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Further insights into the reaction of melatonin with hydroxyl radical

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Abstract

Recent interest has focused on the use of exogenous melatonin as an antioxidant, particularly to scavenge the highly cytotoxic hydroxyl radical ($HO^{\cdot}$) which may be generated in many pathological conditions. However, in vitro and in vivo studies aimed at assessing the antioxidant properties of melatonin have produced conflicting results. While it is known that $HO^{\cdot}$ reacts with melatonin at a diffusion limited rate, very little is known about the products of this reaction. In this investigation it is shown that incubation of melatonin with a Fenton-type $HO^{\cdot}$-generating system at pH 7.4 forms a complex mixture of primary products. These include 2-hydroxymelatonin, which was isolated as its more stable oxindole tautomer, 4- and 6-hydroxymelatonin, *N*-acetyl-N^2-formyl-5-methoxykynurenine and 7,7′-bi-(5-methoxy-*N*-acetyltryptamine-4-one). Reaction pathways that might lead to these products are described. The differing biological effects of these products, while currently incompletely understood, might account for the controversy concerning the antioxidant properties of melatonin.

Keywords: Melatonin; Hydroxyl radical; Antioxidant; Methamphetamine; MPTP

1. Introduction

Brain melatonin (*N*-acetyl-5-methoxytryptamine) is biosynthesized from 5-hydroxytryptamine (5-HT; serotonin) in the pineal gland [1,2]. Melatonin levels follow

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a cyclic pattern with peak concentrations being achieved at night [3]. This variation of melatonin concentration with time appears to be a major factor associated with the maintenance of circadian rhythmicity [4,5]. While endogenously formed melatonin may play a number of physiological roles, much recent interest has focused on its potential role as an antioxidant that scavenges and detoxifies reactive oxygen species (ROS), particularly the highly cytotoxic hydroxyl radical ($HO^{\cdot}$) [6,7], and reactive nitrogen species (RNS) such as peroxynitrite and nitric oxide [8,9]. However, while melatonin reacts very rapidly with $HO^{\cdot}$ [10] its physiological concentrations in blood plasma, cerebrospinal fluid and various tissues (<1 nM) [11–13] are much lower than those of other endogenous antioxidants such as glutathione (GSH), α-tocopherol, and ascorbate. Thus, it is unlikely that a major biological function of melatonin in vivo is as an antioxidant [9], particularly under pathological conditions in which excessive production of ROS and RNS contribute to tissue damage.

A number of in vitro and in vivo studies, however, have reported that exogenously administered melatonin provides protection against oxidative stress [14–17]. This, in part, may be due to the ability of melatonin to directly scavenge $HO^{\cdot}$ and other ROS but also by upregulation of endogenous antioxidant defense mechanisms. For example, melatonin increases the activity of glutathione peroxidase [18] and superoxide dismutase [19]. However, other studies suggest that melatonin is a relatively ineffective antioxidant [20–22] or may even have prooxidant properties [23].

There is substantial evidence that the selective nigrostriatal dopaminergic neurotoxicity of 1-methyl-4-phenylpyridinium (MPP^{+}), the active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in the brain, involves $HO^{\cdot}$ generation [24,25]. Furthermore, elevation of brain levels of $HO^{\cdot}$ scavengers such as GSH [26] or salicylate [27] protect against MPTP/MPP^{+}-induced neurotoxicity. Some studies also indicate that administration of large doses of melatonin to mice and rats fully [28,29] or partially protect dopaminergic neurons against MPTP/MPP^{+}-induced toxicity by scavenging $HO^{\cdot}$ [30]. However, other studies fail to demonstrate that melatonin protects against the neurotoxicity of MPTP/MPP^{+} [31–33]. Methamphetamine (MA) also evokes neurotoxic effects on nigrostriatal dopamine neurons and serotonergic terminals throughout the brain [34] by a mechanism that appears to involve $HO^{\cdot}$ generation [35,36]. Again, however, while some studies report that large doses of melatonin provide full [33] or partial [37] protection against the neurotoxic effects of MA, others suggest that melatonin exacerbates its dopaminergic and serotonergic neurotoxicity [38]. Reports that melatonin fails to block the neurotoxicity of MPTP/MPP^{+} [31–33], exacerbates the neurotoxic effects of MA [38] and may be a prooxidant [23] raise the possibility that under certain circumstances it may react with $HO^{\cdot}$ forming products that potentiate the neurotoxic effects of these drugs. Indeed, administration of high doses of melatonin to human cancer patients induces a form of eosinophilia-myalgia (ESM) [39,40], a syndrome associated with neurotoxic consequences in the brain [41]. It may also be of relevance that partially characterized oxidized forms of melatonin present as contaminants in commercial preparation of this drug may be associated with ESM [42].

Although $HO^{\cdot}$ attack on melatonin in vitro and in vivo forms a number of products, evidence for the structure of only one product, a cyclized form of 3-hydroxymelatonin (1,2,3,3a,8,8a-hexahydro-1-acetyl-5-methoxy-3a-hydroxypyrrolo[2,3-*b*]indole), has

been presented [43]. The present communication describes additional products formed by the HO·-mediated oxidation of melatonin.

2. Materials and methods

Melatonin, ethylenediaminetetraacetic acid (disodium salt, Na_2EDTA), ascorbic acid, 5-hydroxy-2-oxindole, and deuterated acetonitrile (CD_3CN) were obtained from Sigma (St. Louis, MO), ferrous ammonium sulfate from Mallinckrodt (St. Louis, MO), HPLC grade methanol (MeOH) and H_2O_2 (30% by weight) from EM Science (Gibbstown, NJ), HPLC grade MeCN from Fisher Scientific (Fairlawn, NJ) and trifluoroacetic acid (TFA) from Arcos (NJ).

2.1. Preparative HPLC

A Gilson (Middleton, WI) binary gradient HPLC system was used equipped with two model 712 pumps, 10 mL injection loop, preparative reversed phase column (Xpertek Bakerbond C-18, 250 × 22.5-mm, P.J. Cobert Associates, St. Louis, MO) and a Holochrome UV detector set at 254 nm.

HPLC method I was used for the initial separation of the products formed by the HO·-mediated oxidation of melatonin. Solvent A was prepared by adding 100 mL of MeCN to 900 mL of deionized water; the pH of the resulting solution was then adjusted to 2.4 with concentrated TFA. Solvent B was prepared by adding 100 mL of MeOH and 400 mL of MeCN to 500 mL of deionized water; the pH of this solution was then adjusted to 2.4 with TFA. The following gradient was used: 0–3 min, linear gradient from 100% solvent A to 50% solvent B; 3–10 min, linear gradient to 60% solvent B; 10–20 min, linear gradient to 100% solvent B; 21–25 min, 100% solvent B. The flow rate was 10 mL min^{-1}.

Individual products of the HO·-mediated oxidation of melatonin were further purified using HPLC method II. This method differed from HPLC method I only with respect to the mobile phase gradient employed which was: 0–3 min, 100% solvent A; 3–53 min, linear gradient to 100% solvent B; 53–60 min, 100% solvent B. The flow rate was 10 mL min^{-1}.

2.2. Hydroxyl radical-mediated oxidation of melatonin

A stock solution of melatonin (7.6 mM) was prepared by dissolving 52.86 mg of the solid in 30 mL of deionized water with gentle heating and stirring. This stock solution was stable for at least one week when stored in the dark at 4 °C.

A recycling Fenton reaction system developed by Udenfriend et al. [44] was employed to generate HO· which employed a freshly prepared solution of ascorbic acid (9.5 mM), Na_2EDTA (26.6 mM), ferrous ammonium sulfate (2.0 mM), and H_2O_2 (8.70 mM). The sequence of additions and final concentrations for these reagents was as follows: 7.8 mL of pH 7.4 phosphate buffer (50 mM), 1.5 mL of melatonin stock solution (0.8 mM), 1.5 mL of ascorbic acid solution (1.0 mM), 0.15 mL Na_2EDTA (0.28 mM), 1.5 mL Fe^{2+} (0.21 mM) and 1.8 mL of H_2O_2 solution (1.1 mM) to give a

total volume of 14.25 mL. The resulting solution was stirred at ambient temperature for 15 min. Preliminary studies revealed that the latter reaction time was optimal for generation of primary reaction products of the HO·-mediated oxidation of melatonin with minimal secondary reactions which occurred at longer reaction times.

The reaction mixture was filtered through a Millipore (Bedford, MA) type HA (0.45 μm) filter and then injected into the preparative HPLC system and products initially separated using method I. Fractions eluted under each chromatographic peak were collected separately and immediately frozen in a dry-ice/acetone bath. In order to obtain sufficient amounts of each product to permit spectroscopic structure elucidation and other studies, it was necessary to carry out the preceding procedure >50 times. Combined fractions collected under each peak were then freeze-dried and then redissolved in the minimum volume of solvent A and purified using preparative HPLC method II. This latter process was usually repeated several times in order to obtain pure fractions. The solution containing each purified compound was then freeze-dried and subsequently stored in a dessicator at −80 °C.

2.3. Spectroscopy

Electrospray ionization mass spectrometry (ESMS) was carried out on either a Micromass (Beverly, MA) Q-TOF or PE Biosystems (Foster City, CA) Sciex API III spectrometer. ^{1}H NMR spectra were obtained on a Varian (Palo Alto, CA) XL-400 spectrometer. UV–visible spectra were recorded on a Hewlett-Packard (Palo Alto, CA) model 8452A diode array spectrophotometer.

Spectroscopic evidence in support of the proposed structures of major products formed following the HO·-mediated oxidation of melatonin is presented below. The assignments of proton resonances observed in ^{1}H NMR spectra of melatonin and its reaction products were confirmed by two-dimensional correlated spectroscopy (COSY) experiments.

2.4. Melatonin

The UV spectrum of melatonin dissolved in 5% aqueous MeOH at pH 7.4 gave bands at $\lambda_{max} = 295$ (sh), 278 and 228 nm. ESMS gave $m/z = 233$ (MH^+, 100%). ^{1}H NMR (CD_3CN) gave δ: 8.96 (bs, 1H, N(1)–H), 7.29 (d, 1H, $J = 8.8$ Hz, C(7)–H), 7.09 (d, 1H, $J = 2.4$ Hz, C(4)–H), 7.07 (d, 1H, $J = 2.0$ Hz, C(2)–H), 6.79 (dd, 1H, $J = 8.8$ Hz, $J = 2.4$ Hz, C(6)–H), 6.42 (bs, 1H, side-chain N–H), 3.82 (s, 3H, OCH_3), 3.42 (m, 2H, C(β)–H_2), 2.86 (t, 2H, C(α)–H_2), and 1.84 (s, 3H, side-chain –$COCH_3$).

2.5. 6-Hydroxymelatonin

This compound was isolated as an off-white solid that, when dissolved in pH 7.4 phosphate buffer, exhibited a UV spectrum with bands at $\lambda_{max} = 300$, 280 (sh) and 220 nm. ESMS gave $m/z = 249$ (MH^+, 100%). ^{1}H NMR (CD_3CN) gave δ: 8.76 (bs, 1H, N(1)–H), 7.10 (s, 1H, C(7)–H), 6.91 (d, 1H, $J = 2.4$ Hz, C(2)–H), 6.85 (s, 1H, C(4)–H), 6.40 (bs, 1H, side-chain N–H), 3.90 (s, 3H, OCH_3), 3.41 (m, 2H, C(β)–H_2), 2.83 (m, 2H, C(α)–H_2), 1.84 (s, 3H, side-chain –$COCH_3$).

2.6. 5-Methoxy-3-(N-acetyl)-ethylamino-2-oxindole (5-MeO)

This compound was isolated as a white solid. At pH 7.4 it exhibited a UV spectrum with bands at $\lambda_{max} = 298$ and 258 nm. ESMS gave $m/z = 249$ (MH^+, 100%). ^1H NMR(CD_3CN) gave δ: 8.34 (s, 1H, N(1)–H), 6.97 (d, 1H, $J = 2.4$ Hz, C(4)–H), 6.81 (d, 1H, $J = 8.4$ Hz, C(7)–H), 6.77 (dd, 1H, $J = 8.4$, 2.4 Hz, C(6)–H), 6.58 (bs, 1H, side-chain N–H), 3.76 (s, 3H, $–OCH_3$), 3.42 (t, 1H, $J = 6.0$ Hz, C(3)–H), 3.30 (m, 2H, C(β)–H_2), 2.01 (m, 2H, C(α)–H_2), 1.83 (s, 3H, side-chain $–COCH_3$).

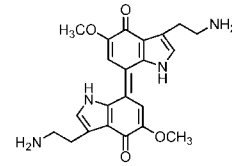
2.7. 4-Hydroxymelatonin

This compound was isolated as an off-white solid. In pH 7.0 phosphate buffer it had a UV spectrum with bands at $\lambda_{max} = 308$ and 238 nm. ESMS gave $m/z = 249$ (MH^+, 100%). It was not possible to isolate sufficient quantities of this compound to obtain a reliable ^1H NMR spectrum. However, its molecular mass (248 g) clearly indicates that this compound is a monohydroxylated derivative of melatonin. Since the keto tautomer of 2-hydroxymelatonin (i.e., 5-MeO) and 6-hydroxymelatonin were unequivocally identified, it seems probable that this compound is 4-hydroxymelatonin. The UV spectrum of this compound is also very similar to that of 4,5-dihydroxytryptamine (λ_{max} at pH 7.0 = 304 and 220 nm) and 7-*S*-glutathionyl-4,5-dihydroxytryptamine [45,46] providing additional indirect support for the conclusion that it is 4-hydroxymelatonin.

2.8. 7,7′-Bi-(5-methoxy-N-acetyltryptamine-4-one) (7,7′-D)

This product was isolated as a dark brown/purple solid that gave bright pink–purple solutions between pH 2–8. The UV–visible spectrum of this compound dissolved in MeOH gave bands at $\lambda_{max} = 566$, 378, 302 and 226 nm. ESMS of a freshly purified solution of this compound (HPLC method I) gave $m/z = 493$ (MH^+, 100%). Attempts to obtain a ^1H NMR spectrum of this compound, however, were unsuccessful owing to its very low solubility and instability in all solvents employed. However, in recent studies, to be described in detail elsewhere, we have isolated and fully characterized a structurally related compound formed from tryptamine-4,5-dione in acidic MeOH solution, 7,7′-bi-(5-methoxytryptamine-4-one). The UV–visible spectrum of 7,7′-bi-(5-methoxytryptamine-4-one) in MeOH, $\lambda_{max} = 554$, 372, 296 and 226 nm is virtually identical to that of 7,7′-D. Furthermore, the molar mass of 7,7′-D (492 g) differs from that of 7,7′-bi-(5-methoxytryptamine-4-one) (408 g) by that expected for two acetyl residues substituted on the ethylamino side-chains of the former compound. We have also synthesized a compound with identical properties to 7,7′-D utilizing the same method employed to obtain 7,7′-bi-(5-methoxytryptamine-4-one). Briefly, *N*-acetyl-5-hydroxytryptamine (30 mg) was oxidized with potassium nitrosodisulfonate (60 mg) in 20 mL of deionized water for 3 h. The product of this oxidation, *N*-acetyltryptamine-4,5-dione (~11 mg), was purified by preparative HPLC method II and then freeze-dried. The latter dry product was dissolved in MeOH (5 mL) containing 50 μL of concentrated TFA and stirred for 45 min. The resultant product solution was injected into the preparative HPLC

system and separated using HPLC method I. The major product of this reaction had an identical retention time (t_R), UV–visible and ESMS spectra to 7,7′-D.



7,7′-bi-(5-methoxytryptamine-4-one)

2.9. *N-Acetyl-N²-formyl-5-methoxykynurenamine (AFMK)*

This compound was isolated as a pale yellow solid which in solution at pH 7.4 exhibited a UV–visible spectrum with bands at $\lambda_{max} = 342$, 262 (sh) and 234 nm. ESMS gave $m/z = 265$ (MH^+, 100%). These properties are in agreement with those expected for AMFK [47].

3. Results

An HPLC chromatogram (preparative method I) of the product mixture formed following incubation of melatonin (0.8 mM) for 15 min with the HO·-generating system employed is presented in Fig. 1. This chromatogram reveals that the HO·-mediated oxidation of melatonin at pH 7.4 forms a complex mixture of products. Preliminary studies revealed that all of the major product peaks in the chromatogram shown in Fig. 1 were present when the reaction solution was sampled within 1 min after addition of the final reactant (H_2O_2) that initiates HO· generation. During the succeeding 15 min each of these major product peaks reached their maximal height. After longer periods, however, secondary reactions began to occur as indicated by the decline of some major product peaks and the appearance of new peaks in chromatograms. Accordingly, in order to gain some insights into the initial steps in the HO·-mediated oxidation of melatonin, several major products formed after a 15 min incubation with the HO·-generating system were isolated and identified.

The major compound eluted under the peak having a t_R of 15 min (Fig. 1), using HPLC method I, was 4-hydroxymelatonin. However, subsequent chromatography of the species eluted under this peak using HPLC method II revealed that 4-hydroxymelatonin was contaminated with two additional compounds although these were not formed in sufficient quantities to permit their isolation and spectroscopic identification. Similarly, the peak at eluting $t_R = 19$ min (Fig. 1) could be resolved into one major component, AMFK, and two unidentified minor components using HPLC method II.

The molar mass of the compound eluted at $t_R = 17$ min was 248 g indicating that it contains one more oxygen substituent than melatonin (molar mass 232 g). The UV spectrum of this compound was virtually identical to that of 5-hydroxy-3-ethylamino-2-oxindole (5-HEO), the major product of the HO·-mediated oxidation of 5-HT at pH 7.4 [48], and an authentic sample of 5-hydroxy-2-oxindole. Furthermore,

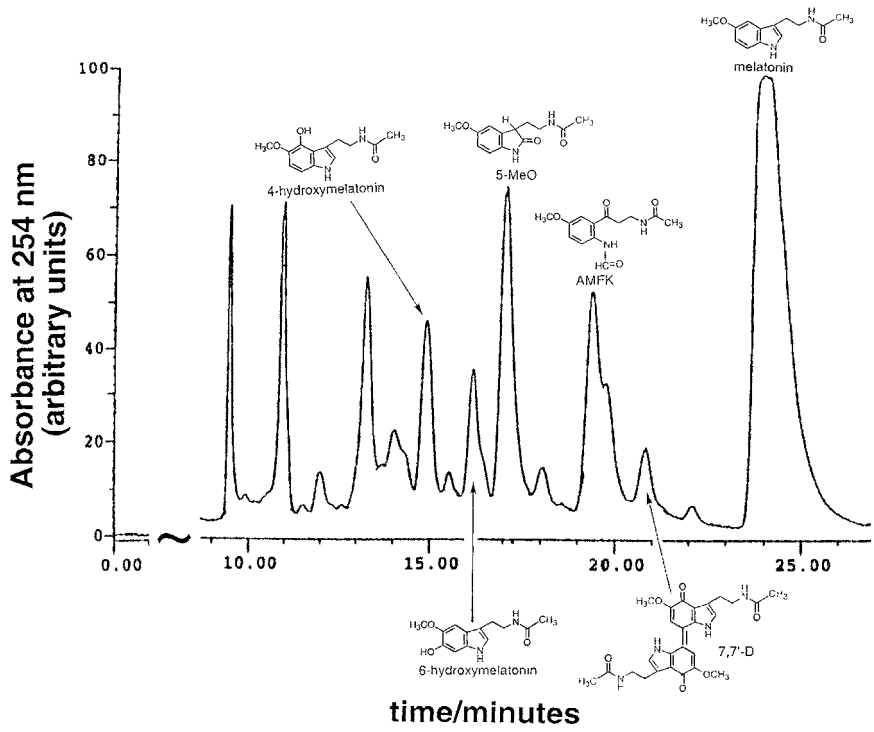
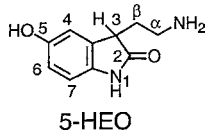


Fig. 1. HPLC chromatogram (preparative method I) of the product mixture formed following incubation of melatonin (0.8 mM) with a recycling Fenton-type HO· generating system.

the characteristic triplet (1H, 3.42 ppm) observed in the ^{1}H NMR spectrum of the compound eluted at $t_R = 17$ min and in that of 5-HEO (1H, 3.52 ppm), which are both coupled to the side-chain C(β)–H_2 protons, clearly indicates that both compounds possess a hydrogen atom substituted at C(3). Furthermore, there are no changes in the N(1)–H, side-chain N–H, C(α)–H_2 and C(β)–H_2 resonances of 5-MeO compared to melatonin indicating that free rotation of the *N*-acetylethylamino side-chain is preserved thus excluding the possibility of a cyclized structure. Taken together, the mass, NMR and UV spectra of the compound that eluted at $t_R = 17$ min and their similarity to those of 5-HEO indicate that the former is the keto tautomer of 2-hydroxymelatonin, i.e., 5-MeO.



5-HEO

The ESMS of the compound eluted under the peak at $t_R = 21$ min (Fig. 1) indicates it has a molar mass of 492 g suggesting it consists of two residues formed by an

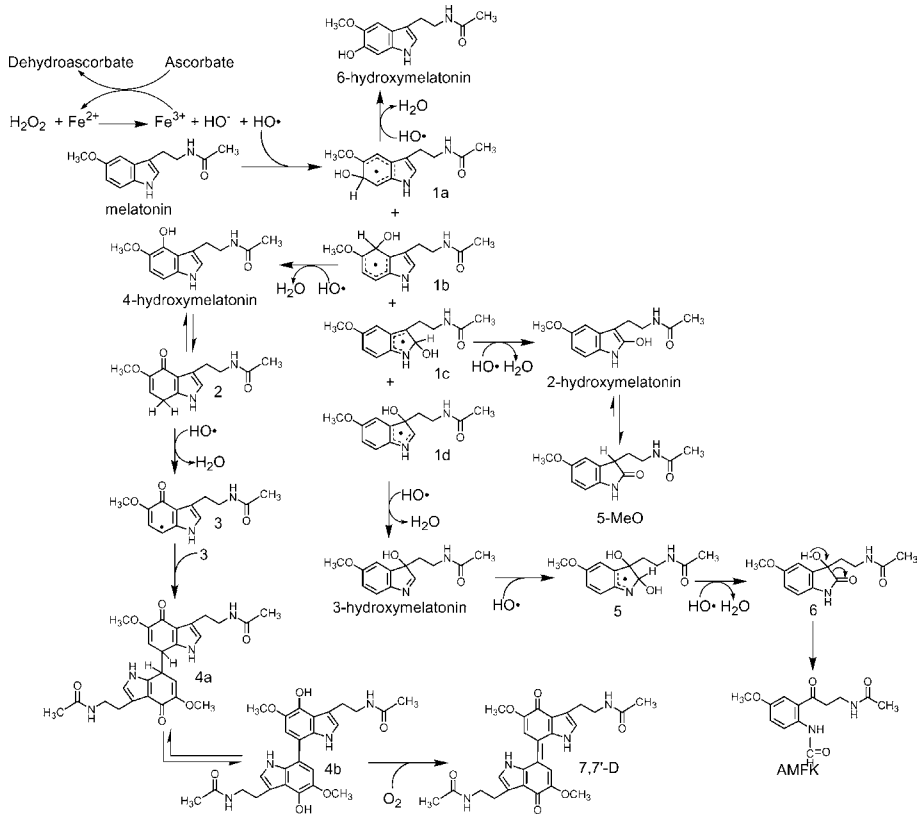
overall $2e/2H^+$ oxidation of a hydroxymelatonin (molar mass 248 g). Unfortunately, attempts to obtain the ^1H NMR spectrum of this compound were not successful. Nevertheless, comparison of the spectral properties of this compound with those of 7,7′-bi-(5-hydroxytryptamine-4-one) and an independent synthesis (see Materials and methods) supports the proposed structure of 7,7′-D.

Attempts to isolate and characterize the products eluted under the peaks prior to 4-hydroxymelatonin (Fig. 1) have not yet been successful owing to their instability.

4. Discussion

Despite a growing, albeit controversial, literature concerning the antioxidant properties of melatonin, surprisingly little is known about the chemistry of its reaction with HO·, the most cytotoxic ROS. Previous studies have established that HO· reacts very rapidly with melatonin in aqueous solution ($k = 2.7 \times 10^{10}\,M^{-1}\,s^{-1}$) [10] although evidence for only a single product of this reaction, a cyclized form of 3-hydroxymelatonin, is available [43]. The present investigation demonstrates that the HO·-mediated oxidation of melatonin in buffered aqueous solution at pH 7.4 results in formation of a complex mixture of primary products. The products that have been identified are clearly formed as a result of hydroxylation of the indolic ring system of melatonin. Monohydroxylated products are 2-hydroxymelatonin (isolated as its more stable 2-oxindole tautomer, 5-MeO), 4- and 6-hydroxymelatonin. Hardeland et al. [49] have proposed a mechanism for the initial reaction of melatonin with HO· in which an electron is abstracted from the aromatic indolic ring system to form a radical cation intermediate. However, a subsequent study concluded that such a reaction is not thermodynamically feasible [50]. More probable pathways involve either abstraction of an indolic hydrogen by HO·, to form a neutral radical intermediate, or direct addition of HO· to the indolic moiety [50]. The latter reaction has been proposed for the attack of HO· on catechols [51] and 5-HT [48]. Thus, it is proposed that HO· initially attacks melatonin to form the neutral radicals **1a**–**1d** (Scheme 1). Subsequent hydrogen atom abstraction from **1a**, **1b**, and **1c** by a second HO·, with elimination of H_2O, results in formation of 6-hydroxy-, 4-hydroxy-, and 2-hydroxymelatonin, respectively, the latter tautomerizing to 5-MeO. In order to account for formation of AMFK, it seems plausible to suggest that the initial attack by HO· on melatonin forms radical **1d** in which a hydroxyl group is added to the C(3)-position. Subsequent hydrogen atom abstraction by HO· would then form 3-hydroxymelatonin (Scheme 1). The latter compound is a possible precursor of cyclized 3-hydroxymelatonin [44] although in the present study neither 3-hydroxymelatonin nor its cyclized forms were identified as a primary product of the HO·-mediated oxidation of melatonin. Attack by HO· on 3-hydroxymelatonin would be expected to form radical **5**, containing a second hydroxyl substituent at C(2), which is further oxidized by HO· to 2-keto-3-hydroxymelatonin (**6**, Scheme 1). Facile ring opening of **6** would then account for formation of AMFK.

A plausible route leading to formation of 7,7′-D involves hydrogen atom abstraction by HO· from 4-hydroxymelatonin forming radical intermediate **3** which



Scheme 1.

dimerizes to **4a/4b**. Subsequent oxidation of **4** by molecular oxygen would then form 7,7′-D (Scheme 1).

Previous studies have reported that 6-hydroxymelatonin is the major metabolite of melatonin in the liver and kidney [52] whereas in brain melatonin is metabolized to AMFK and thence to *N*-acetyl-5-methoxykynurenamine [53]. The present investigation demonstrates for the first time that 6-hydroxymelatonin and AMFK are also formed by non-enzymatic oxidation of melatonin by $HO^\bullet$.

As noted earlier, there is considerable controversy in the literature concerning the antioxidant properties of melatonin. Such controversies are particularly evident in studies aimed at assessing the protective effects of melatonin against the neurotoxicity of MPTP/MPP^+ and MA in which $HO^\bullet$ generation appears to be an important participant in the neurotoxic mechanism evoked by these drugs [24,35,36]. One product of the $HO^\bullet$-mediated oxidation of melatonin, AMFK, based on the present investigation, might be expected to be formed in the brain in response to MPTP/MPP^+ and MA administration. Furthermore, AMFK has been shown to be a potent antioxidant [54]. Thus, formation of AMFK would be expected to potentiate the neuroprotective properties of melatonin. However, another product of the

HO·-mediated oxidation of melatonin, 6-hydroxymelatonin, can function not only as a scavenger of HO· but also as a promoter of HO· generation [10]. Thus, in neutral aqueous solution in the presence of Fe^{2+} and molecular oxygen, 6-hydroxymelatonin can generate large quantities of HO· [10]. By analogy, it is conceivable that another product of the HO·-mediated oxidation of melatonin, 4-hydroxymelatonin, may also potentiate HO· generation by similar reaction pathways. Because of its quinoidal structure, 7,7′-D would also be expected to redox cycle in the presence of endogenous reductants such as ascorbate and/or glutathione with resultant generation of ROS such as $O_2^{-\cdot}$, H_2O_2 and thence HO· by Fenton/Haber–Weiss-type chemistry [44]. Thus, melatonin might potentiate oxidative stress as a consequence of formation of products that mediate ROS generation. Such chemistry may contribute to the controversial results concerning the antioxidant properties of melatonin. It may also be of relevance that oxindoles structurally similar to 5-MeO can evoke a number of pathophysiological effects in vivo including impairment of locomotor activity, hypotension, loss of righting reflex and coma [55]. Furthermore, incompletely identified hydroxylated forms of melatonin that contaminate commercial preparations of this compound have been case-implicated with ESM [42] and possible neurotoxic consequences [41]. Taken together, the controversies concerning the antioxidant properties of melatonin in vitro and in vivo may be dependent on a variety of factors including the doses employed and the nature and relative yields of products formed consequent to its reactions with HO· or other ROS and RNS.

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