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# Nuclear Localization of Melatonin in Different Mammalian Tissues: Immunocytochemical and Radioimmunoassay Evidence

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**Abstract** Melatonin was detected by an improved immunocytochemical technique in the cell nuclei of most tissues studied including several brain areas, pineal gland, Harderian gland, gut, liver, kidney, and spleen from rodents and primates. Cryostat sections from tissues fixed in Bouin's fluid, formalin, or acetone/ethanol were used. The nuclear staining appeared primarily associated with the chromatin. The nucleoli did not exhibit a positive reaction. The melatonin antiserum was used in the range of 1:500 to 1:5,000. Incubation of the antibody with an excess of melatonin resulted in the complete blockade of nuclear staining. Pretreatment of the sections with proteinase K (200–1,000 ng/ml) prevented the positive immunoreaction. In a second aspect of the study, we estimated the concentration of melatonin by means of radioimmunoassay in the nuclear fraction of several tissues including cerebral cortex, liver, and gut. The subcutaneous injection of melatonin (500 μg/kg) to rats resulted, after 30 min, in a rapid increase in the nuclear concentration of immunoreactive melatonin which varied in a tissue-dependent manner. However, samples collected 3 h after the injection showed that melatonin levels had decreased to control values. Pinealectomy in rats resulted in a clear reduction in the nuclear content of melatonin in the cerebral cortex and liver but not in the gut. The results of these studies suggest that melatonin may interact with nuclear proteins and that the indole may have an important function at the nuclear level in a variety of mammalian tissues. 

**Key words:** cell fractionation, pinealectomy, rat, hamster, mouse, baboon, liver, brain, gut

Melatonin (N-acetyl-5-methoxytryptamine) is an ubiquitously acting indolamine which exhibits daily rhythms in the pineal gland and serum of almost all vertebrates investigated [Reiter, 1991a]. The pineal gland, an end organ of the visual system, translates the photoperiodic message into a chemical signal, melatonin, which serves as a messenger to every organ in the body [Reiter, 1993]. Besides its well-documented effects on reproduction, via an action on the hypothalamic mechanisms which control the release of pituitary hormones [Reiter, 1980], melatonin has been implicated in the modulation of the immune system [Maestroni et al., 1986], to have oncostatic actions [Hill and Blask, 1988; Blask, 1993], and to be a potent antioxidant [Tan et al., 1993a; Reiter et al., 1993a,b,c, Poeggeler et al., 1993]. In the pineal gland, melatonin synthesis is determined primarily by the release, at night, of norepinephrine from nerve endings whose cell bodies are located in the superior cervical ganglia. The release mechanisms of melatonin are poorly understood. Because of its high lipophilicity, melatonin presumably simply diffuses out of the pinealocytes into the circulation where melatonin levels parallel pineal concentrations of the indole [Reiter, 1991b]. Thus, blood melatonin is higher during the dark than during the light phase, and since the light:dark ratio changes on a daily basis, the message that the pineal gland sends to the organism also varies daily and seasonally [Reiter, 1993]. The high lipophilicity of melatonin also permits its rapid transfer from the blood into other fluids such as the cerebrospinal fluid [Hedlund et al., 1976] and saliva [Vakkuri, 1985].

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The existence of high affinity melatonin binding sites associated with presumably pure membrane preparations has been reported [Dubocovich and Takahashi, 1987; Reppert et al., 1988]. In mammals, the most frequently mentioned sites for the localization of membrane-bound melatonin receptors include the suprachiasmatic nuclei, hypothalamus, amygdala, brain stem, area postrema, cerebral cortex, and the pars tuberalis [Krause and Dubocovich, 1991; Stankov et al., 1991]. However, melatonin exerts an important role in other areas, such as the liver, the gut, and the heart, where melatonin receptors have not been fully characterized. Although the 24 h rhythm in circulating melatonin seems to be exclusively dependent on pineal melatonin production, there is evidence suggesting that organs in addition to the pineal gland produce melatonin, Thus, melatonin is known to be synthetized in the retina [Pang and Allen, 1986], the Harderian gland [Menendez-Pelaez et al., 1987], the extraorbital lacrimal gland [Mhatre et al., 1988], and the gut [Huether et al., 1992]. Proof that melatonin is not an exclusive product of the pineal gland also comes from the observation that melatonin is found in invertebrates including *Drosophila* [Finocchiaro et al., 1988] and in a unicellular organism, *Gonyaulax polyedra* [Poeggeler et al., 1991]; in the latter species melatonin exhibits a day/night associated rhythm.

The study of the cellular localization of melatonin has been, to a large extent, conditioned by the available antibodies. However, specific antibodies which show very little reactivity with other indoles have been developed [Kennaway et al., 1977; Arendt et al., 1977]. The possible nuclear localization of melatonin in the retina and pineal gland [Mennenga et al., 1991], the possibility that melatonin influences gene expression [Menendez-Pelaez et al., 1991], and the fact that melatonin inhibits DNA adduct production in the liver of rats induced by the administration of the chemical carcinogen safrole [Tan et al., 1993b] prompted us to explore the possibility of a nuclear localization of melatonin in a variety of organs. With this aim in mind, we have studied the subcellular distribution of melatonin in different mammalian tissues by means of radioimmunoassay after cell fractioning and using improved immunocytochemistry techniques.

## METHODS

### Animals and Tissues

Rats and Syrian hamsters were purchased from Harlan and Sasco, respectively. Mice were obtained from a colony maintain at the University of Texas Health Science Center at San Antonio. Animals were housed in plastic cages under either a 12 h light:12 h dark (rats and mice) or a 14 h light:10 h dark (hamsters) lighting regime. Water and food was provided ad libitum. Animals were sacrificed at different times of the light:dark cycle by decapitation. Tissues and organs including several brain areas, gut, liver, kidney, Harderian glands, and spleen were fixed in different fixatives for immunohistochemistry. At the same time, portions of the same tissues were collected and frozen on solid $CO_2$. They were kept at $-70°C$ until assayed. Serum samples were also collected. Liver and kidney samples from baboons (*Papio cynocephalus*) were kindly provided by Southwest Research Institute (San Antonio, TX).

### Antibodies

One antiserum was raised in sheep against N-acetyl-5-methoxytryptophan conjugated through the side chain to bovine thyroglobulin (batch G/S/ 704-8483; Guildhay, Guildford, UK). The specificity of the antiserum has been tested several times by different groups [Webley et al., 1985; this paper, Table I]. The cross-reactivity with all indoles studied was less than 0.01%. The sensitivity of the assay, at the 95% confidence limits, is 2.5–5 pg/tube using a tritiated tracer with a specific activity of 87 Ci/mmol.

A second antiserum was raised in goat against melatonin conjugated to bovine IgG through a 2-carbon link to the N at position 1 (batch G 280) [Kennaway et al., 1977].

### Immunocytochemical Techniques

Bouin's fluid, formalin, and ethanol/acetone mixture were tested as potential fixatives of tissues for the immunocytochemical demonstration of melatonin. Cryostat sections (6–8 μm) and paraffin sections (10 μm) were used. Both fixation by immersion (for 1–6 h) and postfixation of cryostat sections were tested. Once the sections were placed on poly-I-lysine treated slides, endogenous peroxidase activity was quenched with a 30 min incubation in aqueous 3% $H_2O_2$. After a brief wash in water, sections

**TABLE I. Cross-Reactivity of Tryptophan and Tryptophan Metabolites Tested in the Present Study***

| Substance | Cross-reactivity (%) |
|---|---|
| Tryptophan | <0.000003 |
| Hydroxytryptophan | <0.001 |
| Methoxytryptophan | <0.001 |
| Tryptamine | <0.001 |
| Tryptophol | <0.001 |
| Hydroxytryptamine | <0.001 |
| Hydroxytryptophol | <0.001 |
| Hydroxyindole acetic acid | <0.001 |
| Indole acetic acid | <0.001 |
| Methoxytryptamine | <0.001 |
| Methoxyindole acetic acid | <0.001 |
| Indole propyl acid | <0.001 |
| Indole lactic acid | <0.001 |
| Kynuremin | <0.001 |
| Harmine | <0.001 |
| Pinoline | <0.001 |
| Methoxytryptophol | <0.002 |
| N-acetylserotonin | <0.03 |
| N-acetyltryptophan | <0.1 |
| Hydroxymelatonin | <0.33 |

*The $IC_{50}$ of the corresponding compounds was determined and cross-reactivity is expressed in % of melatonin binding to the antibody ($IC_{50}$ for melatonin is 45 pg/tube).

were equilibrated in 0.05 PBS buffer (pH 7.3). Nonspecific antibody binding was blocked by a 30 min incubation with 3% normal rabbit serum. Sheep anti-melatonin (Guilford) was tested in the following dilutions: 1/500; 1/1,000; 1/2,000; 1/5,000; 1/10,000; 1/20,000; and 1/50,000. Incubations in the primary antibody were performed at 20°C for 6–18 h. After extensive washing in PBS, biotinylated rabbit anti-sheep IgG (Vector Laboratories, Burlingame, CA) was applied for 45–60 min. After being washed, sections were treated for 30–45 min with ABC Kit (Vector) prepared in PBS. Sections were extensively washed in PBS and reacted in 0.5 mg/ml diaminobenzidine and 0.02 M imidazole (pH 7.0). Sections were dehydrated and mounted in Flo-Texx.

As controls, antigen-absorbed antisera and sheep serum were used. All control sections were consistently devoid of any staining as a result of endogenous biotin. Internal negative controls were evident in some tissues, such as the Harderian glands from male and female hamsters when the antibody was used at a dilution of 1:10,000. A minimum of three tissue blocks were prepared from each animal. At least three sections were analyzed from each block.

**Nuclear and cytosolic fraction isolation.** Nuclear and cytosolic fractions were isolated according to methods previously described [Blum and Roberts, 1989]. Basically, tissues kept at −70°C were homogenized in a buffer containing 10 mM Tris-HCl, pH 8, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.3 M sucrose, 0.15% Triton X-100 in a glass/teflon homogenizer (50 mg of tissue per milliliter of buffer). The homogenate was carefully layered over 400 μl of the same buffer, except that it contained 0.4 M sucrose rather than 0.3 M sucrose. The tubes were centrifuged at 2,500*g* for 10 min at 4°C. The supernantant, leaving behind the 0.4 M buffer cushion, was removed and used as the cytosolic fraction. The nuclear pellet was resuspended in Tris-NaOH glycine buffer, and thereafter it was used for melatonin radioimmunoassay.

### Radioimmunoassay

The assay is direct and involves a two-phase counting system. Tissue homogenates (50 μl from a dilution of 25 mg of tissue per ml) were incubated with antiserum and $^3$H-melatonin overnight at 4°C. Dextran coated charcoal was used to separate the bound from the free melatonin. Following centrifugation at 4°C, an aliquot of the supernatant was removed into the scintillant and then counted in a β-scintillation counter. Details of the radioimmunoassay have been previously reported [Webley et al., 1985; Fraser et al., 1983].

Validation of the radioimmunoassay was performed in the nuclear fraction of brain. Melatonin-free rat nuclear homogenate was prepared by charcoal-stripping the homogenate. A standard curve was prepared using buffer; the same curve was made using the melatonin-free homogenate. The results show that the assay mantains linearity and there is no cross-interference with homogenate cellular debris. Also, the rat nuclear homogenate was assayed undiluted and diluted with the zero standard. The results showed that the assay maintains the best linearity at the concentration used in our assay. Validation of gut samples have been previously reported [Poeggeler, 1992].

### DNA Measurement

In order to express the amount of melatonin as pg/mg DNA (nuclear content of melatonin),

DNA was measured using the diphenylanine reaction and a spectrophotometer (Beckman-24).

### Statistical Analysis

Data are expressed as mean ± S.E.M. In order to compare the mean from different groups a one-way analysis of variance (ANOVA) followed by the Student Newman-Keuls test was used.

## RESULTS

### Immunocytochemistry

All tissues studied revealed immunoreactive melatonin which appeared preferentially located in the cell nucleus (Figs. 1–6). Tissue differences were observed. The fixation conditions were not critical for the appearance of the nuclear staining. Thus, both unfixed or fixed tissues resulted in positive staining with the antimelatonin serum. The best tissue preservation without losing the immunoreaction was achieved when using Bouin's fixative. Neutral formalin or alcohol/acetone mixtures also provided acceptable results. When tissues were fixed in toto, fixation should not be extended for more than 6 h. Frozen sections from fresh tissues can also be fixed (1 or 2 min). Different negative controls were used with similar results. The substitution of the primary antibody by buffer, sheep serum, or melatonin antibody incubated with excessive melatonin resulted in an absolute lost of the immunoreactive product. Pineal glands from rats collected at night showed the highest positive staining at all dilutions studied. All tissues studied showed positive reaction when the antibody was used at a dilution of 1:1,000 (Table II). When the dilution was augmented to 1:5,000 only pineal gland, Harderian glands, gut, and cerebellum exhibited a positive reaction. Among the tissues studied, cells with different embryological origin and function showed positive immunoreaction. In brain tissue, both glial cells and neurons showed positive reaction. In the cerebellum a particular strong reaction was observed in both granular cells and Purkinje cells (Fig. 1). In the cerebral cortex and brainstem, some cells presented both cytosolic and nuclear localization of the immunoreactive melatonin (Fig. 2). In the gut no differences between cell types were noted (Fig. 3). In the liver, most hepatocytes presented a clear nuclear staining (Fig. 4). The secretory cells of the Harderian gland showed a strong nuclear staining under most assay conditions (Fig. 5). The tissues studied from baboons also showed an intense nuclear staining of the immunoreactive melatonin as illustrated by the kidney (Fig. 6). When observed under high magnification, the nuclear staining with the antimelatonin serum appears mainly related to the chromatin. No positive reaction was observed in the nucleoli.

Pretreatment of unfixed sections by proteinase K (10, 200, and 2,000 ng/ml) for 10 min at 37°C prevented the nuclear localization of melatonin in a dose-dependent manner (Table III). However, the treatment of the unfixed sections by DNAse I (up to 50 μg/ml) did not substantially modify the nuclear staining. The use of paraffin sections did not yield any positive result.

### Radioimmunoassay

Melatonin was detected by radioimmunoassay in all the nuclear fractions assayed. The concentration ranged from 10–150 pg/mg DNA. Validation of the assay for the cerebral cortex nuclear fraction was performed by precipitating the nuclear content of melatonin with charcoal. Parallelism is shown in Figure 7. The assays maintain linearity when the nuclear homogenate is diluted (the ratio between observed and expected values was 97% at the dilution used in the assay).

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**Fig. 1.** Immunocytochemical demonstration of melatonin in the mouse cerebellum. Purkinje cells (arrows) display immunoreactive melatonin both in the cytosol and in the nucleus. The molecular layer which contains few cells (arrowhead) shows very little immunoreaction. Antibody 1:2,000.

**Fig. 2.** Rat brain stem showing positive reaction to the immunocytochemical method both in the cytosol and nucleus of the large neurons. Notice the lack of staining in the white substance. Antibody used at a concentration of 1:2,000.

**Fig. 3.** Low magnification showing a view of mouse intestine where almost all cells present a nuclear staining with melatonin antibody. The antibody was used at a concentration of 1:2,000.

**Fig. 4.** Rat hepatocytes showing positive immunoreaction to melatonin antibody in the nuclei (arrows). Antibody 1:1,000.

**Fig. 5.** Harderian gland from Syrian hamsters showing a clear nuclear (arrowheads) localization of the immunoreaction. The antibody was used at the very low concentration of 1:5,000.

**Fig. 6.** View of a renal glomerulus from a baboon kidney showing the characteristic nuclear localization of melatonin (arrows). Some cell nuclei from the tubules show also positive reaction (arrowheads). Antibody 1:1,000.

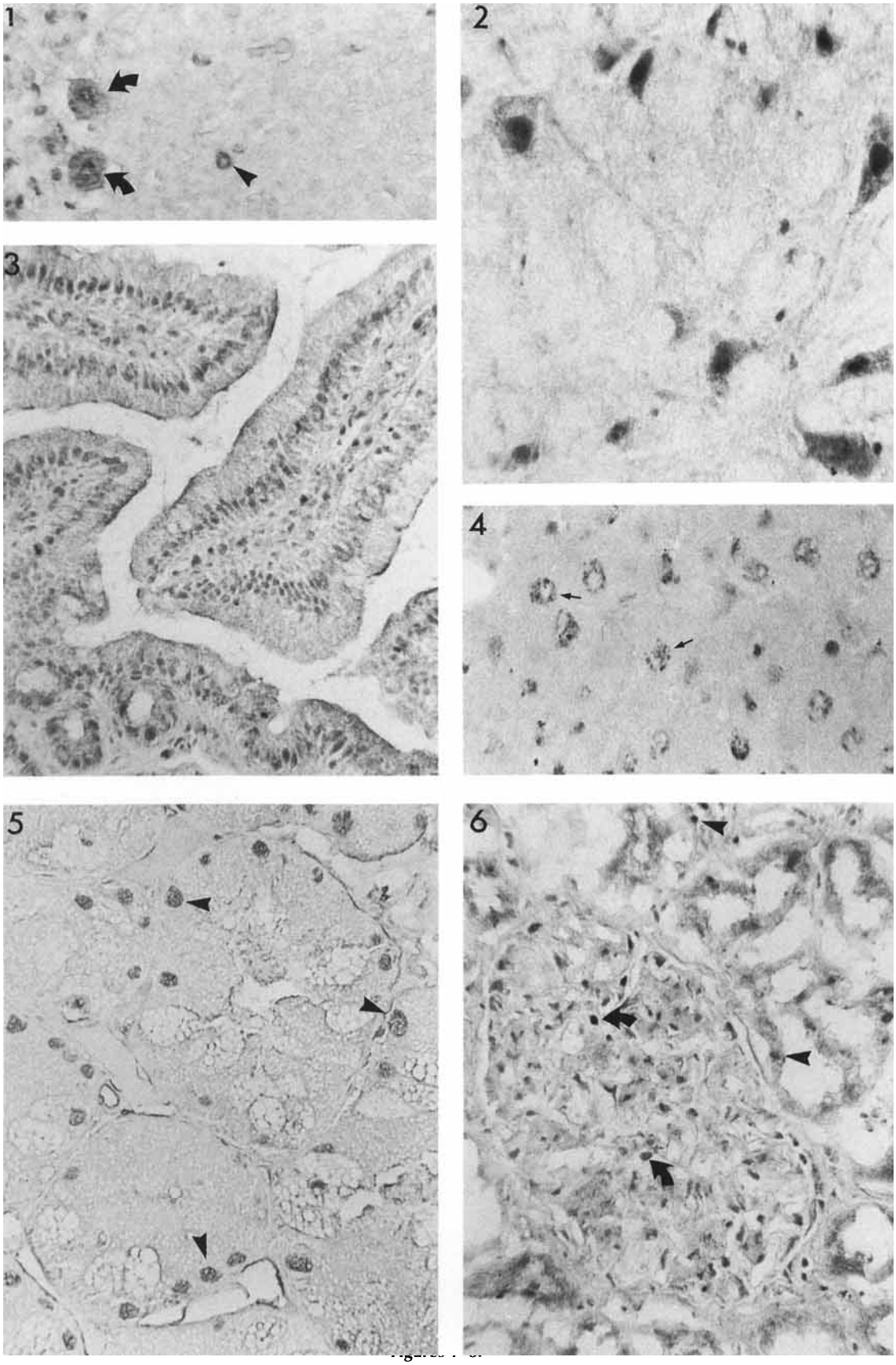
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**TABLE II. Relative Intensity of Nuclear Staining With Guildhay Melatonin Antiserum in Different Tissues of Rats***

| | Dilution | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1:100 | 1:1,000 | 1:2,000 | 1:5,000 | 1:10,000 | 1:20,000 | 1:50.000 |
| HG | +++ | ++ | ++ | +/− | − | − | − |
| Liver | +++ | + | +/− | − | − | − | − |
| Gut | +++ | +++ | ++ | + | − | − | − |
| Kidney | ++ | + | +/− | − | − | − | − |
| Spleen | ++ | +/− | − | − | − | − | − |
| Cerebellum | ++ | + | + | +/− | − | − | − |
| Cortex | ++ | + | +/− | − | − | − | − |
| Pineal | ++++ | ++++ | +++ | ++ | + | +/− | − |

*HG, Harderian gland.

**TABLE III. Effect of Incubation of Brain and Liver Sections From Rats With Different Concentrations of Proteinase K on Nuclear Staining With Melatonin Antiserum (1:1,000)***

| | Proteinase K | | | |
|---|---|---|---|---|
| | 10 ng/ml | 200 ng/ml | 1 μg/ml | 5 μg/ml |
| Brain | +++ | + | − | − |
| Liver | +++ | + | − | − |

*The sections were incubated at 37°C for 10 min.

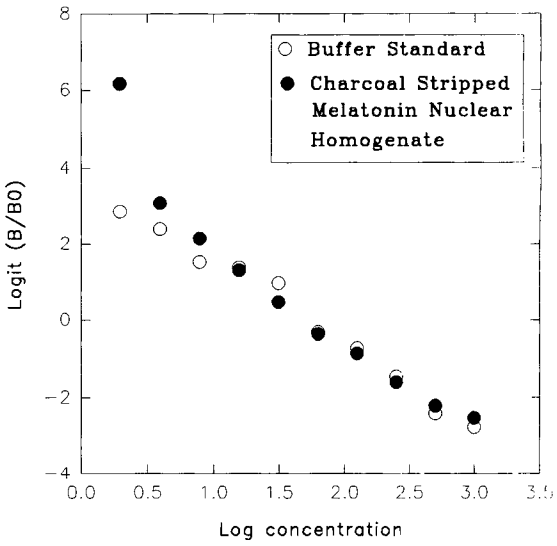


Fig. 7. Validation of the radioimmunoassay in the nuclear fraction of the cerebral cortex. Endogenous nuclear melatonin was precipitated with charcoal. Both the standard curves were performed with buffer or with chacoal-treated nuclear homogenates.

The subcutaneous injection of 500 μg/kg melatonin resulted in a rapid increase in serum levels of the indole (Table IV) within 30 min after the injection. After 90 and 180 min serum samples exhibited a gradual reduction of melatonin levels. By 480 min after the injection, serum melatonin levels had reached control values. Figure 8 represents the increment in immunoreactive melatonin both in serum and in the nuclear fraction of liver and cerebral cortex from rats injected with 500 μg/kg melatonin. After 30 min, the nuclear content of the cerebral cortex and liver increased 8 and 5 times, respectively, relative to levels in control animals that received saline. After 90 min, the nuclear content of melatonin in the cerebral cortex and liver was only 1.5 and 3 times higher, respectively, than control levels. The time-dependent reduction in the nuclear content was slower in the liver than in the cerebral cortex.

In a second experiment, the nuclear content of melatonin was studied in the cerebral cortex, liver, and gut from rats which were killed either in the dark or in the light phase of the light:dark cycle. Also, the effect of pinealectomy and melatonin injection was studied. Figure 9 represents the nuclear content of melatonin in the cerebral cortex of rats killed at 13:00 (during the day) or at 01:00 h (5 h into the dark phase). Melatonin concentration was significantly higher during the night than during the day. Pinealectomy resulted in a dramatic decrease in the nuclear content of melatonin at both time points studied, while the injection of 500 μg/kg, 3 h earlier, restored the levels to control values. The nuclear content of melatonin in the liver followed a different pattern (Fig. 10). No clear day:night differences were observed, but pinealectomy resulted in significant reduction in immunoreactive melatonin levels. The injection of melatonin into pinealectomized animals produced a marked increase in radioimmunoassayable melatonin. The

**TABLE IV. Serum Levels of Immunoreactive Melatonin (pg/ml) After Injection of 500 μg/kg Melatonin**

| | 30 min | 90 min | 180 min | 480 min |
|---|---|---|---|---|
| Control | 45.5 ± 2.9 | 51.5 ± 2.6 | 49.4 ± 5.3 | 52.4 ± 5.3 |
| Melatonin injected | 7,550 ± 1,250 | 1,250 ± 250 | 175 ± 20 | 90 ± 12 |

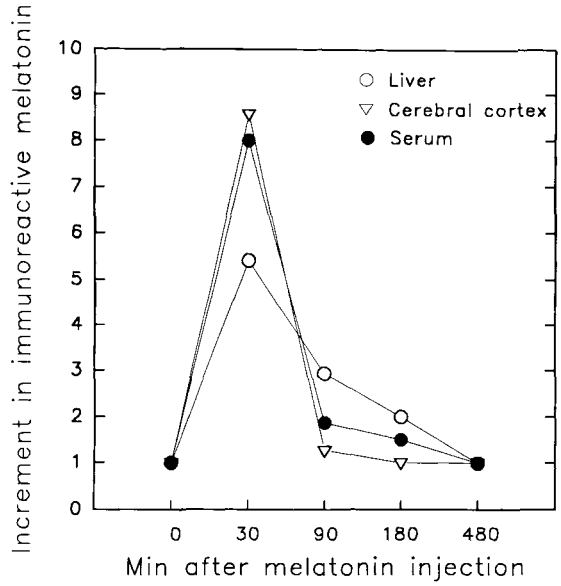


Fig. 8. The subcutaneous injection of 500 μg/kg melatonin resulted, after 30 min, in a marked increase in serum (80 times; the scale for serum is 10 ×) and in the nuclear fraction of cortex (8.5 times) and liver (5 times) relative to levels in animals injected with saline. After 90 min, serum levels were 20 times higher than controls while cortex was 1.5 and liver was 3 times higher than the nuclear fraction of control animals, respectively. After 480 min, the values in the injected animals reached control levels.

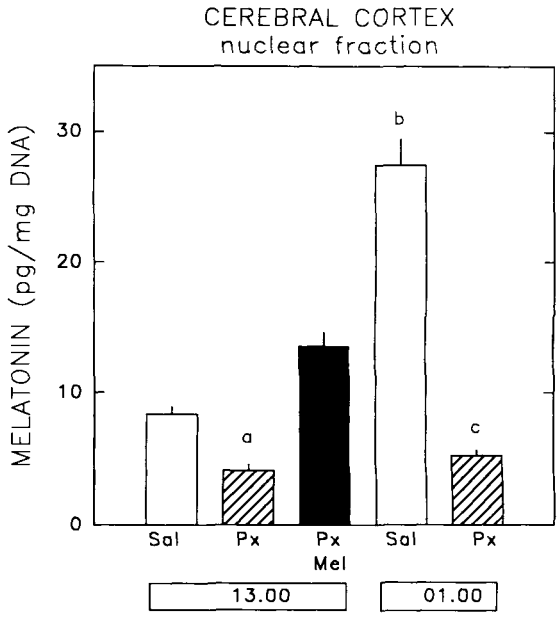


Fig. 9. Immunoreactive melatonin detected by radioimmunoassay in the nuclear fraction of cerebral cortex from rats killed either at 13:00 h or 01:00 h. Px, pinealectomized. Px Mel, pinealectomized, melatonin-injected animals; Sal, saline injected animals. **a:** $P < 0.001$ vs. Sal (13:00 h). **b:** $P < 0.001$ vs. Sal (13:00 h). **c:** $P < 0.001$ vs. Sal (01:00 h).

results obtained in the gut were especially interesting in that, although a day:night rhythm in the nuclear content of melatonin was observed (Fig. 11), this rhythm seemed to be independent of the pineal gland. The injection of melatonin produced a slight, nonsignificant increase in the levels of immunoreactive melatonin detected in the nuclear fraction.

## DISCUSSION

This is the first paper reporting an accumulation of melatonin in the nuclear fraction of cells from different mammalian organs. The immunocytochemical results demonstrated clearly that melatonin is located in the nuclei of almost all tissues and cell systems studied. Through the years, melatonin was thought to be exclusively cytosolic [Bubenik et al., 1976; Vivien-Roels et al., 1981; Meusy-Desolle and Tillet, 1992], but careful examination of some of these pioneering reports revealed a nuclear localization of the immunofluorescence [for a critical review see Menendez-Pelaez and Reiter, in press]. The first evidence for a nuclear localization of melatonin was presented by Mennenga et al. [1990, 1991] while studying the pineal gland and retina of pigeons and rats, organs in which melatonin is known to be produced. In the present study using a much lower concentration of the melatonin antibody (1:2,000 vs. 1:10), we confirm and extend these observations to other tissues. With the use of the immunoprecipitation as a proper control we establish that the immunoreaction corresponds to nuclear melatonin. The intensity of the staining was directly proportional to the concentration of the antibody used. Thus, in those tissues with a very high melatonin content such as the pineal gland, the gut, and the Hard-

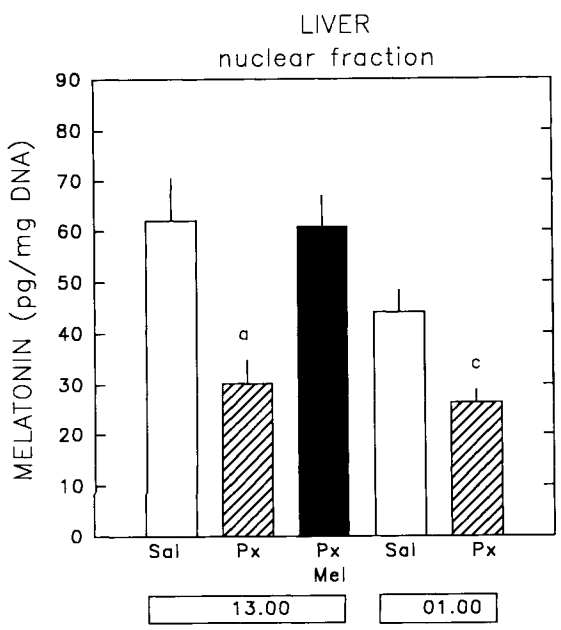


Fig. 10. Melatonin concentrations in the nuclear fraction of rat liver. **a:** $P < 0.05$ vs. Sal (13:00 h). **c:** $P < 0.05$ vs. Sal (01:00 h). Abbreviations are as in Figure 9.

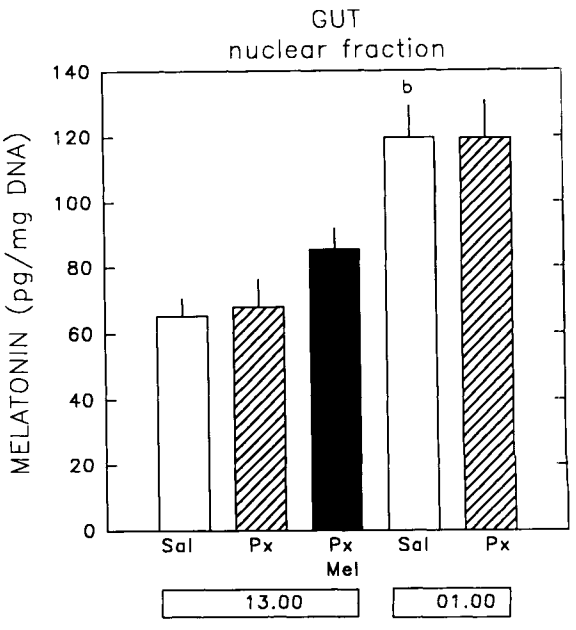


Fig. 11. Melatonin concentrations in the nuclear fraction of the gut from rats. **b:** $P < 0.05$ vs. Sal (13:00 h). Abbreviations are as in Figure 9.

erian glands, a positive reaction was achieved even when extremely low concentrations of the antibody were used [1:5,000–1:10,000]. Although a cytosolic localization of melatonin was observed in some cells such as the large neurons of the brain stem and the cerebellar Purkinje cells, in most cases, an exclusive nuclear localization was found. Radioimmunoassay studies revealed that some melatonin is also found in the cytosolic fraction [Menendez-Pelaez and Reiter, 1993]. This apparent discrepancy may be explained by the fact that nuclear melatonin may be bound to a protein while the cytosolic melatonin is not bound, and therefore it is lost during tissue preparation for immunocytochemistry.

The possible localization of melatonin in the chromatin has been recently proposed [Mennenga et al., 1991]. Our observations corroborate this suggestion. As in the situation for androgen receptor immunodetection, the nucleoli were unstained [Hild-Petito et al., 1991]. The fact that pretreatment of the sections with proteinase K completely abolished the staining while treatment with DNAse caused no apparent change in immunocytochemical localization of melatonin strongly suggests that melatonin binds to a nuclear protein. Binding studies performed in our laboratory have revealed the existence of nuclear binding sites for melatonin in the liver of rats (D. Acuña-Castroviejo, A. Menendez-Pelaez, M.I. Pablos, and R.J. Reiter, unpublished).

The radioimmunoassay results also suggest a strong uptake of melatonin by the nuclei in the liver and cerebral cortex. We also have observed that the melatonin content of the nuclear fraction from other tissues such as the lung, spleen, kidney, and the Harderian glands is increased after melatonin administration [A. Menendez-Pelaez, M.I. Pablos, and R.J. Reiter, unpublished]. The time course study demonstrated that melatonin uptake by the nucleus parallels serum concentrations (Table IV; Fig. 8). However, while the cerebral cortex is loaded rapidly and shows very rapid clearance of melatonin, the liver accumulates melatonin for a longer period of time. This might be one of the reasons for the existence of tissue-associated differences observed in the second experiment. Thus, the cerebral cortex seems to be highly dependent on melatonin produced by the pineal gland, as is shown by the drastic reduction in the nuclear melatonin after pinealectomy. Also, the small increase observed in the pinealectomized-melatonin injected animals after 3 h is in agreement with the rapid reduction in nuclear melatonin observed in the first experiment.

The melatonin content of the hepatocyte nuclei also seems to depend to a large extent on

melatonin derived from the pineal gland. Contrary to the situation found in the cerebral cortex, however, no differences were observed in animals killed at 01:00 (in the light) and at 13:00 h (in the dark). This result should be carefully interpreted. Since the uptake rate by the liver nuclear fraction is slower than that of the cerebral cortex, it may be possible that at 01:00 h the nuclear melatonin content of the liver did not yet reach its peak. On the contrary, at 13:00 h the melatonin that physiologically was accumulating during the night hours may still appear in the nuclear fraction.

The third organ studied represented a different case. It is now known that the gut produces melatonin [Huether et al., 1992; Yaga et al., 1993]. In the present study, the nuclear content of melatonin appeared to be independent of the pineal gland. However, day:night differences, not mediated by the pineal gland, were detected. This phenomenon might be explained by the feeding behavior of the rat; the bulk of feeding occurs, in nocturnal animals such as the rat, at night. An increased intake of tryptophan has been shown to produce large increments in gut melatonin content [Huether et al., 1992; Yaga et al., 1993].

It is clear from these studies that, although the pineal gland is an important source of melatonin in terms of its accumulation in other organs, there is some melatonin that is not of pineal origin since pinealectomized animals showed detectable levels of the indole in the nuclear fraction. Presumably this melatonin is released from the gut or from other melatonin-producing organs, although it could be also locally produced. The observations advance the concept of a nuclear action of melatonin. Heretofore, it was generally felt that melatonin acts more or less exclusively via specific membrane binding sites located at rather discrete locations throughout the organism [Krause and Dubocovich, 1991]. However, the importance of membrane "receptors" has been questioned [Kennaway and Hugel, 1992], and many of the actions of melatonin are not readily explained on the basis of the rather small number of membrane receptors that have been described [Reiter, 1991a]. On the other hand, genomic effects of melatonin have been proposed in tissues as different as the adrenal glands and the Harderian glands [Menendez-Pelaez et al., 1991; Persengiev et al., 1991]. The results described herein provide a further justification for this suggestion since melatonin appears to have specific nuclear binding sites.

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