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Detection of Melatonin, Its Precursors and Related Enzyme Activities in Rabbit Lens

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Melatonin (*N*-acetyl-5-methoxytryptamine) was detected in extracts of albino rabbit lens using radioimmunoassay. Furthermore, melatonin precursors, tryptophan and serotonin (5-hydroxytryptamine), were found in the lens extracts by high-performance liquid chromatography coupled with fluorometric detection. Also, activities of two melatonin-synthesizing enzymes, serotonin *N*-acetyltransferase (NAT) and hydroxyindole-*O*-methyltransferase (HIOMT), were found in the lens. The apparent Michaelis constants (*K*m) for substrates of NAT in the lens were similar to those reported for the pineal gland, although the apparent *K*m values for substrates of HIOMT in the lens were 10-fold higher than those in the pineal gland. When the rabbits were entrained to a 14-hr light : 10-hr dark cycle, melatonin levels and NAT activity in the lens showed significant day/night changes with high levels during the dark period, but HIOMT activity did not show these changes. These findings strongly suggest that the rabbit lens may synthesize melatonin from serotonin by the sequential action of NAT and HIOMT, and that the melatonin synthesis may fluctuate in a diurnal and/or circadian manner.

Key words: melatonin; serotonin; lens; serotonin *N*-acetyltransferase; hydroxyindole-*O*-methyltransferase.

1. Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine), an indoleamine originally found in the pineal gland, is synthesized enzymatically from serotonin (5-hydroxytryptamine) by the sequential action of serotonin *N*-acetyltransferase (acetyl CoA: arylalkylamine *N*-acetyltransferase, EC 2.3.1.87; NAT) and hydroxyindole-*O*-methyltransferase (*S*-adenosyl-L-methionine: *N*-acetylserotonin *O*-methyltransferase, EC 2.1.1.4; HIOMT) (Axelrod, 1974; Namboodiri, Dubbels and Klein, 1987; Sugden, Ceña and Klein, 1987). The synthesis and release of melatonin display a circadian rhythm that is entrained to environmental light:dark cycles (Binkley, 1993; Namboodiri et al., 1987; Tarmarkin, Baird and Almeida, 1985). It is now widely accepted that melatonin is an endogenous mediator of photoperiodic information and a molecular component of circadian time keeping system (Brzezinski, 1997; Dollins et al., 1994; Namboodiri et al., 1987; Reiter, 1993).

Melatonin and its synthesizing enzymes have also been found in the extrapineal organs such as the eye (Heuther, 1993; Tosini and Menaker, 1996), ovary (Itoh et al., 1997a) and Harderian gland (Djeridane et al., 1998). In the eye, melatonin is detectable in the retina, iris, ciliary body and aqueous humor (Pang et al., 1980; Rohde, McLaughlin and Chiou, 1985; Yu et al., 1990), and NAT and HIOMT are present in the retina (Binkley, Hryshchyshyn and Reilly, 1979; Quay, 1965). In addition, retinal melatonin synthesis fluctuates in a circadian fashion (Cahill, 1991; Tosini and Menaker, 1996). It has been shown that retinal melatonin functions as a local regulator of various aspects of rhythmic activities, such as rod photoreceptor disk shedding (Besharse and Dunis, 1983) and dark-adaptive retinomotor movements (Dierce and Besharse, 1985).

Recently, it has been demonstrated that melatonin functions as an antioxidant or free radical scavenger (Reiter et al., 1997a, b). Treatment with buthionine sulfoximine, an inhibitor of glutathione synthesis, increases lipid peroxidation in the lens (Li et al., 1997) and induces cataracts (Abe et al., 1994); melatonin has an anticataract function (Abe et al., 1994) and reduces lipid peroxidation (Li et al., 1997). Thus, it is possible that in the lens, melatonin may function as an antioxidant or free radical scavenger. However, it is not known whether melatonin and its synthesizing-enzymes are present in the lens. We report now that melatonin, its precursors and the activities of NAT and HIOMT are present in the rabbit lens. In addition, the melatonin levels and NAT activity exhibit significant day/night changes with high levels during the dark period of a 24-hr light/dark cycle.

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2. Materials and Methods

Chemicals

All reagents used were of the highest purity available. Melatonin, its structurally related compounds, acetyl CoA and *S*-adenosyl-L-methionine were purchased from Sigma (St. Louis, MO, U.S.A.). *N*-acetyltryptamine was synthesized from tryptamine and acetic anhydride (Itoh et al., 1995). Dibutylamine phosphate was prepared from dibutylamine and phosphoric acid (Lee Chin, 1992). The dye reagent used for protein assay was obtained from Bio-Rad.

Animals

Female albino rabbits (1·5–2·0 kg body weight) were obtained from a domestic distributor and entrained to a 14-hr light: 10-hr dark cycle (light on at 6:00) for a minimum of 1 week before use.

Animals were handled under conditions conforming to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. During the dark period (24:00–01:00) and the light period (12:00–13:00), the animals were anesthetized by administration of an overdose of pentobarbital. Eyes were enucleated, and the lenses were carefully removed using a posterior approach and rinsed with 6·3 mM EDTA phosphate buffer. After absorption of excess buffer, lenses were immediately frozen on solid CO_2. The samples were kept at -70°C before use. Sampling during the dark phase was performed under dim red light. The wet tissue weight (mean $\pm$ S.E.M., $n = 7$) of the lens dissected during the dark and the light period was $0{\cdot}348 \pm 0{\cdot}056$ g and $0{\cdot}344 \pm 0{\cdot}022$ g, respectively. There was no significant difference between the two groups.

Melatonin Radioimmunoassay (RIA)

Lenses were homogenized with a glass homogenizer in an ice-cold 1:5 (w/v) volume of 0·9% KCl. Homogenates were extracted with ice-cold 1:6 (v/v) diethyl ether. After centrifugation at 3000 *g* for 10 min, the samples were kept at -15°C for 60 min and -70°C for 30 min. Diethyl ether phase was decanted to borosilicate glass tube and dried in N_2 gas stream. The melatonin level of samples was determined with RIA using ^{125}I-labeled melatonin and the Kennaway G280 antimelatonin antibody (RIA kit, Bühlmann Laboratories AG, Allschwil, Switzerland) (Vaugham, 1993). After reconstitution in working buffer, 100 μl aliquots of each sample were mixed with 100 μl (about 10000 dpm) of ^{125}I-melatonin and 100 μl of rabbit antiserum against melatonin. After incubation overnight at 4°C, anti-rabbit gamma globulin and latex solution were added. The mixture was incubated for 15 min at room temperature and centrifuged at 3000 *g* for 10 min at 4°C. After aspiration of the supernatant, the radioactivity of the pellet was determined with a gamma counter.

High-performance Liquid Chromatography (HPLC) for Serotonin and Tryptophan Determination

The presence of serotonin, tryptophan and their metabolites was examined by the method described previously (Lee Chin, 1992) with modification. The lens was homogenized with polytron in an ice-cold 1:1 (w/v) volume of 0·15 M $HClO_4$ containing 0·025% each of cysteine and disodium EDTA. The homogenates were centrifuged at 12000 *g* and 4°C for 10 min. The resulting supernatant was filtered through an 0·45 μm filter and the filtrate (50 μl) was subjected to a HPLC system equipped with a Capcell Pak C18 UG80 S-5 μm column ($250 \times 4{\cdot}6$ mm i.d.) (Shiseido, Tokyo, Japan) and a fluorometric detector, RF-550 (Shimadzu, Kyoto, Japan). The detector was used with excitation and emission wavelengths set at 285 and 345 nm, respectively. The mobile phase consisted of 9·5% acetonitrile (v/v), with the aqueous component containing 12·16 mM citric acid, 11·60 mM $(NH_4)_2HPO_4$, 2·54 mM sodium octylsulphonate, 3·32 mM dibutylamine phosphate and 1·11 mM disodium EDTA. The mobile phase was pumped at a flow rate of 1·0 ml min^{-1} and 30°C. Peaks were identified according to the retention times.

NAT Activity Assay

NAT activity in the lens was assayed by measuring the amount of *N*-acetyltryptamine formed from tryptamine and acetyl CoA (Itoh et al., 1995). The lenses were homogenized in various volumes of ice-cold 0·25 M potassium phosphate buffer (pH 6·5) dependent on the sample and protein concentration. Homogenates were centrifuged at 12000 *g* for 10 min at 4°C. Aliquots of 50 μl of the supernatant were mixed with 25 μl of 4·2 mM acetyl CoA and 25 μl of 8·0 mM tryptamine in 0·25 M potassium phosphate buffer (pH 6·5) and incubated for various times at 37°C. The enzymatic reaction was stopped with 20 μl of 6M $HClO_4$. After centrifugation at 12000 *g* at 4°C for 10 min, the supernatant (1–5 μl) was subjected to the HPLC system with a Superiox ODS S-5 μm column ($150 \times 4{\cdot}6$ mm, i.d.) (Shiseido, Tokyo, Japan) and the fluorometric detector. The detector was used at an excitation wavelength of 285 and an emission wavelength of 360 nm. The mobile phase consisted of 50 mM phosphoric acid, 30% methanol (v/v), and 0·65 mM sodium octylsulphonate adjusted to pH 3·5 using NaOH. The flow rate was 1·0 ml min^{-1} at 30°C. Peaks were identified by retention time and *N*-acetyltryptamine was quantified by peak height. For the controls, the mixtures were incubated without tryptamine or without an enzyme source, and then analyzed using HPLC. For kinetic analysis, various

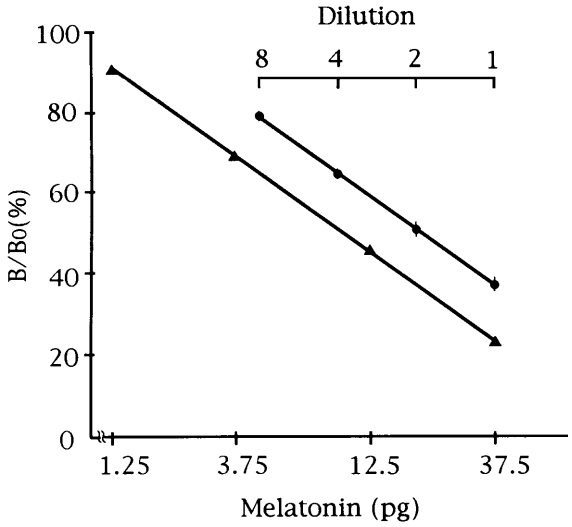

FIG. 1. The inhibition curve of melatonin standard (▲) and the melatonin extracted from the rabbit lens dissected during the dark period (●). Each point and the vertical line represent the mean ± S.E.M. ($n = 4$). For experimental conditions, see Materials and methods.

concentrations of acetyl CoA and tryptamine were used.

HIOMT Activity Assay

HIOMT activity was assayed by measuring the amount of melatonin formed from *N*-acetylserotonin and *S*-adenosyl-L-methionine (Itoh, Hattori and Sumi, 1997b). The lenses were homogenized in various volumes of ice-cold 0·05 M sodium phosphate buffer (pH 7·9) dependent on the sample and protein concentration. Homogenates were centrifuged at 12 000 *g* at 4°C for 20 min. Aliquots (55 μl) of the supernatant were mixed with 25 μl of 3·2 mM *N*-acetylserotonin and 3·2 mM *S*-adenosyl-L-methionine in 0·05 M sodium phosphate buffer (pH 7·9) and incubated for various times at 37°C. The enzymatic reaction was stopped by the addition of 20 μl of 6 M $HClO_4$. After centrifugation at 12 000 *g* at 4°C for 10 min, the supernatants (40–50 μl) were subjected to the same HPLC system as that described in the NAT assay. The fluorometric detector was used at excitation and emission wavelengths of 280 and 340 nm, respectively. The mobile phase consisted of 0·05 M ammonium acetate and 20% methanol (v/v) adjusted to pH 4·3 by acetic acid. The flow rate was 1·0 ml min^{-1} at 30°C. Peaks were identified according to the retention times and melatonin was quantified according to peak height. For the controls, the reaction mixtures were incubated either without *N*-acetylserotonin or without enzyme. For the kinetic analysis, *S*-adenosyl-L-methionine and *N*-acetylserotonin were used at various concentrations.

Protein Assay

Protein concentration was determined by a dye-binding method (Bradford, 1976) with BSA as the standard.

Statistical Analysis

Data are expressed as the mean ± S.E.M. Statistical differences between group means were uncovered using ANOVA followed by the Student's *t*-test. The Michaelis constants (*K*m) were determined by linear regression analysis.

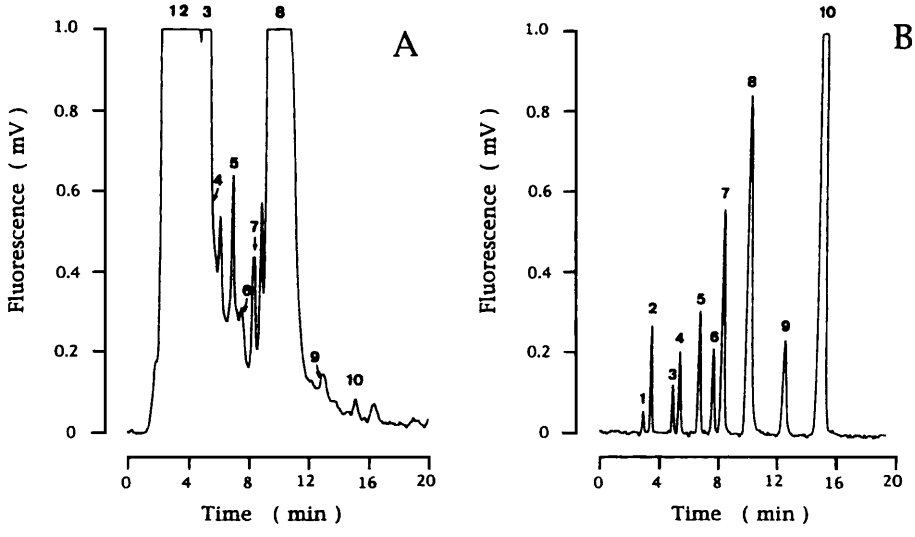

FIG. 2. Representative HPLC chromatograms for tryptophan and serotonin determination of (A) rabbit lens and (B) a standard containing 10 ng of each compounds. The numbers in the figure indicate the elution positions of the following compounds: (1) tyrosine; (2) 4-hydroxy-3-methoxyphenylethylene glycol; (3) norepinephrine; (4) epinephrine; (5) homovanillic acid; (6) dopamine; (7) 5-hydroxyindole-3-acetic acid; (8) tryptophan; (9) 3-methoxytyramine; (10) serotonin. A single lens dissected during the dark period was homogenized in 200 μl of ice-cold $HCLO_4$ containing 0·025% each of cysteine and disodium EDTA. For other experimental conditions, see Materials and methods.

3. Results

Melatonin-immunoreactivity was detected in the rabbit lens extracts using radioimmunoassay (Fig. 1). Displacement of serially diluted samples was parallel to that of the authentic melatonin standard.

In rabbit lens extracts, peaks with retention times identical to those of tryptophan and serotonin, which are melatonin precursors, were found by HPLC with fluorometric detection (Fig. 2). On the HPLC chromatogram of Fig. 2(A), peaks with identical retention times to those of other compounds, such as norepinephrine and 5-hydroxyindole-3-acetic acid, were also found in the extracts.

When rabbit lens homogenates were incubated with tryptamine and acetyl CoA, an *N*-acetyltryptamine peak was detected using HPLC analysis [Fig. 3(A)]. No *N*-acetyltryptamine peak was detected in the control incubations without tryptamine [Fig. 3(B)] or without enzyme source (data not shown). The NAT activity proceeded linearly for 30 min at 37°C [Fig. 4(A)], and showed linearity with the amounts of protein in the lens (1·85–5·55 mg) [Fig. 4(B)]. Kinetic analysis of rabbit lens NAT for its substrates, acetyl CoA and tryptamine, was performed, and the apparent *K*m values determined from Lineweaver-Burk plots were 0·324 mM and 2·19 mM, respectively (Fig. 5).

In the HPLC chromatogram obtained from the reaction mixture of the lens homogenate with *N*-acetylserotonin and *S*-adenosyl-L-methionine, a peak

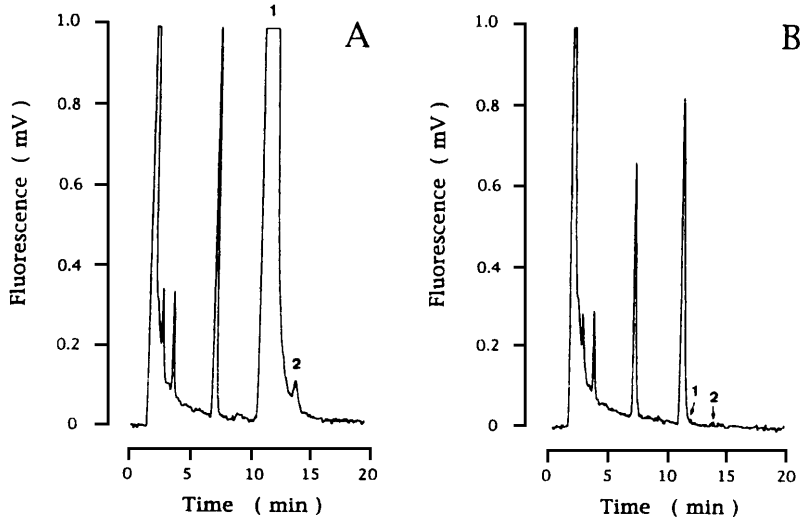

FIG. 3. Representative HPLC chromatograms of (A) the reaction mixture of the homogenates of the rabbit lens with acetyl CoA and tryptamine and (B) the reaction mixture with acetyl CoA and without tryptamine. Indicated the elution positions: (1) tryptamine; (2) *N*-acetyltryptamine. A single lens dissected during the dark period was homogenized in 200 μl of ice-cold 0·25 M potassium phosphate buffer (pH 6·5). After centrifugation, 75 μl of the resulting supernatant was assayed for NAT activity (20 min incubation at 37°C). For other experimental conditions, see Materials and methods.

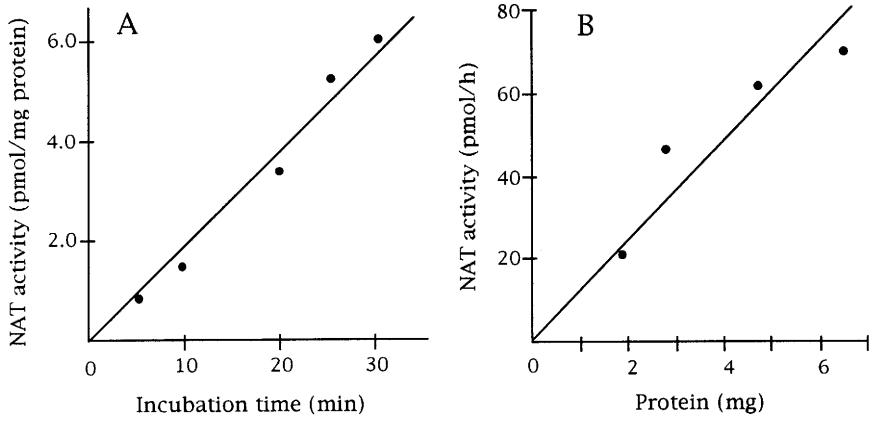

FIG. 4. (A) Time course of *N*-acetylation of tryptamine using rabbit lens homogenate as the enzyme source. Two lenses dissected during the dark period were homogenized in 1·0 ml of ice-cold 0·25 M potassium phosphate buffer (pH 6·5). After centrifugation, the resulting supernatant (protein concentration 111 $\mu g\ \mu l^{-1}$) was used as the enzyme source. The enzyme reaction was carried out for various times at 37°C. (B) Effects of protein concentration of rabbit lens on the amount of enzymatically formed *N*-acetyltryptamine. Two lenses dissected during the dark period were homogenized in 600 μl of ice-cold 0·25 M potassium phosphate buffer (pH 6·5). After centrifugation, the resulting supernatant (protein concentration 185 $\mu g\ \mu l^{-1}$) was diluted at different rations within the homogenization buffer and assayed for NAT activity (20 min incubation at 37°C).

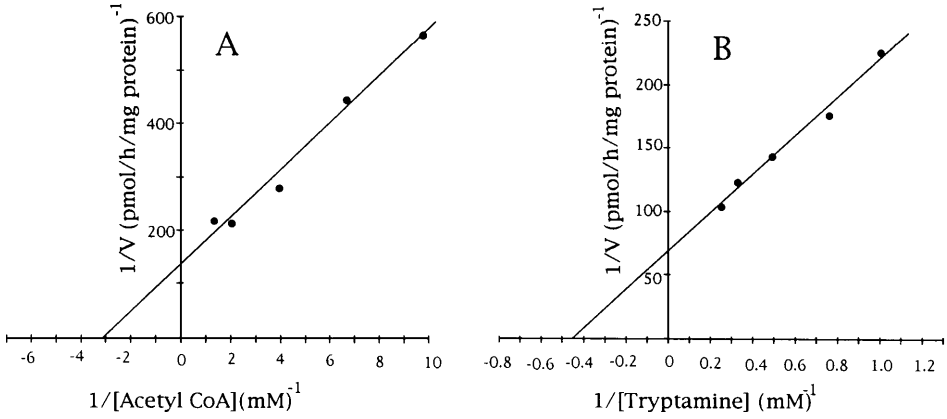

FIG. 5. Lineweaver-Burk plots of the rabbit lens NAT for (A) acetyl CoA and (B) tryptamine. Two lenses dissected at dark period were homogenized in 900 μl of ice-cold 0·25 M potassium phosphate buffer (pH 6·5). After centrifugation, the supernatant (protein concentration 189 μg μl^{-1}) was assayed for NAT activity (20 min incubation at 37°C). For the determination of acetyl CoA *K*m, the tryptamine concentration was fixed at 1·0 mM. For the tryptamine *K*m determination, the acetyl CoA concentration was 1·0 mM.

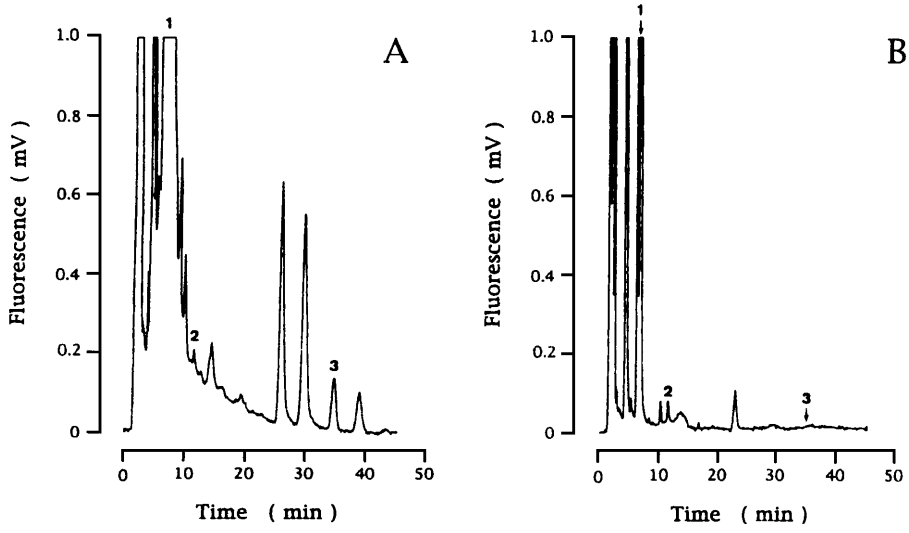

FIG. 6. Representative HPLC chromatograms of (A) the reaction mixture of the rabbit lens homogenate with *N*-acetylserotonin and *S*-adenosyl-L-methionine and (B) the reaction mixture with *S*-adenosyl-L-methionine and without *N*-acetylserotonin. Indicated the elution position: (1) *N*-acetylserotonin, (2) 6-hydroxymelatonin, (3) melatonin. A single lens dissected during the dark period was homogenized in 200 μl of ice-cold 0·05 M sodium phosphate buffer (pH 7·9). After centrifugation, the resulting supernatant was assayed for HIOMT activity (60 min incubation at 37°C). For other experimental conditions, see Material and methods.

with an identical retention time to that of melatonin was detected [Fig. 6(A)]. After incubation without *N*-acetylserotonin in the control, the melatonin peak was not detected [Fig. 6(B)]. However, in the control, a peak with an identical retention time to that of *N*-acetylserotonin, which is the direct melatonin precursor, were detected [Fig. 6(B)], suggesting that the rabbit lens contains *N*-acetylserotonin. Also, in the control, a peak with an identical retention time to that of 6-hydroxymelatonin, which is the melatonin metabolite, was detected [Fig. 6(B)]. The HIOMT activity detected as formed melatonin increased linearly for 60 min at 37°C [Fig. 7(A)]. The amounts of melatonin formed clearly showed linearity with the amounts of protein in the lens (1·85–7·60 mg) [Fig. 7(B)]. A kinetic analysis for substrates of HIOMT, *S*-adenosyl-L-methionine and *N*-acetylserotonin, was performed. The *K*m values for *S*-adenosyl-L-methionine and *N*-acetylserotonin were 0·328 mM and 0·299 mM, respectively (Fig. 8).

When the rabbits were maintained in a 14-hr light: 10-hr dark cycle, the melatonin levels and NAT activity in the lenses were significantly higher during the dark period than during the light period [Figs 9(A) and 9(B). However, no significant day/night change was found in HIOMT activity [Fig. 9(C)].

4. Discussion

The result of this study shows that melatonin is present in the rabbit lens (Fig. 1). Furthermore, the results suggest that the rabbit lens may synthesize

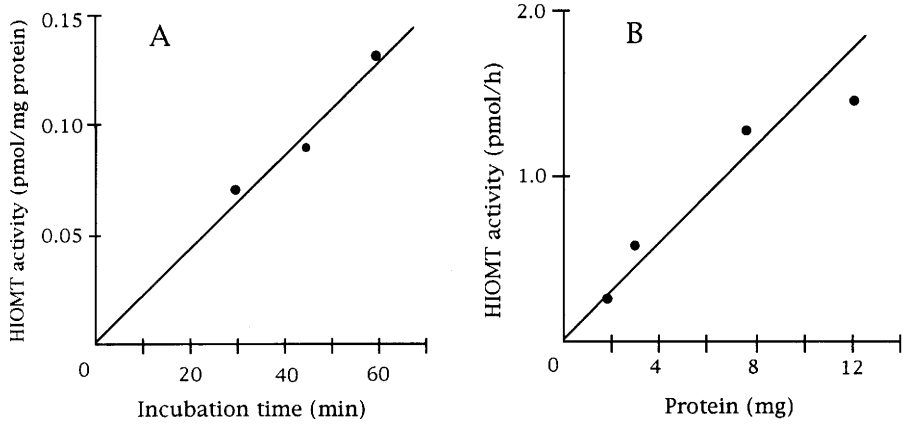

FIG. 7. (A) Time course of *O*-methylation of *N*-acetylserotonin using rabbit lens homogenate as the enzyme source. Two lenses dissected during the dark period were homogenized in 1·3 ml of ice-cold 0·05 M sodium phosphate buffer (pH 7·9). After centrifugation, the resulting supernatant (protein concentration 84·1 μg μl^{-1}) was used as enzyme source. The enzymatic reaction was carried out for various times at 37°C. (B) Effects of protein concentration of rabbit lens on the amount of enzymatically formed melatonin. Two lenses dissected during the dark period were homogenized in 600 μl of ice-cold 0·05 M sodium phosphate buffer (pH 7·9). After centrifugation, the resulting supernatant (protein concentration 185 μg μl^{-1}) was diluted at different ratios with the homogenization buffer and assayed for HIOMT activity (60 min incubation at 37°C).

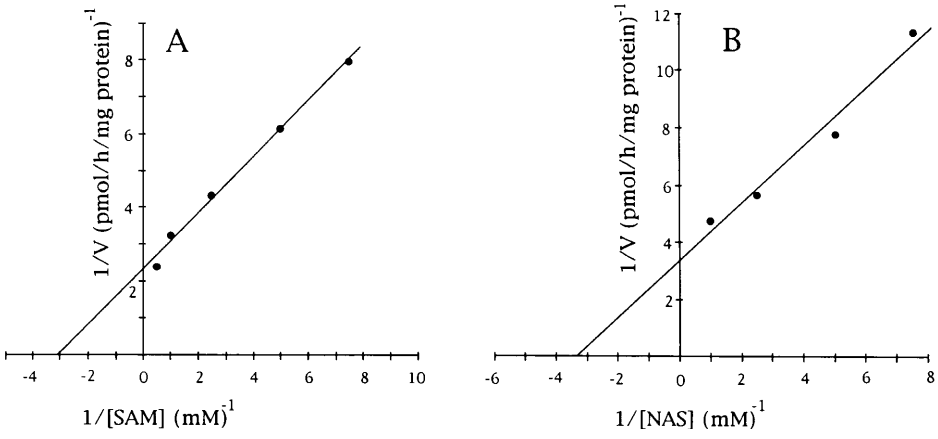

FIG. 8. Lineweaver-Burk plots of the rabbit lens HIOMT for (A) *S*-adenosyl-L-methionine (SAM) and (B) *N*-acetylserotonin (NAS). Two lenses dissected during the dark period were homogenized in 1·3 ml of ice-cold 0·05 mM sodium phosphate buffer (pH 7·9). After centrifugation, the supernatant (protein concentration 125 μg μl^{-1}) was assayed for HIOMT activity (60 min incubation at 37°C). For the SAM *K*m determination, the NAS concentration was fixed at 1·0 mM. For the determination of the NAS *K*m, the SAM concentration was 1·0 mM.

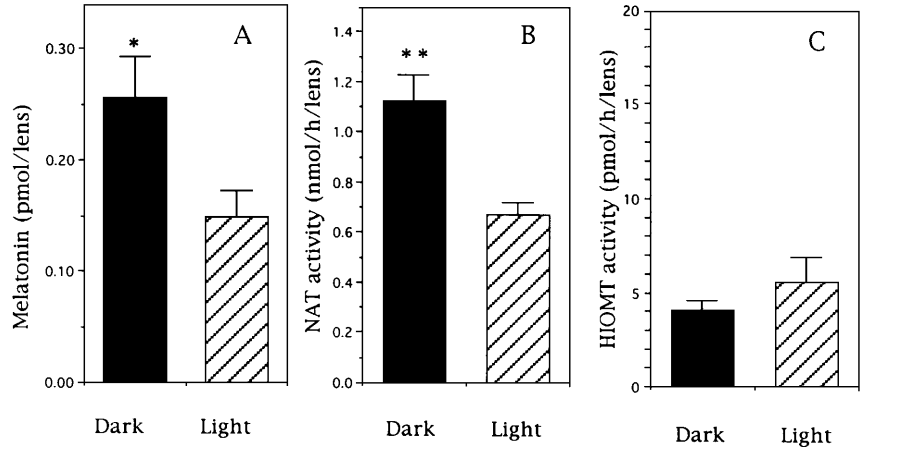

FIG. 9. Day/night changes in (A) melatonin levels, (B) NAT activities, and (C) HIOMT activities in the lens of rabbits kept under a 14-hr light:10 hr dark cycle. Each bar and vertical line show the mean ± S.E.M. (n = 6–8). $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$.

melatonin from serotonin by the sequential action of NAT and HIOMT for the following reasons: (1) serotonin was detected in the rabbit lens homogenates (Fig. 2); (2) NAT and HIOMT activities were detected in the rabbit lens homogenates (Figs 3–8); (3) the apparent *K*m values for substrates of NAT in the rabbit lens were similar to those reported for the pineal gland (Fig. 5) (Heim et al., 1991; Namboodiri et al., 1987; Voisin, Namboodiri and Klein, 1984); and (4) the day/night change in melatonin levels was similar to that of NAT activity [Figs 9(A) and 9(B)]. Furthermore, in the rabbit lens, tryptophan was detectable by HPLC with fluorometric detection (Fig. 2), suggesting that the lens, like the pineal gland and the retina, may synthesize serotonin from tryptophan (Axelrod, 1974; Cahill et al., 1991).

In the rabbit lens, melatonin levels exhibited a significant day/night change with high levels during the dark period [Fig. 9(A)]. A similar day/night change was found in NAT activity, but not in HIOMT activity [Figs 9(B) and (C)]. The results suggest that melatonin synthesis in the lens may occur in a diurnal and/or circadian fashion, as it in the pineal gland and the retina (Axerlod, 1974; Cahill et al., 1991; Tosini and Menaker, 1996). In addition, NAT may be a rate-limiting enzyme in melatonin synthesis in the lens.

The apparent *K*m values for substrates of HIOMT in the rabbit lens were proximately 10-fold higher than those in the pineal gland (Fig. 8) (Cardinali and Wurtman, 1972), suggesting that HIOMT in the lens may not function physiologically as a melatonin-synthesizing enzyme. Similar *K*m values of HIOMT have been reported in rodent Harderian gland (Cardinali and Wurtman, 1972), however, it has been demonstrated that rodent Harderian gland synthesizes melatonin (Djeridane et al., 1998).

In the present study, we cannot rule out another possibility that the lens absorbs melatonin from surrounding fluids. To obtain further evidence for local melatonin synthesis by the rabbit lens, it is necessary to examine *in vitro* synthesis from radiolabelled tryptophan or serotonin.

Little is known concerning the function of melatonin in the lens. It is possible that melatonin in the lens, like in the retina, may function as a local regulator of rhythmic activity, because the melatonin levels show a significant day/night change [Fig. 9(A)]. In addition, melatonin may act its high affinity receptors to elicit its action, as it is in the iris-ciliary body (Osborne and Chidlow, 1994) and retina (Dubocovich, 1985).

Melatonin prevents experimentally-induced cataract (Abe et al., 1994) and possesses antioxidant action (Li et al., 1997). Although melatonin may have an anticataract function in the normal lens, an antioxidant mechanism is unlikely given that micromolar concentrations are required for melatonin to act as an antioxidant (Reiter et al., 1997b) and in the lens, melatonin levels based on the weight of the lenses were the nanomolar range [Fig. 9(A)]. However, the above data indicated that more attention should be given to the functional role of melatonin in the lens.

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