This student paper was written as an assignment in the graduate course

Free Radicals in Biology and Medicine

(77:222, Spring 2003)

offered by the

Free Radical and Radiation Biology Program
B-180 Med Labs
The University of Iowa
Iowa City, IA 52242-1181
Spring 2003 Term

Instructors:
GARRY R. BUETTNER, Ph.D.
LARRY W. OBERLEY, Ph.D.

with guest lectures from:
Drs. Freya Q . Schafer, Douglas R. Spitz, and Frederick E. Domann

The Fine Print:

Because this is a paper written by a beginning student as an assignment, there are no guarantees that everything is absolutely correct and accurate.

In view of the possibility of human error or changes in our knowledge due to continued research, neither the author nor The University of Iowa nor any other party who has been involved in the preparation or publication of this work warrants that the information contained herein is in every respect accurate or complete, and they are not responsible for any errors or omissions or for the results obtained from the use of such information. Readers are encouraged to confirm the information contained herein with other sources.

Melanin: The Pigmented Truth

By

Carolyn J. Fisher

B-180 Medical Laboratories
Free Radical and Radiation Biology Program
The University of Iowa
Iowa City, IA 52242-1181

For 77:222, Spring 2003

13. March 2003

Abbreviations:
DC, Dopachrome
DHI, dihydroxyindoles
DHICA, 5,6-dihydroxylindole-2-carboxylic acid
DOPA, 3,4-dihydrophenylalinine
DQ, DOPA quinone
EPR, electron paramagnetic resonance
RPE, retinal pigmented epithelium
UV, ultraviolet

Table of Contents:

Abstract:

Natural melanin, a pigment produced in melanocytes, does not have a unique structure but comprises a class of conjugated polymers that are commonly referred to collectively as melanins. Melanins (eumelanins and pheomelanins) are synthesized from a two-electron oxidation process involving tyrosine catalyzed by the enzyme tyrosinase. They contain both *in vitro* and *in vivo* free radicals that can interact with hydroxyl radicals, singlet oxygen and superoxide. In humans, melanins are thought to play an important role in photoprotection based on their radical scavenging abilities, especially true of eumelanin. On the other hand, they can also be degraded by oxidations induced by superoxide to produce reactive species such as semiquinones. Since all known melanins contain stable organic free radicals, they can easily be detected by electron paramagnetic resonance spectroscopy. Continued research into the interactions and detection of melanins will advance the studies forward to aid in the determination of their true relevancy as physiologically important cellular protectors.

Introduction:

Melanins are naturally occurring pigments found in animals and plants. They are complex heterogeneous polymers whose chemical structure has not been satisfactorily determined [1-2]. There are two major classes of natural melanins, the black-brown eumelanin found in human black hair and in the retina of the eye and the yellow-red pheomelanin found in red hair and red feathers [1,3]. Two biological functions of melanins have been identified. First, they have been shown to increase optical efficiency of the eye, and secondly, they are responsible for the production of color patterns in hair and superficial epidermis [1].

Melanin synthesis or melanogenesis occurs *via* enzymatic and non enzymatic pathways [1, 4]. During melanin synthesis and polymerization, unpaired electrons are left over from the process and in this respect melanin can be thought of as a free radical [4]. Research has shown that melanins may serves as antioxidants in various ways. For instance, in skin, high melanin levels contributes to protection from melanoma where it serves to absorb ultraviolet (UV) light. Melanins also bind transition metals preventing fenton type reactions and the production of the highly reactive hydroxyl radical ($HO^{\bullet}$) [4]. On the other hand, illumination of eumelanins generates $O_2^{\bullet-}$ that is quickly scavenged, but $O_2^{\bullet-}$ can also reduce melanin intermediates such as quinones to semiquinones (free radicals) as well as oxidizing semiquinones [4]. It is not clear whether melanins are established antioxidants or pro-oxidants in the cell. This review will focus on melanogenesis, antioxidant and pro-oxidant properties and detection of melanins.

Melanogenesis:

Melanogenesis is the production of color pigments eumelanin and pheomelanin in melanocytes. Raper in the 1920's laid the ground work that was later extended by Mason for understanding the mechanisms of tyrosine conversion into melanin by the actions of the enzyme

tyrosinase [1]. The most significant outcome of their work was the derivation of the Raper-Mason scheme of melanogenesis (Figure 1). The two initial steps involve the tyrosine-mediated hydroxylation of tyrosine to 3, 4-dihydrophenyalanine (DOPA), and the oxidation of DOPA to Dopaquinone (DQ). It has been presumed that the backwards reactions from DOPA and Dopaquinone are so small compared to the forward reactions that they can be neglected.

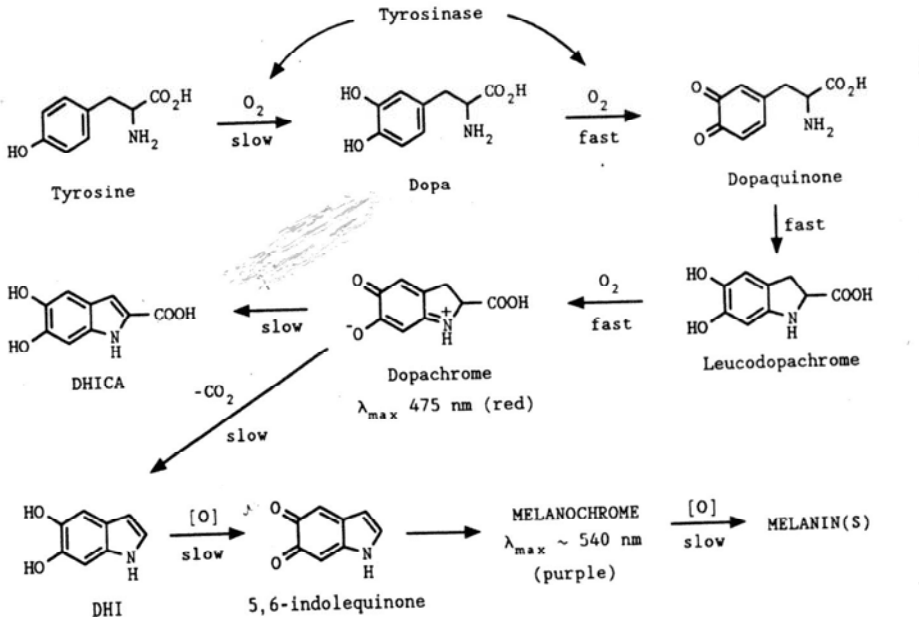

Figure 1. The Raper-Mason scheme of melanogenesis. Adapted from [1].

The Raper-Mason scheme has been further updated as ongoing studies developed new elucidations into the metabolic pathways of melanin synthesis. An updated comprehensive scheme (Figure 2) depicts the two major pathways of synthesis that are divided into eumelanin and pheomelanin pathways.

The major steps in eumelanin formation are the cyclization of dopaquinone to leucodopachrome which is immediately oxidized to form dopachrome (DC). DC is a relatively stable intermediate with a half-life of approximately 30 minutes. After which it is tautomerically rearranged to form 5,6-dihydroxylindole-2-carboxylic acid (DHICA). DC may also

spontaneously decarboxylate to 5,6-dihydroxyindole (DHI) which will rapidly oxidize to form indole 5,6-quinone. The final step is the polymerization to eumelanin (Figures 1, 2).

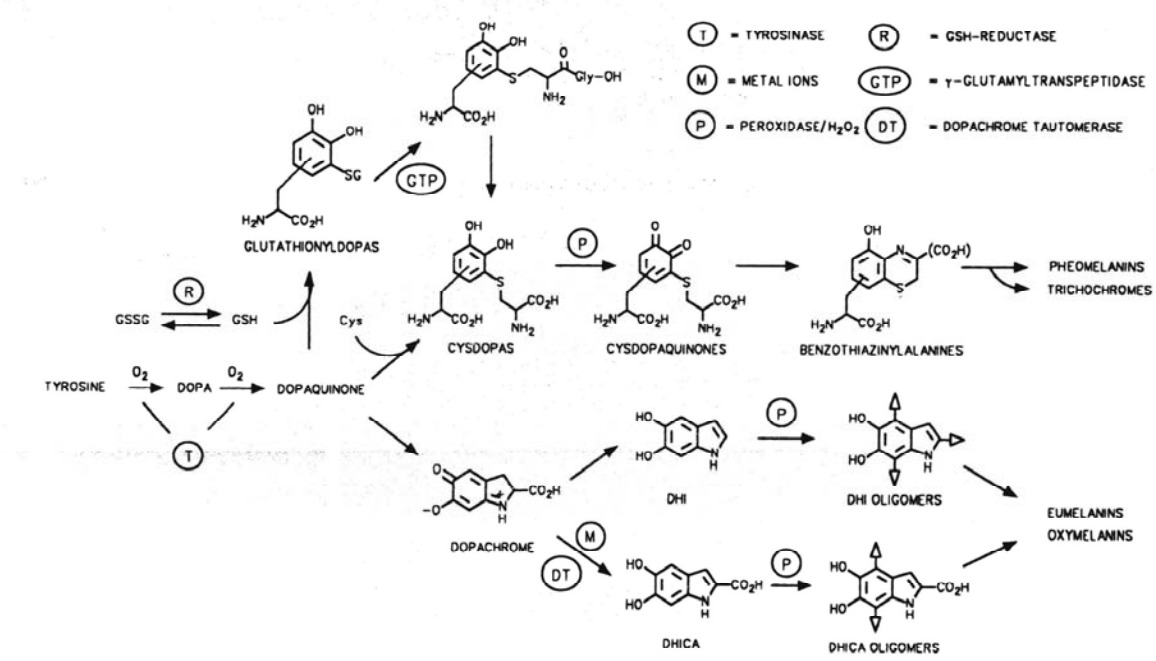

Figure 2. Comprehensive scheme of metabolic pathways leading to melanins and related metabolites. Adapted from [1].

The pheomelanin pathway is initiated by the addition of thiols such as glutathione and cysteine to DQ [1,5]. The glutathionyldopa is rapidly converted to cysteinyldopa by γ-glutamyl transpeptidase (GTP). Oxidation of cysteinyldopa to cyclic quinine-imine intermediates that rearrange into benzothiazine derivatives occur. The benzothiazine derivatives rearrange into pheomelanin (Figure 2). The exact mechanisms that occur in the pheomelanin pathway in the latter conversions are not clearly understood.

Antioxidant and Pro-oxidant Properties:

a. Melanin as an Antioxidant

An important physiological function of melanin is related to its ability to protect against photochemical stress as evidenced by its increased generation in response to UV damage [6, 7].

The chemical basis for melanin's reactivity is believed to be due to the quinol/quinone redox transformation of the DHI form which undergoes two-electron oxidation to the corresponding quinone form (Figure 3) [5]. The DHI form can act as a superoxide dismutase by catalyzing the disproportionation of $O_2^{\bullet -}$ to hydrogen peroxide (H_2O_2) and oxygen (O_2) [6]. Experimentally it has been shown that normal melanocytes exposed to oxidative stress vary in their extracellular buildup of H_2O_2 that was proportional to their initial melanin content. This provided a means for the melanin rich melanocytes to inhibit the initial buildup of H_2O_2. In malignant melanocytes (melanomas), there was a decreased ability for them to neutralize the H_2O_2 indicating a protective effect of melanin.

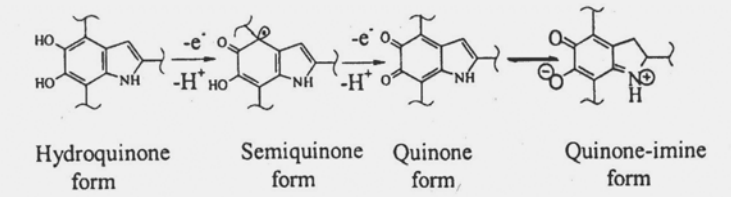

Figure 3. Speciation of monomers within DHI melanin. Adapted from [7].

b. Melanin as a Prooxidant:

The melanocyte whose primary function is to deliver melanin to keratinocytes is under continuous low grade oxidative insult [6]. Within the melanocyte, the synthesis of melanin also results in the production of H_2O_2 that when inefficiently reduced by antioxidant enzymes (catalase, glutathione peroxidase) could lead to deleterious pathological conditions such as cancer. Excess H_2O_2 decomposes melanin in a process known as bleaching *in vitro* and induces higher tyrosinase levels *in vivo* [5, 6].

Melanins also absorb cationic metal ions such as iron and copper *in vivo* that can dramatically affect the redox state of the polymer by promoting the production of the highly reactive $HO^{\bullet}$ in a Fenton Type reaction.

Detection Method:

a. Electron Spin Resonance (ESR)

All known melanins contain free radicals that can be detected by EPR and melanin is the only known biopolymer with a relatively high concentration of free radical centers *in vivo* and *in vitro*. The highly characteristic melanin EPR signal, a single structure-less slightly asymmetric line, is depicted in Figure 4 and is based on results generated in a study concerning the loss of retinal pigmented epithelium (RPE) melanin in human eyes with aging. Signals A, B and C are from 1 mg mL^{-1} of synthetic DOPA melanin (standard), pooled human RPE cell extract and melanosomes from RPE pooled samples, respectively. The similarity of the EPR spectra from the three different sources off melanin rule out artifacts in the detection method.

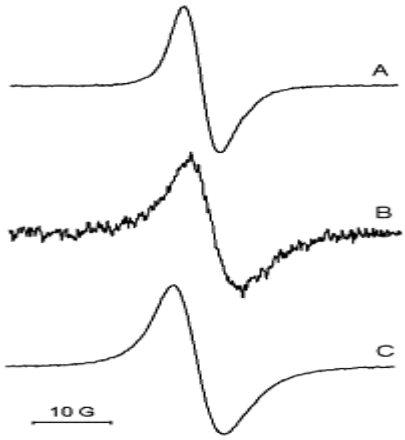

Figure 4. ESR spectra obtained for 1 mg mL^{-1} synthetic DOPA melanin (A), human RPE cell extracts (B) and human melanosomes (C). Adapted from [8].

The amount of melanin was determined by an ESR assay ($h\nu = 2\Delta E = g\mu_B B$) where the spectra of acidified (pH 1) melanin samples are measured at liquid nitrogen temperatures in the g = 2 region using a Bruker ESP 300E ESR spectrometer operating in the X band and equipped with 100 kHz field modulation. The settings were microwave power 20 μW, modulation

amplitude 2.0 G and scan range 50 G. The acidic pH is used to release bound-to-melanin paramagnetic and diamagnetic metal ions thereby minimizing their effects on the EPR signal.

To illustrate that the EPR method is a reliable quantitative measure for melanins, researchers establish a linear relationship between melanosome number and melanin in the particular vehicle that is being tested.

Conclusion:

Melanin's redox activity has been linked to both pro and antioxidant reactions with significant medical implications [7]. In pigmented cells, melanins act as powerful antioxidants in the sequestration of metal ions and in serving as radical sinks for superoxide for which eumelanin is more efficient [3-4]. In humans melanin is considered an efficient photoprotector as reported in cases of skin cancer (melanoma) and retinal damage [1]. Melanins also have the ability to serve as a source of damaging radical intermediates as illumination of pheomelanins generates superoxide that is able to reduce melanin quinones to semiquinones and oxidize hydroquinones to semiquinones [4]. It is still controversial whether melanins that are produced *in vivo* are physiologically important antioxidants. Nevertheless, evidence suggests that melanins serve as photoprotectors in humans.

References:

1. Prota G. (1992) *Melanins and Melanogenesis.* San Diego, CA: Academic Press.

2. Prota G. (2000) Melanins, melanogenesis and melanocytes: looking at their functional significance from the chemist's viewpoint. *Pigment Cell Res.* **13**:283-293.

3. Rozanowska M, Sarna T, Land EJ. Truscot TG. (1999) Free radical scavenging properties of melanin interaction of eu- and pheomelanin models with reducing and oxidizing radicals. *Free Radic Biol Med.* **26**:518-525.

4. Halliwell B, Gutteridge JMC. (1999) *Free Radicals in Biology and Medicine* 3rd ed. New York: Oxford University Press. pp. 198-201.

5. Oyehaug L, Plahte E, Vage DI, Omholt SW. (2002) The regulatory basis of melanogenic switching. *J Theor Biol.* **215**:449-468.

6. Meyskens FL, Farmer P, Fruehauf JP. (2001) Redox regulation in human melanocytes and melanoma. *Pigment Cell Res.* **14**:148-154.

7. Gidanian S, Farmer PJ. (2002) Redox behavior of melanins: direct electrochemistry of dihydroxyindole-melanin and its Cu and Zn adducts. *J Inorganic Biochem.* **89**:54-69.

8. Sarna T, Burke JM, Korytowski W, Rozanowska M, Skumutz CMB, Zareba A, Zareha M. (2002) Loss of melanin from human RPE with aging: possible role of melanin photooxidation. *Exp Eye Res*. **76**:89-98.