modification of pigment structure should be cautioned when acid or base is employed in the isolation procedures.

5 Recent advances in the study of melanogenesis are summarized. In eumelanogenesis, dopachrome rearrangement to DHICA is catalyzed by dopachrome tautomerase (Dct) or by metal ions. DHI and DHICA can copolymerize on the way to eumelanic pigments. In pheomelanogenesis, cysteinyldopaquinone cyclizes to form the *ortho*-quinonimine intermediate, the rearrangement of which gives the benzothiazine derivative(s). Oxidative polymerization of the latter leads to the production of pheomelanins including the trichochrome pigments.

6 The biological significance of melanin-related metabolites, such as 5,6-dihydroxyindoles and cysteinyldopas, is addressed, with special emphasis given to their use as melanoma markers, the mechanism of their cytotoxicity, and their possible role in photoprotection.

7 Melanins are difficult to characterize because of their intractable chemical properties, the heterogeneity in their structural features, and the lack of methods to split melanin polymers into monomer units.

8 Degradation studies carried out in the 1960s provided a number of useful degradation products, such as pyrrole-2,3,5-tricarboxylic acid (PTCA) and 4-amino-3-hydroxyphenylalanine (4-AHP), arising from eumelanins by permanganate or peroxide oxidation and from pheomelanins by hydriodic acid hydrolysis. A rapid and sensitive method for quantitatively analyzing eumelanins and pheomelanins in tissue samples has been developed on the basis of the formation of PTCA and 4-AHP followed by their high-performance liquid chromatography (HPLC) determination.

9 The total amount of melanin (total melanin) in hair samples can be spectrophotometrically assayed by dissolving them in hot Soluene-350 plus water. The PTCA/total melanin and 4-AHP/total melanin ratios are useful in characterizing eumelanins and pheomelanins respectively. The former ratio reflects the DHICA/DHI ratio in various eumelanins.

10 In addition to PTCA and 4-AHP, the significance of which in pigment research has been established, several other degradation products are also useful in characterizing various types of melanin pigments. These products include thiazole-2,4,5-tricarboxylic acid (TTCA) and pyrrole-2,3-dicarboxylic acid (PDCA) as markers of pheomelanic pigments and dopamine-derived melanins respectively. This methodology has been used to analyze natural dopamine melanins, such as neuromelanin. Finally, 6-β-alanyl-2-carboxy-4-hydroxybenzothiazole (BTCA), a product of alkaline peroxide oxidation, may also be a good marker of pheomelanic pigments.

Historical Background

Melanin and melanogenesis have been fascinating subjects for chemists not only because of the widespread presence of pigments in nature but also because of the complexity of their structures and functions. Landmark events in our understanding of the chemistry of melanogenesis are briefly summarized (revised from Prota, 1992; Prota *et al.*, 1998a).

1885 Borquelot and Bertrand discovered the enzyme tyrosinase in fungi and, in 1886, Bertrand identified the amino acid tyrosine as the melanogenic substrate.

1926–27 Raper elucidated the early stages of the oxidation of tyrosine to melanin catalyzed by tyrosinase. He identified the red intermediate as dopachrome and isolated

dopa, 5,6-dihydroxyindole (DHI), and 5,6-dihydroxyindole-2-carboxylic acid (DHICA).

1948 Beer and coworkers first synthesized DHI and DHICA and showed that DHI is more susceptible to oxidation than DHICA.

1948 Mason extended Raper's studies to the later stages of melanogenesis and identified dopachrome by spectroscopy.

1952 Panizzi and Nicolaus identified pyrrole-2,3,5-tricarboxylic acid (PTCA) as the most significant fragment from the degradation of Sepia melanin (PTCA is now used as a specific degradation product of DHICA-derived units in eumelanins).

1955 Leonhardi first recognized that a group of Thormählen-positive urinary melanogens are derivatives of DHI.

1962–66 Based on extensive analytical and degradative studies, Nicolaus, Piatelli, and their associates suggested that Sepia melanin is a heteropolymer derived from copolymerization of various intermediates in the Raper scheme.

1967 Duchón identified the Thormählen-negative melanogens in melanoma urine as metabolites of DHICA.

1967–68 Prota and Nicolaus isolated pheomelanins from red feathers and showed that they contain sulfur, arising by addition of cysteine to dopaquinone. They also isolated trichochromes and showed them to be related to pheomelanins.

1967–70 Fattorusso, Minale, and their associates carried out extensive degradative studies on pheomelanins and suggested that they are complex mixtures of polymers containing benzothiazine, benzothiazole, and isoquinoline units.

1968 Prota and associates synthesized 5-*S*-cysteinyldopa and showed that it is a precursor of pheomelanins and of trichochromes.

1968 Fattorusso, Minale, and their associates identified a number of characteristic degradation fragments of pheomelanins, including aminohydroxyphenylalanines (AHP) (which are now used as specific markers of pheomelanins).

1970–76 Swan and coworkers provided analytical and biosynthetic evidence in support of Nicolaus' heteropolymer model of eumelanins.

1972 Rorsman and Rosengren discovered 5-*S*-cysteinyldopa in the urine of melanoma patients. 5-*S*-Cysteinyldopa is now widely used as a biochemical marker of melanoma progression.

1980 Pawelek and his associates discovered a new factor, now known as dopachrome tautomerase (Dct), which promotes tautomerization of dopachrome to give DHICA.

1985 Ito established microanalytical methods to quantitate eumelanins and pheomelanins, based on HPLC determination of the specific degradation products, PTCA and AHP. The original methods have been improved and are now commonly used as standard methods.

1985 Land, Chedekel, and colleagues introduced a pulse radiolysis method to study the fates of highly reactive *ortho*-quinone intermediates (leading to the determination of kinetic constants for reactions in the early stages of melanogenesis in 2003 by Land and associates).

1986– Prota, d'Ischia, Palumbo, Napolitano, and their coworkers in Naples have carried out a long series of studies on the biogenesis and structure of eumelanins and pheomelanins. Metal ions were shown to modify the course of melanogenesis.

The color of hair, skin, and eyes in animals mainly depends on the quantity, quality, and distribution of the pigment, melanin. Melanocytes are responsible for the synthesis of melanins within membrane-bound organelles, melanosomes, and the transport of melanosomes to surrounding epidermal cells, keratinocytes. Melanocytes in mammals and birds produce two chemically distinct types of melanin pigments, the black to brown eumelanins and the yellow to reddish pheomelanins (Ito, 1998; Ito *et al*., 2000; Prota, 1992; Prota *et al.,* 1998a).

Among the biopolymers, melanins are unique in many respects. The other essential biopolymers, i.e. proteins, nucleic acids, and carbohydrates, are chemically well characterized; their precursors (monomer units) and modes of connection between the monomer units are known, and sequences of their connection can be determined with well-established methodology (Table 15.1). On the other hand, we still do not have, for example, a method to determine accurately the ratio of various units present in melanins. This is due largely to the chemical properties of melanins, such as their insolubility over a broad range of pH, to heterogeneity in their structural features, and to the lack of methods to split melanin polymers into monomer units (all other biopolymers can be hydrolyzed to the corresponding monomer units).

In this chapter, we review advances in the chemistry of melanins and melanogenesis and pay equal attention to the chemical analysis of melanins, with special emphasis on methodology to determine the quantity and quality of melanins present in pigmented tissues and cells (Wakamatsu and Ito, 2002). Characterization of synthetic and natural dopamine melanin is also described briefly. An excellent book (Prota, 1992) is available that deals extensively with the chemistry of melanins and melanogenesis, and more condensed information can also be found in several recent reviews (Ito, 1993a; Prota, 1988, 1993; Prota *et al*., 1998a).

Table 15.1. Comparison of melanins with other biopolymers.

Biopolymer	Monomers	Covalent bond
Protein	Amino acids	Peptide bond (C–N)
Polysaccharide	Glucose	Glucoside bond (C–O)
Nucleic acid	Nucleotide	Phosphate diester bond (P–O)
Melanin	Dihydroxyindoles Benzothiazines	Carbon–carbon bond (C–C)

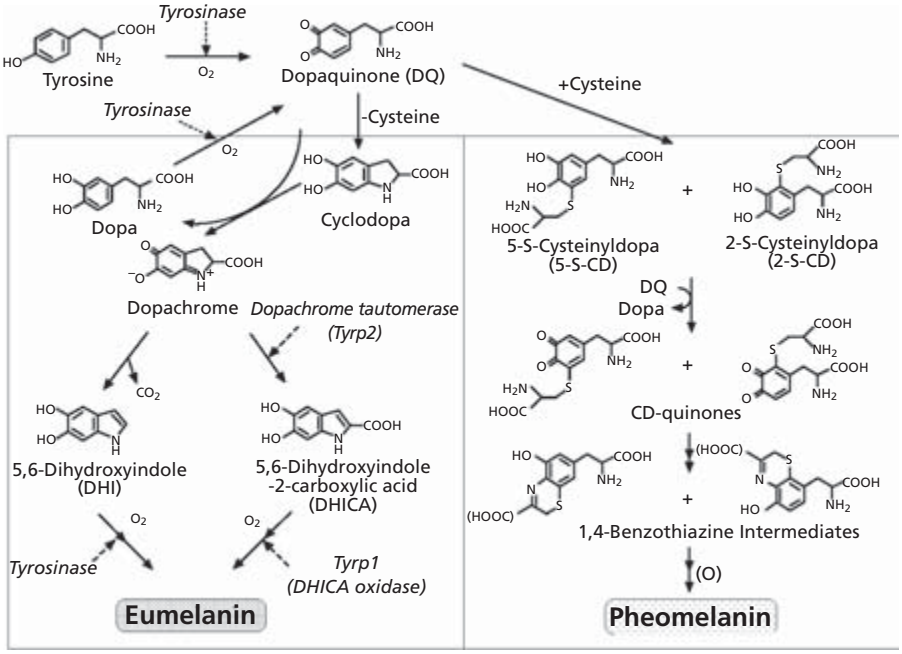


Fig. 15.1. The biosynthetic pathways to eumelanins and pheomelanins. Note that the activities of tyrosinase, Tyrp1, and Tyrp2 are involved in the production of eumelanins, whereas only tyrosinase (and the amino acid cysteine) is necessary for the production of pheomelanins.

Current Concepts

Melanogenesis and Melanins

The Raper–Mason–Prota Pathway of Melanogenesis

It is now well recognized that animal melanins can be classified into two major groups: the brown to black eumelanins that are insoluble in all solvents and the yellow to reddish-brown pheomelanins that are soluble in alkali. Nevertheless, most studies on melanins have so far been conducted on eumelanins. One of the reasons for the scantiness of research on pheomelanins is because it was formerly believed that pheomelanins are produced only in follicular and feather melanocytes. However, it has been shown recently that pheomelanins are also produced in melanomas (Prota *et al*., 1976; Rorsman *et al*., 1979) and in normal epidermis (Thody *et al*., 1991).

Both eumelanins and pheomelanins derive from the common precursor dopaquinone, which is formed by tyrosinase oxidation of the common amino acid L-tyrosine (Fig. 15.1). Until recently, it was generally believed that dopa is first formed on the way to dopaquinone. However, using *N,N*-dimethyldopamine as a tyrosinase substrate, Cooksey *et al*. (1997) showed that *ortho*-quinones such as dopaquinone are formed directly during the initial stage of melanogenesis. Dopaquinone is a highly reactive intermediate and, in the absence of sulfhydryl compounds (thiols), it undergoes the intramolecular addition of the amino group giving cyclodopa (often called leukodopachrome). The redox exchange between cyclodopa and dopaquinone then gives rise to dopachrome, the red intermediate (Mason, 1948; Raper, 1927), and dopa. This latter is considered as a source of dopa formed during melanogenesis (Fig. 15.1). Dopachrome then gradually rearranges to give mostly DHI and, to a lesser extent, DHICA (Palumbo *et al*., 1987a; Raper, 1927). Finally, these dihydroxyindoles are oxidized and polymerized to give eumelanins.

On the other hand, intervention of sulfhydryl compounds (such as cysteine) with this process gives rise exclusively to thiol adducts of dopa, cysteinyldopas, among which 5-*S*-cysteinyldopa (5-*S*-CD) is the major isomer (Ito and Prota, 1977). Further oxidation of the thiol adducts leads to pheomelanin formation via benzothiazine intermediates. In fact, most melanin pigments present in pigmented tissues appear to be mixtures or copolymers of eumelanins and pheomelanins (Prota, 1980; Ito, 1993b).

In addition to tyrosinase, two tyrosinase-related proteins have recently been shown to regulate and promote eumelanogenesis (Hearing, 1993). Dopachrome tautomerase (Dct), the presence of which had been suggested for some years (Pawelek *et al*., 1980), catalyzes the tautomerization of dopachrome to DHICA (Tsukamoto *et al*., 1992a). Recently, this enzyme was shown to be identical to tyrosinase-related protein 2 (Tyrp2) (Jackson *et al*., 1992). Certain metal ions are also known to promote the tautomerization (i.e. the isomerization with a shift of hydrogen atom) of dopachrome to DHICA (Palumbo *et al*., 1987a, 1991). Oxidative polymerization of DHI is known to be catalyzed by mammalian tyrosinase (Tripathi *et al*., 1991). Another tyrosinase-related protein, Tyrp1, isolated from mouse melanoma (the *brown* locus protein) has recently been shown to oxidize DHICA (Jiménez-Cervantes *et al*., 1994; Kobayashi *et al*., 1994), although human Tyrp1 is unable to catalyze the same reaction (Boissy *et al*., 1998). Instead, human tyrosinase is able to oxidize DHICA, as well as tyrosine, dopa, and DHI. It now becomes clear that the activities of these tyrosinase-related proteins greatly affect the

quantity and quality (the ratio of DHI to DHICA and the degree of polymerization) of the eumelanins produced.

The Intrinsic Reactivity of *Ortho*-quinones

Dopaquinone belongs to the category of *ortho*-quinones, and it is essential to summarize the chemistry of *ortho*-quinones for a better understanding of melanogenesis. Figure 15.2 summarizes some of the most important reactions of *ortho*-quinones, which are extremely reactive compounds. In 1976, Tse *et al.* showed that the addition of sulfhydryl compounds proceeds extremely fast to give thiol adducts. In a pulse radiolysis study on the reactivities of 4-substituted *ortho*-quinones with cysteine and glutathione, Cooksey *et al.* (1996) showed that the rate constants of thiol addition are over the range 4×10^5 to 3×10^7/M/s (in the case of cysteine at pH ~7) depending on the nature of the substituents. The reduction to parent catechols through redox exchange proceeds as fast as the thiol addition (Tse *et al.*, 1976) and, therefore, these two reactions are competitive. The reaction with amine compounds does not proceed so fast. However, only when the amino group is present within the same molecule may the amino group fairly rapidly undergo either an addition reaction to give aminochromes (such as dopachrome) or a condensation reaction to give *ortho*-quinonimines. An example of the latter type of reaction is found in the cyclization of cysteinyldopaquinones (see Fig. 15.1). It should be stressed that all these reactions are controlled by the intrinsic *chemical* reactivity of *ortho*-quinones.

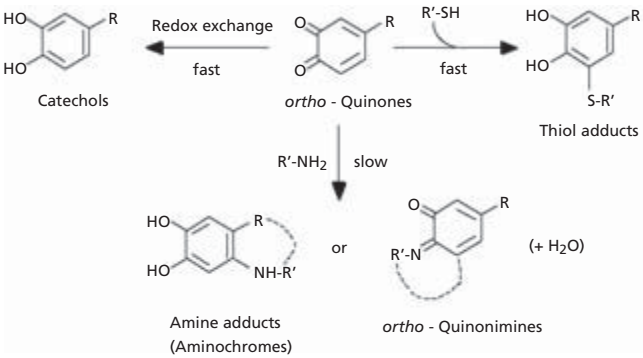


Fig. 15.2. Intrinsic *chemical* reactivities of *ortho*-quinones. Note that, unless the amino group is present in the same molecule, the addition of an amine does not proceed at biologically relevant rates.

Pivotal Roles of Dopaquinone in Controlling Melanogenesis

In 2003, Land *et al.* reported rate constants (r1–r4) for all of the four important steps in the early phase of melanogenesis, based on pulse radiolysis studies (Fig. 15.3; Land and Riley, 2000; Land *et al.*, 2001, 2003). The pulse radiolysis method is a powerful tool to study the fates of highly reactive melanin precursors. The technique depends on the production of bromine radicals $Br_2^{\bullet -}$ by pulse radiolysis of an N_2O-saturated aqueous buffer containing KBr. The bromine radical thus formed oxidizes dopa to dopasemiquinone, which then disproportionates to give dopaquinone (and dopa). This entire process proceeds within 2–3 ms so that the fate of dopaquinone can be followed by spectrophotometry in the presence (or absence) of a targeted molecule.

The first step (r1 = 3.8/s) in eumelanogenesis is the intramolecular addition of the amino group giving cyclodopa, a fairly slow step. However, as soon as cyclodopa is formed, it is rapidly oxidized to dopachrome through a redox exchange ($r2 = 5.3 \times 10^6$/M/s). On the other hand, the first step in pheomelanogenesis ($r3 = 3 \times 10^7$/M/s), the addition of cysteine, proceeds very quickly. The second step, the redox exchange giving cysteinyldopaquinone, proceeds at a slower rate ($r4 = 8.8 \times 10^5$/M/s) (Land and Riley, 2000). From these kinetic data, several important conclusions can be drawn.

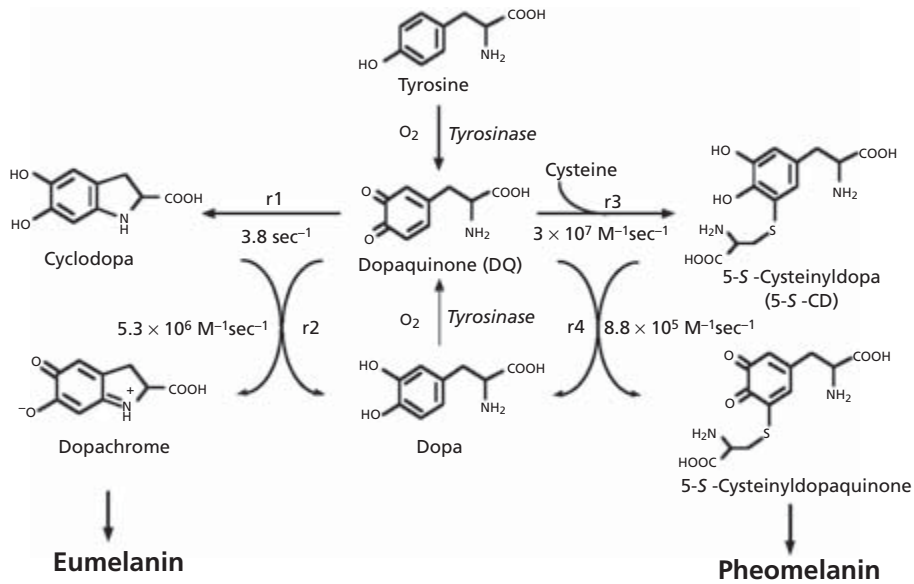


Fig. 15.3. Schematic outline of the branching point of production of eumelanins and pheomelanins (Ito, 2003; Land *et al.*, 2003). Note that the rate constants r1–r4 are all controlled by the intrinsic *chemical* reactivity of dopaquinone. No enzymes other than tyrosinase are necessary to promote these reactions. The intramolecular cyclization of dopaquinone to cyclodopa requires deprotonization of the amino group (actually present in the form of $-NH_3^+$).

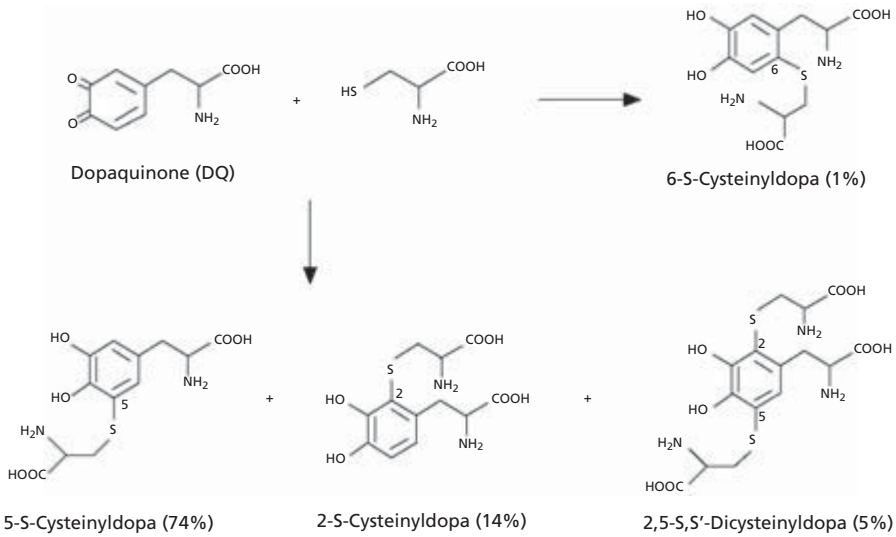


Fig. 15.4. Formation of isomeric cysteinyldopas and 2,5-*S*,*S*′-dicysteinyldopa by the addition of cysteine to enzymically produced dopaquinone (Ito and Prota, 1977).

1 Comparison of the rate constant for the addition of cysteine to dopaquinone (r3) with the rate constant for the intramolecular cyclization (r1) shows that cysteinyldopa formation is preferred over cyclodopa formation as long as the cysteine concentration is higher than 0.13 μM.
2 The redox exchange giving cysteinyldopaquinone (r4) proceeds 30 times more slowly than the addition of cysteine (r3). Cysteinyldopas thus accumulate in the early phase of pheomelanogenesis.
3 Comparison of the rate constant for redox exchange giving dopachrome from dopaquinone (r2) with the rate constant for intramolecular cyclization (r1) shows that dopachrome production becomes faster than cyclodopa production when the cyclodopa concentration is higher than 0.7 μM. Cyclodopa thus does not accumulate in the early phase of eumelanogenesis.
4 Comparison of the rate constant for redox exchange giving cysteinyldopaquinone (r4) with the rate constant for dopachrome formation (2 × r1; Land *et al.*, 2003) shows that pheomelanogenesis is preferred over eumelanogenesis as long as the cysteinyldopa concentration is higher than 9 μM.
5 It is now possible to derive an "index of divergence" between eumelanogenesis and pheomelanogenesis. By taking dopachrome and cysteinyldopaquinone as representatives of the divergent pathways, Land *et al.* (2003) proposed an index of divergence (*D*):

$$D = r3 \times r4 \times [\text{cysteine}]/r1 \times r2$$

This leads to a "crossover value" (i.e. for $D = 1$) for switching between eumelanogenesis and pheomelanogenesis when the cysteine concentration at the site of melanogenesis is 0.8 μM.

The above kinetic data are useful in interpreting the early phase of pheomelanogenesis. Thus, tyrosinase oxidation of dopa in the presence of excess cysteine gives a high yield of 5-*S*-cysteinyldopa (74%) and 2-*S*-cysteinyldopa (14%) in a ratio of about 5:1, together with a minor 6-*S*-isomer (1%) and a diadduct, 2,5-*S*,*S*′-dicysteinyldopa (5%) (Fig. 15.4; Ito and Prota, 1977). This result confirms the interpretation that cysteinyldopa formation is preferred as long as cysteine is present. It may also be pointed out that the ratio of these cysteinyldopa isomers is determined by the intrinsic *chemical* reactivity of dopaquinone.

The ratio of r3 to r4 is ~30, indicating that only after the cysteine concentration decreases to 30 times lower than the 5-*S*-cysteinyldopa concentration does the formation of 5-*S*-cysteinyldopaquinone become predominant. This interpretation is supported by the facts that cysteinyldopa monomers accumulate in the early phase of pheomelanogenesis and that the formation of the diadduct 2,5-*S*,*S*′-dicysteinyldopa is only a minor pathway. This is why high levels of 5-*S*-cysteinyldopa are produced in melanoma tissues and secreted into the blood of melanoma patients, which makes 5-*S*-cysteinyldopa a useful biochemical marker of melanoma progression (Wakamatsu *et al.*, 2002a).

The proposed pathway of pheomelanogenesis was substantiated by the identification of 5-*S*-cysteinyldopa (Bjorklund *et al.*, 1972) and other isomers, along with the diadduct, in melanoma urine (Prota *et al.*, 1977) by Rorsman and his associates. The ratios between various cysteinyldopa isomers found in melanoma urine and tissues (Morishima *et al.*, 1983) are close to that obtained *in vitro* by incubation of dopa with tyrosinase in the presence of cysteine (Ito and Prota, 1977). This indicates that cysteinyldopas originate *in vivo* by a similar route, involving the addition of cysteine to dopaquinone. As this process is the earliest event in the course of pigment metabolism, it is thus likely that a certain portion of cysteinyldopas formed are secreted into body fluid, regardless of the type of melanin eventually formed.

An alternative route has been proposed for the biosynthesis of cysteinyldopas involving the addition of dopaquinone with glutathione (Ito *et al.*, 1985), followed by the hydrolysis of the resultant glutathionyldopas. The latter step requires the action of two enzymes, γ-glutamyltranspeptidase and peptidase, which

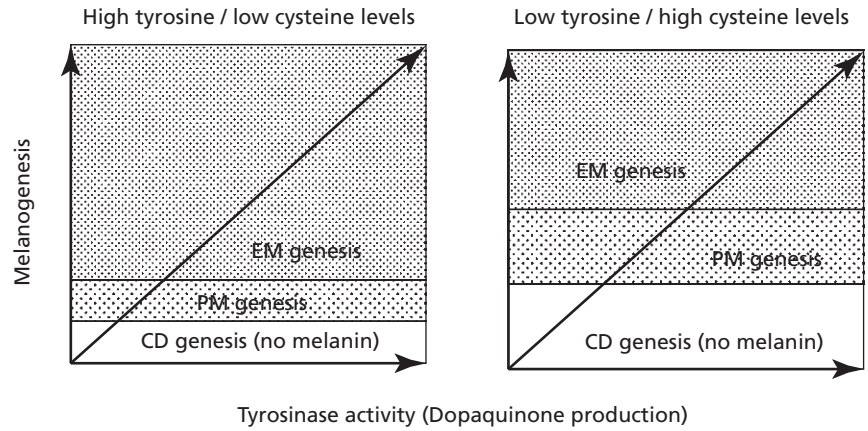


Fig. 15.5. Proposed pathway for mixed melanogenesis. Note that the course of melanogenesis proceeds in three distinct steps: cysteinyldopa genesis, pheomelanogenesis, followed by eumelanogenesis (Ito, 2003; Ito *et al.*, 2000).

are present in melanoma cells (Agrup *et al.*, 1975; Mojamdar *et al.*, 1983). Which of the two pathways for cysteinyldopa formation is actually operative *in vivo* has been addressed. Inhibition of glutathione synthesis by the inhibitor L-buthionine sulfoximine led to a strong reduction in glutathione levels with some increase in cysteine levels in both melanoma cells (Benathan, 1996) and normal melanocytes (Benathan and Labidi, 1996). This modification of thiol levels results in a moderate increase in the cellular level of 5-*S*-cysteinyldopa. Thus, it is now generally believed that glutathione is not directly involved in the production of cysteinyldopas (Potterf *et al.*, 1999).

The Proposed Pathway for Mixed Melanogenesis

From the above interpretation of kinetic data, a pathway for mixed melanogenesis can be proposed as shown in Figure 15.5 (Ito, 2003; Ito *et al.*, 2000). The amount of melanin produced is proportional to dopaquinone production, which is in turn proportional to tyrosinase activity.

Melanogenesis proceeds in three distinctive steps. The initial step is the production of cysteinyldopas, which continues as long as cysteine is present (0.1 μM). The second step is the oxidation of cysteinyldopas to give pheomelanins, which continues as long as cysteinyldopas are present (9 μM). The last step is the production of eumelanins, which begins only after most of the cysteinyldopas (and cysteine) are depleted. Therefore, it appears that eumelanins deposit on the preformed pheomelanin (Agrup *et al.*, 1982) and that the ratio of eumelanins to pheomelanins is determined by the tyrosinase activity and the cysteine concentration. This proposal has been supported by several studies. Ozeki *et al.* (1997a) examined the tyrosinase oxidation of tyrosine in the presence of cysteine and showed that the three steps proceed in sequence. By decreasing the extracellular concentration of cysteine in cultured human melanoma cells, del Marmol *et al.* (1996) showed a shift to more eumelanic cells as a result of a dramatic decrease in intracellular cysteine concentration. By changing concentrations of tyrosine and cysteine in cultured human melanocytes, Smit *et al.* (1997) found a twofold increase in melanin production with a decreased ratio of pheomelanin to total melanin when cultured at a higher concentration of tyrosine.

The proposal that tyrosinase activity plays a major role in controlling melanogenesis is also supported by several studies. It has been shown that tyrosinase activity is lower when pheomelanogenesis proceeds in *viable yellow* mice compared with eumelanogenesis (Burchill *et al.*, 1986, 1993; Movaghar, 1989). Moreover, the switching to pheomelanogenesis is accompanied by a marked decrease in the melanin content of hair (Burchill *et al.*, 1986; Granholm *et al.*, 1990). However, the change in tyrosinase activity itself is not enough for the switching of melanogenesis as seen in *chinchilla* mice where tyrosinase activity is decreased to one-third from the wild type (Coleman, 1962; Lamoreux *et al.*, 2001). The eumelanin content is reduced to one-half in *black chinchilla* without any increase in pheomelanin content compared with *black* mice, whereas the pheomelanin content is reduced nine-fold in *lethal yellow chinchilla* compared with *lethal yellow* mice (Lamoreux *et al.*, 2001). Conversely, the proposal summarized in Figure 15.5 fits well with the above results that pheomelanogenesis is more strongly affected by the decrease in tyrosinase activity than eumelanogenesis. Barsh (1996) has put forth a similar proposal.

Classification and Structure of Eumelanins and Pheomelanins

Table 15.2 summarizes criteria for classifying melanins (Prota *et al.*, 1998a). Extensive studies carried out by Nicolaus' group in Naples (Nicolaus, 1968) and Swan's group in Newcastle (Swan, 1974; Swan and Waggott, 1970) led to the conclusion that eumelanins are highly heterogeneous polymers consisting of different oxidative states of DHI and DHICA units, and pyrrole units derived from their peroxidative cleavage (structure **1** in Fig. 15.6). Interestingly, a recent study indicates a high proportion of pyrrole units in Sepia melanin (Pezzella *et al.*, 1997). However, how much those pyrrole units contribute to the structure of natural eumelanins in mammalian pigmentation remains to be studied.

Most of the present knowledge on pheomelanin chemistry results from work in the late 1960s conducted by Prota, Nicolaus, and collaborators (Prota, 1972; Thomson, 1974). These, combined with some new findings, led us to formulate the representative structure **2** in Figure 15.6 for pheomelanins

Table 15.2. Main types of epidermal melanin pigments (from Prota *et al.*, 1998a).

Melanin	Criteria
Eumelanins	Black or brown nitrogenous pigments, insoluble in all solvents, which arise by oxidative polymerization of 5,6-dihydroxyindoles derived biogenetically from tyrosine via dopaquinone
Pheomelanins	Alkali-soluble, yellow to reddish brown pigments, containing sulfur in addition to nitrogen and arising by oxidative polymerization of cysteinyldopas via 1,4-benzothiazine intermediates
Trichochromes	A variety of sulfur-containing pheomelanins, of low molecular weight, characterized by a pH-dependent bi(1,4-benzothiazine) chromophore

that consist mostly of benzothiazine units with minor contributions from benzothiazole and isoquinoline units. Some portions of the monomer units may be connected through ether bonds (Di Donato and Napolitano, 2003; Napolitano *et al.*, 1996a). However, the contribution of ether bond formation should be minimal, because monomer units connected through ether bonds should be colorless, which is incompatible with the brownish color of natural pheomelanins. The isoquinoline units may be produced by post-polymerization modifications of freshly formed pheomelanin, because isoquinoline units are not found in the earlier stages of pheomelanogenesis (Fig. 15.3). The modification of alanyl side-chain to the aromatic character is consistent with biosynthetic studies using radioisotopes and ^{13}C-NMR spectroscopy (Chedekel *et al.*, 1987; Deibel and Chedekel, 1984).

Trichochromes are the only melanin-like pigments with fully characterized structures (**3–6**; Fig. 15.6). The close similarity in structural features between trichochromes and pheomelanins and their coexistence in pigmented tissues suggest that they are formed oxidatively from the same monomer units,

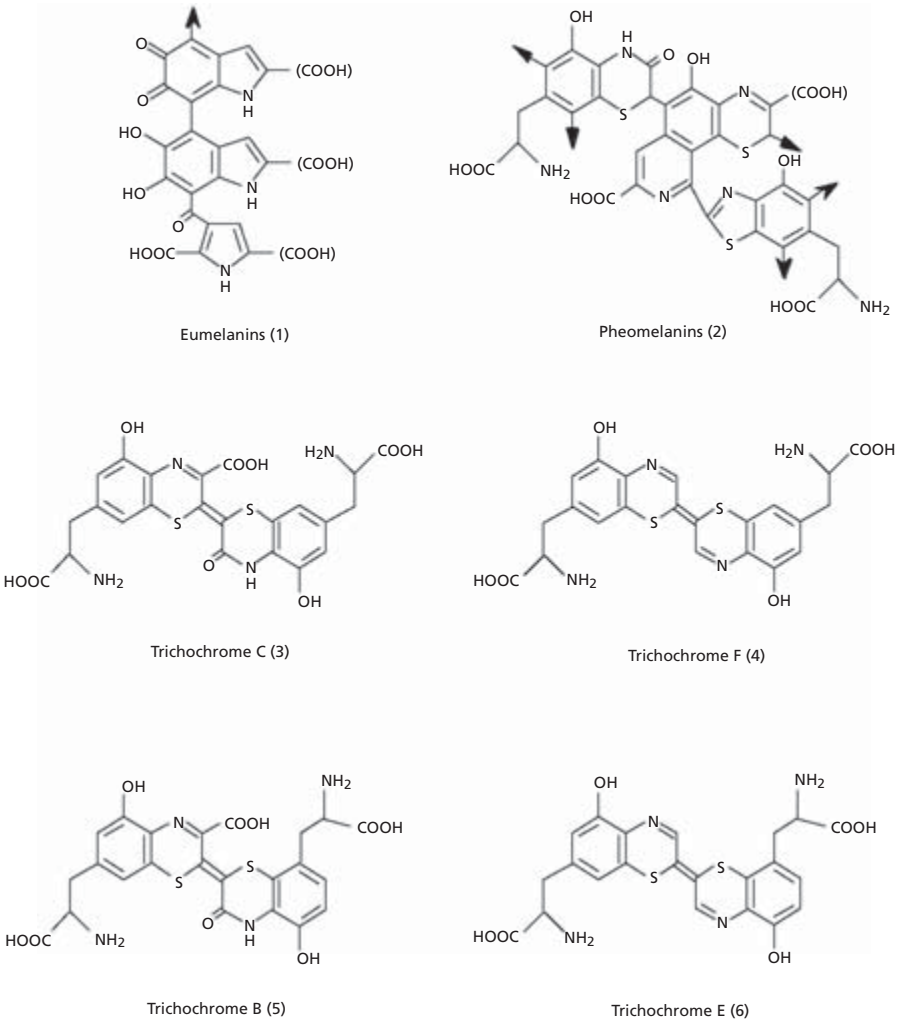


Fig. 15.6. Structures of eumelanins (**1**), pheomelanins (**2**), and trichochromes (**3–6**). Structures of eumelanins and pheomelanins are the only representative ones formulated on the basis of biosynthetic and degradative studies. The positions with (–COOH) are connected either to –H or –COOH; these positions may also be available for attachment to other units. The arrows indicate sites for attachment to other units. The isoquinoline units in pheomelanin structure (**2**) may be produced by the post-polymerization modification of freshly formed pheomelanin, because those units are not produced in the early stages of pheomelanogenesis.

differing only in the mode of polymerization. Therefore, trichochromes may be regarded as a variety of pheomelanic pigment (Table 15.2).

Prota's group conducted a series of biosynthetic studies to clarify the stages beyond DHI and DHICA in eumelanogenesis and those of cysteinyldopas in pheomelanogenesis. Although considerable advances have been made in these respects (Di Donato and Napolitano, 2003; Napolitano *et al.*, 1996a; Prota *et al.*, 1998a), it appears at present unnecessary to change our basic views on the structures of eumelanins and pheomelanins, as depicted in Figure 15.6 (see Degradative Studies below for further discussion).

Isolation and Preparation of Melanins

Natural Eumelanins

One of the major problems in studying melanin is the lack of adequate methods to isolate pure melanin pigments. The most frequently used sources of natural eumelanins are ink sacs of cuttlefish *Sepia officinalis* (Sepia melanin) (Nicolaus, 1968), the uveal tract and the retinal pigment epithelium of bovine eyes (Dryja *et al.*, 1979), and melanoma tumors. Eumelanins are considered to be firmly bound to proteinaceous components, through covalent or ionic bonds (Zeise, 1995). To remove proteins, treatment with hydrochloric acid is used, but complete removal requires a prolonged treatment in concentrated HCl at room temperature or 24 h of boiling in 6 M HCl (Nicolaus, 1968). As early as 1907, von Fürth and Jerusalem isolated "melanin" as an insoluble, black pigment by heating melanoma tissues in concentrated HCl. One should be aware that such harsh treatment causes irreversible structural alterations. For example, carbon dioxide and ammonia molecules are liberated under these conditions from various synthetic eumelanins (Ito, 1986). Also, the indole moiety is known to be labile in strong acid; tryptophan is decomposed during 6 M HCl hydrolysis of proteins. To circumvent these difficulties, treatment with proteinases such as pronase or collagenase may be preferable; but again, treatment with cold acid is often used to remove accompanying residual proteins (Novellino *et al.*, 1981; Prota *et al.*, 1976).

Another approach is to solubilize melanins (melanoproteins) with alkali. Fortner (1910) was the first to report a systemic study to isolate melanin pigment by alkali solubilization. It involves extraction of melanins with NaOH followed by repeated precipitation of the melanins with HCl and redissolution in NaOH (Bolt, 1967). However, proteins cannot be removed by this method; approximately 50% of proteins were found to remain in the melanin preparations from human black hair and from red hair (Menon *et al.*, 1983). In addition, treatment of melanins with alkaline solutions causes irreversible uptake of oxygen (Felix *et al.*, 1978), suggesting that further modification in the melanin structure takes place.

To overcome these difficulties, the use of intact Sepia melanin is preferable. However, some precautions should be taken when one wishes to use it as a representative of natural eumelanins. The intact Sepia melanin consumes little oxygen and displays poor redox properties, suggesting a high degree of breakdown in the indolic moiety (Pezzella *et al.*, 1997). Further, the intact, freshly prepared Sepia melanin differs in surface area depending on the isolation procedure employed (Liu and Simon, 2003). The different degree of aggregation may alter the photochemical properties of melanin granules. If one needs to isolate eumelanins of mammalian origin, preparation of melanosomes from bovine eyes seems to be the choice. Duchón *et al.* (1973) determined the contents of melanin and protein in melanosome preparations from 10 different biological sources and found the melanin contents to be fairly high, ranging between 20% and 70%. If one wishes to remove proteins from the eumelanin preparations, melanosome fractions should be used as starting materials, rather than whole tissues, to reduce the possibility of unnecessary reactions that might occur between melanins and tissue components. An example of such reactions is sulfur incorporation into eumelanins when heated in 6 M HCl in the presence of cysteine or cystine (Ito, 1986).

Recently, Novellino *et al.* (2000) developed a new enzymatic procedure for isolation of eumelanin from black human hair involving digestion with protease, proteinase K, and papain in the presence of dithiothreitol. In an extension of this study, Liu *et al.* (2003) compared two different acid/base extractions and an enzymatic extraction to isolate eumelanin from black human hair. The data indicate that pigments obtained by the acid/base extractions contain significant protein (52% and 40%), destroy the melanosomes, and possess an altered molecular structure. The enzymatically extracted hair melanin (14% protein content), on the contrary, retains the morphology of intact melanosomes and is an excellent source of human eumelanin.

Natural Pheomelanins

As a source of natural pheomelanins, red feathers of New Hampshire chickens have been commonly used (Minale *et al.*, 1967; Prota, 1972; Thomson, 1974). The isolation procedure involves extraction in 0.1 M NaOH, acidification, and dialysis followed by gel chromatography on Sephadex G-50. Although the procedure is much less destructive than those employed for eumelanins, it is still harsh enough to affect the structural features.

Trichochromes (**3–6**), dimeric pheomelanic pigments, have been isolated from red feathers (Prota and Nicolaus, 1967). Interestingly, trichochrome C (and its isomer trichochrome B) was also found in the urine of patients with melanoma metastases (Agrup *et al.*, 1978; Rorsman *et al.*, 1979). It should be noted that trichochrome F (and its isomer E) may be artificially formed from 5-*S*-cysteinyldopa (and a mixture of 5-*S* and 2-*S* isomers) during the extraction and work-up procedures (Prota, 1992; Rorsman *et al.*, 1979).

Synthetic Melanins

Synthetic eumelanins can be prepared by the oxidation of L-tyrosine or L-dopa at neutral pH in the presence of tyrosinase, usually commercially available mushroom tyrosinase (Ito, 1986). Biosynthetic dopa melanin has long been considered as

a good model of eumelanins. However, recent progress in eumelanin chemistry has proved that dopa melanin prepared by tyrosinase oxidation is quite different from natural eumelanins with respect to the carboxyl group content, reflecting a difference in the ratio of DHI to DHICA (Ito, 1986). A synthetic dopa melanin sample finely suspended in neutral buffer and exposed to oxygen showed a marked increase in the carboxyl content over time (Crescenzi *et al.*, 1993). Similar changes may occur during the process of melanin preparation. Dopa melanin prepared by the auto-oxidation of dopa at alkaline pH appears to be degraded to a significant extent by oxidative cleavage of the *ortho*-quinone moiety, and the use of these preparations should be discouraged (Ito, 1986).

Natural eumelanins are now believed to arise from copolymerization of DHI and DHICA in various ratios. Therefore, it is recommended that synthetic eumelanins prepared from DHI, DHICA, and various ratios of their mixture be used as eumelanin standards, at least for structural studies (Ozeki *et al.*, 1997b). A one-step synthesis of DHI and DHICA for such studies is available (Wakamatsu and Ito, 1988).

Synthetic pheomelanins can be prepared by tyrosinase oxidation either of a mixture of L-dopa and L-cysteine or of 5-*S*-cysteinyldopa in the presence of a catalytic amount of L-dopa (Ito, 1989). Degradative experiments suggest that these biosynthetic pheomelanins resemble natural pheomelanins present in the yellow hair of rodents (Ito, 1989). Pheomelanins prepared from 5-*S*- or 2-*S*-cysteinyldopa are shown by isoelectric focusing to be more homogeneous than those prepared from dopa and cysteine (Deibel and Chedekel, 1984).

In contrast to the availability of eumelanin standards, one faces difficulty in obtaining a valid pheomelanin standard, either natural or synthetic. We therefore refined the conditions for preparing pheomelanin by tyrosinase oxidation of L-dopa in the presence of an excess of L-cysteine (Ito, 1989). The method makes it unnecessary to synthesize pure 5-*S*-cysteinyldopa as the starting material for pheomelanin preparation. When one needs to prepare pheomelanins from 5-*S*-cysteinyldopa or other isomers, both enzymic and chemical methods are available for the synthesis of cysteinyldopa isomers (Chioccara and Novellino, 1986; Ito, 1983; Ito and Prota, 1977). Copolymers of eumelanin and pheomelanin can be prepared by tyrosinase oxidation of L-dopa in the presence of varying ratios of L-cysteine (Ito and Fujita, 1985).

When precise analytical data on melanins are required, differences in the water content of melanins prepared can no longer be neglected. It is thus recommended to use melanin standards that are kept in a desiccator of a constant moisture content (Napolitano *et al.*, 1995).

Molecular Weight

The unusual insolubility of eumelanins and their blackness suggest that eumelanins consist of several hundreds of monomeric units. However, there is no direct evidence to support this belief. Because of their insolubility, the direct measurement of molecular weight has been difficult. With soluble DHICA melanins, an estimate of molecular weight using HPLC/molecular sieve analyses gave values in the range of 20 000 to 200 000 (Orlow *et al.*, 1992), although the validity of that is questionable because of a possible interaction of melanins and the chromatographic matrix during the molecular weight determinations (Prota *et al.*, 1998a).

Recent application of matrix-assisted laser desorption ionization (MALDI) mass spectrometry shows that both DHI and DHICA melanins have a large proportion of oligomeric fractions of lower molecular weight, not exceeding 1.5 kDa (Napolitano *et al.*, 1996b, c; Seraglia *et al.*, 1993). However, whether polymeric melanins exceeding 1.5 kDa are not present in the samples or are not detectable by this method remains to be clarified.

In contrast to eumelanins, the measurement of the molecular weights of pheomelanins is more feasible. A molecular weight of less than 2.0 kDa has been obtained for protein-free gallopheomelanin 1, isolated from red chicken feathers (Fattorusso *et al.*, 1968). However, that estimate is also subject to question because of the heterogeneity of the pigment and the irreversible binding of part of the material to the chromatographic gel (Deibel and Chedekel, 1982).

Recent Advances in the Study of Melanogenesis

Late Stages of Eumelanogenesis

Dopachrome accumulates in the early stages of eumelanogenesis, and stages beyond the formation of dopachrome are discussed here. The red pigment dopachrome is a fairly stable molecule with a half-life of about 30 min (first-order rate constant of 4.0×10^{-4}/s), and it spontaneously decomposes to give mostly DHI by decarboxylation at neutral pHs in the absence of Dct (or metal ions). The ratio of DHI to DHICA under these conditions is 70:1 (Palumbo *et al.*, 1987a). On the other hand, in the presence of Dct (Tyrp2), dopachrome is catalyzed to undergo tautomerization preferentially to produce DHICA (Palumbo *et al.*, 1991). The ratio of DHICA to DHI production is thus determined by the activity of Dct. The presence of metal ions, especially Cu^{2+}, accelerates the rate of dopachrome rearrangement and also affects the DHICA/DHI ratio, but Dct seems to be more effective in catalyzing the tautomerization (Palumbo *et al.*, 1987a, 1991).

Until 1980, eumelanins had been believed to be DHI rich, because the decarboxylation of dopachrome is the major pathway taken in the absence of any extrinsic factor. Then, in 1980, Pawelek's group discovered dopachrome conversion factor, now known as dopachrome tautomerase or Dct (Pawelek, 1991; Pawelek *et al.*, 1980). Therefore, we analyzed the contents of DHICA-derived units in various types of eumelanins (Ito, 1986). Two analytical methods were used: acid-catalyzed decarboxylation and permanganate oxidation to give PTCA. The results showed that synthetic dopa melanin contained only trace amounts of DHICA-derived units, while melanins from Sepia, B16 melanoma, and mouse black hair consisted of about equal amounts of DHI- and DHICA-derived units. This study thus confirmed the significance of DHICA-derived units in the structure of natural eumelanins. The same methodology was employed by Palumbo *et al.*

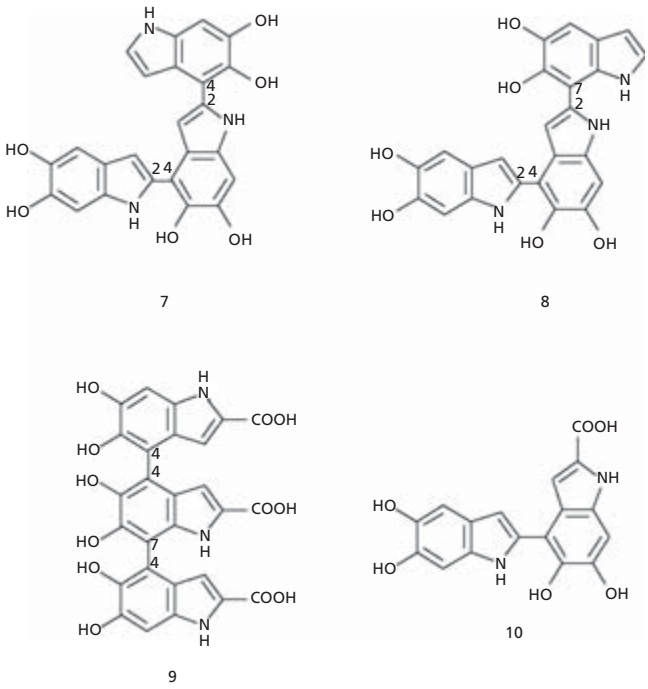


Fig. 15.7. Structures of representative oligomers formed in the early stages of enzymic conversion of DHI and DHICA to eumelanins. The homotrimers **7** and **8** are products of oxidation of DHI whereas the homotrimer **9** is from DHICA (Prota *et al*., 1998a). The heterodimer **10** is among products obtained by oxidation of a mixture of DHI and DHICA.

(1988) to show that dopa melanin prepared in the presence of metal ions, especially Cu^{2+}, are more akin to natural eumelanins than those prepared in the absence of metal ions.

The fact that DHICA is equally significant compared with DHI as a eumelanin precursor prompted Prota and his associates to carry out extensive, biomimetic studies on the mode of polymerization of these 5,6-dihydroxyindoles (for reviews, see d'Ischia *et al.,* 1996; Prota *et al.,* 1998a). Upon enzymic or chemical oxidation, DHI affords in the early stages a number of dimers and trimers (d'Ischia *et al*., 1990). Some representative structures, such as the DHI trimers **7** and **8**, are shown in Figure 15.7. Judged from the structures of oligomers isolated, the most reactive position is the 2-position, followed by the 4- and 7-positions. Oxidation of DHICA, catalyzed by tyrosinase/O_2 or by peroxidase/H_2O_2, produces mixtures of dimers and trimers in which the indole units are mostly linked through the 4- and 7-positions (Palumbo *et al*., 1987b; Pezzella *et al*., 1996). The 4-position appears to be more reactive than the 7-position, as exemplified by the isolation of the trimer **9**. Interestingly, the mode of polymerization is influenced by the oxidant employed; Cu^{2+} ion-catalyzed oxidation of DHICA affords minor 3,4′- and 3,7′-coupled dimers arising from participation of the 3-position, in addition to the major 4,4′-, 4,7′-, and 7,7′-dimers (Pezzella *et al*., 1996). Co-oxidation of DHI and DHICA with peroxidase/H_2O_2 affords, in addition to homo-oligomers, some heterodimers including the dimer **10** (Napolitano *et al*., 1993a).

As shown above, the pulse radiolysis technique is very useful in following the fate of short-lived *ortho*-quinone intermediates. Using this technique, Lambert *et al.* (1989) were able to suggest the possibility that 5,6-indolequinone tautomerizes to its quinone-imine and quinone-methide tautomers, which then undergo nucleophilic addition of water, thus giving trihydroxyindole species.

The role of Dct in melanogenesis has been examined using follicular melanocytes of congenic mice (Lamoreux *et al*., 2001; Ozeki *et al*., 1995). The *slaty* mutation in the mouse is known to decrease the activity of Dct. The effects of tyrosinase, Dct, and Tyrp1 on eumelanogenesis were compared (Ito, 2003). *Chinchilla* and *slaty* mouse hairs had total melanin values about 50% that of black hair, while brown hair had about 30% (Lamoreux *et al*., 2001). *Black*, *chinchilla*, and *brown* mouse hairs give similar PTCA to total melanin ratios. Comparison with synthetic eumelanins indicated that the DHI to DHICA ratios in these three mutants were close to 1:3. On the other hand, *slaty* hair gave a PTCA to total melanin ratio similar to that of synthetic eumelanin having a 3:1 ratio. Thus, Dct accelerates dopachrome tautomerization, increasing the ratio of DHICA in eumelanins and accelerating the production of eumelanins. Human hair (Ozeki *et al*., 1996a) and skin (Alaluf *et al*., 2001) appears to produce DHI-rich eumelanins, similar to mouse *slaty* hair.

Then, what is the role of Tyrp1? There is some controversy regarding the role of Tyrp1 (Hearing, 2000), although it is known that mouse Tyrp1 is able to catalyze the oxidation of DHICA (Jiménez-Cervantes *et al*., 1994). DHICA has a higher oxidation potential than DHI. This means that DHICA-quinone may be able to undergo a redox exchange with DHI yielding DHICA and DHI-quinone (Fig. 15.8). This proposal, however, does not exclude the possibility that DHI adds directly to DHICA-quinone to form heterodimers (Napolitano *et al*., 1993a). Copolymerization of these four intermediates should yield a series of heteropolymers of DHI and DHICA. Thus, the role of Tyrp1 appears to accelerate eumelanogenesis by oxidizing not only DHICA but also DHI indirectly. Our chemical analysis showed that follicular melanocytes of *brown* mice produce a eumelanin with a smaller molecular size compared with black mice (Ozeki *et al*., 1997b). As evidence to show that this type of copolymerization takes place, Prota's group isolated a heterodimer of DHI and DHICA as the acetyl derivative from an oxidation mixture of DHI and DHICA (Napolitano *et al*., 1993a).

Late Stages of Pheomelanogenesis

The early stages of pheomelanogenesis until the formation of cysteinyldopaquinones are well elucidated, as discussed above. In the later stages of pheomelanogenesis, 5-*S*-cysteinyldopaquinone, once formed, then rapidly cyclizes via attack of the cysteinyl side-chain amino group on the carbonyl group to give a cyclic *ortho*-quinonimine intermediate (Fig. 15.9; Napolitano *et al*., 1994). The rate (r5) of quinonimine formation was determined by pulse radiolysis to be 5.5/s (Thompson *et al*., 1985). It should be stressed that the rate of

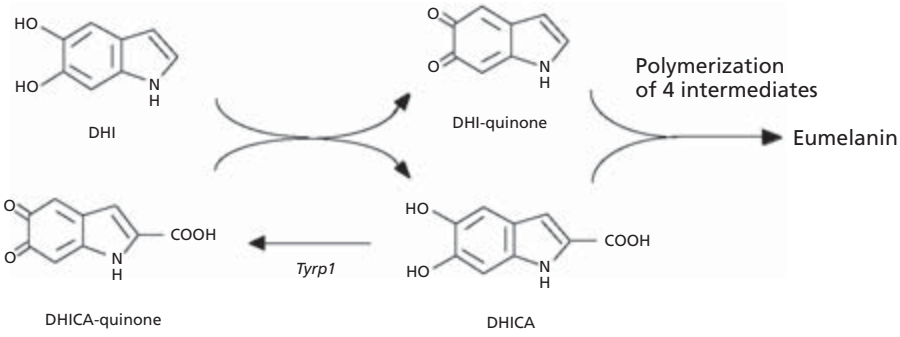


Fig. 15.8. Proposed role of Tyrp1. Copolymerization of DHI and DHICA is suggested (Ito, 2003).

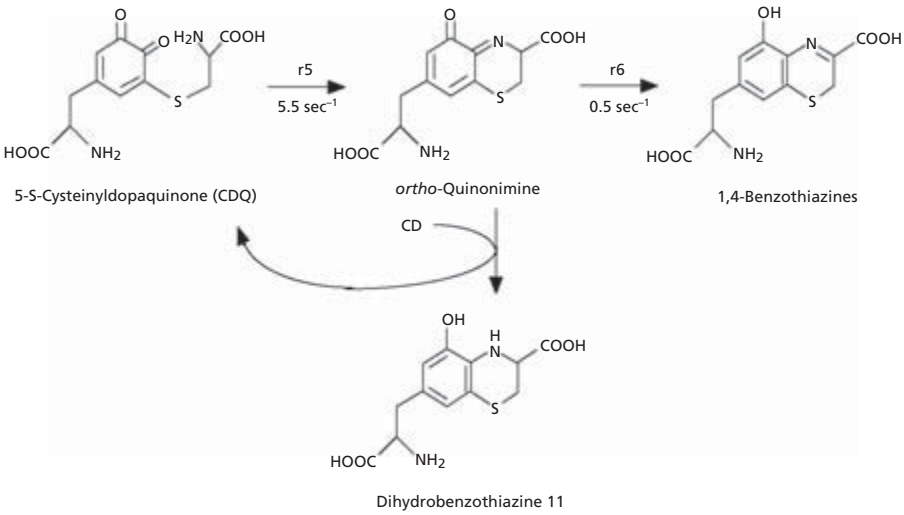


Fig. 15.9. Kinetics in the late stages of the formation of pheomelanins from 5-*S*-cysteinyldopaquinone. Note that the dihydrobenzothiazine **11** is produced via redox exchange whereas benzothiazine intermediates are by rearrangement (with/without decarboxylation).

5-*S*-cysteinyldopaquinone formation is controlled by the rate of dopaquinone formation, because 5-*S*-cysteinyldopa itself is a much poorer substrate for tyrosinase than dopa (Agrup *et al.*, 1982).

An alternative pathway is possible for the metabolism of 5-*S*-cysteinyldopaquinone; this *ortho*-quinone also undergoes the addition of cysteine with a rate constant of 1×10^4/M/s to give the diadduct 2,5-*S*,*S*′-dicysteinyldopa (Thompson *et al.*, 1985). It is thus delineated that, unless the cysteine concentration is higher than 5 mM (an unlikely situation *in vivo*), the quinonimine formation predominates.

The *ortho*-quinonimine then undergoes rearrangement to benzothiazine intermediate(s) with (85%) and without (15%) decarboxylation (Napolitano *et al.*, 1994). The rate (r6) of decay (k = 0.5/s) of the cyclic *ortho*-quinonimine to the benzothiazines was recently determined by pulse radiolysis (Napolitano *et al.*, 1999). As shown in Figure 15.9, an alternative pathway for *ortho*-quinonimine is also possible by redox exchange with 5-*S*-cysteinyldopa, leading to the production of a reduced form of the quinonimine (a 3,4-dihydro-1,4-benzothiazine derivative **11**) and 5-*S*-cysteinyldopaquinone (Napolitano *et al.*, 2000a). Whether the redox exchange or the rearrangement prevails is strongly influenced by many factors, including the nature of the oxidant and the concentration of the precursor 5-*S*-cysteinyldopa. It therefore remains to be seen whether this redox change is a significant pathway *in vivo*.

Reactions beyond the benzothiazines, which lead to pheomelanins, are rather complex, but nevertheless these have also been extensively studied by Prota and associates (reviewed by Di Donato and Napolitano, 2003). It is interesting to note that the presence of metal ions strongly affects the course of later stages; trichochrome-type dimers (reduced form of trichochrome pigments) are produced in the zinc-catalyzed oxidation of 5-*S*-cysteinyldopa (Napolitano *et al.*, 2001), while two monomeric amino acids, the benzothiazinone **12** and the benzothiazole **13** (R = H), are produced by chemical oxidants in the presence of Fe^{3+} or Cu^{2+} at neutral pH (Fig. 15.10; Di Donato *et al.*, 2002). Notably, the formation of benzothiazole (**13**) suggests that the ring contraction of benzothiazines to benzothiazoles is a feasible process under physiological conditions. In this connection, high levels of zinc, iron, and copper ions are detected in melanosomes isolated from human hair (Liu *et al.*, 2003) and in intact melanosomes from the eye (Samuelson *et al.*, 1993). Interestingly, upon irradiation with UVA, the dihydroxybenzothiazine **11** undergoes ring contraction to give 2-methylbenzothiazole **13** (R = CH_3) (Costantini *et al.*, 1994a).

The presence of benzothiazole and isoquinoline units in pheomelanins has been questioned by Prota (Prota *et al.*,

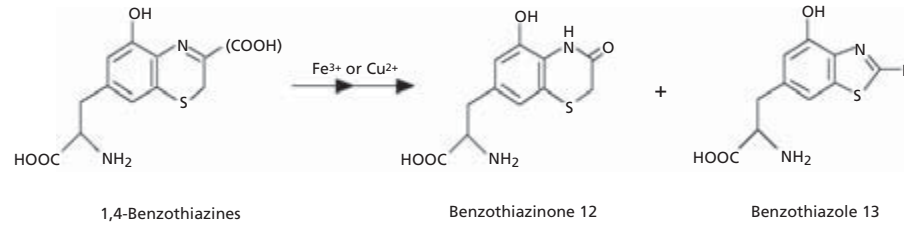


Fig. 15.10. Products of metal-catalyzed oxidation of 5-*S*-cysteinyldopa via the 1,4-benzothiazine amino acid.

1998a) based on the fact that the products derived from benzothiazole and isoquinoline units are obtained only when pheomelanins are subjected to drastic degradative reactions. However, as shown above, the ring contraction of benzothiazines giving benzothiazoles proceeds under very mild conditions that mimic *in vivo* situations. The same argument may hold true for the presence of the isoquinoline units (the unit located at the center of the structure **2**). The isoquinoline structure would be readily formed through the Pictet–Spengler reaction (Manini *et al.*, 2001) when an alanyl side-chain on a benzene ring and a carbonyl group are close to each other.

Chemical Properties of Melanins

A major role of melanins *in vivo* is generally believed to be photoprotection of underlying tissues. However, this concept has been challenged from time to time (Hill, 1992; Wood *et al.*, 1999), and this topic is dealt with extensively in Chapter 17 (Photobiology of melanins). However, it should be pointed out here that the measurement of eumelanin and pheomelanin contents is indispensable in these studies (De Leeuw *et al.*, 2001; Tadokoro *et al.*, 2003). Another important property of melanins is the ability to bind to various chemicals and metal ions. This property is discussed in Chapter 18 (Toxicological aspects of melanin and melanogenesis). Chapter 16 also addresses the physical properties of melanins, including free radical properties, redox state, and photo-oxidation.

Melanin-related Metabolites

Melanin-related Metabolites as Markers of Melanoma (and Melanin Production)

In addition to eumelanins and pheomelanins, normal and malignant melanocytes produce and excrete their precursors, 5,6-dihydroxyindoles and cysteinyldopas. These precursors and their metabolites are found in epidermal and melanoma tissues and in body fluids at variable levels. Therefore, those melanin-related metabolites have been extensively studied as markers of melanoma progression, to detect metastases, evaluate therapeutic effects, and to predict prognosis (for reviews, see Duchón, 1987; Hartleb and Arndt, 2002; Ito, 1992; Rorsman and Pavel, 1990; Rorsman *et al.*, 1983).

As for the 5,6-dihydroxyindoles, DHI, DHICA, and their metabolites in urine were once explored as possible markers for screening of metastasizing melanoma (Duchón *et al.*, 1981). The indolic urinary melanogens were classified into two main groups based on the response to the Thormählen reaction (sodium nitroferricyanide). Thormählen-positive melanogens were first recognized by Leonhardi (1955) as DHI derivatives. Through extensive studies by Duchón and his associates in Prague, this group of melanogens was shown to be glucuronides and sulfates of 5-hydroxy-6-methoxyindole and 6-hydroxy-5-methoxyindole (Matous *et al.*, 1981; Pavel *et al.*, 1981). The Thormählen-negative melanogens were shown to consist mainly of 5-hydroxy-6-methoxyindole-2-carboxylic acid and 6-hydroxy-5-methoxy-indole-2-carboxylic acid (Duchón and Matous, 1967). These indolic melanogens are formed in melanocytes by *O*-methylation (Smit *et al.* 1990), followed by subsequent conjugation with glucuronic acid or sulfuric acid in the liver or kidney. The *O*-methylation appears to be a mechanism of detoxification of these cytotoxic 5,6-dihydroxyindoles.

The potential usefulness of these indole melanogens as markers of melanoma progression (and of melanin production in normal subjects) has attracted the interest of clinicians. 5-Hydroxy-6-methoxyindole-2-carboxylic acid was found to be the best marker of melanin pigmentation in the urine (Westerhof *et al.,* 1987). The level of 6-hydroxy-5-methoxyindole-2-carboxylic acid in plasma was proposed to be more sensitive and reliable than 5-*S*-cysteinyldopa (Hara *et al.,* 1994), whereas other studies have reached the opposite conclusion (Horikoshi *et al.,* 1994). In contrast to 5-*S*-cysteinyldopa, which is still drawing attention (Hartleb and Arndt 2001; Wakamatsu *et al.*, 2002a), it appears likely that clinicians have lost interest in those indolic melanogens as melanoma markers.

The growing interest in DHI and DHICA as equally important precursors of eumelanins and as markers of melanocyte activities has prompted us to establish more efficient methods to prepare these rather labile compounds, because previously reported methods of DHI and DHICA preparation require tedious, multistep procedures (Benigni and Minnis, 1965). Therefore, by taking advantage of the chemical reactivity of dopachrome, we developed biomimetic procedures to prepare DHI and DHICA in subgram quantities (Wakamatsu and Ito, 1988). Dopachrome generated *in situ* by ferricyanide oxidation of dopa at neutral pH undergoes spontaneous decarboxylation to give DHI, while treatment with alkali at pH 13 affords mostly DHICA. After recrystallization, DHI and DHICA are obtained in modest yields.

As for the cysteinyldopas, the major isomer of cysteinyldopas, 5-*S*-cysteinyldopa, was first detected in melanoma tissues and in urine (Bjorklund *et al.*, 1972). Subsequently, other minor isomers, i.e. 2-*S*- and 6-*S*-cysteinyldopas, along with the diadduct 2,5-*S*,*S*′-dicysteinyldopa, have also been

detected in the urine of melanoma patients (Morishima *et al.*, 1983; Prota *et al.*, 1977).

Tyrosinase appears to be primarily responsible for the production of 5-*S*-cysteinyldopa. 5-*S*-Cysteinyldopa has thus been detected not only in melanoma tissues, in the serum, and in the urine of melanoma patients, but also in skin, hair, serum, and urine of normal subjects (Ito, 1992; Rorsman and Pavel, 1990; Rorsman *et al.*, 1983). However, the detection of small amounts of 5-*S*-cysteinyldopa in plasma and urine from human and mouse albinos indicates that tyrosinase-independent routes may also be present (Acquaron *et al.*, 1981; Nimmo *et al.*, 1985). In this connection, it should be noted that some biologically relevant oxidizing systems, in addition to tyrosinase, can mediate the formation of cysteinyldopas from dopa and cysteine, i.e. peroxidase/H_2O_2, ferrous ion, superoxide, and hydroxyl radicals (Ito, 1983; Ito and Fujita, 1981, 1982).

In contrast to 5,6-dihydroxyindoles, 5-*S*-cysteinyldopa is not metabolized to any major extent; decarboxylation does not take place, and *O*-methylation (Agrup *et al.*, 1977) and conjugation with glucuronic and sulfuric acids appear to be only minor pathways. These properties, coupled with the high renal clearance, make 5-*S*-cysteinyldopa a useful marker of pigmentation in normal and malignant melanocytes. Determination of plasma levels of 5-*S*-cysteinyldopa seems to be useful in predicting distant metastases in melanoma patients, and elevated levels are associated with a poor prognosis (Wakamatsu *et al.*, 2002a). However, one of the drawbacks of 5-*S*-cysteinyldopa as a melanoma marker is that the levels often rise two to several-fold in summer, occasionally to pathological levels, due to sun exposure (Rorsman *et al.,* 1976; Wakamatsu and Ito, 1995), although this property may be used as a measure to assess the degree of sun exposure in normal subjects.

The growing demand for 5-*S*-cysteinyldopa, not only for the study of pheomelanogenesis but also for clinical studies, has prompted us to develop convenient laboratory syntheses. For subgram scale preparation, the reaction of cysteine with dopaquinone produced by tyrosinase oxidation of dopa serves the purpose (Ito and Prota, 1977). For gram scale preparation, oxidation of dopa with ceric ammonium nitrate in sulfuric acid in the presence of cysteine seems a better choice (Chioccara and Novellino, 1986).

Cytotoxicity and Related Properties of Melanin Precursors

The concept is generally accepted that melanin precursors are cytotoxic to the cells where melanin pigments are produced, i.e. melanocytes (Hochstein and Cohen, 1963). This concept leads to another concept: that the compartmentalization of melanogenesis in melanosomes represents a strategy by melanocytes to prevent the inherent cytotoxicity of melanin precursors (Prota *et al.*, 1998a).

Wick *et al.* (1977) were the first to explore the possibility of using the cytotoxicity of melanin precursors to develop chemotherapeutic agents against melanoma. They examined the cytotoxic effects of dopa and other related melanin precursors. These studies were followed by studies on the cytotoxicity of melanin precursors, DHI and 5-*S*-cysteinyldopa, to cultured melanoma cells (Fujita *et al.*, 1980; Pawelek and Lerner, 1978). However, the mechanism of cytotoxicity of 5-*S*-cysteinyldopa to melanoma cells soon proved to involve the production of reactive oxygen species, such as H_2O_2, formed by auto-oxidation in culture media (Ito *et al.*, 1983). The inherent cytotoxicity of DHI and DHICA was also re-examined (Urabe *et al.*, 1994). The observed cytotoxic effects were found to be mainly due to the generation of reactive oxygen species outside the cells. It thus appears that DHI and DHICA may be less cytotoxic than one would imagine as long as they are produced and oxidized within melanosomes.

Nevertheless, it is well known that stimulation of melanogenesis leads to the suppression of proliferation and eventually to cell death in cultured normal melanocytes (Hirobe *et al.*, 2003). Further, ectopic expression of tyrosinase in the absence of Tyrp1 or Dct may cause severe cytotoxicity to non-melanocytic cells in which no melanosomal compartmentalization is present (Singh and Jimbow, 1998). Then, what are the more toxic intermediates than DHI and DHICA that are produced in melanocytes? In the process of melanogenesis, a number of highly reactive *ortho*-quinones are produced including dopaquinone, dopachrome, DHI-quinone, and DHICA-quinone (Figs 15.2 and 15.8). Among these quinones, dopaquinone, DHI-quinone, and DHICA-quinone appear to be too reactive and would be polymerized within the melanosomes. Dopachrome is, however, quite stable in the absence of Dct or metal ions (Palumbo *et al.*, 1987a). In fact, a recent study indicates that aminochromes such as dopachrome and dopaminechrome are much more toxic to cultured melanoma and neuroblastoma cells than L-dopa, DHI, and DHICA (Matsunaga *et al.*, 2002). Dopachrome reacts with sulfhydryl compounds at the 4-position (d'Ischia *et al.,* 1987). It now appears that dopachrome, although not extremely reactive, is able to react with sulfhydryl enzymes essential for melanocyte survival and to inactivate them, eventually leading to cell death. Supporting this view, Dct is a melanocyte-specific enzyme considered to be a "rescue" enzyme essential for melanocyte survival (Tsukamoto *et al.*, 1992a). Mutations in Dct that decrease catalytic function affect DHICA production and are generally quite cytotoxic to melanocytes. Melanocytes typically express Dct before any of the other melanogenic enzymes, presumably to minimize such toxicity (Steel *et al.*, 1992). Matsunaga *et al.* (1999) have recently shown that a related enzyme, called macrophage migration inhibitory factor (MIF), is able to catalyze the conversion of dopaminechrome to DHI. MIF is expressed in neuronal tissues and is believed to participate in a detoxification pathway for catecholamine oxidation products.

The cytotoxicity of *ortho*-quinones could potentially lead to chemotherapeutic approaches to treat melanoma using phenolic melanin precursors (Prota *et al.*, 1994; Riley *et al.*, 1997). Phenolic compounds are expected to be less cytotoxic themselves than the corresponding catecholic compounds, yet they can be metabolized in melanocytes to highly reactive

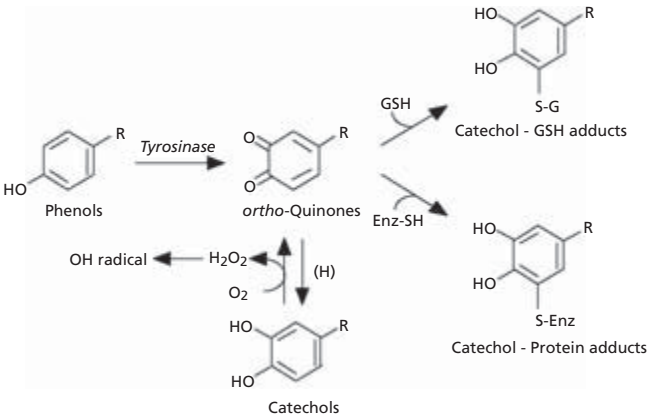


Fig. 15.11. Mechanism of melanocytotoxicity of phenolic and catecholic melanin precursors (Ito, 2003).

ortho-quinones by the action of tyrosinase (Fig. 15.11). The *ortho*-quinones thus formed may be detoxified by glutathione in the cytosol, but those that escape from this detoxification mechanism may enter the nucleus and inactivate sulfhydryl enzymes such as thymidylate synthase, thereby leading to cell death (Prezioso *et al.*, 1992). Among the phenolic compounds so far examined, 4-*S*-cysteaminylphenol and its derivatives appear to be the most promising antimelanoma agents (Alena *et al.*, 1990; Yukitake *et al.*, 2003). The ultimate toxic metabolite of 4-*S*-cysteaminylphenol has been shown to be dihydro-1,4-benzothiazine-6,7-quinone, a sulfur homolog of dopaminechrome (Hasegawa *et al.*, 1997; Mascagna *et al.*, 1994). Catechols, such as dopa and DHI, are cytotoxic through two possible routes: one through the generation of *ortho*-quinones and the other through auto-oxidation producing H_2O_2 and hydroxyl radicals (Graham *et al.*, 1978). To evaluate the binding of *ortho*-quinones to proteins through cysteine residues, we developed a method to measure the catechol–protein adducts. The method is based on the HPLC analysis of cysteinyl–catechols formed on HCl hydrolysis of the modified proteins (Ito *et al.*, 1988a).

Melanin precursors themselves may have protective roles in melanocytes (Prota *et al.*, 1998a). DHI and, to a lesser extent, DHICA and 5-*S*-cysteinyldopa are capable of inhibiting lipid peroxidation in several *in vitro* model systems (Memoli *et al.*, 1997; Napolitano *et al.*, 1993b). These melanin precursors have been shown to have inhibitory effects much greater than those of ascorbic acid and glutathione. 5-*S*-Cysteinyldopa has also been shown to be a potent inhibitor of hydroxylation/oxidation reactions mediated by H_2O_2 and the Fe^{2+}/EDTA complex (Fenton reaction) (Napolitano *et al.*, 1996d). Furthermore, DHI is highly efficient in inhibiting the generation of peroxidation products in *in vitro* models of UV-induced lipid peroxidation compared with 5-*S*-cysteinyldopa as well as eumelanin and pheomelanin samples (Schmitz *et al.*, 1995). A recent kinetic study using laser flash photolysis also shows that the antioxidant properties of 5,6-dihydroxyindoles, in particular DHI, are as good as those of α-tocopherol (Zhang *et al.,* 2000).

The photoprotective role of melanin-related metabolites is also an interesting consideration. Photobiological and photochemical data indicate that DHI and DHICA have protective roles. Upon photoexcitation, these 5,6-dihydroxyindoles undergo photolysis with the generation of semiquinone radicals. DHI semiquinone can react with oxygen and related species, giving rise to hydroxylated oligomer species that can polymerize to eumelanic pigments (d'Ischia and Prota, 1987; Lambert *et al.*, 1989). Thus, DHI and DHICA can contribute significantly to protect the skin from damaging UV radiation by quenching oxygen species and providing an additional amount of photoprotective pigment (Prota *et al.*, 1998a).

In contrast to 5,6-dihydroxyindoles, irradiation of 5-*S*-cysteinyldopa with UVB results in the formation of dopa, arising by photolytic cleavage of the S–CH_2 bond followed by desulfuration (Costantini *et al.*, 1994b; Land *et al.,* 1986). Thus, UV photolysis of 5-*S*-cysteinyldopa affords potentially toxic free radicals, capable of affecting important biological targets such as DNA (Chedekel and Zeize, 1988; Koch and Chedekel, 1986) or membrane lipids (Schmitz *et al.*, 1995). However, how much these photoprotective and phototoxic events are functionally important *in vivo* remains to be studied. The significance of these events should depend on the concentrations of these melanin precursors in epidermal tissues, information that is scarce at present.

Degradative Studies on Melanins

Eumelanins

Biosynthetic and degradative studies indicate that natural eumelanins are highly heterogeneous polymers consisting of various monomer units, including DHI unit **A**, DHICA unit **B**, and the pyrrole units **C** and **D** derived from peroxidative cleavage of units **A** and **B** (Fig. 15.12; Prota, 1992).

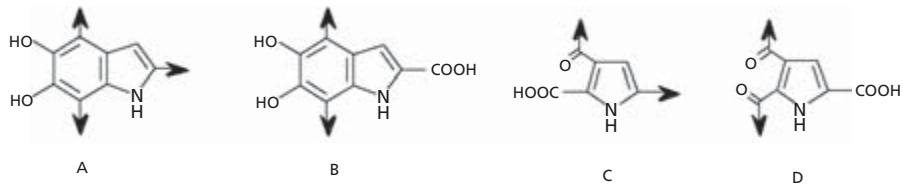
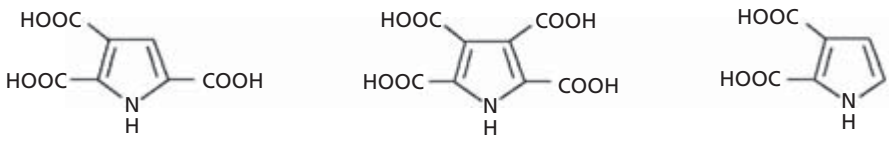


Fig. 15.12. Monomer units present in the eumelanin polymer. The carbon to nitrogen ratios are 8, 9, 6, and 7 for DHI unit (A), DHICA unit (B), pyrrole unit (C), and pyrrole-carboxylic acid unit (D) respectively. DHI and DHICA units may also be present in the oxidized, *ortho*-quinone form.



14 (PTCA) 15 16 (PDCA)

Fig. 15.13. Products of oxidation of eumelanins with acidic potassium permanganate or alkaline H_2O_2.

Extensive degradative studies provided a number of chemical degradative methods (Nicolaus, 1968; Swan and Waggott, 1970). Among them, oxidative degradation by permanganate or H_2O_2 appears to be most informative (Panizzi and Nicolaus, 1952; Piattelli and Nicolaus, 1961). Nicolaus (1968) repeated the oxidation of Sepia melanin with H_2O_2 at pH 7 followed by alkaline hydrolysis, which afforded a 6.5% yield of pyrrole-2,3,5-tricarboxylic acid (PTCA; **14** in Fig. 15.13). In another preparative experiment in which 10 g of Sepia melanin was oxidized by H_2O_2 in acetic acid, 200 mg of PTCA and 61 mg of pyrrole-2,3,4,5-tetracarboxylic acid (**15**) were isolated (Nicolaus, 1968; Piattelli *et al*., 1962). In our studies, permanganate oxidation of natural eumelanins gave about a 2% yield of PTCA (Ito and Fujita, 1985). We also noticed that permanganate oxidation gave a considerable amount of the tetracarboxylic acid **15** only when the oxidation was conducted under alkaline conditions (Ito and Fujita, 1985). This suggests that, during oxidative degradation carried out in an alkaline medium, the 3-position of DHICA unit **B** is connected to another monomer unit, thus giving rise to the artificial formation of the tetracarboxylic acid **15**. This represents an example of possible artifact formation during degradative reactions and calls for more attention to such possibilities.

The origin of PTCA was interpreted in terms of oxidative breakdown of the DHICA-derived structures (units **B** and **D**) in the eumelanin polymer. Another pyrrolic acid, pyrrole-2,3-dicarboxylic acid (PDCA, **16**), can be expected to arise from DHI-derived structures (units **A** and **C**). In fact, small amounts of this pyrrolic acid were detected among oxidation products of Sepia melanin, dopa melanin, and DHI melanin (Piattelli *et al*., 1962). The significance of its formation has recently been re-examined (Napolitano *et al*., 1995).

Other degradative reactions appeared to be much less informative. Alkaline fusion of Sepia melanin afforded both DHI and DHICA, but the yields were too low to make this reaction significant (Piattelli *et al*., 1963). Mild treatment of Sepia melanin with sodium borohydride in 0.1 M NaOH also afforded DHICA (d'Ischia *et al*., 1985). Again, the yield was very low. Some PTCA was also obtained by boiling Sepia melanin in 4% NaOH (Piattelli *et al*., 1962).

Carboxyl Content in Eumelanins

The carboxyl group attached to the indole or pyrrole ring (in units **B** or **D**) is labile and may easily be split off as CO_2 by heating melanin powder at a high temperature or by heating a melanin suspension in Vaseline (Piattelli *et al*., 1962) or in 6 M HCl (Ito, 1986). Our experience indicates that the acid decarboxylation is more reproducible and releases CO_2 quantitatively (Ito, 1986). This methodology has been applied successfully to estimate the content of carboxyl groups in natural and synthetic eumelanins (Ito, 1986; Novellino *et al*., 2000; Palumbo *et al*., 1988; Pezzella *et al*., 1997). Using decarboxylation, substantial amounts of carboxyl groups were found in all the natural eumelanins examined, suggesting the common presence of DHICA-derived units (Prota, 1992; Prota *et al*., 1998a).

Permanganate oxidation of eumelanins gives PTCA that arises from DHICA-derived structures (units **B** and **D**). Comparison of the yields of PTCA from a number of natural and synthetic eumelanins, coupled with results of the acid decarboxylation, indicates that units derived from DHICA comprise only 10% of synthetic dopa melanins, but as much as one-half of intact, natural eumelanins (Ito, 1986).

The first unambiguous demonstration of the involvement of DHICA in eumelanogenesis came from a study in which 1-^{14}C-dopa was injected into melanoma-bearing mice (Tsukamoto *et al*., 1992b). The pigment from the tumor was then isolated, purified, and chemically decarboxylated. Determination of the labeled CO_2 evolved showed that at least 20% of the precursor incorporated into the melanin retains the labile isotope in the form of DHICA-linked carboxyl groups.

In another approach to determine the carboxyl contents in melanins, Zeise and Chedekel (1992) used a titrimetric analysis to quantify the bioavailable carboxyl groups present on the surface of melanin particles. They found that the ratio of moderately acidic (–COOH) to weakly acidic (phenolic OH) groups was 0.86 in Sepia melanin. This method (Zeise and Chedekel, 1992) would be useful in comparing the functional groups present on the surface of various melanin pigments.

Pheomelanins

Biosynthetic and degradative studies carried out on pheomelanic pigments including trichochromes indicate that pheomelanins are also highly heterogeneous polymers arising from the oxidative polymerization of 1,4-benzothiazines derived from cysteinyldopas (Prota, 1992; Prota *et al*., 1998a).

Structural studies on pheomelanic pigments were greatly facilitated by reductive hydrolysis with hydriodic acid that yielded a number of informative degradation products. Although the conditions employed for the degradation were harsh, suggesting the possibility of artifact formation, the relatively high yield of degradation products makes the method indispensable in structural studies of pheomelanic pigments (Patil and Chedekel, 1984; Prota, 1992).

When heated in hydriodic acid, the decarboxy derivative of trichochrome C (**3**) gives as major products 4-amino-3-

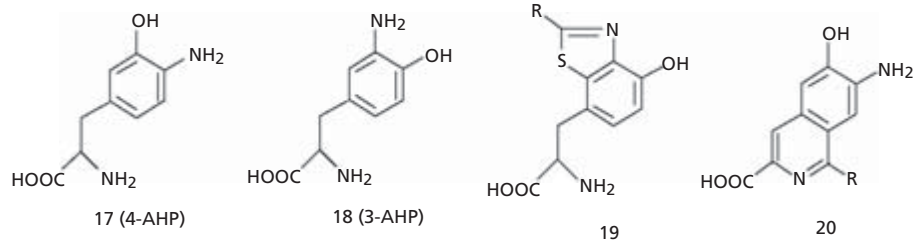


Fig. 15.14. Products of hydriodic acid hydrolysis of trichochromes and pheomelanins. R = H or CH_3.

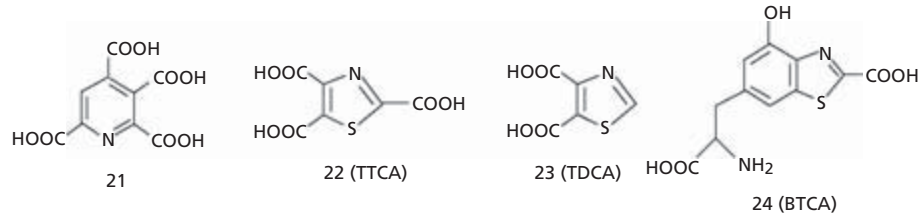


Fig. 15.15. Products of oxidation of pheomelanins with acidic potassium permanganate or alkaline H_2O_2.

hydroxyphenylalanine (4-AHP; **17**) and the benzothiazinone amino acid **12**, along with the benzothiazole amino acid **13** (R = CH_3) (Fig. 15.14; Nicolaus *et al.*, 1969). Trichochrome F (**4**), which occurs concomitantly at a much lower concentration than trichochrome C, gives 4-AHP along with a much smaller amount of the benzothiazole **13** (R = H) on hydriodic acid treatment (Prota *et al.*, 1969). On the other hand, degradation of trichochrome E (**6**) with hydriodic acid gives a mixture of two isomers, 4-AHP (**17**) and 3-AHP (**18**). The benzothiazole amino acids **12** (R = H and CH_3) can arise readily from trichochromes by ring contraction under acidic or alkaline conditions; heating the benzothiazinone **12** in hydrochloric acid affords the benzothiazole **13** (R = H) (Fattorusso *et al.*, 1968).

Gallopheomelanin-1, the major, protein-free, pheomelanic pigment, was isolated from red chicken feathers by alkaline extraction and chromatography on Sephadex (Minale *et al.*, 1967; Prota and Nicolaus, 1967). A most notable feature of gallopheomelanin-1 was its high sulfur content; it contained 9.0% nitrogen and 9.9% sulfur, the molar ratio of nitrogen to sulfur being 2.1:1 (Minale *et al.*, 1967).

On hydriodic acid hydrolysis, gallopheomelanin-1 affords the benzothiazole amino acids **13** and **19** (R = H and CH_3) along with a comparable amount of AHP isomers, 4-AHP (**17**) and 3-AHP (18), with benzothiazole (**19**) and 3-AHP (**18**) being minor isomers (Fattorusso *et al.*, 1968; Minale *et al.*, 1967). These characteristic products were obtained in similar yields (total yields of about 20%) from synthetic pheomelanins prepared either from a mixture of dopa and cysteine or from 5-*S*-cysteinyldopa (Fattorusso *et al.*, 1969a).

Difficulties in interpreting these results arise from the fact that these natural and synthetic pheomelanin preparations were subjected to strongly alkaline pH during the isolation procedure or chromatographic separation. This would suggest that a major part of the benzothiazole units in pheomelanins arise artificially from the benzothiazine units. In support of this view, our recent study has shown that the benzothiazoles **13** and **19** (R = H) were obtained in yields approximately 1/10th those of AHP isomers on hydriodic hydrolysis of both natural and biosynthetic pheomelanins that were not subjected to alkaline conditions (Ito, 1989). The production of AHP isomers occurs specifically at the expense of benzothiazine units, but not benzothiazole units (Fattorusso *et al.*, 1968; Ito, 1989). The reaction involves reductive fission of the aromatic C–S bond induced by the iodide anion.

Other interesting products of reductive degradation with hydriodic acid are the isoquinolines **20** (R = H and CH_3) (Fattorusso *et al.*, 1970). Also, permanganate oxidation of pheomelanins gives a product arising from the isoquinoline unit, pyridine-2,3,4,6-tetracarboxylic acid (**21**), in addition to thiazole-2,4,5-tricarboxylic acid (TTCA; **22**) and thiazole-4,5-tricarboxylic acid (TDCA; **23**), which arise from the benzothiazole units (Fig. 15.15; Fattorusso *et al.*, 1969b).

Red hair was recently found to be closely associated with loss-of-function mutations of the melanocortin-1 receptor (MC1R) (Valverde *et al.*, 1995). After that discovery, there has been a growing interest in red hair as a risk factor for melanoma and for nonmelanoma skin cancers (Rees, 2000). Napolitano *et al.* (2000b) recently described another marker of pheomelanins, i.e. 6-β-alanyl-2-carboxy-4-hydroxybenzothiazole (BTCA; **24**) in addition to TTCA (**22**), both of which are produced by alkaline H_2O_2 treatment of various hair samples. It is suggested that BTCA represents a new biogenetic marker for predicting individuals at high risk for skin cancer and melanoma.

Based on these data, a representative structure (**2**) can be proposed for pheomelanins (Ito, 1993a, 1998). The benzothiazine unit should constitute at least 40% of the monomer units, as hydriodic acid hydrolysis of trichochrome F gives AHP in about a 50% yield, whereas the yields are about 20% from pheomelanins (Ito, 1989; Ito and Fujita, 1985). The other monomer units, such as the isoquinoline and benzothiazole units, may represent only minor constituents. Some of the alanyl and cysteinyl side-chains may be degraded during

Table 15.3. Comparison of chemical and physical properties of eumelanins and pheomelanins.

Property	Eumelanins	Pheomelanins	Specificity
Color of tissue	Dark brown to black	Yellow to reddish brown	Low
Solubility	Insoluble in all solvents	Soluble in alkali	Low
Elemental composition	C, H, O, N (6–9%), S (0–1%)	C, H, O, N (8–11%), S (9–12%)	Low
IR spectrum	No characteristic bands	No characteristic bands	Low
UV-vis spectrum	General absorption	General absorption	Low*
NMR spectrum	Potentially useful	No data	?
EPR spectrum	Single peak	Two peaks	High
Chemical degradation	PTCA	AHP†	High

*Difference between eumelanins and pheomelanins can be used to differentiate them.
†Recently, 4-AHP has been introduced as a more specific marker for pheomelanins (Wakamatsu *et al.*, 2002b).

polymerization (Chedekel *et al.*, 1987; Deibel and Chedekel, 1984).

Chemical Analysis of Melanins

Spectrophotometric Analysis of Melanins: Historical Background

Regulation of melanogenesis has been the subject of extensive studies. In most such studies, quantitation of melanins in pigmented tissues such as hair and skin and in cultured melanocytes is essential. Effects of the genetic background on hair pigmentation in mammals can also be assessed by analysis of the quantity and quality of the melanins produced. Despite these needs, no simple laboratory methods to quantify melanins have been developed. Most laboratory tests to quantify biomolecules such as proteins, carbohydrates, lipids, and nucleic acids utilize specific reagents that develop a characteristic color with a given biomolecule in solution. On the contrary, a major problem in quantifying melanins appears to be how to solubilize the melanin pigment. Once solubilized, melanin can easily be assayed by absorbance in the visible absorption spectra.

Quantitation of melanins present in pigmented tissues is a challenge. By taking advantage of the insolubility of melanins in hydrochloric acid, Oikawa and Nakayasu (1973) reported a spectrophotometric assay of melanins based on solubilizing the deproteinized melanin in Soluene-100 (a 0.5 M solution of dimethyl-*n*-dodecyl-*n*-undecylammonium hydroxide in toluene). Eumelanins can be completely dispersed in Soluene-100 giving a solution that shows no light scattering (Oikawa and Nakayasu, 1975). If the melanin content is sufficiently high, the dry weight of black pigment remaining after HCl hydrolysis also serves the purpose of quantitation (Borovansky, 1978).

On the other hand, melanins present in cultured melanocytes can be solubilized in hot KOH or NaOH of about 1 M concentration, and the resulting solution is analyzed for absorbance between 400 to 500 nm (Whittaker, 1963). Treatments prior to the solubilization include precipitation of melanin (and protein) with perchloric acid or washing with a buffer followed by extraction of lipids with organic solvents. Such pretreatments can be omitted when Soluene-350 is used as a solvent to dissolve all the constituents of cells, including the melanin pigment (Kable and Parsons, 1989). Soluene-350, a tissue solubilizer widely used in liquid scintillation counting, has a higher capacity to dissolve tissue constituents and to retain water than Soluene-100.

Melanins can be oxidatively solubilized by heating in an alkaline, dilute H_2O_2 solution. The resulting solution has a characteristic fluorescence that can be used for quantitation of melanins (Rosenthal *et al.*, 1973). Although the authors claimed that the assay could be applied directly to tissues or cell cultures, it still required a lengthy pretreatment. In another interesting method, sodium borohydride, a mild reducing agent, was used to solubilize melanins (Das *et al.*, 1976), and this method was used to characterize neuromelanin isolated from the substantia nigra (Das *et al.*, 1978).

Comparison of Chemical and Physical Properties of Eumelanins and Pheomelanins

Because of the lack of adequate methods to isolate melanins from biochemical sources, their insolubility at neutral pH, and their structural heterogeneity, full characterization of melanins faces great obstacles. Table 15.3 compares various methods as to whether they can differentially characterize eumelanins and pheomelanins. As the physical analysis of melanins is dealt with in the following chapter, it is discussed here only briefly.

The color of tissue containing eumelanins is considered to be dark brown to black, whereas that containing pheomelanins is yellow to reddish brown. However, one cannot differentiate between dark brown and reddish brown with certainty. In fact, HPLC analysis of hair samples indicates that the visual differentiation is not reliable (Jimbow *et al.*, 1983). Differences in solubility are also not very specific (Prota *et al.*, 1976), as eumelanins appear to be slightly soluble in alkaline solutions, the property serving as a basis for the spectrophotometric assay of melanins (Watt *et al.*, 1981).

Elemental analysis of melanins is often considered unreliable, as repeated analyses of a given preparation of melanin give variable results. However, we have found that this is due to the presence of loosely bound water in the melanin samples;

melanins are hygroscopic, containing from 10% to 20% water (Ito, 1986; Zeise *et al.*, 1992). In addition, duplicate analyses of melanins from various sources showed satisfactory reproducibility (Dryja *et al.*, 1979). Differences in the elemental composition, especially in sulfur content, serve to distinguish pheomelanins from eumelanins. Theoretically, eumelanins contain no sulfur while pheomelanins possess a sulfur to nitrogen molar ratio of 1:2. Novellino *et al.* (1981) attributed the high content of sulfur in some melanins isolated from hair, feathers, and melanomas to copolymerization of the two types of melanin pigments (Prota, 1988). However, the possibility that at least some of the sulfur may arise as an artifact was suggested by the finding that as much as 1–2% of the sulfur was incorporated into melanins during isolation procedures under commonly used acidic conditions (Ito *et al.*, 1988b).

Elemental analysis is also of considerable value in characterizing eumelanins; the carbon to nitrogen molar ratio may be indicative of the extent of retention of the carboxyl group originally present in dopa and of the extent of oxidative cleavage of 5,6-indolequinone units (Ito, 1986) (Fig. 15.12). Chedekel *et al.* (1992) prepared Sepia melanin and tyrosine melanin in the form of K^+ salts under mild conditions. The elemental analyses of these preparations, corrected for amino acid contents, were $C_{7.67}H_{5.33}NO_{3.68}K_{0.18}$ for Sepia melanin and $C_{7.88}H_{4.53}NO_{3.78}K_{0.12}$ for tyrosine melanin. These analytical data indicate that at least 18% and 12%, respectively, of the monomer units contain carboxyl groups (in the form of –COOK), and considerable degrees of peroxidative cleavage of the *ortho*-quinone moiety have taken place.

Infrared absorption spectroscopy appears to be of little value in characterizing melanins (Wilczok *et al.*, 1984). Ultraviolet-visible absorption spectra of both types of melanins exhibit general absorption showing no distinctive absorption maxima. However, it should be pointed out that a solution of black hair melanin dissolved in Soluene-100 showed much higher absorbances at longer wavelengths than those of red hair melanin (Menon *et al.*, 1983). This would suggest a possible use of the absorption spectra to differentiate eumelanins from pheomelanins (see below).

Recently, solid-phase nuclear magnetic resonance (NMR) spectroscopy has been introduced to assess structural features surrounding carbon and nitrogen atoms using natural ^{13}C and ^{15}N as probes (Duff *et al.*, 1988; Hervé *et al.*, 1994). The methodology has thus far been applied to the analysis of synthetic and natural eumelanins; no application has been reported so far for the characterization of pheomelanins. Interestingly, Sepia melanin, partially dissolved in D_2O at pH 10, gives a surprisingly simple and well-resolved ^1H-NMR spectrum (Katritzky *et al.*, 2002). Thus, NMR would potentially be useful in characterizing both synthetic and natural eumelanins as well as pheomelanins.

Among the spectroscopic methods, electron paramagnetic resonance (EPR) spectroscopy has proved most successful in distinguishing between eumelanins and pheomelanins. Both melanins contain radical centers in their polymer matrices; eumelanins are characterized by the *O*-centered *ortho*-semiquinone radical (single peak in the EPR spectrum) and pheomelanins by the *N*-centered *ortho*-semiquinone-imine radical (two peaks). One can estimate the content of pheomelanin in mixtures or copolymers of eumelanins and pheomelanins (Sealy *et al.*, 1982a, b; Vsevolodov *et al.*, 1991).

Extensive studies carried out in Naples resulted in the detection of many degradation products, some of which are specific to one type of melanin. Notably, permanganate oxidation of eumelanins gave pyrrole-2,3,5-tricarboxylic acid (PTCA, **13**) (Nicolaus, 1968; Piattelli *et al.*, 1963), while hydriodic acid hydrolysis of pheomelanins yielded aminohydroxyphenylalanine isomers, 4-AHP (**16**) and 3-AHP (**17**) (Minale *et al.*, 1967; Prota, 1972; Thomson, 1974). We have developed a microanalytical method to quantitate eumelanins and pheomelanins in biological materials, based on HPLC analysis of these specific degradation products (Fig. 15.16; Ito and Fujita, 1985; Ito and Jimbow, 1983). The method is relatively simple and rapid and does not require the isolation of melanins from tissue samples. The details of this HPLC method are discussed below.

Quantitative Analysis of Eumelanins and Pheomelanins by Chemical Degradation

Previous methods for the quantitation of melanins in pigmented tissues required the isolation of melanins. Moreover, none of those methods was suitable for distinguishing between eumelanins and pheomelanins. In 1983, we introduced a rapid method for quantitatively analyzing eumelanins and pheomelanins in tissue samples, which makes the isolation of melanin pigments unnecessary (Ito and Jimbow, 1983). The method is based on the formation of PTCA (**14**) by permanganate oxidation of eumelanins and of AHP isomers (**17**, **18**) by hydriodic acid hydrolysis of pheomelanins respectively (Fig. 15.16). These specific degradation products are determined by HPLC; PTCA is quantified with UV detection while a mixture of AHP isomers is determined as a single peak with electrochemical detection. These degradation products were chosen because not only are they the major products from eumelanins and pheomelanins but they are also easily detected with high sensitivity. A similar approach using paper chromatography had been reported previously by Hackman and Goldberg (1971) and Novellino *et al.* (1981).

The original method was later improved to increase the sensitivity and to reduce the time for pre-HPLC work-up (Ito and Fujita, 1985). It should be noted that the alkaline 1 M K_2CO_3 medium for permanganate oxidation was replaced with acidic 1 M H_2SO_4. With this modification, the artificial formation of pyrrole-2,3,4,5-tetracarboxylic acid (**15**) could be avoided. The improved method requires only 5 mg or less of tissue samples or 10^6 cultured cells for each analysis. Recently, the conditions for permanganate oxidation were refined so that the PTCA values become more linearly correlated to the melanin contents (Ito and Wakamatsu, 1994). The yields of PTCA and AHP (4-AHP and 3-AHP combined) are approximately 2% from natural eumelanins and 20% from synthetic pheomelanins (Fig. 15.16), and the tissue contents of

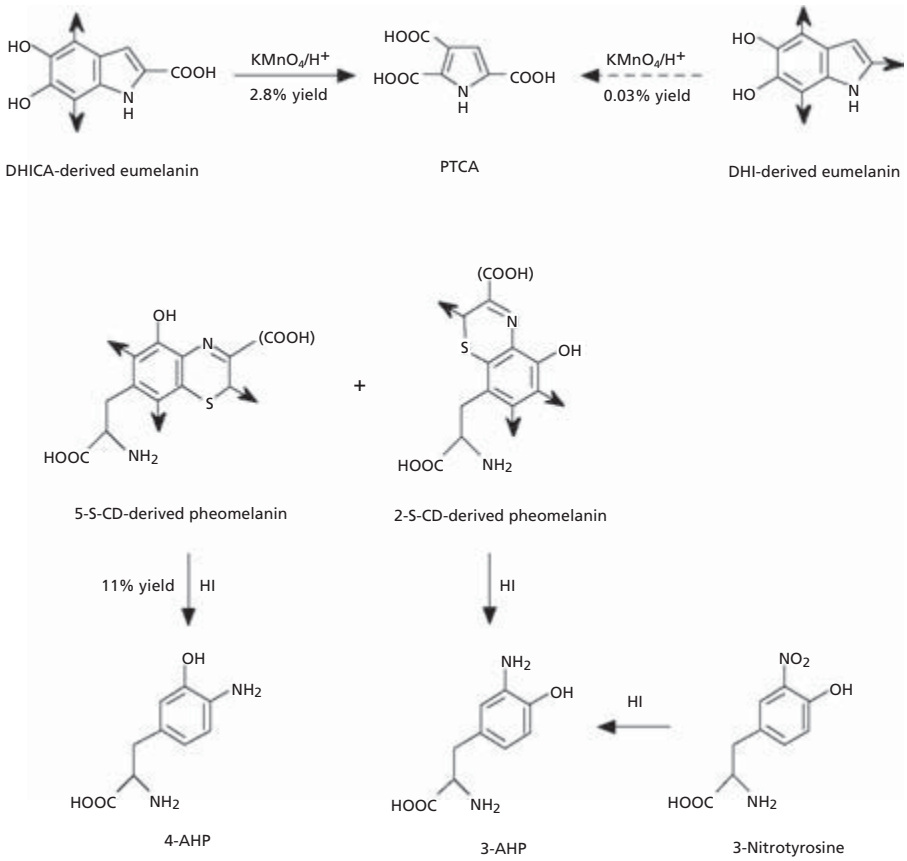


Fig. 15.16. Chemical degradation of eumelanins to form PTCA and of pheomelanins to form 4-AHP and 3-AHP. Note that the yield of PTCA from DHI-derived eumelanins is extremely low, compared with that from DHICA-derived eumelanin. PTCA is thus a specific degradation product of DHICA-derived eumelanin. Reductive hydrolysis of pheomelanins with hydriodic acid gives two isomers, 4-AHP and 3-AHP, arising from 5-*S*- and 2-*S*-cysteinyldopa-derived pheomelanins respectively. 3-AHP also arises from 3-nitrotyrosine-containing proteins (Wakamatsu and Ito, 2002).

eumelanins and pheomelanins can therefore be estimated by multiplying the PTCA and AHP contents by factors of 50 and 5 respectively (Ito and Fujita, 1985). Thus, a PTCA/AHP ratio of 0.1 indicates a mixed melanin consisting of equal amounts of eumelanins and pheomelanin.

Our HPLC method is relatively simple, fairly rapid, and highly sensitive. It has been applied for quantitatively analyzing eumelanins and pheomelanins not only in synthetic melanins, isolated melanosomes, hair, feathers, skin, nevi, and melanomas, but also in human epidermis and in cultured melanocytes (Ito, 1993b, 1998; Ito and Wakamatsu, 2003; Wakamatsu and Ito, 2002). A typical application of the method was to demonstrate that a synthetic analog of α-MSH, $Nle^4DPhe^7\alpha MSH$, induces significant increases in the eumelanin content of cultured human melanocytes (Hunt *et al.*, 1995). Several other laboratories have employed the same method in studies on melanin and melanogenesis (reviewed by Ito and Wakamatsu, 2003). As an example of one study using a similar method, pigments present in iris pigment epithelium of human eyes were found to be eumelanic (Prota *et al.*, 1998b).

The EPR and HPLC methods have been compared in estimating the contents of eumelanins and pheomelanins in hair samples from newborn sheep. The results showed that the EPR method correlates well with the HPLC method, although the former lacks the sensitivity of the latter with respect to the analysis of pheomelanins (Vsevolodov *et al.*, 1991).

The formation of PTCA has been interpreted in terms of the oxidative breakdown of indole units, either linked through the 2-position or bearing a carboxyl group at the same position (Napolitano *et al.*, 1995). Permanganate oxidation of DHI melanin and DHICA melanin in acidic medium afforded PTCA in 0.03% and 2.8% yields respectively (Fig. 15.16; Ozeki *et al.*, 1995; Ito, 1998). A similar result was also reported by Prota's group (Napolitano *et al.,* 1995; Novellino *et al.,* 2000). These results have confirmed our proposal that PTCA is a specific product arising from DHICA-derived structures (units **B** and **D**).

4-Amino-3-hydroxyphenylalanine (4-AHP) as a Specific Marker for Pheomelanins

In previous reports from our laboratory, the HPLC conditions used were such that 4-AHP (**16**) and 3-AHP (**17**) eluted in a single peak. This was based on the assumption that any natural pheomelanin pigment consists of a fixed ratio of 5-*S*-cysteinyldopa and 2-*S*-cysteinyldopa because the ratio of these cysteinyldopa isomers in biological materials is chemically controlled to be approximately 5:1 (Ito and Prota, 1977; Morishima *et al.*, 1983). In fact, the estimation of pheomelanin as the combined amount of 4-AHP and 3-AHP ("total AHP") did not impose any serious problem in most cases. However, small amounts of background values of total AHP were found even in hairs from tyrosinase-negative, albino mice (Lamoreux *et al.*, 2001; Ozeki *et al.*, 1995).

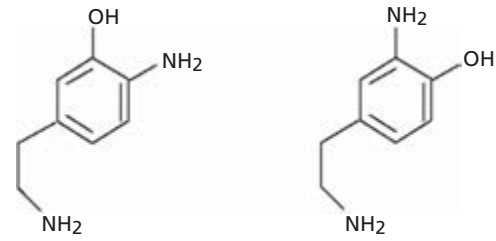
However, one problem with using total AHP as a marker was that background levels of AHP seemed to originate from precursors other than pheomelanin. Considerable and variable amounts of background 3-AHP are produced from other sources, most likely nitrotyrosine residues in proteins. The nitration of tyrosine appears to be a common biological phenomenon originating from nitric oxide.

Kolb *et al.* (1997) and Borges *et al.* (2001) described the separation of 4-AHP and 3-AHP in hydriodic hydrolysates of various tissue samples. However, their reported methods require ion-exchange chromatography prior to the HPLC separation, which is not only time-consuming but also very costly (because of using commercial, disposable ion-exchange columns). In order to overcome this problem, we developed HPLC conditions that enable the direct injection of the hydriodic acid hydrolysis products into the HPLC system allowing separation of 4-AHP and 3-AHP (Fig. 15.16). As the yield of 4-AHP from synthetic pheomelanin is 11%, the content of pheomelanin can be obtained by multiplying the content of 4-AHP by a factor of 9 (Wakamatsu *et al.*, 2002b). We are now using 4-AHP as a more specific marker of pheomelanins in subsequent studies (Naysmith *et al.*, 2004; Tadokoro *et al.*, 2003).

For clinical studies, it is often preferable to use serum or urine specimens to monitor the degree of pigmentation. We recently applied the specific pheomelanin assay method to analyze pheomelanin in the serum and urine from melanoma patients. Wakamatsu *et al.* (2003a) reported that serum levels of 4-AHP in metastatic melanoma patients were sevenfold higher than in control subjects, and they correlated well with serum levels of 5-*S*-cysteinyldopa. Similarly, Takasaki *et al.* (2003) showed significant correlations of 4-AHP and 3-AHP in melanoma urine with the urinary levels of 5-*S*-cysteinyldopa. These results suggest that the levels of 4-AHP in serum and urine could be used to monitor the production of pheomelanin in human skin.

Dopamine Melanin, Cysteinyldopamine Melanin, and Nonmelanocytic Melanins

Dark brown pigments, similar to eumelanins and pheomelanins, are also produced in cells other than melanocytes. For example, humans and primates produce neuromelanin in dopaminergic nigrostriatal neurons (Zecca *et al.*, 2001). Recently, Napolitano *et al.* (1995) showed that peroxide oxidation of DHI melanin in 1 M K_2CO_3 produces PDCA (**16**) in a yield (~0.5%) that is much higher than that produced by acidic permanganate oxidation. To characterize the diverse types of melanins, especially to identify dopamine-derived melanins, we have improved the alkaline H_2O_2 oxidation method of Napolitano *et al.* (1995) in terms of speed and sample size required (Ito and Wakamatsu, 1998). The results with peroxide oxidation show that: (1) PDCA, a specific marker of DHI units in eumelanins, is produced in yields 10 times higher than by acidic permanganate oxidation; (2) PTCA is produced in higher yields as well, but is also artificially produced from pheomelanins; and (3) the PDCA/PTCA ratio may be useful in characterizing eumelanins with various ratios of the monomers DHI and DHICA.



25 (4-AHPEA) 26 (3-AHPEA)

Fig. 15.17. Products of hydriodic acid hydrolysis of cysteinyldopamine-derived melanins.

Analogous to pheomelanins, hydriodic acid hydrolysis of cysteinyldopamine melanin produces a high (12%) yield of a 5:1 mixture of 4-amino-3-hydroxyphenylethylamine (4-AHPEA; **25**) and 3-amino-4-hydroxyphenylethylamine (3-AHPEA; **26**) (Fig. 15.17). 4-AHPEA may thus serve as a specific indicator of cysteinyldopamine-derived melanin (Wakamatsu *et al.*, 1991, 2003a).

It is generally accepted that neuromelanin is produced from dopamine (Zecca *et al.*, 2001). Cysteine may be incorporated into neuromelanin in a mechanism similar to pheomelanin production. However, our group and Rorsman's group reached opposite conclusions as to whether cysteine (via cysteinyldopamine) is actually incorporated (Carstam *et al.*, 1991; Odh *et al.*, 1994a). To solve this discrepancy, a more accurate method was developed to characterize neuromelanin chemically (Wakamatsu *et al.*, 2003b). We prepared synthetic models of neuromelanin by tyrosinase oxidation of dopamine and cysteine in various ratios (Wakamatsu *et al.*, 1991). Alkaline peroxide oxidation of these model neuromelanins produces thiazole carboxylic acids, TTCA (**21**) and TDCA (**22**), in addition to PDCA (**13**) and PTCA (**14**). We found that the yield of PDCA is relatively constant in synthetic melanins with various dopamine and cysteine ratios, whereas the yield of TTCA is higher than that of TDCA and is proportional to the sulfur to nitrogen ratio. It is concluded that the TTCA/PDCA ratio is a useful indicator of cysteinyldopamine-derived units in neuromelanin, and that neuromelanin consists mainly of dopamine melanin with some contribution from cysteinyldopamine melanin (Wakamatsu *et al.*, 2003b). Similarly, Odh *et al.* (1994a, b) used the TDCA/PTCA ratio as an indicator of the benzothiazine units in isolated neuromelanin and in pigment in cultured melanoma cells. These results also suggest that the same methodology should be useful for analyzing eumelanins and pheomelanins in various tissues. Melanin pigments may be characterized by contents of PTCA, PDCA, TTCA, TDCA, and the ratios among them. In particular, TTCA may serve as a specific marker of pheomelanins (Napolitano *et al.*, 2000b).

The dark pigment in butterfly wings is another example of a natural melanin whose chemical nature was mostly

unknown. We applied peroxide oxidation and hydriodic acid hydrolysis to follow the developmental increase in melanin in wings from *Precis coenia* and found that cysteinyldopamine melanin was formed first, followed by more eumelanic, dopamine melanin (Wakamatsu *et al.*, 1998).

Certain bacteria and fungi also produce insoluble, dark brown, melanin-like pigments. *Cryptococcus neoformans* is an opportunistic fungal pathogen that causes life-threatening infections in brains of about 10% of AIDS patients. We have applied the alkaline peroxide oxidation to analyze melanin pigments produced in *C. neoformans* (Williamson *et al.*, 1998). *C. neoformans* produces dark pigments on its cell wall when grown in media containing a diphenolic substrate, such as dopa or dopamine. We performed peroxide oxidation of *C. neoformans* cells grown on dopamine or dopa agar. Cells grown on dopamine agar gave a high ratio of PDCA/PTCA whereas cells grown on dopa agar gave a high ratio of PTCA/PDCA. These data provide direct chemical evidence for the formation of eumelanic pigments by oxidation of catecholic precursors by *C. neoformans* laccase.

Comparison of Permanganate Oxidation and Peroxide Oxidation

Our HPLC methods for assaying eumelanin and pheomelanin are highly sensitive and specific and possess many advantages, but also have certain disadvantages.

The acidic permanganate oxidation method that we have been using for quantitative analysis of eumelanin has a number of advantages (Table 15.4): (1) PTCA is formed primarily from DHICA-derived units in eumelanin, thus making PTCA a specific marker of DHICA content (Wakamatsu and Ito, 2002); and (2) PTCA is not artificially formed from pheomelanin. However, this method also has some disadvantages: (1) the yield of PDCA is too low to be used as a marker of DHI-derived units; and (2) the amount of PTCA formed gives a slightly concave calibration curve against the amount of melanin oxidized despite recent improvements (Ito and Wakamatsu, 1994).

The alkaline peroxide oxidation method has several advantages (Ito and Wakamatsu, 1998): (1) PDCA is an excellent indicator of DHI-derived units in eumelanin, and the PDCA/PTCA ratio is useful in characterizing various types of eumelanin, a ratio greater than 1 indicating dopamine melanin; (2) the calibration curves for PDCA and PTCA are linear; and (3) it is easier to perform than the permanganate oxidation. However, the alkaline peroxide oxidation method also has a certain disadvantage: yields of PTCA and PDCA from 5-*S*-cysteinyldopa melanin are abnormally high compared with those with permanganate oxidation. This indicates that indole units are formed artificially during the oxidation, because the postulated structure of 5-*S*-cysteinyldopa melanin does not contain an indole unit (Prota, 1992). Prota *et al.* (1995) and our group (Ito and Wakamatsu, 1998) also found abnormally high yields of PTCA and PDCA when *lethal yellow* and *recessive yellow* mouse hairs were subjected to alkaline peroxide oxidation. We therefore recommend that special care be taken when alkaline peroxide oxidation is used to analyze pigmented tissues containing pheomelanin.

Table 15.4. Advantages and disadvantages of the two oxidation methods.

Comparison	Acidic $KMnO_4$ oxidation	Alkaline H_2O_2 oxidation
PTCA	Specific for DHICA units	Not specific for eumelanins
PDCA	Little produced	Indicator of DHI units, but not specific for eumelanins
TTCA, TDCA	Produced, but hard to be extracted	Specific for CD-derived and Cys-DA-derived units
Method	Requires some skills	Easier to perform

Combined Use of Spectrophotometric and Degradative Analyses

Although the melanin assay based on chemical degradation and HPLC determination is relatively simple, it still requires an HPLC system with UV and electrochemical detectors. In addition, PTCA arises from DHICA-derived units but not from DHI-derived units. Therefore, we have developed a spectrophotometric method that is specific to eumelanins but does not discriminate between the DHI- and DHICA-derived units (Ito *et al.*, 1993). In this method, hair and melanoma samples are hydrolyzed in hot hydriodic acid to remove pheomelanic components, and the insoluble eumelanic pigments are subsequently solubilized in hot NaOH in the presence of H_2O_2 and analyzed for absorbance at 350 nm. Although much less sensitive, this spectrophotometric method can substitute for the PTCA method to measure eumelanin content when substantial amounts of samples are available.

Total amounts of melanin pigment in tissue samples can be calculated by the use of conversion factors of 50 and 9 for PTCA and 4-AHP respectively. However, the accuracy of this estimation rests on the assumptions that the DHICA/DHI ratios in different eumelanins from various sources are constant and that the 11% yield of 4-AHP from synthetic pheomelanins holds for different natural pheomelanins. The DHICA/DHI ratio appears to vary from one species to another.

These situations made it necessary to introduce a simple, reference method to estimate the total amounts of melanins, even if the method might not give accurate values. We have found that hot Soluene-350 (in the presence of 10–20% water) is able completely to dissolve mouse and human hairs and sheep wool. The resulting brown solutions are analyzed for absorbances at 500 nm (Ito *et al.*, 1996; Ozeki *et al.*, 1995, 1996a, b). Hair samples from different coat color phenotypes of mice and human hairs of various colors were analyzed with this spectrophotometric method. Excellent correlations were found between the absorbance at 500 nm (A_{500}) and the melanin con-

tents calculated from PTCA and AHP levels (Ozeki *et al.*, 1996b). This indicates that the A_{500} value can serve as an indicator of the total amount of eumelanin and pheomelanin combined, regardless of the type of melanin. Using A_{500} values, we obtained a conversion factor of 160 for calculating eumelanin content from the PTCA value in human hair (Ozeki *et al.*, 1996b; Wakamatsu and Ito, 2002). This high conversion factor suggests a low activity of Tyrp2 in humans compared with other species. Alaluf *et al.* (2001) recently reported that the HPLC method underestimates the melanin content in human epidermis by a factor of 3 compared with the spectrophotometric method. This discrepancy can be solved, however, using a conversion factor of 160 instead of 50.

The PTCA/total melanin (A_{500}) ratio appears to be a good indicator of the content of DHICA-derived units in eumelanins. The ratios were at similar levels in mouse *black*, *brown*, *dilute black*, and *pink-eyed black* hairs, whereas they were very low in pheomelanic hairs (Ozeki *et al.*, 1995). In contrast, the opposite holds for the AHP/total melanin ratios. The PTCA/total melanin ratio in *slaty* hair was only one-fifth that of the black counterpart, the result paralleling the decreased activity of Tyrp2 in the *slaty* mutation (Krompouzos *et al.*, 1994). It is interesting to note that black to brown hair eumelanins in humans contain low ratios of DHICA-derived units, comparable to the *slaty* mutation in mouse (Lamoreux *et al.*, 2001; Ozeki *et al.*, 1996a, b). Orlow *et al.* (1992) suggested that DHICA melanins are responsible for brown colors in the animal kingdom. However, our study using coat color mutants of mice indicates that the brown-type eumelanins differ from the black-type eumelanins in the degree of polymerization but not in the ratio of DHICA/DHI (Ozeki *et al.*, 1995, 1997b).

Pheomelanins are soluble in strong alkali solutions. By taking advantage of this property, we were able to solubilize pheomelanin in 8 M urea/1 M NaOH, although not completely, from the yellow hair of pheomelanic mice (Ozeki *et al.*, 1995). Under the same alkaline conditions, brown eumelanins from *brown*, *pink-eyed*, *black*, and *silver* mutants were slightly soluble. On the basis of the ratios of absorbance at 400 nm of the alkaline solution to total melanin (A_{500}), one can differentiate between pheomelanins, brown-type eumelanins, and black-type eumelanins, with the brown-type eumelanins being characterized by their partial solubility in strong alkali. Using a similar approach to characterizing melanins in human epidermis, Alaluf *et al.* (2002) showed that European skin contains as much as 40% alkali-soluble melanin compared with about 15% in African skin.

When classification of melanin pigments into eumelanins and pheomelanins is not a major concern, the solubilization of melanins in Soluene-350 (or NaOH or KOH, if soluble) appears to be the choice for the quantitation. Absorbance at 500 nm of the Soluene-350 solution (total melanin) can be used to quantify melanin contents in hair samples (Ito *et al.*, 1996; Ozeki *et al.*, 1995, 1996a, b) and in cultured melanocytes (Kable and Parsons, 1989) without any pretreatment. However, before it is applied to tissue samples such as melanomas, some refinements in pretreatment are required to remove hemoglobin and other interfering tissue components.

Eumelanins and pheomelanins in hair show significantly different ratios of absorbances at 650 nm to 500 nm when solubilized in Soluene-350. This difference has been used to develop a simple and rapid spectrophotometric method to distinguish eumelanins from pheomelanins, at least for qualitative purposes (Ito *et al.*, 1996; Ozeki *et al.*, 1996b).

Perspectives

The first part of this chapter described the chemistry of melanin pigments and related metabolites.

Two types of melanin production, eumelanogenesis and pheomelanogenesis, have been extensively studied. Most of the pathway at the monomer level has been clarified, using biosynthetic and pulse radiolysis approaches. Both approaches have produced valuable information and have been complementary to each other. The former approach has the strength of isolating various monomeric and oligomeric melanin intermediates, while the latter approach is able to follow rapid reactions involving dopaquinone that could not be studied otherwise.

Some unsolved problems in the chemistry of melanogenesis include: (1) the nature of post-polymerization modifications of eumelanins and pheomelanins; and (2) the nature of copolymerization of eumelanins and pheomelanins. Problem 1 has been addressed only sporadically (Crescenzi *et al.*, 1993; Deibel and Chedekel, 1984), and is also relevant in clarifying the biodegradation of melanins and melanosomes (Borovansky and Elleder, 2003). No study has examined in depth the mode of copolymerization of the two types of melanin pigments (problem 2).

The biological functions of melanin pigments are closely related to their structural features. Therefore, after the great progress in melanin chemistry over the past decade, more intimate cross-talk among specialists from chemistry, biochemistry, biophysics, cell biology, genetics, and dermatology is highly desirable in order to enjoy the fruits of growing chemical knowledge. One example of an unsolved problem in these multidisciplined areas is the mechanism of switching from eumelanogenesis to pheomelanogenesis (Fig. 15.3, 5), which appears to depend on the availability (rate and regulation of uptake) of cysteine in melanosomes.

The latter part of this chapter dealt with the methodology to determine the quantity and quality of melanins present in pigmented tissues, an area that has also enjoyed fruitful collaborative studies (Ito *et al.*, 2000; Ito and Wakamatsu, 2003).

Extensive degradative studies have provided a number of useful (or potentially useful) markers. These include the contents of PTCA and AHP, the PTCA/AHP ratio, the PTCA/total melanin ratio, the AHP/total melanin ratio, the PTCA/TDCA ratio, the PTCA/PDCA ratio, and the TTCA/PDCA ratio. The significance of PTCA, AHP, and their ratio in the study of melanogenesis has been well established (Ito, 1993b; Ito *et al.*,

2000). Recently, AHP ("total AHP") measurement has been replaced by a more specific 4-AHP measurement (Ito and Wakamatsu, 2003; Wakamatsu *et al.*, 2002b).

One problem in using PTCA and 4-AHP as markers of eumelanins and pheomelanins is that two different types of degradation are required for analyzing one sample. When alkaline peroxide oxidation is applied, TTCA and TDCA are formed specifically from pheomelanins, while PTCA and PDCA are derived from both types of melanin pigments (Ito and Wakamatsu, 1998; Wakamatsu and Ito, 2002). It is therefore expected that ratios such as the TTCA/PTCA ratio may be as useful as the 4-AHP/PTCA ratio in characterizing copolymers (or mixtures) of eumelanins and pheomelanins. For the characterization of neuromelanin, the PTCA/TDCA ratio has been used by Odh *et al.* (1994a, b) whereas the TTCA/PTCA ratio was used by Wakamatsu *et al.* (2003b). One problem in applying this approach is that TTCA and TDCA are difficult to extract in organic solvents and should thus be analyzed without purification and concentration (Wakamatsu *et al.*, 2003b). This would lead to a lower sensitivity and specificity, unless melanin pigments are purified prior to the oxidation. Alkaline peroxide oxidation also produces BTCA (**24**) as a specific marker of pheomelanins (Napolitano *et al.*, 2000). How much this marker is useful in pigment research remains to be explored.

In the study of eumelanogenesis, the PTCA/PDCA ratio, analyzed by alkaline peroxide oxidation, may become a useful substitute for the PTCA/total melanin ratio as a marker to estimate the DHICA/DHI ratio in eumelanins. At present, only PDCA is available as an indicator specific for the DHI-derived units in eumelanins. A major problem with the alkaline peroxide oxidation is the artificially high yield of PTCA from pheomelanins present in the hair of genetically pheomelanic mice (Ito and Wakamatsu, 1998; Prota *et al.*, 1995).

The EPR method appears to be highly sensitive and specific in detecting melanin pigments (Enochs *et al.*, 1993). It may also be able to differentiate between eumelanins and pheomelanins (Sealy *et al.*, 1982a, b). Which of the two methods, the EPR method or the HPLC method, is more sensitive and specific has not been fully determined (Vsevolodov *et al.*, 1991), although some groups claim that the HPLC method is not sufficiently sensitive or specific (Sarna *et al.*, 2003). However, it should be stressed that the HPLC method to detect 4-AHP as the measure of pheomelanins is highly specific and sensitive.

Acknowledgments

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