Reactions of Melatonin and Related Indoles with Free Radicals: A Computational Study

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Melatonin is being increasingly promoted as a therapeutic agent for the treatment of jet lag and insomnia and has been recently suggested to act as an efficient free-radical scavenger. In the present work, its mechanisms of action for scavenging hydroxyl radicals have been investigated using semiempirical AM1 and density functional theory (DFT) computational tools. Two different reactions were proposed as follows: one involving the abstraction of an indolic hydrogen to yield a neutral radical and another involving the addition of the hydroxyl radical to the indolic moiety. Our results show that, from a thermodynamical standpoint, melatonin may directly scavenge hydroxyl radicals both in vacuum and in aqueous solution. The structural requirements for free-radical-trapping ability have been examined comparing melatonin with related indoles. Computational data suggest that 5-methoxy and *N*-acetyl groups of melatonin do not significantly affect its thermodynamical capacity of free-radical trapping. The present results support experimental data on the potential of melatonin as a physiological or pharmacological antioxidant agent.

Introduction

The hormone melatonin, mainly produced by the pineal gland during the hours of darkness, mediates a variety of cellular, neuroendocrine, and physiological processes. The most striking features of pineal melatonin regulation are its diurnal variability and its precise response to changes in environmental lighting. Thus, melatonin is considered as a neurohormone with important functions in circadian biology.[1] Moreover, recent evidence suggests that melatonin, being a highly lipophilic molecule, has pleiotropic non-receptor-mediated functions that are not related to circadian biology and may influence also peripheral tissues as direct targets.[2] In this context, there have been multiple proposals that melatonin, as an antioxidant, can protect against damage caused by free radicals.[3]

Antioxidant effects of a compound in vivo can occur by at least two mechanisms. The compound itself may exert direct antioxidant effects, scavenging free radicals and/or inhibiting their formation. Alternatively, or in addition, the compound can act by upregulating endogenous antioxidant defenses. Available data in the literature suggest that melatonin may be able to act in both ways. It has been shown that melatonin protects the whole cell antioxidant defense system by increasing the activity of glutathione peroxidase[4] and by raising the mRNA for superoxide dismutase.[5] It has also been shown that melatonin greatly reduces DNA adduct formation in animals treated with the free-radical-generating chemical carcinogen safrole,[6] and it totally abolishes lipopolysaccharide-induced increase both in lipid peroxidation[7] and in oxidative damage in phenobarbital-treated animals.[8] These results are in agreement with other observations showing that melatonin protects against oxidative damage induced by the herbicide paraquat.[9] Taken together, these findings provide strong, albeit indirect, evidence that melatonin is a potent free-radical scavenger.

However, it was unequivocally demonstrated that melatonin in vitro scavenged •OH radicals in experiments using the spin-trapping reagent 5,5-dimethylpyrroline *N*-oxide (DMPO) and the UV photolysis of H_2O_2.[10] Moreover, melatonin was also shown to scavenge •OH generated by a Fenton reaction system. In these investigations, melatonin turned out to be the most potent scavenger of hydroxyl radicals ever detected. It has been proposed that the mechanism of action of melatonin in scavenging •OH involves the abstraction of an electron to give an indolyl cation radical.[3,11] However, the thermodynamic feasibility of this reaction has not been examined.

As •OH is the most reactive species produced in biological systems, causing oxidative damage to DNA, amino acids, protein, and membrane lipids, and since the hypothesis that melatonin may scavenge free radicals is only based on limited experimental data, we considered it worthwhile to assess, by computational tools, the free-energy changes associated with the scavenging of •OH by melatonin and related indoles. More specifically we deal herein with the following questions: (1) Is melatonin itself thermodynamically able to scavenge hydroxyl radicals in vacuum and aqueous solution? (2) Is the pathway proposed by Reiter et al.[3,11] for reaction of melatonin with •OH and O_2^- thermodynamically feasible in vacuum and aqueous solution? (3) Should melatonin be compared with

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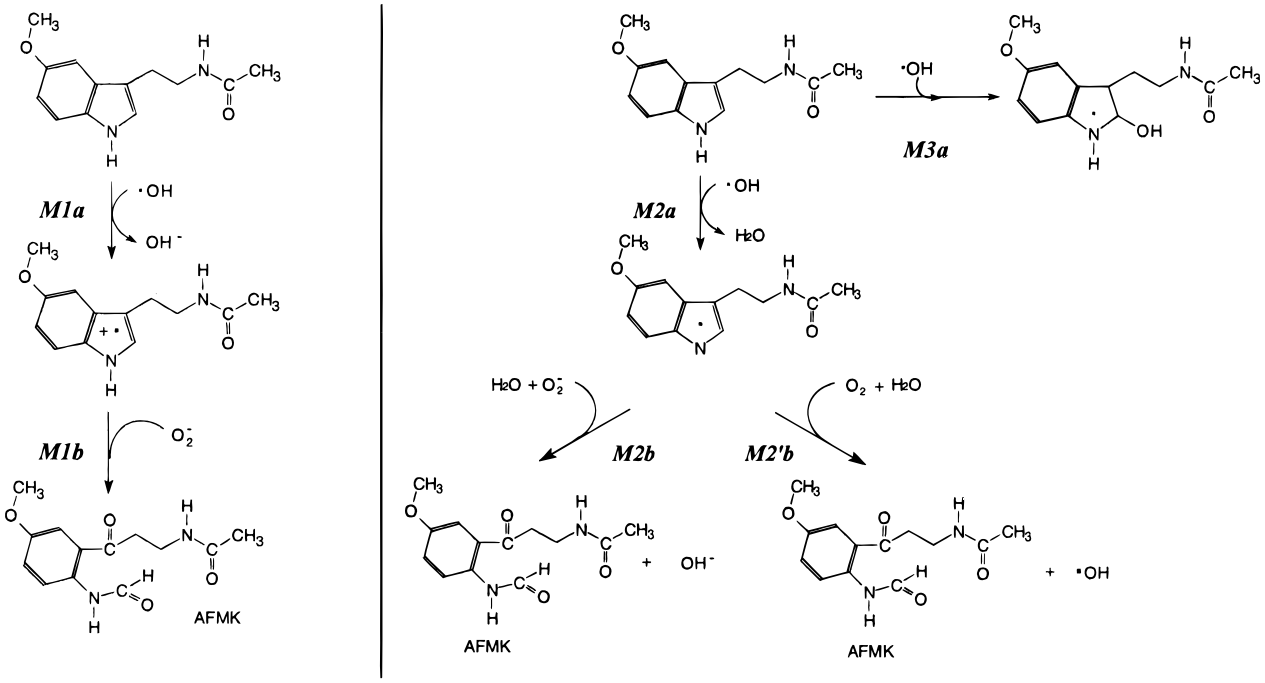
Scheme 1. M1, M2, M2′, and M3 Pathways for Reaction of Melatonin with •OH, O_2^-, and O_2



related indoles, which are the structural requirements for effective antioxidant activity?

Methods

The geometry of the isolated intermediates, reactants, and products of the investigated mechanisms was optimized by AM1[12,13] semiempirical calculations using the AMSOL package.[14] Restricted Hartree−Fock (RHF) calculations were performed for closed shell species, and an unrestricted Hartree−Fock (UHF) scheme was used for the radical states. In addition to the isolated systems AM1 computations, single-point density functional calculations (DFT) at the AM1-optimized geometries were performed using the DFT-Molecole program[15,16] at the local density approximation (LDA)[17] and the generalized gradient approximation (GGA)[18] levels. Gaussian basis sets of double ζ plus polarization quality were used for C, N, and H atoms.[19] A more detailed description of the technical aspects of the DFT calculations is given in ref 15. Radical states were calculated by unrestricted spin-polarized DFT. All closed shell species were treated with a standard restricted scheme.

The evaluation of energetic changes of the relevant processes involved computing the difference in electronic energies and zero-point energies (ZPE) between reactants and products. Since electronic energies computed at the optimized geometries correspond to 0 K, thermal effects were estimated by adding corrections which take into account the change in population of vibrational, rotational, and translational levels of reactants and products with temperature, using standard formulas of statistical mechanics[20] and the rigid-rotor, harmonic oscillator and ideal gas approximations. The change in enthalpy for a given process is

$$\Delta H^\circ = \Delta E^\circ + \Delta ZPE + \Delta H_v(T) + \Delta H_r(T) + \Delta H_t(T) \quad (1)$$

The first term (ΔE°) was evaluated from the difference in electronic energies between reactants and products. Since this term is very sensitive to the level of theory used, computations were performed using AM1 and DFT techniques at the LDA and GGA levels. The other terms were computed by using a normal modes analysis performed at the semiempirical level. The temperature was set to 37 °C. Gas-phase free-energy changes were evaluated by adding the thermal entropic contributions, estimated using the same approximations mentioned previously, based on the equipartition of energy, rigid-rotor, and harmonic oscillator approximations.

$$\Delta G^\circ(g) = \Delta H^\circ - T\Delta S^\circ \quad (2)$$

Since melatonin has been shown to have a moderate aqueous media solubility,[21] besides the well-known high-lipid solubility due to the indole moiety, we have considered the lipidic nonpolar environment situation, using an isolated system model, and the aqueous media situation, modeling solvent effects using the AMSOL-AM1-SM2.2 model of aqueous solvation.[22] The method consists of a scheme in which a generalized Born continuum model is included in the AM1 Hamiltonian. It has been parametrized to estimate not only the electrostatic contributions but also dispersion terms and energetic and entropic terms associated with solvent reorganization. This scheme has been shown to provide reliable solvation free energies for compounds containing second-row heteroatoms.[23]

As illustrated in the following thermodynamic cycle,

$$\begin{array}{ccc} & \Delta G^\circ(g) & \\ A(g) & \longrightarrow & B(g) \\ -\Delta G^\circ(\text{solv A}) \uparrow & & \downarrow \Delta G^\circ(\text{solv B}) \\ A(aq) & \longrightarrow & B(aq) \\ & \Delta G^\circ(aq) & \end{array}$$

the free-energy change of the process A → B in aqueous solution is computed by:

$$\Delta G^\circ(aq) = \Delta G^\circ(g) + \Delta G^\circ(\text{solvation products}) - \Delta G^\circ(\text{solvation reactants})$$

Results

Scheme 1 represents the mechanism proposed by Reiter et al.[11] for melatonin reaction with •OH and O_2^- radicals. The ΔG° results calculated by AM1 and DFT methodologies for the individual steps and for the global

Table 1. Free-Energy Changes ($\Delta G°$) at 310 K in Vacuum and Aqueous Solution for the Reactions of Pathways M1, M2, M2′, and M3[a]

	$\Delta G°$, kcal/mol					
	AM1		LDA		GGA	
reaction	vac	aq	vac	aq	vac	aq
M1a	161	8				
M1b	−236	−103				
M1T	−75	−95	−114	−134	−95	−113
M2a	−33	−30	−27	−23	−24	−20
M2b	−42	−65	−87	−111	−72	−96
M2T	−75	−95	−114	−134	−95	−113
M2′b	−24	−27	−47	−50	−30	−33
M2′T	−57	−57	−74	−73	−54	−53
M3a	−40	−31	−43	−34	−21	−12

[a] M1T, M2T, and M2′T are the global reactions.

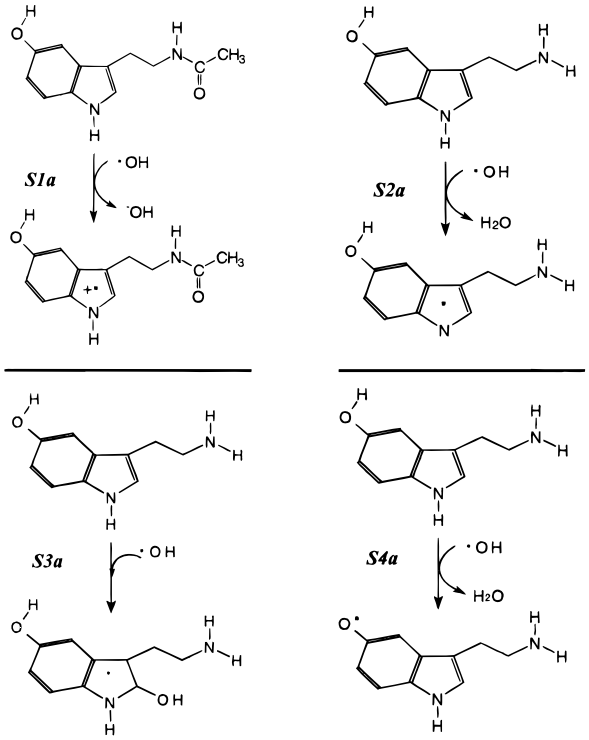
reactions are shown in Table 1. Both techniques predicted that the global process is highly exergonic both in vacuum and in aqueous solution, presumably due to the stability of the final product (N^1-acetyl-N^2-formyl-5-methoxykynuramine, AFMK). However, the critical step in scavenging •OH by melatonin (M1a reaction, Scheme 1) turns out to be an endergonic process. The $\Delta G°$ values predicted by the AM1 method for this step were 161 and 8 kcal/mol in vacuum and aqueous solution, respectively, while DFT calculations failed in converging for this intermediate. These results suggest that this may not be a feasible mechanism even though the M1b (Scheme 1) step is highly exergonic. Thus, two other pathways for melatonin conversion to AFMK, both involving the abstraction of a hydrogen atom and yielding a neutral radical as a first step, were proposed (named M2 and M2′, respectively, also shown in Scheme 1). The difference between mechanisms M2 and M2′ lies in the fact that in mechanism M2, melatonin effectively scavenges •OH and $[O_2]^-$ radicals, while the global reaction associated with mechanism M2′, denoted by M2′T, involves a futile cycle in which free-radical scavenging or production by melatonin is not involved.

The first reaction for both mechanisms (M2a) calculated by AM1 and DFT is thermodynamically feasible in vacuum and aqueous solution, but the M2b step is slightly less exergonic than M1b as the latter involves a reaction between species of opposite charge. As for the M2′ pathway, the second step (M2′b) involves the reaction of the intermediate with O_2, a more abundant species than $[O_2]^-$, its global $\Delta G°$ being smaller than the corresponding value for process M2T, in both vacuum and aqueous solution.

Alternatively, •OH may react with melatonin through addition mechanisms.[24] In this sense, we have considered the addition of •OH to the indolic moiety, as shown in Scheme 1 (M3a). This process is also predicted to be exergonic (Table 1).

To analyze the structural requirements of melatonin in its reaction with •OH, the $\Delta G°$ values of possible reactions of this radical with several related indoles (serotonin, *N*-acetylserotonin, and 5-methoxytryptamine) were computed in vacuum and aqueous solution. Four possible reactions of serotonin with •OH (S1a, S2a, S3a, and S4a, Scheme 2) were chosen: the first, second, and third are analogous to M1a, M2a, and M3a steps for melatonin, respectively, while the fourth involves the abstraction of the phenolic hydrogen, yielding a product

Scheme 2. S1, S2, S3, and S4 Reactions of Serotonin with •OH



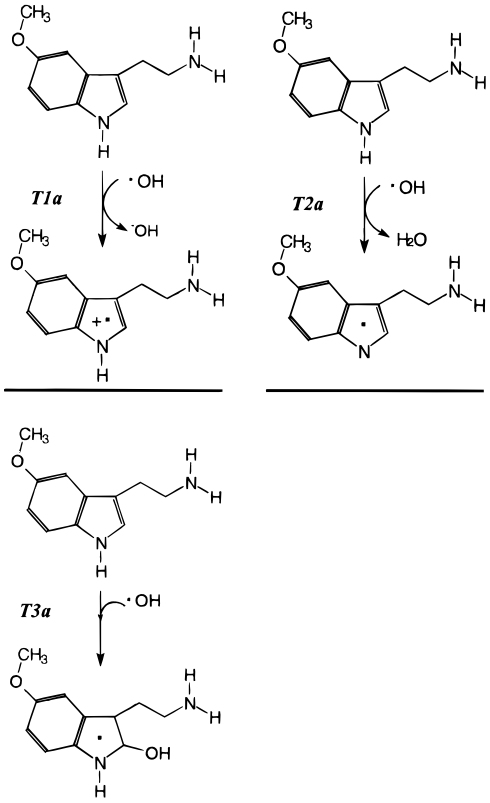
that has been reported in an EPR study.[25] The results with serotonin were very similar to those obtained for melatonin, the S1a reaction being thermodynamically not feasible, while the S2a and S4a, which involve the abstraction of an indolic and phenolic H, respectively, and the S3a addition reaction were all exergonic. Moreover, the computed $\Delta G°$ values for S2a and S4a were close to the value obtained for the M2a reaction, even though the phenolic H is more labile than the indolic H, in agreement with experimental EPR results.[25] According to our results, it seems that substitutions on lateral chains in the indolic ring do not have relevant effects on •OH thermodynamical scavenging ability, as suggested by the fact that the computed $\Delta G°$ values for the reactions of 5-methoxytryptamine (T2a and T3a, Scheme 3) and *N*-acetylserotonin (N2a and N3a, Scheme 4) with •OH were also similar to the values obtained for melatonin (M2a and M3a) and serotonin (S2a and S3a) (Table 2). Similar to M1a and S1a, both T1a and N1a reactions turned out to be endergonic.

Comparison of the computed $\Delta G°$ for the N2a and N3a reactions indicated that, as in the case of serotonin, the phenolic hydrogen of the *N*-acetylserotonin molecule is more labile than the indolic hydrogen (Table 2).

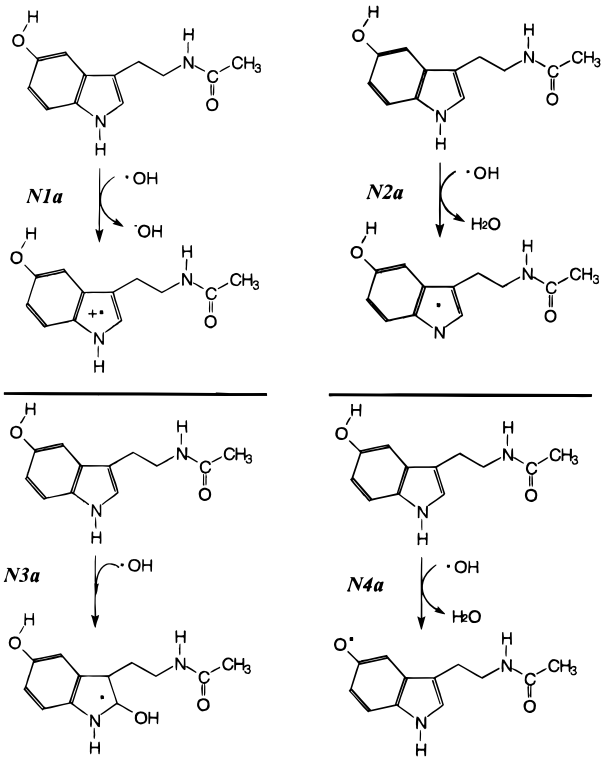
Discussion

Considerable evidence supporting the fact that melatonin, physiologically or pharmacologically, can exert antioxidant effects in vivo and in vitro has been reported.[4−11] For the first time, the present results indicate that, from the thermodynamic standpoint, melatonin may effectively scavenge free radicals (in particular •OH). Melatonin conversion to AFMK has been shown to be highly exergonic in both vacuum and aqueous solution. This result further demonstrates

Scheme 3. T1, T2, and T3 Reactions of 5-Methoxytryptamine with •OH



Scheme 4. N1, N2, N3, and N4 Reactions of *N*-Acetylserotonin with •OH



that besides its interaction with the endogenous antioxidant defense system,[4,5] melatonin itself may exert direct antioxidant effects. Although the computational methodology used is not intended to be quantitatively accurate, it is encouraging that at the different levels of theory used (AM1, DFT-LDA, and DFT-GGA) the predicted results are qualitatively similar.

Table 2. Free-Energy Changes ($\Delta G°$) at 310 K in Vacuum and Aqueous Solution for Scavenging of •OH by Melatonin, Serotonin, *N*-Acetylserotonin, and 5-Methoxytryptamine

	$\Delta G°$, kcal/mol					
	AM1		LDA		GGA	
reaction	vac	aq	vac	aq	vac	aq
M1a	161	8				
M2a	−33	−30	−27	−23	−24	−20
M3a	−40	−31	−43	−34	−21	−12
S1a	187	32				
S2a	−34	−29	−36	−31	−32	−28
S3a	−38	−30	−46	−38	−21	−13
S4a	−38	−34	−46	−42	−42	−37
N1a	163	6				
N2a	−30	−26	−37	−32	−33	−29
N3a	−40	−27	−42	−29	−28	−20
N4a	−37	−35	−47	−44	−43	−39
T1a	131	7				
T2a	−35	−31	−37	−33	−34	−30
T3a	−40	−27	−42	−29	−22	−9

Despite the accumulation of experimental evidence, little attention has been focused on the mechanism of melatonin free-radical-scavenging behavior. Hitherto, there has been only one pathway postulated by Reiter et al.[3,11] A reaction that as a first and critical step in •OH scavenging involves the abstraction of an electron to yield an indolyl cation radical (M1a). However, the $\Delta G°$ of this reaction computed by the AM1 method resulted to be positive, suggesting that this may not be a feasible mechanism.

As the conversion of melatonin to AFMK is highly exergonic, two other pathways (M2 and M2′) with the same first step and that yield the same final product, were proposed. The first reaction involves the abstraction of an indolic hydrogen yielding an indolyl neutral radical, which eventually may react through many other pathways. Among them, we have considered two alternative steps (M2b and M2′b). M2b consists of the trapping of O_2^-, while M2′b involves the reaction of the indolyl neutral radical with O_2, regenerating •OH in a futile cycle. Alternatively to M2′b, melatonin may directly react with O_2, probably through the following chain of events: indole-e^- → indole radical → indole peroxyl radical → endoperoxide → ring opening and formation of dioxiethanes → kynurenines. As this pathway has the same reactants and products as those of the M2′T reaction, the global $\Delta G°$, and therefore the thermodynamical feasibility, is the same for both processes. To assess the relative relevance between these two pathways, kinetical considerations should be taken into account.

The M2a step turned out to be exergonic in vacuum as well as in aqueous solution. According to our computed results, we can conclude that the •OH trapping by melatonin may occur by the formation of a neutral indolyl radical but not by the M1a reaction proposed by Reiter et al.[3,11] However, the possibility that, in the process of hydrogen abstraction by the hydroxyl radical to yield a neutral stable indolyl radical, the transition state has cationic character cannot be ruled out. Besides the M2a process, the scavenging of •OH by melatonin may involve an addition mechanism, as in the M3a, that turned out to be thermodynamically feasible in both vacuum and aqueous solution.

The M2T process is predicted to be more exergonic than M2′T. However, the relative relevance of these two competitive reactions also relies on the abundance of the species involved in the second step of the mechanism, O_2^- and O_2, respectively, and on other species able to react with the indolyl radical, which may vary in different physiological microenvironments. Moreover, both pathways may also compete with addition-type mechanisms.

No significant differences on the computed $\Delta G°$ for the M2a and M3a steps were found when going from vacuum to aqueous solution, probably due to the fact that no charged species were involved. The same holds for the $\Delta G°$ for the M2′b step. Larger solvent effects are observed for the M2b reaction, favoring this process in water, presumably because it involves a reaction with an ionic species. In any case, the overall reaction for the M2 and M2′ pathways is predicted to be thermodynamically spontaneous in vacuum as well as in aqueous solution, suggesting that they may be feasible in different biological media (e.g., cytoplasm and membranes) as has been previously proposed.[11]

Regarding the structure−antioxidant activity relationship, our study revealed that the 5-methoxy and the *N*-acetyl group of melatonin do not seem to significantly affect its thermodynamical capacity of radical trapping, the global $\Delta G°$ for the reaction of •OH with related indoles being very similar to that obtained for melatonin. Indoles have long been known to possess antioxidant properties,[26−28] which is consistent with our computed results. In particular, serotonin has recently been shown to react with •OH through a manifold of pathways.[29] To compare melatonin with related indoles in the present study, we have only considered the mechanisms shown in Scheme 1−4. Taking into account the available information at present, the possibility that melatonin and related indoles may operate by different mechanisms cannot be ruled out.

The thermodynamical investigation performed in this work does not take into account the kinetical aspects of the considered pathways. However, since most of the reactions investigated involve free radicals, which are expected to react at near-diffusion-controlled rates, it seems likely that the efficiency as a free-radical trapper of a compound relies mainly on the thermodynamics of the reaction and on the activities of the relevant species. In this sense, it has been demonstrated that the rate constant for the reaction of melatonin with hydroxyl radicals in aqueous solution is very high (of the order of 10^{10} M^{-1} s^{-1})[30] and that related indoles also react with •OH radicals with high rate constants, similar to those of melatonin, consistent with a diffusion-controlled regime.

Despite its thermodynamical ability to scavenge free radicals reported herein, the physiological relevance of melatonin as an antioxidant agent still remains an open question. The likelihood that melatonin will protect cells in vivo through •OH scavenging depends on the concentrations of both species in different physiological microenvironments within the cell. The endogenous concentrations of pineal melatonin are very low (the maximal serum concentrations in man being in the nanomolar range), but it may be concentrated in tissues to about 10^3 times higher than circulating levels.[31]

In addition, it may also be produced in organs other than the pineal gland such as in the retina.[32] Several experimental reports show that high concentrations of melatonin are needed to effectively scavenge free radicals, suggesting that these are unlikely mechanisms of action of physiological levels of melatonin in vivo.[30,33] However, since the predicted equilibrium constant for melatonin conversion to the indolyl neutral radical is very high (of the order of 10^{14}), each molecule of melatonin could be able to scavenge one •OH radical. Thus, it can be assumed that melatonin, even at physiological levels, would protect the cell, but as it is irreversibly oxidized, the magnitude of that protection is limited by its concentration. Therefore, in view of the lack of toxicity of melatonin, the administration of pharmacological doses of this compound may significantly improve its efficiency as an antioxidant agent.

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