

Kinetic Radical-scavenging Activity of Melatonin

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Abstract. Carbon-centred free radicals can be involved in damage to biological systems under hypoxic/anoxic conditions as well as in ischaemia/reperfusion injury. The antioxidant activities of melatonin against carbon-centred radicals are poorly understood. The aim of this study was to investigate the antioxidant properties of melatonin against carbon-centred radicals in a biomimetic model system consisting of growing methyl methacrylate (MMA) radicals (poly-MMA radicals, PMMA[·]). The kinetics of the polymerization of MMA initiated by thermal decomposition of 2,2'-azobis(isobutyronitrile) (AIBN; R[·] radical) or benzoyl peroxide (BPO; PhCOO[·] radical) in the presence of melatonin were investigated by the induction period method under nearly anaerobic conditions. As melatonin concentrations increased, the length of the induction period (IP) increased, but for the BPO system the IP reached a plateau at a molar ratio of BPO to melatonin of 5:1, indicating that the oxidation of melatonin by PhCOO[·] was limited. At low concentrations of melatonin, the stoichiometric factor (n, the number of free radicals trapped by the antioxidant moiety) for melatonin was approximately 2, but as the melatonin concentration increased the n value decreased markedly to 0.1. These observations suggest that melatonin may possess catalytic activity contributing to radical avoidance. The initial rate of polymerization (Rp) in the BPO system was markedly suppressed by high concentrations of melatonin, suggesting a strong interaction between oxidative end-products formed from melatonin and PMMA[·]. Under conditions where n was about 2, the k_{inh} values for melatonin in the BPO system and the AIBN system were $6.58 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$ and $2.49 \times 10^3 \text{ M}^{-1} \text{s}^{-1}$, respectively. In the BPO system, the k_{inh} of melatonin was of a similar magnitude to that of α -tocopherol, whereas in the AIBN system the k_{inh} of

melatonin was 100-fold greater than that of tocopherol. The present findings suggest that melatonin may be able to scavenge harmful carbon-centred radicals *in vivo*.

The pineal hormone melatonin [compound **1**] was previously found to be a potent free radical scavenger that scavenges a variety of reactive oxygen species (ROS) including the hydroxy radical, hydrogen peroxide, singlet oxygen, nitric oxide and the peroxy nitric anion (1-6). Melatonin also scavenges peroxy radicals derived from an azoinitiator (7) and lipid peroxy radicals (8). Ischaemia/reperfusion is a frequently encountered phenomenon in organisms. Prolonged ischaemia followed by reperfusion results in severe oxidative injury to tissues and organs. Carbon-centred free radicals can be involved in damage under hypoxic/anoxic conditions as well as in ischaemia/reperfusion injury. Melatonin plays an important role as a member of the antioxidant defense system that protects against potential oxidative injury induced by physiological ischaemia/reperfusion (9). However, the kinetics of the reaction of melatonin with carbon-centred radicals are virtually unknown.

We have previously proposed a quantitative model rationalizing the radical-scavenging activity of phenolic compounds such as butylated hydroxytoluene-related compounds in polymerization of methyl methacrylate (MMA) initiated by thermal decomposition of 2,2'-azobis(isobutyronitrile) (AIBN) or benzoyl peroxide (BPO) under nearly anaerobic conditions (10). The model was well able to explain the mechanism of radical-scavenging activity and to predict the chain-breaking activity of bioactive agents such as the polyamines (11), β -carotenes (12), oestrogens (13), ebselen (14), vitamin E (15) and diphenylamine-related compounds (16). The advantage of this model system is that measurements using a differential scanning calorimeter (DSC) are highly sensitive; furthermore, the use of anaerobic conditions makes this system relatively biomimetic, since oxygen is sparse in living cells (17). In the present study, the investigation was extended to melatonin, reporting kinetic studies of the radical-scavenging activity of melatonin.

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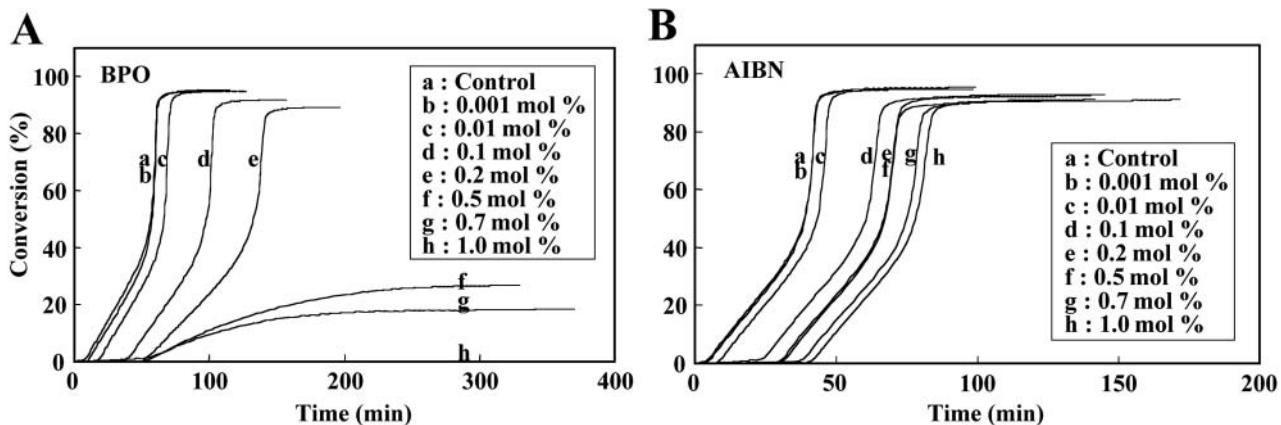


Figure 1. Time-conversion curves for polymerization of MMA 9.4 M at 70°C initiated by BPO (1 mol%) (A) or AIBN (1 mol%) (B) in the presence of 0, 0.001, 0.01, 0.1, 0.2, 0.5, 0.7 or 1.0 mol% melatonin.

Materials and Methods

Melatonin was obtained from Tokyo Kasei Chem. Co., Tokyo, Japan. MMA (Tokyo Kasei Chem. Co.) was purified by distillation. AIBN and BPO (Tokyo Kasei Chem. Co.) were recrystallized from methanol and methanol/chloroform (1:1 v/v), respectively.

Induction period and initial rate of polymerization. The induction period (IP) and initial rate of polymerization in the presence ($R_{p,inh}$) or absence ($R_{p,con}$) of an antioxidant were determined by the method previously reported (10). In brief, the experimental resin consisted of MMA and AIBN (or BPO) with or without additive (melatonin). AIBN (or BPO) were added at 1.0 mol%, and melatonin was used at 0, 0.001, 0.01, 0.1, 0.2, 0.5, 0.7 and 1.0 mol%. Approximately 10 μ l of the experimental resin (MMA: 9.12-9.96 mg) was loaded into an aluminum sample container and sealed by applying pressure. The container was placed in a differential scanning calorimeter (model DSC 3100; MAC Science Co., Tokyo, Japan) kept at 70°C, and the thermal changes induced by polymerization were recorded for the appropriate periods. The heat due to polymerization of MMA was 13.0 kcal/mole in this experiment. The conversion of samples, as calculated from the DSC thermograms, was 0-95%. Polymerization curves were derived from the DSC thermograms using the integrated heat evoked by the polymerization of MMA. Polymerization curves break when an inhibitor is consumed (Figure 1). These breaks are sharp and provide a reliable measure of the IP of the inhibitor. The presence of oxygen retards polymerization because oxygen reacts with MMA radicals activated by the initiator and then, subsequently, produces a non-radical product. Thus, polymerization of the control was slightly inhibited, even though the reaction was carried out in a sealed DSC pan, because the pan contained a small amount of oxygen, having been sealed in air. Tangents were drawn to polymerization curves at an early stage in the run. The IP of the test compounds was determined from the length of time between the zero point on the abscissa and the point of intersection of tangents drawn to the early stage of polymerization. The IP was calculated from the difference between the induction period of specimens and that of controls. The initial rates of polymerization in the absence

($R_{p,con}$) and presence ($R_{p,inh}$) of melatonin were calculated from the slope of the plots of the first linear line of the conversion rate of MMA polymerization (tangent drawn at the early polymerization stage).

Measurement of stoichiometric factor (n). The relative n value in Eq. 1 can be calculated from the induction period in the presence of an inhibitor:

$$n = R_i[IP]/[IH] \quad 1$$

where [IP] is the induction period in the presence of an inhibitor and R_i is the rate of initiation. The number of moles of peroxy (or alkyl) radicals trapped by the antioxidant was calculated with respect to 1 mole of inhibitor moiety unit. The R_i values of AIBN and BPO were 5.66×10^{-6} Ms^{-1} and 2.28×10^{-6} Ms^{-1} , respectively (10).

Measurement of inhibition rate constant (k_{inh}). When R_i is constant, i.e., when new chains are started at a constant rate, a steady-state treatment can be applied and the initial rate of polymerization of MMA is given by Eq. 2 (10):

$$R_{p,con} = \{k_p[MMA] R_i^{1/2}\} / (2k_t)^{1/2} \quad 2$$

where MMA represents methyl methacrylate and k_p and k_t are the rate constants for chain propagation and termination, respectively. The $k_p/(2k_t)^{1/2}$ rate of polymerization of MMA (9.4 M) initiated by AIBN (1 mol%) or BPO (1 mol%) at 70°C was a constant value, $9.86 \times 10^{-2} \text{ M}^{-1/2} \text{ s}^{-1/2}$ (10). The $R_{p,inh}$ rates are determined by Eq. 3:

$$R_{p,inh} = \{k_p[MMA] R_i\} / \{n k_{inh} [IH]\} \quad 3$$

in which $R_{p,inh}$ is the initial rate of inhibited polymerization, [MMA], n , [IH] and k_p are defined above, and k_{inh} is the rate constant for scavenging (inhibiting) of MMA radicals by an antioxidant. From Eq. 2 and Eq. 3, k_{inh}/k_p can be calculated (Eq. 4):

$$k_{inh}/k_p = [MMA] / \{[IP] \times [R_{p,inh}]\} \quad 4$$

The kinetic chain length (KCL) is given by Eq. 5.

$$KCL = R_{p,con}(\text{or } R_{p,inh}) / R_i \quad 5$$

Computational details. Theoretical calculations of HOMO (highest occupied molecular orbital) and heats of formation were carried out by the semi-empirical molecular orbital (MO) method PM5, as implemented in the MOPAC program (Fujitsu CaChe 5.0).

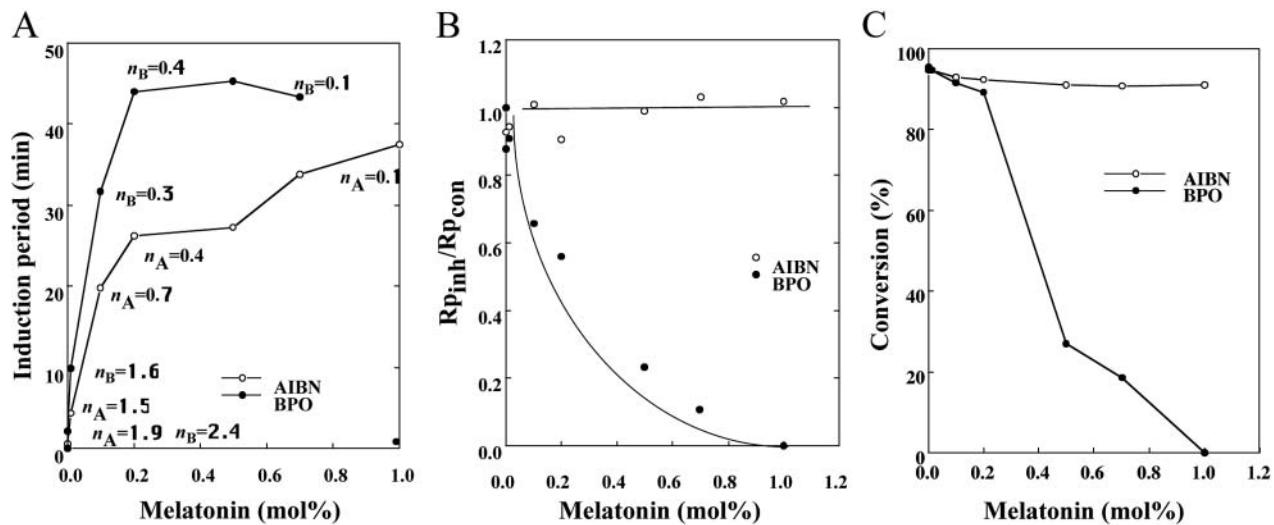


Figure 2. Plots of induction period (A), Rp_{inh}/Rp_{con} (B) and conversion (C) versus concentrations of melatonin in the BPO and AIBN systems.

Results

IP. Time-conversion curves for the BPO (A) and AIBN (B) systems in the presence of melatonin are shown in Figure 1. The concentration-effect curves for melatonin in the BPO and AIBN systems were clearly different. Values for IP, Rp_{inh}/Rp_{con} and conversion were calculated from the curves, and the relationships between IP, Rp_{inh}/Rp_{con} or conversion and concentration of melatonin are shown in Figures 2A, B and C, respectively. For the BPO system, the IP increased with increasing concentrations of melatonin, but reached a plateau at concentrations of 0.2 mol%. An IP value could not be calculated for the highest melatonin concentration (1.0 mol%) because polymerization did not occur, even after curing for 396 minutes. BPO ($\text{PhCOO}-\text{OOCPh}$) decomposes into two benzoate radicals ($\text{PhCOO}-\text{OOCPh} \rightarrow 2\text{PhCOO}^\cdot$) in the presence of MMA. Similarly, AIBN decomposes into two radicals ($(\text{CH}_3)_2\text{C}^\cdot-\text{C}\equiv\text{N}$). The observation that polymerization of MMA did not occur in an equimolar mixture of BPO and melatonin suggests that melatonin scavenges two PhCOO^\cdot radicals. Although initiation by BPO may also occur *via* further decomposition ($\text{PhCOO}^\cdot \rightarrow \text{Ph}^\cdot + \text{CO}_2$), such a decomposition has been well-known to preferably occur in the absence of methacrylates. The n values were calculated from Eq. 1 and are shown in Figure 2A. At low melatonin concentrations (0.001 mol%), the n value for both systems was about 2. As melatonin concentrations increased, the n values decreased, reaching about 0.1 at the highest concentration investigated.

Rp_{con}/Rp_{inh} The results are shown in Figure 2B. The Rp_{inh}/Rp_{con} of melatonin for the BPO system decreased as the melatonin concentration increased. At a melatonin

concentration of 1.0 mol%, Rp_{inh}/Rp_{con} was close to zero. In contrast, the Rp_{inh}/Rp_{con} of melatonin for the AIBN system was about 1, similar to that of the control. The kinetic chain length (KCL) was calculated from Eq. 4. The KCL of the BPO system in the presence of 0, 0.1, 0.2, 0.5, 0.7 and 1.0 mol% melatonin was 611, 450, 386, 160, 139 and 0, respectively, whereas for the AIBN system the KCL was independent of the melatonin concentration and was almost constant, with values of 251-277. Melatonin oxidized by PhCOO^\cdot markedly suppressed PMMA^\cdot . The KCL of the control in the BPO system was twice that in the AIBN system, showing an inverse relationship to the corresponding R_i values.

Conversion. In the BPO system, increasing concentrations of melatonin greatly decreased conversion (Figure 2C). In contrast, conversion in the AIBN system was not affected by the concentration of melatonin and was similar to that of the control. Melatonin oxidized by PhCOO^\cdot acted as both an inhibitor and a retarder. In contrast, melatonin oxidized by R^\cdot acted as an inhibitor alone.

k_{inh} The k_{inh} was calculated from Eq. 3. At a 1 to 0.001 molar ratio of initiator to melatonin, the k_{inh} of melatonin for the AIBN and BPO systems was $6.58 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ and $2.49 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$, respectively. (Note: k_p is $405 \text{ M}^{-1}\text{s}^{-1}$ (14).) The k_{inh} of melatonin for reaction with PMMA^\cdot in the AIBN system was an order of magnitude greater than the corresponding value for the BPO system.

Discussion

Since biological systems have low oxygen tensions (17), the effectiveness of antioxidants *in vivo* may be considerably

different from that in model systems. Many of the studies of the antioxidant activity of melatonin have been performed in the presence of oxygen (7, 8). Radicals can react with scavengers in different ways, by abstraction of an electron or a hydrogen atom, or by addition. For melatonin, radical addition has been observed or predicted theoretically only for interactions with hydroxy radicals (18) and nitric oxide (18, 19). Electron/hydrogen abstraction, however, may be a common mechanism for the interaction of melatonin with highly reactive oxidizing radicals such as PhCOO^\cdot and R^\cdot . Electron/hydrogen abstraction is a primary step of melatonin oxidation (Figure 3). The singlet transfer reaction of melatonin possibly plays a role in suppressing the reactivity of PhCOO^\cdot and R^\cdot under nearly anaerobic conditions. The ability of melatonin to scavenge PhCOO^\cdot and R^\cdot was decreased by increasing concentrations of melatonin, as reflected by the greatly decreased n value at high concentrations. This suggests that the antioxidant activity of melatonin depends on a balance of free radical concentration and quantity of melatonin. Melatonin showed antioxidant activity at low concentrations, but prooxidant activity at higher concentrations. A melatonin intermediate with melatonyl radicals (compound 3) may prevent further oxidation of other melatonin molecules, suggesting that melatonin possesses radical avoidance activity.

In the present study, the radical-scavenging capacity of melatonin was evaluated in terms of both IP and R_p , allowing its activity to be represented as k_{inh} . The k_{inh} values of melatonin for the AIBN and BPO systems under conditions when n was about 2 were $6.58 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ and $2.49 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$, respectively. The k_{inh} values of α -tocopherol for the AIBN and BPO systems, obtained by a method similar to that of the present study, were estimated to be $494 \text{ M}^{-1}\text{s}^{-1}$ and $6.08 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$, respectively (15). The scavenging activity of melatonin in the system initiated by AIBN was about 100-fold greater than that of α -tocopherol. In contrast, the scavenging activity of melatonin in the system initiated by BPO was of a similar magnitude to that of α -tocopherol. The radical-scavenging activity of melatonin was previously reported to be twice that of α -tocopherol (20, 21). The radical-scavenging activity of melatonin with lipid peroxy radicals derived from radiation-induced oxidation of linoleate initiated by hydroxy radicals was previously reported to be $2.8 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, suggesting that the antioxidant activity of melatonin-related compounds is related to their lipophilicity (8).

In general, the destructive ability of carbon-centred radicals is underestimated because of their lower reactivity compared with that of hydroxy radicals. Nevertheless, aliphatic carbon-centred radicals are highly reactive in breaking supercoiled plasmid DNA (22). Carbon-centred free radicals can be involved in damage under hypoxic/anoxic conditions as well as in ischaemia/reperfusion injury.

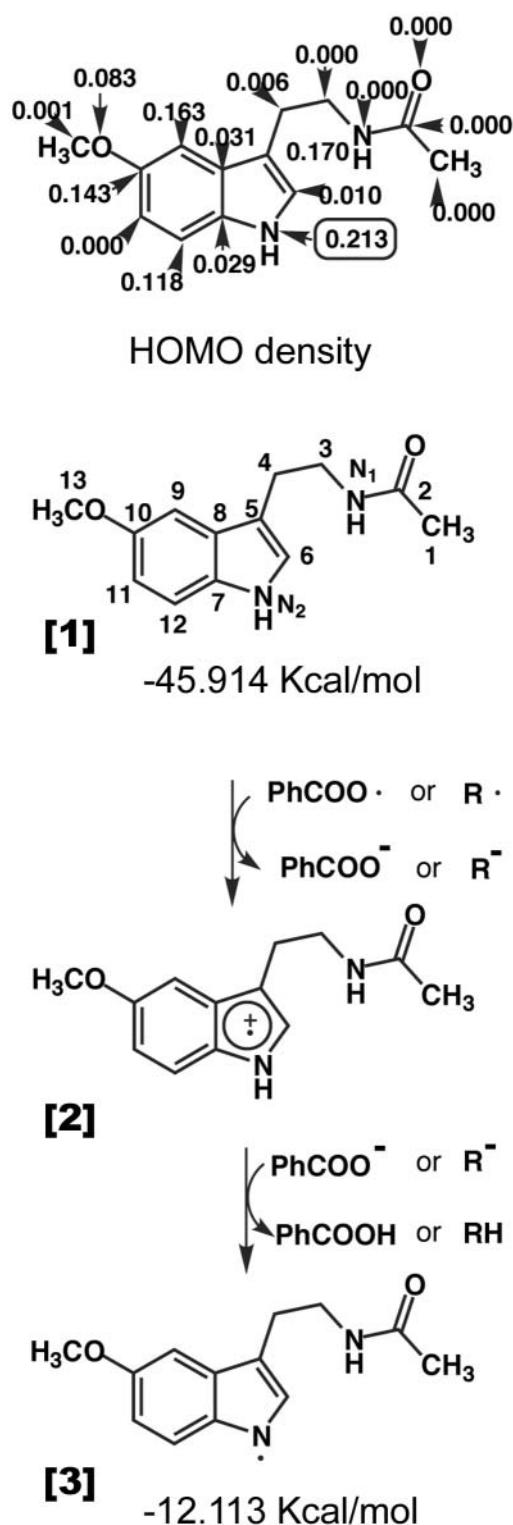


Figure 3. A possible pathway of the one-electron transfer reaction of melatonin and heat of formation energies for its intermediates. PhCOO^\cdot , benzoate radical derived from BPO; R^\cdot , cyanoisopropyl radical derived from AIBN. Compound 1, melatonin.

Melatonin was previously reported to be useful against ischaemia/reperfusion injury and has been shown to attenuate cardiac arrhythmias and to reduce oxidized lipids in the ischaemic heart (23). Whether lipid carbon-centred radicals generated from polyunsaturated fatty acids *via* chemical or enzymatic hydrogen abstraction are able to attack pathologically important targets remains to be clarified, but the present findings suggest that melatonin may be capable of scavenging such potentially harmful radicals.

The antioxidant action of melatonin involves the donation of two electrons, not one electron, and melatonin (compound **1**) scavenges two hydroxy radicals (3). One melatonin molecule has the potential to scavenge up to four or more reactive species (7). However, in the present study, the *n* value was 2 or less, suggesting the possibility of a singlet-electron transfer reaction in addition to a two-electron transfer reaction. The chemistry of the interaction of melatonin with reactive species in the presence of oxygen has previously been reported (5). In the present study, the interaction of melatonin with the non-biological radicals PhCOO[·] and R[·] was examined to clarify the reaction pathway of melatonin. A PM5 method was used to estimate the mechanism of oxidation of melatonin by such non-biological radicals. The HOMO for melatonin is shown in Figure 3. Targeting sites for reaction exist in N2 (the highest density, 0.213) and in carbons C5 (0.170), C9 (0.163), C10 (0.143) and C12 (0.118). Possible reaction pathways with the target carbon atoms and the corresponding radical adducts for PhCOO[·] or R[·] were identified by computational calculations, although indoleamine failed to act as an antioxidant. There are studies showing that compounds containing an NH group in a 5-membered pyrrole ring are not generally peroxy radical-trapping antioxidants (25, 26). Alkylperoxy and alkyl radicals are much more reactive, explaining why melatonin showed radical-scavenging activity in the present study. To further understand the antioxidant mechanism of melatonin, it would be of great interest to analyze melatonin oxidation end-products. Although we attempted to detect such end-products, we were unable to analyze them because of their very small quantity, suggesting that melatonin limits its own oxidation. Carbon-centred radicals such as PMMA[·] are scavenged by addition to oxygen or carbon. In the present study, melatonin reduced the KCL of the BPO system, indicating that the end-products formed by the reaction of melatonin with PhCOO[·] could be targeted by PMMA[·]. In contrast, melatonin did not affect the KCL of the AIBN system. This marked difference in the chain termination activity of melatonin between the AIBN and BPO systems suggests that different end-products of melatonin were formed by reaction with BPO or AIBN. Enzymatic and non-enzymatic reactions of melatonin with free radicals are

known (24). Apart from enzymatic metabolism, melatonin represents the most potent physiological scavenger of hydroxy radicals. The ability of melatonin to protect against hydroxy radical-induced carcinogenesis and neurodegeneration may be a significant part of the biological roles of this indoleamine (18, 25). The present findings extend these potential biological roles by suggesting that melatonin can react with and detoxify harmful carbon-centred radicals.

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