Melanogenesis using tyrosinate (not tyrosinase).

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Abstract We present our initial observations regarding the effect of the presence of L-tyrosinate (= L-tyrosine disodium salt) on the auto- or Fe²⁺/H₂O₂-mediated oxidation of various catecholic substances into melanin-like pigments. We observed that L-tyrosinate inhibited the Fe²⁺/H₂O₂-mediated oxidation. In contrast, L-tyrosinate promoted the auto-oxidation of ortho-diphenols like L-DOPA, dopamine, epinephrine, norepinephrine, catechol or pyrogallol, but not a meta-diphenol like resorcinol. In addition, we briefly demonstrated the melanogenic properties of cell culture media containing L-tyrosinate. The reactions were monitored using UV-Vis spectroscopy and size exclusion chromatography. For a reaction between L-tyrosinate and L-DOPA, a large scale experiment was set up allowing us to isolate, purify and characterize using FT-IR spectroscopy the melanin-like material obtained. We discuss our observations in the context of the *in vitro* and *in vivo* study of melanogenesis and provide some directions for future research efforts.

Keywords: melanin; tyrosine; tyrosinate; DOPA; catecholamines; catechol; pyrogallol; resorcinol

1. Introduction

Melanins (MNs) are a heterogeneous and ubiquitous class of pigments found in all kingdoms of life.[1, 2] MN-like pigments can be generated from a broad variety of precursors and different biosynthetic pathways can lead to the formation of the darkly-colored pigment.[2] Because of this, MNs can not be defined by a precise chemical structure. D'Ischia et al. have proposed a standardization of the nomenclature and described MNs as “pigments of diverse structure and origin derived by the oxidation and polymerization of tyrosine in animals or phenolic compounds in lower organisms”.[3] The main MN pigments found in mammalian cells are eumelanin and pheomelanin. Eumelanin is generated through the oxidation and polymerization of L-tyrosine ((**1**) in Figure 1) and has a dark brown to black color. Pheomelanin is generated through the oxidation and polymerization of (**1**) in the presence of L-cysteine or other thiol-containing biomolecules and is typically reddish in color.[2] In the epidermis, MNs are present in melanocytes, stored in specialized organelles, the melanosome.[4] The formation of melanosomes is a complex, multi-stage process.[5] The biosynthesis and enzymology of MNs in melanocytes has been extensively studied and reviewed.[6] In this context the enzyme tyrosinase (TYR) and tyrosinase-related proteins (TYRP-1 and -2) have received a lot of attention.[7] The transformation from (**1**) into eumelanin involves multiple steps and many intermediates like L-DOPA ((**2**) in Figure 1), dopaquinone (DQ), cyclodopa (CD), dopachrome (DC), 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA). This process of melanogenesis is often presented as shown in Scheme 1 based upon the so-called Raper-Mason pathway.[2, 8-10]

Scheme 1: Proposed sequence of reactions converting L-tyrosine (**(1)**) into eumelanin. In this scheme (**(2)**) = L-DOPA, DQ = dopaquinone, CD = cyclodopa, DC = dopachrome, DHI = 5,6-dihydroxyindole (DHI) and DHICA = 5,6-dihydroxyindole-2-carboxylic acid. Adapted from [8].

Early studies of TYR suggested that the first step during melanogenesis involves the formation of (**(2)**) from (**(1)**).[11] However, it is now generally accepted that the formation of DQ is the first step and that the redox exchanges between DQ and other intermediates of the melanogenesis process are the sources of (**(2)**) during the enzymatic action of TYR; with (**(2)**) then serving as an additional substrate for TYR (see Scheme 1).[8, 12-14] Other enzymes, e.g., TYRP-1, may be involved in the melanogenesis process, although the enzymatic activity of e.g., TYRP-1 has been described as “enigmatic”.[7] The *in vitro* evaluation of the enzymology of the melanogenesis process is complicated by the fact that TYR obtained from mushrooms is sometimes used[15] and TYR from lower organisms may differ from mammalian TYR. In addition, TYR obtained from different mammalian species, e.g., mouse vs. human, appears to display different enzymatic activities.[7, 16] Throughout the history of the literature regarding the action of TYR on (**(1)**), observations have been presented involving the presence of a “lag phase” in the reaction of the enzyme on (**(1)**) and the fact that the addition of (**(2)**) to the TYR/(**(1)**) combination can reduce the length of this lag phase. In addition, multiple observations indicate that some steps in the process of melanogenesis as shown in Scheme 1 can occur without the need of any enzymes.[8, 11-13, 17-19]

As (**(1)**) is the natural substrate for TYR and similar enzymes, it is logical to use (**(1)**) for studies focused on the enzymology involved in the process of melanogenesis. Commercially, (**(1)**) is available in two forms shown as (**(1A)**) and (**(1B)**) in Figure 1. Tyrosinate, the disodium salt of (**(1)**) ((**(1B)**) in Figure 1), has a higher solubility in water than (**(1A)**) and provides the phenolate form of (**(1)**). The structure and associated function of human TYR and related proteins was recently reviewed.[7] Of these human proteins, a crystal structure is available only for TYRP-1, but sufficient homology exists between TYRP-1 and TYR or TYRP-2 that the structure of TYRP-1 can serve as a model for the other proteins. It appears that TYR and related proteins bind their monophenolic substrate in the unprotonated, phenolate form; deprotonation being mediated by a water molecule bridged between two metal ions inside the enzyme.[16, 20] A similar mechanism of substrate binding was described for insect phenoloxidase[21] and catechol oxidase[22]. Thus, (**(1B)**) was used for all the studies described in this report and (**(1B)**) was also used in an earlier set of experiments.[23] In this earlier report we detailed the $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ -mediated oxidation of a variety of catecholic precursors into MN-like pigments and showed that (**(1B)**) did not undergo auto-oxidation nor was it susceptible to $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ -mediated oxidation.[23] We initiated a set of experiments to evaluate whether (**(1B)**) would inhibit the $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ -mediated oxidation of catecholic compounds and the results of these initial observations are presented in this report. However, in the process of these experiments we observed an unexpected pro-oxidizing effect of (**(1B)**) on the auto-oxidation of many catecholic compounds. Thus, in addition to the effect of (**(1B)**) on the $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ -mediated oxidation of a broad variety of precursors, we studied the effect of the presence of (**(1B)**) on the auto-oxidation of these catecholic compounds. Figure 1 presents the chemical structures of the compounds discussed in this report. In addition, as (**(1B)**) is a common ingredient in many cell culture media, we briefly explored the interaction between two commonly used cell culture media and select catecholic compounds.

Figure 1: Chemical structures of the compounds discussed in this report: **(1A)** = L-tyrosine, **(1B)** = L-tyrosine, disodium salt (= tyrosinate), **(2)** = L-DOPA, **(3)** = dopamine, **(4)** = norepinephrine, **(5)** = epinephrine, **(6)** = catechol, **(7)** = pyrogallol and **(8)** = resorcinol

2. Materials and Methods

2.1. Materials

L-tyrosine, L-Tyrosinate (L-tyrosine disodium salt), L-DOPA, dopamine.HCl, norepinephrine.HCl, epinephrine.HCl, catechol, pyrogallol and resorcinol were obtained from Sigma-Aldrich (St Louis, MO). FeCl₂.2H₂O, RPMI-1640 and DMEM cell culture media were obtained from Fisher Scientific. H₂O₂ solution at 3% (v/v) was obtained from Kroger Co (Cincinnati, OH).

2.2. UV_Vis spectroscopy

UV/Vis spectroscopic measurements were made in wells of a 96-well microplate using the SynergyHT microplate reader from Biotek (Winooski, VT).

2.3. Size exclusion chromatography (SEC)

SEC analyses were performed as described elsewhere.[24]

2.4. FT-IR spectroscopy

FT-IR spectroscopic scans were made as described elsewhere.[24]

2.5. Dialysis and lyophilization

Dialysis and lyophilization were performed as described elsewhere.[24]

3. Experimental results

3.1. Preliminary observations involving L-tyrosinate and L-DOPA, dopamine, epinephrine or norepinephrine.

Equimolar amounts (5mM) of (1B) and (2), (3), (4) or (5) were mixed in 100mM acetate buffer pH=6.5 at RT. In the absence of any (1B), the mixtures containing (2), (3), (4) or (5) exhibited minimal auto-oxidation as determined from visual observations and SEC analyses (results not shown). When mixed with (1B), changes in color appeared rapidly (within minutes) for (2) or (3) and more slowly (within hours) for (4) or (5). After overnight reaction, mixtures containing (1B) and (2) or (3) were black in outlook without any precipitations. Mixtures containing (1B) and (4) or (5) were dark-brown or golden in outlook respectively without any precipitations. In the following days, these colors appeared to be darkening upon standing at RT. Figure 2 presents a photograph of the reaction mixtures involving (1B) and (3), (4) or (5) after one week standing at RT.

Figure 2: Photograph of mixtures containing equimolar amounts of (1B) and, from left to right, (3), (4) or (5) in 100mM acetate buffer (pH=6.5) after one week at room temperature.

Figure 3, panel A, presents the SEC profiles of tenfold diluted aliquots of the mixtures shown in Figure 2, while Figure 3, panel B, presents the UV-Vis spectra of the peak with retention time around 13 minutes of the SEC profiles shown in Figure 3, panel A.

Figure 3: SEC profiles (panel A) of tenfold diluted aliquots from the reaction mixtures shown in Figure 2 and UV-Vis spectra (panel B) of the peak with retention time of about 13 minutes.

The SEC profiles presented in Figure 3, panel A, are similar to the profiles presented in all our previous reports on the synthesis of MN-like materials.[23, 25-32] As discussed in these reports, the lower limit of exclusion in our SEC analyses is between 14 and 15 minutes. Thus, the peaks within the SEC profiles with a retention time around 13 minutes represent oligo- or polymeric molecules that are not present at the start of the reaction or when (3), (4) or (5) are left at RT without any (1B). The peak with retention time around 13 minutes typically represents the MN-like material generated in our experiments. The UV-Vis spectra of the MN-like material shown in Figure 3, panel B,

indicate the presence of multiple absorbance maxima, particularly in the cases of (4) or (5), and are a major departure from the UV-Vis profiles obtained from SEC analyses of MN-like materials generated from (2).[26]

In general, these preliminary experiments indicated that the presence of (1B) promoted the air-mediated oxidation of (3), (4) and (5) leading to the formation of darkly- or golden-colored mixtures.

3.2. Preliminary observations involving cell culture media

Select compounds were incubated with two types of cell culture media that contain (1B): RPMI 1640 and DMEM (with glucose). When between 1 and 2mM test compounds like (2), (3), (5), (6) or (7) were mixed with both types of cell culture media (threefold diluted), darkening of the mixtures could be observed over the course of a few days when kept at RT. Figure 4 presents Vis spectra of mixtures containing (7), RPMI 1640 medium and (7) mixed with RPMI 1640 medium after five days standing at RT.

Figure 4: Vis spectra of (7) in water (1mM), RPMI 1640 medium (threefold diluted) and (7) (1mM) mixed with RPMI 1640 medium (three fold diluted) after five days standing at RT.

Apart from the absorbance maximum around 550nm because of the presence of the indicator, cell culture medium does not exhibit any absorbance in the visible region. Under the conditions tested, (7) appeared to have undergone a minimal amount of auto-oxidation as exhibited by the slightly increased absorbance between 400 and 500nm. The mixture containing RPMI 1640 medium and (7) turned very dark and the Vis spectrum of this mixture exhibited strongly enhanced absorbance over the entire visible range of the electromagnetic spectrum. This effect of RPMI 1640 or DMEM media was not limited to (7). It was also observed for other compounds like (2), (3), (5) or (6), although there were notable differences in color intensities (from gold to black and anything in between). Figure 5 presents photographs taken from mixtures containing 10mL water, RPMI 1640 or DMEM media and 50mg (2) or (7) kept at RT for one or five days.

Panel A

Panel B

Figure 5: Photographs taken from mixtures containing 50mg (7) (top row) or (2) (bottom row) mixed with 10mL, from left to right, water, RPMI 1640 and DMEM after one (panel A) or five (panel B) days at RT.

The media/pigment mixtures obtained from (2) were dialyzed and lyophilized and FT-IR spectra of the dried materials were collected. Figure 6 presents these FT-IR spectra.

Figure 6: FT-IR spectra of pigment generated through the incubation of (2) with RPMI 1640 or DMEM cell culture media.

The FT-IR spectra shown in Figure 6 contain the typical features of MN-like materials obtained from (2) or other catecholic compounds through air- or $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ -mediated oxidation.[23, 26, 30] At this stage it can not be proven that the presence of (1B) in these cell culture media is responsible for the pigment formation from (2), (7) or the other compounds. However, it is clear that these cell culture media are not chemically inert towards catecholic compounds.

3.3. Experiments involving L-tyrosinate and the air- or $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ -mediated oxidation of various compounds

Reaction mixtures were prepared in 100mM acetate buffer pH=6.5 and kept at RT. Four different reaction conditions, I through IV, were set up in triplicate and are presented in the table below.

Table 1: Overview of the reaction conditions set up for the reactions involving (1B) and select “other compounds”.	
I	5mM “other compound”
II	5mM “other compound” + 5mM (1B)
III	5mM “other compound” mixed with 0.3mM Fe^{2+} and 0.075% v/v H_2O_2
IV	5mM “other compound” + 5mM (1B) mixed with 0.3mM Fe^{2+} and 0.075% v/v H_2O_2

At select time points, aliquots from the reaction mixtures were diluted tenfold with SEC solvent and analyzed. The AUC of the peaks corresponding to the “other compound” and tyrosine were determined whenever possible. These AUCs were compared to the AUC of the peaks corresponding to the “other compound” and (1B) from analyses of aliquots of reaction mixture II analyzed at the start of the experiments. At select time points Vis spectra of the crude reaction mixtures were recorded.

3.3.1. Experiments involving L-tyrosinate and epinephrine

As illustrated in Figures 2 and 3, compared to (3) or (4), the interaction between (1B) and (5) resulted in a mixture with a much lighter color and with a more complex pattern in the SEC profiles. Thus, more studies were initiated involving (5). Figure 7, panels A and B, illustrate typical SEC results obtained for the reactions involving (1B) and (5).

Panel A

Panel B

Figure 7: SEC profiles for select reactions involving **(1B)** and **(5)** in 100mM acetate buffer pH=6.5 at the start of the reaction or after one (panel A) or three (panel B) days standing at RT. See Table 1 for the description of reaction conditions **II** and **IV**.

In these SEC profiles, the peak with a retention time of about 20 minutes corresponds to **(5)**, the peak with retention time of about 15 minutes corresponds to **(1B)** and the peak with retention time of about 13 minutes corresponds to the material that exhibits absorbance for wavelengths above 300nm. Figure 8, panels A through C, provide the UV-Vis spectra of the signal with retention times around 13 minutes from the SEC analyses of select reaction mixtures involving **(1B)** and **(5)**.

Panel

Panel B

Panel C

Figure 8: UV-Vis spectra of peaks with retention time around 13 minutes for the SEC analyses of reaction mixture **II** (panel A), **III** (panel B) or **IV** (panel C) for reactions involving **(5)** in 100mM acetate buffer pH=6.5 after three days of reaction at RT. See Table 1 for the description of reaction conditions **II**, **III** and **IV**.

The spectrum shown in Figure 8, panel A, is similar to the one shown in Figure 3, panel B, featuring a broad absorbance range with maxima around 250, 275 and 325nm. In addition, the spectrum at a retention time of about 12.75 minutes is distinctly different from the spectra at retention times of about 13, 13.25 or 13.5 minutes. This suggests that the material eluting at a retention time of about 13 minutes represents a mixture of compounds with distinctly different physico-chemical properties. In contrast, the results presented in Figure 8, panel B, for reaction mixture **III** suggests the presence of a single type of molecule with a broad absorbance range and a distinct absorbance maximum at 275nm. The results presented in Figure 8, panel C, for reaction mixture **IV** suggests a mixture of compounds featuring a broad absorbance range with maxima around 250 or 275nm. Overall, the results presented in Figure 8 indicate that the different reaction conditions evaluated for the interaction between **(1B)** and **(5)** lead to distinctly different endproducts.

Figure 9 illustrates the % **(5)** or **(1B)** remaining after one day of reaction at RT as judged from the AUC calculations of the SEC profiles from aliquots of the various reaction mixtures. The results presented are the average $\pm$ standard deviation of three mixtures for conditions **I** through **IV**.

Figure 9: % **(5)** or **(1B)** remaining estimated from the AUC of the SEC profiles of reactions involving **(5)** in 100mM acetate buffer pH=6.5 after one day standing at RT. The results presented are the average $\pm$ standard deviation (n=3). See Table 1 for the description of reaction conditions **I** through **IV**.

The AUC corresponding to **(1B)** was estimated only for reaction mixture **II** as in our SEC analyses H_2O_2 exhibits a retention time similar to that of **(1B)**. In the presence of Fe^{2+} and H_2O_2 , **(5)** readily reacts away forming a darkly colored pigment. The presence of **(1B)** appeared to inhibit the reaction of **(5)** with Fe^{2+} and H_2O_2 , as judged from the % **(5)** remaining, although reaction mixture **IV** still exhibited a very dark color. However, after three days of reaction, most of **(5)** in mixture **IV** had reacted away (see Figure 7, panel B). In the absence of any Fe^{2+} and H_2O_2 , **(5)** underwent no significant amount of auto-oxidation (about 100% remaining after one or three days). The presence of **(1B)** resulted in an average loss of 20% **(5)** and the emergence of a gold color in the reaction mixture. In general, the presence of **(1B)** promoted the auto-oxidation of **(5)** with the formation of a colored substance. This process appeared to be less efficient than the $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ -mediated oxidation of **(5)**. On the other hand, the presence of **(1B)** has an inhibitory effect on the $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ -mediated oxidation of **(5)**.

Interestingly, the AUC of the peak corresponding to **(1B)** in reaction mixture **II** had increased with about 40% over the course of the reaction. To explain this apparent anomaly we considered the possibility that **(1B)** may have been converted into **(2)** as both compounds have similar retention times in our SEC analyses. Figure 10 compares the UV spectra of the peak with retention time of about 15 minutes from reaction mixture **II** to the UV spectra of **(1B)** and **(2)**. In addition, the SEC profile of reaction mixture **IV** (see Figure 7, panel A) indicates the presence of a distinct peak with a retention time of about 16 minutes; a peak that is not present after three days of reaction (see Figure 7, panel B). The UV spectrum of this distinct peak at a retention time of about 16 minutes is included in Figure 10.

Figure 10: UV-spectra of **(1B)** and **(2)** and peaks with retention time of about 15 or 16 minutes for the SEC analyses of select reaction mixtures involving **(5)** in 100mM acetate buffer pH=6.5 after one day standing at RT. See Table 1 for the description of reaction conditions **II** and **IV**.

The results indicate that, for reaction mixture **II**, the peak at 15 minutes in the SEC profiles has the features of **(1B)** and none of **(2)**, but the spectrum does indicate the presence of an additional, broad absorbance band around 310nm; possibly due to the presence of a reaction intermediate. The peak with a retention time of 16 minutes in reaction mixture **IV** is distinctly different from **(1B)** or **(2)** and very likely represents a reaction intermediate.

Figure 11, panels A and B, illustrates the Vis spectra of the crude reaction mixtures after one or three days of reaction at RT. The results presented are the average of three mixtures for each reaction condition, but standard deviations are omitted from the figures in order to maintain clarity within these figures.

Figure 11: VIS spectra of aliquots of crude reaction mixtures involving **(5)** in 100mM acetate buffer pH=6.5 after one day (panel A) or three days (panel B) standing at RT. Reaction mixtures **III** and **IV** were diluted two-fold. See Table 1 for the description of reaction conditions **I** through **IV**.

The results indicate that after one day of reaction, little absorbance is to be observed in mixture **I**, while mixture **IV** exhibited a distinct absorbance band around 500nm (weakly present in reaction mixture **I**). Reaction mixtures **II** and **IV** exhibited the typical broad-range absorbance profiles common for MN-like materials. After three days of reaction at RT, all reaction mixtures exhibited absorbance over the entire visible range of the electromagnetic spectrum, resulting in the appearance of a very dark color, but with significant differences in the intensities of these absorbancies (see Figure 11, panel B).

3.3.2. Experiments involving L-tyrosinate and catechol

Figure 12 illustrates select SEC analyses obtained for aliquots from reaction mixtures involving **(1B)** and **(6)**.

Figure 12: SEC profiles for reactions involving **(1B)** and **(6)** in 100mM acetate buffer pH=6.5 at start of the reaction or after one day of standing at RT. See Table 1 for the description of reaction conditions **II** and **IV**.

In these SEC profiles, the peak with a retention time of about 77 minutes corresponds to **(6)**, the peak with retention time of about 15 minutes corresponds to **(1B)** and the peak with retention time of about 13 minutes corresponds to the material that exhibits absorbance for wavelengths above 300nm. In the SEC profile of mixture **II** an additional peak with a retention time of about 21 minutes can be observed. Figure 13, panels A through C, provide the UV-Vis spectra of the signal with retention times around 13 minutes from the SEC analyses of select reaction mixtures.

Figure 13: UV-Vis spectra of peaks with retention time around 13 minutes for the SEC analyses of the reaction mixtures **II** (panel A), **III** (panel B) or **IV** (panel C) involving **(6)** and **(1B)** in 100mM acetate buffer pH=6.5 after one day of reaction at RT. See Table 1 for the description of reaction conditions **II**, **III** and **IV**.

The spectra shown in Figure 13, panels A through C, exhibit a similar pattern: a broad range absorbance profile with a distinct absorbance peak around 250 to 275nm. The presence of this distinct absorbance peak is less pronounced for the reaction mixture **III**. A detailed view of the spectra obtained for reaction mixtures **I** and **IV** does suggest that the peak with retention time around 13 minutes does not constitute a single type of molecule, but rather a mixture of molecules with different physic-chemical properties. However, the pattern of results obtained for **(6)** appear less complex compare to the results obtained for **(5)** (see Figures 3 or 8).

Figure 14 illustrates the % **(6)** or **(1B)** remaining after one day of reaction at RT as judged from AUC calculations of the SEC profiles of aliquots of the various reaction mixtures. The results presented are from the analysis of single aliquots from reactions mixtures **I** through **IV**. Although the reaction mixtures were set up in triplicate, no triplicate analyses were performed as SEC analyses of mixtures containing **(6)** are time consuming.

Figure 14: % (6) or (1B) remaining estimated from the AUC of the SEC profiles of reactions involving (6) and (1B) in 100mM acetate buffer pH=6.5 after one day standing at RT. See Table 1 for the description of reaction conditions I through IV.

The results presented in Figure 14 indicate that (6) underwent minimal auto-oxidation, but reacted away readily in the presence of Fe^{2+} and H_2O_2 as observed before.[23] The presence of (1B) appeared to enhance the auto-oxidation of (6), but inhibited the $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ -mediated oxidation of (6). As for the reactions involving (5) and (1B) (see Figure 10), in Figure 15 we compare the UV spectrum of the peak with a retention time of about 15 minutes of the SEC profiles obtained for reaction mixture II to the UV spectra of (1B) and (2). Figure 15 includes the UV spectrum of the peak with a retention time of about 21 minutes as observed in reaction mixture II (see Figure 12).

Figure 15: UV-spectra of (1B) and (2) and peaks with retention time of about 15 or 21 minutes for the SEC analyses of reaction mixture II involving (6) and (1B) in 100mM acetate buffer pH=6.5 after one day standing at RT. See Table 1 for the description of reaction condition II.

The results presented in Figure 15 indicate that the peak with retention time of about 15 minutes in reaction mixture II has all the features of (1B), while the peak with retention time of about 21 minutes represents a reaction intermediate as its UV spectrum is distinctly different.

Figure 16, panels A and B, illustrates the Vis spectra of the crude reaction mixtures after one or three days of reaction at RT. The results presented are the average of three mixtures for each reaction condition, but standard deviations are omitted from the figures in order to maintain clarity within these figures.

Figure 16: VIS spectra of aliquots of crude reaction mixtures involving **(6)** and **(1B)** in 100mM acetate buffer pH=6.5 after one day (panel A) or three days (panel B) standing at RT. Reaction mixtures **III** and **IV** were diluted two-fold. See Table 1 for the description of reaction conditions **I** through **IV**.

The results indicate that after one day of reaction, little absorbance is to be observed in mixture **I**, while mixture **IV** exhibited a distinct absorbance band around 520nm. After three days of reaction at RT, all mixtures, with the exception of **I** exhibited a very broad-band absorbance over the entire visible range of the electromagnetic spectrum, resulting in the appearance of a very dark color, but with significant differences in the intensities of these absorbancies.

3.3.3. Experiments involving L-tyrosinate and pyrogallol

Figure 17 illustrates typical SEC results obtained for the reactions involving **(1B)** and **(7)**.

Figure 17: SEC profiles for reactions involving **(1B)** and **(7)** in 100mM acetate buffer pH=6.5 at start of the reaction or after one day of standing at RT. See Table 1 for the description of reaction conditions **II** and **IV**.

In these SEC profiles, the peak with a retention time of about 58 minutes corresponds to **(7)**, the peak with retention time of about 15 minutes corresponds to **(1B)** and the peak with retention time of about 13 minutes corresponds to the material that exhibits absorbance for wavelengths above 300nm. Figure 18, panels A through C, provide the UV-Vis spectra of the signal with retention times around 13 minutes from the SEC analyses of select reaction mixtures.

Panel A

Panel B

Panel C

Figure 18: UV-Vis spectra of peaks with retention time around 13 minutes for the SEC analyses of the reaction mixtures **II** (panel A), **III** (panel B) or **IV** (panel C) involving **(7)** and **(1B)** in 100mM acetate buffer pH=6.5 after one day of reaction at RT. See Table 1 for the description of reaction conditions **II**, **III** and **IV**.

All the spectra shown in Figure 18, panels A through C, exhibit very similar patterns and the peak with retention time around 13 minutes appears to be homogenous as the spectra are similar for all the retention times surrounding the 13 minutes mark. The pattern of results obtained for **(7)** appear less complex compare to the results obtained for **(5)** (see Figure 3 or 8).

Figure 19 illustrates the % **(7)** or **(1B)** remaining after one day of reaction at RT as judged from AUC calculations of the SEC profiles of aliquots of the various reaction mixtures. The results presented are from the analysis of single aliquots from reactions mixtures **I** through **IV**. Although the reaction mixtures were set up in triplicate, no triplicate analyses were performed as SEC analyses of mixtures containing **(7)** are time consuming.

Figure 19: % (7) or (1B) remaining estimated from the AUC of the SEC profiles of reaction mixtures involving (7) and (1B) in acetate buffer pH=6.5 after one day standing at RT. See Table 1 for the description of reaction conditions I through IV.

The results presented in Figure 19 indicate that, under the experimental conditions, (7) does not undergo any significant amount of auto-oxidation and readily reacts in the presence of Fe^{2+} and H_2O_2 as studied before.[23] When mixed with (1B), (7) underwent an extensive amount of auto-oxidation; possibly with a consumption of (1B) during this reaction. On the other hand, the presence of (1B) appeared to inhibit the $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ -mediated of (7). Figure 20 provides the UV spectra of peaks with retention time of about 15 minutes for the SEC analyses of select reaction mixtures.

Figure 20: UV-spectra of peaks with retention time of about 15 minutes for the SEC analyses of (1B), (2) and reaction mixture II involving (7) and (1B) in acetate buffer pH=6.5 after one day standing at RT. See Table 1 for the description of reaction condition II.

The results presented in Figure 20 indicate that the peak with retention time of about 15 minutes in reaction mixture II has all the features of (1B).

Figure 21 illustrates the Vis spectra of the crude reaction mixtures after one or three days of reaction at RT. The results presented are the average of three mixtures for each reaction condition, but standard deviations are omitted from the figures in order to maintain clarity within these figures.

Figure 21: VIS spectra of aliquots of crude reaction mixtures involving (7) and (1B) in 100mM acetate buffer pH=6.5 after one day standing at RT. Reaction mixtures III and IV were diluted two-fold. See Table 1 for the description of reaction conditions I through IV.

The results presented in Figure 21 indicate that, apart from the auto-oxidation (mixture I), (7) reacts readily in the presence of (1B) and in the presence of $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ leading to darkly colored mixtures. Mixture IV does exhibit a distinct absorbance peak at around 560nm.

3.3.4. Experiments involving L-tyrosinate and resorcinol

Figure 22 illustrates typical SEC results obtained for select reactions involving (1B) and (8).

Figure 22: SEC profiles for reactions involving (1B) and (8) in 100mM acetate buffer pH=6.5 at start of the reaction or after one day of standing at RT. See Table 1 for the description of reaction conditions II and IV.

In these SEC profiles, the peak with a retention time of about 98 minutes corresponds to (8), the peak with retention time of about 15 minutes corresponds to (1B) and the peak with retention time of about 13 minutes corresponds to the material that exhibits absorbance for wavelengths above 300nm. In the SEC profile of mixture IV an additional peak with a retention time of about 20 minutes can be observed. Figure 23, panels A and B, provide the UV-Vis spectra of the signal with retention times around 13 minutes from the SEC analyses of select reaction mixtures.

Figure 23: UV-Vis spectra of peaks with retention time around 13 minutes for the SEC analyses of the reaction mixtures **III** (panel A) or **IV** (panel B) involving **(8)** and **(1B)** in 100mM acetate buffer pH=6.5 after one day of reaction at RT. See Table 1 for the description of reaction conditions **III** and **IV**.

All the spectra shown in Figure 23, panel A, exhibit a similar pattern: absorbance over a broad range of wavelengths with a distinct absorbance maximum around 275nm. The spectra shown in Figure 23, panel B, exhibit a more complex pattern with a distinct absorbance maximum around 250nm.

Figure 24 illustrates the % **(8)** or **(1B)** remaining after one day of reaction at RT as judged from AUC calculations of the SEC profiles of aliquots of the various reaction mixtures. The results presented are from the analysis of single aliquots from reactions mixtures **I** through **IV**. Although the reaction mixtures were set up in triplicate, no triplicate analyses were performed as SEC analyses of mixtures containing **(8)** are time consuming.

Figure 24: % **(8)** or **(1B)** remaining estimated from the AUC of the SEC profiles of reaction mixtures involving **(8)** and **(1B)** in 100mM acetate buffer pH=6.5 after one day standing at RT. See Table 1 for the description of reaction conditions **I** through **IV**.

The results presented in Figure 24 indicate that, under the experimental conditions, **(8)** does not undergo any significant amount of auto-oxidation, even in the presence of **(1B)**, and readily reacts in the presence of Fe^{2+} and H_2O_2 . In addition, the presence of **(1B)** appeared to inhibit the $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ -mediated of **(8)**.

Figure 25 provides the UV spectrum of the peak with a retention time of about 20 minutes as observed in the SEC profile of reaction mixture **IV** (Figure 22).

Figure 25: UV-Vis spectrum of the peak with retention time of about 20 minutes for the SEC analyses of reaction mixture **IV** involving **(8)** and **(1B)** in 100mM acetate buffer pH=6.5 after one day standing at RT. See Table 1 for the description of reaction condition **IV**.

The results presented in Figure 25 indicate that the peak with retention time of about 20 minutes represents a reaction intermediate as its UV spectrum is distinctly different from **(1B)** or **(8)**.

Figure 26 illustrates the Vis spectra of the crude reaction mixtures after one day of reaction at RT. The results presented are the average of three mixtures for each reaction condition, but standard deviations are omitted from the figures in order to maintain clarity within these figures.

Figure 26: VIS spectra of aliquots of crude reaction mixtures involving **(8)** and **(1B)** in 100mM acetate buffer pH=6.5 after one day standing at RT. Reaction mixtures **III** and **IV** were diluted two-fold. See Table 1 for the description of reaction conditions **I** through **IV**.

The results presented in Figure 26 indicate that the various reaction conditions resulted in varying absorbance profiles for the reactions involving **(8)**; in line with the limited reactivities (with the exception for mixture **III**) observed for **(8)** (see Figure 24)

3.4. Large scale reactions involving the air-mediated oxidation of L-DOPA in the presence of L-tyrosinate

In our SEC analyses (1) and (2) have similar retention times and quantitative types of comparisons as were done for compounds (3) through (8) (see Figures 9, 14, 19 and 24) could not be performed. A multitude of preliminary experiments had indicated that, as for all the other compounds tested, (1B) promotes the auto-oxidation of (2). Thus a large scale experiment was set up in order to isolate and purify the MN-like material generated through the reaction between (1B) and (2). Thus, about 500mg (1B) was mixed with about 250mg (2) in 100mL acetate buffer pH=6.5 and kept at RT for three days. Upon mixing (1B) and (2) the mixture turned intensely black within the hour. Figure 27 presents the SEC profile of a 40-fold diluted aliquot of this mixture.

Figure 27: SEC profile of a reaction mixture containing (1B) and (2) in 100 mM acetate buffer pH=6.5 after two days of standing at RT.

In this SEC profile, the peak with a retention time of about 15 minutes corresponds to (1B) and/or (2) and the peak with retention time of about 13 minutes corresponds to the material that exhibits absorbance for wavelengths above 300nm.

Figure 28, panels A and B, provides the UV-Vis spectra of the signal with retention time around 13 and 15 minutes respectively from the SEC analysis shown in Figure 27.

Figure 28: UV-spectra of peaks with retention time of around 13 minutes (panel A) or about 15 minutes (panel B) for the SEC analysis shown in Figure 27. Panel B includes the UV spectra for the SEC analyses of (1B) and (2).

The results presented in Figure 28, panel A, show the presence of a material with a broadrange absorbance and a distinct absorbance maximum between 270 and 300nm. A detailed view of the spectra shown in Figure 28, panel A, does suggest that the peak with retention time around 13 minutes does not constitute a single type of molecule, but rather a mixture of molecules with different physic-chemical properties. The results presented in Figure 28, panel B, indicate that the peak with retention time around 15 minutes from the reaction mixture containing (**1B**) and (**2**) is subtly different from (**1B**) and (**2**) and may well represent a mixture of both compounds.

After three days standing at RT, the reaction mixture was divided in two fractions and kept frozen till further processing. One half of this mixture was dialyzed against distilled water with multiple changes of water over the course of three days. The second half of the reaction mixture was first dialyzed against 2L water containing 0.3M NaCl for two days. This was followed by dialysis against distilled water with multiple changes of water for an additional day. Throughout the dialysis processes aliquots from the reaction mixture were diluted fivefold and analyzed using SEC. Figure 29 presents the SEC profiles of the mixture at the end of the reaction and after dialysis against water or salt.

Figure 29: SEC profile of a reaction mixture containing (**1B**) and (**2**) in 100mM acetate buffer pH=6.5 after three days of standing at RT and after dialysis against water or dialysis against 0.3M NaCl followed by water.

The results presented in Figure 29 indicate that the dialysis process was very effective in removing the unreacted (**1B**) or any other low molecular mass component that may have been present at the end of the reaction. At the end of the dialysis process, only the peak with a retention time of about 13 minutes, corresponding to the MN-like material, remains. Figure 30 presents the UV-Vis spectra of the peaks with retention time of about 13 minutes in the SEC profiles shown in Figure 29.

Figure 30: UV-spectra of peaks with retention time of around 13 minutes for the SEC analyses shown in Figure 29.

The results presented in Figure 30 indicate that following dialysis, the UV-Vis spectrum of the MN-like material lost its distinct absorption maximum around 275nm; with dialysis against salt possibly being more effective than dialysis against water. Both dialyzed fractions were lyophilized and characterized using FT-IR spectroscopy. These spectra are shown in Figure 31.

Figure 31: FT-IT spectra of the MN-like material generated through a reaction between (1B) and (2) in 100mM acetate buffer pH=6.5 and dialyzed against distilled water or salt, followed by distilled water as discussed in the body of the text.

The results presented in Figure 31 indicate that the different dialysis processes used yielded similar endproducts. The scans presented in Figure 31 are in line with FT-IR scans taken of other MN-like materials derived from (2).[26, 30]

4. Discussion

In general, the following patterns of observations were made for the experiments reported here.

- 1) The presence of (1B) enhanced the auto-oxidation of all compounds tested, with the exception of (8), while the presence of (1B) appeared to slow down the $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ -mediated oxidation of all compounds tested. The auto-oxidation of compound (7) was particularly enhanced in the presence of (1B). The auto-oxidation reactions were performed in the absence of any enzymes or redox-sensitive cations, although we can not exclude the possibility that trace amounts of redox sensitive cations were present in our mixtures. All reactions were performed at RT and it is not unreasonable to expect that higher temperatures, e.g., 37°C, would enhance the observed reactivities.
- 2) Whether (1) is reactive only in its phenolate form ((1B) in Figure 2) and not in its (1A) form remains to be determined. The solubility of (1A) is much lower than (1B) and direct comparisons are hard to make. However, a single mixture was prepared consisting of equal amounts of (1A) and (2) in 100mM acetate buffer pH=6.5 (with (1A) present as a suspension) and left standing at RT. Only after an overnight reaction did this mixture turn very dark with much of the darkly-colored pigment precipitated (results not shown).
- 3) The physic-chemical properties of the endproducts of the reaction appeared to be different depending on the precursor used. The reaction between (1B) and (5) yielded a gold-colored mixture, rather than the dark-brown to black color for the other compounds, and the UV_Vis profile of the oligomeric or polymeric substance produced was much more complex (see Figure 3, panel B and Figure 8, panels A and C). In our SEC analyses, the MN-like material generated in our experiments consistently eluted with a retention time close to 13 minutes; independent of the type of precursor used or whether auto-oxidation or $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ -mediated oxidation was studied. In SEC, the retention time is related to the hydrodynamic volume of the compound (often related to its molecular mass): the lower the retention time the higher the hydrodynamic profile. The SEC analyses of purified MN-like material obtained from (2) (see Figure 29) show significant “tailing” at the right side of the peak. This observation suggests that, in addition to hydrodynamic interactions, hydrophobic interactions are determining the retention of

the MN-like materials in our SEC results and that retention times based purely upon hydrodynamic interactions would be lower. Nevertheless, evaluating the results obtained in this and our previous reports[23, 25-28, 30, 32], there is a remarkable consistency in the SEC retention times, and thus hydrodynamic volumes, of the MN-like materials generated; independent of the precursor or the mode of melanogenesis studied.

- 4) Many of our observations indicate that one or more intermediates are generated during the transformation of compounds (**3**) through (**7**) into MN-like materials. It is unclear at this stage whether any of these possible intermediates resemble the compounds shown in Scheme 1. With the possible exception for the reaction involving (**7**), the effect of the presence of (**1B**) appeared to be catalytic in nature as (**1B**) seemed not to be consumed in the reaction, while the other compounds, given enough time, reacted away almost completely.
- 5) Cell culture media like RPMI-1640 or DMEM can promote melanogenesis of many catecholic compounds. Our results imply that (**1B**) present in these media might be responsible for the reactivity associated with these cell culture media.
- 6) The UV-Vis spectra of the MN-like materials generated in our experiments show broad range absorbance over the entire UV range of the electromagnetic spectrum superimposed with one or more distinct sharp absorbance peaks (see Figures 8, 13, 18, 23, 28). These sharp absorbance bands could be due to specific chemical features within the MN structure. Alternatively, they could be due to the presence of precursors or intermediates from the reaction physically embedded within the MN structure. Our experience from the large scale reaction between (**1B**) and (**2**) does suggest the latter possibility. Dialysis of the MN material generated from (**2**) reduced the presence of the distinct absorbance peak around 275nm (see Figure 30).

Based upon the results presented in this report we would like to make suggestions for any current and/or future research regarding melanogenesis.

- a) Compared to the other compounds tested, (**8**) is a meta-diphenol rather than an ortho-diphenol and this may explain the difference in auto-oxidation reactivity that we observed. Whether para-diphenols, e.g., homogentisic acid, react with (**1B**) has not been addressed yet. In addition, whether the auto-oxidation reaction promoted by (**1B**) has stereoselectivity, e.g., no reaction with D-DOPA or no reaction when using D-tyrosinate, has not been evaluated yet. Other compounds like 5-hydroxyindoles, e.g., serotonin, can be converted into MN-like materials[23, 27] and the possible reaction between (**1B**) and 5-hydroxyindoles has not been studied yet. All the experiments reported here could be executed in the presence of L-cysteine or other thiol-containing compounds to simulate the synthesis of pheomelanin-like pigments; similar to other studies.[33]
- b) Any study of the enzymes involved in the conversion of (**1**) into MN-like materials should include a careful evaluation of the non-enzymatic contribution of (**1**) to the rates of the various downstream reactions shown in Scheme 1.[13-15] A specific point that could be addressed is whether the non-enzymatic contribution of (**1**) has any effect on the ratio of DHI to DHICA (see Scheme 1) as this ratio may influence the physical properties of the MN material.[9]
- c) This report suggests a third, non-enzymatic, mechanism to generate darkly colored pigments from a multitude of catecholic precursors. In previous reports we detailed how the presence of polysaccharides[27, 29, 31] or the action of $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ [23, 26, 28, 30] can generate darkly colored pigments from compounds like the ones shown in Figure 1. Thus the balance between pro-oxidizing or anti-oxidant factors or the presence or absence of non-enzymatic, melanogenic biomolecules like polysaccharides (or proteins, e.g., PMEL[34, 35]), may make significant contributions to the quantity and quality, including an influence on the color, of the MN generated. However, all these non-enzymatic mechanisms require the presence of a catecholic compound like (**2**), as (**1**) by itself appears to be unreactive. Thus, enzymes like TYR are still essential to initiate the melanogenesis process. However, we feel that non-enzymatic mechanisms need to be taken into account when studying melanogenesis in any tissue, organ or organism[36], when studying the genetics behind pigmentation[37] or when studying the inhibition of the melanogenesis process[38].
- d) The reactivity of (**1B**) towards catecholic compounds or any of the intermediates shown in Scheme 1 should be studied in the presence of redox-sensitive cations; particularly in light of the reports that TYR and related proteins contain Cu or Zn cations in their active sites.[7, 39] A single observation made in our laboratory indicated that (**1B**) promotes the auto-oxidation of (**3**), (**4**) or (**5**) in the presence of Fe^{2+} , Cu^{2+} or Zn^{2+} (results not shown).
- e) Melanogenesis studies involving whole cells should be executed with a careful choice of cell culture medium, particularly when catecholic compounds are added externally.[40] There is a possibility that the externally added catecholic compound reacts with (**1B**) present as a component of the cell culture medium and generates reaction

products that may skew any experimental results. The possible impact of **(1)** as a component of the cell culture medium on the behavior of isolated melanoma cells has been suggested before.[41]

- f) To the standard protocols proposed for the purification of MN materials[3], we would like to stress the importance of including a step involving the dialysis of the MN material against a salt solution to effectively remove unreacted precursors or intermediates that may have been embedded inside the MN structure. As discussed before, the MN materials generated in our experiments often exhibit the presence of a distinct, sharp absorbance peak superimposed on the broad range absorbance profile of the MN material. This feature has been reported elsewhere for MN materials generated from DHICA.[9] In this report we have shown that the presence of this feature in the UV-Vis spectra of MN can be reduced through dialysis against a NaCl solution.
- g) The relationship between the chemical and physical properties of MN has been and is continued to be studied extensively.[9, 42, 43] We would recommend using compounds like **(6)** or **(7)** to study such relationships as their chemical structures are much simpler. E.g., no intramolecular ring closure can occur or no uncertainties exist regarding the loss of carboxylic acid groups during the melanogenesis process. Yet, despite the simplicity of their structure, compounds like **(6)** or **(7)** readily yield darkly-colored pigments with the typical broadband absorbance of MN materials as shown in this and one of our previous reports.[23]

As mentioned earlier, it is unclear whether **(1)** is reactive only in its **(1B)** form (phenolate form; see Figure 1) and not its **(1A)** form. Any basic textbook in biochemistry will indicate that the pK_a value of the phenol group in **(1)** is about 10.1. Thus, at pH=6.5 (the pH value of our experiments) about 0.025% of **(1)** should be in its phenolate form. The solubility of **(1)** in water at 25°C is 479mg/L (2.64mM).^{*} The typical concentration of **(1B)** used in our experiments was 5mM, thus we assume that, when dissolving **(1B)** in buffer pH=6.5, the fraction that remains in the phenolate form is significantly larger than 0.025%. The fraction of **(1)** in **(1B)** form should increase with increasing pH values. However, if **(1B)** acts in a catalytic fashion, as some of our observations suggest, then even minute amounts of **(1B)** may promote the process of melanogenesis.

At this stage we can not explain why **(1B)** promotes the auto-oxidation of catecholic compounds and inhibits their Fe^{2+}/H_2O_2 -mediated oxidation into MN-like materials. The combination of Fe^{2+} and H_2O_2 leads to reactive oxygen species (ROS) and **(1B)** may interact differently towards molecular oxygen (O_2) than towards ROS. Although the observation that **(1B)** promotes the auto-oxidation of many catecholic compounds was unexpected, the importance of the redox properties of **(1)** inside proteins is very well documented.[44] As a matter of fact, the presence of **(1)** and its redox properties is crucial for some of the most fundamental biochemical processes of Life: the oxidation of water to oxygen in photosystem II[45], the reduction of oxygen to water in cytochrome c oxidase[46] and others.[44]. Thus, the reactivity of **(1B)** discussed in this report should not be surprising. One of the functions ascribed to the process of melanogenesis, particularly the synthesis of NM, is its capacity to remove catecholamine-derived, cytotoxic orthoquinones from the cell's environment.[47] If the presence of free **(1)** inside a cell's environment may lead to uncontrolled and nonspecific pro-oxidizing reactions, than the process of melanogenesis may well represent a mechanism to remove excess **(1)** from a cell's environment in addition to other toxins.

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