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MELATONIN: MECHANISMS AND ACTIONS AS AN ANTIOXIDANT*

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INTRODUCTION

Indoles and their derivatives have long been known to possess redox properties (Jovanovic & Simic, 1985; Al-Kazwini, O'Neill, Adams, Cundall, Jacquet, Lang & Junino, 1990). Melatonin is an indoleamine synthesized from tryptophan in the pineal gland and other organs in vertebrates (Reiter, 1991). Roughly 10 years ago this molecule was found to exhibit significant chemical reducing activity and it was shown to neutralize the highly reactive hydroxyl radical ($^{\cdot}OH$) (Tan, Chen, Poeggeler, Manchester & Reiter, 1993).

In the intervening years, a large number of studies have shown melatonin and its metabolites to be highly effective free radical scavengers (Reiter, Tan, Manchester & Qi, 2000c; Tan, Manchester, Reiter, Qi, Karbownik & Calvo, 2000; Livrea, Tesoriere, Tan & Reiter, 2001) and ubiquitously acting antioxidant (Reiter 1995, 1998; Hardeland, 1997; Pappolla, Chyan, Poeggeler, Frangione, Wilson, Ghiso & Reiter, 2000). These findings have provided a wealth of new information related to a molecule, i.e., melatonin, which was initially thought to only function as a endogenous chemical signal which synchronized circadian and seasonal rhythms (Armstrong & Redman, 1993; Reiter, 1993). Furthermore, the investigations have uncovered evidence that melatonin has a significant role in reducing oxidative stress under a wide variety of experimental settings where free radical generation is known to be high (Hardeland, Reiter, Poeggeler & Tan, 1993; Hardeland, Balzer, Poeggeler, Fuhrberg, Uria, Behrmann, Wolf, Meyer & Reiter, 1995; Reiter, Oh & Fujimori, 1996; Reiter, Tang, Garcia & Muñoz-Hoyos, 1997; Reiter, Tan, Osuna & Gitto, 2000b). Both physiological (Benot, Molinero, Soutto, Goberna & Guerrero, 1998; Benot, Goberna, Reiter, Garcia-Maurino, Osuna & Guerrero, 1999; Reiter, Tan, Qi, Manchester, Karbownik & Calvo, 2000a) and pharmacological (Hardeland *et al.*, 1995; Reiter, 1995; Reiter *et al.*, 1997) evidence supports melatonin's status as an antioxidant.

This brief review summarizes what is known concerning the interactions of melatonin with strong reactants and free radical species. Where they have been identified, the products of these interactions are also mentioned along with the rate constants of the reactions. Unexpectedly, melatonin neutralizes a large number of reactive oxygen and nitrogen species.

CHEMICAL REACTIONS OF MELATONIN WITH REACTIVE OXYGEN AND NITROGEN SPECIES

The successive addition of electrons to ground state oxygen (O_2) eventually leads to the generation of water. However, the intermediates are highly reactive species, particularly those that have an unpaired electron in their valence orbital; these

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are classified as free radicals. The terminal enzyme in the mitochondrial respiratory chain, i.e., cytochrome oxidase, adds four electrons to O_2 rather efficiently to generate water and ATP. During this process, however, some reactive oxygen species (ROS) are also formed. It has been estimated that up to 4% of the O_2 utilized by mitochondria may eventually be chemically reduced to the superoxide anion radical ($O_2^{\cdot}$) (Fig. 1) (Halliwell, 1994). Considering this, a 70 kg individual will produce on the order of 2 kg $O_2^{\cdot}$ on an annual basis; fortunately, only a small percentage of this eventually ends up as the highly toxic $^{\cdot}OH$.

It is the function of a complex antioxidative defense system to protect cellular macromolecules from the abuse inflicted by ROS and free radicals. When essential molecules are mangled by reactive species they function less efficiently which then can lead to pathophysiology and disease. One component of the antioxidative defense system is melatonin which has the capability of neutralizing a number of reactive oxygen and nitrogen species (Reiter, 1998; Reiter *et al.*, 2000b; Tan *et al.*, 2000; Livrea *et al.*, 2001).

Singlet oxygen ($^1O_2$)

When O_2 absorbs energy it becomes excited and forms $^1O_2$, a non-radical ROS (Fig. 1). We initially showed that riboflavin catalyzed the oxidation of melatonin upon autooxidation induced by exposure to bright white light (Poeggeler, Saarela, Reiter, Tan, Chen, Manchester & Barlow-Walden, 1994). Similarly, Zang, Cosma, Gardner and Vallynathan (1998) found that melatonin reduced $^1O_2$-dependent 2,2,6,6-tetramethylpiperidine oxide radical generation during photodynamic excitation with the photosensitive dye, rose bengal. More recently, Roberts, Hu, Martinez and Chignell (2000) determined that melatonin scavenged UV light-induced $^1O_2$ with a rate constant of 2.2×10^7 $M^{-1}s^{-1}$. These findings are consistent with the observations that melatonin prevents damage to primary cerebellar neurons incubated with rose bengal and exposed to light (Cagnoli, Atabay, Kharlamova & Manev, 1995), a treatment which is known to generate $^1O_2$.

Superoxide anion radical ($O_2^{\cdot}$)

The addition of one electron to O_2 gives rise to the $O_2^{\cdot}$ (Fig. 1). This reactant is produced during the respiratory burst of phagocytic cells and is accidentally generated in mitochondria when oxygen is reduced to water. Assuming a 2% leakage of electrons from mitochondria and the consumption of 10^{12} O_2 molecules per cell per day, it has been estimated that as many as 2×10^{10} $O_2^{\cdot}$ may be

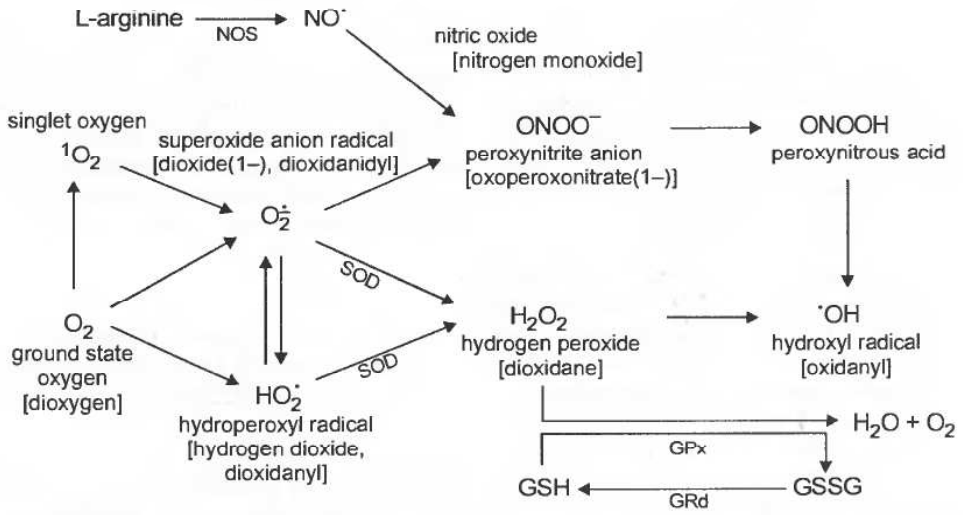

Fig. 1. One to four percent of the ground state oxygen (O_2) utilized by aerobes is reduced to reactive oxygen species (ROS), some of which are free radicals (molecules with an unpaired electron in their valence orbital). Some of the products of O_2 metabolism are shown in this figure. Of the oxygen-based products that are generated, the $^{\cdot}OH$ is considered to be the most damaging. It is estimated that in excess of 50% of the molecular clutter that accumulates as a consequence of the damage by reactive species is due to the $^{\cdot}OH$, Other highly reactive products include the $ONOO^-$ and its metabolites. $ONOO^-$ is a nitrogen-based species that is formed when $NO^{\cdot}$ couples with $O_2^{\cdot}$. Enzymes involved in the metabolism of these species include, among others, the superoxide dismutases (SOD), the glutathione peroxidases (GPx) and glutathione reductase. GSH, reduced glutathione; GSSG, oxidized glutathione (glutathione disulfide)

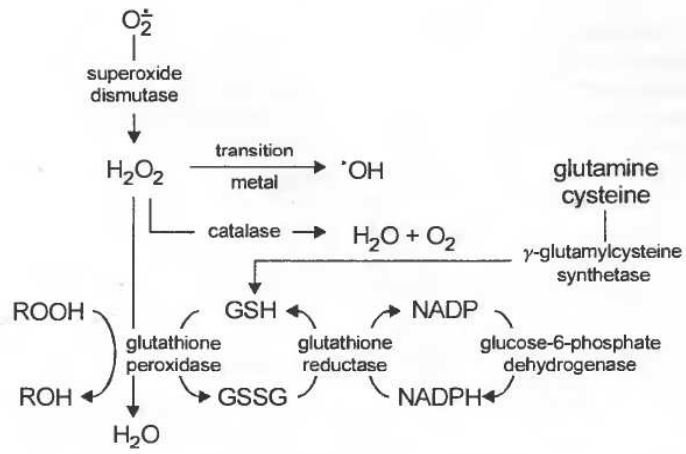

Fig. 2. Removal of H_2O_2 from cells is essential in the protection of macromolecules from damage inflicted by the highly toxic $^{\cdot}OH$. One means by which steady state concentrations of H_2O_2 within cells are held in check is via its enzymatic degradation by glutathione peroxidase and catalase. These enzymes metabolize H_2O_2 to innocuous products. Besides its enzymatic removal, H_2O_2 may also react directly with melatonin (see text) thereby also lowing intracellular concentrations of the peroxide. Reduced glutathione levels, a required substrate for glutathione peroxidase, is also regulated by melatonin since the rate limiting enzyme in glutathione synthesis, i.e., γ-glutamylcysteine synthetase, is also reportedly regulated by the indoleamine

generated in each cell every 24 hours (Chance, Sies & Boveris, 1979). Besides its formation as a natural consequence of mitochondrial oxidative phosphorylation, $O_2^{\cdot}$ is also formed during the autooxidation of catecholamines, folates, toxins, some drugs and bipyridinium herbicides. As a reactant, the $O_2^{\cdot}$ is not considered to be highly toxic. The major pathophysiological consequences of its formation relate to is metabolism to more highly reactive species.

The possible scavenging of the $O_2^{\cdot}$ by melatonin has been investigated by several groups. Marshall, Reiter, Poeggeler, Aruoma and Halliwell (1996) found melatonin ineffective as a direct $O_2^{\cdot}$ scavenger; in these studies the radical was generated by a hypoxanthine/xanthine (HX/XO) system in the presence of either cytochrome C or nitroblue tetrazolium. The reactivity of melatonin with the $O_2^{\cdot}$ also was examined in a competition study wherein DL-epinephrine autooxidation to adrenochrome was induced in the HX/XO system. Again, this study failed to provide evidence that melatonin neutralizes the $O_2^{\cdot}$ (Chan & Tang, 1996). One study has shown that melatonin quenched the electron spin resonance (ESR) signal from a spin trap 5,5-dimethyl-pyrroline-*N*-oxide (DMPO)-$O_2^{\cdot}$ adduct (Zang *et al.*, 1998); the efficacy of melatonin in doing so was only modest, however.

That melatonin may secondarily scavenge the $O_2^{\cdot}$ has been proposed (Hardeland *et al.*, 1993). In this scheme the radical product formed when melatonin scavenges a $^{\cdot}OH$ i.e., the melatonyl radical, then theoretically quenches the $O_2^{\cdot}$ to form N^1-methyl-N^2-formyl-5-methoxykynuramine (AFMK). While feasible, this metabolic pathway of melatonin has not been unequivocally documented.

Hydrogen peroxide (H_2O_2)

H_2O_2 is formed during the dismutation of $O_2^{\cdot}$ by a family of enzymes, the superoxide dismutases (SOD), as well as via several other enzyme reactions. This non-radical peroxide is also generated as a consequence of the redox cycling of catecholamines, from mitochondrial respiration, during the respiratory burst of phagocytes and from microsomal cytochrome P-450. Intracellular concentrations of H_2O_2 are believed to be controlled by its enzymatic degradation, i.e., via the action of catalase (CAT) and several glutathione peroxidases (GPx) (Fig. 2).

Early studies indicated that melatonin may not directly interact with H_2O_2. Consistent with the claims of Poeggeler *et al.* (1994), Chan and Tang (1996) found that melatonin had no direct reactivity with H_2O_2 as indicated by the lack of color formation in the peroxidase/guaiacol system.

In contrast to these reports, Zang *et al.* (1998) reported that melatonin does neutralize H_2O_2 in a system which depends on the oxidation of phenol red. We recently investigated the potential interaction of melatonin with H_2O_2 more directly and concluded that melatonin does detoxify this non-

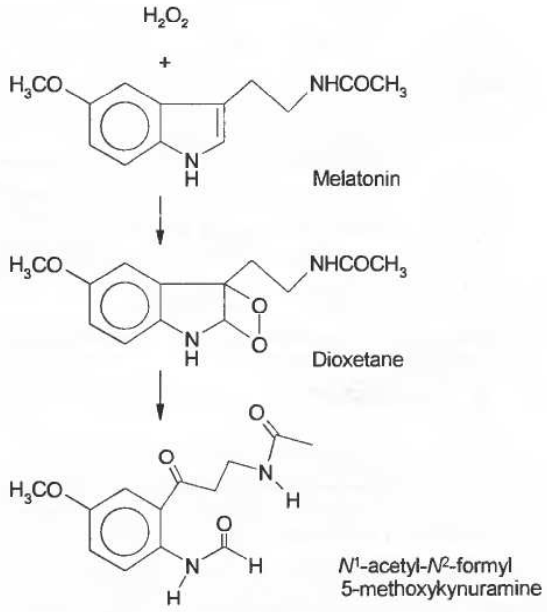

Fig. 3 We have proposed the following pathway for the direct detoxification of H_2O_2 by melatonin. The product formed, i.e., N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK), may also be an effective scavenger. Thus, not only is melatonin an effective antioxidant but the products it generates appear also to reduce oxidative damage. This cascade of reactions likely contributes to the overall antioxidative potential of melatonin. Dioxetane is one proposed intermediate product although there may be others as well

radical ROS (Tan *et al.*, 2000; Tan, Manchester, Reiter, Plummer, Limson, Weintraub & Qi, 2001). In the process of neutralizing H_2O_2, melatonin is converted to AFMK, a product identified by mass spectrometry and proton and carbon nuclear magnetic resonance (Fig. 3). In the test system which contained melatonin, both the reduction in H_2O_2 and the accumulation of AFMK were monitored. Melatonin scavenging of H_2O_2 was dose-dependent and appeared to occur in two distinctive phases. An initial rapid reaction phase reaches equilibrium within 5 s and the calculated rate constant for melatonin scavenging of H_2O_2 during this phase is 2.3×10^6 $M^{-1}s^{-1}$. During the second phase (the slow reaction phase) the rate of scavenging of H_2O_2 by melatonin gradually decreases. One intermediate that is formed when melatonin interacts with H_2O_2 is proposed to be dioxetane (Andrisano, Bertucci, Battaglio & Cavrini, 2000; Tan *et al.*, 2000).

The studies in which melatonin has been shown to directly scavenge H_2O_2 have all utilized pure chemical, cell-free systems. Assuming a similar interaction occurs intracellularly, melatonin could play a significant role in maintaining steady state levels of H_2O_2 and complementing the actions of CAT and GPx (Tan *et al.*, 2001).

Hydroxyl radical ($^{\cdot}OH$)

The $^{\cdot}OH$ (Fig. 1) is normally generated via two primary mechanisms, i.e., by the homolytic scission of the water molecule during exposure to ionizing radiation and during the interaction of H_2O_2 with a transition metal in what is referred to as the Fenton reaction, represented by the following equation:

$$metal^{n+} + H_2O_2 \rightarrow metal^{(n+1)} + {}^{\cdot}OH + OH^-$$

Under *in vivo* conditions, the metal involved is most often iron but it can also be a number of others, e.g., copper, chromium, vanadium, etc. The $^{\cdot}OH$ may also be a degradation product of peroxynitrous acid (Pryor & Squadrito, 1995) and may be formed in the reaction of $O_2^{\cdot}$ with hypochlorous acid (HOCl) (Candeias, Patel, Stratford & Wardman, 1993).

Among the oxygen-derived species, the $^{\cdot}OH$ is considered to be the most aggressive in damaging neighboring macromolecules. It reacts at a diffusion-controlled rate with every molecule it encounters and indiscriminately defiles DNA, RNA, lipids, proteins and carbohydrates. The second order rate constant for the interaction of the $^{\cdot}OH$ with these molecules is in the range of 10^9–10^{10}

$M^{-1}s^{-1}$. Because it is so reactive, its damage is site specific, i.e., very near to the locus at which it is produced.

To protect against the ·OH, organisms have evolved several means of defense including, a), reducing the formation of the ·OH by removing H_2O_2 either enzymatically (by CAT and GPx) or non-enzymatically (by melatonin) or by chelating the divalent metal ions that participate in the Fenton reaction, b), by scavenging the ·OH (by many antioxidants) once it is produced and, c), by repairing or eliminating molecules that have been damaged by the ·OH.

In 1993, melatonin was found to efficiently scavenge the ·OH (Tan *et al.*, 1993). In these studies we used the photolysis of H_2O_2 to generate ·OH and then captured the radicals with the spin trapping agent, DMPO, as in the following reaction:

$$H_2O_2 \xrightarrow[254\ mm]{\text{UV light}} OH^- + \text{DMPO}-\dot{O}H$$

The specificity and the quantity of the DMPO-·OH adducts were confirmed using electron spin resonance (ESR) spectroscopy. In this system melatonin, in a dose-dependent manner, quenched the ESR signal indicating it had successfully competed with DMPO for the ·OH. In this pure chemical system, melatonin was shown to be 5 times more effective than glutathione and 21 times better than mannitol in scavenging the ·OH. From these and other data, we calculated the rate constant for the melatonin/·OH interaction to be 0.6×10^{11} $M^{-1}s^{-1}$ (Poeggeler, Reiter, Hardeland, Tan & Barlow-Walden, 1996). Using similar methodologies, except ·OH were generated using Fenton agents, Matuszek, Reszka and Chignell (1997) confirmed melatonin's efficacy as a ·OH scavenger. The rate constant they calculated, i.e., 2.7×10^{10} $M^{-1}s^{-1}$, is similar to that determined by Poeggeler *et al.* (1996).

Others have also studied melatonin's ability to detoxify the ·OH. Using the pulse radiolysis of water to generate the radical and by monitoring the absorption spectrum of the indolyl (or melatonyl) radical, Stasica, Ulanski and Rosiak (1998a) determined that melatonin neutralized the ·OH with a rate constant of 1.2×10^{10} $M^{-1}s^{-1}$. Similarly, with the aid of similar techniques and data, two additional groups (Chyan, Poeggeler, Omar, Chain, Frangione, Ghiso & Pappolla, 1999; Mahal, Sharma & Mukherjee, 1999) calculated rate constants for the interaction of melatonin with the highly reactive ·OH to be in the range of those estimated by the earlier workers. These rate constants are 4.0×10^{10} $M^{-1}s^{-1}$ and 1.25×10^{10} $M^{-1}s^{-1}$ in the reports of Chyan *et al.* (1999) and Mahal *et al.* (1999), respectively.

Other *in vitro* and *in vivo* studies have been carried out in which melatonin was documented to detoxify the ·OH. In these studies, summarized in Table 1, a variety of different methodologies were utilized to estimate the efficiency of melatonin in removing ·OH, thereby reducing the damage they would normally inflict. Of particular interest

Table 1. In addition to the reports summarized in the text where the rate constants were calculated, the studies summarized herein have also documented melatonin's ability to directly interact with the ·OH. The studies are presented in the order of their publication

Source of ·OH	Method of measurement	References
Photolysis of H_2O_2	Spin trapping and ESR	Tan *et al.* (1993)
Fenton reagents	Reduction in melatonin fluorescence	Poeggeler, Reiter, Hardeland, Tan & Barlow-Walden (1996)
Fenton reagents	Spin trapping and ESR	Susa, Ueno, Furukawa, Ueda & Sugiyama (1997)
Fenton reagents	Kinetic study with terephthalic acid	Pähkla, Zilmer, Kullisar & Rägo (1998)
Pulse radiolysis of water	Absorption spectrum of indolyl radical	Stasica, Ulanski & Rosiak (1998b)
Ionizing radiation	Formation of cyclic 3-hydroxymelatonin	Tan, Manchester, Reiter, Plummer, Hardeis, Weintraub, Vijayalaxmi & Shepherd (1998)
Dopamine autooxidation	Production of 2,3-dihydroxybenzoate	Khaldy, Escames, Leon, Vives, Luna & Acuña-Castroviejo (2000)
Cu^{2+}/ascorbic acid system	Kinetic study with methane sulfonic acid	Bandyopadhyay, Biswas, Bandyopadhyay, Reiter & Banerjee (2000)
Glutathione/alloxan/iron system	Spin trapping and ESR	Brömme, Mörke, Peschke, Ebelt & Peschke (2000)

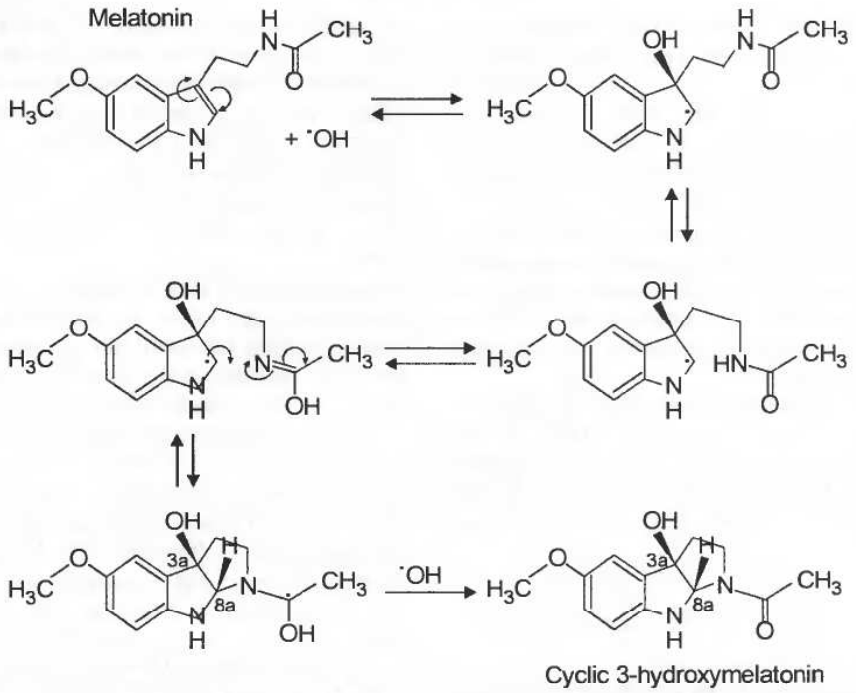

Fig. 4 Schematic representation of the scavenging of the two ·OH by one molecule of melatonin. When melatonin interacts with the initial ·OH it, by necessity, becomes a radical, in this case the indolyl (melatonyl) radical. This reactant has very low toxicity so there is a net gain when melatonin scavenges the ·OH, i.e., a highly toxic reactant is replaced by a radical with low toxicity. After some molecular rearrangement the indolyl radical scavenges a second ·OH to form cyclic 3-hydroxymelatonin. We have identified this product by electron ionization mass spectrometry and protein nuclear magnetic resonance

among these are the reports of Li, Zhang, Gu, Zhang and Sun (1997) and Khaldy *et al.* (2000). In both of these reports, salicylate trapping of the ·OH with the formation of 2,3-dihydrobenzoate (2,3-DHBA) was used to monitor melatonin's scavenging ability. When melatonin was peripherally administered to rats whose brain had been subjected to ischemia/reperfusion injury (a condition known to generate ·OH), the quantity of 2,3-DHBA was reduced in the fluid collected from microdialysis tubes placed into the striatum. This is consistent with melatonin detoxifying ·OH and thus reducing the formation of 2,3-DHBA. Using the same method for estimating the scavenging effect of melatonin, Khaldy *et al.* (2000) also found 2,3-DHBA to be reduced when melatonin was added to dopamine which was induced to undergo autooxidation. In this case, melatonin was more effective than either vitamin E or deprenyl in lowering the formation of 2,3-DHBA.

We have identified one product that results from the interaction of melatonin with the ·OH (Tan *et al.*, 1998). The proposed scheme indicates that each melatonin molecule has the capability of scavenging two ·OH resulting in the formation of 1,2,3,3a,8,8a-hexahydro-1-acetyl-5-methoxy-3a-hydroxypyrrolo [2,3-b] indole or cyclic 3-hydroxymelatonin (cyclic 3-OHM) (Fig. 4). This metabolite has a molecular weigh of 248.16 mass units greater than melatonin itself, and its structure was elucidated with the aid of proton nuclear magnetic resonance (^{1}H NMR) and COSY ^{1}H NMR. Cyclic 3-OHM occurs in both the rat and human urine and its urinary content increases after melatonin-treated rats are subjected to whole body ionizing radiation, a procedure which exaggerates ·OH generation. These findings indicate that cyclic 3-OHM is an index of the number of ·OH detoxified by melatonin and its excretion in the urine makes it a convenient biomarker of *in vivo* ·OH production. It is possible that this metabolite could be used to estimate the progression of free radical-based disease processes and/or the degree of oxidative damage in humans and in animals.

Reactive nitrogen species

Because of its electronic structure, nitrogen is polyvalent with the principle valences being 3 and

5. As a consequence, nitrogen can form a variety of compounds, several of which are of great biological importance. The nitrogen-based molecules that have been investigated relative to their interactions with melatonin include nitric oxide ($NO^{\cdot}$), the peroxynitrite anion ($ONOO^{-}$) and peroxynitrous acid (ONOOH) (Fig. 1).

$NO^{\cdot}$ is formed from the guanidino nitrogen of L-arginine and during the process it consumes five electrons; the enzyme catalyzing this reaction is nitric oxide synthase (NOS) of which there are several forms. $NO^{\cdot}$ is known to be very labile with an estimated half-life of < 1 s in blood while in tissue it presumably exists for 6–10 s. It has a number of important functions in mammals with its primary roles relating to the regulation of blood pressure, destruction of pathogens in the immune system and functioning as a retrograde neurotransmitter in the consolidation of long-term memory. $NO^{\cdot}$ is, however, known as a "double-edged sword". Besides its obvious beneficial effects, it can be highly toxic as documented in several neurodegenerative processes, e.g., ischemia/reperfusion injury (Beckman, 1991).

There are two reports claiming that melatonin can directly scavenge $NO^{\cdot}$. Noda, Mori, Liburty and Packer (1999) compared the abilities of melatonin, serotonin, *N*-acetyl-5-methoxytryptamine and 5-hydroxytryptophan to neutralize $NO^{\cdot}$ which was generated during the decomposition of 1-hydroxy-3-oxo-3-(*N*-methyl-3-aminopropyl)-3-methyl-1-triazene in a cell-free system. Of these compounds, melatonin was the best. The next best molecule for scavenging $NO^{\cdot}$ was serotonin but its IC_{50} was more than 5 times greater than that of melatonin. These findings are in line with those of Mahal *et al.* (1999) who also found melatonin to detoxify $NO^{\cdot}$; the calculated rate constant for this reaction was $3.0 \times 10^7\ M^{-1}s^{-1}$.

$NO^{\cdot}$ quickly couples with $O_2^{\cdot}$ to form $ONOO^{-}$. The reaction occurs a near a diffusion controlled rate and is roughly three times greater than the dismutation rate of $O_2^{\cdot}$ by SOD. $ONOO^{-}$ is a nitrating agent and a potent oxidant. At physiological pH, $ONOO^{-}$ is quickly protonated to form peroxynitrous acid (ONOOH), a product that produces some very strong oxidants (Crow & Beckman, 1995). Both $ONOO^{-}$ and ONOOH (as well as its activated isomer ONOOH*) attack a variety of biological targets (Radi, Beckman, Bush & Freeman, 1991).

Gilad, Cuzzocrea, Zingarelli, Salzman and Szabo (1997) were the first to suggest that melatonin reduced the toxicity of $ONOO^{-}$. They found that the oxidation of dihydrorhodamine due to exposure to $ONOO^{-}$ was reduced in the presence of melatonin. The action of melatonin in this system was equivalent to that of glutathione and cysteine. Subsequently, two elegant studies (Zhang, Squadrito & Pryor, 1998; Zhang, Squadrito, Uppu, & Pryor, 1999) showed that both $ONOO^{-}$ and ONOOH react with melatonin but, at physiological pH melatonin almost exclusively reacts with ONOOH*. One of the products of this interaction was found to be 6-hydroxymelatonin, which is also an enzymatic degradation metabolite of melatonin.

Most recently, Blanchard, Pompon and Ducrocq (2000) also examined the chemistry of melatonin in relation to $ONOO^{-}$. In a phosphate-buffered solution they showed that melatonin reacts with $ONOO^{-}$ and they noted the nitrosation and oxidation of the indoleamine at the pyrrole nitrogen leads to the formation of 1-nitrosomelatonin and 1-hydroxymelatonin, respectively; in addition small amounts of other metabolites are also formed. While the kinetics of these transformations were directly correlated with $ONOO^{-}$ decay, the yields of the metabolites were pH and CO_2 dependent. In general, the outcomes of the studies that have investigated the chemical interactions of melatonin with nitrogen-based reactants are consistent with the biological evidence showing that melatonin reduces the nitrosation of tyrosine in tissues (Cuzzocrea, Zingarelli & Caputi, 1999; El-Sokkary, Reiter, Cuzzocrea, Caputi, Hassanein & Tan, 1999).

Peroxyl radicals

Shortly after melatonin was discovered to detoxify the $^{\cdot}OH$ (Tan *et al.*, 1993), evidence was published suggesting it is also a powerful chain breaking antioxidant. According to Pieri, Marra, Moroni, Recchioni and Marcheselli (1994), in the conditions under which the tests were performed they concluded that melatonin was twice as effective as vitamin E (Trolox) in scavenging the peroxyl radical ($ROO^{\cdot}$). In their system the thermal decomposition of water soluble 2,2'-azo-bis (2-amidinopropane) dihydrochloride (AAPH) was the source of the $ROO^{\cdot}$ and the fluorescence of β-phycoerythrin was the indicator protein. A follow-up study by the same group also suggested melatonin was superior to vitamin E in neutralizing the $ROO^{\cdot}$ (Pieri, Moroni, Marra & Marcheselli, 1995). The results of these studies were somewhat startling considering vitamin E is universally accepted as the most effective natural chain breaking antioxidant. Subsequent work, however, has not been totally successful in confirming the work of Pieri and colleagues.

We examined melatonin in the context of its ability to scavenge the lipoperoxyl radical (Marshall *et al.*, 1996). Using ox brain phospholipid liposomes that were incubated with $FeCl_3$ and ascorbic acid to induce free radical generation, melatonin did reduce, in a concentration-dependent manner, the breakdown of lipids as measured by the thiobarbituric acid test; the IC_{50} value for the action of melatonin was 210 μM. Direct comparisons were not made with vitamin E in this report.

The interaction of melatonin with lipoperoxyl radicals generated in soybean phosphatidylcholine (PC) liposomes was investigated by Livrea, Tesoriere, D'Arpa and Morreale (1997) and it was their conclusion that the efficacy of melatonin in scavenging lipoperoxyl radicals in this cell-free system was less than that of α-tocopherol. Livrea *et al.* (1997) used unilamellar liposomes from the non-peroxidable lipid dimirystoyl phosphatidylcholine in comparison with soybean PC liposomes with AAPH as the radical initiator. In this mixture, they were able to distinguish reactivity of melatonin with AAPH-initiated peroxyl radicals from that of melatonin with the lipoperoxyl radical. Melatonin consumption, estimated using fluorescence measurements, was at the rate of 0.13×10^{-8} $M^{-1}s^{-1}$ while that of α-tocopherol was at the rate of 0.17×10^{-8} $M^{-1}s^{-1}$. These workers also showed that melatonin also interacted with lipoperoxyl radicals in multilamellar liposomes.

Like Livrea *et al.* (1997), Longoni, Salgo, Pryor and Marchiafava (1998) also checked the lipoperoxyl scavenging activity of melatonin. This group used several systems in which to examine these interactions; these included peroxidation of linoleic acid micelles by either AAPH (system 1) or by Fe^{2+}-EDTA (system 2) or Fe^{2+}-EDTA induction of the peroxidation of dilinoleoyl phosphatidylcholine (DLPC) multilamellar liposomes (system 3). In systems 1 and 2 the production of conjugated dienes was monitored spectrophotometrically while a colorimetric assay was used for system 3. While a concentration of 20 nM melatonin reduced the formation of lipid peroxides in the two Fe^{2+} + EDTA systems, in system 1 melatonin proved to be inept in altering the peroxidation of lipids. The authors (Longoni *et al.*, 1997) felt that these findings indicated that whereas melatonin was likely a good $^{\cdot}OH$ scavenger it was considerably less effective than α-tocopherol in neutralizing the peroxyl radical.

Some of the most complete studies designed to examine the lipoperoxyl trapping efficacy of melatonin were carried out by Antunes, Barclay, Ingold, King, Norris, Scaiano and Xi (1999). Using a variety of different systems in which α-tocopherol was used as a positive control, the finding did not support the conclusion that melatonin is highly efficient as a trap of the lipoperoxyl radical. Livrea *et al.* (1997) have shown, however, that melatonin can synergistically act with vitamin E to reduce lipoperoxyl radical concentrations.

The outcomes of the studies summarized above are clearly inconsistent and no explanation for the apparent contradictions has been provided. Melatonin lacks phenolic hydrogens and its indole hydrogen more readily undergoes electron transfer rather than hydrogen atom transfer (Solar, Getoff, Surdhar, Armstrong & Singh, 1991). Rather than abstracting hydrogen atoms from melatonin, peroxyl radicals may add to the indoleamine. When the product(s) of the interaction of melatonin with the lipoperoxyl radical is identified, a clearer picture will emerge as to if and how melatonin neutralizes this radical species.

Due to the electron-deficient nature of halide ions, haloperoxyl radicals are highly reactive. As a consequence, they oxidize substrates by electron transfer (Valgimigli, Ingold & Lusztyk, 1996). We have shown that melatonin is a potent trap of the trichloromethylperoxyl radical ($CCl_3OO^{\cdot}$) (Marshall *et al.*, 1996). In this study the direct interaction of melatonin with the $CCl_3OO^{\cdot}$ formed by radiolysis of an aqueous mixture of propanol-2-ol and carbon tetrachloride; the second order rate constant for the melatonin/$CCl_3OO^{\cdot}$ interaction was determined to be 2.7×10^8 $M^{-1}s^{-1}$. This is comparable to the rate constant (6×10^8 $M^{-1}s^{-1}$) reported by Mahal *et al.* (1999). The rate constant data are summarized in Table 2.

In vitro studies in which comparisons were made between melatonin and vitamin E in reducing lipid peroxidation have almost always shown the vitamin is superior to melatonin (Escames, Guerrero, Reiter, Garcia, Muñoz-Hoyos, Ortiz & Oh, 1997; Siu, Reiter & To, 1998). In contrast, *in vivo* melatonin is often significantly better in limiting the peroxidative breakdown of lipids and reducing oxidative damage (Abdel, Wahab, Akoul & Abdel-Aziz, 2000). Clearly, *in vitro* and *in vivo* conditions differ and until the mechanisms by which melatonin restricts lipid peroxidation in organisms are identified, the relative efficacies of melatonin and α-tocopherol in reducing the breakdown of lipid moities *in vivo* remain unknown.

Alkoxyl radicals

Melatonin has been shown to interact with the photoinduced alkoxyl radical with a rate constant

Table 2. A summary of the rate constants for the reactivity of melatonin with peroxyl and alkoxyl radicals

Source of radial (radical species)	Method of measurement	K_d ($m^{-1}s^{-1}$)	Reference
Radiolysis of aqueous CCl_4 and propanol-2-ol (trichloromethyl peroxyl radical)	Measurement of $CCl_3OO^{\cdot}$	2.7×10^8	Marshall *et al.* (1996)
Electron pulse of aqueous mixture of isopropanol, acetone and CCl_4 (trichloromethyl peroxyl radical)	Absorption spectrum of melatonyl radical	6.0×10^8	Mahal *et al.* (1999)
Laser photolysis of di-*tert*-butoxyl peroxide in acetonitrile (*tert*-butoxyl radical)	Absorption spectrum of melatonyl radical	3.4×10^7	Scaiano (1995)
Pulse radiolysis of aqueous mixture of *tert*-butanol and *tert*-butoxyl-hydroperoxide (*tert*-butoxyl radical)	Absorption spectrum of melatonyl radical	2.8×10^9	Mahal *et al.* (1999)
Laser photolysis of di-*tert*-cumyloxyl peroxide in acetonitrile (*tert*-cumyloxyl radical)	Absorption spectrum of melatonyl radical	6.7×10^7	Scaiano (1995)

of 3.4×10^7 $M^{-1}s^{-1}$ (Scaiano, 1995). This was determined in a system in which laser flash photolysis was used to cause the cleavage of di-*tert*-butoxyl and di-*tert*-cumyloxyl peroxide into the *tert*-butoxyl radical (*t*-butO$^{\cdot}$). Spectrophotometric analysis of the transient spectra was used to estimate the scavenging action of melatonin. As with the *t*-butO$^{\cdot}$, melatonin also reacted with the *tert*-cumyloxyl radical with a rate constant of 6.7×10^7 $M^{-1}s^{-1}$. Subsequent studies by Mahal *et al.* (1999) confirmed that melatonin scavenged the t-butO$^{\cdot}$; in this case the calculated rate constant was 2.8×10^9 $M^{-1}s^{-1}$. Finally, according to Barsacchi, Kusmic, Damiani, Carboni, Greci and Donati (1998) melatonin is incapable of quenching the ESR signal in a system in which galvinoxyl radicals (in CH_2Cl_2) were used as a hydrogen abstractor. The rate constants for the reactions of melatonin with alkoxyl radicals are summarized in Table 2.

Hypochlorous acid (HOCl)

HOCl is produced in neutrophils (and other cells that contain myloperoxidase) and are involved in the killing of invading organisms. During severe inflammatory responses, this reactant inflicts extensive tissue damage. The first evidence that melatonin interacts with HOCl was provided by Marshall *et al.* (1996). We conducted competition studies in which the oxidation of 5-thio-2-nitrobenzoic acid (TNB) by HOCl was assessed at a wavelength of 412 nm. Melatonin added in advance of TNB curtailed the loss of absorbance indicating it had detoxified HOCl. Chan and Tang (1996) concurrently provided evidence for a protective effect of melatonin against HOCl. They observed that melatonin reduced β-carotene quenching induced by HOCl. Finally, on the basis of their studies Dellegar, Murphy, Boune, Di Cesare and Purser (1999) calculated the rate constant for the deactivation of HOCl by melatonin to be 7.0×10^3 $M^{-1}s^{-1}$. This complete report should be consulted for the factors which influence the interactions of melatonin and HOCl.

CONCLUDING REMARKS AND PERSPECTIVES

Studies to date have likely only unraveled a portion of the mechanisms by which melatonin reduces oxidative damage. The processes by which this indoleamine controls oxidative abuse include, a), the direct scavenging of a variety of radicals and reactive species (as summarized herein and elsewhere; Reiter, 1995; Reiter *et al.*, 1997; Tan *et al.*, 2000; Livrea *et al.*, 2001), b), by inducing antioxidative enzymes which reduce steady state levels of reactive species (Pablos, Reiter, Ortiz, Guerrero, Agapito, Chuang & Sewerynek, 1998; Urata, Honma, Goto, Todoroki, Ueda, Cho, Honma & Kondo, 1999), c), inhibiting a proxidative enzyme which generates NO$^{\cdot}$, a generally beneficial molecule that can contribute to the oxidative challenge which organisms face (Pozo, Reiter, Calvo & Guerrero, 1997; Crespo, Macias, Pozo, Escames, Martin, Vives, Guerrero & Acuña-Castroviejo, 1999; Wakasuki & Okatani, 2000), d), by stabilizing cell membranes which assist them in resisting oxidative damage (Garcia, Reiter, Ortiz, Oh, Tang, Yu & Escames, 1998), e), by increasing the efficiency of mitochondrial oxidative phosphorylation thereby likely reducing free radical generation (Martin, Macias, Escames, Reiter, Agapito, Ortiz & Acuña-Castroviejo, 2000a; Martin, Macias, Escames, Leon & Acuña-Castroviejo, 2000b), f), by inhibiting the synthesis of

adhesion molecules, e.g., P-selectin and ICAM, which increase leucocyte infiltration into damaged tissue thereby exaggerating molecular destruction (Cuzzocrea, Costantino, Mazzon, Micoli, De Sarro & Caputi, 2000) and, g), perhaps most importantly, the metabolites, e.g., AFMK, that are formed when melatonin detoxifies a reactive species are also potent direct free radical scavengers (Tan *et al.*, 2000). We refer to this as the cascade action of melatonin as a free radical scavenger.

That melatonin protects cells, organs, organ systems and organisms from damage is indisputable (Acuna-Castroviejo, Escames, Macias, Muñoz-Hoyos, Molina Carballo, Arauzo, Montes & Vives, 1995; Reiter, 1995, 1998; Hardeland, 1997; Reiter *et al.*, 2001). Indeed, this indoleamine has been shown to reduce damage to nuclear and mitochondrial DNA (Reiter, Tan, Kim & Qi, 1998; Pappolla, Chyan, Poeggeler, Bozner, Ghiso, Le Doux & Wilson, 1999; Reiter 1999; Qi, Reiter, Tan, Garcia, Manchester, Karbownik & Calvo, 2000), proteins (Cuzzocrea *et al.*, 1999; El-Sokkary *et al.*, 1999; Tesoriere, D'Arpa, Conti, Giaccone, Pintaudi & Livrea, 1999) and lipids (Reiter, *et al.*, 1997; Reiter *et al.*, 1998; Coto-Montes & Hardeland, 1999; Ohta, Kongo, Sasaki, Ishiguro & Harada, 2000). Additionally, melatonin reduces tissue destruction induced by a very wide variety of chemical and physical agents (Acuna-Castroviejo *et al.*, 1995; Reiter *et al.*, 2001). Indeed, the overwhelming number of reports related to its function as a cell protector have shown melatonin to be highly effective in doing so. The actions of melatonin are certainly of pharmacological relevance but may also be of physiological importance (Marchiafava & Longoni, 1999; Reiter *et al.*, 2000b).

Antioxidants not uncommonly can, under certain conditions, also be prooxidative. This probably is most obvious for ascorbic acid; when the vitamin encounters free iron it is capable of inflicting massive oxidative damage. In reference to melatonin, Ianas, Olinescu and Badescu (1991) claimed that, while melatonin was an antioxidant, it also possessed prooxidative capabilities. Unfortunately the experimental details are not well defined in this report so the validity of this claim is in doubt. When Marshall *et al.* (1996) examined melatonin for potential proxidative capabilities, they found none.

The protective capability of antioxidants against free radical damage is increased if the scavenging molecule can be recycled. Although this has been rarely tested, Mahal *et al.* (1999) reported regeneration of melatonin from the one-electron oxidized melatonyl radical by both ascorbate and urate. The calculated rate constants for the restoration of melatonin were 7.0×10^7 $M^{-1}s^{-1}$ and 4.0×10^7 $M^{-1}s^{-1}$ for ascorbate and urate, respectively. We also have evidence that melatonin can be regenerated from an intermediate in the detoxification of H_2O_2 (unpublished, Tan, Manchester & Reiter).

Without a well integrated antioxidative defense system, aerobic organisms would quickly succumb to the persistent bludgeoning that essential macromolecules sustain as a consequence of their exposure to oxygen and nitrogen-based toxic species. A large number of molecules participate in the defense of organisms against this never-ending onslaught by reactants. The complexity of this defense system is yet not totally understood and the specific role of melatonin in the system remains undefined. Considering the multiple actions by which this indoleamine combats macromolecular mutilation by oxygen and nitrogen reactants, however, it would be difficult to dismiss it as being inconsequential. Further study will likely define additional processes by which melatonin acts to minimize oxidative stress and identify its position in the hierarchy of the antioxidative defense system.

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