DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service
Food and Drug Administration

Memorandum

0429 '03 JAN 27 P2:21

Date: January 16, 2003

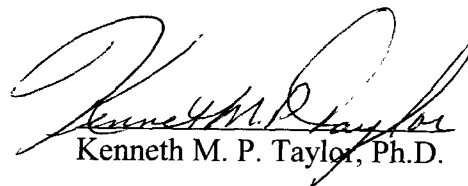
From: Chemist, Division of Standards and Labeling Regulations, Office of Nutritional Products, Labeling and Dietary Supplements, HFS-821

Subject: 75-Day Premarket Notification of New Dietary Ingredients

To: Dockets Management Branch, HFA-305

Subject of the Notification:	N-acetylserotonin (Normelatonin)
Firm:	Heartmind Nutrition
Date Received by FDA:	July 26, 2002
90-Day Date:	October 24, 2002

In accordance with the requirements of section 413(a) of the Federal Food, Drug, and Cosmetic Act, the attached 75-day premarket notification and related correspondence for the aforementioned substance should be placed on public display in docket number 95S-0316 as soon possible since it is past the 90-day date. Thank you for your assistance.



Kenneth M. P. Taylor, Ph.D.

Attachments

95S-0316

RPT 146

DEPARTMENT OF HEALTH AND HUMAN SERVICES

Public Health Service

Food and Drug Administration
College Park, MD

OCT -4 2002

Mr. Clayton A. Prepsky
Heartmind Nutrition
1015 North 2nd Avenue
435 B
Phoenix, Arizona 85003

Dear Mr. Prepsky:

This is in response to your submission of a new dietary ingredient notification, dated July 21, 2002, to the Food and Drug Administration (FDA) pursuant to 21 U.S.C. 350b(a)(2) and 21 Code of Federal Regulations (CFR) Part 190.6. FDA received your notification on July 26, 2002, of your intent to market a product containing the ingredient N-acetylserotonin (normelatonin) from a botanical source.

In accordance with 21 U.S.C. 350b(a)(2), the manufacturer or distributor of a dietary supplement that contains a new dietary ingredient that has not been present in the food supply as an article used for food in a form in which the food has not been chemically altered must submit to FDA, at least 75 days before the dietary ingredient is introduced or delivered for introduction into commerce, information that is the basis on which the manufacturer or distributor has concluded that a dietary supplement containing such new dietary ingredient will reasonably be expected to be safe. FDA reviews this information to determine whether it provides an adequate basis for such a conclusion. Under 21 U.S.C. 350b(a)(2), there must be a history of use or other evidence of safety establishing that the new ingredient, when used under the conditions recommended or suggested in the labeling of the dietary supplement, will reasonably be expected to be safe. If this requirement is not met, the dietary supplement is deemed to be adulterated under 21 U.S.C. 342 (f)(1)(B) because there is inadequate information to provide reasonable assurance that the new dietary ingredient does not present a significant or unreasonable risk of illness or injury.

Your submission indicates that you intend to market N-acetylserotonin as a tablet containing 1 mg of the ingredient with a recommended serving of 1 to 2 tablets taken at bedtime. You also intend on marketing a second related product containing 2 mg N-acetylserotonin with 100 mg alpha/gamma tocopherol. You indicate that a 2 mg portion of this product is to be taken once daily at bedtime.

FDA has carefully considered the information in your submission, and the agency has significant concerns about the evidence on which you rely to support your conclusion that

dietary supplements containing N-acetylserotonin, when used under the conditions recommended or suggested, will reasonably be expected to be safe. You state in your submission that "Millions of people have consumed melatonin as a dietary supplement over the last ten years in dosages from 1-20 mg. The recommended dosage of 1-2 mg N-acetylserotonin is based on the approximate quantities of exposure thousand of consumers have had to this antioxidant, as a metabolite of 9-20 mg of melatonin supplementation. The dosage is also based on the theoretical formation of 1-2 mg of melatonin from the formation of 1-2 mg of N-acetylserotonin." However, your notification contains no information to support these statements nor establishes that historical use, if any, is relevant to reaching a conclusion that your product when used under the conditions recommended or suggested in the labeling of the dietary supplement, will reasonably be expected to be safe. Your submission contains twenty-five reference articles, the majority of which focus on melatonin and N-acetylserotonin metabolism. You indicate that N-acetylserotonin was administered to healthy human males in doses of 25, 50 and 75 mg in reference 3. However, the reference provided is incomplete as only the abstract is provided. You also state, in your notification, that N-acetylserotonin was administered to human subjects in 75 mg doses for 90 days with no adverse responses but you provide no documentation to support this assertion. Studies that document use of melatonin, which you include, do not establish evidence of safety for the N-acetylserotonin, which is the subject of your notification. Furthermore, you stated in your submission that you relied on a rat study in which L-5-hydroxytryptophan, not N-acetylserotonin, was used to induce serotonin syndrome. However, you did not describe the relevance of this study to N-acetylserotonin and how it establishes safety of the new dietary ingredient.

Furthermore, your notification is incomplete because it does not comply with 21 CFR 190.6 (copy enclosed). (You may view FDA's web site at http://www.cfsan.fda.gov/~dms/ds-ingrd.html for additional details on new dietary ingredient notification requirements.) For example, your notification:

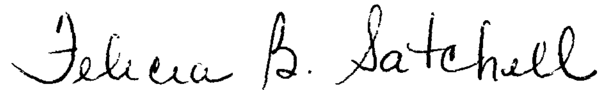
- Does not include the Latin binomial name (stating the author) of the herb or botanical [21 CFR 190.6 (b)(2)].
- Does not indicate the intended form of ingestion of the N-acetylserotonin/tocopherol mixture and FDA is unable to determine the form of the supplement from the information supplied [(21 CFR 190.6 (3)(ii) and 321 U.S.C. (ff)].

For the reasons discussed above, the information in your submission does not provide an adequate basis for safety to conclude that N-acetylserotonin, when used under the conditions recommended or suggested, will reasonably be expected to be safe. Therefore, your product may be adulterated under 21 U.S.C. 342 (f)(1)(B) as a dietary supplement that contains a new dietary ingredient for which there is inadequate information to provide reasonable assurance that such ingredient does not present a significant or unreasonable risk of illness or injury. Introduction of such a product into interstate commerce is prohibited by 21 U.S.C. 331 (a) and (v).

Your notification will be kept confidential for 90 days after the filing date. After October 24, 2002, the notification and related correspondence from FDA will be placed on public display at FDA's Dockets Management Branch in docket number 95S-0316. However, any trade secret or otherwise confidential commercial information that is in the notification will not be disclosed to the public.

If you have any questions concerning this letter, please contact me at (301) 436-2371.

Sincerely yours,



Felicia B. Satchell
Director
Division of Standards
and Labeling Regulations
Office of Nutritional Products, Labeling
and Dietary Supplements
Center for Food Safety
and Applied Nutrition

Enclosure

Premarket Notification: Normelatonin (N-acetylserotonin)
July 21, 2002 New Dietary Ingredient

Division of Standards and Labeling Regulations
Office of Nutritional Products, Labeling, and Dietary Supplements (HFS-820)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD, 20740-3835

Dear Sir or Madam:

Pursuant to Section 8 of The Dietary Supplement Health and Education Act 1994, Heartmind Nutrition wishes to inform the FDA of our intention to manufacture and distribute N-acetylserotonin (normelatonin) as a new dietary ingredient in a dietary supplement to promote human health. N-acetylserotonin (normelatonin) is a metabolite of melatonin, a dietary ingredient on the market before October 15, 1994.

Heartmind Nutrition has used due diligence in evaluating the potential health benefits and any possible health risks in the use of N-acetylserotonin (normelatonin) as a new dietary ingredient. All available information forms the basis for our conclusion that the use of N-acetylserotonin (normelatonin) is reasonably expected to be safe in the dosages recommended on the label.

Heartmind Nutrition has developed a method for producing 99.9% pure N-acetylserotonin (normelatonin) from a botanical source under non-microbial conditions. The contract manufacture will only use a GMP facility.

N-acetylserotonin (normelatonin) will be marketed in1mg dosages as a tablet.The recommended dosage is 1 to 2mg at bedtime as an antioxidant. A Heartmind Nutrition product containing 2mg N-acetylserotonin (normelatonin) as a dietary ingredient in a dietary supplement containing 100mg alpha/gamma tocopherol mixture will also be marketed. The dietary supplement containing normelatonin 2mg as a dietary ingredient is to be taken once daily at bedtime.

The label will have a warning:
"Take this product only at bedtime. If you are taking prescription medication consult with your physician before taking any dietary supplement. Do not take this product if you have been diagnosed with low blood pressure, are pregnant or breast feeding, or are diagnosed with depression. This product is not intended to treat, cure, prevent, or mitigate disease. "

Your time and attention in the evaluation of this notification is greatly appreciated. If you have any questions concerning the manufacturing procedures developed by Heartmind Nutrition we would be pleased to provide additional information. We would request such information be kept confidential, as it is a proprietary process.

Respectfully Yours,
Clayton Prepsky

Phone (602-795-6348)
Contact: Clayton A. Prepsky

BJS

Background on N-acetylserotonin (Normelatonin)

N-acetylserotonin is the biosynthetic precursor and a hepatic metabolite of the natural substance melatonin. N-Acetylserotonin may also be referred to as normelatonin (Lezoualc'h et al.1998 ref. 1.) (Sigma specification ref. 22). Melatonin (N-acetyl-O-methylserotonin) is a dietary ingredient on the market before October 15, 1994. Melatonin is consumed in the diet and has been detected in bananas, rice, and cherries (Reiter 2001 ref. 2.).

Oral administration of N-acetylserotonin in humans increases N-acetyl-O-methylserotonin (melatonin) production in a dose dependent manner (Shteinlukht et al.1998 ref.3). Dietary supplementation with normelatonin to maintain physiological levels of melatonin would be an effective way to balance the total antioxidant status of the blood (Reiter et al.1999 ref. 4.). The potential health benefits of N-acetylserotonin (normelatonin) as a new dietary ingredient are not limited to its role as the biosynthetic precursor of melatonin. N-acetylserotonin is a potent antioxidant independent of its conversion to melatonin in the body. N-acetylserotonin is neuroprotective in human neurons (ref.1.) and is 10x more potent than melatonin in protecting human low density lipoprotein (LDL) from free radical mediated oxidation (Seeger et al. 1997 ref. 5.) Serum levels of N-acetylserotonin are 10-100 times higher than melatonin in humans (Wolfer et al.1999 ref.6.).

Normelatonin Histoy of Use:

1. ***N-Acetylserotonin (Normelatonin) is a metabolite of melatonin supplementation.***

2. ***Oral N-acetylserotonin (Normelatonin) supplementation increases melatonin formation in healthy humans.***

Reasonable expectation of safety for recommended dosage 1-2 mg N-acetylserotonin:

1. ***N-Acetylserotonin 1-2mg orally as an antioxidant before bedtime correlates to quantities of long term human exposure to N-acetylserotonin (Normelatonin) as hepatic metabolite of exogenous melatonin in healthy humans.***

2. ***Long term oral supplementation with 1mg/kg N-acetylserotonin in animal models (mice) for 22 months resulted in only beneficial effects.***

3. ***L-5-Hydroxytryptophan supplementation increases N-acetylserotonin and melatonin levels***

Melatonin is a ubiquitous molecule first isolated from bovine pineal glands in 1958. In the last two decades an exponential amount of scientific research has elucidated the role of this hormone in maintaining oxidative homeostasis in organisms as diverse as algae and man. In the last decade the distribution of melatonin in a large number of edible plants has been quantified. Ingestion of foodstuffs containing melatonin elevated plasma levels of the hormone in vertebrates (Hattori et al.1995 ref. 7.).

Millions of people have consumed melatonin as a dietary supplement since 1992 in recommended dosages from 1 to 20mg. No significant toxicity or adverse reactions have been reported from the long-term human consumption of melatonin as a dietary supplement.This directly correlates with the increased long-term exposure millions of consumers have had to its metabolite N-acetylserotonin. It is established that the supplemental use of melatonin produces N-acetylserotonin by O-demethylation in the liver.

In man, N-acetylserotonin represents an average 15% of melatonin metabolites formed by exogenous melatonin (Young et al.1985 ref. 8.). Melatonin is metabolized one way in the liver of humans and another in the central nervous system (Lerner et al.1978 ref.19.).The low bioavailability of melatonin supplementation has been explained in terms of a large first pass metabolism (Facciola et al. 2001 ref. 9.). Demuuro et al. (reference 20) report approximately 15% of the oral dosage of melatonin is bioavailable with 2 and 4mg in human volunteers. Approximately 85% is metabolized in first pass metabolism. In human liver there are two principle metabolites N-acetylserotonin and 6-hydroxymelatonin. Human liver microsomes and expressed human cytochrome P-450 convert melatonin to N-acetylserotonin in vitro (ref. 9).

In a typical 10 mg dosage of melatonin as much as 1.5 mg (± 15%) of N-acetylserotonin would be formed in the liver from first pass and circulating melatonin. Anecdotally many consumers take several supplements of melatonin sold in 10mg dosages at bedtime. Melatonin is commercially available in 20mg dosages (reference 23). A dosage of 20 mg would correlate to the approximate formation of 3.0 mg (±15%) N-acetylserotonin in human liver. N-acetylserotonin conferred antioxidant protection to rat liver in vivo (Calvo et al. 2001 ref. 10.). N-acetylserotonin maintains membrane fluidity and reduces lipid peroxidation in rat hepatic microsomes (Garcia et al 2001 ref.21.).

Supplementation with exogenous melatonin increases the hepatic formation of N-acetylserotonin as evidenced by increased urine excretion of sulphate and glucuronide conjugates. Oral administration of 1g melatonin allows the isolation of the metabolic N-acetylserotonin conjugates from human urine (Leone et al. 1987 ref. 11.). Leone et al. were able to isolate 226 mg of NAS (normelatonin) conjugates from the pooled urine of three volunteers who each ingested 1000mg melatonin. Investigations of acute dosages of melatonin 6g orally in humans have shown no clinically significant toxicity (Lerner 1978 ref. 19.) The oral dosage of 6g melatonin correlates with the hepatic formation of approximately 500-900 mg (±15%) N-acetylserotonin.

Oral administration of N-acetylserotonin to healthy human males in dosages of 25, 50, and 75mg increased melatonin levels in a dose dependent manner, as evidenced by urinary excretion of 6-hydroxymelatonin as sulphate conjugates (Sheinlukht et al.1998 ref. 3.). The substantial production of N-acetylserotonin as a metabolite from dietary supplementation with melatonin and the formation of melatonin from supplementation of moderate doses of N-acetylserotonin (normelatonin) support the reasonable expectation of safety for normelatonin as a new dietary ingredient. No LD-50 for melatonin has ever been established as it one of the safest and least toxic molecules known (Barchas Dacosta et al. 1967 ref. 24). Injections of 800mg/kg in mice failed to produce death.

Millions of people have consumed melatonin as a dietary supplement over the last ten years in dosages from 1-20 mg. The recommended dose of 1-2 mg N-acetylserotonin (normelatonin) is based on the approximate quantities of exposure thousands of consumers have had to this antioxidant, as a metabolite of 9-20 mg of melatonin supplementation. The dosage is also based on the theoretical formation of 1-2 mg of melatonin from the ingestion of 1-2 mg N-acetylserotonin. The molecular weights of N-acetylserotonin and melatonin are 218.26 and 232.3 respectively.

Long term oral administration of N-acetylserotonin in several strains of mice 2.5 mg/kg for up to 22 months further indicates the intrinsic safety of this molecule (Oxenkrug et al. 1999 ref. 13.) in small oral doses. Life span was not significantly increased in female mice. In male C3H mice life span was increased by 20% by N-acetylserotonin 1mg/kg(p.o.) in drinking water.The administration of N-acetylserotonin increased the antioxidant capacity of kidney and brain using in vivo models. Aged animals administered N-acetylserotonin had shiny coats and less balding than animals fed a control diet devoid of this antioxidant. N-acetylserotonin and melatonin independently conferred neuroprotective and cognition enhancing effects against neurotoxins in vivo when administered to rats orally at 1mg/kg and 3mg/kg respectively (Bachurin et al. ref. 12.).

The biosynthesis of serotonin occurs via the tryptophan pathway in humans. Hundreds of thousands of people benefit from the supplemental use of L-5-hydroxytryptophan (5HTP). N-acetylserotonin is formed in vivo by the acetylation of serotonin derived from the decarboxylation of L-5-hydroxytryptophan. The ingestion of this widely used supplement (5-HTP) increases peripheral and cerebral serotonin, and its metabolites N-acetylserotonin and melatonin. Dietary supplementation with 5-HTP increases N-acetylserotonin production and this further demonstrates the safety of small dosages of N-acetylserotonin as a new dietary antioxidant.

Heartmind Nutrition has considered all available information in its determination of a reasonable expectation of safety for N-acetylserotonin as a new dietary ingredient. The scientific literature and premarket consumption continue to support the intrinsic safety of this endogenous antioxidant.

Evaluation of Health Risks:
Acute dosages of 5-HTP produce serotonin syndrome in rats; 75mg normelatonin orally for 90 days resulted in no adverse responses in healthy men and women

Heartmind Nutrition has carefully considered two rat studies, conducted by the same authors, on the pharmacologic regulation of “serum N-acetylserotonin” levels which reported behavioral changes in rats characteristic of the “serotonin syndrome”(Burns et al. 1982 ref. 14.) and (Brown and Burns et al. 1984 Ref.15.). These studies are of questionable relevance to the use of low oral dosages of N-acetylserotonin (normelatonin) as a dietary ingredient in humans. Serum levels of N-acetylserotonin are 10-100 times higher than melatonin in humans (Wolfer et al.1999 ref. 6.).

The experimental design relied on several strategies to manipulate serum N-acetylserotonin levels. Humans and rats produce serotonin by the decarboxylation of L-5-hydroxytryptophan via tryptophan decarboxylase.This enzymatic process is inhibited by the synthetic compound carbidopa. The authors pretreated rats with100mg/kg carbidopa followed ten minutes later by nialamide. One hour later rats were injected with 28mg/kg L-5-hydroxytryptophan or a synthetic compound N-acetyl-L-5-hydroxytryptophan 33mg/kg. These dosages are equivalent to injection of over a gram of L-5-hydroxytryptophan in humans. The animals were killed thirty minutes later and serum levels of N-acetylserotonin were measured. After injections "rapid" behavioral changes characteristic of the serotonin syndrome were observed. The rat studies injected L-5-hydroxytryptophan not N-acetylserotonin (normelatonin). Most importantly, " serotonin syndrome" is not a significantly seen side effect in individuals using L-5-hydroxytryptophan as an oral supplement in recommended dosages of 100mg-200mg. N-acetylserotonin is not metabolized to serotonin. Fillion et al.1994 injected mice i.p. with N-acetylserotonin at higher levels e.g. 30mg/kg and no behavioral symptoms characteristic of the serotonin syndrome were observed (ref. 25). Injection of N-acetylserotonin proper leads to its rapid conversion to melatonin in rats (ref. 3.). The rate limiting step in melatonin synthesis is N-acetylation.

In premarket tests, conducted in the Netherlands, administration of N-acetylserotonin 75 mg orally in male and female subjects for 90 days resulted in no adverse responses. Intravenous administration of 3mg N-acetylserotonin in a human volunteer nightly for one month produced no adverse behavioral or mental changes. Over a year later no adverse effects are reported.

Dietary supplementation with melatonin 2 and 4mg in humans results in physiological levels of melatonin (ref. 20). Serum levels of N-acetylserotonin are higher than melatonin throughout the life cycle in men (Pang et al. 1985 ref. 16. abstract and data in English). Serum N-acetylserotonin levels are significantly higher in samples of women and men (Manz et al.1985 ref. 17.) (Wolfer et al. ref. 6.).

In vitro studies using human lymphocytes indicate N-acetylserotonin to be a physiological antioxidant superior to its metabolite melatonin (Wolfer et al.1999 ref. 6.). Endogenous antioxidants such as ascorbate (vitamin C), N-acetylserotonin, and tocopherols (vitamin E can be prooxidants in high concentrations under some in vitro conditions. This oxidant potential is through the classic mechanism of hydrogen donation. In human red blood cells N-acetylserotonin reduced consumption of Vitamin E in models of oxidative stress (Barrachi et al. 1998 ref. 18).

Heartmind Nutrition has considered all available information in its determination of a reasonable expectation of safety for N-acetylserotonin as a new dietary ingredient. The scientific literature and premarket consumption continue to support the intrinsic safety of this endogenous antioxidant in small oral doses.

References:

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4. **Physiologial levels of melatonin effect the Total Antioxidant Status TAS of the blood**
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5. **Effect of melatonin and metabolites on copper mediated oxidation of low density lipoprotein**
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6. **N-Acetylserotonin is a better extra- and intracellular antioxidant than melatonin**
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10. ***Characterization of the protective effect of melatonin and related indoles against alpha-napthylisocynate-induced liver injury in rat***
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Journal of Clinical Chemistry and Clinical Biochemistry (1985) 23:657-662 Manz and Kosfeld

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22. ***Sigma Chemical specification***

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N-acetyl-serotonin (normelatonin) and melatonin protect neurons against oxidative challenges and suppress the activity of the transcription factor NF-κB

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Lezoualc'h F, Sparapani M, Behl C. N-acetyl-serotonin (normelatonin) and melatonin protect neurons against oxidative challenges and suppress the activity of the transcription factor NF-κB. J. Pineal Res. 1998; 24:168–178. © Munksgaard, Copenhagen

Frank Lezoualc'h, Mauro Sparapani, and Christian Behl
Max-Planck-Institute of Psychiatry, 80804 Munich, Germany

Abstract: It is now well established that the formation of free radicals and oxidative stress-induced neuronal cell death can be involved in various neurodegenerative disorders, including Alzheimer's disease and Parkinson's disease. The pineal hormone melatonin has been suggested to be a neuroprotective antioxidant. To better understand the molecular mechanism of this activity, we compared the ability of melatonin and its precursor, N-acetyl-serotonin (normelatonin), to protect human neuroblastoma SK-N-MC cells and primary cerebellar granular neurons against oxidative stress. We found that normelatonin and melatonin have differential neuroprotective effects depending on the neuronal cell type. Normelatonin was more protective against hydrogen peroxide (H_2O_2) and glutamate-induced cell death in SK-N-MC cells compared to melatonin which was more effective to protect primary cerebellar granular neurons against the toxicity of H_2O_2, glutamate and N-methyl-D-aspartate when compared to normelatonin. At the molecular level, we tested the capacity of normelatonin and melatonin to inhibit the oxidative stress-induced NF-κB activation in both neuronal systems. Whereas normelatonin was more potent in the suppression of the activation of NF-κB by H_2O_2 in SK-N-MC cells compared to melatonin, no apparent differences in the extent of suppression could be detected in primary neurons. Normelatonin's and melatonin's neuroprotective activity in SK-N-MC neuroblastoma cells may be mediated by the suppression of NF-κB activation.

Key words: oxidative stress – NF-κB – melatonin – N-acetyl-serotonin – N-methyl-D-aspartate (NMDA) – glutamate – neuroprotection

Address reprint requests to Christian Behl, Max-Planck-Institute of Psychiatry, Neurodegeneration Group, Kraepelinstrasse 10, 80804 Munich, Germany. E-mail: chris@mpipsykl.mpg.de

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Introduction

Eukaryotic cells continuously produce reactive oxygen intermediates (ROIs) as byproducts of electron transfer reactions [Halliwell and Gutteridge, 1989]. Among these ROIs are superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^\cdot$) [Hall and Braughler, 1989a,b]. In healthy cells, there is a balance between the generation of ROIs and the different enzymatic and non-enzymatic antioxidant defense systems [Halliwell and Gutteridge, 1989; Ames et al., 1993]. An imbalance leading to the accumulation of ROIs is defined as oxidative stress [Olanow, 1993]. Oxygen free radicals have been shown to be associated with certain neurological disorders, such as Parkinson's disease (PD) and Alzheimer's disease (AD) [Coyle and Puttfarcken, 1993; Behl et al., 1994; Behl, 1997]. The amyloid-β protein (Aβ), which accumulates in the brain of AD patients, can induce oxidative stress and lipid peroxidation via the induction of H_2O_2 accumulation [Behl et al., 1994]. Furthermore, the excitatory amino acid glutamate may induce oxidative stress in neurons and has also been implicated in various neurodegenerative diseases including AD [Choi, 1988; Coyle and Puttfarcken, 1993]. Indeed, cerebellar granule neurons produce O_2^- after brief

exposure to N-methyl-D-aspartate (NMDA), a selective agonist for ionotropic glutamate receptors [Lafon-Cazal et al., 1993]. Moreover, NMDA can induce the generation of H_2O_2 in hippocampal neurons [Mattson et al., 1995]. Another possible mechanism of glutamate-induced oxidative toxicity is the glutamate receptor-independent reduction of the level of the intracellular antioxidant glutathione [Murphy et al., 1989]. As a consequence of the observation that oxidative stress-mediated events can be involved in neurodegenerative disorders, antioxidants have been proposed and used as potential therapeutic agents against oxidative stress-induced neuronal cell death [Behl et al., 1992; 1997; Zhao et al., 1995; Sano et al., 1997].

One potent natural antioxidant is the pineal hormone, melatonin [Reiter et al., 1995; Reiter et al., 1996]. Indeed, this neurohormone has been shown to protect against various oxidative stressors, such as glutamate and Aβ [Reiter, 1995; Lezoualc'h et al., 1996; Pappolla et al., 1997]. We have recently shown that melatonin's precursor, N-acetyl-serotonin (normelatonin), is also a neuroprotectant for clonal hippocampal cells and primary cortical neurons against oxidative cell death [Moosmann et al, 1997]. However, in contrast to melatonin's well-studied antioxidant activity [Reiter et al., 1995], melatonin's potential intracellular effects are presently largely unknown, e.g., possible interactions with transcription factors.

The nuclear transcription factor κB (NF-κB) was the first eurkaryotic transcription factor shown to respond directly to ROIs [Schreck et al., 1991]. NF-κB was initially identified as an enhancer of the expression of the immunoglobulin κ light chain gene in B-lymphocytes [Sen and Baltimore, 1986]. Recently, it has been shown that melatonin can decrease NF-κB DNA binding activity in vitro in Hela cells [Mohan et al., 1995] and in vivo in the rat spleen [Chuang et al., 1996]. With respect to the central nervous system, several neurotoxins can activate NF-κB in neurons, including Aβ and glutamate [Behl et al., 1994; Kaltschmidt et al., 1995]. At present it is still under discussion whether the activation of NF-κB is casually involved in mechanisms of neuroprotection or neurodegeneration [Lezoualc'h and Behl, 1997b; Lipton, 1997]. To investigate a possible interaction with NF-κB, we examined the ability of normelatonin and melatonin to modulate NF-κB's activity in oxidative stress-induced neuronal cell death.

First, we directly compared the potential neuroprotective antioxidative activity of normelatonin and melatonin against the toxicity of H_2O_2 and glutamate in human neuroblastoma SK-N-MC cells and in primary cultures of rat cerebellar granule neurons. Furthermore, the protective effects of a preincubation with these molecules against the NMDA toxicity in cerebellar granule neurons were studied. Second, possible genomic effects of normelatonin and melatonin on the DNA binding and transcriptional activities of NF-κB were investigated after a challenge with oxidative neurotoxins.

Materials and methods

Reagents

All media, sera, and media supplements were from Gibco (Eggenstein, Germany). Melatonin (N-acetyl-5-methoxy-tryptamine), N-acetyl-serotonin (normelatonin), H_2O_2, and other chemicals were purchased from Sigma (Deisenhofen, Germany). Glutamate and NMDA were purchased from RBI (Biotrend, Köln, Germany). Melatonin and normelatonin stock solutions were dissolved in 100% ethanol. Subsequent dilutions were prepared in water. Glutamate and NMDA were also dissolved in water. SK-N-MC cells were purchased from ATCC (Rockville, USA) and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum under standard culture conditions.

Cell cultures

Primary cultures of rat cerebellar granule neurons were prepared from 8-day-old Sprague-Dawley (Charles River, Sulzfeld, Germany) rat pups, as previously described [Sparapani et al., 1997]. Neurons dissociated from cerebella were plated at different densities (see below) on plastic dishes coated with poly-L-lysine (10 μg/ml), and grown in basal modified Eagle's medium (BMEM) containing 10% heat-inactivated fetal calf serum, 25 mM KCl, 2 mM glutamine, and gentamicin (100 μg/ml). Cultures were placed in a humidified incubator at 37°C under 95% air/5% CO_2 atmosphere. Experiments were performed after 2, 3, or 8 days in vitro (as indicated below). Glial proliferation was prevented by addition of 10 μM cytosine arabino-furanoside, an inhibitor of cell proliferation, 16 hr after plating.

Cell survival analysis

Cell viability was assessed using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay, exactly as previously described [Behl et al., 1994]. All MTT assays were at least repeated four times in quadruplicate.

Drug treatments

SK-N-MC cells and primary cerebellar neurons were seeded at a density of 10^4 and 10^5 cells per well in 96-well microtiter dishes with 100 µl medium per well, respectively. After 2–3 days in vitro, the cells were preincubated for 24 h with different concentrations of melatonin and normelatonin. Then, the different toxins were added for 16 hr and cell viability was determined using the MTT assay. For glutamate and NMDA toxicity, primary cerebellar neurons were seeded in 24-well dishes at a density of 5×10^5 cells per well and after 8 days in vitro the cultures were washed in Locke's buffer (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5.6 mM glucose, and 5 mM Hepes, pH 7.4), preincubated in the same buffer for 15 min at room temperature and then exposed to 100 µM glutamate or 500 µM NMDA in Mg^{++}-free Locke's buffer for 20 min or 50 min, respectively. Melatonin or normelatonin were typically present during the incubation with glutamate receptor agonists. At the end of the treatment, cultures were washed in Locke's buffer and returned to BMEM medium without serum, until the MTT assay was performed 16 hr later. Control cultures were treated in the same way, with the exception that the toxins were not present in Mg^{++}-free Locke's buffer.

Electrophoretic mobility shift assay (EMSA)

Cytoplasmic and nuclear extracts for the EMSAs were prepared using a mini-extraction protocol [Schreiber et al., 1989]. The NF-κB double-stranded oligonucleotide corresponding to the NF-κB consensus sequence in the κ light chain enhancer in B cells (5′-AGT TGA *GGG GAC TTT CCC* AGG C-3′) was from Promega/Serva (Heidelberg, Germany) and was end-labeled with γ-[^{32}P]ATP (3000 Ci/mmol; Amersham, Braunschweig, Germany), T4 polynucleotide kinase (Promega/Serva, Heidelberg, Germany), and purified on a G-25 column. Nuclear extracts (8 to 12 µg) were incubated for 20 min at room temperature with 20 µl of 2 µg of poly(dI.dC) (Pharmacia, Freiburg, Germany), 10% glycerol, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride and 15,000 to 25,000 counts per minute of ^{32}P-oligonucleotides. For competition studies and prior to the addition of NF-κB-labeled probe, nuclear extracts were incubated for 10 min at room temperature with an 100-fold excess of NF-κB unlabeled probe. DNA-protein complexes were resolved on 6 % non-denaturing polyacrylamide gels at 20 mA for 3 hr in 0.5 × TBE (45 mM Tris-borate, and 1 mM EDTA).

Transfection and plasmids

For transient transfection, SK-N-MC cells were seeded at 120,000 cells per well in 24-well tissue culture dishes and were transfected with polyethylenimine (PEI), as previously described [Boussif et al., 1995]. PEI was used at 10 equivalents (10 amino groups per phosphate group, 3 µl of a 10 mM PEI aqueous solution per µg of plasmid). Practically, DNA (1 µg/well) and PEI (25 kDA, Aldrich, Germany) were first diluted in 50µl of 150 mM NaCl. PEI/DNA particles were, thereafter, obtained by gently mixing the 2 solutions and then, after 10 min, the transfectant solution mixture was diluted into 450 µl of DMEM without serum and applied to the cells for 3 hr. The cells were rinsed and cultured with fresh DMEM medium supplemented with 10% fetal calf serum, and glutamate with or without the hormones (melatonin or normelatonin) was applied for 12 to 15 hr. Luciferase activity was monitored, as previously reported [DeWet et al., 1987]. Each transfection experiment was done in quadruplicate, repeated two times and was normalized per mg of cell protein using the Bio-Rad protein reagent (Bio-Rad, München, Germany). As previously described, the transfection procedure did not interfere with the end result [Lezoualc'h and Behl, 1997a]. In addition, transfection efficiency is high and very regular. Thus, there has never been a need for internal normalization of reporter gene expression or for transfection efficiency when using the PEI method [Boussif et al., 1995; Boussif et al., 1996].

NF-κB-Luc plasmid construct used in this transfection study was generously provided by Dr. P. Bäuerle (Tularik, Inc., San Francisco, CA).

Statistical analysis

For statistical comparisons, the Student's t-test or analysis of variance (one-way ANOVA) followed by an appropriate post hoc test (Fisher PLSD) was used as indicated. P values <0.05 were considered as significant.

Results

Normelatonin is more protective against H_2O_2- and glutamate-induced cell death in SK-N-MC cells compared to melatonin

To measure cell viability, we used the MTT assay that is a sensitive first indicator of oxidative damage [Behl et al., 1994; Liu et al., 1997]. SK-N-MC cells were pretreated for 24 hr with different concentrations of normelatonin and melatonin and then, after this hormonal pretreatment, the cells were challenged for 16 hr with increasing concentrations of H_2O_2 (Fig. 1) and with 60 mM glutamate (Fig.

2). A high concentration of glutamate was used to induce a glutamate receptor-independent oxidative toxicity, as described [Murphy et al., 1989]. As shown in Figures 1 and 2, addition of H_2O_2 and of glutamate to the culture medium reduced SK-N-MC cell survival; 100 μM of normelatonin was highly protective against H_2O_2 and glutamate toxicity (Figs. 1A, 2) and 10 μM normelatonin still had some neuroprotective effects against these oxidative stressors. Lowering the normelatonin concentration to 1 μM could not significantly enhance the cell survival (Fig. 1A, 2). Parallel experiments were carried out

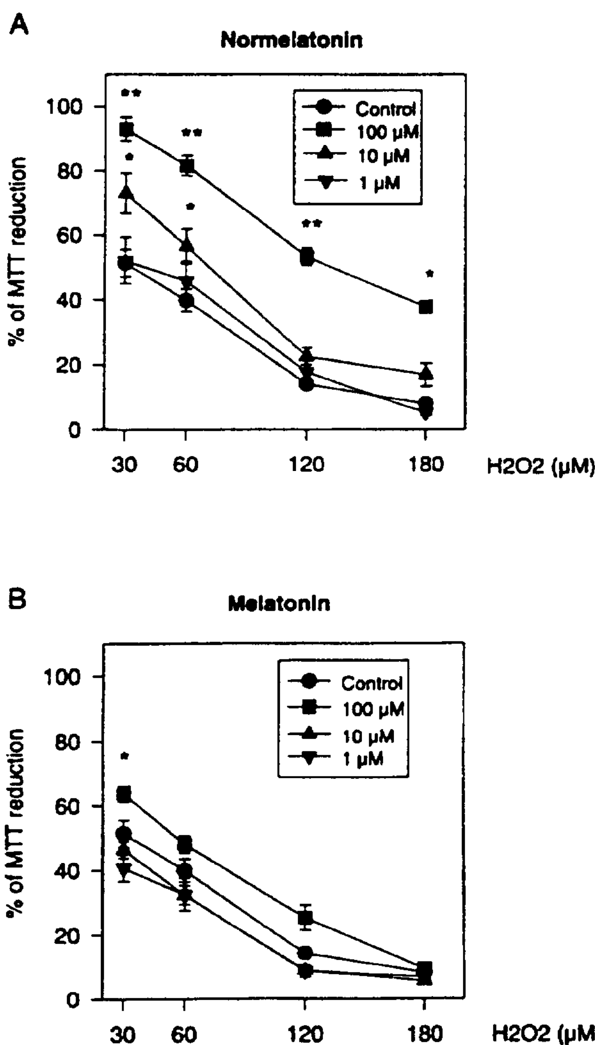


Fig. 1. Protective effects of normelatonin (A) and melatonin (B) against the toxicity of H_2O_2 in SK-N-MC neuroblastoma cells. After 2 days in vitro, the cultures were preincubated with normelatonin for 24 hr and then exposed to increasing concentrations of H_2O_2 for 16 hr. After additional 24 hr, cell viability was determined using the MTT assay. Results are the means ± SEM of at least four different experiments in quadruplicate. **$P < 0.01$ compared to control condition, *$P < 0.05$ compared to control condition. P values were determined using Fischer's PLSD after ANOVA.

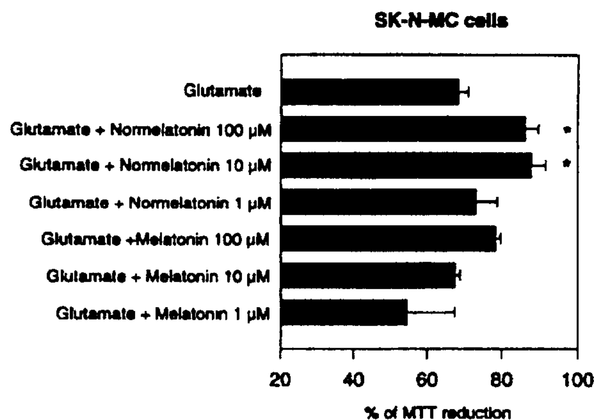


Fig. 2. Effects of normelatonin and melatonin against glutamate-induced cell death in SK-N-MC neuroblastoma cells. After 2 days in vitro, the cultures were preincubated with normelatonin or melatonin (1–100 μM) for 24 hr and then exposed to 60 mM glutamate for 16 hr. MTT assays were performed as described in Materials and Methods. Results are the means ± SEM of at least 4 different experiments in quadruplicate. *$P < 0.05$ compared to control condition. P values were determined using Fischer's PLSD after ANOVA.

using melatonin (Figs. 1B, 2). As previously reported for glutamate toxicity in murine hippocampal HT22 cells [Lezoualc'h et al., 1996], pretreatment of human SK-N-MC cells with 100 μM of melatonin afforded protection against cell death induced by H_2O_2 (Fig. 1B). Overall, in SK-N-MC cells, melatonin was less effective in preventing H_2O_2 and glutamate toxicity compared to normelatonin (Fig. 1 and Fig. 2). While 100 μM of normelatonin increased the cell viability by approximately 42% after a toxic challenge with 30 μM H_2O_2 (Fig. 1A), the same concentration of melatonin enhanced cell viability only by approximately 12% (Fig. 1B). For the glutamate toxicity, 100 μM melatonin did also not significantly enhance cell viability, whereas normelatonin significantly increased the cell viability by approximately 18 ± 4% ($P<0.05$) (Fig. 2).

Melatonin is more protective against H_2O_2-, glutamate and NMDA-induced cell death in primary cerebellar neurons compared to normelatonin

Using primary cultures of rat cerebellar granule neurons, we studied whether-melatonin and its precursor, normelatonin, can also act as antioxidative neuroprotectants in this neuronal system. Hydrogen peroxide can be toxic for primary cerebellar neurons as assessed with the MTT assay (Fig. 3). When the cerebellar neurons were preincubated for 24 hr with 100 μM normelatonin or 100 μM melatonin before the addition of H_2O_2, neuronal viability was significantly increased. In contrast to the protection ob-

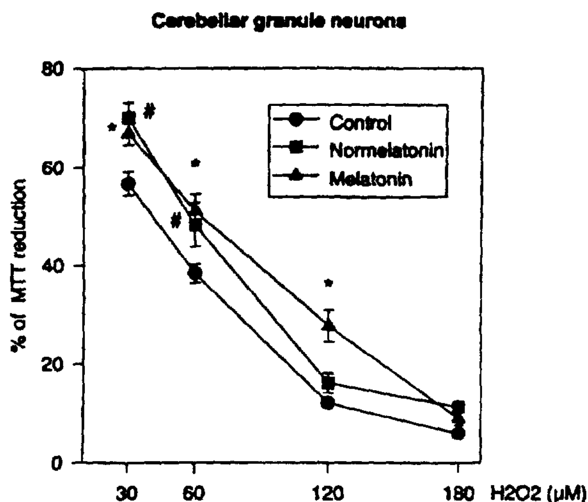


Fig. 3. Normelatonin and melatonin protect against H_2O_2-induced cell death in cerebellar granule neurons. After 3 days in vitro, the cultures were preincubated with normelatonin or melatonin (100 µM) for 24 hr and then exposed to H_2O_2 for 16 hr. Cell viability was determined by performing the MTT assay. Results are the means ± SEM of at least four different experiments in quadruplicate. *$P < 0.05$ compared to control condition. P values were determined using Fischer's PLSD after ANOVA.

served in SK-N-MC cells, melatonin was more protective against H_2O_2 toxicity in primary cerebellar neurons. Even after the treatment with 120 µM H_2O_2, only melatonin still increased cell survival by approximately 16% (Fig. 3). Lower concentrations of melatonin and normelatonin did not afford any protection from H_2O_2 challenge (data not shown).

Because granular neurons develop a sensitivity to glutamate receptor-mediated toxicity after several days in culture, which is similar to that of mature neurons [Schramm et al., 1990; Resink et al., 1994], we tested the neuroprotective effects of melatonin and normelatonin on glutamate and NMDA toxicities in primary cerebellar neurons. Here, we found that melatonin is effectively protecting against glutamate-induced cell death (Table 1). Melatonin's protective activity against glutamate is also shown in Figure 4. Moreover, melatonin is more protective against the toxicity triggered by 500 µM NMDA compared to normelatonin (Table 1). Normelatonin increased the cell survival only by 16 ± 4.5% and melatonin by approximately 38 ± 8.1% (P<0.05).

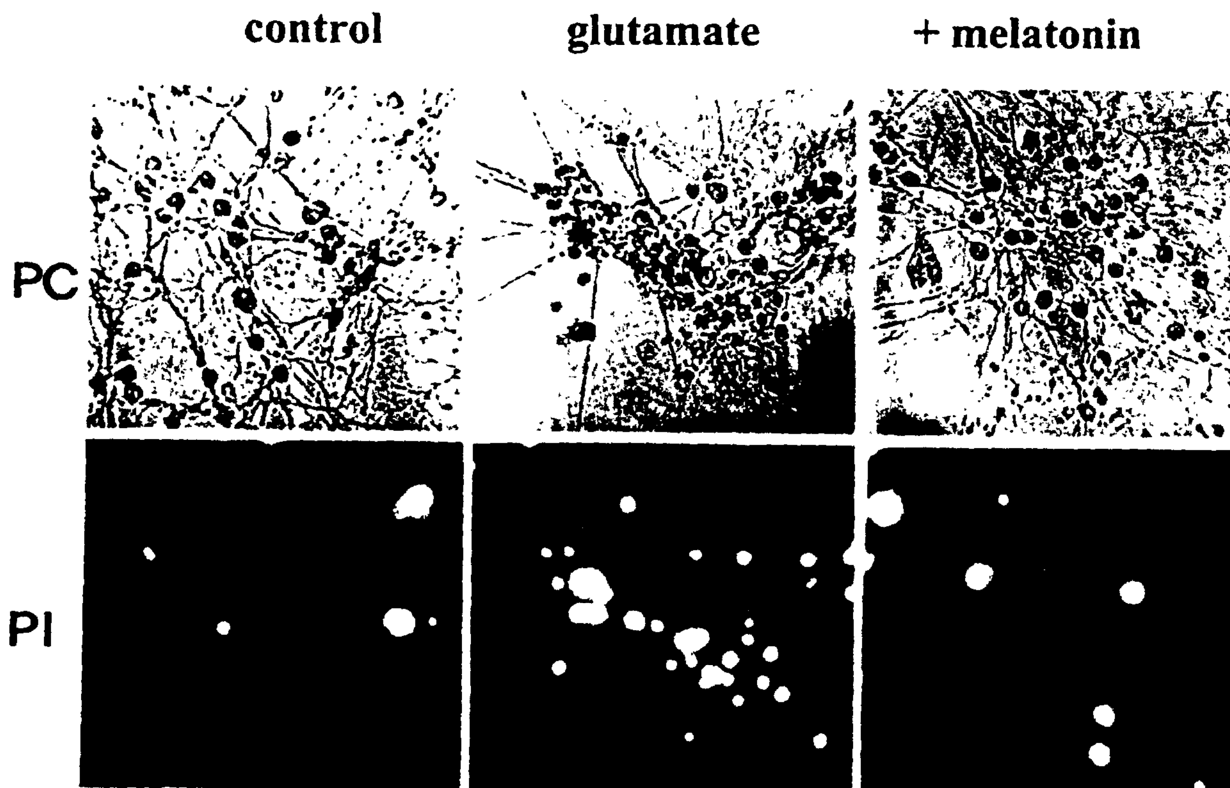


Fig. 4. Protection from glutamate toxicity by melatonin as detected by propidium iodide (PI) analysis. Cerebellar granule neurons were pretreated for 24 hr with 100 µM melatonin, before an oxidative challenge with 100 µM glutamate. After 16 hr, cultures were stained with PI and then viewed using phase contrast (PC) and fluorescence microscopy. Magnification was 200-fold.

Table 1. Protective effect of normelatonin and melatonin against neurotoxicity induced by exposure to glutamate or NMDA in cerebellar granule neurons[a]

Control	100
100 μM glutamate alone	47.1 ± 3.9
100 μM glutamate + 100 μM melatonin	73.8 ± 4.1[b]
100 μM glutamate + 100 μM normelatonin	54.9 ± 3.0
500 μM NMDA alone	50.9 ± 2.5
500 μM NMDA + 100 μM melatonin	88.7 ± 8.1[b,c]
500 μM NMDA + 100 μM normelatonin	66.5 ± 4.5[b]

[a]Primary cultures were pretreated with the hormones for 24 hr at 8 days in vitro. Then, cultures were challenged with glutamate or NMDA in Mg^{++}-free Locke's buffer for 20 min or 50 min, respectively. After 16 hr, neuronal survival was assessed with the MTT assay. Viability of cerebellar granule neurons is indicated in percentage ± SEM. Results are the means ± SEM of 4–10 independent experiments.
[b]$P < 0.05$ melatonin + glutamate compared to glutamate alone. $P < 0.05$ melatonin or normelatonin + NMDA compared to NMDA alone.
[c]$P < 0.05$ melatonin + NMDA compared to normelatonin + NMDA. P values were determined using Fischer's PLSD after ANOVA.

Effects of normelatonin and melatonin on NF-κB's activity in SK-N-MC cells and in primary cerebellar neurons

To determine whether the neuroprotective effects of melatonin and normelatonin are correlated with any change in the activity of the redox-sensitive transcription factor NF-κB, we first studied the ability of these compounds to modulate H_2O_2- and glutamate induced NF-κB's DNA binding activity by EMSA. According to the observation that NF-κB is a transcription factor whose regulation precede the death of neurons [Baichwal and Baeuerle, 1997], we selected a window of time which precedes the cell death to examine its activity. We found that both toxins can induce NF-κB's DNA binding activity in SK-N-MC cells (Fig. 5A). Because H_2O_2 is a more rapid and potent inducer of NF-κB DNA binding activity than glutamate (Fig. 5A), we chose H_2O_2 as a paradigm of oxidative stress to study the effects of melatonin and normelatonin on NF-κB's activity at the DNA binding level. Consistent with previous results [Schreck et al., 1991, Meyer et al., 1993], NF-κB's DNA binding activity was increased upon treatment of SK-N-MC cells with 120 μM H_2O_2 for 1 or 2 hr (Fig. 5A, lanes 5,6; Fig. 5B, lane 2). Preincubation of the cells for 6 hr with 100 μM normelatonin completely prevented H_2O_2-induced NF-κB activation (Fig. 5B, lane 4). The DNA binding activity could also be inhibited by the addition of 10 μM normelatonin, but to a less extent (Fig. 5B, lane 6).

In stark contrast but consistent with the results of the toxicity assay, 10 μM and 100 μM melatonin were less effective than the same concentrations of normelatonin in the suppression of NF-κB's activation in SK-N-MC cells (Fig. 5B, lanes 3 and 5). Normelatonin and melatonin added to the cells alone did not influence NF-κB's base line activity (Fig. 5B, lanes 7–10).

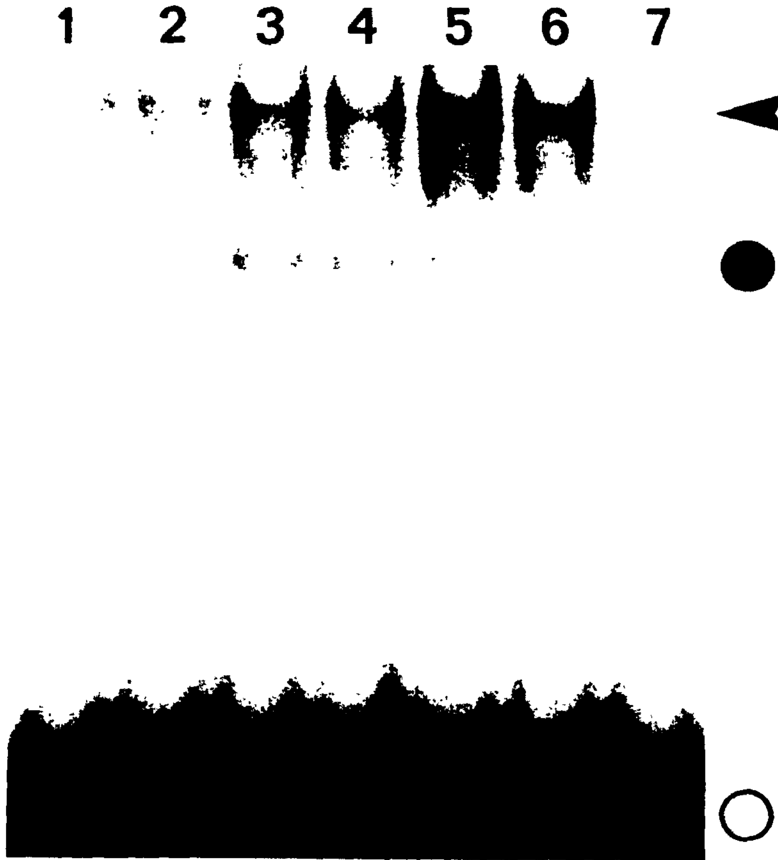
For transfection experiments that require at least an overnight exposure with the toxin, we selected glutamate that is less toxic than H_2O_2 (compare Fig. 1 to Fig. 2) to analyze the effects of melatonin and normelatonin on NF-κB transcriptional activity. Therefore, we transiently transfected SK-N-MC cells with a plasmid construct containing a promoter composed of six NF-κB-binding DNA consensus sites linked to a luciferase reporter gene (NF-κB-Luc). Figure 6 shows that the luciferase activity is significantly increased after exposure to glutamate. Coincubation of the cells with 1 mM glutamate and either with 100 μM normelatonin or with 100 μM melatonin abolished glutamate-induced NF-κB activity (Fig. 6). Consistent with the suppression of the DNA binding activity of NF-κB, normelatonin was more efficient than melatonin to inhibit NF-κB's transcriptional activity ($P< 0.05$, Student's t-test) (Fig. 6). Cell treatment with only normelatonin or melatonin did not significantly influence NF-κB transcriptional base line activity (Fig. 6). These results suggest that the ability of normelatonin and melatonin to protect against oxidative stress-induced SK-N-MC cell death is correlated with their capacity to inhibit NF-κB activation in these cells.

Next, we measured the effects of normelatonin and melatonin on NF-κB activity also in primary cerebellar neurons by EMSAs. Hydrogen peroxide induced the activity of NF-κB (Fig. 7, lane 2). Preincubation of the cells with either 100 μM melatonin (Fig. 7, lane 3) or 100 μM normelatonin (Fig. 7, lane 4) for 6 hr inhibited NF-κB's activation to a similar extent, in contrast to SK-N-MC cells.

Taken together, these observations suggest that at least one mechanism of normelatonin's and melatonin's neuroprotective activity in SK-N-MC cells is due to the suppression of the activation of NF-κB.

Discussion

Melatonin and its precursor, N-acetyl-serotonin (normelatonin), protect neurons against oxidative challenge with different efficiencies, depending on the neuronal system examined. Melatonin can prevent cell death induced by H_2O_2 in SK-N-MC neuroblastoma cells and in primary cerebellar granular neurons. Our results are consistent with previous reports showing the neuroprotective properties of this pineal hormone against different oxidative stress paradigms in different neuronal and non-neuronal cellular systems [Tan et al., 1993; Giusti et al., 1995;



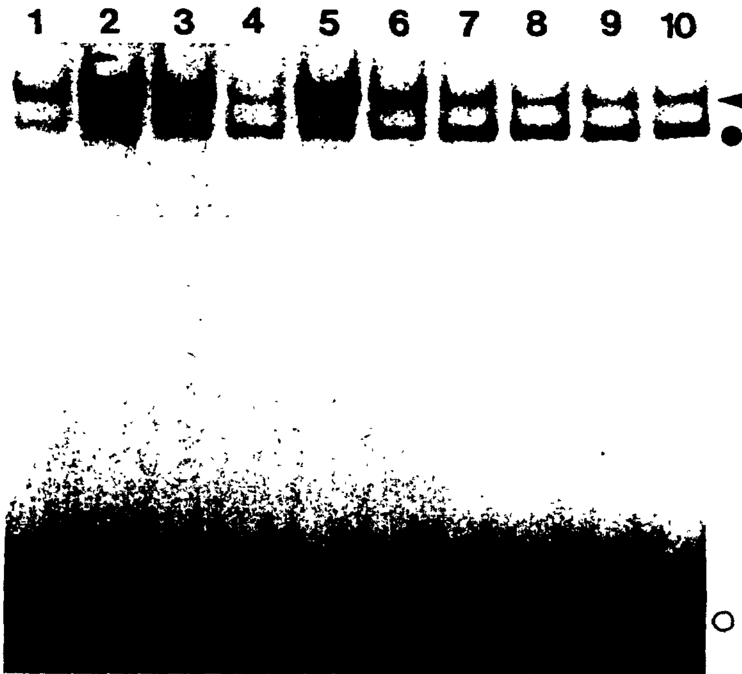


Fig. 5. A: Kinetics of induction of NF-κB's DNA binding activity upon treatment of SK-N-MC cells with glutamate and H_2O_2. Nuclear extracts were prepared from SK-N-MC cells treated with either 2.5 mM glutamate or 120 μM H_2O_2 after different time points and analysed by EMSA. Autoradiograph of a native gel is shown. Lane 1, control untreated cells; lane 2, 2.5 mM glutamate for 2 hr; lane 3, 2.5 mM glutamate for 7 hr; lane 4, 2.5 mM glutamate for 12 hr; lane 5, 120 μM H_2O_2 for 1 hr; lane 6, 120 μM H_2O_2 for 2 hr, lane 7, nuclear extracts from H_2O_2 treated cells were coincubated with the labeled probe and 200-fold excess of unlabeled specific nucleotides (B) Effects of normelatonin and melatoninon NF-κB's DNA binding activity in SK-N-MC cells. Cell cultures were pretreated for 6 hr with different concentrations of normelatonin and melatonin and then were treated with 120 μM H_2O_2 for 1 h. EMSA was performed as described in Materials and Methods. Autoradiograph of a native gel is shown. Lane 1, control untreated cells; lane 2, 120 μM H_2O_2; lane 3, 100 μM melatonin and 120 μM H_2O_2; lane 4, 100 μM normelatonin and 120 μM H_2O_2; lane 5, 10 μM melatonin and 120 μM H_2O_2; lane 6, 10 μM normelatonin and 120 μM H_2O_2, lane 7, 100 μM melatonin; lane 8, 100 μM normelatonin; lane 9, 10 μM melatonin; lane 10, 10 μM normelatonin. The filled arrowhead indicates the position of specific NF-κB/DNA complexes, the filled circle the position of non-specific complexes, and the circle shows the positions of free DNA probe.

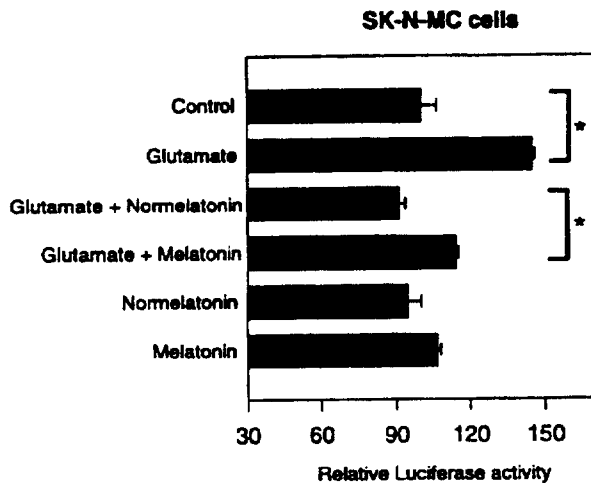


Fig. 6. Normelatonin and melatonin inhibit NF-κB's transcriptional activity induced by glutamate. SK-N-MC cells were transfected with 1 μg of NF-κB-Luc plasmid and were then exposed to either 1 mM glutamate or 1 mM glutamate and 100 μM normelatonin or 100 μM melatonin for 12 to 15 hr prior to harvesting. Results are shown in arbitrary units of luciferase activity (relative luciferase activity) corrected for identical protein amounts. All data are means ± S.E.M. for one representative quadruplicate determination. Luciferase activity of untreated control cells was defined as 100%. The results represent one of two independent experiment.

Fig. 7. Normelatonin and melatonin suppress H_2O_2-induced NF-κB's DNA binding activity in cerebellar granule neurons. Primary neurons were pretreated for 6 hr with different concentrations of normelatonin and melatonin and then were treated with 120 μM H_2O_2. EMSA was performed as described in Materials and Methods. Autoradiograph of a native gel is shown. Lane 1, control untreated cells; lane 2, 120 μM H_2O_2; lane 3, 100 μM melatonin and 120 μM H_2O_2; lane 4, 100 μM normelatonin and 120 μM H_2O_2; Lane 5 nuclear extracts from H_2O_2 treated cells were coincubated with the labelled probe and 100-fold excess of unlabeled specific nucleotides. The filled arrowheads indicate the position of specific NF-κB/DNA complexes, the filled circle the position of non-specific complexes and the circle shows the positions of the free DNA probe.

Reiter et al., 1995; Giusti et al., 1996; Lezoualc'h et al., 1996; Pappolla et al., 1997]. In addition, we report that normelatonin also afforded protection against H_2O_2 in both neuronal systems employed. Moreover, normelatonin efficiently protected the SK-N-MC against the glutamate receptor-independent toxicity. The aromatic alcohol normelatonin showed a higher protective potential compared to melatonin in the SK-N-MC cells. This is consistent with our most recent observations employing clonal hippocampal HT22 cells [Moosmann et al., 1997]. There, we have shown that one reason for this lower protective potential of melatonin could be the ether modification of the hydroxyl group at the C-5 of the indole scaffold at the melatonin molecule. The phenolic group, as structural prerequisite for the neuroprotective activity, has previously been identified also for estrogens [Behl et al., 1997].

It is well known that the excitotoxicity triggered by the glutamate agonist in the cerebellar granule neurons involves the production of ROIs [Atlante et al., 1997]. Using this model of excitotoxic injury, we showed that melatonin and normelatonin have a differential protection potential, as melatonin appeared to be more efficient than normelatonin. In contrast to the results of Giusti et al. [1995], we found that melatonin can also protect against NMDA toxicity in primary cerebellar cultures. This could be explained because Giusti and colleagues [1995] applied the melatonin only during the 60 min NMDA treatment and in the present study, we added the hormone 24 hr before as well as throughout the NMDA challenge.

Another factor that can influence the neuronal sensitivity and that has recently gained great inter-

est for its potential role in neuroprotection is the oxidative-stress sensitive transcription factor NF-κB [Lezoualc'h and Behl, 1997b; Lipton, 1997]. To elucidate a potential role for NF-κB in normelatonin's and melatonin's neuroprotective activity, we examined their effects on the induction of NF-κB activity by oxidative stress. We found that both compounds, normelatonin and melatonin, inhibited the activation of NF-κB in both neuronal systems employed. These results are consistent with the observation that exogenous addition of melatonin in HeLa cells blocks the activation of NF-κB by tumor necrosis factor-α, phorbol 12-myristate 13 acetate, or ionizing radiation [Mohan et al., 1995]. In addition, we showed that normelatonin, which is more protective than melatonin against H_2O_2- and glutamate-induced cell death in SK-N-MC cells, also led to a more potent inhibition of NF-κB DNA binding activity. These observations were also confirmed at the transcriptional level, suggesting that normelatonin is a more efficient than melatonin to inhibit the expression of NF-κB target genes in this cellular system. According to these results, we can speculate that a decrease in NF-κB's activity in this system is causally related to normelatonin's and melatonin's neuroprotective effect. The support of this hypothesis comes from the recent study of Grilli and colleagues [1996]. They reported that several different anti-inflammatory drugs, such as aspirin and salicylate, block excitotoxic damage in neurons by the inhibition of glutamate-induced NF-κB activation. On the other hand, some reports also support a potential role for the activation of NF-κB in neuroprotection [Lipton, 1997].

In the primary cultures, we did not see any difference in the inhibition of NF-κB's activity by melatonin and normelatonin, despite the higher protective potential of melatonin against oxidative stress in the cerebellar granule neurons. Therefore, in this neuronal system the protective activity does not appear to be directly correlated with the inhibition of the NF-κB induction. While other mechanisms might be involved, such as the direct inhibition of lipid peroxidation, at least for melatonin, additional hormonal activities that could play a role in the neuroprotection have to be considered (see below).

In our present study, we found that normelatonin and melatonin can have differential neuroprotective effects depending on the neuronal cell type. Herein, normelatonin is shown to be less effective than melatonin to protect primary cerebellar granular neurons against H_2O_2, NMDA and glutamate excitotoxicity. The neuroprotective effects of melatonin in the cerebellar granule neurons do not seem to be related to a direct action of melatonin on ionotropic glutamate receptors [Giusti et al., 1995]. A cell-specific neuroprotective activity of melatonin may be due to additional, probably hormonal effects of this compound besides its pure antioxidant activity. For instance, melatonin has been found to stimulate the activity of the H_2O_2-detoxifying antioxidant enzyme gluthatione peroxidase (GSH-Px) in rat brain [Barlow-Walden et al., 1995]. GSH-Px is a major antioxidative enzyme in many tissues and is believed to be a major antioxidative enzyme system in the brain [Sinet et al., 1980]. Moreover, it has been reported that melatonin can confer marked protection against paraquat-induced oxidative toxicity in both, the lung and the liver, by preventing a drop in one of the most powerful nonenzymatic cellular antioxidants, glutathione [Melchiorri et al., 1995]. Finally, in the cerebellum melatonin is reported to reduce the activity of nitric oxide synthase (NOS), probably also via its genomic activity [Pozo et al., 1994; Reiter et al., 1996]. Therefore, one could speculate that depending on the cellular system melatonin may act either as a pure antioxidant or as both, a stimulator of the intracellular antioxidant defense system and a free radical scavenger. The latter mechanism could also make the physiological hormone, melatonin, more effective in neuroprotection against oxidative stress in the primary cultures than its precursor, normelatonin.

In conclusion, we present evidence that melatonin and its precursor normelatonin can prevent oxidative stress-induced by glutamate (receptor-mediated or receptor-independent neurotoxicity) and H_2O_2 in neurons. The neuroprotective potential of the two molecules is dependent on the time of incubation, the model of oxidative stress, and the neuronal cell type. In SK-N-MC cells, the protective activity may reside in their ability to inhibit NF-κB induction, at least in part. On the other hand, in cerebellar granule neurons, the protective potential for melatonin may be due to the combination of the antioxidant free radical scavenging activity and to the capacity to stimulate the intracellular defense system. This latter mechanism may be mediated via a nuclear melatonin receptor with subsequent action on gene expression [Barlow-Walden et al., 1995; Reiter et al., 1996]. This point remains to be elucidated in detail, in order to better understand the molecular mechanism of melatonin's neuroprotection and to develop new strategies against oxidative stress-induced neuronal death.

Acknowledgments

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Melatonin in Plants

Russel J. Reiter, Ph.D., Dun Xian Tan, M.D., Susanne Burkhardt, Ph.D., Lucien C. Manchester, Ph.D.

Once thought to be exclusively a molecule of the animal kingdom, melatonin has now been found to exist in plants as well. Among a number of actions, melatonin is a direct free radical scavenger and an indirect antioxidant. Melatonin directly detoxifies the hydroxyl radical (•OH), hydrogen peroxide, nitric oxide, peroxynitrite anion, peroxynitrous acid, and hypochlorous acid. The products from each of these reactions have been identified in pure chemical systems and in at least one case in vivo; the interaction product of melatonin with the •OH, i.e., cyclic 3-hydroxymelatonin, is found in the urine of humans and rats. Some of the products that are produced when melatonin detoxifies reactive species are also highly efficient scavengers. As a result, a cascade of scavenging reactions may enhance the antioxidant capacity of melatonin. Additionally, melatonin increases the activity of several antioxidative enzymes, thereby improving its ability to protect macromolecules from oxidative stress. Melatonin is endogenously produced and is also consumed in edible plants. In animal experiments, feeding melatonin-containing foods raised blood levels of the indole. Because physiologic concentrations of melatonin in the blood are known to correlate with the total antioxidant capacity of the serum, consuming foodstuffs containing melatonin may be helpful in lowering oxidative stress.

Introduction

Interest in dietary antioxidants that relieve tissues of the oxidative burden imposed by reactive oxygen, nitrogen, and chlorine species has increased exponentially in the last two decades.[1–4] The molecular damage inflicted by these reactive species predisposes cells to a number of age-related, free radical–based diseases. Given that antioxidants detoxify, neutralize, and/or metabolize reactive species, they have the potential to reduce the incidence and/or severity of some degenerative conditions. Whereas some antioxidants are endogenously produced, others are only obtained through the diet, and the role of ingested antioxidants and free radical scavengers in maintaining humans and animals in a healthy state has been the subject of numerous scientific publications.

Melatonin, *N*-acetyl-5-methoxytryptamine, was long thought to be an endogenously generated molecule found exclusively in vertebrates that synchronizes circadian and circannual rhythms.[5] In recent years, however, melatonin has also been discovered in insects, unicellular organisms, and bacteria.[6–8] Because the unicells in which melatonin is found bridge the animal and plant kingdom, the search for melatonin in plants was inevitable. These studies have successfully demonstrated that melatonin also exists in the plant kingdom.

This brief review considers the antioxidant properties of melatonin, the distribution of the indole in plants, and its fate and actions once it is ingested.

Melatonin as an Antioxidant and Free Radical Scavenger

In 1993, melatonin was found to neutralize the highly reactive hydroxyl radical (•OH) in a purely chemical system.[9] Indeed, each molecule of melatonin scavenges two •OH, thereby generating the urinary excretion product cyclic 3-hydroxymelatonin, which serves as a biomarker of in vivo •OH scavenging by melatonin.[10] This scavenging action of melatonin is potentially important because the •OH is highly devastating to virtually all macromolecules in the organism. It is not known, however, of the total oxidative burden inflicted on organisms, what proportion is actually a consequence of the •OH, but it is surmised to be high as 50%.

The direct scavenging actions of melatonin were subsequently found to extend to other reactive species and, in several cases the resulting products have been identified (Table 1). Of these actions, the ability of melatonin to neutralize the peroxynitrite anion ($ONOO^-$) and hydrogen peroxide (H_2O_2) are especially noteworthy inasmuch as

Dr. Reiter is a Professor in the Department of Cellular and Structural Biology, University of Texas Health Science Center, San Antonio, TX 78229-3900, USA. Dr. Tan is an NIH postdoctoral fellow (T32AG00165-13). Dr. Burkhardt is a postdoctoral fellow supported by The Deutsche Forschungsgemeinschaft. Dr. Manchester is a professor in the Department of Biology, St. Mary's University, San Antonio, TX 78228, USA.

Table 1. Publications in which the interactions of melatonin with toxic reactants are described along with the potential end products generated[a]

Reactant Scavenged	Proposed Product	Reference
Hydroxyl radical (•OH)	Cyclic 3-hydroxymelatonin	10
Hydrogen peroxide (H_2O_2)	N^1-Acetyl-N^2-formyl-5-methoxytryptamine	11
Singlet oxygen ($^1O_2$)	N^1-Acetyl-N^2-formyl-5-methoxytryptamine	12
Nitric oxide (NO)	*N*-Nitrosomelatonin	13
Peroxynitrite anion ($ONOO^-$) and peroxynitrous acid (ONOOH)	6-Hydroxymelatonin; cyclic 2-hydroxymelatonin; cyclic 3-hydroxymelatonin; 1-nitromelatonin; 1-hydroxymelatonin	14–16
Hypochlorous acid (HOCl)	2-Hydroxymelatonin	17

[a]The original articles can be consulted for the rate constants for these reactions. Besides these publications, there are numerous reports documenting the ability of melatonin to limit molecular damage, both in vivo and in vitro, under conditions in which these reactive oxygen-, nitrogen-, or chlorine-based species are present.

the $ONOO^-$, like the •OH, causes widespread molecular mutilation and has been linked to a number of diseases. Melatonin's ability to react with H_2O_2 is important because this molecule, although it is not highly toxic, is the precursor of the •OH and lowering its steady-state concentration also reduces the generation of the •OH. Of additional interest and potential importance is the fact that the product that is generated when melatonin interacts with H_2O_2, i.e., N^1-acetyl-N^2-formyl-5-methoxykynuramine, is also a powerful radical scavenger. This cascade of antioxidative reactions is believed to greatly increase the protective action of melatonin against free radicals.[18]

Besides the direct free radical–scavenging functions summarized in Table 1, melatonin also has indirect actions on enzymes related to the antioxidative defense system, which would be beneficial in terms of reducing oxidative molecular damage.[19,20] Either the mRNA levels and/or the activities of several antioxidative enzymes have been shown to be stimulated by melatonin; these include the superoxide dismutases, glutathione peroxidase, glutathione reductase, and catalase. Maintaining high levels of activity of these enzymes would generally reduce overall oxidative stress. Additionally, melatonin inhibits one proxidative enzyme, i.e., nitric oxide synthase (NOS).[21] This enzyme generates nitric oxide (NO) from arginine. Once formed, NO can couple with the superoxide anion radical ($O_2^{\cdot -}$) to produce the $ONOO^-$.

Recent studies have generated data that may broaden melatonin's ability to protect tissues against free radical damage. Thus, melatonin stimulates Complex I and Complex IV of the electron transport chain in the inner mitochondrial membrane, thereby likely reducing electron leakage and limiting the formation of the $O_2^{\cdot -}$ at this level.[22] Because the $O_2^{\cdot -}$ is the precursor of •OH and $ONOO^-$, reducing its generation would also lower the production of these highly toxic reactants. Finally, within cell membranes, melatonin may position itself between the polar heads of polyunsaturated fatty acids,[23] thereby reducing lipid peroxidation and maintaining the optimal fluidity of the membranes.[24]

Whereas the direct scavenging actions of melatonin are obviously receptor-independent, it is possible that melatonin's ability to augment the activities of antioxidative enzymes may be receptor-mediated. Also, the suppression of NOS by melatonin reportedly requires melatonin's binding to calmodulin (CaM); the activities of some isoforms of NOS are known to be CaM-dependent.

Whereas melatonin's actions as an antioxidant seem ubiquitous, the specific significance of each of its functions in reducing molecular damage owing to oxygen-, nitrogen-, and chlorine-based reactants remains unknown. There are, however, numerous reports documenting melatonin's ability to limit oxidative destruction of macromolecules both in vitro and in vivo.[19,20,25,26]

Melatonin in Plants

Until recently, melatonin was thought to exist exclusively in the animal kingdom. Approximately a decade ago, however, melatonin was discovered in a photosynthetic dinoflagellate (*Gonyaulax polyedra*).[8] In this species, physiologic levels of melatonin are relevant in terms of antioxidant protection.[27] The discovery of melatonin in this phylogenetically ancient species prompted studies related to the examination of edible plants for the presence of this indoleamine.

To date, only a small number of plants have been tested for melatonin.[28] The results of these studies have, however, uncovered several facts: melatonin is present in a wide number of plant products; the concentration of melatonin varies extremely widely in different plants; and melatonin is unequally distributed in plant parts. In these studies, melatonin was extracted and typically identified and quantified by high performance liquid chromatography and in a few cases by radioimmunoassay. Using these methods, melatonin has been identified in a large number of plant taxa.[29–32] Among angiosperms, melatonin has been

found in more than 30 species belonging to 19 different families and in both mono- and dicotyledons. Within plant tissues, melatonin has been found in roots, stems, leaves, fruit, and seeds. Most of the plants that have been analyzed are edible.[29,30]

The concentrations of melatonin in plant products vary widely. These differences are likely real but they may in part reflect variations in the ability of the extraction methods used to recover melatonin from plant material. In roots, stems, leaves, and fruits from various plants, melatonin levels varied from being undetectable to concentrations of 5 ng/g of tissue.[33,34] In some so-called medicinal plants, e.g., *Tancetum parthenium* (fever few), *Hypericum perforatum* (St. John's wort), and *Scutellaria biocalensis* (huang-quin), melatonin levels are reported to be much higher, i.e., up to 7 μg/g tissue.[33] These investigators have subsequently provided preliminary evidence that these plants may actually synthesize the indoleamine.[34] Whereas plants that contain melatonin may produce it, it is also possible that they are able to take up melatonin from the soil in which they are growing.

In a study that examined the melatonin content of the seeds of 15 edible plants, Manchester et al.[35] documented measurable amounts of the indoleamine in all products investigated. As with other studies, they showed that the concentrations of melatonin in seeds varied widely, i.e., from 2 ng/g dry seed for milk thistle to 189 ng/g dry seed for white mustard. Other representative melatonin levels from these products included 7 ng/g (celery), 29 ng/g (sunflower), and 103 ng/g (wolf berry). This group suggested that melatonin in seeds, as in animals, may function as an antioxidant to protect against lipid peroxidation that would reduce the likelihood of the seeds undergoing successful germination. The idea that melatonin protects against free radical damage in seeds is consistent with the suggestion of Dubbels and co-workers[29] in other plant parts.

Among the few fruits that have been examined, melatonin has been found in bananas (236 pg/mg protein)[29] and in Montmorency tart cherries (*Prunus cerasus*) (16–18 ng/g weight) (Burkhardt S, Tan DX, Reiter RJ, unpublished data, 2001). In cherries, the orchard of origin of the fruit does not influence the melatonin concentration. Likewise, the degree of ripeness of the cherries was not correlated with the melatonin levels that were measured.

Melatonin Absorption and Bioavailability

Pure melatonin taken in any known form and by any route is readily absorbed into the blood from which it has access to all tissues. Thus, melatonin is readily taken up from the gastrointestinal tract,[36] from a sublingual deposit, or when given as an intranasal spray.[37] There are no known morphophysiologic barriers to melatonin; for example, unlike vitamin E, it readily crosses the blood-brain barrier[38] and the placenta.[39] Melatonin also seemingly has ready access to all cellular organelles although it may not be distributed in equal concentrations in all subcellular compartments. There is evidence that the nucleus[38] and mitochondria[40] may have higher concentrations of melatonin than do other organelles.

The bioavailability of melatonin from foodstuffs has been sparingly investigated. Hattori et al.[30] did, however, show that feeding birds melatonin-containing grains increased circulating levels of melatonin. Furthermore, they reported that dietary melatonin competed with [I^{125}] iodomelatonin for binding to cell membrane receptors indicating it is bioactive.

Physiologic blood levels of melatonin, which can be achieved by consuming foods containing this indole, also are adequate in mediating the antioxidant actions of this molecule. This is documented by studies in which endogenous melatonin levels are depressed by surgical removal of the pineal gland. Under such conditions, oxidative damage induced by ischemia/reperfusion injury,[41,42] as well as the normal age-related accumulation of oxidatively damaged products is exaggerated.[43] Thus, loss of normal physiologic levels of melatonin leads to enhanced free radical damage. Supplementing melatonin-deficient animals with the indole reduces the induced oxidative damage.

These findings are consistent with the fact that endogenous melatonin levels, which can be achieved by consuming melatonin in the diet, contribute to the antioxidant potential of the blood. In support of this, the concentration of endogenously produced melatonin in the blood correlates with the blood's total antioxidant status (TAS) in humans.[44] Thus the nocturnal rise in circulating melatonin positively correlates with a rise in the TAS of the blood. Likewise, if the nighttime increase in blood melatonin is inhibited by exposing subjects to bright light, the TAS is proportionally reduced.

Because the concentration of melatonin in edible plants varies widely, and there may be foodstuffs that contain much higher levels than have been reported to date, it is difficult to calculate how a specific diet may influence functions such as those regulated by the biologic clock, i.e., the suprachiasmatic nuclei (SCN), which modulate the sleep-wake cycle, jet lag, etc. The SCN in many animals, including the human, contains membrane receptors for melatonin that presumably modify the circadian functions mentioned.[45] Also, because the sensitivity of the melatonin receptor for its ligand may vary over a 24-hour period, the time of day a melatonin-rich product is consumed could be important in determining the response. As already noted, consuming grains, which have comparatively low levels of melatonin, increases blood concentrations of the indole, which binds to the receptors.[30] In addition, the level of melatonin in the blood is proportionate to its ability to combat oxidative damage.[44]

The estimated total amount of melatonin in the blood of an average human (with roughly 5 liters of blood) at any one time during the day (average of 5–10 pg melato-

nin/mL) is equivalent to the quantity of melatonin in one Montmorency tart cherry. Variables such as absorption from the gut and extraction by the liver during the first pass,[36] however, make calculating how much of a given melatonin-containing foodstuff would have to be consumed to alter receptor-mediated functions of the indole somewhat enigmatic at this time. On the other hand, any melatonin consumed would increase the total free radical scavenging capacity of the organism.

Conclusion

The data obtained to date indicates that melatonin is a broad-spectrum direct free radical scavenger and indirect antioxidant. Thus, besides directly detoxifying a variety of reactive species (Table 1) that inflict damage on all biomolecules, melatonin also indirectly enhances antioxidative defense mechanisms by increasing the activities of several antioxidative enzymes[19,25] and by stimulating the synthesis of another important intracellular antioxidant, glutathione.[46] Furthermore, the findings show that melatonin is available in edible fcodstuffs and that melatonin-containing diets increase circulating concentrations of the indole. The findings also show that dietary-derived melatonin is adequate to contribute to the total antioxidative capability of the blood.[47]

Whereas these findings are enticing, what are needed are more extensive studies to define the levels of melatonin in a wider variety of normally consumed foodstuffs. There may well be yet untested foods that contain higher levels of melatonin than those that have been reported to date. Additionally, further investigations on the role of diet-derived melatonin in reducing tumor growth and enhancing the total antioxidative capacity of the organism are needed. Only after the results of such studies are available, could any recommendations concerning the dietary intake of melatonin be made. Indeed, it is one purpose of this brief review to stimulate further interest in research relating to melatonin in the diet.

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173. N-ACETYLSEROTONIN ELEVATES HUMAN URINE 6-SULFATOXYMELATONIN

T.P. Shteinlukht[1], P.J. Requintina[1,2] & G.F. Oxenkrug[1]

[1]Department of Psychiatry and Pineal Research Laboratory, St. Elizabeth's Medical Center of Boston/Tufts University, Boston, MA, 02135; [2]Providence VA Medical Center, Providence, R.I.

Melatonin is synthesized from serotonin via formation of N-acetylserotonin in the pineal gland. Decline of melatonin production has been associated with numerous pathological conditions while administration of the exogenous melatonin was claimed to be beneficial. Stimulation of the endogenous melatonin production by the precursors of serotonin and noradrenaline and by monoamine oxidase A type inhibitors and some other pharmacological agents targets the rate-limiting step of melatonin biosynthesis: N-acetylation of serotonin, the process dependent upon the pineal β-1-adrenoceptors responsiveness which is decline with aging and during some periods of light phase. The second step of melatonin biosynthesis, methylation of N-acetylserotonin, is not considered to be the rate-limiting step and is less subjective to the circadian variations. We have previously reported that N-acetylserotonin increased rat pineal melatonin formation in dose- and time-dependent manner (Oxenkrug & Requintina, Intern. J. Neurosci., 1994). The present study aimed to evaluate the effect of N-acetylserotonin on 6-sulfatoxymelatonin (6-SM) urine excretion in healthy human subjects. N-acetylserotonin was administered (30, 50 and 75 mg, p.o.), and 6-SM was detected in urine by the ELISA method. N-acetylserotonin caused dose-dependent increase of urine 6-SM concentration. The present results demonstrate that N-acetylserotonin can be used to stimulate melatonin production in humans. N-acetylserotonin-induced stimulation of melatonin production might be used as the physiological way of pineal gland activation for diagnostic and therapeutic purposes.

174. GENDER ASSOCIATED COGNITIVE DIFFERENCES IN PATIENTS WITH DEMENTIA

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Physiological levels of melatonin contribute to the antioxidant capacity of human serum

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Abstract: This work evaluates whether physiological concentrations of the pineal secretory product melatonin contribute to the total antioxidant status (TAS) of human serum. Day and nighttime serum samples were collected from healthy volunteers ranging from 2 to 89 years of age and used to measure melatonin and TAS. Results showed that both melatonin and TAS in human serum exhibited 24 hr variations with nocturnal peak values at 01:00 hr. Moreover, exposure of volunteers to light at night resulted in clear decreases of both TAS and melatonin. Furthermore, when melatonin was removed from sera collected at night, the TAS value of the sample was reduced to basal daytime values. In aging studies, it was found that nocturnal serum values of TAS and melatonin exhibited maximal values during the first four decades; thereafter, these values decreased as age advanced. In 60-year-old individuals, day/night differences in serum melatonin and TAS levels were clearly diminished, by more than 80%, with these differences being completely abolished in older individuals. Our results suggest that melatonin contributes to the total antioxidative capability of human serum. This antioxidant contribution of melatonin is reduced as age advances correlating with the age-related reduction of melatonin.

Soledad Benot[1], Raimundo Goberna[1], Russel J. Reiter[2], Sofia Garcia-Mauriño[1], Carmen Osuna[1] and Juan M. Guerrero[1]

[1]Department of Medical Biochemistry and Molecular Biology, The University of Seville School of Medicine and Virgen Macarena Hospital, Seville, Spain; [2]Department of Cellular and Structural Biology, The University of Texas Health Science Center at San Antonio, San Antonio, Texas

Key words: aging – antiaging – circadian rhythm – oxidative stress – pineal gland

Address reprint requests to: Juan M. Guerrero, Department of Medical Biochemistry and Molecular Biology, The University of Seville School of Medicine, Avda Sanchez Pizjuan 4, 41009-Seville, Spain
Email: juanxyz@arrakis.es

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Introduction

Melatonin (N-acetyl-5-methoxytryptamine) is synthesized by the pineal gland of vertebrates including humans [Reiter, 1991; Brzezinski, 1997]. Melatonin participates in several important physiological functions, including the control of seasonal reproduction in animals [Goldman, 1983], circadian rhythm regulation [Lewy et al., 1992], and possibly normal patterns of sleep [Haimov et al., 1995]. Also, melatonin may play a fundamental role in neuroimmunomodulation in rodents and humans [Guerrero and Reiter, 1992; Maestroni, 1993; Rafii-El-Idrissi et al., 1996, 1998; Pozo et al., 1997a; Garcia-Mauriño et al., 1997], and it influences the growth of spontaneous and induced tumors in animals [Hill and Blask, 1988; Bartsch et al., 1989; Panzer and Viljoen, 1997].

Recently, melatonin was reported to be an effective free radical scavenger [Reiter et al., 1995]. Both in vitro [Tan et al., 1993a] and in vivo [Tan et al., 1993b] studies have indicated that melatonin directly scavenges the highly toxic hydroxyl radical and other oxygen centered radicals. As a result, it has been speculated that melatonin may provide protection against diseases that involve degenerative or proliferative changes by shielding macromolecules (DNA, lipids, and proteins) from free radical damage [Reiter et al., 1995]. The bulk of the studies to date have tested the efficacy of pharmacological levels of melatonin to prevent oxidative damage suggesting that at physiological concentrations melatonin may be irrelevant as an antioxidant. A modicum of evidence suggests otherwise, however. Thus, Tan et al. [1994] showed that the nighttime rise in endogenous blood melatonin was sufficient to counteract a portion of the DNA damage normally inflicted by the carcinogen safrole. This indicates that melatonin at physiological concentrations may also be relevant as an antioxidant in vivo.

A significant factor in endogenous melatonin availability is age. In all species where it has been studied, advancing age is associated with a progressive reduction of the circadian melatonin rhythm derived from the pineal gland [Reiter,

1992]. Assuming melatonin at physiological concentrations is important in preventing oxidative damage, the protection of macromolecules from free radical attack could be significantly compromised in advancing age. Certainly, the onset of many degenerative and proliferative diseases are age-related. Whether this rise in these diseases relates in any way to the diminished antioxidant protection potentially provided by melatonin remains unknown.

In this paper, we show for the first time a nyctohemeral rhythm of serum total antioxidant status (TAS) in humans which parallels and is apparently related to the 24 hr melatonin cycle. During the nighttime, both melatonin and TAS exhibit their highest values. When melatonin is removed from nighttime serum, TAS decreases to the basal daytime values. Additionally, the day–night differences in melatonin and TAS change with aging. Maximal day–night differences are observed during the first four decades, thereafter exhibiting a significant decrease in individuals between 40 and 60 years of age and virtually disappearing in individuals 60 years and older. Results suggest that melatonin is relevant physiologically together with other compounds, in terms of the antioxidative capacity of the serum of humans, and its diminished nocturnal production during aging is likely responsible of the lack of day–night differences in TAS in the elderly.

Materials and methods

Serum samples

Serum samples were collected from healthy volunteers ranging from 2 to 89 years of age. Donors fasted a minimum of 3 hr before the samples were collected and alcoholic drinks were prohibited during the study. When blood samples were collected at night in darkness the subjects were exposed to a dim light (intensity of 100 lux). This light had no noticeable effect on nocturnal melatonin levels. Samples were frozen at −20°C until assayed. TAS of the serum was measured within 48 hr of sample collection. In addition to TAS, melatonin levels were estimated in all serum samples. A basic biochemical profile was also run to determine the general health of the individuals.

When required, melatonin was removed from serum using the microtiter plates of a melatonin enzyme immunoassay (EIA) which uses a capture second antibody technique (Bühlmann Laboratories AG, Postfach, Switzerland). Serum (50 μL) aliquots were incubated in wells coated with a polyclonal antibody specific for rabbit immunoglobulin; then, a rabbit anti-melatonin antibody was added with the corresponding incubation buffer. The melatonin–antibody complex and free anti-melatonin antibodies were then captured by the second antibody on the coated well. After 24 hr incubation, melatonin-free supernatants were immediately used for TAS determinations. Controls were incubated in wells lacking coated polyclonal antibody for rabbit immunoglobulin and an incubation buffer with no anti-melatonin antibody. The absence of measurable melatonin after this procedure was confirmed using the mentioned EIA and confirmed by a competitive enzyme immunoassay (Melatonin ELISA, ICN Pharmaceuticals, Costa Mesa, CA).

Total antioxidant status determination

Total antioxidant status (TAS) was measured using a kit purchased from Randox Laboratories (Crumlin, Northern Ireland). In this assay, metmyoglobin reacts with H_2O_2 to form the radical species ferrylmyoglobin. A chromogen (2,2′-azino-di-[ethylbenzthiazoline sulfonate]; ABTS) is incubated with the ferrylmyoglobin to produce the radical cation species $ABTS^{\bullet +}$. This has a relatively stable blue-green color which is measured at 600 nm. Antioxidants in the added sample cause suppression of this color production to a degree which is proportional to their concentration. The assay is calibrated using 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid and results are expressed as mmol L. The assay range is 0–2.5 mmol L. Intra- and inter-assay precision studies showed coefficients of variation lower than 2 and 3%, respectively.

Melatonin determinations

Melatonin was determined by a competitive enzyme immunoassay (Melatonin ELISA, ICN Pharmaceuticals, Costa Mesa, CA) using serum without extraction. For melatonin measurement in serum the samples are treated enzymatically to free the antigen from its binding proteins. The assay procedure follows the basic principle of competitive ELISA whereby there is competition between a biotinylated and a non-biotinylated antigen for a fixed number of antibody binding sites. Quantification of unknown samples is achieved by comparing the enzymatic activity of the unknowns with a response curve prepared by using known standards. The lowest detectable level that can be distinguished from zero standards is 2.6 pg/mL. Intra- and inter-assay studies had coefficients of variation lower than 7 and 12%, respectively.

Statistical analyses

Results are expressed as means ± SEM. Data were statistically analyzed using an ANOVA followed by a Student-Newman-Keul multiple range test.

Results

To determine whether there was a relationship between circulating melatonin concentrations and the antioxidant status of the blood, the nyctohemeral profiles of both TAS and melatonin in humans were compared. Fig. 1 shows that both TAS and melatonin in human serum exhibit 24 hr variations with nocturnal peak values at 01:00 hr. When volunteers were maintained under bright light (> 1,500 lux) from 19:00 to 01:00 hr, both serum TAS and melatonin remained at basal daytime values. The results show a clear correlation between melatonin and TAS in human serum, suggesting a likely relationship between these two parameters.

In another experiment we further attempted to link serum melatonin levels with its antioxidative capacity. As shown in Fig. 2, serum samples obtained at night (01:00 hr) again exhibited higher TAS levels than serum samples obtained during the day (13:00 hr). However, when melatonin was

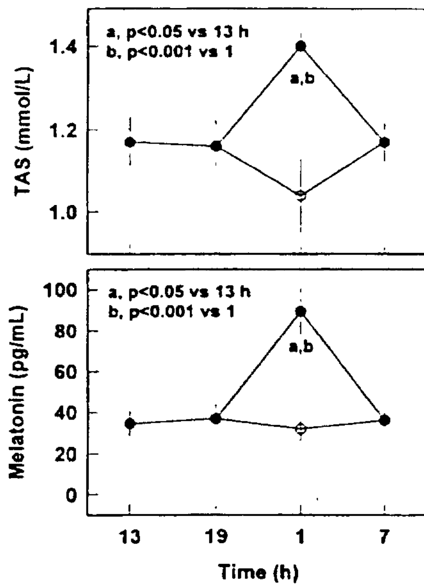

Fig. 1. Day-night differences in TAS (top) and melatonin (bottom) in human serum (●). Serum samples were collected from healthy volunteers ranging in age from 25 to 40 years at the indicated times and used for TAS and melatonin determinations. The day after, the same individuals were maintained under bright light (1,500 lux) from 19:00 hr until 01:00 hr, and serum samples were collected (○). Each point is the mean ± SE of 12 individuals.

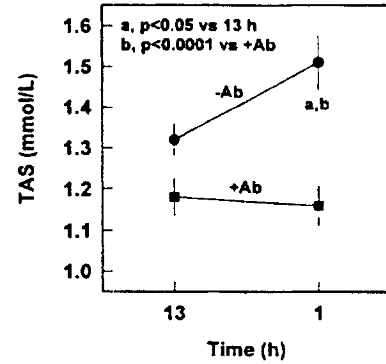

Fig. 2. Effect of removing melatonin from serum samples on the TAS. Serum samples were obtained either during the day (13:00 hr) or at night (01:00 hr) from 11 healthy volunteers ranging in age from 25 to 40 years. TAS was measured after removing melatonin (+ Ab) using the microtiter plates of a melatonin enzyme immunoassay which uses a capture second antibody technique as described in the Methods. Control samples containing melatonin (− Ab) were incubated in microtiter plates lacking of coated polyclonal antibody for rabbit immunoglobulin with an incubation buffer with no anti-melatonin antibody. Each point is the mean ± SE of 11 individuals.

removed from sera (by the addition of antibody) collected at night, the TAS level of the sample was similar to that of the basal daytime value. These findings suggest that melatonin is responsible for the nocturnal increase in TAS observed in human serum.

Day-night differences in TAS and melatonin in human serum were also compared during development and aging in a study in which 120 individuals were studied. As shown in Fig. 3 (bottom), daytime serum melatonin concentrations remained unchanged during aging. Nocturnal levels of the indole, however, exhibited maximal values during the first decade; thereafter, nocturnal melatonin concentrations decreased during the following five decades, with the most rapid reduction occurring during the fourth and fifth decades of life. In 60-year-old individuals day-night differences in serum melatonin were clearly diminished by more than 80%, while in individuals greater than 60 years of age, the nocturnal melatonin increase had totally disappeared. A similar pattern was observed when serum TAS was studied throughout life (Fig. 3, top). As with melatonin, day-night differences in serum TAS exhibited maximal values during the first four decades of life; thereafter, nocturnal serum TAS decreased dramatically such that by 60 years of age the levels of TAS at night did not differ from daytime levels. In even older volunteers, the day-night TAS differences were also absent. These findings further suggest that the nocturnal rise in serum TAS is related to the

nighttime melatonin rise and, likewise, the age-associated decline in the antioxidative capacity of human serum may be a consequence of an associated reduction in circulating melatonin levels.

Discussion

The present paper shows for the first time that physiological melatonin levels in human serum exhibit significant antioxidative capacity. In addition to in vitro experiments showing the antioxidative properties of melatonin, studies on human serum showed diurnal variations in both TAS and melatonin, with maximal nocturnal values for both parameters. Moreover, the nocturnal increase in serum TAS is apparently due to the nocturnal melatonin increase since suppressing the nighttime rise in melatonin in humans exposed to bright light also prevented the nocturnal increase in serum TAS, and depleting the melatonin by the addition of antibody also reduced the TAS of the serum. Additionally, the well-known age-related loss of the day–night differences in serum melatonin concentrations [Reiter, 1992; Miguez et al., 1998] correlates with a similar loss of day–night differences in TAS.

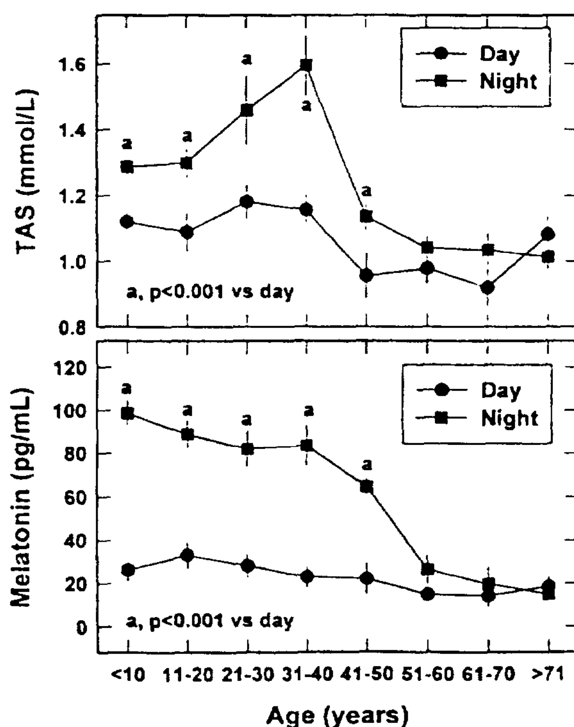

Fig. 3. Day and night values of TAS and melatonin concentration in serum samples during aging. Samples were obtained from 120 volunteers either during the day (13:00 hr; ●) or at night (01:00 hr; ■) at the ages indicated. Each point is the mean ± SE of 12 individuals.

The results strongly suggest that melatonin is responsible for the nocturnal increase in serum TAS and for its age-related decrease. The results confirm previous results of our group showing a nyctohemeral rhythm in serum TAS of the rat which, like in humans, parallels the 24 hr melatonin cycle [Benot et al., 1998].

Melatonin has been reported to be an efficient scavenger of both the hydroxyl (•OH) [Tan et al., 1993a,b; Susa et al., 1997; Stasica et al., 1998] and peroxyl radical (ROO•) [Pieri et al., 1994], although the latter finding has been questioned [Marshall et al., 1996]. The initial findings suggested that melatonin is unexpectedly effective in these roles as indicated by the fact that when compared with the intracellular scavenger glutathione, melatonin proved five times better at neutralizing •OH and, when compared to vitamin E, melatonin was twice as effective in neutralizing ROO•. Glutathione [Meister, 1992] and vitamin E [Packer, 1994] are considered to be premier antioxidants. Besides these direct scavenging actions of melatonin, there are indirect antioxidant effects as well. Thus, melatonin stimulates glutathione peroxidase (GSH-Px) activity [Barlow-Walden et al., 1996; Pablos et al., 1997] and inhibits nitric oxide synthase (NOS) [Pozo et al., 1994, 1997b; Bettahi et al., 1996, 1998]. GSH-Px is an important antioxidative enzyme because it metabolizes hydroperoxides including hydrogen peroxide (H_2O_2), thereby reducing the formation of •OH [Liochev and Fridovich, 1994]. By inhibiting NOS, melatonin reduces the formation of the free radical nitric oxide (NO•) [Palmer et al., 1988]. NO• may itself be toxic but additionally it can degrade into peroxynitrite anion [Cuzzocrea et al., 1998] and eventually into the highly toxic •OH. In these studies, pharmacological concentrations have usually been used to demonstrate melatonin's antioxidative ability.

The present results suggest that physiological levels of melatonin in human serum may well be relevant in terms of total antioxidant of the serum. Herein we show day–night variations in both TAS and melatonin is serum. Both parameters exhibited a similar pattern, with basal values during the day and maximal levels at night. The circadian melatonin rhythm has been extensively studied [Reiter, 1986, 1991]. However, this is the first demonstration of a diurnal variation in serum TAS. This coincidence was also observed when volunteers were maintained in light (> 1,500 lux) at night. Bright light exposure not only prevented the nocturnal increase in serum melatonin levels but also the increase in TAS.

Additionally we show that nighttime serum TAS levels are diminished when melatonin is removed from the samples. Thus, nocturnal melatonin-free sera exhibited TAS values similar to those obtained in samples collected during the day. The results strongly suggest that melatonin is the cause of the nocturnal increase in TAS. The implication is that physiologically melatonin contributes, in addition to other known antioxidants, to the total antioxidative capacity of human serum, especially at night.

In a final experiment, day–night differences in melatonin and TAS during development and aging were studied. As previously described, throughout at least the first five decades the pineal gland of all animals, including humans, produces melatonin more abundantly at night than during the day. As advanced age approaches, the ability of the pineal to synthesize melatonin gradually wanes such that, in old age, the pineal melatonin rhythm is only a vestige of that in younger individuals [Sack et al., 1986; Reiter, 1992, 1998]. Herein, we observed a similar pattern when serum TAS was determined. While day–night differences in serum TAS exhibited maximal values in young individuals, nocturnal TAS decreased as age advanced. Indeed, in the oldest individuals tested, day–night differences in TAS were absent. Inasmuch as the nocturnal increase in melatonin is likely the cause of the nocturnal increase in TAS, the loss of the nocturnal antioxidative capacity of the serum during aging is apparently related to the inability of the pineal gland to synthesize melatonin. A number of studies have shown that other antioxidants may also change with age. For example, there is an age-related reduction in glutathione in plasma samples collected during the day [Michelet et al., 1995], which may explain the slight reduction in daytime TAS values we have observed during aging (Fig. 3, top). Since they are consumed in the diet, antioxidants such as α-tocopherol, β-carotene, and ascorbic acid do not exhibit these age-related changes [Schmuck et al., 1995].

In conclusion, these results suggest that melatonin contributes, in addition to other compounds, to the antioxidant properties of human serum, especially at night. This antioxidant effect of melatonin disappears as age advances since, in the elderly, melatonin production is reduced. Since in physiological concentrations melatonin contributes to the total antioxidant capacity of human serum, the loss of melatonin with age could be associated with age-related free radical-based degenerative conditions [Reiter, 1995; 1998].

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Effect of melatonin and metabolites on copper-mediated oxidation of low density lipoprotein

Harald Seeger, Alfred O. Mueck & Theodor H. Lippert
Section of Clinical Pharmacology, Dept OB/GYN, University Tubingen, Tubingen, Germany

Aims A prophylactic use of melatonin as an anti-ageing drug has recently gained public interest due to its radical scavenging property *in vitro*. The present study was designed to investigate a possible antiatherogenic effect of melatonin and its physiological metabolites by examining their action on the radical-initiated formation of oxidized LDL, which is known to possess a high atherogenic potency. The metabolites investigated were the precursors serotonin and *N*-acetyl-serotonin and the main breakdown product 6-hydroxymelatonin.
Methods The effect of the test substances on the *in vitro* oxidation of LDL (increase in conjugated diene formation) was investigated at concentrations of 1, 5, and 10 μM.
Results Melatonin increased the lag time of formation of oxidized LDL only at a concentration of 10 μM. In contrast, 6-hydroxymelatonin, serotonin and *N*-acetyl-serotonin as well as vitamin E showed inhibitory effects starting at 1 μM. Thus the antioxidative action of melatonin was negligible compared with the effect of its main metabolite, its precursors and of vitamin E.
Conclusions The present results indicate that the pineal hormone melatonin appears to have little antiatherogenic property as regards the oxidation of LDL. Its main breakdown product 6-hydroxymelatonin, however, inhibits LDL-oxidation comparable to vitamin E. The precursors of melatonin, *N*-acetyl-serotonin and serotonin may also play a role in the inhibition of LDL oxidation *in vivo*

Keywords: melatonin metabolites LDL-oxidation

Introduction

The pineal hormone melatonin is believed to play a role in the synchronization of day and night rhythm. Therefore it may have a clinical role in alleviating sleeping disorders occuring in night workers or after long trans flights (jet lag syndrome) [1]. Recently, melatonin has been shown to have a radical scavenging effect and therefore may have an anti-ageing property [2]. Since oxidation of low density lipoprotein (LDL) is initiated by radicals, the present study was undertaken to investigate the influence of melatonin as well as its metabolites, i.e., the main breakdown product 6-hydroxymelatonin and the precursors, 5-hydroxytryptamine (serotonin) and *N*-acetyl-5-hydroxytryptamine (*N*-acetyl-serotonin) on this reaction. Vitamin E, a nutritive antioxidant, was also tested, for comparison reasons.

Methods

Melatonin, 6-hydroxymelatonin, *N*-acetyl-5-hydroxytryptamine (*N*-acetyl-serotonin), 5-hydroxytryptamine (serotonin) and vitamin E were purchased from Sigma (Deisenhofen, Germany).

LDL oxidation was investigated as described by Esterbauer *et al.* [3] using pooled blood serum from healthy premenopausal women containing the antioxidants EDTA ($1\ mg\ ml^{-1}$) and butylated hydroxytoluene (BHT) ($4.4\ \mu g\ ml^{-1}$). The collected blood was stored at 4° C and LDL was obtained by ultracentrifugation within 4 h after the collection of blood samples. In brief, 1.638 g sodium bromide was added to 3 ml serum which was overlayed with saline. After ultracentrifugation of this solution at 105 000 *g* for 9 h (fixed angle rotor), the LDL-layer (density $1.02-1.05\ g\ ml^{-1}$) was aspirated by a syringe. Oxidation of LDL was immediately started after ultracentrifugation as follows: LDL-solution was separated from BHT and EDTA by gelfiltration (Sephadex G-25, column 10×1.5 cm, eluants: saline, fraction volumes. 500 μl). Samples of 150 μl of LDL (adjusted to 300 μg protein ml^{-1}) were mixed each with 850 μl saline containing 10 μM $CuCl_2$ and the test substances. The substances, dissolved in ethanol, were added to this mixture to give final concentrations of 1, 5 and 10 μM. Control values were obtained by the addition of alcohol alone in the same concentrations as in the test substances (final ethanol concentration in all samples approximately 1%).

The increase in conjugated diene formation, characteristic for the oxidation of LDL, was monitored spectrometrically at 234 nm. Tangents were drawn to the segments of the absorption curve corresponding to the lag phase and propagation phase of LDL oxidation The length of the lag phase = lag time was determined as the intercept of the two tangents.

The antioxidative effect of the substances tested is expressed as the elongation of the lag time after the ox-LDL

Correspondence. Professor T. H. Lippert, Section of Clinical Pharmacology, Department of Obstetrics and Gynecology, Schleichstrasse 4, 72 076 Tübingen, Germany

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formation of the control. The increasing lag time was estimated up to 300 min. Each concentration of the substances was tested in nine different experiments.

Protein content of LDL was determined by a colorimetric protein assay (Bio-Rad, München, Germany).

Statistical analysis was performed using the Student's *t*-test.

Results

The control values for ox-LDL formation showed an average lag time of 105.4±21.8 min.

The increases of the lag time in minutes by melatonin, 6-hydroxymelatonin, *N*-acetyl-serotonin, serotonin and vitamin E at the concentrations of 1, 5 and 10 μM are depicted in Table 1.

Melatonin had no effect at 1 and 5 μM and significantly elongated the onset of the diene formation at the highest concentration of 10 μM, for 21.8±4.3 min. 6-hydroxymelatonin inhibited the LDL-oxidation at all three concentrations tested. The values were: 27.8±4 1 min at 1 μM, 92.5±10.2 min at 5 μM and more than 300 min at 10 μM.

Both *N*-acetyl-serotonin and serotonin significantly increased the lag time at all concentrations: At 1 μM with values of 33.8±4.9 min and 28.2±3.8 min, respectively, at 5 μM for 182.6±19.3 min and 111.8±15.8 min, respectively, and at 10 μM of more than 300 min for both substances. Vitamin E enhanced the lag time at 1 μM for 23.6±3.9 min, at 5 μM for 85.9±9.1 min, and at 10 μM more than 300 min.

Discussion

The oxidation of LDL renders this molecule into a highly potent atherogenic substance [4]. Different radicals seem to be involved in this reaction and radical scavenging substances like vitamin E or probucol are supposed to exert an antiatherogenic effect [5]. Melatonin has been shown to be a radical scavenging agent with respect to *in vitro* generated hydroxyl radicals with an IC_{50} value of 21 μM [2]. The present study indicates that melatonin in a similar concentration, i.e 10 μM, shows only a weak inhibitory effect on the oxidation of LDL. Furthermore the melatonin effect is lower by far than that observed by its main hepatic metabolite and its precursors as well as by vitamin E at the same concentration. Pierrefiche *et al.* [6] using mouse brain homogenates also found that melatonin exerts a weaker inhibitory effect on lipid peroxidation than its main metabolite 6-hydroxymelatonin and serotonin However, since *in vivo* melatonin is rapidly metabolized to 6-hydroxymelatonin [7] and our results indicate that this metabolite is able to inhibit LDL-oxidation, exogenously applied melatonin may demonstrate an anti-atherogenic property.

The precursors serotonin and *N*-acetyl-serotonin may also play an important role as antiatherogenic substances as well.

The current results suggest that the presence of a hydroxy group may be essential for the inhibition of copper-mediated oxidation of LDL. Such a hydroxy group is present in 6-hydroxymelatonin, serotonin as well as in *N*-acetyl-serotonin but not in melatonin. For scavenging hydroxyl radicals generated *in vitro* by hydrogen peroxide exposed to u v. light [2], where melatonin had a distinct inhibitory effect, such a hydroxy group seems to be less important

Although melatonin *per se* may be a potent radical scavenging agent with respect to diverse radical generating reactions, its inhibitory effect on LDL oxidation seems to be rather mediated by its main metabolite 6-hydroxymelatonin

However, conclusions as to the antioxidative effects of the tested substances *in vivo* cannot be drawn on the basis of our *in vitro* results as LDL oxidation takes place within the artery wall and nothing is known about the concentrations of the tested substances within this environment

Table 1 Increase of the lag time (min) of LDL transformed into oxidated LDL by $CuCl_2$ after addition of different concentrations of melatonin, 6-hydroxymelatonin (6-OH-Mel), *N*-acetyl-5-hydroxytryptamine (5-NAcHT), 5-hydroxytryptamine (5-HT) and vitamin E. The values are expressed as means±s.d., $n=9$. *$P<0.05$, **$P<0.01$ compared with control value.

	Increase of the lag time compared to control values (min)		
Concentration	1 μM	5 μM	10 μM
Melatonin	<5	<5	21 8±4.3*
6-OH-Mel	27 8±4 1*	92 5±10.2**	>300**
5-NAcHT	33 8±4.9*	182 6±19.3**	>300**
5-HT	28.2±3 8*	111.8±15 8**	>300**
Vitamin E	23 6±3.9*	85.9±9 1**	>300**

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N-acetylserotonin is a better extra- and intracellular antioxidant than melatonin

Albert Wölfler[a], Peter M. Abuja[b], Konrad Schauenstein[a], Peter M. Liebmann[a,*]

[a]*Institute of General and Experimental Pathology, University of Graz, Heinrichstraße 31a, A-8010 Graz, Austria*
[b]*Institute of Biochemistry, University of Graz, Halbärthgasse 5/1, A-8010 Graz, Austria*

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Abstract **Both melatonin and its precursor *N*-acetylserotonin have been reported to exert antioxidant properties both in vitro and in vivo. Since little is known about their antioxidant activity in lymphocytes, we investigated their effects on spontaneous and on oxidant-induced reactive oxygen species formation in human peripheral blood lymphocytes in comparison to the antioxidant trolox, a water-soluble analogue of α-tocopherol. Both melatonin and *N*-acetylserotonin exhibited antioxidant properties against t-butylated hydroperoxide- and diamide-induced reactive oxygen species formation in peripheral blood lymphocytes. *N*-acetylserotonin turned out to be about three times more effective than melatonin. In resting cells, the intracellular reactive oxygen species concentration was only decreased by *N*-acetylserotonin and trolox, melatonin had no effect. In t-butylated hydroperoxide-mediated cell death, *N*-acetylserotonin was as effective as trolox in protecting peripheral blood lymphocytes from cell death and required 10-fold lower concentrations than melatonin. Furthermore, in an aqueous cell-free solution, the capacity of *N*-acetylserotonin to scavenge peroxyl radicals was much higher than that of melatonin. These results clearly indicate *N*-acetylserotonin to be a much better antioxidant than melatonin.**
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Key words: Reactive oxygen species; Melatonin; *N*-acetylserotonin; Antioxidant activity; Lymphocyte

1. Introduction

Melatonin (MEL), the chief secretory product of the pineal gland, has been claimed to exhibit distinct antioxidant features in vitro as well as in vivo (for review see [1]). Since MEL was described to be a more effective scavenger of peroxyl and hydroxyl radicals than α-tocopherol and glutathione [2,3], it was postulated to be an essential element of the mammalian antioxidant defense system and to exert its physiological effects at least in part via influencing the cellular redox status [4]. Because of its low toxicity, MEL was considered to have therapeutic implications in deferring aging processes [5,6], where reactive oxygen species (ROS) are known to play an important pathogenetic role [7]. More recent studies, however, point to a rather limited direct antioxidant potency of MEL: to some extent it can scavenge hydroxyl [8] and peroxyl radicals [3], but not the less reactive superoxide anion [9]. A recent study has demonstrated that MEL acts as a retarder of the lipid peroxidation but not as a chain-breaking antioxidant [10]. This is consistent with results from lipid peroxidation assays, where the antioxidant capacity of MEL was much lower than that of α-tocopherol [11,12] and required markedly supraphysiological concentrations [13].

Indoleamines in general are known to influence biological oxidation processes [14] and *N*-acetylserotonin (NAS), the immediate precursor as well as a major product of in vivo back-transformation of MEL, has been reported to exert antioxidative properties [15,16,17]. To further differentiate the role and the physiological significance of MEL in the cellular antioxidant defense system, it was the aim of the present study to clarify the radical scavenging potency of MEL in comparison to NAS and the well-characterized antioxidant trolox (TX), a water-soluble analogue of α-tocopherol. Since little is known about antioxidant activities of MEL and NAS in lymphocytes, which are sensitive to oxidative stress [18,19], we investigated their effects on the spontaneous and oxidant-induced ROS formation, as well as on the ROS induced cell death in human peripheral blood lymphocytes (PBL).

*Corresponding author. Fax: (43) (316) 380 9640.
Internet: http://www.kfunigraz.ac.at/fupwww/leibmann.htm;
E-mail: peter.liebmann@kfunigraz.ac.at

Abbreviations. PBL, peripheral blood lymphocyte; ROS, reactive oxygen species; MEL, melatonin; NAS, *N*-acetylserotonin; TX, trolox; t-BHP, t-butylated hydroperoxide; PBS, phosphate buffered saline; DHR, dihydrorhodamine; FCS, fetal calf serum; PI, propidium iodide, DMSO, dimethyl-sulfoxide; AAPH, 2,2′-azobis(amidinopropane hydrochloride)

2. Materials and methods

If not otherwise specified, substances were purchased from Sigma (St. Louis, MO, USA) and were of analytical grade or better. Dihydrorhodamine (DHR) was purchased from Molecular Probes (Eugene, OR, USA). 2,2′-azobis (amidinopropane hydrochloride) (AAPH) was obtained from Polysciences (Warrington, PA, USA).

2.1 *Isolation of lymphocytes*

Peripheral blood lymphocytes (PBL) from venous blood of healthy adult male donors were obtained by Ficoll-Hypaque gradient centrifugation. Isolated PBL were washed three times with PBS (10 mM sodium phosphate, 160 mM NaCl, pH = 7.4) and finally suspended in RPMI 1640 (PAA Laboratories, Exton, USA) plus 10% fetal calf serum (FCS, PAA Laboratories, Exton, USA) at a concentration of 1×10^6 cells/ml. Cells were then stimulated with the mitogen concanavalin A (Con A, 5 μg/ml) for 72 h, washed three times with PBS and cultured in RPMI 1640/10% FCS and 10 U/ml recombinant human interleukin-2 (IL-2, Boehringer Mannheim, Germany) at a concentration of 1×10^6 cells/ml. Medium and IL-2 were replaced every two days. For the experiments, activated PBL from day five to day 11 after stimulation were taken. The cell viability as determined with the trypan blue exclusion test was >95%.

2.2 *Determination of ROS formation*

Formation of ROS was monitored using the oxidation sensitive dye DHR, the uncharged and non-fluorescent reduction product of the cationic fluorescent dye rhodamine 123, which is suitable for ROS measurements in aqueous solutions [20]. In cellular systems, this dye passively diffuses across cell membranes and is oxidized within the cell to rhodamine, which is then located in the mitochondria. Thus, it is a

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useful tool for the detection of intracellular ROS formation as well [21].

2.2.1. Monitoring of the rhodamine fluorescence in an aqueous solution. 10 μM DHR (20 mM stock solution in dimethyl-sulfoxide (DMSO)) in PBS was incubated at 37°C. The ROS-induced rhodamine fluorescence was continuously monitored at 500 nm/560 nm (excitation/emission) after the addition of 1 mM AAPH as a radical-generating system using a Perkin-Elmer LS50-B spectrofluorimeter. The effect of MEL and NAS at the final concentration indicated (all solutions adjusted to a final concentration of 0.5% ethanol, required for the initial dispersion of MEL and NAS) on the peroxyl radical-induced oxidation was determined. Pure solvent (0.5% ethanol) was used as a negative control.

2.2.2. Monitoring of the rhodamine fluorescence in PBL. PBL (2×10^6 cells/ml) were incubated at 37°C in the presence of 2 μM DHR (20 mM stock solution in DMSO) for 10 min. After washing with PBS, cells were resuspended in RPMI (1×10^6 cells/ml) and left untreated or were incubated with 100 μM t-butylhydroperoxide (t-BHP), which forms peroxyl- and alkoxyl radicals in the presence of traces of transition metal ions [22] or 100 μM diamide, which oxidises sulfhydryl groups [23]. At the same time, the indicated concentrations of MEL, NAS and TX were added as a 500 mM stock solution in ethanol. The final ethanol concentration was 0,1%. Pure solvent (0.1% ethanol) was used as a negative control. After the indicated time periods, the rhodamine fluorescence was analyzed at 488 nm/525 nm (excitation/emission) using a Becton-Dickinson FACScan.

2.3. Determination of cell death

Cell death was monitored by means of the propidium iodide (PI) uptake [24]. After 4 h of treatment with t-BHP and various concentrations of MEL and NAS, PBL were washed twice with an ice-cold HEPES buffer (10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7,4), 10 μl of PI (50 μg/ml) was added to the cell pellet and incubated for 15 min at room temperature. The PI uptake, which occurs in dead cells, was analyzed using a Becton-Dickinson FACScan at an emission wavelength of 600 nm.

2.4. Statistical analysis

A data analysis was performed by a repeated measures ANOVA with a two factor repetition followed by a Tukey post-hoc test. The analysis was done with the SigmaStat 2.03 Software package.

3. Results

3.1. Formation of rhodamine in the presence of MEL and NAS in a cell-free system

In a cell-free system, we used 1 mM AAPH, which thermally decomposes to yield peroxyl radicals at a constant rate, as a ROS-generating system. Incubation of a solution of DHR with 1 mM AAPH led to a time-dependent increase of the rhodamine fluorescence (Fig. 1). Addition of MEL led to a reduced rate of fluorescence generation, indicating inefficient scavenging of peroxyl radicals, however, its respective capacity was much lower than that of NAS. Fig. 1 shows the relative efficiency of MEL, NAS and TX in inhibiting the peroxyl radical-induced oxidation of DHR. It is evident that NAS at 10 μM was an even more efficient radical scavenger than TX at the same concentration, while MEL was comparatively weak in retarding the oxidation. Ethanol alone did not affect the DHR oxidation by peroxyl radicals.

3.2. MEL and NAS effects on the intracellular DHR oxidation in human PBL

Resting PBL showed almost no change in their spontaneous intracellular ROS formation over a period of 4 h, as shown in Fig. 2. Nevertheless, the content of intracellular ROS in these cells can be reduced by the addition of TX and NAS. Interestingly, while NAS had an effect comparable to TX, MEL did not decrease the intracellular ROS content. After 4 h of incubation, even a slight enhancing effect of MEL on the ROS formation in resting PBL could be observed.

When PBL were incubated with the membrane-permeable oxidant t-BHP (100 μM), which forms peroxyl- and alkoxyl radicals [22], the intracellular rhodamine fluorescence showed a time-dependent increase with a maximum after 2 h of incubation (Fig. 3A). Addition of 1 mM MEL, NAS or TX, respectively, reduced the DHR oxidation, indicating an antioxidant effect of all substances. However, the capacity of MEL and NAS to scavenge t-BHP-induced ROS showed marked differences (Fig. 3B). While MEL significantly inhibited the ROS formation in a concentration range of 100 μM–1 mM with a maximal inhibition of 44.5 ± 1.9% compared to controls, NAS significantly decreased the DHR oxidation already at 30 μM with a maximal inhibition of 78.8 ± 0.9% at 1 mM, which is comparable to the antioxidant capacity of an equimolar concentration of TX (Fig. 3A).

Incubation with the sulfhydryl-oxidizing agent diamide (100 μM) also induced an increase of the intracellular DHR oxidation in PBL (Fig. 4) which was counteracted by MEL and NAS. The lowest concentrations of MEL and NAS tested, which showed an effect, were 300 μM and 100 μM, respectively. Maximal inhibition of the diamide-induced ROS for-

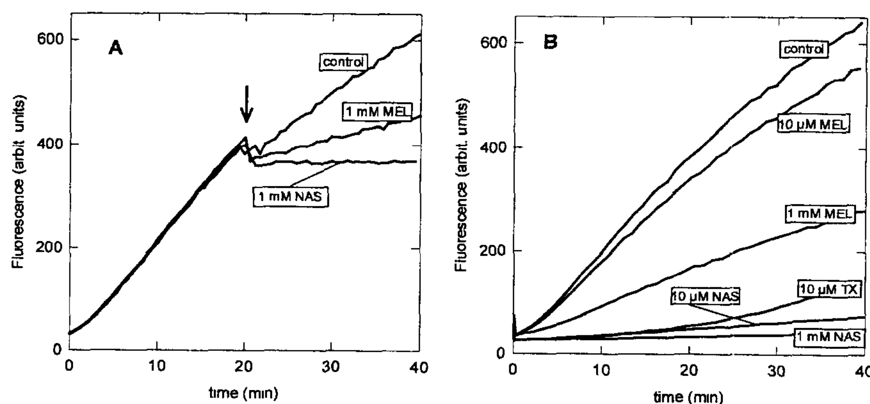


Fig. 1. Effects of MEL, NAS or TX on ROS formation in aqueous solution. 10 μM DHR in PBS was incubated at 37°C and the rhodamine fluorescence was monitored at 500 nm/560 nm (excitation/emission). Peroxyl radicals were generated by 1 mM AAPH and the effect of 10 μM TX, 10 μM and 1 mM MEL, 10 μM and 1 mM NAS on the DHR oxidation was monitored. All solutions contained 0.5% ethanol.

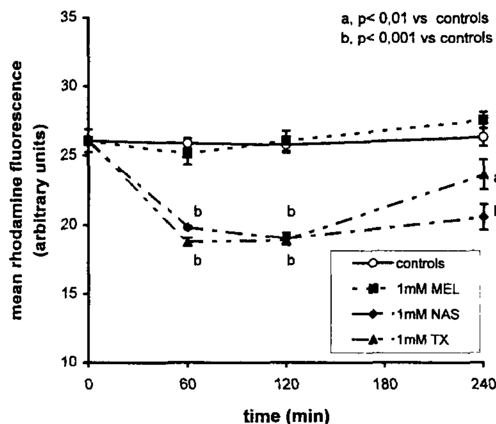


Fig. 2 Effects of MEL, NAS and TX on ROS formation in resting human PBL PBL (2×10^6 cells/ml) were loaded with 2 μM DHR for 10 min at 37°C. After washing with PBS, cells were resuspended in RPMI (1×10^6 cells/ml) and incubated with the indicated concentrations of MEL, NAS, TX or solvent (0.1% ethanol in RPMI). After the indicated time periods, the rhodamine fluorescence was measured at 488 nm/525 nm (excitation/emission) using a Becton-Dickinson FACScan. Data represent mean ± S.E.M., $n = 4$, each done in triplicate

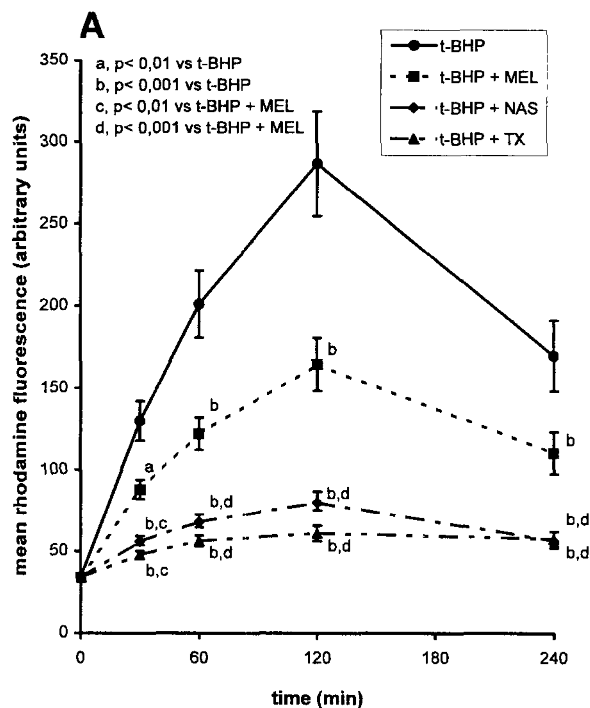
mation by MEL (1 mM) and NAS (1 mM) was 54.1 ± 7.7% and 84.0 ± 2.9%, respectively.

3.3. *Influence of MEL and NAS on the ROS-induced cell death in human PBL*

Since oxidants are known to induce lymphocyte cell death, possibly by an apoptotic pathway [25], we investigated the implications of the above described antioxidant features of MEL and NAS on the t-BHP-mediated cell death in PBL. As shown in Fig. 5, MEL as well as NAS were able to decrease the percentage of non-viable PBL after incubation with t-BHP. However, 100 μM NAS but MEL only in concentrations of more than 300 μM were effective in protecting against t-BHP-induced cell death and 100 μM NAS was as effective as 1 mM MEL, indicating that MEL is by far less efficient in protecting PBL against oxidative stress than NAS.

4. Discussion

Since MEL and NAS can easily cross cell membranes due to their amphiphilicity [26,27], we were interested to what extent these indoleamines influence intracellular redox processes. Our results clearly point to a considerably higher ROS-scavenging activity of NAS compared to that of MEL. In terms of protective effects against radical-induced cell death, the difference between NAS and MEL was even more pronounced. While 300 μM NAS decreased the t-BHP-induced cell death in PBL by 63%, the same concentration of MEL only showed a reduction by 18%. Furthermore, 100 μM NAS



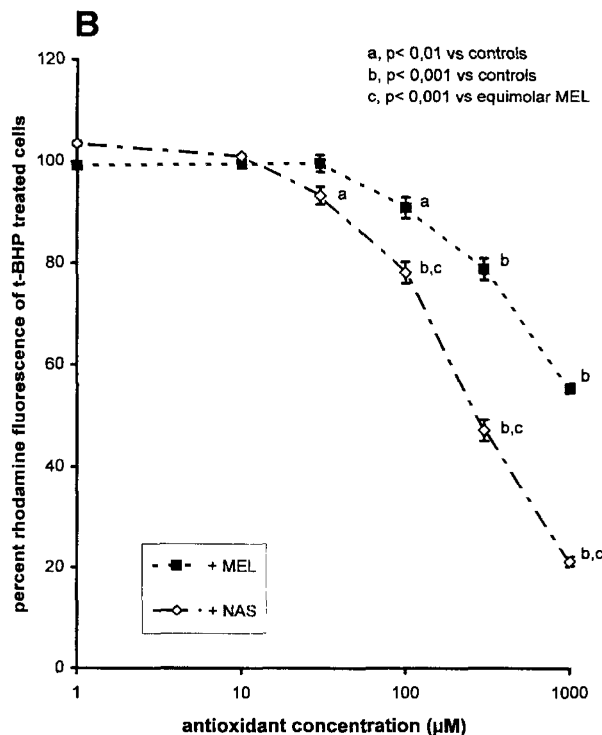


Fig. 3. Effects of MEL, NAS and TX on ROS formation in t-BHP-treated PBL. PBL (2×10^6 cells/ml) were loaded with 2 μM DHR for 10 min at 37°C. After washing with PBS, cells were resuspended in RPMI (1×10^6 cells/ml). (a) Cells were left untreated or were incubated with 100 μM t-BHP and 1 mM MEL, NAS, TX or solvent (0.1% ethanol in RPMI). After the indicated time periods, the rhodamine fluorescence was measured at 488 nm/525 nm (excitation/emission) using a Becton-Dickinson FACScan Data represent mean ± S.E.M., $n = 4$, each done in duplicate. (b) Cells were left untreated or were incubated with 100 μM t-BHP and the indicated concentrations of MEL, NAS or solvent (0.1% ethanol in RPMI). After 2 h of incubation, the rhodamine fluorescence was analyzed at 488 nm/525 nm (excitation/emission) using a Becton-Dickinson FACScan. Data represent mean ± S.E.M., $n = 3$, each done in duplicate

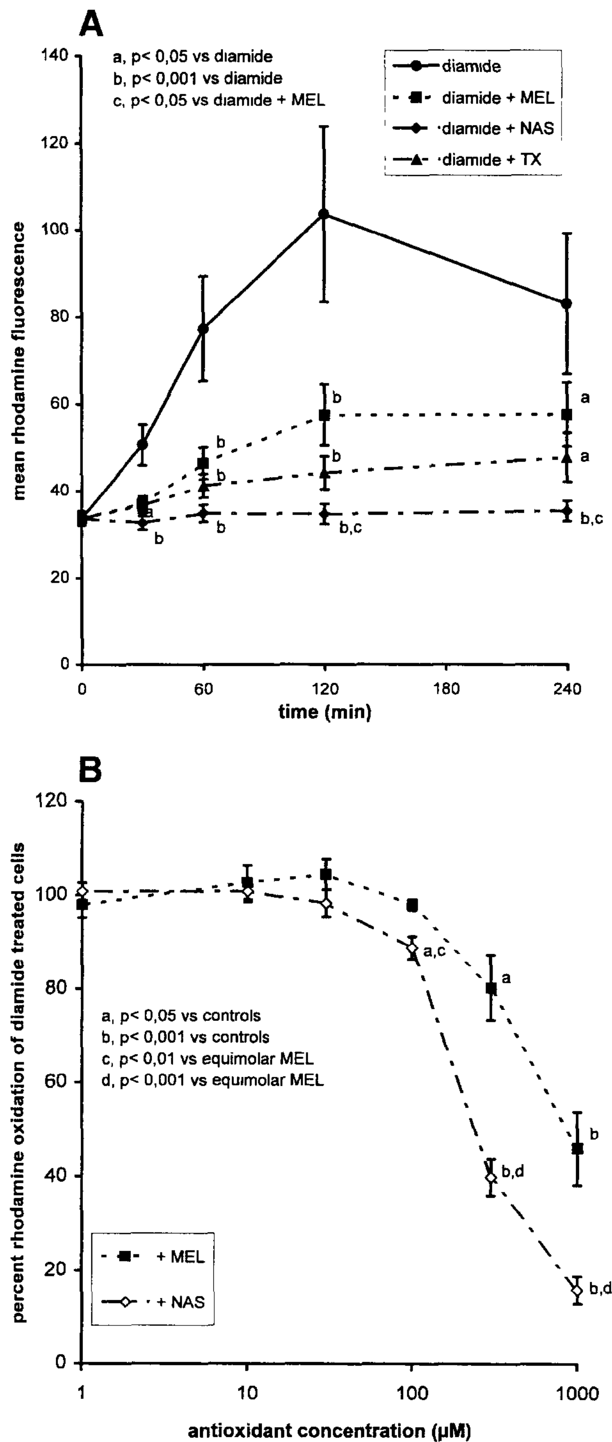


Fig. 4. Effects of MEL, NAS and TX on ROS formation in diamide-treated PBL. PBL (2×10^6 cells/ml) were loaded with 2 μM DHR for 10 min at 37°C. After washing with PBS, cells were resuspended in RPMI (1×10^6 cells/ml). (a) Cells were left untreated or were incubated with 100 μM diamide, which oxidizes sulfhydryl groups, and 1 mM MEL, NAS, TX or solvent (0.1% ethanol in RPMI). After the indicated time periods, the rhodamine fluorescence was measured at 488 nm/525 nm (excitation/emission) using a Becton-Dickinson FACScan. Data represent mean ± S.E.M., $n=4$, each done in duplicate. (b) Cells were left untreated or were incubated with 100 μM diamide and the indicated concentrations of MEL, NAS or solvent (0.1% ethanol in RPMI). After 2 h of incubation, the rhodamine fluorescence was measured at 488 nm/525 nm (excitation/emission) using a Becton-Dickinson FACScan. Data represent mean ± S.E.M., $n=3$, each done in duplicate.
←

was as effective as 1 mM MEL to protect PBL from ROS-induced cell death. These results indicate that MEL exerts only a little protective effect against oxidative stress in cells as compared to NAS.

Furthermore, in aqueous cell-free solutions, the capacity of MEL to scavenge peroxyl radicals was much lower than that of NAS. While 10 μM NAS was more effective than the same concentration of TX, MEL, even at millimolar concentrations, had only minor effects on the peroxyl radical-induced DHR oxidation. This is in contrast to Pieri et al. [3], who reported the capacity of MEL to scavenge AAPH-derived peroxyl radicals to be twice that of TX. More recent studies, however, are in line with our findings. MEL showed only a weak protection of low density lipoproteins against oxidative damage as compared to α-tocopherol [11] and a lack of antioxidant activity of MEL against the peroxyl radical-induced lipid peroxidation in model membranes has been demonstrated recently [10]. According to Seegar et al. [15], who also investigated effects of MEL on the LDL oxidation, the antioxidative activity of MEL was negligible compared to other indoleamines, like serotonin, NAS and 6-hydroxymelanin. For serotonin, a higher efficiency to prevent lipid peroxidation than for MEL was reported [9]. A possible reason for this difference in their reactivity towards peroxyl radicals

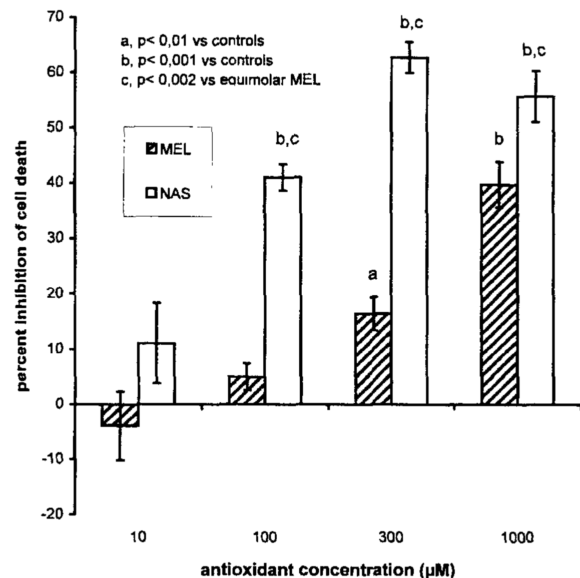


Fig. 5. The influence of MEL and NAS on the t-BHP-induced cell death in PBL. PBL (1×10^6 cells/ml) were left untreated or were incubated with 100 μM t-BHP and the indicated concentrations of MEL, NAS or solvent (0.1% ethanol in RPMI). After 4 h of treatment, PBL were washed twice with ice-cold HEPES buffer and incubated with PI (10 μg/ml) for 15 min at room temperature. The percentage of PI positive cells was determined using a Becton-Dickinson FACScan at an emission wavelength of 600 nm. Data represent mean ± S.E.M. of one experiment out of three giving identical results, $n=3$, each done in duplicates.

might be the phenolic OH-group in NAS and serotonin which is absent in MEL.

Although pinealectomy in rats, which abolishes circulating levels of MEL, has been reported to exaggerate the oxidative damage after treatment of rats with ROS-generating agents [28], the physiological significance of its antioxidant activity, which is observed only at micromolar concentrations, remains questionable, since the nocturnal peak serum concentration of MEL in humans as well as in other mammals is in the picomolar range [29]. Beside its endocrine release from the pineal gland, MEL was suggested to be produced and released by human PBL [30] as well as by cultured skin cells [31], indicating a paracrine or intracellular role as well. However, the concentrations of paracrine and intracellular MEL in lymphoid tissues or in the skin are not yet known.

In human serum, NAS is found in nanomolar concentrations [32], which are about 10–100-fold higher than MEL and NAS is produced and released by cultured skin cells [31] and by human PBL [30]. In skin cells, the amount of released NAS after serotonin addition was about 10 times higher than that of MEL. Furthermore, NAS is a major product of the in vivo back-transformation of MEL in various tissues [33], including human PBL [34]. Therefore, the concentration of NAS in various tissues is presumably much higher than that of MEL. This issue has to be clarified in further studies.

Our finding that MEL, in contrast to NAS and TX, is not able to decrease the intracellular ROS formation in resting PBL, but even led to a slight increase, points to a qualitative difference between MEL and NAS in influencing intracellular redox processes. This notion is corroborated by findings that MEL enhances the ROS formation in resting Jurkat cells, a human leukemic cell line, while NAS reduced it [35] and by Barsacchi et al. [36] reporting that vitamin E consumption in erythrocytes exposed to oxidative stress was reduced by NAS but enhanced by MEL.

Our results in a biochemical and a cellular experimental system demonstrate that NAS, the immediate precursor of MEL, is a better antioxidant than MEL itself. NAS exhibited antioxidative effects at 3–10-fold lower concentrations than MEL. Together with the fact that extracellular and intracellular NAS concentrations in vivo are considerably higher than those of MEL, these results clearly indicate NAS to be a physiologically more relevant antioxidant. Thus, an outstanding role of MEL as well as its physiological significance as anti-ageing principle due to its antioxidative features has to be questioned.

Acknowledgements: This study was supported by the Austrian Science Foundation (P-12679-Med) and SFB00709.

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IDENTIFICATION OF MELATONIN IN PLANTS AND ITS EFFECTS ON PLASMA MELATONIN LEVELS AND BINDING TO MELATONIN RECEPTORS IN VERTEBRATES

Atsuhiko Hattori, Hiro Migitaka, Masayuki Iigo, Masanori Itoh, Koji Yamamoto, Ritsuko Ohtani-Kaneko, Masayuki Hara, Takuro Suzuki and Russel J. Reiter[a,*]

Department of Anatomy, St. Marianna University School of Medicine, Kawasaki 216, Japan
[a]Department of Cellular and Structural Biology, The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284-7762

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Summary

Twenty-four edible plants were investigated for the presence of melatonin, heretofore considered to be a molecule found only in the animal kingdom. The amount of melatonin in different plants varied greatly with highest melatonin being present in plants of the rice family. Melatonin was identified by radioimmunoassay and verified by high performance liquid chromatography with fluorescence detection. Feeding a diet containing plant products rich in melatonin to chicks increased radioimmunoassayable levels of melatonin in their blood. Likewise, melatonin extracted from plants inhibited binding of [^{125}I]iodomelatonin to rabbit brain. Thus, melatonin ingested in foodstuffs enters the blood and is capable of binding to melatonin binding sites in the brain of mammals.

Introduction

Most living organisms use annual changes in seasonal environmental variations, i.e., photoperiod, temperature, rainfall, and food supply, to regulate physiological functions such as reproduction, migration, diapause, molt, plumage color and fur quality. In temperature zone species, it has been clearly shown that the changing photoperiod serves as an important environmental cue for these processes (1,2). Melatonin, a hormonal product of the pineal gland, is known to mediate the changing photoperiodic message via its daily pattern of secretion (3,4).

In some animals photoperiod does not serve as the critical seasonal cue. For example, Bergert et al (5) reported that the springtime ingestion of the young seedlings of winter wheat,

*Corresponding author
Fax#: 210 567-6948
Phone #: 210 567-3859

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rather than seasonal photoperiodic changes, triggers the annual reproductive cycle of the vole. A chemical component of winter wheat identified as 6-methoxy-benzoxanlinone (6-MBOA) was found to be the active ingredient which regulated seasonal reproduction in this species (6). This was the first illustration of a plant factor capable of inducing the annual reproductive cycle in a rodent.

Exogenously administered melatonin alters reproductive activity and entrains the circadian rhythms a variety of fishes, amphibians, reptiles, birds, and mammals including the human (2,7-9). Melatonin administration to humans can change mood performance, sleepiness and sleep onset (10) and it alleviates the symptoms of jet lag (11). The introduction of a high affinity, high specific activity ligand [^{125}I]iodomelatonin has permitted the identification of melatonin-binding sites in the retina, hypothalamus, and pituitary gland (12,13). A recent report indicates that high affinity melatonin binding sites also may be found in the gastrointestinal tract of duck, chick and human (14,15).

To date there are no studies related to foodstuffs derived from the plants as a possible source of melatonin. This possibility does not seem unlikely since melatonin has been shown to be present in photosynthetic algae (16). The present paper reports the presence of melatonin in plants as determined by radioimmunoassay (RIA) and high pressure liquid chromatography (HPLC). Furthermore, we show that plasma melatonin concentrations in birds increase after feeding them plant products rich in melatonin and we also demonstrate that plant-derived melatonin binds to melatonin receptors in the rabbit brain.

Materials and Methods

The plants of twenty-four representative species and their sampling portions were selected on the basis of being edible. The following plants, were studied: Indian spinach (Basella alba), cucumber (Cucumis sativus), kiwi fruit (Actinidia chinensis), cabbage (Brassica oleracea), Japanese radish (Bassica campestris), Chinese cabbage (Raphanus sativus), Japanese ashitaba (Angelica keiskei), carrot (Paucus carota), strawberry (Fragaria magna), apple (Malus domestica), shungiku (Chrysanthemum coronarium), Japanese butterbur (Patasites japonicus), tomato (Lycopersicon esculentum), rice (Oryza sativa japonica), barley (Hordeum vulagare), sweet corn (Zea mays), oat (Abena sativa), tall fescue (Sestvca arundinacea), taro (Colocasisesculenta), ginger (Zingiber officinale), pineapple(Ananas comosus), asparagus (Asparagus officinalis), onion (Allium cepa), Welsh onion (Allium fistulosum). The tissue from each species was homogenized by Polytron or Food Mill Millser with 10mM PBS, centrifuged and the supernatant was assayed for melatonin by means of radioimmunoassay (Table 1). The limit of detection of this assay was 1 pg/tube, and the interassay variation was 4.6%. The antiserum was highly specific for melatonin; cross-reactivity was less than 0.005% (17), and its cross-reactivity with indole-3-acetic acid and 6-MBOA was less than 0.025%.

Results and Discussion

Immunologically-identifiable melatonin was found in all plants studied (Table 1). Substantial amounts of melatonin were present in the rice family, Gramineae. Parallel inhibition was obtained with the standard curve of melatonin and serially diluted samples (representative species shown in Fig. 1A). Inhibition curves of chloroform extracts of the same representative species were also parallel as seen in figure 1B. In radish, radioimmunoassayable melatonin was found in the leaf, stem and root with the concentrations in each portion being approximately 1 ng/g wet tissue. Different portions of the root had essentially the same concentrations of melatonin. To further verify that immunoreactive material measured in plants was melatonin, we subjected radish extracts to high performance liquid chromatographic (HPLC) analysis with fluorescence detection. The HPLC retention time of synthetic melatonin was 16.6 min which is the same as the material found in radish (Fig. 2A). Co-injection of synthetic melatonin (250 pg) with extracts of radish caused increasing peak levels with retention times of 16.6 min (fig. 2B). Using several different chromatographic conditions, the substance in radish extracts had the same retention time as authentic melatonin.

We also tested whether plant-derived melatonin would influence plasma melatonin levels after feeding to chicks. Two week-old female chicks (Gallus domesticus) were food restricted for 48 hours. Following this period, chicks were fed ad libitum either normal chick food (Chubushiryo Comp., Chita) composed of corn, milo, beans, and rice (melatonin concentration of 3.5 ng/g) or a low melatonin-food (melatonin concentration of less than 100 pg/g) after extraction of melatonin from the normal chick food with PBS. Mean melatonin concentrations in plasma increased after feeding of the melatonin-rich food, but not following low melatonin-food (Fig. 3). Statistically significant differences ($p < 0.01$) between the two groups (two way ANOVA) in terms of plasma melatonin concentrations were noted with the chicks receiving the unextracted melatonin-rich food having significantly higher plasma melatonin values at all times tested. Administration of as little as 0.1mg melatonin orally to human, has been shown to increase plasma melatonin concentrations (10). In view of the similar composition of chick food in both groups (except for the PBS soluble substances, including melatonin) the results show that melatonin derived from ingested plant food passes through the gastrointestinal wall and enters the blood thereby increasing plasma melatonin levels.

To examine whether plant melatonin would be active at the level of the melatonin receptors in mammals, we investigated the ability of the plant extract to bind melatonin receptors

Table 1. Concentrations of radioimmunoassayable melatonin levels in various plants. Plants were selected on the basis of their being edible. Results are expressed as the mean ± SEM.

Family	Species	pg/g tissue
Basellaceae	indian spinach	38.7 ± 5.5
Cucurbitaceae	cucumber	24.6 ± 3.5
Actinidaceae	kiwi fruit	24.4 ± 1.7
Cruciferae	cabbage	107.4 ± 7.3
	Japanese radish	657.2 ± 29.0
	Chinese cabbage	112.5 ± 10.3
Umbelliferae	Japanese ashitaba	623.9 ± 63.1
	carrot	55.3 ± 11.9
Roseceae	strawberry	12.4 ± 3.1
	apple	47.6 ± 3.1
Compositae	chungiku	416.8 ± 54.6
	Japanese butterbur	49.5 ± 5.6
	tomato	32.2 ± 2.4
Cramineae	rice	1006.0 ± 58.5
	barley	378.1 ± 25.8
	sweet corn	1366.1 ± 465.1
	oat	1796.1 ± 43.3
	tall fescue	5288.1 ± 368.3
Araceae	taro	54.6 ± 23.0
Zingiberaceae	ginger	583.7 ± 50.3
Bromeliaceae	pineapple	36.2 ± 8.4
Liliaceae	asparagus	9.5 ± 3.2
	onion	31.5 ± 4.8
	Welsh onion	85.7 ± 8.0

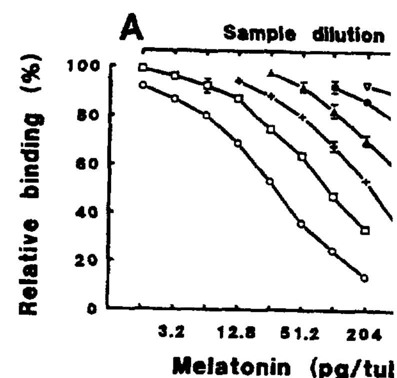

Fig 1. Comparative displacement c
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determined by radioimmunoass
fescue; ▲, Japanese radish; •, J
represents the mean ± SEM of fi
The slopes of the displacement c
radish, Japanese ashitaba, rice, an

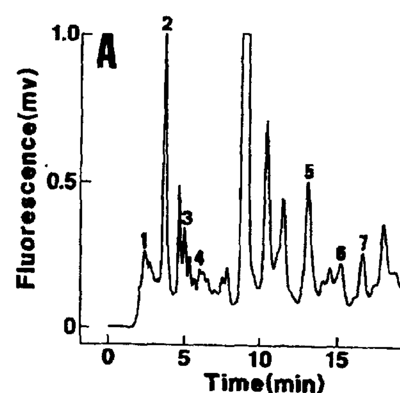

Fig 2. RP-HPLC elusion profiles of t
authentic melatonin (B). Aliquo
extraction after perchloric acid
system, which was equipped wit
and a RF-550 fluorometric de
chromatographic conditions, see I
50 mM ammonium acetate; flow-
wavelength, 340 nm. Peaks: 1
hydroxytryptophol; 3 = 5-me
methoxyindole acetic acid; 6 = 5-
the co-injection of 250 pg of synth

onin levels in various plants. Plants
: expressed as the mean ± SEM.

pg/g tissue
38.7 ± 5.5
24.6 ± 3.5
24.4 ± 1.7
107.4 ± 7.3
657.2 ± 29.0
112.5 ± 10.3
623.9 ± 63.1
55.3 ± 11.9
12.4 ± 3.1
47.6 ± 3.1
416.8 ± 54.6
49.5 ± 5.6
32.2 ± 2.4
1006.0 ± 58.5
378.1 ± 25.8
1366.1 ± 465.1
1796.1 ± 43.3
5288.1 ± 368.3
54.6 ± 23.0
583.7 ± 50.3
36.2 ± 8.4
9.5 ± 3.2
31.5 ± 4.8
85.7 ± 8.0

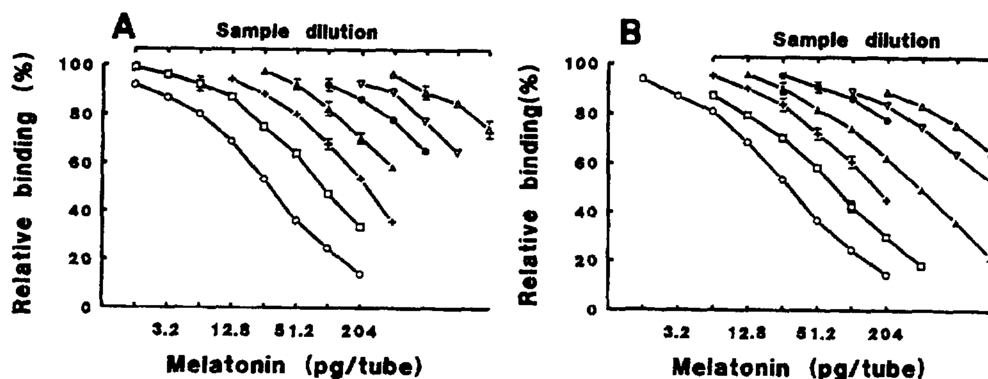

Fig 1. Comparative displacement curves obtained with synthetic melatonin and representative plants extracted with PBS (A) or chloroform (B). Melatonin content in each tissue was determined by radioimmunoassay. Symbols: o, synthetic melatonin; □, ginger; +, tall fescue; ▲, Japanese radish; •, Japanese ashitaba; ∇, rice; Δ, shungiku. Each data point represents the mean ± SEM of five to six individual determinations performed in triplicate. The slopes of the displacement curves for authentic melatonin, ginger, tall fescue, Japanese radish, Japanese ashitaba, rice, and shungiku were not statistically significantly different.

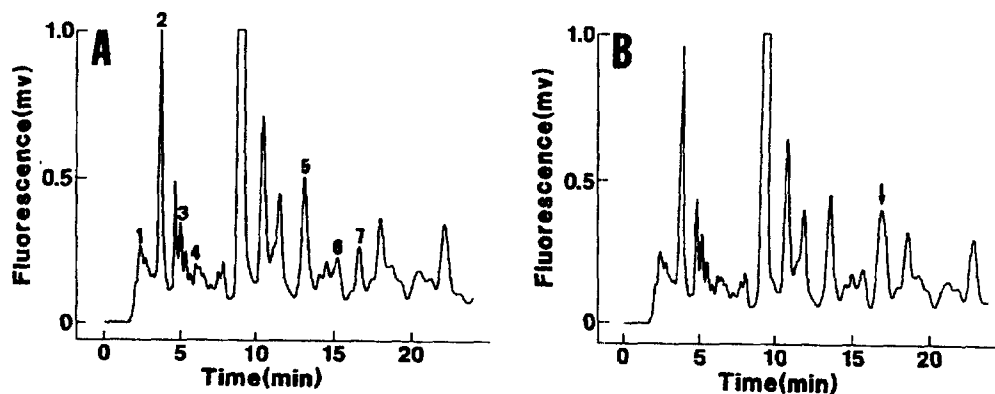

Fig 2. RP-HPLC elusion profiles of the extract of Japanese radish (A) and with co-injection of authentic melatonin (B). Aliquots of 20 µl of the tissue solution obtained by chloroform extraction after perchloric acid homogenate were directly injected into the RP-HPLC system, which was equipped with a Superior ODS S-5 µm column (Shiseido Co., Tokyo) and a RF-550 fluorometric detector (Shimadzu Co., Kyoto). For details on the chromatographic conditions, see Itoh et al (18). Conditions: mobile phase, 30% methanol in 50 mM ammonium acetate; flow-rate, 1 ml/min; excitation wavelength, 280 nm; emission wavelength, 340 nm. Peaks: 1 = Serotonin; 2 = 5-hydroxyindole-3-acetic acid and 5-hydroxytryptophol; 3 = 5-methoxytryptamine; 4 = 6-hydroxymelatonin; 5 = 5-methoxyindole acetic acid; 6 = 5-methoxytryptophol; 7 = melatonin. An arrow (B) indicates the co-injection of 250 pg of synthetic melatonin.

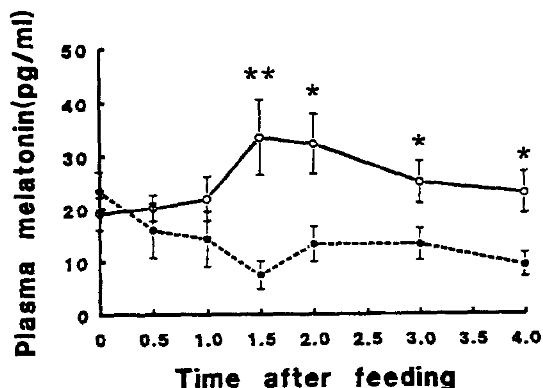

Fig.3. Changes in plasma melatonin concentrations in chicks after feeding a melatonin-rich food (o; N=10) and low-melatonin food (•; N=7). Two week-old female chicks (Gallus domesticus) were fed either melatonin-rich chick food (3.5 ng/g melatonin concentration) or low-melatonin food (less than 100 pg/g) ad libitum after they had been food restricted for 48 hours. Blood samples were collected serially from all birds from the brachial vein using heparinized tubes. Similar results were obtained in another experiment (data not shown). *$p < 0.05$; **$p < 0.01$

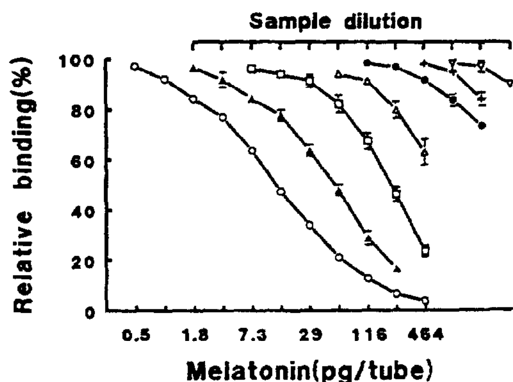

Fig 4. Competition curves for various plant extracts in inhibiting [^{125}I]iodomelatonin binding (50 pM) to rabbit brain membranes at 37°C. Whole brains from 3-month old rabbits were dissected and homogenized in 50 mM Tris-HCl buffer including 4 mM CaC12 (pH 7.4). The homogenate was centrifuged twice at 28,000 Xg for 20 min at 4°C. The membrane pellet was finally resuspended in Tris-HCl buffer to yield a tissue concentration of 8 mg wet weight/tube. Binding assays were performed as described by Iigo et al. (19). Each data point represents the mean ± SEM of five individual determinations performed in triplicate. Symbols are the same as in Fig. 1.

in the rabbit brain. The extracts o
clearly inhibited [^{125}I]iodomelatonin

These findings suggest that a
melatonin in foodstuffs to influenc
animals in the wild could contain
reproductive events.

Food derived-melatonin co
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because of its high lipophilicity (4) m

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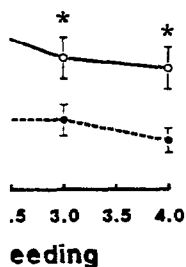
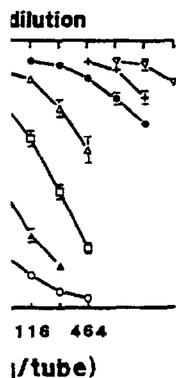


ıicks after feeding a melatonin-rich food Two week-old female chicks (Gallus ›d (3.5 ng/g melatonin concentration) or ıfter they had been food restricted for 48 ı all birds from the brachial vein using another experiment (data not shown).



ıhibiting [^{125}I]iodomelatonin binding (50 brains from 3-month old rabbits were ıffer including 4 mM CaC12 (pH 7.4). Xg for 20 min at 4°C. The membrane yield a tissue concentration of 8 mg wet escribed by Iigo et al. (19). Each data l determinations performed in triplicate.

in the rabbit brain. The extracts of Japanese radish, ginger, shungiku and Japanese ashitaba clearly inhibited [^{125}I]iodomelatonin binding in the rabbit brain (Fig. 4).

These findings suggest that animals including human, may ingest sufficient quantities of melatonin in foodstuffs to influence physiological processes. In this context, food eaten by animals in the wild could contain melatonin in sufficient quantities that may alter seasonal reproductive events.

Food derived-melatonin could also be involved in post-prandial sleepiness when melatonin-rich foods are ingested considering that melatonin is a sleep inducing agent (10,20). Melatonin is known to be rapidly taken up from the gastrointestinal tract when it is administered orally (21,22). The sleep enhancing action of melatonin is presumably mediated by membrane-bound receptors found in various locations in the brain.

Considering the potent antioxidant activity of melatonin (21-23), the ingestion of foods selected for their high melatonin content also could play a role in protection against oxygen radical mediated cellular damage. Melatonin has been proposed, because of its antioxidant potential, to possibly be protective against some age-related diseases as well as against aging itself (24-26). The antioxidant actions of melatonin within the cell do not require a binding site; because of its high lipophilicity (4) melatonin readily enters all cells in the organism.

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AN IMPROVED SIMPLE CO
OF NON-TRANS

Daxian Zhang, Shigeru Ok

Department of Pathology an
Okayama Universit

Received December 20, 1994

A simple method for direct qua
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(NTA) to serum to form an Fe-NTA
NTBI in the ultrafiltrate was quantitated
detection condition and several potent
measurements to samples *in vivo* and *in*

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Melatonin Is Metabolized to *N*-Acetyl Serotonin and 6-Hydroxymelatonin in Man

I. M. YOUNG, R. M. LEONE, P. FRANCIS, P. STOVELL, AND R. E. SILMAN*

Mass Spectrometry Unit, Department of Reproductive Physiology, St Bartholomew's Hospital Medical College, 51-53 Bartholomew Close, London EC1A 7BE, United Kingdom

ABSTRACT. To investigate whether melatonin (aMT) can be metabolized to *N*-acetyl serotonin (NAS), a low dose of deuterated aMT was administered to four normal subjects, and their urine samples were analyzed for the presence of deuterated NAS and deuterated 6-hydroxymelatonin (6-HaMT). In one set of experiments, the urine samples were subjected to column chromatography to separate the glucuronide and sulfate conjugates for independent analysis. In another, an internal standard (NAS-sulfate) was used for quantification and total conjugate analysis. Measurement was by gas chromatography-mass spectrometry, and the molecular ions of deuterated and nondeuterated NAS and 6-HaMT were monitored. Deuterated aMT was metabolized to deuterated NAS and deuterated 6-HaMT. The proportion of NAS was less in the sulfate than in the glucuronide conjugates and, overall, represented 15% of the total. Since demethylation is not a pathway that occurs with other pineal methoxyindoles, even at a much larger dose, it seems to be a significant finding with regard to aMT. Thus, it may be important to elucidate the differential metabolism of aMT at different time points and in different age groups. (*J Clin Endocrinol Metab* **60:** 114, 1985)

IT RECENTLY was reported that the pineal hormone, melatonin (aMT), can be differentially metabolized to produce *N*-acetyl serotonin (NAS) in addition to 6-hydroxymelatonin (6-HaMT; Fig. 1) (1). The study was conducted in rats, and the dose of melatonin was high (~100 mg/kg BW). The question, therefore, arises as to whether this metabolic pathway exists in man and at concentrations that more nearly approximate physiological levels.

To investigate this problem, we injected normal men with a small dose of deuterated melatonin (10–15 μg/kg BW) and collected their urine. Using gas chromatography-mass spectrometry (GCMS), the urinary products were analyzed for the presence of deuterated NAS and deuterated 6-HaMT.

Materials and Methods

Organic solvents

The organic solvents were of Analar grade (BDH Chemical Co., Poole, Dorset, United Kingdom) and were freshly redistilled before use.

Synthesis of deuterated aMT

Acetyl-deuterated aMT was synthesized using a previously described method (1). Fifty milligrams of the white powder were dissolved in 5 ml ethanol and made up to 50 ml with normal saline. Five-milliliter aliquots of the solution then were filtered through disposable filters (Millipore, Bedford, MA) into glass bottles for autoclave sterilization. One milliliter from each bottle was taken for injection.

Sample collection

The study was conducted on four normal men, aged 25-43 yr. On day 1, the men emptied their bladders at 1030 h, and the urine was discarded. Urine then was collected at the next voiding, between 1230 and 1430 h. Volumes ranged between 160–270 ml. The urine samples were labeled pretest A, B, C, and D and were stored frozen at −20 C.

On day 2, the men emptied their bladders at 1030 h, and the urine was discarded. Immediately afterward, 1 ml of a sterile 1 mg/ml solution of acetyl-deuterated aMT in ethanol and normal saline (1:10) was injected iv via a forearm vein. Urine was collected at the next voiding, between 1230 and 1430 h. Volumes ranged between 115–270 ml. The urine samples were labeled posttest A, B, C, and D and were stored frozen at −20 C.

Sample preparation

The urine samples were prepared for analysis in two ways. The first method used column chromatography to separate the glucuronide and sulfate conjugates for independent analysis. The second method was used for total conjugate analysis.

Separation of glucuronide and sulfate conjugates. Column chromatography: Four K16/20 columns (200 mm long; id, 16 mm; Pharmacia, Hounslow, United Kingdom) were filled with Bio-

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Address all correspondence and requests for reprints to: Dr. R. E. Silman, Department of Reproductive Physiology, St. Bartholomew's Hospital, Medical College, Mass Spectrometry Unit, 51-53 Bartholomew Close, London EC1A 7BE, United Kingdom.
* Wellcome Trust Senior Lecturer.

Melatonin

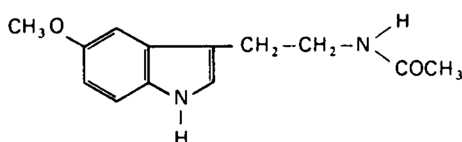
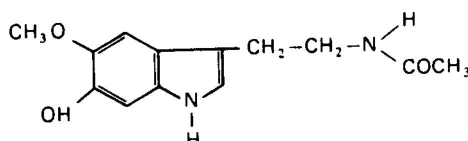
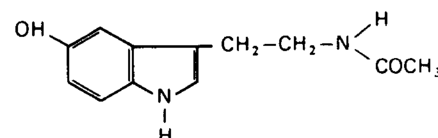


FIG. 1. The structure of aMT and its two principal urinary metabolites, 6-HaMT and NAS.

6-hydroxy melatonin



N-acetyl serotonin



Gel P-2 (200–400 mesh; fine; Bio-Rad Laboratories, Watford, United Kingdom) swollen in 0.01 M acetic acid. The flow rate was set at 10 ml/h, and fractions were collected every 40 min (Ultrorac, LKB Instruments, Croydon, United Kingdom).

The columns were calibrated using 1.5-ml aliquots of urine containing large concentrations of 6-sulfatoxymelatonin (SaMT), 5-sulfatoxy-NAS (SNAS), 6-glucuronide aMT (GaMT), and 5-glucuronide NAS (GNAS). Urine was obtained from rats in the morning after the administration of 20 mg melatonin, ip, the previous evening. The salt fractions were determined by conductivity (CMD 400 digital conductivity meter WPA Ltd., Saffron Walden, United Kingdom). The urea, indole-glucuronide, and indole-sulfate fractions were determined by thin layer chromatography. Merck 5554 plates (Merck, Rahway, NJ) were used, with solvent: butan-2-ol-acetic acid-water (4:1:1) and a front of 70 mm. Urea gave a yellow spot. The indole conjugates were determined by spraying the thin layer chromatographic plates with Ehrlich's reagent. Rf values for GaMT and GNAS were 30% and 33%, respectively; for SaMT and SNAS, they were 48% and 53%, respectively. The glucuronide metabolites eluted in fractions 5 and 6; the sulfate metabolites eluted in fractions 12–17.

Aliquots (1.5 ml) from the pretest urines A, B, C, and D were loaded onto the 4 Bio-Gel columns and eluted with 0.01 M acetic acid. After chromatography was complete (~24 h), fractions 5 and 6 were pooled, as were fractions 12–17. All other fractions were discarded. Aliquots (1.5 ml) from the posttest urines A, B, C, and D were treated similarly. Thus, for each individual urine, there were two sample pools corresponding to the glucuronide and sulfate fractions. The 16 samples (4 pretest and 4 posttest indole-glucuronide and indole-sulfate samples) were freeze-dried (Mini-Fast 3400, Edwards Alto Vuoto, Trezzano sul Naviglio, Italy).

Glucuronide fractions: The samples were reconstituted in 2 ml H_2O and enzymically hydrolyzed. To each sample were added 125 μl 0.85 M ascorbic acid, 125 μl 0.053 M EDTA solution, 0.5 ml acetate buffer (pH 4.5), 5 mg barium hydroxide, and 0.5 ml HA3F, a β-glucuronidase enzyme preparation containing sulfatase activity (Sigma, Poole, United Kingdom). The solutions were mixed for 10 sec and then incubated for 3 h in a water bath maintained at 37 C.

After hydrolysis, the urines were extracted twice with 3 ml ethyl acetate. Gentle shaking was employed, since vigorous mixing tended to create a gel. The organic phase was transferred to a glass vial by Pasteur pipette and dried under oxygen-free nitrogen on a heating block maintained at 40 C. Two hundred microliters each of ethyl acetate and pentafluoroproprionic acid (PFPA; Phase Separations, Queensferry, Clwyd, United Kingdom) were added to each dried extract. The vials were capped, mixed, and left for 10 min in a heating block maintained at 40 C, after which they were dried under oxygen-free nitrogen and stored dry until assayed by GCMS.

Sulfate fractions: The sulfate metabolite was both deconjugated and derivatized as a single step procedure in the reaction with PFPA. Two hundred microliters each of ethyl acetate and PFPA were added to each dried extract. The vials were capped, mixed, and left for 1 h at room temperature, after which they were dried under oxygen-free nitrogen and stored dry until assayed by GCMS.

Total conjugate analysis. Internal standard: Nondeuterated SNAS (494 ng; the equivalent of 380 ng deconjugated NAS) was added to 1-ml aliquots of urine taken from each of the four pretest and four posttest urine samples. One-milliliter aliquots from each urine sample were also analyzed without the addition of internal standard.

Purification of conjugates: Each aliquot of urine was added

to an extraction tube and mixed with 5 g XAD-2 (BDH Ltd.) for a few minutes. The tubes then were placed in an oven and heated at 120 C for approximately 1 h until the XAD was dry and granular. The XAD then was washed with 5 ml distilled water, the tubes were mixed, and the water was discarded. The wash was repeated, and then the conjugates were eluted with 5 ml methanol and mixed, and after letting the XAD settle, the alcohol layer was pipetted to a clean tube and dried under oxygen-free nitrogen.

Enzymic hydrolysis: The dried samples were reconstituted in 1 ml distilled water with the addition of 0.25 ml ascorbic acid (0.85 N), 0.25 ml EDTA (0.053 M), and 0.4 ml HA3F β-glucuronidase containing sulfatase activity (Sigma). The samples were mixed gently and left to incubate at 37 C in a water bath overnight.

Extraction of deconjugated products: The next morning, 5 ml ethyl acetate were added to each tube and mixed gently. The ethyl acetate phase was pipetted into a clean tube and dried under oxygen-free nitrogen. A further 5 ml chloroform then were added to the aqueous phase and mixed. After the phases had separated, the chloroform phase was transferred to the corresponding tube containing the dried ethyl acetate residue, and the tube was dried again. After the second drying, the tubes contained a slightly gummy residue.

Derivatization: The samples were derivatized by the addition of 200 μl ethyl acetate and 200 μl PFPA. After mixing, they were left for 15 min at 40 C, and then dried under oxygen-free nitrogen. The derivatives then were cleaned using small silica Sep-pak columns (Waters Associates, Inc., Milford, MA). The columns were first washed with chloroform, then each derivatized sample was reconstituted in 0.5 ml chloroform and applied to the washed column. A further 0.5 ml chloroform then was added to the columns, and the effluents were discarded. The derivatized samples were eluted from the columns with 2.25 ml chloroform. The eluted chloroform was dried under oxygen-free nitrogen until assay by GCMS.

GCMS. Gas chromatograph (GC): Samples were introduced onto a Carlo Erba GC fitted with an on-column splitless injection port. A 25-meter capillary column (Carlo Erba Strumentazione) was used, with an internal coating of OV-1 phase. It was operated at a temperature of 180 C for 11 min after injection, then raised at a rate of 3°/min to a maximum of 210 C. The helium flow rate was 1.6 ml/min, and the volume of sample injected was 1 or 2 μl.

Mass spectrometer (MS): The GC was interfaced to a Kratos MS 25S MS controlled by a Nova 4 computer (Data General). MS operating conditions were: filament, 1 mA; electron voltage, 45 eV; and source temperature, 200 C. The widest source and collector slit settings were used, giving a resolution of approximately 1200 ppm.

GCMS of NAS and 6-HaMT: One milligram each of standard crystalline NAS and 6-HaMT (Sigma) was derivatized by the addition of 200 μl redistilled ethyl acetate and 200 μl PFPA. The vials were capped and left at room temperature for 10 min. The solutions then were dried under oxygen-free nitrogen and redissolved in 1 ml ethyl acetate. One-microliter aliquots were injected onto the GC. The retention time for NAS was 10 min and 52 sec; the mass spectrum showed a prominent molecular ion with a mass of 492 and a major fragment with a mass of 345 (Fig. 2A). The retention time for 6-HaMT was 19 min and 20 sec; the mass spectrum showed a prominent molecular ion with a mass of 522 and a major fragment with a mass of 375 (Fig. 2B). Dilutions were made of derivatized NAS and 6-HaMT, and the MS was set up in the multiple peak monitoring mode (MPM) to monitor the molecular ions 492.05 and 522.05. A standard curve was thus obtained using the peak height of the molecular ion of each compound. Both curves were linear down to 3 ng compound on column.

Assay of samples: The dried derivatives were made up in 10 μl ethyl acetate, of which 1 or 2 μl were taken for injection. The MS was set up in the MPM mode, and four masses were monitored: 492.05, the molecular ion of NAS; 494.06, the molecular ion of deuterated NAS; 522.05, the molecular ion of 6-HaMT; and 524.06, the molecular ion of deuterated 6-HaMT. NAS and deuterated NAS eluted at 10 min and 52 sec; 6-HaMT and deuterated 6-HaMT eluted at 19 min and 20 sec. Quantification of the deuterated compounds was obtained by measuring peak height and reading values from the standard curves for NAS and 6-HaMT. When there was an internal standard, quantification was based on the ratio of the peak height of the molecular ion of the deuterated compounds with that of the peak height of the molecular ion of the internal standard.

Results

Glucuronide and sulfate fractions

Pretest urine samples. Assay of the glucuronide and sulfate fractions in the pretest urine samples showed no peaks that corresponded to NAS and 6-HaMT or deuterated NAS and deuterated 6-HaMT. In other words, for any of these compounds, less than 3 ng had been injected on column.

Posttest urine samples. Sulfate fractions: All sulfate fractions contained two peaks, corresponding to deuterated NAS and deuterated 6-HaMT. Figure 3 shows the results obtained in subject A. There were no significant peaks of endogenous (*i.e.* nondeuterated) NAS or 6-HaMT. In all cases, the quantity of NAS was less than that of 6-HaMT.

Table 1 shows the value of the deuterated sulfate conjugates for all subjects after the on-column value had been calculated by reference to a standard curve and corrected to take account total urine volume. The urine content of the deuterated NAS sulfate ranged between 4 and 8 μg, whereas that of the deuterated 6-HaMT sulfate ranged between 20 and 58 μg.

Glucuronide fractions: As with the sulfate fractions, all of the glucuronide fractions contained two peaks corresponding to deuterated NAS and deuterated 6-HaMT. There was no nondeuterated material. However, unlike the sulfate fractions, the proportion of deuterated NAS was increased relative to that of deuterated 6-HaMT, and in three of four samples, it was the major peak.

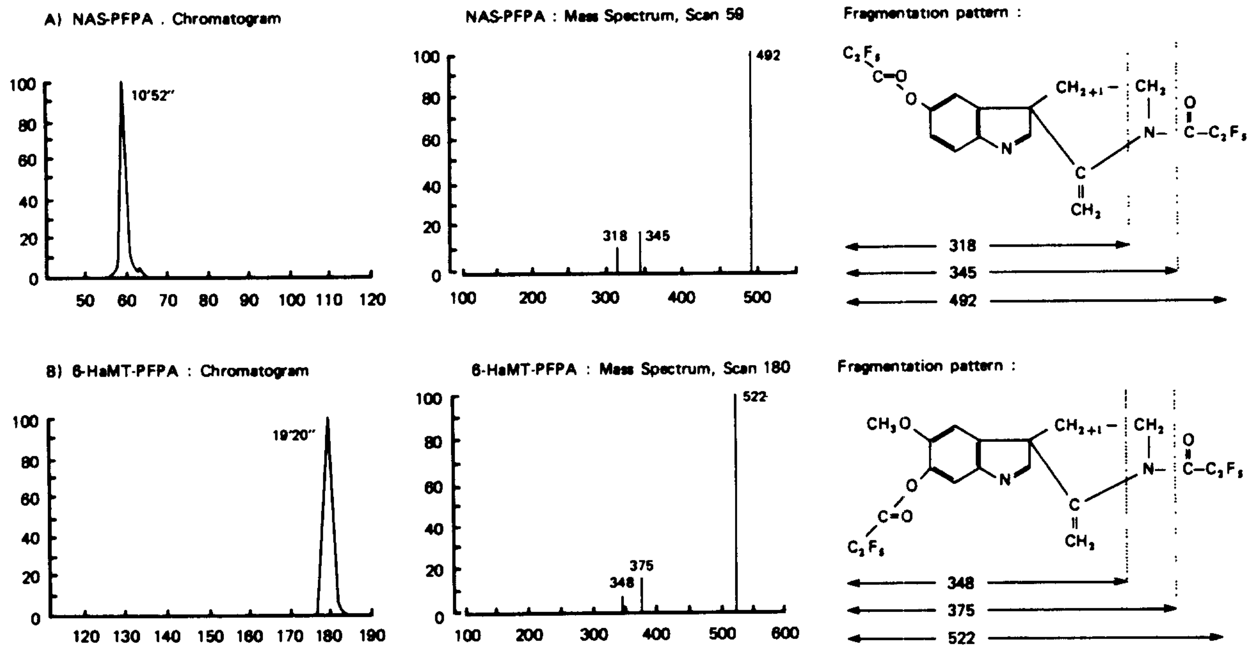


FIG. 2. A, Retention time and mass spectrum of standard PFPA-derivatized NAS; B, Retention time and mass spectrum of standard PFPA-derivatized 6-HaMT.

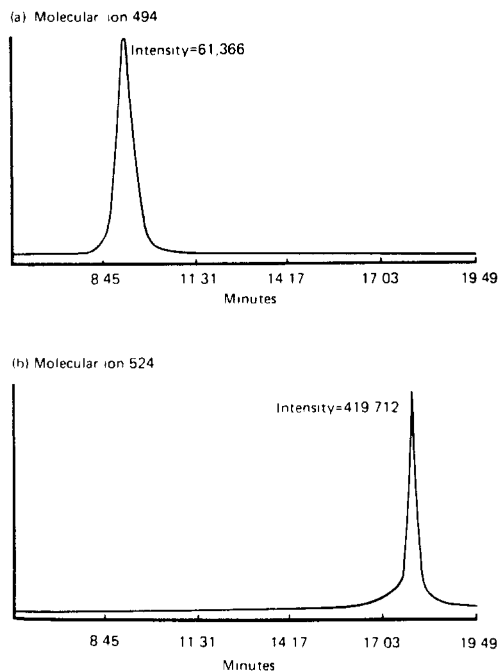


FIG. 3 Posttest urine A: sulfate fraction showing the presence of: a peak eluting at 10 h and 50 min with a molecular ion of 494 corresponding to deuterated NAS (a) and a peak eluting at 19 h and 18 min with a molecular ion of 524 corresponding to deuterated 6-HaMT (b).

Table 1 shows the value of the deuterated glucuronide conjugates for all subjects after the on-column value had been calculated by reference to a standard curve and corrected for total urine volume. The urine content of the deuterated NAS glucuronide ranged between 3 and 19 µg, whereas that of the deuterated 6-HaMT glucuronide ranged between 2 and 7 µg.

TABLE 1. Urinary deuterated NAS (D-NAS) and deuterated 6-HaMT (D-6-HaMT) sulfate and glucuronide conjugates in 4 men injected iv with 1 mg acetyl-deuterated aMT

Sample	Sulfates		Glucuronides		Total
	D-NAS	D-6-HaMT	D-NAS	D-6-HaMT	
Posttest A	5	34	19	6	64
Posttest B	8	58	3	2	71
Posttest C	4	20	5	7	36
Posttest D	8	51	10	4	73

Values are expressed as micrograms in the total urine sample.

Total conjugate analysis. Pretest urine samples: There were no peaks that corresponded to deuterated NAS or deuterated 6-HaMT. In those samples that had no internal standard, there were small peaks corresponding to endogenous (*i.e.* nondeuterated) NAS and 6-HaMT. However, these peaks were not quantified and represented less than 3 ng on the column. In those samples that contained an internal standard, there was a large peak corresponding to nondeuterated NAS.

Posttest urine samples: In all samples, there were significant peaks corresponding to deuterated NAS and deuterated 6-HaMT. In each sample that received an internal standard, there was also a large peak corresponding to nondeuterated NAS.

Table 2 shows the values of deuterated NAS and deuterated 6-HaMT after the on-column value had been calculated by reference to the internal standard and corrected for total urine volume. The urine content of

TABLE 2. Total urinary deuterated NAS (D-NAS) and deuterated 6-HaMT (D-6-HaMT) sulfate and glucuronide conjugates in 4 men injected iv with 1 mg acetyl-deuterated aMT

Sample	D-NAS	D-6-HaMT	Total	% NAS in recovered metabolite
Posttest A	45	169	214	21
Posttest B	57	470	527	11
Posttest C	13	55	68	19
Posttest D	46	446	492	9

Values are expressed as micrograms in the total urine sample.

deuterated NAS ranged between 13 and 57 μg, and that of deuterated 6-HaMT ranged between 55 and 470 μg. The total recovery of deuterated urinary metabolites from 1 mg deuterated aMT ranged from 7–53%, with a mean of 33%. The proportion of NAS to 6-HaMT ranged from 9–21%, with a mean of 15%.

Discussion

NAS is a metabolite of aMT in man

One milligram of deuterated aMT injected iv in man was followed by the excretion of two urinary metabolites, deuterated NAS and deuterated 6-HaMT. The two metabolites were excreted as either sulfate or glucuronide conjugates. The proportion of 6-HaMT was greater than NAS in the sulfate conjugates, whereas there was a greater proportion of NAS in the glucuronide conjugates. However, the sulfate conjugates represented a larger total value, and therefore, more 6-HaMT was excreted than NAS overall.

The recovery of the deuterated metabolites in urine after column separation of the sulfate and glucuronide conjugates was less than that achieved without prior column separation. There are probably two reasons for this. First, recovery was decreased due to considerable losses on the columns. Second, internal standards were not used for the column separation analysis, and therefore, losses, using this procedure, could not be normalized against those of an internal standard. However, the pattern of recovery in the two methods was similar, *i.e.* two individuals (B and D) had relatively high values, and one (C) had a lower value. Hence, the differences between individuals probably represent differences in metabolism and excretion rather than methodological variables in the preparation and processing of the samples.

There was no attempt in this study to determine total recovery of the deuterated metabolites, and only the first urine voiding was examined. The reason for this is that in previous (unpublished) experiments using tritiated melatonin, we found that over 50% of the recoverable metabolites were excreted in the first urine voiding. Since our methods of analysis were not sufficiently sensitive to measure the residual metabolites, especially as they must be diluted in larger volumes of urine, we limited our investigation to a study of the metabolic products of aMT in the first 2–4 h after the dose. However, it is worth considering that in the overall metabolism of aMT the proportion of 6-HaMT and NAS may be somewhat different from that in the first 2–4 h.

The significance of NAS as a metabolite of aMT

One milligram of deuterated aMT is a pharmacological dose, and the fact that we demonstrated a metabolic pathway whereby it can be demethylated to form NAS does not prove that this occurs physiologically. We clearly identified, in the pretest urines, peaks that represent endogenous (*i.e.* nondeuterated) NAS and 6-HaMT, and this may prove that endogenous NAS is metabolized from endogenous aMT. However, this is not necessarily so. Whereas 6-HaMT is only produced as a metabolite of aMT, NAS can be produced by other routes, *e.g.* direct synthesis and secretion by the pineal gland.

Nonetheless we demonstrated that aMT can be demethylated even at a comparatively modest dose. The original observation had been made using rats, where dosages as high as 100 mg/kg BW were given (1). Reducing the dosage of aMT reduced the proportion of NAS so it might have seemed as if demethylation did not occur at much lower doses. However, in the experiments reported here, the dosages were at least 1000-fold lower and demethylation was still observed.

These observations are in striking contrast to those where other pineal methoxyindoles have been used. For example, when high doses of 5-methoxytryptophol or 5-methoxytryptamine were administered to rats, there was no evidence of demethylation (3). Thus, it appears that demethylation, like 6-hydroxylation, is a specific pathway in the metabolism of aMT.

Demethylation is an unexpected metabolic pathway. First, it is curious that aMT should be metabolized to a product that is its own precursor. aMT is synthesized in the pineal gland, and the final step is the conversion of NAS to aMT by the action of the enzyme hydroxyindole-*O*-methyl transferase. The fact that there is a specific metabolic pathway that can convert aMT back to NAS suggests that there may be a complex feedback mechanism in the control of aMT production. Second, NAS is considered to be a hormone in its own right (4–6). Hence, the fact that aMT can be metabolized to a product that has different biological potency from that of aMT may give some clue as to why the biological actions of aMT can be altered at different times of the day (7) and different seasons of the year (8, 9). In this respect, it would be particularly interesting to know whether the

differential metabolism of aMT has any role in human puberty (10–13).

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Second International Symposium on The Fetus as a Patient: Diagnosis and Therapy

The Second International Symposium on the Fetus as a Patient: Diagnosis and Therapy will be held May 26 to 31, 1985 at the Jerusalem Hilton Hotel, Jerusalem, Israel. The program will include State-of-the-Art Lectures, panel discussions and oral and poster presentations. The deadline for submission of abstracts is February 15, 1985.

For further information please contact:

Ms. Iris Rosen
Congress Secretariat
P.O.B. 50006
Tel Aviv 61500, Israel

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PHARMACOKINETICS AND DISPOSITION

Gabriella Facciolá · Mats Hidestrand
Christer von Bahr · Gunnel Tybring

Cytochrome P_{450} isoforms involved in melatonin metabolism in human liver microsomes

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Abstract *Objective*: The present study was carried out to identify the cytochrome P_{450} enzyme(s) involved in the 6-hydroxylation and *O*-demethylation of melatonin.
Methods: The formation kinetics of 6-hydroxymelatonin and *N*-acetylserotonin were determined using human liver microsomes and cDNA yeast-expressed human enzymes (CYP1A2, 2C9 and 2C19) over the substrate concentration range 1–1000 μM. Selective inhibitors and substrates of various cytochrome P_{450} enzymes were also employed.
Results: Fluvoxamine was a potent inhibitor of 6-hydroxymelatonin formation, giving $50 \pm 5\%$ and $69 \pm 9\%$ inhibition at concentrations of 1 μM and 10 μM, respectively, after incubation with 50 μM melatonin. Furafylline, sulphaphenazole and omeprazole used at low and high concentrations substantially inhibited both metabolic pathways. cDNA yeast-expressed CYP1A2, CYP2C9 and CYP2C19 catalysed the formation of the two metabolites, confirming the data obtained with specific inhibitors and substrates.
Conclusions: Our results strongly suggest that 6-hydroxylation, the main metabolic pathway of melatonin, is mediated mainly, but not exclusively, by CYP1A2, the high-affinity enzyme involved in melatonin metabolism, confirming the observation that a single oral dose of fluvoxamine increases nocturnal serum melatonin levels in healthy subjects. Furthermore, the results indicate that there is a potential for interaction with drugs metabolised by CYP1A2 both at physiological levels and after oral administration of melatonin, while CYP2C19 and CYP2C9 are assumed to be less important.

Key words Cytochrome P_{450} · Melatonin metabolism · CYP1A2

G. Facciolá · G. Tybring (✉)
Department of Medical Laboratory Sciences and Technology, Division of Clinical Pharmacology, Huddinge University Hospital, Karolinska Institutet, 14186 Stockholm, Sweden
E-mail: gunnel.tybring@labtek.ki.se
Tel.: +46-8-58587888
Fax: +46-8-58581070

M. Hidestrand
National Institute of Environmental Medicine, Division of Molecular Toxicology, Karolinska Institutet, 171 77 Stockholm, Sweden

C. von Bahr
Department of Medicine, Division of Clinical Pharmacology, Stockholm Söder Hospital, 11883 Stockholm, Sweden

Introduction

Melatonin, *N*-acetyl-5-methoxytryptamine, is a hormone produced and released by the pineal gland. The daily light–dark cycle regulates melatonin synthesis and thereby the secretion into the blood flow, and in the vertebrate species investigated, the hormone levels are low during the day and high during the night (Arendt 1988). The main function of melatonin is to co-ordinate circadian rhythms, but increasing evidence suggests that it may have a role in the regulation of several processes such as sleep, mood, reproduction, body temperature, immune response, tumour growth and ageing (Brzezinski 1997).

Both in vitro (Tan et al. 1993a) and in vivo (Tan et al. 1993b) studies have shown that melatonin is a potent scavenger of the highly toxic hydroxyl radical and other oxygen-centred radicals and seems to be more effective than other known antioxidants in protecting against oxidative damage (Reiter 1995). Furthermore, a recent study (Wolfler et al. 1999) has indicated that *N*-acetylserotonin (NAS), which is both a precursor and a metabolite of melatonin, is a better extra- and intracellular antioxidant than melatonin itself. For this reason, as well as for its presumed effects on sleep and circadian rhythms (jet-lag; Petrie et al. 1993), the use of melatonin as a dietary supplement has increased significantly over the past few years, exceeding in the USA the use of vitamin C (Bonn 1996).

Melatonin is rapidly metabolised in man by the hepatic cytochrome P_{450} (CYP) system mainly to 6-hydroxymelatonin (6-OH-melatonin), followed by

conjugation with sulphuric (70%) or glucuronic (30%) acid. Additional metabolites such as 2-hydroxymelatonin, NAS and kynurenamine derivatives have been identified, but they constitute a negligible part compared with 6-OH-melatonin (Arendt 1988). Although NAS has not been shown to be a significant metabolite of endogenous melatonin (Di et al. 1999), Leone and Silman (1984) demonstrated that exogenous melatonin has two principal urinary metabolites, NAS and 6-OH-melatonin, the former being excreted as 5-sulphate and 5-glucuronide (Fig. 1).

In vivo studies have shown that a single dose of fluvoxamine, a selective serotonin reuptake inhibitor (SSRI), increases the nocturnal serum level of melatonin (Skene et al. 1994). This was recently confirmed by Härtter et al. (2000) and von Bahr et al. (2000), who demonstrated that fluvoxamine increases serum melatonin concentrations in vivo. This effect was not seen after intake of paroxetin (Härtter et al. 2000) or citalopram (von Bahr et al. 2000), two other SSRIs. Since these two SSRIs have the same pharmacodynamic action as fluvoxamine but differ in the effect on CYP enzymes, the authors suggested that the fluvoxamine effect on melatonin plasma concentrations is likely due to an inhibition of the CYP1A2 enzyme. Fluvoxamine was early shown to be a potent inhibitor of this enzyme (Brøsen et al. 1993).

Although oral administration of synthetic melatonin appears safe, its bioavailability varies widely (Waldhauser et al. 1984; Lane et al. 1985; Di et al. 1997). The absolute bioavailability after oral intake of 2 mg and 4 mg was approximately 15% compared with 2 mg intravenously administered. No difference in serum half-life was seen, indicating a high degree of hepatic first-pass extraction, which converts melatonin to its metabolites, and not due to the absorption phase (DeMuro et al. 2000). The variation in bioavailability is probably due to the inter-individual differences in the activity of the CYP enzymes involved in its hepatic metabolism. The understanding of the reasons for this variability is important both for endogenous melatonin and for the determination of the appropriate individual dosage of exogenous melatonin.

The aim of the present investigation was to identify the specific human CYP enzymes responsible for melatonin metabolism. For this purpose, we have measured the formation kinetics of both 6-OH-melatonin and NAS in human liver microsomes and in single yeast-expressed enzymes. A study with different selective inhibitors was also performed.

Materials and methods

Drugs and chemicals

Melatonin, 6-OH-melatoninatonin, NAS, sulphaphenazole, caffeine, theophylline and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sigma Chemicals Co. (St Louis, Mo.). Fluvoxamine maleate was obtained from Solvay Duphar (Weesp, Netherlands), quinidine from Apoteksbolaget (Stockholm, Sweden), ketoconazole from Janssen Biotech (Olen, Belgium), omeprazole from Astra-Zeneca R&D (Mölndal, Sweden) and citalopram from Lundbeck (Copenhagen, Denmark). Furafylline was purchased from Research Biomedicals International (RBI, Mass.). All other chemicals were purchased from commercial sources and were of analytical grade quality.

Human liver microsomes

Microsomes were obtained from six human livers (HL 47, 49, 51, 54, 55 and 67) of healthy organ donors, prepared as described previously (von Bahr et al. 1980), and suspended in 50 mM K_2HPO_4 buffer (pH 7.4). The protein content was estimated according to Lowry et al. (1951). The microsomal preparations were stored at −80°C until used for incubation.

Incubation conditions

Different quantities of melatonin in a methanol solution were added to a series of incubation tubes, and the solvent was evaporated to dryness under N_2 atmosphere at room temperature. Microsomes were incubated in 500 µl 50 mM TRIS buffer (pH 7.4) using 0.5 mg microsomal protein at 37°C for 15 min. The reaction was started by adding 100 µl 5 mM NADPH solution in 50 mM TRIS buffer and stopped by the addition of 50 µl acetonitrile. After a rapid cooling, the samples were centrifuged for 5 min at 3500 rpm.

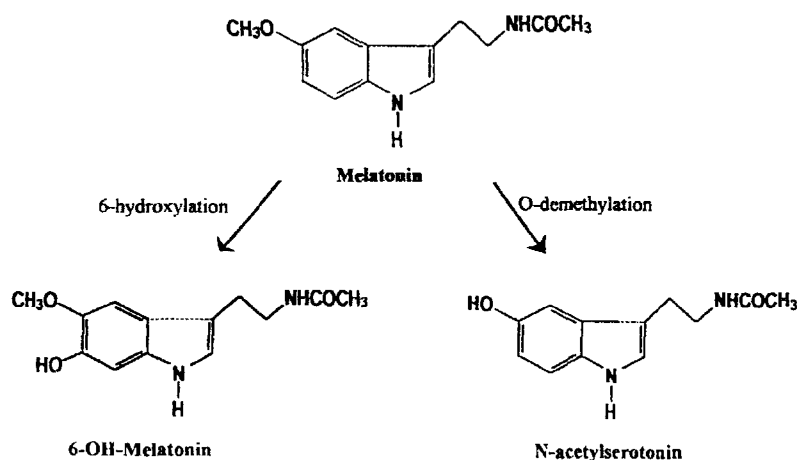

Fig. 1 Structural formula of melatonin and its principal metabolites 6-hydroxymelatonin (from both endogenous and exogenous melatonin) and *N*-acetylserotonin (after melatonin administration)

A fraction of the supernatant was transferred to an auto-sampling vial and was within 1 h injected onto the high-performance liquid chromatography (HPLC) column.

The formation kinetics of 6-OH-melatonin and NAS were determined by incubating melatonin in duplicate at eleven different concentrations (1, 5, 10, 25, 50, 100, 200, 300, 500, 750 and 1000 μM) in microsomes prepared from six individual human livers. All reactions were performed in the linear range with respect to protein concentration and incubation time. The solubility of melatonin in the incubation mixture after evaporation of the methanol stock solution was verified using HPLC analysis. Incubations without microsomes were used to verify that formation of the observed metabolic products required microsomal enzymes. The same experiment was performed without NADPH. The reaction velocities were calculated in units of picomoles of product formed per minute per milligram of microsomal protein.

HPLC analysis

Concentrations of 6-OH-melatonin and NAS were determined using reverse-phase HPLC analysis. The system consisted of a Kontron model 422 isocratic pump (Kontron Instruments), a Gilson automatic injector 231 XL equipped with a 7010 Rheodyne injection valve (Berkeley, Calif.) and a Shimadzu spectrofluorometric detector RF-5 (Shimadzu, Kyoto, Japan) set at 284 nm and 345 nm excitation and emission wavelengths, respectively.

Chromatographic separations were carried out on a Zorbax SB-C18 analytical column (4.6 mm × 7.5 cm, DuPont, Wilmington, Del.) connected with a Zorbax SB-C18 guard column (4.6 × 12.5 mm). The mobile phase consisted of di-sodium hydrogen phosphate (25 mM, pH 3.5) and acetonitrile 85:15 (v/v), with a flow rate of 1.0 ml/min. The retention times of NAS, 6-OH-melatonin and melatonin were 2.7, 3.7 and 12.3 min, respectively. Integration of peak areas was performed using the laboratory data system ELDS 900 (Chromatography Data System, Kungshög, Sweden).

Standard solutions of melatonin, 6-OH-melatonin and NAS were prepared in methanol and stored at –20°C in N_2 atmosphere for 1 month. A standard curve was prepared in water over the concentration range of 0.1–20 μM and 0.002–1 μM for 6-OH-melatonin and NAS, respectively, and injected prior to each analysis. Regression analysis of the peak areas versus the standard concentrations was used for the calculations.

The intra-day coefficients of variation (CVs, $n=6$) for 6-OH-melatonin and NAS at low (0.5 μM, 0.01 μM) and high (10 μM, 0.5 μM) concentrations were less than 3% for both metabolites. The inter-day CVs ($n=6$) at the same low and high concentrations were 14% and 7% for 6-OH-melatonin and 6% and 8% for NAS. The limits of quantitation (LOQ) were 100 nM and 5 nM for 6-OH-melatonin and NAS, respectively.

Inhibition study

Nine different selective inhibitors or substrates were used in order to define the role of individual CYP enzymes in melatonin metabolism. The experiments were performed in four different microsomal preparations obtained from livers HL47, 54, 55 and 64 at a substrate concentration of 50 μM.

Fluvoxamine (1, 10 μM) and furafylline (2, 20 μM) were used as CYP1A2 inhibitors, ketoconazole (0.5, 5 μM) for CYP3A4, sulphaphenazole (2, 20 μM) for CYP2C9, quinidine (0.5, 5 μM) for CYP2D6 and omeprazole (10, 100 μM) for CYP2C19 (Newton et al. 1995; Bourrié et al. 1996). Furthermore, citalopram (1, 10 μM), a substrate of CYP2C19, and caffeine and theophylline (100, 200 μM), substrates of CYP1A2 (Tjia et al. 1996; Kobayashi et al. 1997; Rasmussen et al. 1998), were also used in order to study their potential effect on melatonin metabolism.

Fluvoxamine, furafylline, citalopram, ketoconazole, omeprazole, sulphaphenazole and theophylline were dissolved in methanol. The methanol solution was evaporated to dryness under N_2 atmosphere before each incubation. Caffeine and quinidine were dissolved in water.

Furafylline, a selective mechanism-based inhibitor of CYP1A2 that requires NADPH-mediated complexes for inactivation was pre-incubated for 15 min with microsomal protein and NADPH before addition of melatonin. All the other inhibitors or substrates were co-incubated with melatonin for 15 min (Newton et al. 1995). The inhibitors were also incubated without substrate under the same conditions to ensure that their presence during the incubation would not interfere with the quantification of melatonin metabolites. Incubation with omeprazole and caffeine resulted in peaks interfering with the HPLC analysis of 6-OH-melatonin and NAS, respectively.

Assay with yeast-expressed human P450s

The expression of human recombinant CYP1A2, 2C9 and 2C19 was carried out in the *Saccharomyces cerevisiae* strain W (R), which has been genetically modified to overexpress the yeast reductase by integrating a galactose-inducible promotor in front of the yeast reductase gene (Krynetski et al. 1995). The expression vector used was pYeDP60 (V60), a galactose-inducible yeast expression vector carrying the 2μ origin of replication and the URA3 and ADE2 selection markers. In order to achieve spectrally quantifiable amounts of the CYP2C19 enzyme, a triplet of adenine bases were inserted just prior to the initiating ATG codon of the cDNA (Truan et al. 1993). No such modification was necessary for the expression of CYP1A2 or CYP2C9 enzymes. After transformation of the V60 plasmid into the W (R) yeast strain, selection of clones was achieved by growth in adenine- and uracil-deficient media. The yeast was subsequently grown to high density before expression was initiated by adding galactose. The cells were harvested and mechanically disrupted with glass beads, and microsomes containing the recombinant enzymes were collected after several differential centrifugations. A more detailed description of the expression and preparation of microsomes can be found elsewhere (Oscarson et al. 1997).

Incubations with microsomes from yeast-expressed CYP1A2, 2C9 and 2C19 were carried out as described for human liver microsomes. The formation kinetics of 6-OH-melatonin and NAS were determined by incubating melatonin at eight different concentrations (1, 5, 10, 50, 100, 200, 500 and 1000 μM) with each enzyme. Microsomes from yeast-expressed CYP1A2 (12.5 pmol) were incubated for 15 min, while microsomes from yeast-expressed CYP2C9 and CYP2C19 (10 pmol) were incubated for 5 min. The reactions were linear with regard to time and added amount of enzyme within the ranges used in the study. Enzymatic activity was defined as picomoles of product formed per minute per picomole of CYP enzyme.

Data analysis

The velocities of formation of 6-OH-melatonin and NAS were investigated with microsomes from all six livers and with the three yeast-expressed enzymes. The mean of duplicate data points representing metabolites formation velocity (v) as a function of the substrate concentration (C) were fitted, without weighting and using an iterative curve-fitting program (Sigma plot.) based on non-linear regression analysis, to the following equations:

- One-enzyme model, Michaelis–Menten (MM):

$$V = V_{max} \times C/(K_M + C) \tag{1}$$

- Two-enzymes model, both MM:

$$V = V_{max(1)} \times C/(K_{M(1)} + C) + V_{max(2)} \times C/(K_{M(2)} + C) \tag{2}$$

where V_{max} is the apparent maximal velocity and K_M the apparent Michaelis constant, the substrate concentration at which the reaction velocity is equal to 50% of V_{max}. The kinetic curves obtained with yeast-expressed microsomes were fitted only with the one MM equation.

K_M and V_{max} values were obtained initially using graphical analysis of Eadie–Hofstee plots (v versus v/[S]). The results were used as first estimates for iterative nonlinear regression analysis. Goodness of fit to a particular model was determined using evaluation of criteria that included visual inspection of Eadie–Hofstee and Lineweaver–Burk plots of the data, the random distribution of residuals, the sum of squares of the residual (F-test) and the standard error (SE) of parameter estimates (Schmider et al. 1996).

Results

Kinetic analysis

Incubation with melatonin in six human liver microsome preparations resulted in the formation of 6-OH-melatonin and NAS. Eadie–Hofstee plots, for 6-hydroxylation and *O*-demethylation reactions, over the concentration range of 1–1000 μM, were curvilinear in all the livers tested (Fig. 2). This shows that the formation of the two metabolites could be mediated by at least two CYP isoforms. Fitting the data points, related to formation of the two metabolites in human liver microsomes, with the two MM equation, it was not possible to get an accurate estimation of the low K_M and V_{max} values. This is due to the difficulty of measuring the amount of metabolites produced at low concentrations nearing the low K_M value of the high affinity site with the assay used (Kato et al. 1994). The kinetic parameters shown in Table 1 are for this reason only calculated with Eq. 1. The V_{max} and K_M values obtained for the two metabolites formation clearly show that 6-OH-melatonin is the main metabolite formed from melatonin.

Table 1 Apparent Michaelis–Menten kinetic parameters of melatonin 6-hydroxylation and *O*-demethylation in human liver microsomes. V_{max} apparent maximal velocity; K_M apparent Michaelis constant

	V_{max} (pmol min^{-1} mg^{-1} enzyme)	K_M (μM)
6-OH-melatonin		
HL 47	1480	216
HL 49	2151	385
HL 51	2207	114
HL 54	3828	237
HL 55	1348	164
HL 67	2462	179
N-acetylserotonin		
HL 47	67	172
HL 49	126	244
HL 51	102	138
HL 54	125	227
HL 55	83	208
HL 67	92	154

Inhibition study

The effects of various inhibitors/substrates on the formation of 6-OH-melatonin and NAS at a substrate concentration of 50 μM are shown in Fig. 3. Fluvoxamine, a potent inhibitor of CYP1A2, with a moderate inhibitory effect also on CYP2C9 and CYP2C19 activity, showed a strong inhibition on the formation of both metabolites in the four livers tested. Furafylline (CYP1A2 inhibitor) strongly inhibited the 6-hydroxylation, while a lower degree of inhibition was observed for the *O*-demethylation reaction. Inhibitors selective for

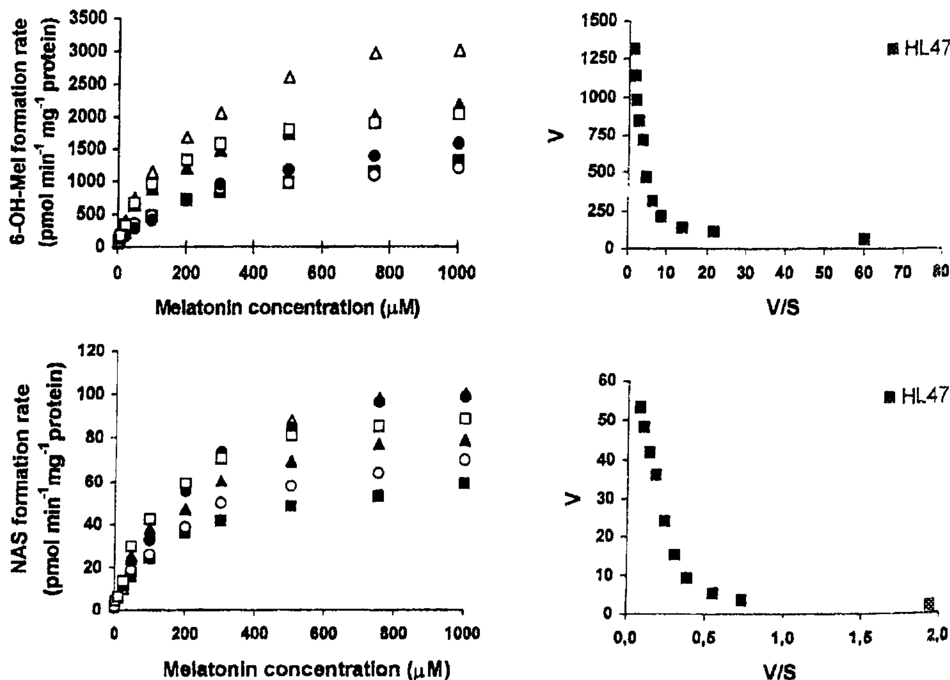

Fig. 2 *Left panels* show the formation kinetics of 6-OH-melatoninatonin and *N*-acetylserotonin in microsomes from six human livers. Eadie–Hofstee plots in the *right panel* show the corresponding melatonin 6-hydroxylation and *O*-demethylation in one representative liver (HL 47). Each *point* represents the mean of duplicates

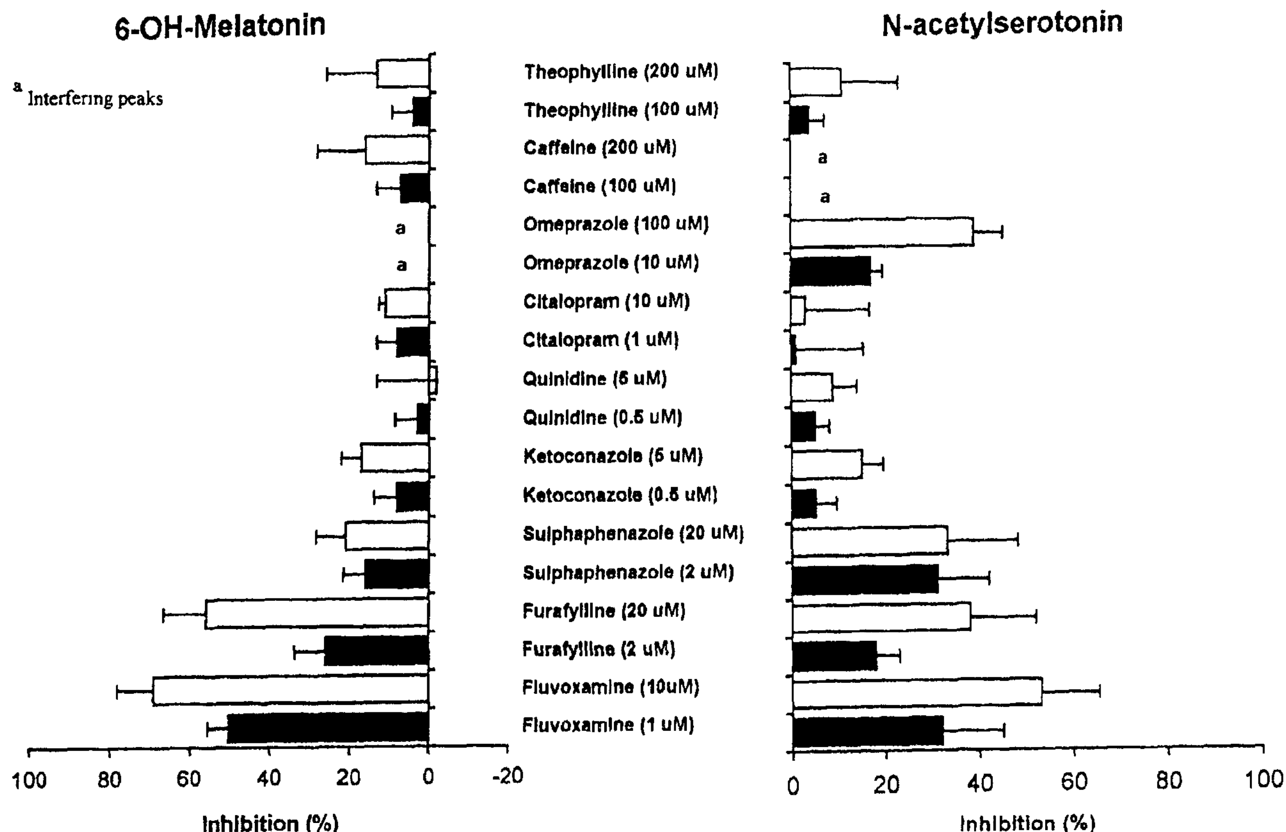

Fig. 3 Effect of different cytochrome P_{450} (CYP) enzymes inhibitors or substrates on melatonin 6-hydroxylation and *N*-demethylation in human liver microsomes at a substrate concentration of 50 μM. Data are expressed as percentage of inhibition (mean ± SD, $n=4$)

CYP2D6 (quinidine) and CYP3A4 (ketoconazole) had essentially no effect on either of the two pathways studied. Citalopram did not affect any of the two metabolic pathways, and sulphaphenazole 20 μM (CYP2C9 inhibitor) inhibited 6-hydroxylation and *O*-demethylation by 21% and 29%, respectively. Although omeprazole is not a strong and selective inhibitor of CYP2C19, it affected NAS formation (17% at 10 μM and 39% at 100 μM). A negligible inhibitory effect was obtained with both caffeine and theophylline, probably due to the concentrations used.

Yeast-expressed P_{450} isoforms study

The kinetic curves of melatonin metabolite formation obtained with each expressed enzyme (Fig. 4) indicate that CYP1A2 and CYP2C19 are the main enzymes involved in the 6-hydroxylation and *O*-demethylation reactions, respectively. At melatonin concentrations lower than 50 μM, CYP2C9 and CYP2C19-mediated formation of 6-OH-melatonin was not quantifiable, confirming that CYP1A2 represents the high affinity site for 6-OH-melatonin formation. The 6-hydroxylation capacity of CYP2C19 was not significant at any melatonin concentration.

The kinetic results for the formation of melatonin metabolites after incubations with CYP1A2 and CYP2C19 yeast-expressed microsomes are listed in Table 2. All the three enzymes selected for the study were able to 6-hydroxylate and *O*-demethylate melatonin, but the K_M and V_{max} values for CYP2C9- and CYP2C19-mediated formation of 6-OH-melatonin and CYP2C9-mediated formation of NAS were undefined because, as previously stated, it was impossible to quantify the metabolites for substrate concentrations lower than 50 μM. Thus, with only five velocity values, we could not estimate the kinetic parameters.

Discussion

The in vivo metabolism of the pineal hormone melatonin consists mainly of 6-hydroxylation, followed by sulphate or glucuronide conjugation (Arendt 1988). It is not clear whether NAS is formed from endogenous melatonin (Di et al. 1999), but it was shown that exogenous melatonin has two principal urinary metabolites, NAS and 6-OH-melatonin (Leone and Silman 1984). Our in vitro study showed that the biotransformation of melatonin by human liver microsomes yielded two metabolites formed by 6-hydroxylation and *O*-demethylation reactions.

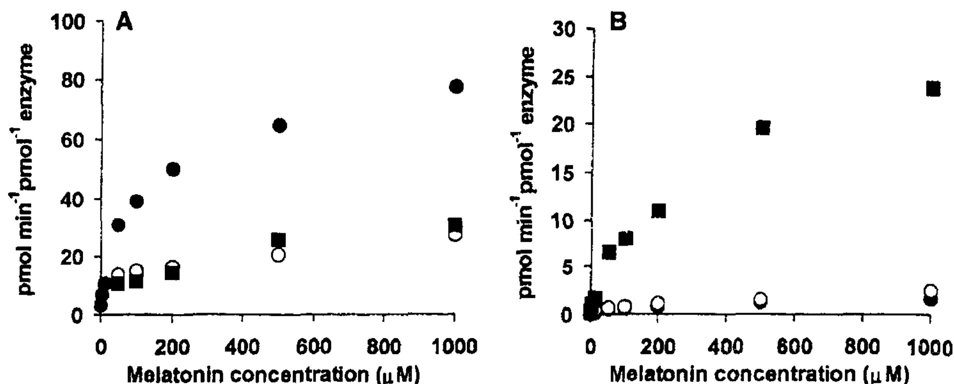

Fig. 4 Rates of formation of 6-OH-melatonin (A) and *N*-acetylserotonin (NAS; B) in yeast-expressed CYP1A2(●), CYP2C9 (○) and CYP2C19 (■)

The substrate concentrations required (1–1000 μM) to produce accurately detectable levels of metabolic products are considerably higher than those that would be observed in vivo for endogenous melatonin plasma levels in normal young adults (40 pM and 260 pM, daytime and peak night-time, respectively; Brzezinski 1997). In an in vivo study on bioavailability of oral melatonin in humans, Di et al. (1997) reported a wide variation between subjects with melatonin plasma concentrations in the range of 2–40 nM after a low oral dose of 0.5 mg. Furthermore, a low bioavailability of approximately 15% after oral administration of 2 mg and 4 mg melatonin is reported (DeMuro et al. 2000). The sleep-promoting effects of melatonin have been tested using a broad range of doses (0.1–2000 mg; Sack et al. 1997). In another study, Waldhauser et al. (1984) reported that an oral dose of 80 mg produces a range of melatonin concentrations between 0.2 μM and 5 μM (25-fold variation among subjects).

Our kinetic analysis after incubations with human liver microsomes indicates that there could be more than one enzyme involved in the formation of melatonin metabolites at the used substrate concentrations range. Obviously only the high-affinity site will be of any physiological relevance, and in vivo results from other investigators suggest that it is most likely an enzyme of CYP inhibited by fluvoxamine.

Results from the inhibition study show that the SSRI fluvoxamine is a potent inhibitor of melatonin biotransformation to its principal metabolite 6-OH-melatonin in human liver microsomes, confirming the previous findings in healthy subjects in vivo (Skene et al. 1994; Härtter et al. 2000; Von Bahr et al. 2000). It also inhibits in vitro, but to a minor extent, the formation of NAS. Although fluvoxamine has been proposed as a relatively specific index inhibitor of CYP1A2 (Brøsen et al. 1993), it is also a moderately strong inhibitor of CYP3A isoforms (Perucca et al. 1994; von Moltke et al. 1995) as well as 2C9 (Schmider et al. 1997) and 2C19 (Xu et al. 1996; Jeppesen et al. 1997). The higher degree of inhibition of 6-hydroxylation and *O*-demethylation reactions obtained with fluvoxamine in comparison with furafylline could therefore be explained by the additional inhibitory actions on cytochromes other than CYP1A2. However, incubations with yeast-expressed enzymes confirmed the involvement of CYP2C9 and CYP2C19 in both reactions. Although a strong and selective inhibitor of CYP2C19 is not available, the CYP2C19 substrate and inhibitor omeprazole (100 μM; Andersson et al. 1990; Funck-Brentano et al. 1997) inhibited NAS formation by 39% (Table 2). Unfortunately, it was not possible to evaluate omeprazole inhibition of 6-hydroxylation reaction due to the presence of an interfering peak after melatonin and omeprazole co-incubation.

The potent CYP2C9 inhibitor sulphaphenazole was a moderate inhibitor of the formation of both 6-OH-melatonin and NAS, although the effect on *O*-demethylation activity was higher. The involvement of CYP2C9 was also confirmed by using the yeast-expressed enzyme.

Quinidine was not an inhibitor of either 6-hydroxylation or *O*-demethylation reactions. Also ketoconazole had minimal effects. This suggests that CYP2D6 and CYP3A4 are not important enzymes in melatonin metabolism. Furthermore, the absence of inhibition obtained with citalopram suggests that the observed in vivo effect of fluvoxamine on endogenous melatonin plasma concentrations is probably due to a metabolic interaction and not due to serotonin uptake inhibition (Härtter et al. 2000; von Bahr et al. 2000).

Table 2 Apparent Michaelis–Menten kinetic parameters of melatonin 6-hydroxylation and *O*-demethylation in yeast-expressed enzymes. V_{max} apparent maximal velocity; K_M apparent Michaelis constant

Parameter	Metabolite	CYP1A2	CYP2C19
V_{max} (pmol min^{-1} mg^{-1} enzyme)	6-Hydroxymelatonin	84.2 ± 4.3	–[a]
	N-acetylserotonin	1.5 ± 0.1	10.1 ± 0.8
K_M (μM)	6-Hydroxymelatonin	85.5 ± 15.6	-[a]
	N-acetylserotonin	143.9 ± 34.0	282.2 ± 54.9

[a] Not calculated metabolite was formed

In view of our results, the finding that melatonin bioavailability varies widely between subjects can be explained by the involvement of CYP1A2, and to a lesser extent, of CYP2C9 and CYP2C19 in its metabolism. These enzymes are in fact characterised by genetic polymorphism (CYP2C9 and CYP2C19, Wilkinson et al. 1992) and show a large inter-individual variability in activity (CYP1A2; Kalow et al. 1991; CYP2C9, Miners and Birkett 1988). Our results strongly suggest that 6-hydroxylation, the main metabolic pathway of melatonin, is mediated mainly, but not exclusively, by CYP1A2, the high-affinity enzyme involved in melatonin metabolism. They also indicate that there is a potential for interaction with drugs metabolised by CYP1A2, the main enzyme responsible for melatonin metabolism both at physiological and pharmacological plasma concentrations, in patients taking melatonin. Moreover, if the hormone is administered at high doses an additional potential for interactions with drugs metabolised by CYP2C9 and CYP2C19 could not be excluded. Furthermore, we have confirmed that fluvoxamine, unlike citalopram, is a potent inhibitor of melatonin metabolism in vitro and that this mechanism could be the source of the observed augmentation of endogenous melatonin plasma concentrations in subjects taking fluvoxamine.

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Characterization of the Protective Effects of Melatonin and Related Indoles Against α-Naphthylisothiocyanate-Induced Liver Injury in Rats

J.R. Calvo,[1,2] R.J. Reiter,[1*] J.J. García,[1,3] G.G. Ortiz,[1,4] D.X. Tan,[1] and M. Karbownik[1,5]

[1]Department of Cellular and Structural Biology, University of Texas Health Science Center, San Antonio, Texas 78229
[2]Department of Medical Biochemistry and Molecular Biology, University of Sevilla, Sevilla 41009, Spain
[3]Department of Pharmacology and Physiology, University of Zaragoza, Zaragoza 50009, Spain
[4]CIBO-IMSS, Guadalajara, Mexico
[5]Department of Thyroidology, Institute of Endocrinology, University of Lodz, Lodz 91-425, Poland

Abstract The protective effect of melatonin, 6-hydroxymelatonin and *N*-acetylserotonin against α-naphthylisothiocyanate (ANIT)-induced liver injury was investigated and compared in rats injected once with the hepatotoxicant (75 mg/kg body weight). In rats injected with ANIT alone, liver injury with cholestasis developed within 24 h, as indicated by both serum levels of alanine aminotransferase (SGPT) and aspartic acid aminotransferase (SGOT) activities and serum total bilirubin concentration. The administration of melatonin or 6-hydroxymelatonin (10 mg/kg body weight) to ANIT-injected rats reduced significantly the serum levels of both SGPT and SGOT and the serum total bilirubin concentration. For all hepatic biochemical markers, melatonin was more effective that 6-hydroxymelatonin. By comparison, the administration of *N*-acetylserotonin (10 mg/kg body weight) to ANIT-injected rats did not reduce the serum levels of either hepatic enzymes or the serum total bilirubin concentration. In ANIT-injected rats, hepatic lipid peroxidation (LPO) was significantly higher than in control animals and this increase was significantly reduced by either melatonin, 6-hydroxymelatonin or *N*-acetylserotonin. Furthermore, ANIT treatment caused a significant reduction in liver microsomal membrane fluidity and this reduction was completely reversed by the three indoles. The liver from ANIT-injected rats showed several histopathological alterations; above all there was an acute infiltration of polymorphonuclear neutrophils and an increase in the number of apparent apoptotic hepatocytes. The concurrent administration of melatonin reduced the severity of all morphological alterations, specially the neutrophil infiltration and the number of presumed apoptotic cells. On the contrary, the administration of 6-hydroxymelatonin or *N*-acetylserotonin did not provide any protective effect in terms of the histopathological alterations. These results indicate that melatonin protects against ANIT-induced liver injury with cholestasis in rats, and suggests that this protective effect is likely due to its antioxidant properties and above all to its capacity to inhibit liver neutrophil infiltration, a critical factor in the pathogenesis of ANIT-induced liver injury. 6-hydroxymelatonin, although able to provide partial protection against the ANIT-induced hepatic injury, probably through its antioxidant properties by mechanisms that are unclear, was unable to reduce neutrophil infiltration. Finally, *N*-acetylserotonin in the experimental conditions of this study, only exhibited some antioxidant protection but had no protective effect against ANIT-induced hepatic damage. J. Cell. Biochem. 80:461–470, 2001. © 2001 Wiley-Liss, Inc.

Key words: melatonin; indoles; α-naphthylisothiocyanate; liver injury; lipid peroxidation; membrane fluidity

α-naphthylisothiocyanate (ANIT) is a well-known toxic substance that produces a cholangiolitic hepatitis characterized by intrahepatic cholestasis, hepatocellular and biliary epithelial cell necrosis, and bile duct obstruction [Plaa and Priestly, 1977; Roth and Dahm, 1997]. An inflammatory response in the periportal regions of liver lobules leads to edema and a pronounced infiltration of polymorphonuclear neutrophils into hepatic tissue occurs before the onset of overt liver damage in rats intoxi-

*Correspondence to: Russel J. Reiter, Department of Cellular and Structural Biology, Mail code 7762, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900.
E-mail: reiter@uthscsa.edu

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cated once with ANIT [Goldfarb et al., 1962]. ANIT has attracted attention because it produces, in certain animal species, hepatic lesions that resemble those occurring in biliary cirrhosis in humans [Plaa and Priestly, 1977]. In addition, cholestasis and hepatic lesions resulting from the administration of certain drugs (e.g., erythromycin estolate, chlorpromazine, and others) to humans are mimicked by ANIT administration to rats, so animal models of ANIT hepatotoxicity may by useful for studying the mechanisms of drug-induced cholestasis [Plaa and Priestly, 1977].

The mechanisms of ANIT-induced liver injury have been proposed but they have not been proven [Roth and Dahm, 1997]. In this context, Calcamuggi et al. [1992] suggested that endotoxemia may play a pathogenic role in ANIT-induced liver injury in rats and other studies demonstrated that glutathione plays an important role in ANIT hepatotoxicity [Jean and Roth, 1995]. On the other hand, it has been clearly demonstrated that the pathogenesis of ANIT hepatotoxicity is neutrophil-dependent. Thus, Dahm et al. [1991] reported that neutrophil depletion protects against liver injury in rats treated once with ANIT, while administration of oxygen radical scavengers, such as superoxide dismutase and catalase, do not protect against this hepatic injury. Thus, these authors suggested that the contribution of neutrophils to the development of ANIT-induced liver injury involves a mechanism independent of the production of oxygen radicals. Ohta et al. [1997] reported, however, that in rats intoxicated once with ANIT, serum lipid peroxide (LPO) level increases with the formation and progression of liver injury and that two different Chinese herbal products, which work as oxygen radical scavengers and have anti-inflammatory actions, prevent the progression of ANIT-induced hepatic injury and inhibit the rise in serum lipid peroxidation products. In addition, recently Kongo et al. [1999] suggested that lipid peroxidation might be associated with ANIT-induced liver injury in rats and that the damage to lipids probably occurs via oxygen radicals derived from neutrophils which infiltrate the liver tissue of ANIT-intoxicated rats.

Melatonin, the main pineal secretory product, is known to function as an efficient antioxidant molecule in vitro and in vivo [Reiter, 1996]. Thus, it has been shown that in animals and tissues exposed to agents which induce lipid peroxidation, melatonin provides substantial protection against this oxidative destruction [Reiter, 1996; Longoni et al., 1998]. Additionally, melatonin exerts a protective effect against acute hepatic injuries induced by endotoxic shock [Sewerynek et al., 1995] and ischemia-reperfusion [Sewerynek et al., 1996] in rats through its antioxidant and its inhibitory effects on neutrophil infiltration into the liver tissue. These findings suggest that melatonin may protect against ANIT-induced liver injury with cholestasis in rats because of its antioxidant action and/or its inhibitory effect on neutrophil infiltration. Similar suggestions have been made recently by Ohta et al. [2000a].

In addition to melatonin, other related indoles such as 6-hydroxymelatonin and *N*-acetylserotonin have antioxidant properties in in vitro studies [Chan and Tang, 1996; Matuszak et al., 1997; Wölfler et al., 1999]. However, few studies have investigated and compared the antioxidant properties of different indoles under in vivo conditions [Hara et al., 1997]. In the current study, we investigate and compare the protective effect of melatonin, 6-hydroxymelatonin and *N*-acetylserotonin against ANIT-induced liver injury in rats.

MATERIALS AND METHODS

Chemicals

ANIT, melatonin, 6-hydroxymelatonin, *N*-acetylserotonin, and bovine serum albumin were purchased from Sigma (St. Louis, MO) and 1-(4-trimethyl-ammoniumphenyl)-6-phenyl-1,3,5-hexatriene-p-toluene-sulfonate (TMA-DPH) was purchased from Molecular Probes (Eugene, OR). The Bioxytech LPO-586 kit, purchased from Cayman Chemical (Ann Harbor, MI), was used for measuring the lipid peroxidation products, malonaldehyde (MDA) and 4-hydroxyalkenals (4-HDA). Apoptosis was morphologically detected in situ by terminal deoxynucleotidyl transferase assay (Apoptag Kit, Oncor, Inc. USA). Total serum bilirubin, serum alanine aminotransferase (SGPT) and serum aspartic acid aminotransferase (SGOT) were assayed using commercial test kits from Bayer Diagnostic. All other chemicals used were of analytical grade and were purchased from commercial sources.

Animals

All animal procedures were in strict accordance with the NIH Guide for the Care and Use of Laboratory animals. Male Sprague-Dawley rats, weighing 200–250 g, were purchased from Harlan (Houston, TX) and housed three per plexiglass cage. The animal rooms were windowless with automatic temperature control ($22 \pm 2°C$) and lighting (light on at 0700 hours and off at 2100 hours; 14 h light and 10 h dark). Animals received standard laboratory chow and water ad libitum.

Experimental Procedures

After 1 week of acclimation, the animals were divided in eight groups as follows: group I (control), group II (melatonin), group III (6-hydroxymelatonin), group IV (*N*-acetylserotonin), group V (ANIT), group VI (ANIT+melatonin), group VII (ANIT+6-hydroxymelatonin), and group VIII (ANIT+*N*-acetylserotonin). Prior to the injections, the rats were fasted for 12 h, but had free access to water. For the experiment, groups V–VIII were intraperitoneally injected with ANIT dissolved in olive oil at a dose of 75 mg/kg body weight [Ohta et al., 2000a; Kongo et al., 1999]. The groups I–IV received an intraperitoneal injection of the same volume of olive oil. Thirty minutes before the ANIT injections and at 6, 12, and 18 h after toxin injection, melatonin (groups II and VI), 6-hydroxymelatonin (groups III and VII) or *N*-acetylserotonin (groups IV and VIII) was intraperitoneally administered at a dose of 10 mg/kg body weight. The three indoles were dissolved in ethanol and thereafter diluted in saline (the final concentration of ethanol was 1%). Groups I (control) and V (ANIT) received the dose intraperitoneally and at the same time the same volume of ethanol/saline solution. This scheme of indole administration has been previously used and found effective in reducing oxidative damage [Sewerynek et al., 1995; Carneiro and Reiter, 1998]. Beginning at the time of ANIT administration and continuing to the end of the study all animals had free access to food and water. The animals were sacrificed by decapitation. After decapitation, blood and liver samples were quickly collected. The collected blood was centrifuged to obtained serum. Serum and liver samples were frozen at $-80°C$ until use.

Assay of Serum Enzymes and Total Bilirubin

Serum alanine aminotransferase (SGPT) and the aspartic acid aminotransferase (SGOT) were measured automatically (COBAS INTEGRA, Roche) using a commercially prepared kit (Roche). These enzyme activities are expressed as international units/litre (IU/L). Total serum bilirubin also was measured automatically (COBAS INTEGRA, Roche) using a kit from Roche. The serum SGPT and SGOT were used as indices of hepatic cell damage and the total bilirubin as an index of cholestasis.

Assay of Hepatic LPO and Protein

To analyze for lipid peroxidation, a portion of each liver was homogenized in ice-cold 20 mM Tris buffer, pH 7.4, with a Polytron-like stirrer to produce a 1/10 homogenate. Homogenates were centrifuged at 3,000 rpm for 30 min at 4°C and the supernatant was collected and immediately tested for products of lipid peroxidation. Lipid peroxidation products [malonaldehyde (MDA) and 4-hydroxyalkenals (4-HDA)] were estimated in the supernatants by a colorimetric assay using the Bioxytech LPO-586 kit. In this technique, MDA and 4-HDA react with a chromogenic reagent at 45°C yielding a stable chromophore with maximal absorbance at 586 nm wavelength and provide a convenient index of lipid peroxidation [Esterbauer and Cheeseman, 1990]. The light wavelength and the low temperature of incubation used for the measurements eliminate interference and undesirable artefacts. Results are expressed as nmol MDA+4-HDA mg^{-1} microsomal protein. Liver protein concentrations were measured by the Bradford [1976] method using bovine serum albumin as standard.

Microsome Isolation

The microsomal fraction was isolated as described previously [García et al., 1997]. Briefly, the liver was homogenized 1/10 w/v in 140 mM KCl, 20 mM HEPES buffer, pH 7.4. The suspension was centrifuged at $1{,}000 \times g$ for 10 min and the resulting supernatant was centrifuged at $105{,}000 \times g$ for 60 min. The pellet obtained was re-suspended in the same buffer and centrifuged at $10{,}000 \times g$ for 15 min. Then, the supernatant was re-centrifuged at $105{,}000 \times g$ for 60 min and the final pellet was re-suspended 1/1 v/v in the same buffer and stored at $-80°C$ until assay. After isolation,

microsomal membrane fluidity and MDA+4-HDA concentrations were determined.

Membrane Fluidity

Membrane fluidity was measured using the TMA-DPH as probe as described by García et al. [1997]. Briefly, a suspension of 0.5 mg microsomal protein in 50 mM Tris–HCl buffer, pH 7.4 (3 ml final volume) was vigorously mixed on a vortex with TMA-DPH (66.7 nM) for 1 min and incubated with shaking for 30 min at 37°C to ensure the uniform distribution of the fluorescent probe in the microsomes. Polarization parameters (average of 30 observations for each determination) were carried out in a Perkin-Elmer LS-50 luminiscence spectrometer equipped with a circulatory water bath to maintain the temperature of 22 ± 0.1°C. TMA-DPH was excited at 360 nm and its emission recorded at 430 nm. The degree of polarization (P) was calculated using following equation:

$$P = \frac{I_{V_V} - GI_{V_H}}{I_{V_V} + GI_{V_H}}$$

I_{V_V} and I_{V_H} are the emission intensity of vertically polarized light detected by an analyzer oriented parallel or perpendicular, respectively, to the excitation plane and G is a correction factor for the optical system. Results of membrane fluidity were expressed as the inverse of P [García et al. 1997].

Morphological Analysis

For the histological studies, a small portion of six livers was obtained from rats of each group. These were fixed in 4% paraformaldehyde buffered with phosphate solution (0.1 M, pH 7.4) at room temperature. Liver fragments were washed in phosphate buffer and dehydrated in graded concentrations of ethanol (70, 80, 90 and 100%); the fragments were embedded in Paraplas-Plus. From each liver, 4 µm thick sections were obtained and stained with hematoxylin-eosin to evaluate hepatic morphology. Polymorphonuclear neutrophils were counted in 10 randomly selected high-power fields (×650) using an image analysis system (analySIS 2.0).

Morphological Apoptosis Analysis

Apoptosis is often preceded by internucleosomal DNA fragmentation. Apoptotic cells were morphologically detected in situ by terminal deoxynucleotidyl transferase assay and counted under a light microscope at a magnification of 250 ×; in each field the number of apoptotic cells was assessed. A total of 50 periportal areas per rat liver were randomly examined using an image analysis system (analySIS 2.0) in six rats each group.

Statistical Analysis

Data were analyzed using one-way analysis of variance (ANOVA). If the values were significant, the Bonferroni test was used to compare the treated and controls groups. The level of significance was accepted at $P < 0.05$.

RESULTS

Serum Levels of Hepatic Enzymes and Total Bilirrubin

Serum SGPT and SGOT activities were determined as indices of hepatic cell damage. In rats injected with ANIT the serum levels of both SGPT and SGOT activities were significantly higher than those determined in the control group (Fig. 1A,B). The administration of melatonin and 6-hydroxymelatonin to ANIT-injected rats reduced significantly the serum levels of SGPT and SGOT activities (Fig. 1A,B). However, in both cases melatonin was more efficient that 6-hydroxymelatonin in reducing the effect of ANIT. On the other hand, the administration of *N*-acetylserotonin did not lower serum activity levels of either SGPT or SGOT (Fig. 1A,B). The injection of melatonin, 6-hydroxymelatonin and *N*-acetylserotonin alone did not affect the activities of these hepatic enzymes in the serum (Fig. 1A,B).

Total bilirubin concentration, which is an index of cholestasis, in the ANIT-injected rats was significantly higher than values obtained in control rats (Fig. 1C). When rats were treated with melatonin, 6-hydroxymelatonin or *N*-acetylserotonin, respectively, melatonin and 6-hydroxymelatonin, but not the *N*-acetylserotonin, reduced significantly total serum bilirubin (Fig. 1C). Melatonin was again more efficient that 6-hydroxymelatonin in reducing the high levels of total bilirubin obtained in ANIT-injected rats. Treatment of rats with melatonin, 6-hydroxymelatonin or *N*-acetylserotonin alone did not affect the total bilirubin concentrations compared to that of control rats (Fig. 1C).

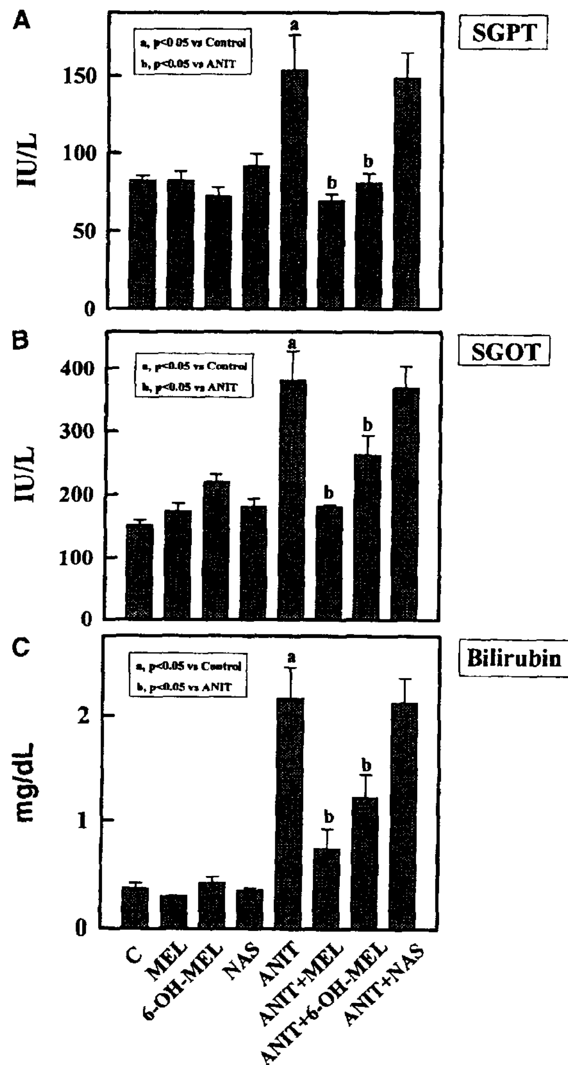

Fig. 1. Effect of intraperitoneal injections of melatonin, 6-hydroxymelatonin or *N*-acetylserotonin (10 mg/kg body weight) on activities of SGPT (**A**), SGOT (**B**), and total bilirubin (**C**) in sera of rats given a single intraperitoneal injection of ANIT (75 mg/kg body weight). Serum SGPT, SGOT, and total bilirubin were assayed in control rats (C), rats injected with melatonin (MEL), 6-hydroxymelatonin (6-OH-MEL), *N*-acetylserotonin (NAS), ANIT (ANIT), ANIT plus melatonin (ANIT+MEL), ANIT plus 6-hydroxymelatonin (ANIT+6-OH-MEL), and ANIT plus *N*-acetylserotonin (ANIT+NAS) as described in Material and Methods. Each value represents the mean ± SEM.

Lipid Peroxidation and Membrane Fluidity

Hepatic lipid peroxidation in the ANIT-injected rats was significantly higher than that in the control group (Fig. 2). When rats were treated with melatonin, 6-hydroxymelatonin or *N*-acetylserotonin, the increased MDA+4-HDA levels induced by ANIT were significantly reduced (Fig. 2). The administration of melatonin, 6-hydroxymelatonin or *N*-acetylserotonin alone did not influence the level of liver lipid peroxidation products (Fig. 2).

As is shown in Figure 3, ANIT caused a significant reduction in microsome membrane fluidity, quantitated as changes in 1/P, with respect to the values of the control group. This reduction in microsome membrane fluidity was completely reversed by melatonin, 6-hydroxymelatonin or *N*-acetylserotonin (Fig. 3). Melatonin, 6-hydroxymelatonin or *N*-acetylserotonin alone did not influence membrane fluidity (Fig. 3).

Morphological Studies

The livers of the control group and the rats injected only with melatonin, 6-hydroxymelatonin or *N*-acetylserotonin exhibited the normal structure (data not shown). Microscopic examination of liver after ANIT administration

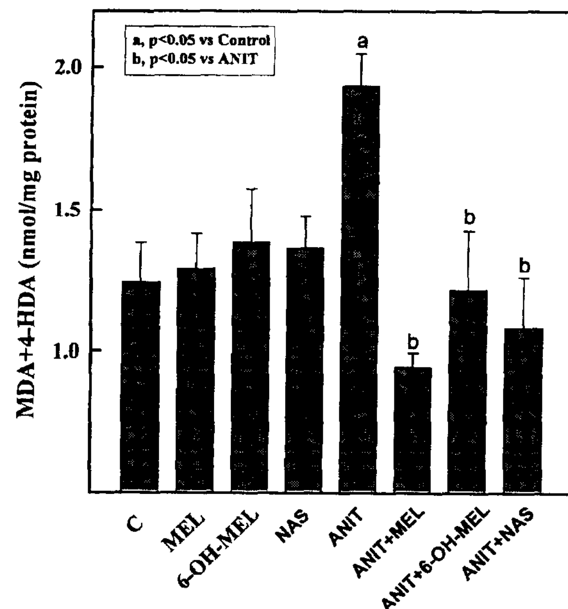

Fig. 2. Effect of intraperitoneal injections of melatonin, 6-hydroxymelatonin or *N*-acetylserotonin (10 mg/kg body weight) on hepatic lipid peroxidation in rats given a single intraperitoneal injection of ANIT (75 mg/kg body weight). Lipid peroxidation is expressed by MDA and 4-HDA concentrations. Hepatic lipid peroxidation was assayed in control rats (C), rats injected with melatonin (MEL), 6-hydroxymelatonin (6-OH-MEL), *N*-acetylserotonin (NAS), ANIT (ANIT), ANIT plus melatonin (ANIT+MEL), ANIT plus 6-hydroxymelatonin (ANIT+6-OH-MEL), and ANIT plus *N*-acetylserotonin (ANIT+NAS) as described in Material and Methods. Each value represents the mean ± SEM.

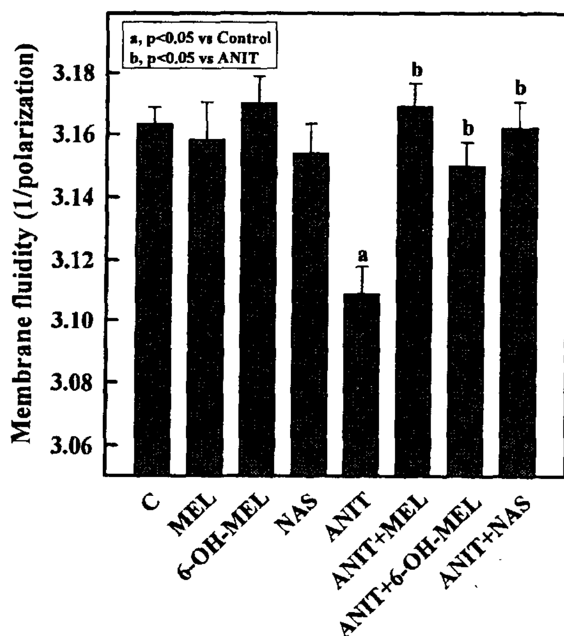

Fig. 3. Effect of intraperitoneal injections of melatonin, 6-hydroxymelatonin or *N*-acetylserotonin (10 mg/kg body weight) on microsomal membrane fluidity (1/P) in rats given a single intraperitoneal injection of ANIT (75 mg/kg body weight). Microsomal membrane fluidity was assayed in control rats (C), rats injected with melatonin (MEL), 6-hydroxymelatonin (6-OH-MEL), *N*-acetylserotonin (NAS), ANIT (ANIT), ANIT plus melatonin (ANIT+MEL), ANIT plus 6-hydroxymelatonin (ANIT+6-OH-MEL), and ANIT plus *N*-acetylserotonin (ANIT+NAS) as described in Material and Methods. Each value represents the mean ± SEM.

showed an acute infiltration with polymorphonuclear neutrophils (Table I). Concurrent melatonin administration reduced the severity of polymorphonuclear neutrophil infiltration (Table I). Injection of 6-hydroxymelatonin or *N*-acetylserotonin did not reduce the polymorphonuclear neutrophil infiltration caused by ANIT (Table I).

Morphological Apoptosis Analysis

The apoptotic cells were found as individual cells in close proximity to the ducts and among the cells comprising the ductual epithelium. Quantification of the number of cells undergoing apoptosis in the periportal area revealed an increase in ANIT-injected rats (Table I). Although apoptotic cells were only quantified in the portal areas, they were detectable in other areas of the liver acinus as well. Concurrent melatonin administration reduced significantly the number of apoptotic cells (Table I). Rats treated with 6-hydroxymelatonin or *N*-acetylserotonin were not protected

TABLE I. Effect of Melatonin and Related Indoles on the Number of Polymorphonuclear Neutrophils (PMNs) and Apoptotic Cells in the Liver After α-naphthylisothiocyanate Administration

Treatment	PMNs	Apoptotic cells
C	10 ± 1.63	6 ± 1.22
MEL	8 ± 1.22	4 ± 0.82
6-OH-MEL	9 ± 2.04	6 ± 0.82
NAS	10 ± 2.04	6 ± 0.82
ANIT	98 ± 8.16	15 ± 1.22
ANIT+MEL	47 ± 5.31	9 ± 0.82
ANIT+6-OH-MEL	87 ± 6.12	16 ± 1.22
ANIT+NAS	96 ± 8.16	15 ± 1.22

The number of polymorphonuclear neutrophils and apoptotic cells were assayed in control rats (C), rats injected with melatonin (MEL), 6-hydroxymelatonin (6-OH-MEL), *N*-acetylserotonin (NAS), ANIT (ANIT), ANIT plus melatonin (ANIT+MEL), ANIT plus 6-hydroxymelatonin (ANIT+6-OH-MEL), and ANIT plus *N*-acetylserotonin (ANIT+NAS). The number of polymorphonuclear neutrophils were assayed in 10 randomly selected high power fields. The number of apoptotic cells were assayed from a total of 50 periportal areas randomly selected per rat liver. Values are expressed as mean ± SEM in six animals in each group.

from ANIT in terms of the number of apoptotic cells (Table I). Control rats and rats treated with only melatonin, 6-hydroxymelatonin or *N*-acetylserotonin exhibited a small number of apoptotic cells (Table I).

DISCUSSION

In the present paper we investigated and compared, under in vivo conditions, the protective effect of melatonin, 6-hydroxymelatonin or *N*-acetylserotonin against the hepatotoxicity of ANIT, a well-characterized hepatotoxin that produces a cholangiolitic hepatitis characterized by intrahepatic cholestasis, necrosis of both hepatocytes and biliary epithelial cells and bile duct obstruction [Plaa and Priestly, 1977; Roth and Dahm, 1997]. Rats injected once with ANIT (75 mg/kg body weight) exhibited liver injury and cholestasis 24 h after the injection, as indicated by the serum activity levels of SGPT and SGOT and total serum bilirubin concentration; this is consistent with previous reports [Kongo et al., 1999; Ohta et al., 2000a]. In our experimental model, melatonin, 6-hydroxymelatonin or *N*-acetylserotonin was intraperitoneally administered to the rats at a dose of 10 mg/kg body weight 30 min before the ANIT injection and 6, 12, and 18 h after ANIT

injection. This experimental injection schedule for melatonin and related indoles has been used in other experimental models and been found effective in reducing oxidative damage [Sewerynek et al., 1995; Carneiro and Reiter, 1998]. Melatonin, and to a lesser degree 6-hydroxymelatonin, as able to protect against ANIT-induced liver injury with cholestasis as demonstrated by the serum values of both SGPT and SGOT activities and total bilirubin concentration. However, *N*-acetylserotonin was unable to protect against the hepatotoxic effects of ANIT. This is the first study in which 6-hydroxymelatonin and *N*-acetylserotonin have been tested as to their potential protective effects against ANIT toxicity.

Previous reports [Kongo et al., 1999; Ohta et al., 2000a] have shown that in rats given a single injection of ANIT, an increase in hepatic LPO levels occurs before discernible morphological injury which is enhanced with the progression of hepatic damage. Accordingly, they have proposed that lipid peroxidation is a significant feature of ANIT-induced hepatic injury [Kongo et al., 1999; Ohta et al., 2000a]. In the present study, hepatic LPO products were consistent with morphological evidence of damage. When rats were treated with either melatonin, 6-hydroxymelatonin or *N*-acetylserotonin, interestingly, the increased hepatic lipid peroxidation products induced by ANIT were reduced in a similar manner by all three indoles.

With respect to melatonin, it is well known that this molecule has antioxidant properties under both in vitro and in vivo conditions [Reiter, 1996; Longoni et al., 1998]. Furthermore, melatonin has been shown to protect against hepatic injury induced by endotoxic and non-endotoxic shock [Sewerynek et al., 1995; El-Sokkary et al., 1999a], ischemia-reperfusion [Sewerynek et al., 1996], ethanol administration [El-Sokkary et al., 1999b], carbon tetrachloride injection [Ohta et al., 2000b], and ligature of extra-hepatic biliary duct [Montilla et al., 2000]. In all these situations, lipid peroxidation induced by oxygen radicals is involved and the protective role of melatonin is believed to result from its antioxidant properties. Consequently, we speculate that the reduction of lipid peroxidation seen in this study, at least in part, was a consequence of the antioxidant properties of melatonin. Moreover, very recently our group has reported the existence of high levels of melatonin in the bile of rats and other mammals [Tan et al., 1999] and we speculate that these high levels of melatonin in the bile may be involved in the protective antioxidant effects of melatonin observed in this study.

With respect to 6-hydroxymelatonin and *N*-acetylserotonin, previous studies have demonstrated that these indoles also have antioxidant properties in several in vitro conditions [Chan and Tang, 1996; Matuszak et al., 1997]. However, few studies have been carried out to investigate the antioxidant properties of these indoles under in vivo conditions [Hara et al., 1997]. Furthermore, in the in vitro studies the results obtained with different indoles have been variable [Wölfler et al., 1999]. In our study, the results obtained with liver LPO were supported by the studies on membrane fluidity. Thus, studies in a variety of membrane systems have demonstrated that free radicals disturb the order and lipid dynamics of the membrane and the biophysical measurements of membrane fluidity are a good method to document membrane structural alterations as a consequence of lipid peroxidation [Curtis et al., 1984]. In this context, two basic mechanisms have been proposed to explain loss in membrane fluidity due to lipid peroxidation. Firstly, the reduction in the unsaturation/saturation ratio of membrane fatty acids [Curtis et al., 1984] and, secondly, the formation of cross-linking of lipid–lipid and lipid–protein moieties [Eichenberger et al., 1982]. Hence, antioxidant molecules that inhibit lipid peroxidation also prevent membrane rigidity induced by oxidative stress. The results obtained in this study, using TMA-DPH as a fluorescence probe, are consistent with these observations. Thus, ANIT caused a significant reduction in hepatic microsome membrane fluidity and this reduction was completely prevented by melatonin, 6-hydroxymelatonin or *N*-acetylserotonin. With respect to melatonin, similar results have been reported from several in vitro studies which used other methods to induce LPO [García et al., 1997, 1998]. However, this is the first study that compared the effects of the melatonin, 6-hydroxymelatonin, and *N*-acetylserotonin on hepatic microsome membrane fluidity under in vivo condition.

In the present study the liver injury induced by ANIT, as indicated by the serum values of SGPT and SGOT activities and total bilirubin concentration, was efficiently reversed by mel-

atonin and to a lesser degree by 6-hydroxymelatonin, but not by *N*-acetylserotonin. On the other hand, membrane oxidative injury induced by ANIT, as shown by both LPO and membrane fluidity was prevented to a similar degree by all three indoles. To explain these differences the pathogenic mechanisms of hepatic injury due to ANIT are summarized. Several mechanisms for the pathogenesis of ANIT-induced liver injury have been proposed; these include endotoxemia [Calcamuggi et al., 1992], the formation of a reversible S-conjugate between the glutathione and the ANIT [Jean and Roth, 1995], and the infiltration of polymorphonuclear neutrophils into the hepatic parenchyma all of which induce hepatic damage. Although glutathione may play a role in ANIT hepatotoxicity, neutrophil infiltration is critical to hepatic pathogenesis caused by ANIT. Thus, in rats given one injection of ANIT, it has been shown by histological studies that polymorphonuclear neutrophils prominently infiltrate the hepatic tissue before the onset of liver injury [Goldfarb et al., 1962]. In this context, Dahm et al. [1991] reported that in rats pretreated with antineutrophil serum, the development of ANIT-induced liver damage was prevented. Because of this they suggested that neutrophil infiltration is a major aspect of ANIT-induced liver injury. Also, it has been demonstrated under in vitro conditions that ANIT stimulates neutrophils to release superoxide anions and proteolytic enzymes that are toxic to hepatic parenchyma cells [Roth and Hewett, 1990; Hill and Roth, 1998]. However, Dahm et al. [1991] found that in rats pretreated with a combination of the antioxidative superoxide dismutase and catalase, the degree of ANIT-induced hepatic damage is not attenuated. They, therefore, suggested that neutrophil-derived oxygen radicals are not involved in the development of ANIT-induced liver injury. Recent reports [Kongo et al., 1999; Ohta et al., 2000a] indicate otherwise. These workers believe that hepatic lipid peroxidation associated with ANIT-induced liver injury involves oxygen radicals derived from neutrophils which infiltrate the liver. Neutrophils are known to mediate lipid peroxidation through the production of superoxide anion via activated NADPH oxidoreductase [Casini et al., 1997]. In the present study histological analyses of liver tissue after ANIT administration showed the infiltration of neutrophils. Furthermore, in the ANIT-injected rats the number of apoptotic hepatocytes was also increased. Concurrent melatonin administration reduced the polymorphonuclear neutrophil infiltration as well as the number of apoptotic hepatocytes. With respect to melatonin, these results are in good accord with previous reports showing that melatonin protects against acute liver injuries induced in rats by endotoxic shock and ischemia-reperfusion, not only through its direct antioxidant action but also by inhibiting the infiltration of polymorphonuclear neutrophils [Sewerynek et al., 1995, 1996]. Collectively these findings indicate that melatonin exerts a protective effect against ANIT-induced liver injury through not only its antioxidant properties, but also because of its inhibitory effect on neutrophil infiltration into the liver tissue.

In contrast to melatonin, the administration of 6-hydroxymelatonin or *N*-acetylserotonin did not protect against neutrophil infiltration and hepatocyte apoptosis. With respect to 6-hydroxymelatonin, its partially protective actions must have been due to actions other than a reduction of neutrophil infiltration. With regard to *N*-acetylserotonin, the current results are in good accordance with previous published findings. Thus, its protective effects appear minimal. However, another possibility is that the results observed in this study with *N*-acetylserotonin may be ascribed to melatonin produced after the administration of *N*-acetylserotonin. It has been proposed that *N*-acetylserotonin stimulates pineal melatonin biosynthesis after its administration [Oxenkrug and Requintina, 1994]. Consequently, the in vivo effects of *N*-acetylserotonin might be better studied in pinealectomized animals or in strains of animals with a defective melatonin biosynthesis system.

The acute hepatotoxicity induced by ANIT in rats is manifested, as mentioned above, as neutrophil-dependent necrosis of not only hepatocellular cells, but also of bile epithelial cells (BECs). In fact, BECs are the primary targets of ANIT-induced toxicity and injury to these cells occurs prior to hepatocellular damage [Connolly et al., 1988]. Furthermore, it has been demonstrated that after exposure to ANIT, BECs produce a factor(s) that causes neutrophil chemotaxis and neutrophil-dependent hepatocellular injury [Hill et al., 1999]. A recent study has demonstrated that human BECs exposed to certain proinflammatory

cytokines (IL-1 and TNFα) rapidly express IL-8 and monocyte chemotactic protein-1 (MCP-1), potent chemotactic agents for neutrophils and monocytes or T cells, respectively [Morland et al., 1997].This is not unusual, since epithelial cells of various types are known to produce chemotactic factors. For example, after exposure to certain toxicants, pulmonary airway epithelial cells and renal epithelial cells produce several chemokines (e.g., macrophage inflammatory protein [MIP], cytokine-induced neutrophil chemoattractant [CINC], and MIP-1) capable of inducing neutrophil chemotaxis and activation [Driscoll et al., 1993; Schmouder et al., 1993]. BECs have active inmunological roles in both innate and adaptive immune responses. In fact, it has been demonstrated that BECs are able to secrete chemokines and cytokines, to express cell adhesion molecules, and to carry out functions as professional antigen-presenting cells [Reynoso-Paz et al., 1999].

In conclusion, from the observations summarized herein and from the results of the present study, we speculate that melatonin protects against ANIT-induced liver injury because of its antioxidant properties and additionally due to its capacity to inhibit hepatic neutrophil infiltration, probably by inhibiting the production in BECs due to one or more factors (probably chemokines) that are responsible for neutrophil chemotaxis. It is important to note that neutrophil infiltration is the first event in inflammation and melatonin has been postulated as an anti-inflammatory agent [Cuzzocrea et al., 1997]. Furthermore, it has been demonstrated that melatonin is able to modulate the production of several cytokines [García-Mauriño et al., 1997]. However, the exact nature of this factor(s) requires further investigations. With respect to 6-hydroxymelatonin, although this indole is able to provide partial protection against ANIT-induced liver injury, probably through its antioxidant properties, its mechanism of action in unclear. Finally, *N*-acetylserotonin, in the experimental conditions of this study, showed less pervasive effects, only reducing ANIT-induced hepatic LPO and the effects on microsomal membrane fluidity.

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The Isolation, Purification, and Characterisation of the Principal Urinary Metabolites of Melatonin

A.M. Leone, P.L. Francis, and R.E. Silman

Department of Reproductive Physiology, St. Bartholomew's Hospital Medical College, London, England

Melatonin is metabolised by hydroxylation to form 6-hydroxy-melatonin and by demethylation to form N-acetyl-serotonin, which are excreted as sulphate and glucuronide conjugates. We required these metabolites as pure powders and therefore undertook their isolation and characterisation. Three volunteers ingested 1 g each of melatonin, and their urine was collected and pooled. For the sulphate conjugates, a Lichoprep column was used to concentrate the metabolites and to remove most of the urea. The sulphate conjugates were separated from the glucuronides on a Florisil column and further purified on a fractogel column. They were separated by high-performance liquid chromatography (HPLC) resulting in white powders of 6-hydroxy-melatonin sulphate (SaMT) and N-acetyl-serotonin sulphate (SNAS). For the glucuronide conjugates, an aliquot of the pooled urine was taken to dryness, the residue was dissolved in methanol, and the solution was filtered. The methanol filtrate was taken to dryness, and the residue was applied to a Florisil column. The isolated glucuronide conjugates were recrystallized prior to separation by HPLC, which gave pure white powders of N-acetyl-serotonin glucuronide (GNAS) and 6-hydroxy-melatonin glucuronide (GaMT) Characterisation was achieved by using infrared and ultraviolet spectroscopy, thin-layer chromatography (TLC), and gas chromatography-mass spectrometry (GCMS) These techniques unambiguously confirmed the assigned structures for SaMT and SNAS and fully supported the assigned structures for GNAS and GaMT. Three TLC solvent systems were used, and in each case the individual conjugated metabolite appeared as a discreet spot. Purity, as assessed by GCMS, was shown to be greater than 95% for SNAS, SaMT, and GaMT and to be 88% for GNAS.

Key words: metabolism, GCMS, urinary analysis, HPLC

INTRODUCTION

The pineal gland secretes a number of methoxyindoles of which melatonin (N-acetyl-5-methoxy-tryptamine) has been the most extensively stud-

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Address reprint requests to A.M. Leone, Department of Reproductive Physiology, St. Bartholomew's Hospital Medical College, London EC1, UK.

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ied. Shortly after its isolation and identification in 1958 [Lerner et al., 1958], a number of studies concerned with its metabolic fate were undertaken [Kveder and McIsaac, 1961; Kopin et al., 1960,1961]. Whereas most of the methoxy-indoles, such as methoxytryptamine, methoxytryptophol, and O-acetyl-methoxytryptophol were metabolised by side-chain oxidation to a single product, methoxyindole acetic acid, melatonin was shown to give rise to several metabolic products, principally two forms of conjugated 6-hydroxy-melatonin. More recently it has been shown that another pathway exists for the metabolism of melatonin, i.e., demethylation, which gives rise to N-acetyl-serotonin, the biosynthetic precursor of melatonin [Leone and Silman, 1984; Young et al., 1985a].

To date, only one study out of the many performed has actually isolated any of the urinary metabolites of melatonin. Kveder and McIsaac isolated 6-sulphatoxy-melatonin (SaMT) as a solid from urine [Kveder and McIsaac, 1961], but it is unlikely that it was pure since it was described as an orange powder.

We wished to establish a gas chromatography-mass spectrometry (GCMS) assay for the urinary metabolites of melatonin. To ensure we had an accurate assay we required SaMT, N-acetyl-serotonin sulphate (SNAS), 6-hydroxy-melatonin glucuronide (GaMT), and N-acetyl-serotonin glucuronide (GNAS) as pure powders. For this reason we have undertaken the isolation and purification of these compounds and have attempted to characterise them.

MATERIALS AND METHODS

All indoles were obtained from Sigma Chemical Co., Poole, Dorset. Solvents were of Analar grade, and unless otherwise indicated they were obtained from BDH, Poole, Dorset. Pentafluoroproprionic anhydride (PFPA), used in the GCMS analysis, was purchased from Phase Separations, Clywd, Wales. All methoxyindoles were methoxylated at the five position.

Analytical Techniques

Thin-layer chromatography (TLC). Sections (10 × 5 cm) of silica-coated, aluminium-backed plates (Merck 5554) were used. The analysate was spotted onto the origin, usually 1 cm from the bottom, dried, and placed into a chromatography tank. Except where indicated, all plates were developed in butan-1-ol:acetic acid:water (BAW) (4:1:1). Visualization was achieved either with ultraviolet light or with Ehrlich's reagent.

TLC analysis was used to locate and identify the metabolites after each chromatographic step. Aliquots of the column fractions were spotted onto TLC plates and run in BAW. This system clearly separates the glucuronide from sulphate metabolites; Reference to front (Rf) assignment was based on previously reported work [Leone and Silman, 1984; Young et al., 1985b].

Lichoprep chromatography. A 20 × 1.2-cm glass column was packed with Lichoprep RP18 (25–40 μm) as a water slurry. The solid phase was allowed to settle, and then 50 ml of methanol was added to the column. Finally 100 ml of water was eluted through the column.

Silica gel chromatography. A 20 × 1.2-cm glass column was packed with Florisil silica gel (60–100 mesh size) as a chloroform:methanol slurry (70/30 v/v).

Fractogel chromatograph. A 20 × 1.2-cm glass column was packed with Fractogel TSK HW-40(S) (25–40 μm particle size) as a water slurry.

Infrared (IR) spectrometry. IR analysis was performed on a Perkin-Elmer 237 grating spectrometer. Two milligrams of each compound was mixed with 100 mg of dry potassium bromide, ground together, and pressed into a disc in a die under 10 tons of pressure in vacuo. Spectra were recorded from 4,000 cm^{-1} to 625 cm^{-1}.

Ultraviolet (UV) spectrometry. UV spectra were recorded on a Perkin-Elmer 550 spectrophotometer. The compounds were dissolved in distilled water, and spectra were taken from 320 nm to 200 nm.

High-performance liquid chromatography (HPLC). HPLC analysis was performed on a Pye-Unicam LC-XP series HPLC set, with an on-line UV detector. Injections of up to 500 μl were made onto a semipreparative column (250 × 10 mm) packed with Ultrasphere ODS. The flow rate was set to 4 ml/min, and the mobile phase (using HPLC grade solvents) was either water or 3% methanol/water. The effluent was monitored at 280 nm.

Gas chromatography-mass spectrometry (GCMS). GCMS analysis was performed on a Kratos MS25S mass spectrometer (MS) in the electron impact (EI) mode, which was interfaced to a Carlo Erba gas chromatograph. MS conditions were as follows: filament 1 mA, electron voltage, 45 eV and source temperature, 200°C. The widest source and collector slit settings were used, giving a resolution of approximately 1,200 ppm. Samples were injected onto a Quadrex 25 m × .32-mm, methyl silicone-coated capillary column. The column was held at 80°C for each injection and then heated ballistically at 40°C/min until a temperature of 250°C was obtained. This temperature was maintained for the rest of the run.

Sample preparation for GCMS. As the isolated metabolites, SaMT, SNAS, GNAS, and GaMT are too involatile to be directly analysed, they had to be first deconjugated. This was achieved either during derivatisation with PFPA or by enzymic hydrolysis prior to derivatisation. After deconjugation and derivatisation, the samples were blown down under nitrogen and cleaned. The residue was dissolved in 0.5 ml of chloroform and then applied to a silica sep-pak cartridge that had previously been washed with 2 ml of chloroform. The initial eluate was discarded, and the derivative was eluted in a further 1.5 ml of chloroform. This fraction was taken to dryness, the residue was dissolved in 1 ml of chloroform, and 1-μl aliquots were injected onto the GC. The mass spectra for 6-hydroxy-melatonin (HaMT) and N-acetyl-serotonin (NAS) after derivatisation with PFPA were as described elsewhere [Young et al., 1985b].

Isolation and Purification

Urine pool from three volunteers. Three adult volunteers voided their bladders prior to taking 1 g of melatonin by mouth. The next two voidings were collected, and all six collections were pooled to give a total volume of 1.57 litres.

Concentration of metabolites and partial separation from urea. A 100-ml aliquot of the pooled urine was applied to a Lichoprep column. The first 80 ml of the eluate was discarded, and the last 20 ml was collected. The conjugates were eluted from the column in 20 ml of 40% methanol:chloroform. The two fractions were combined and stored at 4°C. The column was cleaned with 20 ml of methanol, and activity was regenerated by washing with 60 ml of water. A further 100 ml of pooled urine was applied, and the cycle was repeated twelve times, ie, until 1,300 ml of pooled urine had been processed in like manner.

The retained combined fractions from each cycle were pooled and rotary-evaporated at 40°C to give a brown gum. Five ml of methanol was added, which resulted in a fine white precipitate under a brown mother liquor. The mixture was filtered, and the precipitate was discarded. The methanol solution was rotary-evaporated again, and the residue, a brown gum, was dissolved in approximately 4 ml of 30% methanol:chloroform.

Partial separation of sulphate and glucuronide metabolites. The solution was applied to a silica column. The column was eluted with 30% methanol:chloroform, and 5-ml fractions were collected. TLC analysis showed that fractions 2–10 contained mostly SNAS and SAMT, and fractions 21–30 contained mostly GNAS and GaMT.

Isolation of the sulphate conjugates. Fractions 2–10, obtained from the silica column step, were combined and rotary-evaporated to give a brown gum. The gum was dissolved in a minimum volume of methanol, and acetonitrile was added dropwise, resulting in a white precipitate. The mother liquor was removed, and the precipitate was redissolved in 2 ml of methanol. Ethyl acetate (1 ml) was added slowly, whereupon a white precipitate was again formed. The liquor was aspirated off, the precipitate was dissolved in 1.5 ml of methanol, and 3.5 ml chloroform was added (ie, 30% methanol:chloroform).

The solution was then applied to a silica column, eluted with 30% methanol:chloroform, and 5-ml fractions were collected. TLC analysis showed that fractions 3–33 contained mostly SNAS and SAMT. These fractions were combined and rotary-evaporated to dryness. Two millilitres of methanol was added to the residue, and then 1 ml of ethyl acetate was slowly added to give a brown powder.

The powder was dissolved in 5 ml of HPLC grade water. The solution was applied to a Fractogel column, eluted with water, and 7.5-ml fractions were collected. TLC analysis indicated that fractions 19–26 contained most of the sulphate conjugates. These were combined, rotary-evaporated, and redissolved in 10 ml of water. Five hundred-microlitre aliquots were then injected onto the HPLC, and the resultant chromatogram showed two major peaks. TLC analysis of these peaks showed that the first (3'55") was SNAS and that the second (7'45") was SaMT. The cycle was repeated a further nine times until all 5 ml had been processed in like manner. The fractions corresponding to SNAS and SaMT were individually bulked.

These pools were rotary-evaporated to dryness, each residue was dissolved in 2 ml of methanol, and the sulphated conjugates were precipitated by the dropwise addition of 1 ml of ethyl acetate. The precipitates were

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RESULTS

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recovered and dried in vacuo. This resulted in a yield of 260 mg of SaMT, as a white powder, and 205 mg of SNAS also as a white powder.

Isolation of the glucuronide conjugates. One hundred fifty millilitres of the original pooled urine, which had been stored at −20°C, was rotary-evaporated to give a dark brown gum. Ten millilitres of methanol was added, which resulted in a white precipitate under a dark brown mother liquor. The mixture was filtered, the solid was discarded, and the methanol solution was taken to dryness by rotary evaporation. The residue was washed three times with 5 ml of acetonitrile, and the solvent was removed. The gum was then dissolved in 2 ml of methanol, and 1 ml of ethyl acetate was slowly added to give a brown precipitate.

The brown precipitate was dissolved in 5 ml of 30% methanol:chloroform and applied to a silica column. The column was eluted with 30% methanol:chloroform, and 5-ml fractions were collected. The fractions containing the glucuronide conjugates, as shown by TLC, were bulked and taken to dryness, yielding a light brown powder.

The powder was dissolved in 5 ml of water, and 500-μl aliquots were injected onto the HPLC and eluted in 3% methanol:water. The resulting chromatograph showed two major peaks. TLC analysis of the peaks showed that the first was GNAS and that the second was GaMT. On rotary evaporation, both fractions gave white powders. Both were recrystallized from methanol and ethyl acetate. The precipitates were recovered and dried in vacuo. This procedure gave 21 mg of a white powder corresponding by TLC to GNAS and 15 mg of a white powder corresponding by TLC to GaMT.

RESULTS

Characterisation of the Isolated Metabolites

IR analysis. IR analysis was performed on the four powders (Figs. 1–4) and compared with nitrophenyl glucuronide (NPG), indoxyl sulphate (ISO4), NAS, and HaMT. The results of the analyses are given in Table 1.

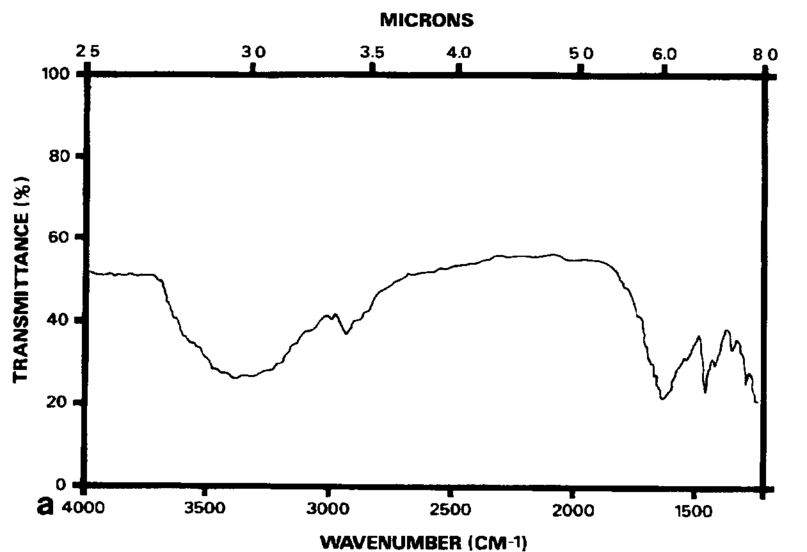
All samples gave a band in the O-H region, ranging between 3,410 cm^{-1} and 3,200 cm^{-1}. Also every sample, with the exception of ISO4, gave a band in the C=0 region, in this case ranging from 1,715 cm^{-1} to 1,610 cm^{-1}. Only the sulphate-conjugated compounds, ie, ISO4, SNAS, and SaMT, gave three bands in the S=0 region, at 1,280–1,270 cm^{-1}, 1,230–1,220 cm^{-1}, and 1,060–1,050 cm^{-1} (Figs. 1, 2).

UV analysis. All compounds were dissolved in water and read against a water blank. The concentration of the test compounds was standardised at 55.6 μg/ml.

GNAS and GaMT were compared with NPG, and SNAS and SaMT were compared with kynurenine sulphate (KSO4) and with ISO4.

For NPG, maximum absorbance (λmax) was seen at 223 nm, with an extinction coefficient (E) of 10,300. For GNAS, λmax was seen at 204 nm, with an E of 15,700. GaMT gave a λmax at 205 nm and an E of 15,100.

ISO4 gave a λmax of 223 nm, with an E of 12,300. For KSO4, λmax was at 227 nm, with an E of 16,200. SaMT gave a λmax at 223 nm, with an E of 16,200. SNAS gave a λmax of 225 nm, with an E of 14,200.



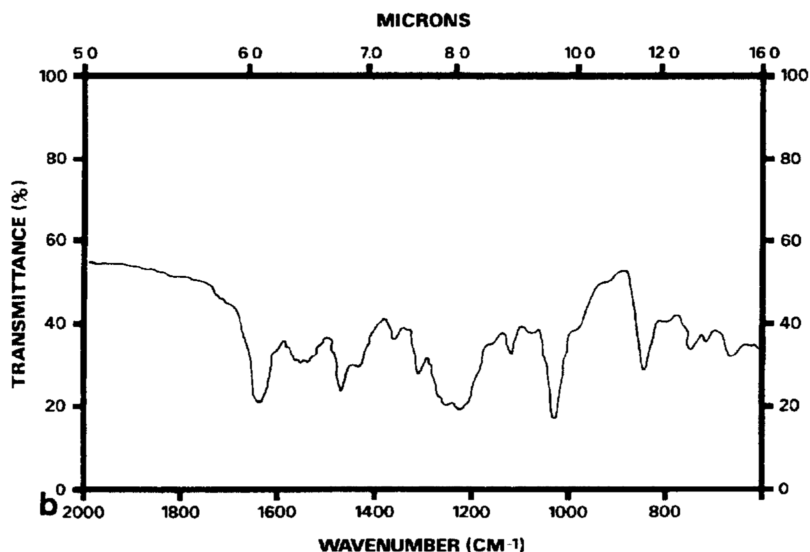
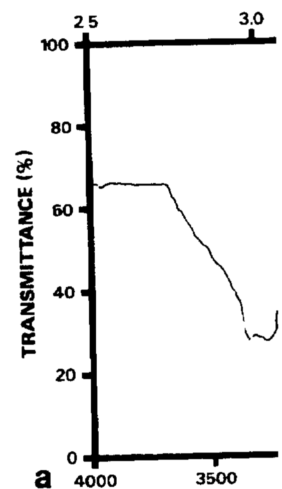


Fig. 1. **a:** The infrared spectrum of 6-hydroxy-melatonin sulphate (SaMT) from 4,000 cm^{-1} to 1,250 cm^{-1}. **b:** The infrared spectrum of SaMT from 2,000 cm^{-1} to 625 cm^{-1}.



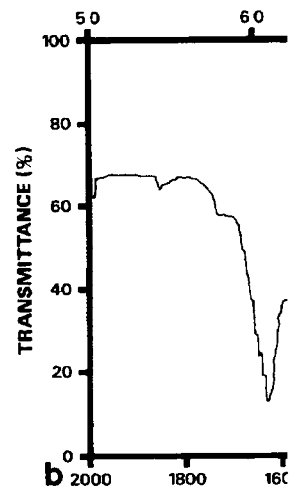


Fig. 2. **a:** The infrared spectru 1,250 cm^{-1}. **b:** The infrared spe

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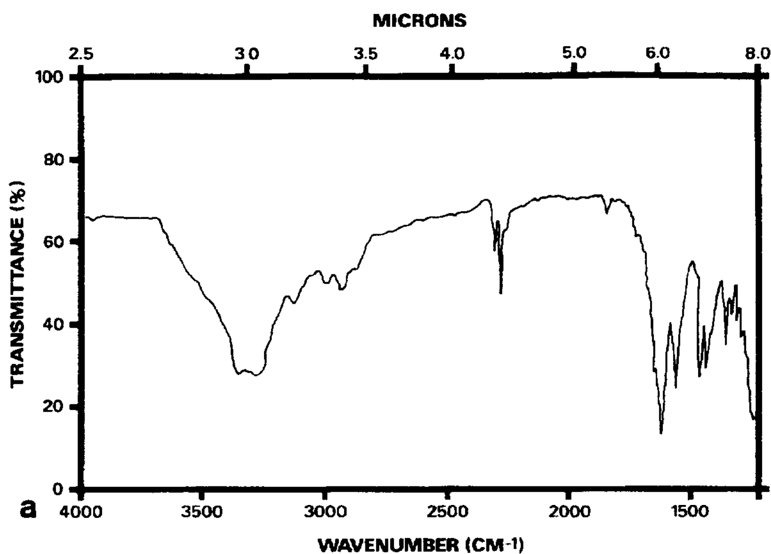


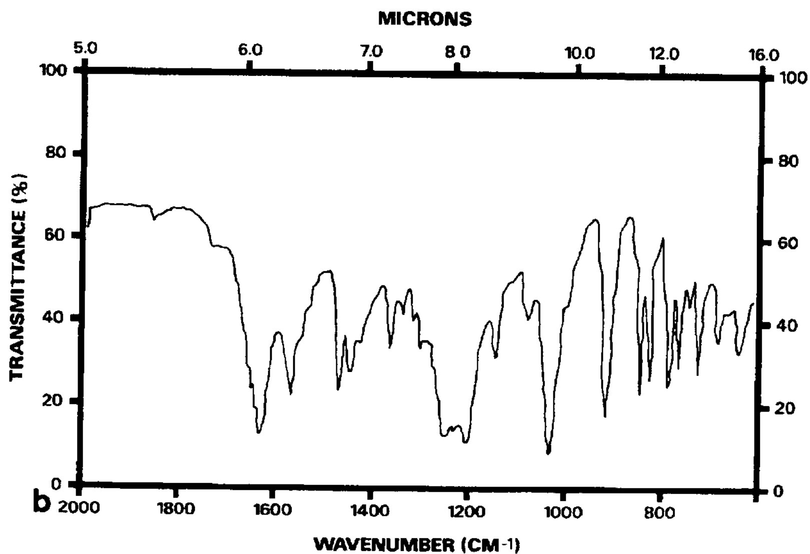
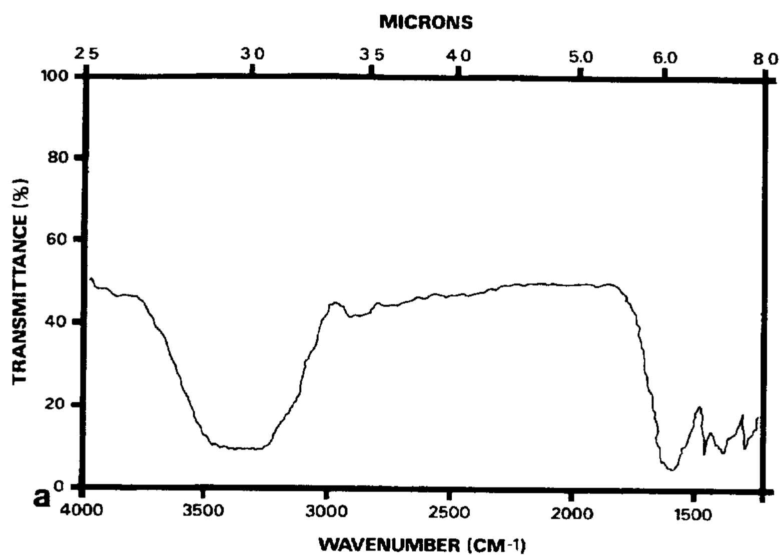


Fig. 2. **a:** The infrared spectrum of N-acetyl-serotonin sulphate (SNAS) from 4,000 cm^{-1} to 1,250 cm^{-1}. **b:** The infrared spectrum of SNAS from 2,000 cm^{-1} to 625 cm^{-1}.



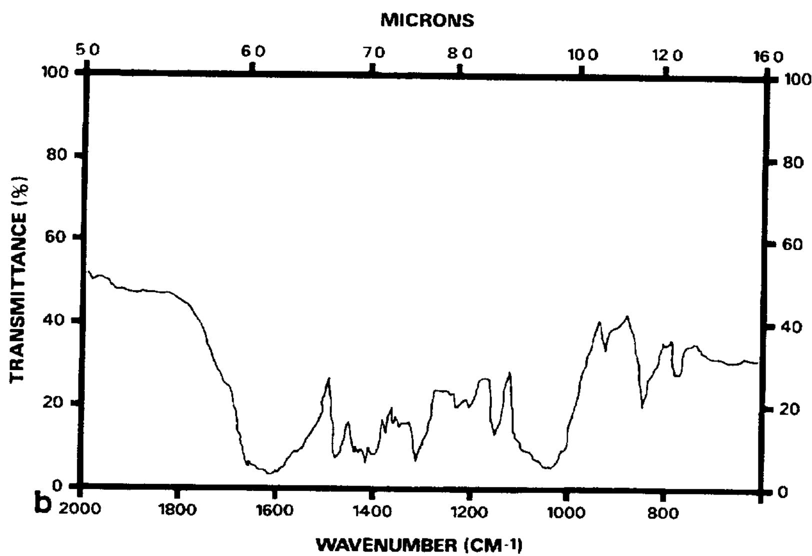
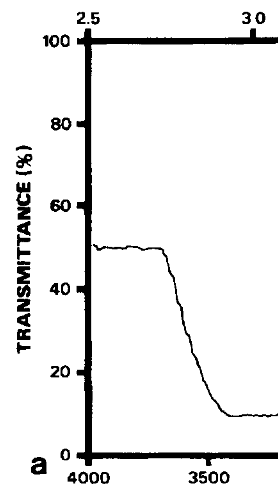


Fig. 3. **a:** The infrared spectrum of 6-hydroxy-melatonin glucuronide (GaMT) from 4,000 cm^{-1} to 1,250 cm^{-1}. **b:** The infrared spectrum of GaMT from 2,000 cm^{-1} to 625 cm^{-1}.



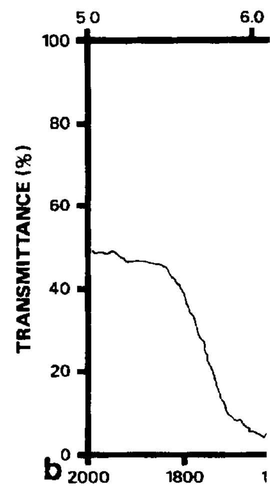
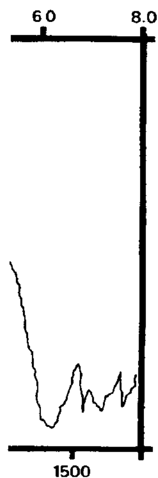
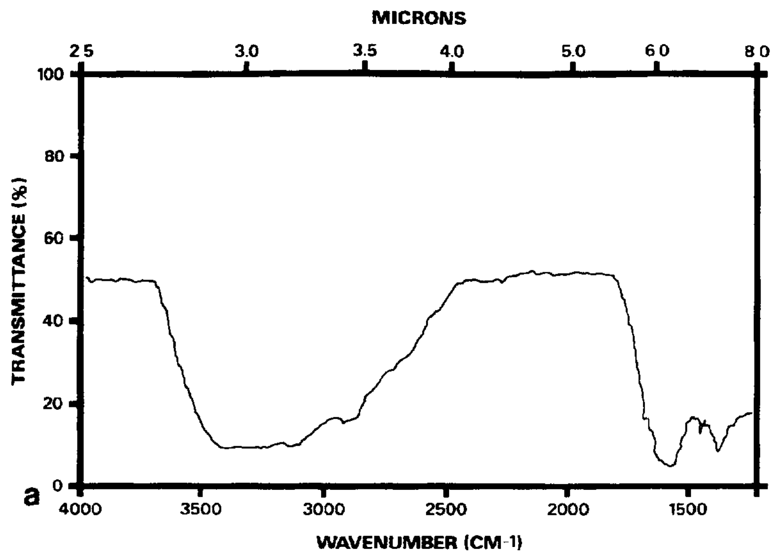
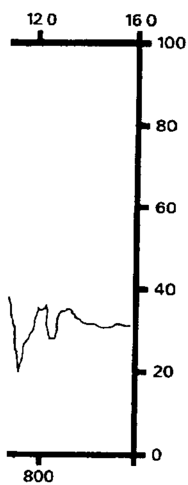
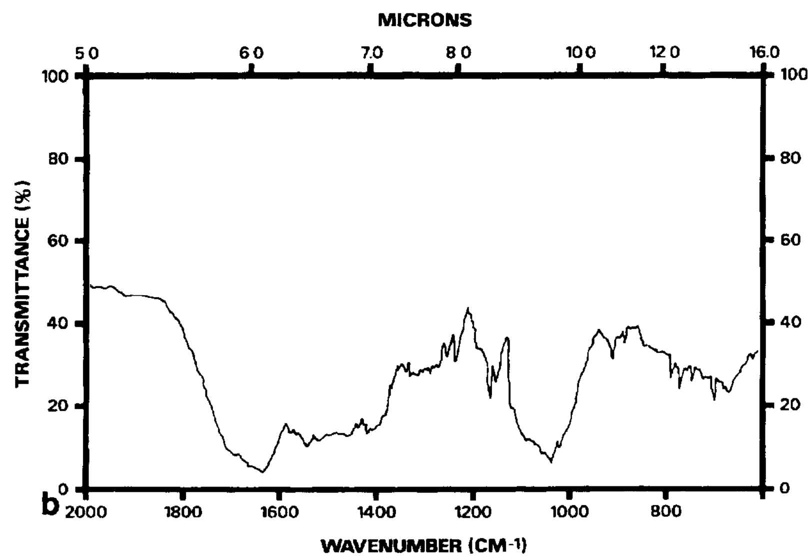


Fig. 4. **a:** The infrared spectru to 1,250 cm^{-1} **b:** The infrared









ıide (GaMT) from 4,000 cm^{-1} to 625 cm^{-1}.

Fig. 4. **a:** The infrared spectrum of N-acetyl-serotonin glucuronide (GNAS) from 4,000 cm^{-1} to 1,250 cm^{-1}. **b:** The infrared spectrum of GNAS from 2,000 cm^{-1} to 625 cm^{-1}

TABLE 1. The Absorbance Bands From Specific Regions in the IR Spectrum[1]

Compound	Absorbance O-H	C=0	S=0
NPG	3,300 (br)[2]	1,715 (sh)	—
NAS	3,200 (br)	1,635 (sh)	—
GNAS	3,250 (br)	1,650 (br)	—
HaMT	3,305 (sh)[3]	1,610 (sh)	—
GaMT	3,300 (br)	1,620 (br)	—
ISO4	3,410	—	1,280, 1,220, 1,060
SaMT	3,350 (br)	1,645	1,270, 1,230, 1,050
SNAS	3,320 (br)	1,640	1,270, 1,230, 1,060

[1] Obtained from the four conjugate metabolites and two reference compounds
[2] br, broad
[3] sh, sharp

TLC analysis. All four metabolites were run together in three different solvent systems. Compounds were visualized under UV light and then with Ehrlich's reagent. Unless otherwise stated, each compound gave a single coincident spot with UV and Ehrlich's reagent.

BAW. In this system the Rfs were as follows: SaMT, 47%; SNAS, 51%; SaMT, 25%; and GNAS, 31%.

Acetonitrile. In this system the Rfs were SaMT, 65%; SNAS, 70%; GaMT, 9%; and GNAS, 16%. A weak spot corresponding to free HaMT was seen for SaMT (Rf = 91%).

30% Methanol/chloroform. In this system the RFs were SaMT, 57%; SNAS, 57%, GaMT, 12%; and GNAS, 9%. Again, a weak spot corresponding to HaMT was seen for SaMT (Rf = 86%).

GCMS analysis

Direct derivatisation. One hundred microlitres of ethyl acetate and 100 μl of acetonitrile were added to 1-mg samples of SaMT, SNAS, HaMT, and NAS followed by 200 μl of PFPA. The vials were capped and left for 1 h at 50°C. The derivatives were then cleaned, and 1 μl aliquots were injected onto the GC.

HaMT gave a single peak that eluted at 11'27", and its spectrum showed a base peak at m/z 522, whose mean intensity was 265,553. SaMT gave a single peak that eluted at 11'27", and its spectrum showed a base peak at m/z 522 whose mean intensity was 189,784. Comparison of the two sets of data shows that the mean purity of SaMT, after allowing for molecular weight differences, was >95%.

NAS gave a single peak that eluted at 8'33", and its spectrum showed a base peak at m/z 492, whose mean intensity was 104,104. SNAS gave a single peak that eluted at 8'29", and its spectrum showed a base peak at m/z 492 whose mean intensity was 77,130. Comparison of the two sets of data shows that the mean purity of SNAS, after allowing for molecular weight differences, was >97%.

Deconjugation by enzymic hydrolysis. One-milligram samples of SNAS, SaMT, GNAS, GaMT, NAS, and HaMT were dissolved in 1 ml of distilled water. Four hundred-microlitre aliquots were dispensed, in duplicate, into

screw-top vials. To each tub
μl of 0.053 M ethylenediami
buffer at pH 4.5, and 250 μl
were gently mixed and place
were removed, 3 ml of chlo
for 30 s. The phases were s
min. The upper, aqueous
organic phase were transfer
under nitrogen, and 100 μl
The tubes were capped, v
derivatives were cleaned, ar
HaMT gave a single peal
showed a base peak at m/z
gave a single peak that elute
at m/z 522, whose mean int
eluted at 11'25", and its sp
mean intensity was 189,082
Comparison of the thre
differences, showed SaMT
to HaMT.
NAS gave a single peak th
a base peak at m/z 492, who
peak that eluted at 8'29", a
492, whose mean intensity
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intensity was 31,161.
Comparison of the thre
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was greater than 70% pure.

DISCUSSION

Problems of Isolation and

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Sulphate conjugate isol
achieved both sample con
tively unretained urea. Sam

screw-top vials. To each tube was added 250 μl of 0.85 M ascorbic acid, 250 μl of 0.053 M ethylenediamine tetraacetic acid (EDTA), 500 μl of 1 M acetate buffer at pH 4.5, and 250 μl of beta-glucuronidase enzyme (HA3F). The tubes were gently mixed and placed in a water bath set to 60°C. After 3 h the tubes were removed, 3 ml of chloroform was added, and the tubes were vortexed for 30 s. The phases were separated by centrifugation at 2,500 rpm for 20 min. The upper, aqueous phase was aspirated, and 1-ml aliquots of the organic phase were transferred to clean vials. These were taken to dryness under nitrogen, and 100 μl of ethyl acetate and 100 μl of PFPA were added. The tubes were capped, vortexed, and heated at 50°C for 10 min. The derivatives were cleaned, and 1 μl aliquots were injected onto the GCMS.

HaMT gave a single peak that eluted at 11'25", and its mass spectrum showed a base peak at m/z 522, whose mean intensity was 259,824. GaMT gave a single peak that eluted at 11'24", and its spectrum showed a base peak at m/z 522, whose mean intensity was 140,780. SaMT gave a single peak that eluted at 11'25", and its spectrum showed a base peak at m/z 522, whose mean intensity was 189,082.

Comparison of the three results, after allowing for molecular weight differences, showed SaMT and GaMT to be greater than 96% pure, relative to HaMT.

NAS gave a single peak that eluted at 8'31", and its mass spectrum showed a base peak at m/z 492, whose mean intensity was 66,044. SNAS gave a single peak that eluted at 8'29", and its mass spectrum showed a base peak at m/z 492, whose mean intensity was 34,098. GNAS gave a single peak that eluted at 8'29", and its mass spectrum showed a base peak at m/z 492, whose mean intensity was 31,161.

Comparison of the three results, after allowing for molecular weight differences, showed that GNAS was greater than 88% pure and that SNAS was greater than 70% pure.

DISCUSSION

Problems of Isolation and Purification

The major problem in the isolation of the conjugates is the separation of SNAS from SaMT and GNAS from GaMT. The presence of the conjugate moiety markedly diminishes any inherent polarity differences between the free indoles, ie, NAS and HaMT. HPLC was the only method found capable of separating the two sulphate and the two glucuronide conjugates in a reproducible and reliable manner. However, urea, a major component of urine, has a similar polarity on HPLC to that of the conjugates and in particular to the glucuronides; therefore, it had to be excluded as far as possible prior to separation by HPLC. In addition, optimum performance on HPLC is greatly dependent on sample purity, and, in this case, considerable peak broadening with resultant loss of resolution was seen without extensive prepurification.

Sulphate conjugate isolation. The initial step, C18, was useful in that it achieved both sample concentration and removed about 80% of the relatively unretained urea. Sample loading was limited to 100 ml per cycle since

higher load volumes resulted in poor retention of the conjugates. The second step, silica gel column chromatography, largely separated the sulphate conjugates from the glucuronide conjugates. However, the sulphates were still sufficiently impure to cause peak broadening on HPLC and so a third purification step for these conjugates was developed, namely a Fractogel column. The sulphate conjugates were well retained in this system and in the process removed most of the residual urea. This step markedly improved peak shape on HPLC, which facilitated the separation of SNAS from SaMT.

Glucuronide conjugate isolation. The glucuronides were well retained on silica, and this step removed sufficient urea and general impurities to enable them to be adequately resolved and separated on HPLC without the prior C18 column. In fact the degree of purification was such that further processing, apart from recrystallization, was not found to be necessary.

Characterisation

Four techniques were used to characterise the isolated metabolites, UV, IR, TLC, and GCMS. Since the conjugated metabolites are not readily analysed intact by GCMS, this method was used to identify the indole part of the molecule. Conversely, IR was most useful for the identification of the conjugate moieties, since these gave rise to distinctive bands. UV and TLC analysis of the whole, intact molecule served to assist in the determination of purity.

GCMS analysis of the four metabolites, after enzymic hydrolysis, gave rise to the correct mass spectra at the correct retention time of NAS or HaMT, fully confirming the indole structure assignment of each conjugate. The fact that SNAS or SaMT, when derivatised directly with PFPA, also gave rise to their correct mass spectra at the correct retention times of NAS and HAMT strongly supports their assignment as sulphate conjugates, since this reagent is known to be able to replace sulphate groups with perfluoropropionyl groups [Fellenberg et al., 1980].

IR was of most use in the analysis of the sulphate conjugates. ISO4, the reference compound, showed three intense bands in the S=O absorbance region: 1,280 cm^{-1}, 1,220 cm^{-1}, (seen as a doublet), and 1,060 cm^{-1}. Similar intense bands were seen for both SaMT and SNAS (Figs. 1, 2) but not for GaMT or GNAS (Figs. 3 ,4) or any of the other nonsulphate reference compounds. Although IR proved less conclusive for the glucuronide conjugates, it was nonetheless supportive of the assigned structures. A small shift in the position of the hydroxyl band was seen for GNAS and GaMT as compared with NAS and HaMT. Also, the carbonyl band was shifted and broadened in both GNAS and GaMT, relative to their free indoles. It would seem likely that this effect was due to the overlap of the carbonyl band from the glucuronide moiety (1,715 cm^{-1}, NPG) and the carbonyl band from the indole N-acetyl side chain (1,635 cm^{-1}, NAS and 1,610 cm^{-1}, HaMT; both sharp).

UV analysis showed that extinction coefficients varied little among all the compounds tested. Maximum absorbance was seen at about 223 nm, with the exception of GNAS and GaMT. Interestingly, this did not seem to be directly related to conjugation, since NPG absorbed maximally at 223 nm also.

TLC analysis showe
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Purity Assessment

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TLC analysis showed that, with the exception of SaMT, the metabolites were free from other indoles and general UV absorbing material. The Rf values obtained were in good agreement with previous work, both in this laboratory [Leone and Silman, 1984] and elsewhere [Kopin et al., 1961]. The presence of some free HaMT in the SaMT is likely to be due to degradation after isolation, since they have hugely different retention times on HPLC in the operating system we have used. This would tend to confirm the view held by Jones et al. that SaMT may be relatively unstable as a powder [Jones et al., 1969].

Purity Assessment

GCMS was the method of choice for the determination of the purity of the metabolites. Known amounts of the four metabolites were compared with equal quantities of the standard nonconjugated indoles. Peak intensities of the base peak in the mass spectrum, m/z 492 for NAS-PFP and m/z 522 for HaMT-PFP, were compared, and the ratio was used to determine purity relative to the standards. Sulphate conjugates were analysed either by direct derivatisation with PFPA or after enzymic deconjugation followed by conventional derivatisation with PFPA. The glucuronide metabolites were analysed after enzymic hydrolysis followed by derivatisation with PFPA.

The sulphate metabolites, SNAS and SaMT, when analysed by direct derivatisation were shown to be $>97\%$ and $>95\%$ pure, respectively. When analysed after enzymic hydrolysis, SaMT and GaMT were both $>96\%$ pure. However GNAS and SNAS were shown to be only 88% and 70% pure, respectively. This is in contrast to the value of 97% purity obtained for SNAS following direct derivatisation. Naturally, the assumption made in the enzyme method is that there is complete turnover of the metabolite. The discrepancy between the two purity values for SNAS suggests that this may well not be the case. We may therefore conclude that the purity values for SNAS and GNAS are underestimates. Further, it suggests that NAS conjugates as compared with HaMT conjugates are less readily hydrolysed enzymically. Presumably this is due to some neighbouring group effect from the five-position methoxyl in the HaMT conjugates.

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INTRODUCTION

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Address reprint requests to Dr. P.S\
Bldg., University of California, Be

Dr. Catalá's present address is Dep\
Universidad de Valencia. Valencia

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N-Acetylserotonin, Melatonin and Their Derivatives Improve Cognition and Protect against β-Amyloid-Induced Neurotoxicity

S. BACHURIN, G. OXENKRUG,[a] N. LERMONTOVA, A. AFANASIEV, B. BEZNOSKO, G. VANKIN, E. SHEVTZOVA, T. MUKHINA, AND T. SERKOVA

Institute of Physiologically Active Compounds, Russian Academy of Sciences, Chernogolovka 142432, Russia

Pineal Research Laboratory, Department of Psychiatry, St. Elizabeth's Medical Center/ Tufts University, Boston, Massachusetts, USA

ABSTRACT: After a single injection of cholinergic neurotoxin ethylcholine aziridinium (AF64A, 3 nmol intracerebroventricularly (i.c.v.)), rats failed to perform the tasks in the active avoidance (learning and retention paradigms) and water maze tests. *N*-Acetylserotonin (NAS), melatonin and their newly synthesized derivatives, CA-15 and CA-18, (0.3–3.0 mg/kg daily for 12–14 days) reversed the effect of AF64A in a dose-dependent manner with CA-18 being the most active. Melatonin and NAS caused sedation absent in CA-18-treated rats. The studied compounds (25–500 μM for 72 hr) protected against β-amyloid peptide (βAP) fragment 25–35-induced neurotoxicity in cerebellar granule cell culture. Our results suggest that neuroprotecting properties of these compounds might mediate their cognition-enhancing effects. The results obtained warrant the further search for the novel types of safe neuroprotectors among the synthetic NAS/melatonin derivatives.

INTRODUCTION

It is known that dysfunction of circadian rhythms in Alzheimer's disease (AD) can be compensated by exogenous melatonin.[1] It was also suggested that the positive effect of chronic prophylactic administration of melatonin as gerontoprotector is based on its antioxidative properties.[2] Numerous studies indicate that melatonin as free radical scavenger displays pronounced neuroprotective effects against neurotoxic action of the excitatory amino acids (excitotoxicity) and toxic effect of beta-amyloid peptide (βAP)—one of the specific hallmarks of AD.[3] Recently neuroprotective activity was revealed for the melatonin precusor *N*-acetylserotonin (NAS).[4] Since neurodegenerative processes in AD are associated with the decreased cognitive functions, it was reasonable to study the effect of melatonin, NAS and their newly synthesized derivatives on cognitive functions in animal models of AD-type neurodegeneration.

[a]Corresponding author: Prof. G. F. Oxenkrug, M.D., Ph.D., Pineal Research Laboratory, Department of Psychiatry, St. Elizabeth's Medical Center, QN-3P, 736 Cambridge St., Boston, MA, 02135. Phone, 617/789-2925; fax, 617/789-2066.
e-mail, Oxenkrug@SEMC.ORG

In the present study both *in vivo* and *in vitro* models were used. The *in vivo* study involved the neurotoxin-induced animal model of AD, based on observation that intracerebroventricular (i.c.v.) administration of ethylcholine aziridinium ion (AF64A) produced chronic cholinergic hypofunction and learning and memory impairment in rat analogous to that observed in AD.[5] Currently this model is also used for screening of the compounds for their potential cognition-enhancing properties.[6,7] At the cellular level (*in vitro*) the adequate model of AD-type degeneration is believed to be the neuronal cell culture degeneration induced by βAP fragment 25–35.[8] In the present study we examined cognition-enhancing and neuroprotective properties of melatonin, NAS and their newly synthesized derivatives CA-15 and CA-18 in the above-mentioned animal and cell models of AD-type neurodegeneration.

MATERIALS AND METHODS

Chemicals

NAS and melatonin derivatives CA-15 and CA-18 were identified by H1-nuclear magnetic resonance (NMR) and elemental analysis. βAP fragment 25–35 was purchased from Bachem; AF64 was purchased from RBI. All other reagents were purchased from Sigma. Melatonin, NAS and CA-15 and CA-18 were preliminarily dissolved in dimethyl sulfoxide (DMSO) and diluted by water each day prior to use.

Animals

Male Wistar rats (12–16 weeks old, 280–450 g) were used in behavioral experiments. Rats were kept at 12 hr light:12 hr dark schedule (lights on/off at 4:00/16:00 hr) with free access to water and food.

Rats were anesthetized with ether and placed into a stereotaxic frame before the surgery. Freshly prepared from AF64 solutions of AF64A (3 nmol/3 ml) or vehicle (cerebrospinal fluid, CSF) were injected into each of lateral cerebral ventricles. After surgery, rats were given a recovery period (12 days) before being tested in behavioral experiment. Melatonin, NAS and their derivatives were administrated orally (in starch solutions) once a day around the time of circadian light-off during the whole recovery period.

Behavioral Studies

Active Avoidance Test

Training was conducted in a two-chamber shuttle box according to the procedure described earlier.[7] The conditioned stimulus was a 5-sec light followed by the unconditioned stimulus, a 1-mA shock, which was delivered to the grid in the lit chamber. The rat avoided the shock by crossing through to the other (dark) chamber. The avoidance during the conditioned stimulus was considered as a correct response. Training procedure consisted of 35 trials (learning test). Fifteen further trials were given 24 hr later (retention test).

TABLE 1. The influence of melatonin, NAS, and its *o*-benzil homologs on active avoidance performance of the AF64A-treated rats

No.	Compounds	Daily Dose mg/kg	Number of Rats	Correct Responses, %	
				Learning Test	Retention Test
1	Control (i.c.v. CSF)	solvent	33	79.3 ± 5.4*	76.3 ± 6.3*
2	AF64A alone	solvent	31	37.4 ± 9.8	43.8 ± 8.1
3	Melatonin	3	9	75.4 ± 7.0**	72.2 ± 5.8*
	Melatonin	0.3	11	79.0 ± 6.0*	83.0 ± 3.2*
4	NAS	1	10	65.4 ± 8.8*	56.0 ± 9.2
5	CA-15	3	9	61.4 ± 7.4	68.0 ± 8.4**
6	CA-18	1	10	50.0 ± 8.8	48.1 ± 9.8
	CA-18	0.3	11	84.0 ± 5.4*	82.7 ± 5.0*

NOTE: Rats were treated i.c.v. AF64A (3 nmol/3μl) or CSF (control groups) and were given a recovery period (12–14 days) before being tested in the behavioral experiment. The experimental rats were orally given the studied compounds during the recovery period. Shuttle-box avoidance performance: *Learning test.* Following 20 acquisition trials, rats were given 3 blocks of 5 trials. *Retention test.* Two blocks of 5 trials were given 24 hr after the learning trial. Data are presented as the mean ± SEM percentage of correct responses summarized over each block and were analyzed by a statistical test (ANOVA); *p <0.001, **p <0.05 vs the AF64A-treated group.

Statistical Treatment

Each experimental group, i.e., control, AF64A-treated groups and groups for each concentration of the tested compounds, contained 9–11 rats. Data of the number of correct responses from the last 15 trials of the first 35 trials (learning test) or first 15 trials of the retention test were collected for each rat. The mean ± SEM of correct responses was calculated for total number of rats in groups. Data were analyzed as the mean ± SEM in percentage of the maximum possible number of correct responses (= 100%) by anaylsis of variance (ANOVA) followed by post hoc comparisons.

Morris Water Maze Test

The test was started 2 days after the last injection of tested compounds and was performed daily for the period from 3 to 9 days. Round swimming pool (1.8 m diameter and 0.45 m high) with 22°C water was placed in the center of the room. The platform was located 1 cm below the surface of the water. Starting points for the swims were at the cardinal compass points (N, S, E, W), which were selected in a semirandom fashion for each rat on each trial.

Statistical Treatment

Each experimental group, i.e., control, AF64A-treated groups and groups for each concentration of the tested compounds, contained 9–11 rats. Results were estimated as time required for a rat having fallen into the water pool to reach the plat-

form (a sum of 2 trials from a different position every day). Data were analyzed as the mean ± SEM (ANOVA followed by post hoc comparisons).

Neuronal Cell Culture

Cerebellar granule cells (CGC) were prepared from the postnatal rats (7–8 days old) by the following procedure based on the generally accepted methods.[9] The pieces of cerebellum were digested with 0.25 mg/ml trypsin for 25 min at 37°C and incubated for 5 min in 0.1% soybean trypsin inhibitor. After washing, cells were dissociated by triturating. Following 2 centrifugation-resuspension steps, the cells were plated at a density of 2.5–5 × 10 cells per ml on polylysine-coated 24-well plates (Corning) and maintained at 37°C in a humidified incubator with 5% CO_2/ 95% room air. The medium was composed of Eagle's minimum essential medium and fetal calf serum (10%) supplemented with 20 mM potassium chloride, 10 mM glucose, 2 mM glutamine, and 50 µg/ml gentamycin sulfate. Cytosine arabinoside (10 µM) was added 24–48 hr later to prevent the replication of nonneuronal cells.

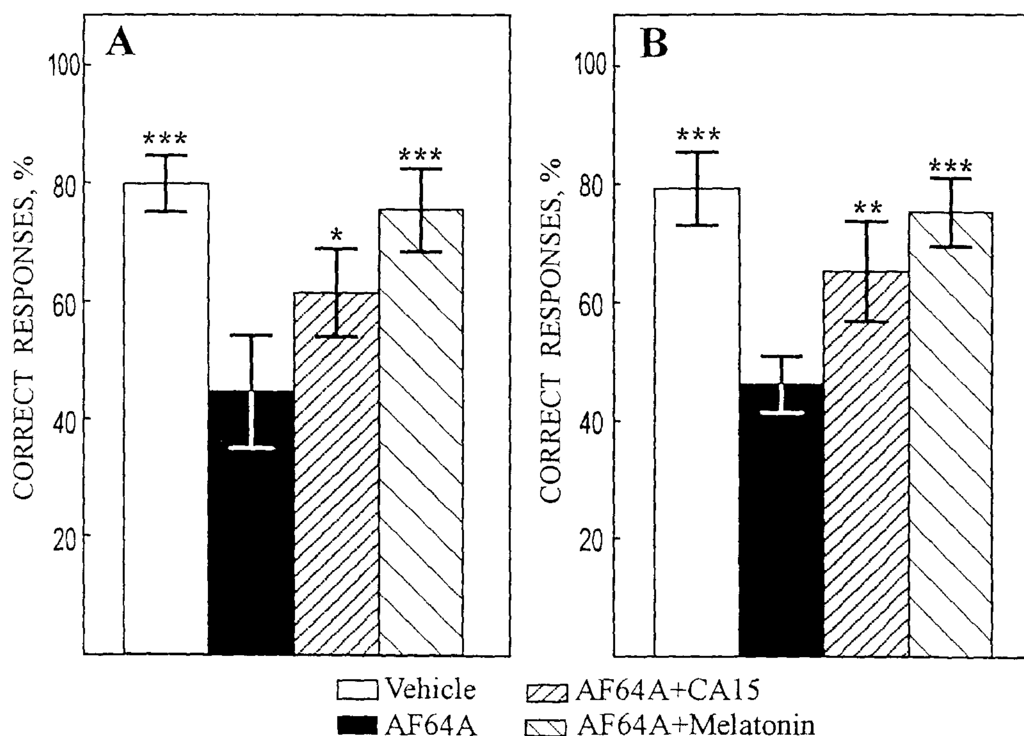

FIGURE 1. Cognition-enhancing effect of melatonin and CA15. Shuttle-box avoidance performance of vehicle-treated rats (n = 11), AF64A-treated rats and AF64A-treated rats receiving melatonin or CA-15 (3 mg/kg, once daily, 12–14 days, orally; n = 10), where n is the number of rats in each group. (**A**) Learning test. Following 20 acquisition trials, rats were given 3 blocks of 5 trials. (**B**) Retention test. Two blocks of 5 trials were given 24 hr after the learning trial. Data are presented as mean ± SEM percentage of correct responses summarized over each block; $**p < 0.05$, $***p < 0.001$ vs AF64A-treated group, where p is the significance level; *not significant (post hoc ANOVA).

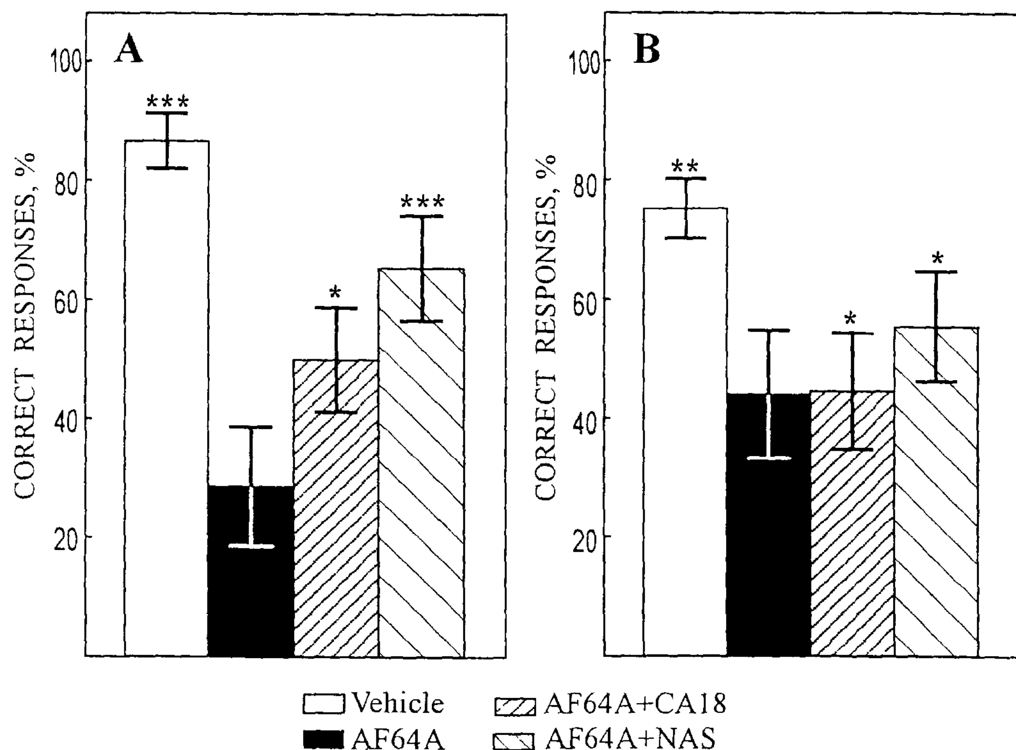

FIGURE 2. Cognition-enhancing effect of CA18 and NAS. Shuttle-box avoidance performance of vehicle-treated rats ($n = 11$), AF64A-treated rats and AF64A-treated rats receiving NAS or CA-18 (1 mg/kg, once daily, 12–14 days, orally; $n = 10$), where n is the number of rats in each group. (**A**) Learning test. Following 20 acquisition trials, rats were given 3 blocks of 5 trials. (**B**) Retention test. Two blocks of 5 trials were given 24 hr after learning trial. Data are presented as mean ± SEM percentage of correct responses summarized over each block; $^{**}p < 0.05$, $^{***}p < 0.001$ vs AF64A-treated group, where p is the significance level; *not significant (post hoc ANOVA).

Toxic Assays

The neurotoxic and neuroprotective effects of NAS, melatonin and their derivatives were tested in mature cultures at 7–8 days *in vitro* (7–8 DIV) after changing of medium to a fresh medium without serum with Supplement N1 (Sigma). The βAP 25–35 (Bachem) was dissolved by sonication in sterilized distilled water at a concentration of 1 mM. Solutions of all reagents were added to the wells with cultures at 25 μM, and the effect was observed during the next days by microscopy.

Quantitative Assessment

Neuronal viability was evaluated by morphometric cell counting using the presence of neurites and smooth, round cell bodies as criteria of survival. The cells were examined under the phase-contrast microscope Axiovert 25C with videocamera and Software miroMedia PCTV VideoCap program for image scanning and photography. Cell survival was quantified by counting the number of viable neurons in premarked microscope fields prior to, and 4 days after the exposure. The difference in numbers of living neurons before and after 4 days of treatment was determined.

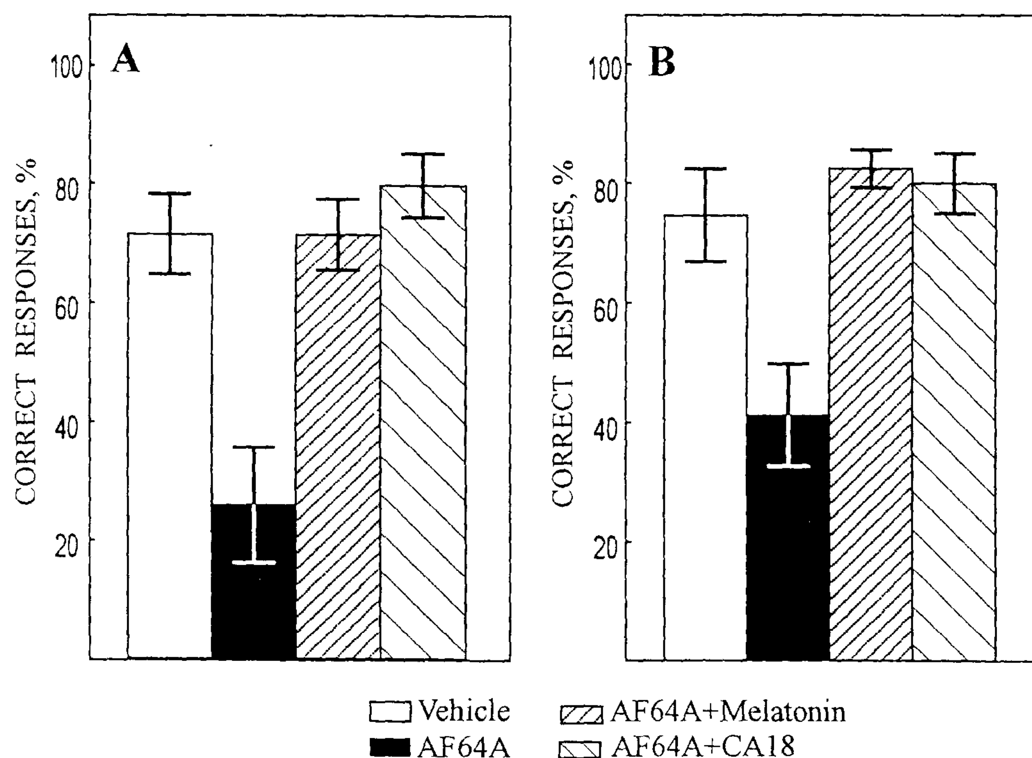

FIGURE 3. Cognition-enhancing effect of melatonin and CA18. Shuttle-box avoidance performance of vehicle-treated rats ($n = 11$), AF64A-treated rats ($n = 10$) and AF64A-treated rats receiving melatonin or CA-18 (0.3 mg/kg, once daily, 12–14 days, orally; $n = 11$), where n is the number of rats in each group. **(A)** Learning test. Following 20 acquisition trials, rats were given 3 blocks of 5 trials. **(B)** Retention test. Two blocks of 5 trials were given 24 hr after learning trial. Data are presented as the mean ± SEM percentage of correct responses summarized over each block; $p < 0.001$, where p is the significance level. The values of p illustrate that there was a significant difference in avoidance performance vs AF64A-treated group (post hoc ANOVA).

Statistical Analysis

For each experiment, we used 4–6 separate wells of a 24-well multiwell plate for control (with 0.05% DMSO) and for each concentration of compound or composition of compound with βAP. Each well equaled one observation (average of 25 cells per microscope field). Experiments are repeated 2–3 times ($n = 12$–18). For graphical presentation, average data from representative experiments were expressed as a percentage of survival cells in comparison with control ± SEM and analyzed by ANOVA and Student t-test.

RESULTS

Active Avoidance Test

AF64A (3 nmol/3 μl i.c.v.) dramatically decreased rats performance in learning and retention paradigms of the active avoidance test (FIGS. 1–3, TABLE 1).

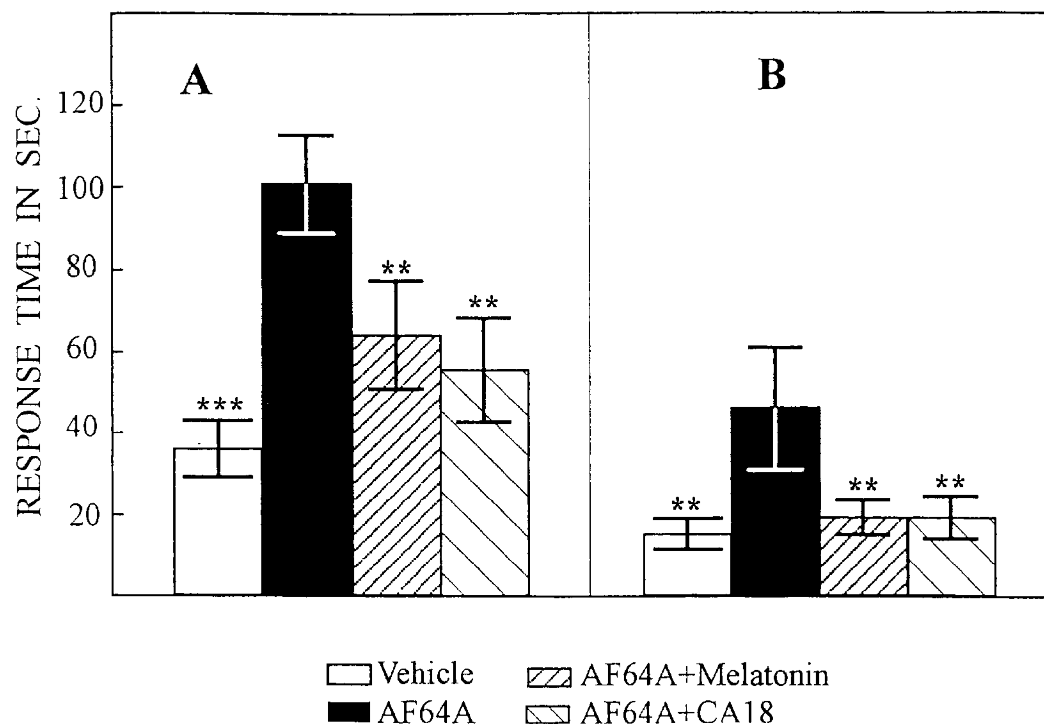

FIGURE 4. Average swim latency made by rats, when trained **(A)** 1 and **(B)** 5 days to find a platform in the Morris water maze test. Performance of vehicle-treated rats (n = 11), AF64A-treated rats (n = 10) and AF64A-treated rats receiving melatonin or CA-18 (0.3 mg/kg, once daily, 12–14 days, orally; n = 11) was started 3 days after the last injection of compound and was performed daily during the period from the 3rd to the 7th day (n is the number of rats in each group). Results were estimated as time required for rat having fallen into the water pool to reach a platform; $^{**}p < 0.05$, $^{***}p < 0.001$ vs the AF64A-treated group, where p is the significance level (post hoc ANOVA).

NAS (1 mg/kg per os (p.o.) daily) improved the performance of AF64A-treated rats in learning (FIG. 2A) but not in retention (FIG. 2B) paradigms of the active avoidance test.

The effect of melatonin was studied in 2 doses: 0.3 and 3 mg/kg (p.o. daily). The lower dose of melatonin (0.3 mg/kg) completely restored rats performance in the learning (FIG. 3A) and retention (FIG. 3B) paradigms of the active avoidance test. The cognitive-enhancing effect of the higher dose of melatonin (3 mg/kg) was significant but somewhat less pronounced than the effect of the lower dose (0.3 mg/kg) (FIG. 1A and B).

CA-15 (3 mg/kg p.o. daily) improved the performance of AF64A-treated rats in retention paradigm of the active avoidance test (FIG. 1B). CA-15 demonstrated a strong tendency (although not reaching the level of statistical significancy) towards improvement of the learning ability of the AE64A-treated rats (FIG. 1A).

The effect of CA-18 was studied in 2 doses: 0.3 and 1 mg/kg (p.o. daily). The lower dose of CA-18 (0.3 mg/kg) completely restored rats' performance in the learning (FIG. 3A) and retention (FIG. 3B) paradigms of the active avoidance test. The higher dose of CA-18 (1 mg/kg) demonstrated a strong tendency (although not

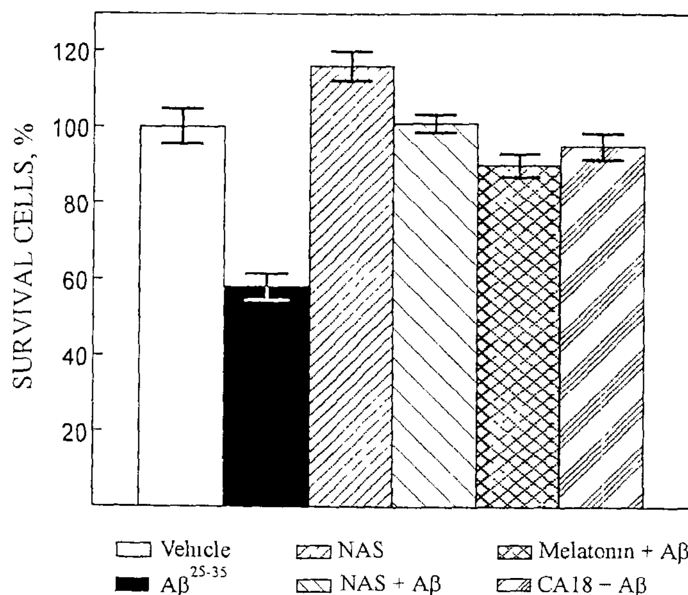

FIGURE 5. Protective effect of *N*-acetylserotonin (NAS), melatonin and CA-15 against the toxicity of βAP 25–35 in mature cultures of cerebellar granule cells. Cultures 8 DIV were treated with vehicle, 25 mM βAP, 25 mM βAP and 25 mM NAS, or 25 mM melatonin, or 25 mM CA-15 for 4 days. The difference in numbers of living neurons before and after 4 days of treatment were determined. The amount of viable neurons is expressed as mean (%) above viable in preliminary photography of the same place. Experiments were repeated 2–3 times (n = 12–18). For graphical presentation, average data from representative experiments are converted to percentages of control group's viability. Results are expressed as a percentage of survival cells to compare with control ± SEM and were analyzed by a statistical test (ANOVA).

reaching the level of statistical significance) towards the improvement of the learning (FIG. 2A) but not in the retention (FIG. 2B) ability of the AF64A-treated rats.

Although we did not plan to perform the special evaluation of the general locomotor activity, we did notice the unspecified sedative effect in rats treated with melatonin (3 mg/kg) starting from the third to fourth day of treatment and in rats treated with NAS (1 mg/kg) starting from the sixth day of treatment.

Melatonin (0.3 mg/kg) and CA-18 (0.3 and 1 mg/kg) did not induce sedation in rats.

Morris Water Maze Test

AF64A significantly increased the response time in comparison to rats treated with the vehicle (CSF) (FIG. 4A and B). Melatonin and CA-18 (0.3 mg/kg daily) decreased the response time of AF64A-treated rats. Only the results observed on day 1 (FIG. 4A) and day 5 (FIG. 4B) are presented.

Neuronal Cell Culture

Earlier it was shown that exposure of mature cultures of CGC against the fragment of βAP reduced the cell viability in a dose-dependent manner (IC_{50} = 25 μM).

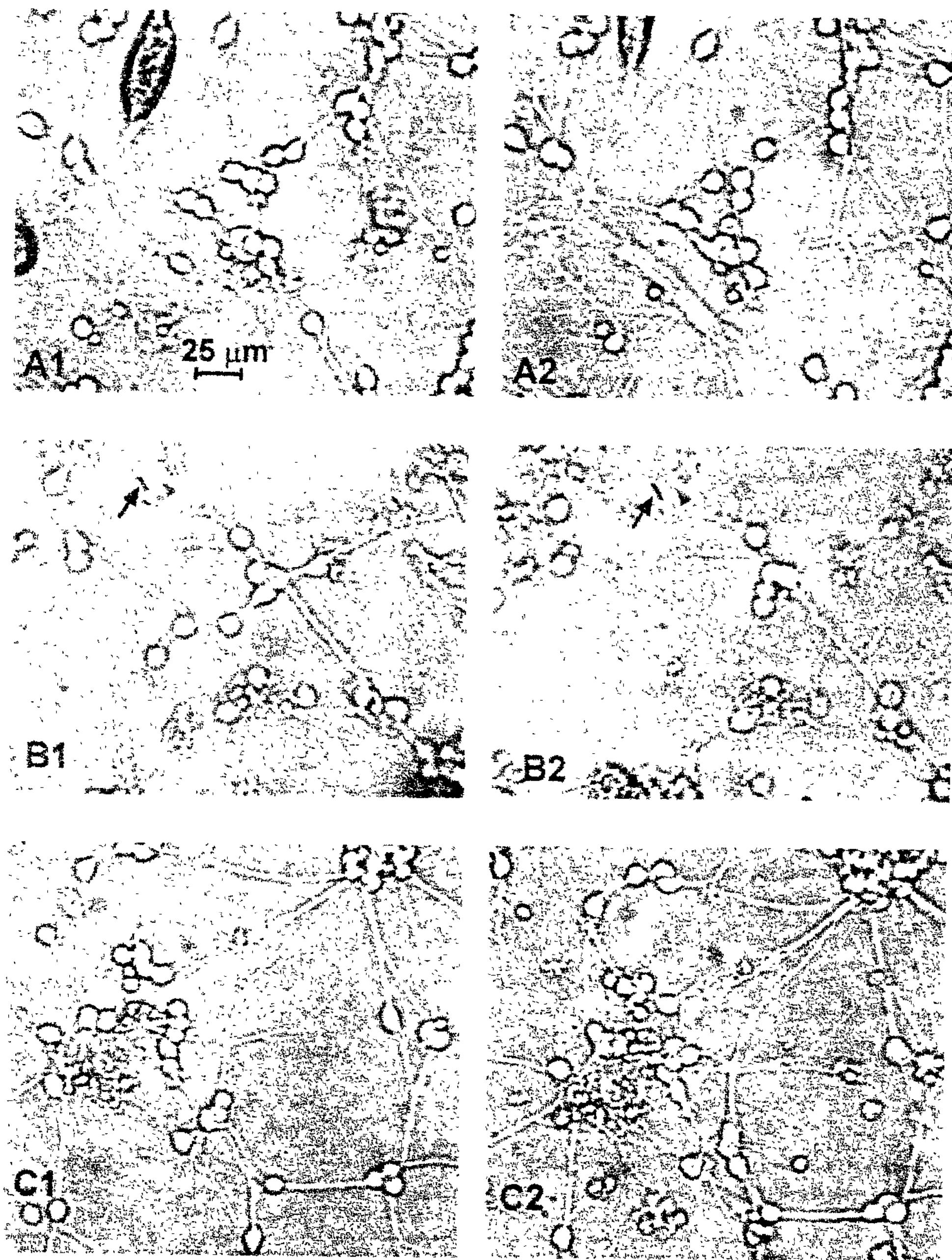

FIGURE 6. Protective effect of *N*-acetylserotonin (NAS) against the toxicity of βAP 25–35 in mature cultures of cerebellar granule cells. Cultures 8 DIV were treated with vehicle **(A1)**, 25 mM βA **(B1)** or 25 mM βA and 25 mM NAS **(C1)** for 4 days, at which time photographs of the same part of the microscope fields were taken **(A2)**, **(B2)** and **(C2)**, respectively. Living cultures, "darkfield" method (*arrowhead* shows the mark on a plate).

Morphological changes (shrinkage of the body and fragmentation of neurites) were observed only when cells were exposed to βAP for 3 days or longer.[10] In the present study we used βAP in concentration of 25 µM to evaluate the ability of the compounds to protect neurons against β-amyloid (βA) neurotoxicity. Incubation of cultures only with NAS or melatonin in the concentration range 25–200 µM increased the amount of living CGCs by 16–28% or 3–10% of control, respectively. The via-

bility of neurons exposed to 25 μM βAP was 58 ± 7% (mean ± SEM) of control, while coincubation of cultures with 25 μM NAS and 25 mM βAP resulted in an increase of viable neurons up to 101 ± 5% of control (FIGS. 5 and 6) in the same conditions. Melatonin and CA-18 at 25 μM protected neurons against 25 μM βAP (90% and 93%, respectively) as well. Melatonin was less effective in preventing βA toxicity compared to NAS (FIG. 5).

DISCUSSION

The obtained results indicate that chronic administration of NAS, melatonin, and their newly synthesized derivatives, CA-15 and CA-18, improve cognitive performance of AF64A-treated rats in active avoidance and in water-maze tests. What is more, these compounds exerted the neuroprotective effect against the βA (25–35)-induced neurotoxicity in the cerebellar granule cell culture. It is known that the neurotoxic action of AF64A related, in part, to oxidative stress, and its indexes are persisting up to 4 months.[11] In this vein, one might suggest that antioxidative properties of melatonin, NAS and, possibly, their derivatives, CA-15 and CA-18, are responsible for their neuroprotective effects. Recent studies indicate that the antioxidant ability of melatonin is inferior in comparison to NAS,[24] and, at least in one model, melatonin exerted a prooxidant effect, while NAS exerted strong antioxidant action.[12] Traditionally, NAS was considered only as the precursor of melatonin in the process of melatonin biosynthesis from serotonin. Very few researchers pointed out the effects of NAS independent from melatonin, i.e., its memory facilitating,[13] hypothermic,[14] analgesic,[15] antihypertensive,[16–18] antidepressant[19] and antioxidative action.[20] NAS, therefore, might be considered not only as melatonin precursor but as endogenous indolamine with its own biological properties. Since about 30% of melatonin is demethylated back into NAS,[21] the antioxidant effect of supraphysiological concentrations of melatonin[22]might be ascribed to NAS formed from melatonin. It is noteworthy that the very first indication of the NAS involvement in the cognitive processes came from the observation that scotophobin A, the memory neuropeptide, increased dark avoidance behavior in goldfish via inhibition of NAS methylation into melatonin.[13]

Under the *in vivo* conditions rapid methylation of NAS into melatonin[23] might limit the effect of NAS. Therefore, the availability of NAS derivatives that would not undergo the *in vivo* transformation into melatonin might be of therapeutic advantage. Our preliminary experiments indicated that systemic administration of CA-15 and CA-18 did not change the rat pineal levels of NAS and melatonin (Oxenkrug & Requintina, unpublished data). Although all studied compounds attenuated the AF64A-induced cognitive impairment, there were noticeable differences between the effects of NAS, melatonin, and their derivatives (TABLE 1). NAS was apparently the weakest among the studied compounds in the active avoidance test, while it was the strongest in the attenuating of βA-induced neurotoxicity. Since NAS is rapidly converted into melatonin in rats,[23] the effect of NAS in our *in vivo* experiments could not be attributed only to NAS but rather to the mixture of NAS and melatonin.

The occurrence of the sedative effect in rats treated with NAS and melatonin, and somewhat delayed appearance of sedation in NAS- than in melatonin-treated rats

suggest that melatonin but not NAS is responsible for the sedative action. The absence of the sedative effect in rats treated with CA-18 might be of therapeutic advantage of the synthetic NAS/melatonin derivatives.

We have found that CA-15 and CA-18 in addition to their positive effect on cognition exerted antihypertensive and antidepressant-like effects.[24] The antidepressant-like activity (decreasing the duration of immobility in the mouse tail suspension test) was more pronounced in CA-18- than in CA-15-treated rats. The combination of cognition-enhancing and antidepressant effect in the one and the same compound might be of additional therapeutic advantage.

The results of our studies warrant the further search of the novel types of safe neuroprotectors among the synthetic NAS/melatonin derivatives.

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