Reaction of Peroxynitrite with Melatonin: A Mechanistic Study

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The pH profile of the peroxynitrite/melatonin reaction suggests that both peroxynitrous acid (ONOOH) and its anion ($ONOO^-$) are reactive toward melatonin, but at physiological pH most of the reaction with melatonin involves ONOOH and the activated form of peroxynitrous acid (ONOOH*). The formation of hydroxylated products (mainly 6-hydroxymelatonin) suggests that melatonin also reacts with ONOOH*. The overall peroxynitrite/melatonin reaction is first-order in melatonin and first-order in peroxynitrite, but the hydroxylation of melatonin is presumed to be zero-order in melatonin. Melatonin is metabolized in the liver, mainly to 6-hydroxymelatonin, so we do not think this metabolite is a useful biomarker for melatonin's antioxidant activity; however, 6-hydroxymelatonin is a better chain-breaking antioxidant than melatonin and may contribute to the beneficial effects of melatonin in vivo. As is now well-known, CO_2 modulates the reactions of peroxynitrite. The reaction of peroxynitrite with melatonin in the absence of added bicarbonate produces mainly 6-hydroxymelatonin and 1,2,3,3a,8,8a-hexahydro-1-acetyl-5-methoxy-8a-hydroxypyrrolo[2,3-*b*]indole, with some isomeric 1,2,3,3a,8,8a-hexahydro-1-acetyl-5-methoxy-3a-hydroxypyrrolo[2,3-*b*]indole. In the presence of added bicarbonate, product yields decrease and 6-hydroxymelatonin is not formed. These facts suggest that melatonin scavenges reactive species (such as $CO_3^{\bullet-}$ and $^{\bullet}NO_2$) that are produced from the peroxynitrite/CO_2 reaction. The spectrum of the melatoninyl radical cation is observed both in the absence and in the presence of added bicarbonate, suggesting that the melatoninyl radical cation is the initial product and the hydroxypyrrolo[2,3-*b*]indole products are derived from it. Unlike tyrosine, where both nitrated and hydroxylated products can be isolated, nitromelatonin is not found in the final products from the melatonin/peroxynitrite reaction in either the absence or presence of added bicarbonate. However, we suggest that 2-hydroxy-3-nitro- and/or 2-hydroxy-3-peroxynitro-2,3-dihydromelatonin are formed as intermediates and subsequently decompose to give 1,2,3,3a,8,8a-hexahydro-1-acetyl-5-methoxy-8a-hydroxypyrrolo-[2,3-*b*]indole. Since peroxynitrite/CO_2 governs the reactions of peroxynitrite in vivo, we suggest that the hydroxypyrrolo[2,3-*b*]indole products are the main products from the oxidation of melatonin by peroxynitrite-derived species in vivo, and that these products may serve as indexes for melatonin's antioxidant activity.

Introduction

Peroxynitrite,[1] which is formed in vivo via the coupling of NO and $O_2^{\bullet-}$, is able to oxidize a variety of biomolecules, including thiols, lipids, ascorbate, DNA, sulfides, and aromatic species (*1*−*7*). These reactions can involve the transfer of either one or two electrons, and can be either zero- or first-order in substrate (*8*). However, in aerobic biological systems, the reactions of peroxynitrite are generally modulated by CO_2 due to the fast reaction of peroxynitrite with CO_2 and the abundance of CO_2 (*9*−*12*), and few biomolecules can compete with CO_2 for peroxynitrite (*13*, *14*).

Melatonin (MLT[2]), the main secretory product of the pineal gland, has been proposed to protect against damage caused by radicals in vivo (*15*−*20*). Melatonin also has been reported to cause a dose-dependent inhibition of the oxidation of dihydrorhodamine 123 by peroxynitrite (*21*), suggesting that it is a scavenger of peroxynitrite. Recently, we reported the observation of the spectrum of the melatoninyl radical cation ($MLT^{\bullet+}$) during the peroxynitrite/MLT reaction, providing direct evidence of an initial one-electron transfer from MLT to peroxynitrite (*22*). The reaction of MLT with peroxynitrite does not yield nitromelatonin in either the absence or presence of CO_2 (*22*), despite the fact that CO_2 is known to catalyze nitrations by peroxynitrite (*9*−*11*, *23*, *24*).

Herein we report kinetic studies of the reaction of MLT with peroxynitrite in the absence and presence of carbon dioxide. The possible mechanisms and the biological relevance of these reactions also are discussed in terms of the reaction kinetics and product analyses.

Experimental Procedures

Materials. MLT (97%) and C18 TLC plates (10 cm × 10 cm) were purchased from Aldrich (Milwaukee, WI), and sodium

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[1] The sum of ONOOH and $ONOO^-$ is termed peroxynitrite, and the sum of CO_2, H_2CO_3, HCO_3^-, and CO_3^{2-} is termed bicarbonate unless otherwise indicated.

[2] Abbreviations: MLT, melatonin; 3-HO-MLT, 3-hydroxymelatonin; 6-HO-MLT, 6-hydroxymelatonin.

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azide and melatonin were from Sigma (St. Louis, MO). Deionized water (>15 MΩ/cm) was used in the preparation of buffers and reagents.

Instrumentation. The stopped-flow instrument that was used for the measurement of reaction rates was from On-Line Instrument Systems (Bogart, GA), and it was equipped with a UV/vis rapid scan spectrophotometer and a 2 cm cell. The Sander 200 ozonizer was from Erwin Sander (Uetze-Eltze, Germany). The HP8451A diode array UV/vis spectrophotometer with a 1 cm cell was from Hewlett-Packard (Willmington, DE).

The GC/MS model HP5890/5973 apparatus was from Hewlett-Packard. The column that was used was a SGE BPX5 column (25 m); the temperatures of the injection port and detector were 250 and 280 °C, respectively. The column was initially set at 200 °C (5 min), and then the temperature was increased to 230 °C (10 min) and finally to 250 °C (5 min) at speeds of 10 °C/min.

Synthesis of Peroxynitrite. Peroxynitrite was synthesized by the ozonation of an aqueous solution of sodium azide (*25*, *26*). Briefly, 0.25 g of sodium azide was dissolved in 25 mL of water preadjusted to pH 11 using 1 N sodium hydroxide. After cooling to <5 °C, the solution was ozonized by feeding oxygen to the ozonator at a flow rate of 100 mL/min. The concentration of peroxynitrite was monitored by measuring the A_{302nm} of samples diluted 100 times using pH 11 water. It requires about 60 min for the absorbance to reach the maximum (ca. 1.1), and the ozonation was stopped after the absorbance decreased to ca. 0.9 (*25*, *26*). The resulting solution was purged for 10 min with nitrogen and then stored at −12 °C until it was used.

Kinetics. Peroxynitrite solutions (pH 11) were mixed on the stopped-flow spectrophotometer with equal volumes of 50 mM phosphate buffer containing varying amounts of MLT (ionic strength adjusted to 0.3 M using NaCl). The pH values were measured both before and after mixing of the reactants. A small pH jump (ca. 0.15) was usually observed on mixing, and the final pH was reported unless otherwise stated. The temperature was maintained to within ±0.1 °C.

For pseudo-first-order reactions, the molar ratio of MLT to peroxynitrite was greater than 4:1. The decay of peroxynitrite in the absence of MLT is usually analyzed at 302 nm, but since MLT absorbs at this wavelength, the peroxynitrite/MLT reaction was analyzed at 400 nm where the interference from both peroxynitrite and MLT is minimal. Data are the average of at least three runs.

Preparation of Melatonin Solutions. Melatonin has a relatively low solubility. To prepare 25 mL of a 10 mM aqueous MLT solution, MLT (58 mg) was dissolved in 1.5 mL of 5 N HCl and the resulting solution diluted with 0.05 M phosphate buffer and then adjusted to the desired pH using 50% NaOH. The samples were monitored by ^{1}H NMR, and it was found that MLT is stable during this procedure. However, to minimize autoxidation, only freshly prepared solutions of MLT were used in these investigations.

Product Analysis. Aliquots of peroxynitrite (1 mL each) were flow-mixed with an equal volume of 0.05 M phosphate buffer (pH 6.85−7.20) containing various amounts of MLT and sodium bicarbonate. The reaction mixtures were incubated for 20 min and then dried on a rotary evaporator at 60 °C. The residue was extracted with methanol, and the methanolic extracts were analyzed by GC/MS and/or ^{1}H NMR.

Reactions at Preparative Scale. Stock solutions of peroxynitrite (ca. 50 mM) were added dropwise to 2 mL of 0.05 M phosphate buffer (pH 7.2) containing 4 mM MLT and saturated sodium bicarbonate until an overall molar ratio of peroxynitrite to MLT of 5:1 was reached. Throughout the course of addition of peroxynitrite, the contents were constantly stirred using a vortex mixer. The reaction mixture were dried on a rotary evaporator at 60 °C; the residue was extracted into methanol, and the products were separated by reversed phase TLC (C_{18} silica developed with water/methanol in a ratio of 80:20).

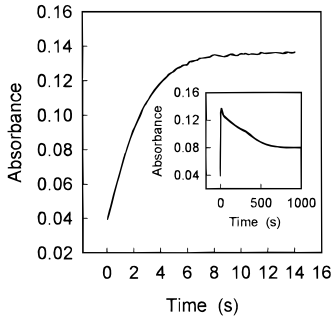
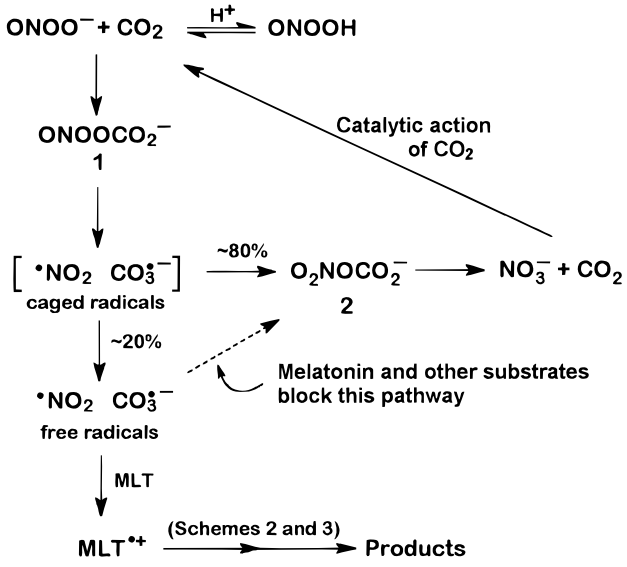


Figure 1. Typical kinetic trace for the reaction of peroxynitrite with melatonin (MLT). The reaction was carried out using a stopped-flow apparatus at 25.0 ± 0.1 °C by mixing equal volumes of peroxynitrite solution with a buffer solution containing MLT. After the mixing but before the reaction, the mixture contained 0.125 mM peroxynitrite and 4.9 mM MLT. The pH measured at the outlet was 7.0. The curve was fitted to the first-order formation equation to calculate k_{obs} values (see the inset of Figure 2). Data are shown at 400 nm. (Inset) Data for the same reaction as observed with longer times.

Scheme 1



Results

Kinetics. The stopped-flow reaction kinetics analyzed at 400 nm suggest that the peroxynitrite/MLT reaction occurs in two stages. In the first stage, the absorbance increases rapidly to a maximum, revealing the formation of intermediates derived from melatonin (Figure 1). In the second stage, the absorbance decreases slowly, showing the decay of these intermediates (inset of Figure 1). As we determined previously (*22*), the melatoninyl radical cation is the main intermediate; however, the reaction of melatonin with peroxynitrite is complex, and we cannot rule out small contributions of secondary intermediates, such as unstable melatonin adducts (Scheme 1), to the absorbance.

The spontaneous decomposition of peroxynitrite cannot be followed in the presence of MLT due to the overlap of absorption of MLT and peroxynitrite (see Experimental Procedures), and it is only possible to measure the rate of formation of products at 400 nm. Since the reactions were performed under pseudo-first-order conditions, the observed rate constant, k_{obs}, was calculated by fitting the rising phase of the kinetic traces (the first stage) to an equation that is first-order in peroxynitrite. At a given

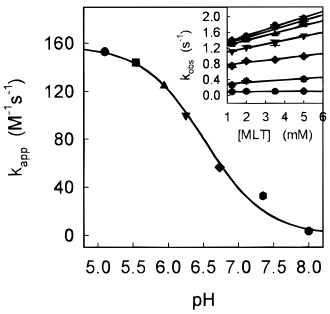


Figure 2. Influence of pH on the peroxynitrite/melatonin reaction. The plotted values of k_{app} are calculated from the slopes of the lines shown in the inset. Nonlinear fitting of k_{app} to eq 6 gives the value of the second-order rate constants and the acidity constant of peroxynitrite ($k_2 = 159 \pm 5$ M^{-1} s^{-1}, $k_2' = 5 \pm 5$ M^{-1} s^{-1}, and $pK_{PN} = 6.5$). (Inset) The reactions were performed on the stopped-flow instrument (see the legend of Figure 1). After the mixing but before the reaction, the mixture contained 0.25 mM peroxynitrite and 0, 1.25, 2.0, 3.5, or 5.0 mM melatonin. The pH values as measured at the outlet were 5.10, 5.54, 5.94, 6.25, 6.73, 7.35, and 8.00. For other reaction conditions, see the legend of Figure 1.

pH, k_{obs} increases linearly with the concentration of MLT, indicating that the reaction also is first-order in MLT (inset of Figure 2). The reactions were performed using relatively high concentrations of MLT to minimize the influence of adventitious CO_2. [CO_2 modulates the reactions of peroxynitrite (*13*, *14*, *23*, *24*).] Equation 1 depicts the acid dissociation of peroxynitrous acid ($pK_{PN} = 6.8$), and eq 2 depicts the overall spontaneous decomposition of peroxynitrite.

$$ONOOH \overset{K_{PN}}{\rightleftharpoons} H^+ + ONOO^- \quad (1)$$

$$ONOOH \xrightarrow{k_1} H^+ + NO_3^- \quad (2)$$

Thus, the observed rate constant for the formation of products described in eqs 3 and 4 can be written as shown in eq 5, where the constant C reflects the zero-order component due to the reaction of ONOOH* with MLT (see below).

$$ONOOH + MLT \xrightarrow{k_2} \text{products} \quad (3)$$

$$ONOO^- + MLT \xrightarrow{k_2'} \text{products} \quad (4)$$

$$k_{obs} = \frac{k_2[H^+] + k_2'K_{PN}}{[H^+] + K_{PN}}[MLT] + C \quad (5)$$

For the sake of convenience, we define an apparent second-order rate constant, k_{app}, in eq 6.

$$k_{app} = \frac{k_2[H^+] + k_2'K_{PN}}{[H^+] + K_{PN}} \quad (6)$$

The value of k_{app} is pH-dependent and was calculated from the slopes of the plots such as those shown in the inset of Figure 2. In eqs 3 and 4, it is assumed that both ONOOH and $ONOO^-$ react with MLT; see below. The second-order rate constant, k_2 (for the ONOOH/MLT reaction), k_2' (for the $ONOO^-$/MLT reaction), and the acidity constant of peroxynitrite, pK_{PN}, are determined to be 159 M^{-1} s^{-1}, 5 M^{-1} s^{-1} and 6.5, respectively (Figure 2), by nonlinear fitting of k_{app} to eq 6 at different pHs.

Table 1. Rate and Equilibrium Constants

constant	value	comment
k_1	1.34 s^{-1}	rate constant for the spontaneous decomposition of peroxynitrite (*31*)
k_2	159 ± 5 M^{-1} s^{-1}	rate constant for the ONOOH/MLT reaction
k_2'	5 ± 5 M^{-1} s^{-1}	rate constant for the $ONOO^-$/MLT reaction
pK_{PN}	6.5	acidity constant of ONOOH based on the k_{app} for the peroxynitrite/MLT reaction

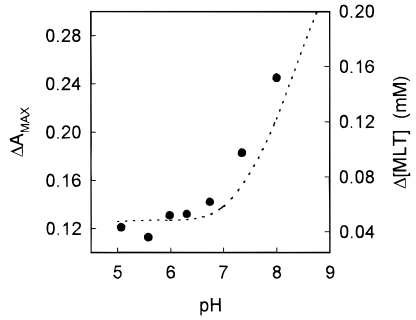


Figure 3. Influence of pH on product formation. The circles represent the values of ΔA_{max} (the difference between the initial absorbance and the absorbance at the maximum in a plot like that shown in Figure 1) which are shown for the reaction of 0.25 mM peroxynitrite with 2.0 mM MLT performed on the stopped-flow instrument. The dotted line represents the loss of MLT (Δ[MLT]) calculated using eq 8, with a pK_{PN} of 6.5, a k_1 of 1.34 s^{-1}, a k_2 of 159 M^{-1} s^{-1}, and a k_2' of 5 M^{-1} s^{-1}.

The rate and equilibrium constants discussed here and in later sections are summarized in Table 1.

Influence of pH on Product Yields. Surprisingly, the value of ΔA_{max} ($\Delta A_{max} = A_{max} - A_{t=0}$; see Figures 1 and 3) increases with pH, as shown in Figure 3 (circles) despite the fact k_{app} decreases with pH. This reflects the complexity of the peroxynitrite/MLT reaction and suggests the peroxynitrite anion also reacts at basic pH. A mechanism that can explain these data is given below.

Activation Parameters. Activation parameters were obtained using the Eyring equation (eq 7) applied to k_{app} at pH 6.0, and the study was conducted by stopped flow by varying the temperature from 7 to 45 °C (Figure 4).

$$\ln\left(k_{app}\frac{[H^+] + K_{PN}}{[H^+]}\frac{h}{k_B T}\right) = \frac{\Delta S^‡}{R} - \frac{\Delta H^‡}{RT} \quad (7)$$

At pH 6, in eq 6, the term $k_2'K_{PN}$ is small relative to $k_2[H^+]$, and can be neglected. Thus, in eq 7, $k_{app}([H^+] + K_{PN})/[H^+]$ is approximately k_2, and the activation parameters obtained at pH 6 approximate those for the reaction of ONOOH with MLT. However, it must be kept in mind that k_{app} is a composite that includes several reactions, and the activation parameters obtained from k_{app} will have contributions from all these processes. In eq 7, the value of K_{PN} is 6.5 at 25 °C and varies with temperature with a $\Delta H°$ of 2.7 ± 1.0 kcal/mol (*27*) [other investigators have confirmed that the enthalpy of dissociation of ONOOH is small and positive (*28*)] and h and k_B are the Planck and Boltzmann constants, respectively. The values for the activation enthalpy, $\Delta H^‡$, and entropy, $\Delta S^‡$, are calculated to be 8.1 ± 0.6 kcal/mol and -20 ± 2 eu, respectively.

Peroxynitrite/Melatonin Reaction in the Presence of Added Bicarbonate. Upon mixing of peroxynitrite with MLT and bicarbonate in the stopped-flow

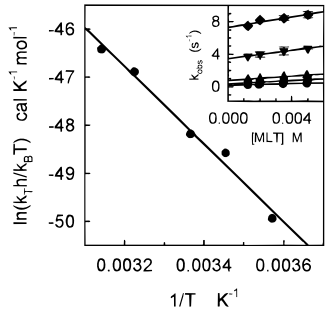


Figure 4. Eyring plot for the reaction of peroxynitrite with melatonin. The data points are the slopes of the plots shown in the inset vs $1/T$. The temperatures were 7.0, 16.5, 24.1, 37.1, and 45.2 ± 0.1 °C. The activation parameters are as follows: $\Delta H^{\ddagger} = 8.1 \pm 0.6$ kcal/mol and $\Delta S^{\ddagger} = -22 \pm 2$ eu. (Inset) The reactions were conducted on the stopped-flow instrument and were monitored at 400 nm. After the mixing but before the reaction, the mixture contained 0.25 mM peroxynitrite and 0, 1.22, 2.0, 3.5, or 5.0 mM MLT. The pH as measured at the outlet was 6.02 ± 0.06. For the other reaction conditions, see Figure 1.

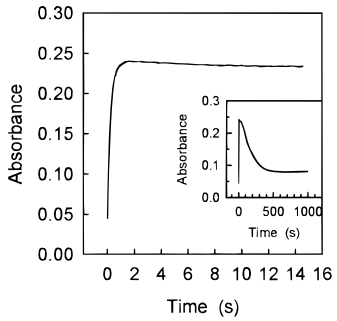


Figure 5. Typical kinetic trace for the reaction of peroxynitrite with melatonin in the presence of added bicarbonate. The reactions were performed on the stopped-flow instrument and were observed at 400 nm and 25.0 ± 0.1 °C. After mixing but before the reaction, the mixture contained 0.2 mM peroxynitrite, 1.0 mM melatonin, and 2.0 mM sodium bicarbonate. (Inset) The same reaction as observed at a longer period of time. For the other reaction conditions, see Figure 1.

instrument, the absorbance at 400 nm initially increases rapidly to reach a maximum, and then decreases at a much slower rate (inset of Figure 5). The rising phase of the absorption trace is fitted to a first-order equation to calculate the value of k_{obs}.

At a given concentration of MLT, the value of k_{obs} increases nonlinearly with the concentration of bicarbonate (Figure 6). However, ΔA_{max}, which reflects the amount of the intermediate(s) formed in the reaction, decreases with added bicarbonate (Figure 7). At a given concentration of bicarbonate, increasing the concentration of MLT causes the value of k_{obs} to decrease (Figure 8) but ΔA_{max} to increase (Figure 9). These observations can be explained in terms of the competition between MLT and CO_2 for peroxynitrite, and the ability of MLT to capture intermediates and interfere with the catalytic cycle of CO_2. We previously observed that tyrosine also decreases the fraction of CO_2 that undergoes a true catalytic cycle (*29*).

Discussion

Reactive Species. In the absence of added bicarbonate, the pH profile of k_{app} for product appearance in the peroxynitrite/MLT reaction (Figure 2) closely resembles the pH profile for the decomposition of peroxynitrite in

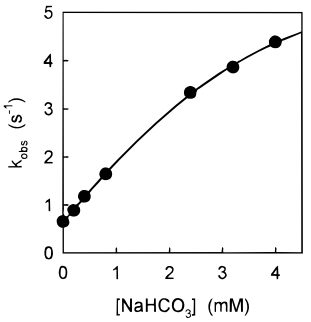


Figure 6. Effect of CO_2 on k_{obs}. The values of k_{obs} were calculated by fitting kinetic traces similar to the one shown in Figure 5 to a first-order equation. The reactions were performed on the stopped-flow instrument at 25.0 ± 0.1 °C (data shown at 400 nm). After mixing but before the reaction, the mixture contained 0.2 mM peroxynitrite, 1.0 mM MLT, and 0, 0.1, 0.2, 0.4, 1.4, 1.6, or 2.0 mM bicarbonate. The pH as measured at the outlet was 7.01 ± 0.03. For the other reaction conditions, see Figure 1.

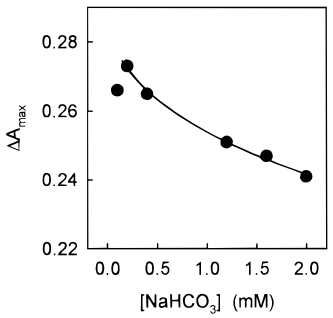


Figure 7. Effect of CO_2 on ΔA_{max}. The values of ΔA_{max} (see Figure 3 for definition) are plotted vs the concentration of bicarbonate for the reactions shown in Figure 6.

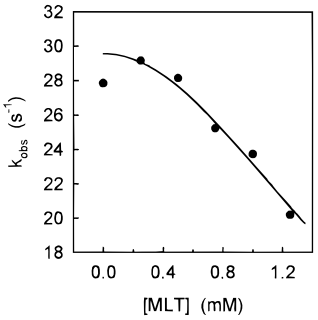


Figure 8. Effect of melatonin on the k_{obs} of the peroxynitrite/CO_2 reaction. The values of k_{obs} were calculated by fitting kinetic traces similar to the one shown in Figure 5 to a first-order equation. The reactions were performed on the stopped-flow instrument at 25.0 ± 0.1 °C (data shown at 400 nm). After mixing but before the reaction, the mixture contained 0.5 mM peroxynitrite, 5.0 mM bicarbonate, and 0, 0.25, 0.5, 0.75 1.0, or 1.25 mM MLT. The pH as measured at the outlet was 7.11 ± 0.02. For the other reaction conditions, see Figure 1.

the absence of MLT from pH 5.0 to 8.0. Also, the value of pK_{PN} (6.5; see Table 1) obtained from Figure 2 is in reasonable agreement with the value of 6.75 previously determined using other methods (*8*, *30*). These facts suggest that ONOOH is the reactive species toward MLT (Figure 2 and eq 3).

Strikingly, the product yield (Figure 3) increases with pH. This can be explained by suggesting that the peroxynitrite anion ($ONOO^-$) also reacts with melatonin to yield oxidation products at basic pH (eq 4). The rate constant for the $ONOO^-$/MLT reaction ($k_2' = 5\ M^{-1}\ s^{-1}$, eq 4) is smaller than that for the ONOOH/MLT reaction ($k_2 =$

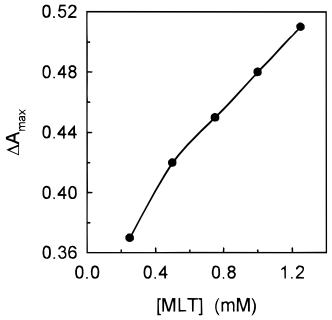


Figure 9. Effect of melatonin on ΔA_{max}. Values of ΔA_{max} (see Figure 3 for definition) are plotted vs the concentration of MLT for the reactions shown in Figure 8.

159 M^{-1} s^{-1}, eq 3), and under physiological conditions, the reaction of MLT with $ONOO^-$ is about 10 times slower than with ONOOH.

We derived[3] eq 8 to calculate the loss of MLT (Δ[MLT]) and explain the pH dependence we observe for the yields of products (Figure 3).

$$-\frac{k_1[H^+] \times \ln\left(1 - \frac{\Delta[MLT]}{[MLT]_0}\right)}{k_2[H^+] + k_2'K_{PN}} + \Delta[MLT] = [peroxynitrite]_0 \quad (8)$$

(A value of pK_{PN} of 6.5, and the values of k_2 and k_2' given above were used for this calculation since these values were obtained directly from fitting the data in Figure 2.) In eq 8, $[MLT]_0$ and $[peroxynitrite]_0$ are the initial concentrations of the two reactants. This equation only approximately predicts Δ[MLT] since it does not take into account minor processes such as the spontaneous decomposition of the peroxynitrite anion or reaction of peroxynitrite with traces of adventitious CO_2. Despite its simplicity, eq 8 predicts, as shown in Figure 3 (dotted lines), that the yield of MLT oxidation will increase with pH, in agreement with the experimental data (Figure 3, solid circles).

Kinetics of the Peroxynitrite/Melatonin Reaction. The second-order rate constant for the reaction of MLT with ONOOH is relatively small (159 M^{-1} s^{-1}) and very close to that of tryptophan (160 M^{-1} s^{-1}) (*31*), indicating that the methoxy group at C-5 does not activate MLT toward peroxynitrite. The insensitivity of the peroxynitrite/melatonin reaction to electron-donating groups (MLT bears a methoxy substituent on C-5, while tryptophan bears a hydrogen atom on the same position) is substantiated by a Hammett study of 5-substituted indole-3-carboxylic acids, where σ_m was used and electron-donating groups had only small effects on the p*K* of the carboxylic acids (*32*). These facts taken together support a mechanism in which an electron is transferred from MLT to ONOOH, and the reaction center is the 3-position of MLT. This mechanism will be discussed below in more detail.

Table 2. Activation Parameters for Some Peroxynitrite Reactions Involving ONOOH

reaction	$\Delta H^‡$ (kcal/mol)	$\Delta S^‡$ (eu)	ref
ONOOH/MLT	8.1 ± 0.6	−20 ± 2	this work[a]
ONOOH/ascorbate	9.3 ± 0.5	−16 ± 2	*46*
ONOOH/tryptophan	9.1 ± 0.3	−19 ± 1	*31*
$ONOOH/I^-$	4.6 ± 0.4	−23 ± 1	*33*
ONOOH/methionine	7.7 ± 0.4	−22 ± 2	*30*

[a] The activation parameters for melatonin were calculated using a $\Delta H°$ of 2.7 ± 1.0 kcal/mol for the acid dissociation of ONOOH, as we reported previously (*27*). All the other values do not take into account this enthalpy of dissociation, but the correction is small. For example, the uncorrected values for melatonin are 7.4 ± 0.6 kcal/mol for $\Delta H^‡$ and 23 ± 2 eu for $\Delta S^‡$.

The values of $\Delta H^‡$ and $\Delta S^‡$ for the peroxynitrite/MLT reaction are in good agreement with those of other reactions of peroxynitrite (Table 2). The large, negative $\Delta S^‡$ value (−20 ± 2 eu) suggests a restricted configuration of ONOOH, and MLT is required for the reaction to occur. For example, a hydrogen bond between peroxynitrite and the indolic hydrogen has been suggested for the peroxynitrite/tryptophan reaction (*31*), and association with the involvement of some bond making has been proposed for the peroxynitrite/iodide reaction (*33*).

Protection of Melatonin by CO_2 from Peroxynitrite-Mediated Oxidation. Carbon dioxide provides partial protection of some biomolecules, such as glutathione (*34*) and selenomethionine (*35*), against peroxynitrite. Therefore, we investigated whether CO_2 also protects MLT. Figures 6 and 7 show k_{obs}, the observed rate constant for the formation of the products from MLT, and ΔA_{max}, a measure of the yield of products form the peroxynitrite/MLT reaction in the presence of bicarbonate at pH 7.0, respectively. Values of k_{obs} increase with increasing concentrations of bicarbonate (Figure 6), showing that the reaction of peroxynitrite with CO_2 is the rate-limiting step. However, the yields of the products from MLT decrease with increasing concentrations of bicarbonate (Figure 7), indicating protection of MLT by CO_2. Nevertheless, CO_2 does not provide complete protection of MLT, as discussed below.

Catalytic Action of CO_2 and the Formation of the Free Radicals. Carbon dioxide reacts with peroxynitrite initially to form **1** (Scheme 1) (*9*, *10*, *13*, *14*). We have shown that ca. 80% of **1** in the presence of a substrate such as tyrosine is converted to **2**; **2** then rapidly decomposes to regenerate CO_2 (*29*). (The remaining 20% of **1** becomes free $CO_3^{\bullet-}$ and $^{\bullet}NO_2$, and these radicals are trapped by the substrate.) In the absence of a substrate, >90% of **1** is converted to **2** and subsequently regenerates CO_2 (*36*). The regeneration of CO_2 in this pathway is fast, so CO_2 is a true catalyst for the decomposition of peroxynitrite (*29*, *34*, *36*). This also means that in the presence of CO_2 only about 20% of the peroxynitrite is available to oxidize a substrate such as melatonin. In contrast, in the absence of CO_2, and for a substrate that

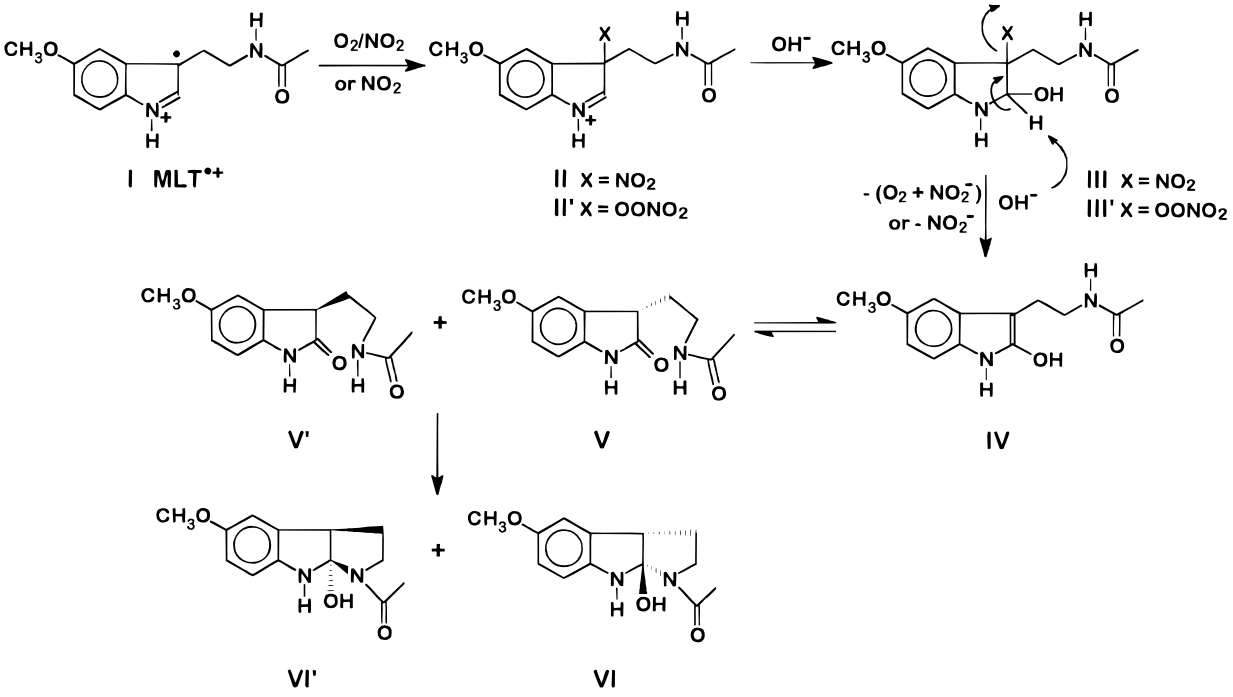
[3] Equation 8 is derived from eq iii which, in turn, is obtained by dividing the differential rate laws for the disappearance of MLT (eq i) and peroxynitrite (eq ii).

$$-\frac{d[MLT]}{dt} = \frac{k_2[H^+] + k'_2K_{PN}}{[H^+] + K_{PN}}[MLT][peroxynitrite] \quad (i)$$

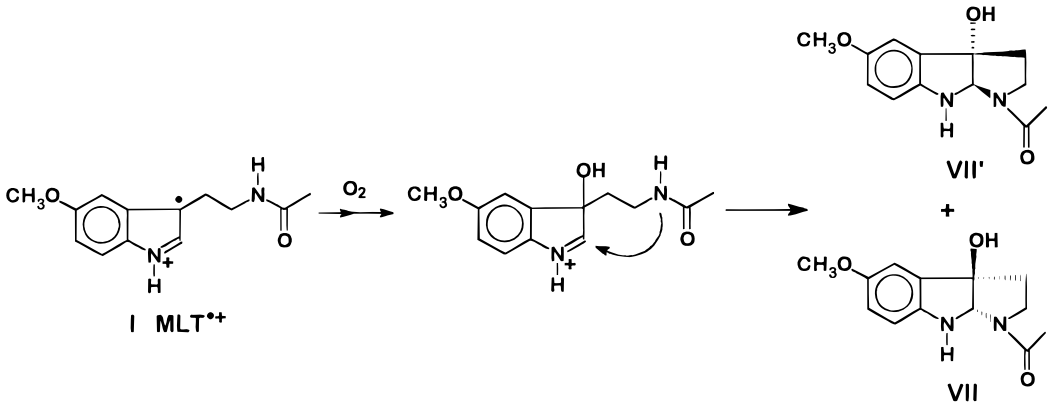
$$-\frac{d[peroxynitrite]}{dt} = \left(\frac{k_1[H^+]}{[H^+] + K_{PN}} + \frac{k_2[H^+] + k'_2K_{PN}}{[H^+] + K_{PN}}[MLT]\right)[peroxynitrite] \quad (ii)$$

$$\int_{[MLT]_0}^{[MLT]} \left(\frac{k_1[H^+]}{k_2[H^+] + k'_2K_{PN}} \times \frac{1}{[MLT]} + 1\right) d[MLT] = \int_{[peroxynitrite]_0}^{0} d[peroxynitrite] \quad (iii)$$

Scheme 2



Scheme 3



follows second-order kinetics, all of the peroxynitrite that undergoes this reaction is capable of oxidizing the substrate.

When peroxynitrite reacts with CO_2, intermediate species such as $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ are formed (*9*, *10*, *13*, *14*, *36*), as shown in Scheme 1. We therefore evaluated the ability of melatonin to react with the radicals derived from the peroxynitrite/CO_2 reaction. We previously suggested glutathione (*34*) and tyrosine (*29*) scavenge $CO_3^{\bullet-}$ and $^{\bullet}NO_2$. The rate constant for the reaction of MLT with $CO_3^{\bullet-}$ (eq 9) is unknown, but probably is similar to that for the reaction of tryptamine with $CO_3^{\bullet-}$, 1.3×10^9 M^{-1} s^{-1} (*37*).

$$CO_3^{\bullet-} + \text{MLT} \rightarrow CO_3^{-} + \text{MLT}^{\bullet+} \qquad (9)$$

Recently, we reported the formation of melatoninyl radical cation ($MLT^{\bullet+}$) in a MLT/peroxynitrite/CO_2 system due to the reaction of MLT with $CO_3^{\bullet-}$ (*22*). Figure 8 shows that with limiting CO_2, k_{obs} decreases with increasing concentrations of MLT; this results since any $CO_3^{\bullet-}$ that reacts with MLT cannot be converted back to CO_2, resulting in a decrease in the CO_2 concentration and lower k_{obs} values (Scheme 1; MLT and other substrates block the reaction shown as a dotted arrow). If MLT were able to compete with CO_2 for peroxynitrite, k_{obs} would increase rather than decrease with increasing concentrations of MLT. In fact, melatonin reacts much more slowly than CO_2 with peroxynitrite. The reaction rate constants for the $ONOO^-/CO_2$ and ONOOH/MLT reactions are 30 000 (*9*) and 159 M^{-1} s^{-1}, respectively; these values predict that <1% of the peroxynitrite reacts directly with MLT under our reaction conditions, as described in the legend of Figure 8. However, we expect as much as 20% of the peroxynitrite reacts indirectly with MLT via the peroxynitrite/CO_2 reaction pathway (*29*). Figure 9 shows that ΔA_{max} (a measure of the yield of products) increases in a MLT concentration-dependent manner, resulting from the scavenging of the free radicals $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ by MLT.

Possible Mechanisms for the Formation of Products. Here we analyze the pathways (see Schemes 2 and 3) that lead to the main products of the reaction, the hydroxypyrrolo[2,3-*b*]indoles 1,2,3,3a,8,8a-hexahydro-1-acetyl-5-methoxy-8a-hydroxypyrrolo[2,3-*b*]indole (**VI/VI′**) and 1,2,3,3a,8,8a-hexahydro-1-acetyl-5-methoxy-3a-hy-

droxypyrrolo[2,3-*b*]indole (**VII/VII′**), and 6-hydroxymelatonin (6-HO-MLT).

(1) Formation of VI/VI′ and VII/VII′. The peroxynitrite/MLT reaction yields 6-HO-MLT and the hydroxypyrrolo[2,3-*b*]indoles **VI/VI′** and **VII/VII′** as the major final products, but only the hydroxypyrrolo[2,3-*b*]indoles are formed if CO_2 also is present (*22*). The melatoninyl radical cation is observed as an intermediate both in the presence and in the absence of CO_2 (*22*). Therefore, we assume that $MLT^{\bullet+}$ leads to the hydroxypyrrolo[2,3-*b*]-indoles **VI/VI′** and **VII/VII′** and not to 6-HO-MLT.

Since the reaction depicted in eq 9 is responsible for the formation of $MLT^{\bullet+}$ in the presence of CO_2, we suggest that the ground state of peroxynitrous acid (ONOOH) is responsible for the formation of $MLT^{\bullet+}$ in the absence of CO_2 (eq 10). This suggestion is supported by the fact that the peroxynitrite/MLT reaction is first-order in peroxynitrite and first-order in MLT, and the high one-electron reduction potential for ONOOH/$^{\bullet}NO_2$ (1.6 V at pH 7) (*38*), 0.4 V higher than that of most indolic compounds (*39*). In line with this explanation, an electron transfer pathway has been suggested for the very similar reaction of peroxynitrite with tryptophan (*31*).

$$\text{ONOOH} + \text{MLT} \xrightarrow{\text{ET}} \text{MLT}^{\bullet+} + {}^{\bullet}\text{NO}_2 + \text{OH}^- \qquad (10)$$

Scheme 2 shows a pathway in which $MLT^{\bullet+}$ is converted to **VI/VI′**. In this mechanism, $MLT^{\bullet+}$ captures $^{\bullet}NO_2$, which is formed both in the absence and in the presence of CO_2 (Scheme 1 and eq 10), to form the unstable adduct 2-hydroxy-3-nitro-2,3-dihydromelatonin (**II**). Alternatively, $MLT^{\bullet+}$ can first couple with O_2 and then with $^{\bullet}NO_2$ to form 2-hydroxy-3-peroxynitro-2,3-dihydromelatonin (**II′**). Under strongly acidic conditions, the NO_2 or $OONO_2$ groups at C-3 could migrate to C-2 in MLT (*40*), but at neutral pH, addition of hydroxide ion is more likely, resulting in **III/III′**. Because of the acidity of the proton at C-2 in **III/III′**, NO_2^- (or O_2 and NO_2^-) is eliminated, to yield an oxindole derivative (**V/V′**), which undergoes ring closure to give the final product **VI/VI′**. A related mechanism for the formation of oxindole from 3-chloroindole has been previously suggested (*41*). The NMR spectra reveal that two isomers are formed in an approximately 10:1 ratio.[4] We suggest the other isomer is a positional isomer in which the hydroxyl group is on C-3a rather than on C-8a (**VII/VII′** in Scheme 3). In this regard, 1,2,3,3a,8,8a-hexahydro-3a-hydroxypyrrolo[2,3-*b*]-indole-2-carboxylic acid has been reported together with 2-hydroxytryptophan during the oxidation of tryptophan with hydrogen peroxide (*42*), and the *tert*-butoxycarbonyl derivatives of these products had been reported during the oxidation of (*tert*-butoxycarbonyl)-tryptophan with peroxynitrite (*43*). (2-Hydroxytryptophan is the analogue of **IV**, the open form of **VI** and **VI′** in Scheme 2, when the original substrate is tryptophan instead of melatonin.) The formation of hydroxypyrrolo[2,3-*b*]indoles is emerging as an important reaction pathway during the oxidation of tryptophan and tryptophan metabolites. Moreover, Kato et al. (*43*) also found peroxynitrite does not nitrate tryptophan.

(2) Formation of 6-Hydroxymelatonin (6-HO-MLT). This major product is formed only in the absence of CO_2 and thus is not derived from $MLT^{\bullet+}$. Its formation probably involves the reaction of MLT with ONOOH* (eq 11), an activated form of peroxynitrite (*8*, *44*). This species also has been suggested to be responsible for the hydroxylation of phenol, which is first-order in peroxynitrite but zero-order in phenol. [The rate-determining step is the formation of ONOOH* (*23*).] Thus, the hydroxylation of MLT also should be zero-order in MLT; this also is suggested by the fact that the extrapolated *y*-intercept of traces of k_{obs} versus MLT concentration (inset of Figure 2) is larger than k_{obs} in the absence of MLT. The reactions of peroxynitrite with methionine, ascorbate, and tryptophan also exhibit a similar pattern (*3*, *7*, *31*), and this kinetic behavior was attributed to the reactions of ONOOH* that can be detected at low substrate concentrations. However, these reactions of ONOOH*, including the formation of 6-HO-MLT, are unimportant in vivo. This is true because nearly all the peroxynitrite formed in vivo will not form ONOOH* because ground state peroxynitrite (ONOOH and $ONOO^-$) will undergo fast bimolecular reactions, generally with CO_2 or with heme-containing proteins such as hemoglobin (*14*), before it can form ONOOH*. Nevertheless, melatonin is metabolized by the liver mainly to 6-HO-MLT (*45*) in a peroxynitrite-independent pathway.

$$\text{ONOOH*} + \text{MLT} \rightarrow\rightarrow \text{MLT-OH} \qquad (11)$$

Summary and Conclusions

The kinetics of the reaction of melatonin with peroxynitrite has a first-order component (involving ONOOH), and probably also a zero-order component (involving ONOOH*), as previously observed for methionine, ascorbate, and tryptophan (*3*, *7*, *31*). Melatonin also reacts with $ONOO^-$ with first-order kinetics; however, the rate constant for this reaction is much smaller than that for the melatonin/ONOOH reaction, and the reaction is unimportant at physiological pH. The combination of these reactions of melatonin with peroxynitrite gives rise to a pH profile in which the yields of the products from melatonin increase with pH. A mechanism is presented that is in agreement with these observations.

The peroxynitrite/melatonin reaction produces 6-hydroxymelatonin, 1,2,3,3a,8,8a-hexahydro-1-acetyl-5-methoxy-8a-hydroxypyrrolo[2,3-*b*]indole (**VI/VI′**), and 1,2,3,3a,8,8a-hexahydro-1-acetyl-5-methoxy-3a-hydroxypyrrolo[2,3-*b*]indole (**VII/VII′**), but in the presence of added bicarbonate, 6-hydroxymelatonin is not formed. The melatoninyl radical cation ($MLT^{\bullet+}$) is formed when the peroxynitrite/melatonin reaction occurs in either the presence or absence of added bicarbonate (*22*). These observations indicate that the hydroxypyrrolo[2,3-*b*]indole products are formed from the secondary reactions of $MLT^{\bullet+}$. Carbon dioxide lowers the yield of oxidation of melatonin and also markedly lowers the yield of 6-hydroxymelatonin, but the peroxynitrite/CO_2 reaction also gives $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ that can oxidize melatonin to hydroxypyrrolo[2,3-*b*]indole products.

[4] The ^1H NMR data (in $CDCl_3$) of the major isomer have been reported previously (*20*). The major isomer was named 5-methoxy-2-hydropyrroloindole in ref *20*; the current name, 1,2,3,3a,8,8a-hexahydro-1-acetyl-5-methoxy-8a-hydroxypyrrolo[2,3-*b*]indole, is consistent with IUPAC and CAS nomenclature. The NMR data for the minor isomer, 1,2,3,3a,8,8a-hexahydro-1-acetyl-5-methoxy-3a-hydroxypyrrolo-[2,3-*b*]indole, are as follows: (δ in parts per million and J values in hertz) δ 6.900 (1H, d, $J = 2.5$), 6.806 (1H, dd, $J_1 = 2.5$, $J_2 = 8.6$), 6.651 (1H, d, $J = 8.6$), 3.775 (3H, s), 3.736−3.652 (2H, m), 3.299 (1H, dd, $J_1 = 7.1$, $J_2 = 9.6$), 2.560−2.339 (2H, m), 2.153 (3H, s). Note: When resonances of the minor and major isomers overlap, the chemical shifts of the major isomer are used.

The peroxynitrite/melatonin reaction does not produce a nitrated product that is stable and can be isolated, whether bicarbonate is present. Nitration probably occurs both in the presence and in the absence of added bicarbonate, but we suggest that the nitroproduct is converted to an oxindole that then rearranges to give 1,2,3,3a,8,8a-hexahydro-1-acetyl-5-methoxy-8a-hydroxypyrrolo[2,3-*b*]indole (**VI/VI′**). The hydroxylation of melatonin by peroxynitrite to give 6-hydroxymelatonin, on the other hand, likely occurs by reaction of melatonin with the activated form of peroxynitrous acid, ONOOH*, but this reaction is unimportant in vivo because nearly all peroxynitrite formed in vivo does not form ONOOH* (*14*).

It is unlikely that the major biological function of melatonin is to scavenge peroxynitrite or peroxynitrite-derived species, since the physiological concentrations of melatonin are very low, much lower than those of other antioxidants and potential scavengers, such as ascorbate, glutathione, and urate that react rapidly with peroxynitrite and/or peroxynitrite-derived species. Thus, most peroxynitrite and peroxynitrite-derived species will react with other antioxidants and not with melatonin. Nevertheless, melatonin is expected to react with the carbonate radical[5] produced from the reaction of peroxynitrite with CO_2 at a near diffusion-controlled rate, as do many other tryptophan and tryptamine derivatives. Since the peroxynitrite/CO_2 reaction governs the reactions of peroxynitrite in vivo (*13*, *14*), we suggest the hydroxypyrrolo[2,3-*b*]indoles **VI/VI′** and **VII/VII′** are the main product from the oxidation of melatonin by peroxynitrite-derived species in vivo.

Melatonin is metabolized in the liver mainly to 6-hydroxymelatonin, but we do not think this metabolite is a useful biomarker for melatonin's antioxidant activity. However, 6-hydroxymelatonin may be a better chain-breaking antioxidant than melatonin, because of its readily transferable phenoxyl hydrogen analogous to that in α-tocopherol, and this metabolite may contribute to the beneficial effects of melatonin in vivo. We suggest that the formation of 1,2,3,3a,8,8a-hexahydro-1-acetyl-5-methoxy-8a-hydroxypyrrolo[2,3-*b*]indole (**VI/VI′**) and/or its interconverting forms (**IV**, **V**, and **V′** in Scheme 2) and the isomeric 1,2,3,3a,8,8a-hexahydro-1-acetyl-5-methoxy-3a-hydroxypyrrolo[2,3-*b*]indole (**VII/VII′**) may serve as indices for melatonin's antioxidant activity, and for evidence of its role as a scavenger of radicals derived from the reaction of peroxynitrite with carbon dioxide. To our knowledge, no attempt has been made to measure these compounds in vivo, and further research in this area is needed.

[5] Melatonin is predicted to react with $CO_3^{\bullet-}$ produced by the reaction between peroxynitrite and CO_2 despite the low concentration of melatonin in vivo. This can be demonstrated by using the known rate constants for the reaction of $CO_3^{\bullet-}$ with glutathione (5.3×10^6 M^{-1} s^{-1}) and ascorbate (1.4×10^9 M^{-1} s^{-1}) and assuming the rate constant for the reaction of $CO_3^{\bullet-}$ with melatonin, which is unknown, is similar to that of tryptamine (1.3×10^9 M^{-1} s^{-1}), an analogue and precursor of melatonin. When the physiological concentrations of ascorbate (61 μM), glutathione (1.1 mM), and melatonin (211 nM) are considered (*12*), one can calculate the corresponding pseudo-first-order rate constants for ascorbate (8.5×10^4 s^{-1}), glutathione (5.8×10^3 s^{-1}), and melatonin (270 s^{-1}). Since the pseudo-first-order rate constants are proportional to the amount of these antioxidants that will react with $CO_3^{\bullet-}$, if one assumes 20 μM $CO_3^{\bullet-}$ derived from peroxynitrite is produced in this environment, one can calculate that $CO_3^{\bullet-}$ reacts mostly with ascorbate (74%), but also that enough $CO_3^{\bullet-}$ reacts with melatonin to consume 28% of its initial concentration. A more rigorous simulation using the Bio/chemical Kinetics Simulator software package GEPASI (Pedro Mendes, University of Wales Aberystwyth) affords similar results.

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