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The acid-catalyzed interaction of melanin with nitrite ions. An EPR investigation

Zenon Matuszak, Collin F. Chignell†, Krzysztof J. Reszka†

Abstract. The interaction of synthetic dihydroxyphenylalanine (DOPA) melanin (DM) with nitrite ions, NO_2^-, in the pH 3.6–7.0 range, has been investigated using electron paramagnetic resonance (EPR). We found that especially at pH <5.5 (from ca. 5.5 to 3.6) the reaction of DM with nitrite generated large quantities of new melanin radicals, which implies the involvement of nitrous acid, HNO_2, in the radical formation process. Measurements carried out at constant pH of 3.6 showed that the melanin signal increased together with nitrite concentration, reaching a plateau level which was more than fourfold larger compared to the initial signal amplitude observed in a nitrite-free buffer of the same pH. The effects of nitrite and DM concentrations on the melanin-free radical content were also investigated. It is proposed that the radicals are generated by one electron oxidation of melanin *ortho*-hydroquinone groups to *ortho*-semiquinones by HNO_2 or related nitrogen oxides such as $NO_2^{\bullet}$ radicals. The possible involvement of nitric oxide ($^{\bullet}NO$) and peroxynitrite ($ONOO^-$) in DM oxidation was also examined. In air-free solutions, nitric oxide *per se* did not generate melanin radicals; however, in the presence of oxygen a marked increase in the melanin EPR signal intensity was observed. This result is interpreted in terms of the generation of radicals via the oxidation of DM by peroxynitrite. Our findings suggest that melanin can function as a natural scavenger of nitrous acid and some nitrous acid-derived species. This property may be relevant to physiological functions of melanin pigments *in vivo*.

Key words: EPR • melanin • nitrite • nitrogen dioxide • nitrous acid • radicals

Z. Matuszak✉
Department of Medical Physics and Biophysics,
Faculty of Physics and Applied Computer Science,
AGH-University of Science and Technology,
30 Mickiewicza Ave., 30-059 Krakow, Poland,
Tel.: +48 12 617 4454, Fax: +48 12 634 0010,
E-mail: Zenon.Matuszak@fis.agh.edu.pl

C. F. Chignell, K. J. Reszka
Laboratory of Pharmacology and Chemistry,
National Institute of Environmental Health Sciences (NIH),
P. O. Box 12233, Research Triangle Park,
North Carolina 27709, U.S.A.

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Introduction

Melanins are natural pigments responsible for the coloration of skin and hair in animals and humans. The biosynthesis of melanins is confined to highly specialized cells, melanocytes, which contain tyrosinase, a unique enzyme capable of hydroxylating the amino acid tyrosine to dihydroxyphenylalanine (DOPA) and oxidizing DOPA to DOPA-quinone [1]. The latter is rapidly converted to 5,6-dihydroxyindole (5,6DHI) and 5,6-dihydroxyindole-2-carboxylic acid 5,6DHI(2CA), which upon oxidation and condensation yields eumelanin, the brown-to-black pigment (Fig. 1) [1, 2]. An alternative route to the pigment, not involving tyrosinase, is via the oxidation of DOPA, or 5,6DHI(2CA), by peroxidase/H_2O_2 systems (Fig. 1) [3].

EPR investigations have shown that melanin contains free radicals, mostly, *ortho*-semiquinone ($Q^{\bullet-}$) in nature [2]. It is believed that these radicals

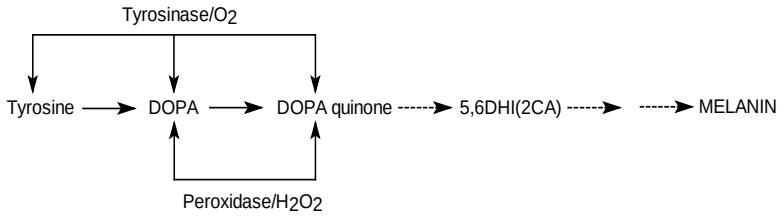

Fig. 1. Schema of melanin synthesis.

are derived primarily from two structural subunits of eumelanin, 5,6DHI and 5,6DHI(2CA), which are readily oxidized to semiquinone and quinone forms. The content of the radicals in melanin can be changed by changing pH of the medium, by reacting melanin with oxidizing and reducing species, and by interaction with metal ions [2, 4–7]. Using EPR it has been shown that melanin scavenges free radicals [8] and deactivates other reactive species, such as excited states of photosensitizers and singlet oxygen [9].

It is believed that the most important biological role of melanin is the protection of the skin against ultraviolet (UV) light-induced damage. In this respect an important role is played by the ability of melanin to absorb and scatter the light, and the capacity to react with free radicals and to deactivate other reactive species produced in the course of photochemical/photosensitized reactions. Specifically, the ability of eumelanin to scavenge free radicals has been linked to the high resistance to chemo- and radiotherapy of melanoma, a form of pigmented skin cancer.

Some findings suggest that the development and growth of tumors including melanoma may be regulated by nitric oxide ($^{\bullet}NO$) and NO-derived species. It has been reported that both melanoma and normal melanocytes contain constitutive nitric oxide synthase (NOS), an enzyme responsible for the formation of $^{\bullet}NO$ from arginine, and that the activity of NOS in melanoma is significantly greater than in normal melanocytes [10]. In addition, it has been found that in contrast to nonmetastatic melanoma cells, highly metastatic melanoma cells express a high level of inducible nitric oxide synthase and nitric oxide production [11–13]. $^{\bullet}NO$ regulates many physiological processes, including anticancer and antibacterial action of macrophages [14]. It is involved, presumably, in the control of the growth and metastasis of tumors through the regulation of angiogenesis and platelet aggregation [10, 15–17]. $^{\bullet}NO$ is also the natural precursor of other reactive nitrogen species (RNS) such as the nitrogen dioxide radical ($NO_2^{\bullet}$) and *in vivo* produced nitrite and nitrate [18]. It is therefore important to know whether melanin may react with radical and nonradical species produced along the nitric oxide pathway and whether by doing so, it can affect the course of the disease. Before now it has not been known whether melanin can react with any of these reactive species.

In the present work we have investigated the interaction between synthetic DOPA-melanin (DM) and nitrite anions, NO_2^-, as a function of pH. We found that the reaction of DM with nitrite generates melanin radicals but only in acidic solutions, which suggests the involvement of nitrous acid, HNO_2, in the radical formation process. The rationale behind this study is that: (i) the intracellular pH in cancer cells is acidic [17] and, in particular, the intramelanosomal pH can be as low as 3–4 [19, 20]; (ii) the NO_2^- ions present *in vivo* undergo protonation in the acidic environment of cancer cells to form HNO_2, $pK_a(HNO_2/NO_2^-) = 3.35$ [21]; (iii) the melanin pigment and products of NOS activity, including HNO_2, may be present simultaneously in melanoma where they may react with each other *in situ*. Because DM is considered to be an adequate model of the natural eumelanin pigment present in the skin this study suggests that endogenous melanin may scavenge nitrous acid *in vivo*. HNO_2 is a powerful toxin and mutagen and its scavenging by melanin may be pertinent to the cytoprotective action of the pigment. Possible participation of related oxygen oxides has also been considered based on the dependence of radical formation on the presence of oxygen, superoxide dismutase, and catalase (CAT).

Materials and methods

DOPA-melanin was obtained from Dr. C. C. Felix (Medical College of Wisconsin, Milwaukee, WI, U.S.A.) and the method of its preparation was described in [7]. Sodium nitrite, CAT, superoxide dismutase (SOD), and DEA/NO (sodium 2-(*N*, *N*-diethylamino)-diazenolate-2-oxide) were purchased from Sigma-Aldrich Chemical Company (Milwaukee, WI, U.S.A.). The stock solution of DEA/NO (10 mM) was prepared in 20 mM NaOH and the release of NO by DEA/NO was controlled by NO-electrode (ISO-NOP200 electrode, World Precision Instruments, Sarasota, FL, U.S.A.).

Peroxynitrite was synthesized according the method described in [22], its concentration was controlled spectrophotometrically (ε_{302} = 1670 $M^{-1} \cdot cm^{-1}$) [23]. The reaction was initiated by the addition of a small aliquot (100 μL) of the nitrite solution (5 mM) to DM dissolved in a buffer of appropriate pH. Buffers used in this work were: sodium acetate/acetic acid (0.1 M) for pH 3.6, 4.0, 4.7, 5.3, and 5.8, and phosphate buffers (0.1 M) for pH 6.5 and 7.0. EPR measurements were performed using a Varian E-Line Century Series EPR spectrometer operating at 9.4 GHz with a 100 kHz modulation frequency and equipped with a TM_{110} cavity. Measurements were performed in a flat quartz aqueous cell at room temperature. The deoxygenated samples were prepared by purging with nitrogen in the EPR cell for 5 min prior to the addition of an oxygen-free solution of $NaNO_2$, after which bubbling was continued for another 30 s.

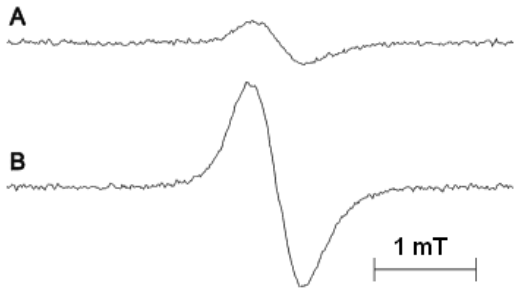

Fig. 2. EPR spectra of DM at pH 3.6 in the absence (A) and the presence of 0.5 mM nitrite (B).

Typical instrumental settings for the recording the melanin EPR signals were: microwave power 10 mW, modulation amplitude 0.165 mT, time constant 0.25 s, scan rate 4 min/10 mT, and the appropriate receiver gain level. At 10 mW the melanin EPR signals were partially saturated, especially in air-free solutions, but this power level was chosen because it allowed the prompt detection of relatively low concentrations of the pigment. We have found that for the purpose of this work this partial saturation can be ignored because experiments carried out on selected samples using the nonsaturating power of 0.5 and 1 mW have led to identical conclusions as those carried out at 10 mW. Because neither line shape nor line width of melanin EPR signal changed in the course of the reaction, concentrations (relative) of melanin radicals were determined by measuring amplitudes of the respective EPR signals. This approach and a double integration method yielded similar results. Simulation of the spectra was performed using the software described in [24].

Results

The EPR spectrum of DM in pH 3.6 buffer is a symmetrical single line with a peak-to-peak line width (ΔB_{pp}) of approximately 0.5 mT (Fig. 2A). After the addition of $NaNO_2$ (final concentration, 0.5 mM), the amplitude of the signal increased by a factor of about 4.4, indicating the formation of a large number of new free radical centers in DM.

This change in the signal intensity occurred rapidly (in less than 1 min) so the time course of this initial increase could not be recorded in static (offline) EPR experiments. Under applied conditions this significant increase in the radical content in DM did not cause any noticeable changes in the line shape or ΔB_{pp} of the melanin EPR signal. Accordingly, signals recorded prior to and after the addition of nitrite could be simulated assuming the existence of only one type of radical center in DM (r = 0.98–0.99).

Dependence on pH

The generation of melanin radicals by nitrite is strongly pH dependent. When the pH of the sample containing DM and nitrite was decreased from 7.0 to 3.6, the amplitude of the melanin EPR signal increased about threefold (Fig. 3A, line ●). Note that in the control (nitrite omitted), the decrease in pH alone induced a moderate, about 38%, decrease in signal amplitude (Fig. 3A, line ■). It is known that changes in pH affect the intensity of the melanin signal. A significant increase in radical concentration has been observed only in alkaline solutions, while at acidic pH the radical content is only slightly smaller than that at neutral pH. Thus the action of H^+ alone is the opposite of the action of HNO_2. Figure 3B shows the net dependence of the melanin signal on pH after the correction for the effect of H^+ alone. In the presence of nitrite the relationship between the EPR signal amplitude of DM and the pH is linear in the pH range 3.6–5.5 (r = –0.99).

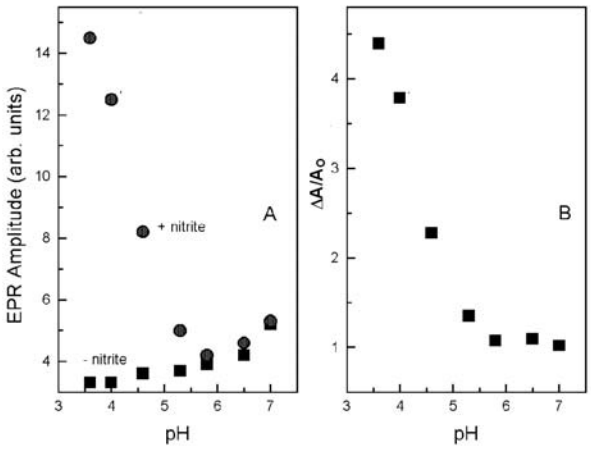

Fig. 3. Dependence of the melanin (DM ~ 0.55 mg/mL) EPR signal amplitude on pH. (A) Sample containing DM and nitrite ($NaNO_2$ = 0.5 mM), line ●; nitrite omitted, line ■. (B) The net dependence of the melanin signal on pH after correction for the effect of H^+ alone. The amplitudes are expressed relative to the amplitude measured at a given pH in the absence of $NaNO_2$, $\Delta A/A_O$, where $\Delta A = A_N - A_O$; A_N and A_O correspond to EPR amplitudes measured in the presence and absence of nitrite, respectively.

The constant level of the signal amplitude was attained approximately 2 min from the start of the reaction and the signals did not change over the period of several minutes.

Dependence on $NaNO_2$

The dependence on $NaNO_2$ was studied at pH = 3.6 because at this pH the generation of radicals was most efficient. When the concentration of $NaNO_2$ increased the melanin signal also gradually increased reaching plateau at a fourfold higher level compared to control (Fig. 4).

Dependence on melanin concentration

The amplitude of EPR signal increased linearly with DM concentration (Fig. 5, line ●). In the presence of $NaNO_2$ the increase was considerably larger and it was still proportional to DM concentration (Fig. 5, line ■).

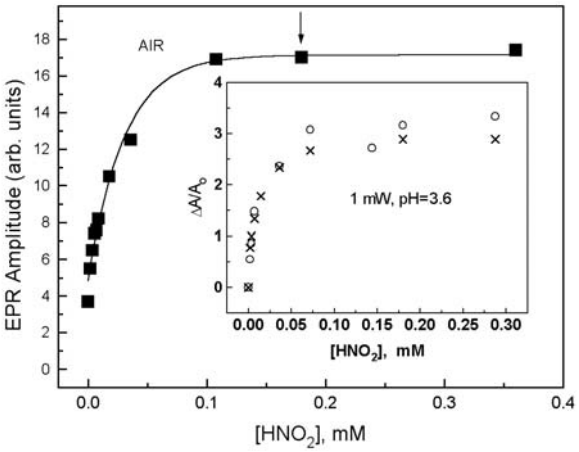

Fig. 4. Dependence of the melanin EPR signal amplitude on HNO_2 in air-saturated buffers (pH 3.6), line ■. The signals were measured using 10 mW power. The arrow indicates a data point used as a reference in the investigation of the effect of melanin concentration on the nitrite-induced radical generation (Fig. 5). Inset shows the EPR amplitude plotted vs. HNO_2 measured using 1 mW microwave power for melanin samples in air-saturated (o) and air-free (×) pH 3.6 buffers. The experimental data points represent the relative increase of the signal amplitude, $\Delta A/A_O$, where $\Delta A = A_N - A_O$; A_N and A_O correspond to EPR amplitudes measured in the presence and absence of nitrite, respectively. Concentrations of nitrous acid HNO_2 refer to NO_2 calculated using the expression $HNO_2 = [T]/(1+10^{pH-pKa})$ where [T] is the total concentration of nitrite (0.5 mM). $pK_a(HNO_2/NO_2^-) = 3.35$ [21].

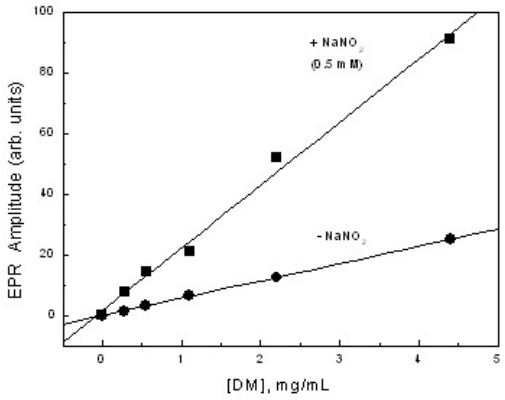

Fig. 5. Dependence of the melanin EPR signal amplitude on melanin concentration in the absence (●) and presence (■) of $NaNO_2$ ($NaNO_2$ = 0.5 mM, HNO_2 = 0.18 mM) in aerated pH 3.6 buffer. The concentration of nitrite used was sufficient to reach the plateau level of the EPR signal for a DM concentration of 0.5 mg/mL (point marked with an arrow in the plateau region in Fig. 4).

Reaction of DM with nitric oxide and peroxynitrite and the effects of SOD and CAT

It is possible that some RNS other than HNO_2 also participate in the growth of EPR melanin signal after adding nitrite. In order to check this possibility, taking into account some aspects of melanin chemistry, the additional experiments were performed. Results are presented in Fig. 6.

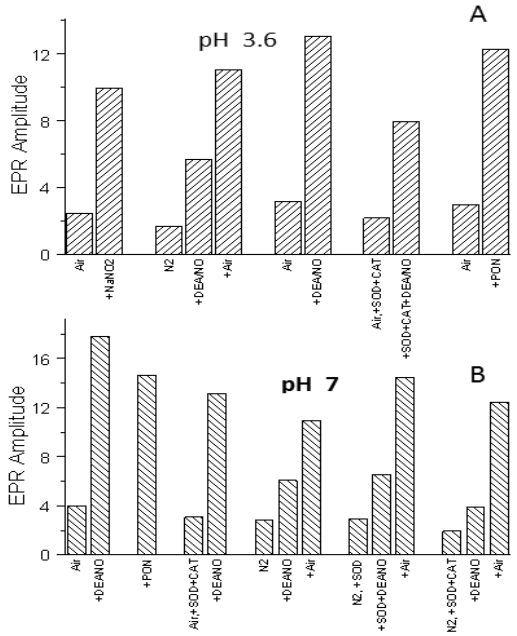

Fig. 6. (A) Effect of $NaNO_2$ (0.5 mM), DEA/NO (1 mM) and peroxynitrite (PON) (2 mM) on melanin (0.55 mg/mL) EPR signal amplitude in aerated and deaerated pH = 3.6 buffer. (B) Effect of DEA/NO (1 mM) and PON (2 mM) on melanin (0.55 mg/mL) EPR signal amplitude in aerated and deaerated pH = 7.0 buffer. In some experiments CAT (0.2 mg/mL) and SOD (0.1 mg/ml) were present.

Effect of H_2O_2

It is known that in aerated solutions melanin undergoes autooxidation, producing superoxide and hydrogen peroxide which exist in thermodynamic equilibrium with the pigment [25].

(1) $\quad Mel + O_2 \leftrightarrows Mel\ semiquinones + O_2^{\bullet -}$

(1A) $\quad QH_2 + O_2 \leftrightarrows Q^{\bullet -} + O_2^{\bullet -} + 2H^+$

where QH_2 represents melanin *ortho*-hydroquinone groups.

However, it is anticipated that at acid pH the actual concentration of superoxide will be markedly decreased because of its protonation (pK_a = 4.7, [26]) and subsequent rapid dismutation to H_2O_2, and also because of a diminished capability of DM to reduce O_2. To find out whether H_2O_2 is involved in the nitrite-dependent generation of melanin radicals, CAT (100 μg/mL) was added to DM solution in water (250 μL) and the sample was incubated for 5 min. Subsequently 250 μL of pH 3.6 acetate buffer (0.1 M) was added followed by the addition of nitrite. Although in the presence of CAT the reference signal (nitrite omitted) was about 20% less than without CAT, the ratio of the amplitudes from samples containing (DM + nitrite)/(DM) was almost the same as that for (DM + CAT + nitrite)/(DM + CAT) suggesting that at this pH any residual H_2O_2 had no apparent effect on the generation of radicals in DM by nitrite. Similar result was obtained when CAT was substituted for SOD which implies that the generation of radicals in DM by nitrite is

independent on the presence of superoxide/hydroperoxyl in the system.

Because CAT and H_2O_2 alone did not affect the melanin signal, this result suggests that H_2O_2 (at acid pH) may be involved in the nitrite-mediated oxidation of DM, probably through *in situ* produced peroxynitrite (ONOOH/$ONOO^-$), and perhaps $O_2^{\bullet-}$ (at near neutral pH).

Effect of $^{\bullet}NO$ and $ONOO^-$

To gain further insight into the possible mechanisms of oxidation of DM by HNO_2 the effects of nitric oxide ($^{\bullet}NO$) and peroxynitrite ($ONOO^-$) on the melanin EPR signal were examined. HNO_2 exists in an equilibrium with its precursors, nitrogen oxides $^{\bullet}NO$ and $^{\bullet}NO_2$ (Eqs. (2) and (3)), and both react with a number of biological electron donors causing their oxidation. Therefore, their involvement should also be considered.

(2) $$2HNO_2 \leftrightarrows N_2O_3 + H_2O$$

(3) $$N_2O_3 \leftrightarrows {}^{\bullet}NO + {}^{\bullet}NO_2$$

In addition, it is known that $^{\bullet}NO$ reacts very rapidly with superoxide forming the strong oxidant peroxynitrite ($k_4 = 6.7 \times 10^9\ M^{-1}\cdot s^{-1}$) [27].

(4) $$^{\bullet}NO + O_2^{\bullet-} \leftrightarrows ONOO^-$$

The possible involvement of NO in the generation of melanin radicals was studied using a NO donor DEA/NO at pH 3.6 and 7.0. When a $^{\bullet}NO$ donor, DEA/NO, was added to DM in aerated pH 7.0 buffer, the EPR signal amplitude increased fourfold (Fig. 6). In contrast, when a nitrogen-saturated solution of DEA/NO was added to a N_2 gassed bubbled DM sample, the increase was, approximately, twofold (Fig. 6). When a deaerated DM sample was preincubated with SOD and CAT (pH 7), and then DEA/NO was added anaerobically, the increase was about 55% (Fig. 6), suggesting that $^{\bullet}NO$ *per se* does not produce radicals in DM. The small increase was presumably due to residual oxygen in the DM solution. DEA/NO preincubated at pH 7 for 30 min at room temperature and then added to DM induced no changes in the melanin EPR signal, in agreement with the short half lifetime (15 min) of this $^{\bullet}NO$ donor [28]. SOD and CAT (combined) only partially inhibited (ca. 40%) the generation of new radicals in DM (Fig. 6). (The relationship between these experiments and those carried out using HNO_2 is that $^{\bullet}NO$ and ONOOH/$ONOO^-$ can be present in our systems (*vide infra*)).

When $ONOO^-$ (2 mM) was added to DM at pH 3.6 and 7.0, the melanin signal increased by a factor of 4 (Fig. 6), suggesting the peroxynitrite and peroxynitrous acid might participate in melanin oxidation. The result at pH 3.6 might be questionable because it is known that peroxynitrite solutions are always contaminated with nitrite [22] and, therefore, the observed increase might be attributed to nitrous acid (*vide supra*, Fig. 2), and not to the action of peroxynitrite itself. However, the result obtained at pH 7, where nitrite alone is inactive, clearly points out to peroxynitrites a likely oxidant of DM.

Discussion

We have shown that in acidic solutions, especially at pH <5.5, $NaNO_2$ reacts with DOPA-melanin to produce large quantities of melanin radicals (Figs. 2 and 3). This result suggests that nitrous acid, HNO_2 ($pK_a = 3.35$, [21]) is involved in the radical generation process. This suggestion is further supported by the observation that at constant $NaNO_2$ the increase in the melanin EPR signal is larger at more acidic pH, and that in the 5.5–3.6 pH range, the EPR amplitude is linearly dependent on pH (Fig. 3B). It is also consistent with the results that show that at a given acidic pH, higher concentration of nitrite induced larger increase in the melanin EPR signal. It is seen, that in the condition of experiment (Fig. 4) the high concentrations of $NaNO_2$ had no effect on the DM EPR signal (saturation), suggesting melanin quinones are inaccessible for oxidation by HNO_2 due to localization inside the polymer matrix. Additionally, it appears that oxygen plays minimal role in melanin radical formation, because it was found that the concentration of nitrite-induced radicals is only slightly higher in aerated samples compared to air-free samples (Fig. 4, inset). The mechanism of reaction is consistent with the model of radical formation in which the concentration of radical-generation sites in melanin is proportional to its concentration (Fig. 5).

We propose that the interaction of DM with HNO_2 involves the oxidation of *ortho*-hydroquinone groups in the pigment, as described by Eqs. (5)–(7).

(5) $$QH_2 + HNO_2 \rightarrow QH^{\bullet} + {}^{\bullet}NO\ (+ H_2O)$$

(6) $$QH^{\bullet} + HNO_2 \rightarrow Q + {}^{\bullet}NO\ (+ H_2O)$$

the overall reaction being

(7) $$QH_2 + 2HNO_2 \rightarrow Q + 2{}^{\bullet}NO\ (+ 2H_2O)$$

The formation of new melanin radicals can be explained by reaction (5) and by comproportionation of hydroquinone and quinone groups in DM (Eq. (8)), which produces a new equilibrium characterized by the higher content of semiquinone radicals.

(8) $$QH_2 + Q \leftrightarrows 2Q^{\bullet-} + 2H^+$$

The proposed mechanism is supported by our observation that the exposure of a melanin precursor, such as DOPA, or related catechol(amine)s, to HNO_2 produces the respective semiquinone radicals (not shown). This mechanism is consistent with the published literature data on the oxidation of *para*- and *ortho*-hydroquinones by HNO_2 [29].

In addition to the oxidation of melanin hydroquinones directly by HNO_2, species derived from

HNO_2 decomposition might also be involved. In moderately acidic solutions HNO_2 exists in equilibrium with $^{\bullet}NO$ and $^{\bullet}NO_2$ radicals (Eqs. (2), (3)) and we considered the possibility that they also could react with DM.

Nitric oxide, an intermediate in HNO_2-dependent oxidation (Eqs. (5)–(7)), appears to be without any effect on the generation of melanin radicals. Results of our experiments, in which DEA/NO was used as $^{\bullet}NO$ donor, revealed that $^{\bullet}NO$ does not react chemically or physically with DM, unless air was present (double integrals of melanin EPR signals recorded in the absence and presence of the NO donor, DEA/NO (1 mM) were identical within experimental error). The requirement that oxygen be present in order to produce melanin radicals by DEA/NO can be explained assuming that the process is mediated by the *in situ* formed $^{\bullet}NO_2$ or/and $ONOO^-$ species. While $^{\bullet}NO_2$ does indeed react with melanin and efficiently oxidizes melanin generating new melanin radicals [30] (in aerated buffer (pH = 7.0) the generation of radicals in DM by DEA/NO is a very fast process), its role in the present system is rather inefficient because the rate of the NO_2 formation from $^{\bullet}NO$ and O_2 is slow, being second order in $^{\bullet}NO$ and first order in O_2 [31, 32]. In agreement with this we have found that the concentration of O_2 did not change appreciably during the reaction (not shown).

However, participation of the $^{\bullet}NO_2$ radical in melanin oxidation could not be verified under applied conditions. This aspect of melanin chemistry is under investigation.

The possibility of the involvement of peroxynitrite stems from the known fact that in aerated solutions DM reduces O_2 to $O_2^{\bullet-}$ (Eq. (1), (1A)), and that the $O_2^{\bullet-}$ radicals react very fast with $^{\bullet}NO$ to produce $ONOO^-$ (Eq. (4), [27]).

At acid pH the peroxynitrous acid (pK_a 6.8 [32]) could be formed from HNO_2 and H_2O_2 (Eqs. (10) and (11)).

(10) $$O_2^{\bullet-} + {}^{\bullet}O_2H + H^+ \rightarrow H_2O_2 + O_2$$

(11) $$HNO_2 + H_2O_2 \rightarrow ONOOH + H_2O \leftrightarrows 2\ ONOO^- + 2H^+$$

Peroxynitrite is a powerful oxidizing species, resembling in many respect $^{\bullet}OH$ radical [33]. The involvement of this species in DM oxidation by nitrite at low pH is suggested by the observation that CAT partially prevented the generation of melanin radicals (Fig. 6). In aerated solutions melanin slowly reduces O_2 to $O_2^{\bullet-}$, which subsequently dismutates to H_2O_2. This dismutation is accelerated in acidic pH, and as a result H_2O_2 and melanin coexist, even if the pigment consumes some H_2O_2 molecules [34]. Nitrous acid reacts with H_2O_2 to form peroxynitrous acid, ONOOH (Eq. (10)).

Although at the present time no exact mechanism(s) of the oxidation of DM by HNO_2 can be given, certain reaction pathways, based on the results of our experiments and the existing literature data, can be proposed. The proposed reaction mechanism (Eq. (7)) is consistent with the mechanism of oxidation by HNO_2 of *para*-hydrobenzoquinone [22] and catechols [35].

Conclusions

In conclusion, we have shown that the interaction of melanin with nitrite at acidic pH (pH <5.5) leads to melanin oxidation and is accompanied by the formation of large number of melanin radicals. The strong dependence of the radical formation on pH suggests that nitrous acid, HNO_2, and/or HNO_2-derived species are involved. The reaction occurs at the expense of melanin hydroquinone groups, which are converted into semiquinone radicals. As a result of this oxidation the concentration of the radicals in melanin could be markedly – more than fourfold – increased. This large increase in the radical content indicates that melanin contains a very high concentration of centers responsible for the deactivation of HNO_2.

Because melanin and the RNS may be present simultaneously in melanocytes, cells possessing the capacity of melanin synthesis, our studies suggest that the pigment could function as a natural scavenger of HNO_2 and related species *in vivo*. This property may be pertinent to the biological function of melanin. This suggestion is supported by the report that melanized *Cryptococcus neoformans* cells exposed to $NaNO_2$ in acidic media show significantly higher survival than nonmelanized cells [36].

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