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Melatonin, its precursors, and synthesizing enzyme activities in the human ovary

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The presence of melatonin (*N*-acetyl-5-methoxytryptamine) and its precursors, serotonin (5-hydroxytryptamine) and *N*-acetylserotonin, was demonstrated in extracts of human ovary using reverse-phase high-performance liquid chromatography coupled with fluorometric detection. In addition, activities of two melatonin-synthesizing enzymes, arylalkylamine *N*-acetyltransferase (NAT) and hydroxyindole-*O*-methyltransferase (HIOMT), were found in human ovary homogenates. The apparent Michaelis constants for the substrates of NAT and HIOMT in the human ovary were similar to those reported for the pineal glands of humans and other mammals. These findings strongly suggest that the human ovary, like the pineal gland, may synthesize melatonin from serotonin by the sequential action of NAT and HIOMT.

Key words: arylalkylamine *N*-acetyltransferase/hydroxyindole-*O*-methyltransferase/melatonin/ovary/serotonin

Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine) is an indoleamine originally identified in the pineal gland, where it is synthesized enzymatically from serotonin (5-hydroxytryptamine) by the sequential action of arylalkylamine *N*-acetyltransferase (EC 2.3.1.87; NAT) and hydroxyindole-*O*-methyltransferase (EC 2.1.1.4; HIOMT) (Figure 1; Axelrod, 1974; Namboodiri *et al*., 1987; Sugden *et al*., 1987). The synthesis and secretion of melatonin fluctuate in a circadian rhythm that is entrained to a 24 h light/dark cycle (Axelrod, 1974; Tamarkin *et al*., 1985; Bals-Pratsch *et al*., 1997; Brzezinski, 1997). It is now widely accepted that melatonin is an endogenous mediator of photoperiodic information, and a molecular component of the circadian timekeeping system (Tamarkin *et al*., 1985; Dollins *et al*., 1994; Brzezinski, 1997).

Melatonin acts directly on ovarian cells (Brzezinski, 1997; Sirotkin and Schaeffer, 1997), and the putative receptor for melatonin is known to be present in both human and rat ovaries (Cohen *et al*., 1978; Yie *et al*., 1995a). Melatonin stimulates progesterone production by ovarian granulosa cells in several mammalian species, including humans, *in vitro* and acts synergistically with human chorionic gonadotrophin (HCG) to increase the production of progesterone (Fiske *et al*., 1984; Webley *et al*., 1988; Brzezinski *et al*., 1992; Yie *et al*., 1995b). Furthermore, melatonin is detectable in human preovulatory follicular fluid, its concentration in the fluid being significantly higher than that in peripheral serum (Brzezinski *et al*., 1987; Ronnberg *et al*., 1990). Although it has been assumed that melatonin detected in the human preovulatory follicular fluid is derived from the general circulation (Wurtman *et al*., 1964; Brzezinski *et al*., 1987; Ronnberg *et al*., 1990), it may also be synthesized in the human ovary. Recently, we have found that melatonin and the activities of its synthesizing enzymes, NAT and HIOMT, are present in the rat ovary (Itoh *et al*., 1997a). Enzyme activity has previously been detected in the Hardelian gland and lens of mammals (Cardinali and Wurtman, 1972; Djeridane *et al*., 1998; Abe *et al*., 1999), while *HIOMT* mRNA has been detected in the retina and whole brain (Rodriguez *et al*., 1994) and *NAT* mRNA in several brain regions and the pituitary of humans (Coon *et al*., 1996).

In addition, kinetic analyses have revealed that the apparent Michaelis constants (K_m) for the substrates of rat ovary NAT and HIOMT were similar to those of the pineal gland (Sugden

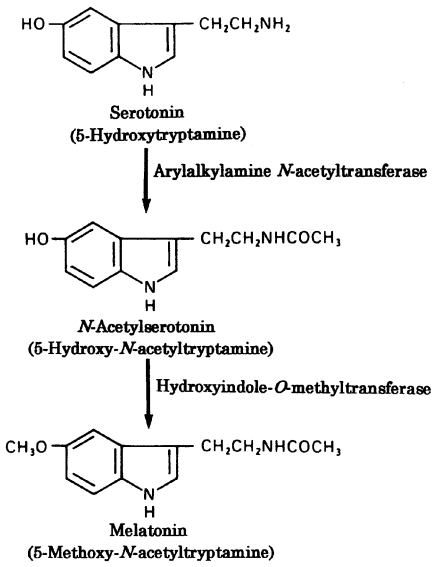

Figure 1. Melatonin is synthesized from serotonin by the sequential action of arylalkylamine *N*-acetyltransferase and hydroxyindole-*O*-methyltransferase (Axelrod, 1974; Namboodiri *et al*., 1987; Sugden *et al*., 1987).

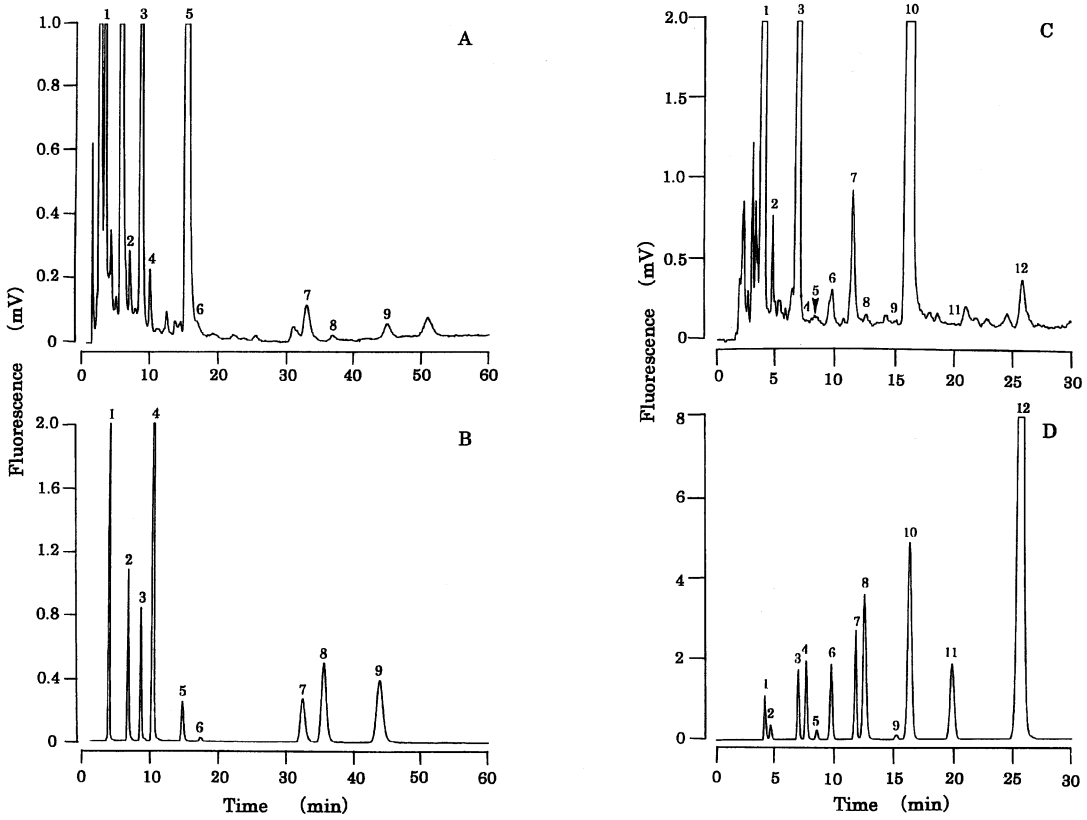

Figure 2. The presence of melatonin and its precursors in the human ovary. (**A**) and (**B**) Representative chromatograms for determination of melatonin in (**A**) human ovary extracts and (**B**) a standard solution containing 250 pg (1.01–1.41 pmol) of each of several indole compounds. The numbers in the figure indicate the elution positions of the following compounds: (1) serotonin; (2) 5-hydroxyindole-3-acetic acid; (3) 5-hydroxytryptophol; (4) *N*-acetylserotonin; (5) 5-methoxytryptamine; (6) 6-hydroxymelatonin; (7) 5-methoxyindole-3-acetic acid; (8) 5-methoxytryptophol; (9) melatonin. (**C**) and (**D**) Representative chromatograms for tryptophan and serotonin determinations of (**C**) human ovary extracts and (**D**) a standard solution containing 1.0 ng (3.00–5.95 pmol) of each of several compounds. The numbers in the figure indicate the elution positions of the following compounds: (1) tyrosine; (2) 4-hydroxy-3-methoxyphenylglycol; (3) noradrenaline; (4) adrenaline; (5) 3,4-dihydroxyphenylacetic acid; (6) normetanephrine; (7) dopamine; (8) 5-hydroxyindole-3-acetic acid; (9) homovanillic acid; (10) tryptophan; (11) 3-methoxytryptamine; (12) serotonin. See text for experimental conditions.

and Klein, 1983; Namboodiri *et al*., 1987; Bernard *et al*., 1995; Itoh *et al*., 1997a), suggesting that rat ovary NAT and HIOMT function physiologically as melatonin-synthesizing enzymes. Therefore, we examined whether melatonin, its precursors, and activities of NAT and HIOMT are present in the human ovary.

Materials and methods

Chemicals

Melatonin, serotonin and their structurally related compounds, acetyl coenzyme A and *S*-adenosyl-L-methionine, were purchased from Sigma (St Louis, MO, USA). *N*-Acetyltryptamine was synthesized from tryptamine and acetic anhydride, and elemental analysis of the product was in agreement with the predicted values for *N*-acetyltryptamine (Itoh and Sumi, 1998). Dibutylamine phosphate was prepared from dibutylamine and phosphoric acid (Lee Chin, 1992). The dye reagent used for protein assay was obtained from Bio-Rad.

Samples

Ovarian tissue was obtained from two women (aged 27 and 28 years) with endometriosis, two (aged 36 and 46 years) with dermoid cysts and three (aged 35, 50 and 51 years) with uterine myoma by unilateral wedge resection at the time of laparoscopic or open surgery. Two women (aged 35 and 51 years) had received gonadotrophin-releasing hormone (GnRH) agonist therapy for 3 months before surgery. The remaining five women had regular menstrual cycles of 28–30 days and had not received any hormonal therapy or ovarian suppression for at least 3 months before surgery. The ovarian tissue was obtained at different phases of the menstrual cycle; during the follicular phase in one subject and during the luteal phase in the others. Microscopic examination demonstrated no growing follicles in the tissue obtained from two women (aged 28 and 51 years), while in the tissue obtained from the remaining five women, follicles or corpora lutea of 5–14 mm in diameter were observed. Ovarian tissue was rinsed with saline to remove all visible evidence of blood. After absorption of excess saline, the tissue was stored at –80°C until assayed. Informed consent was obtained from all subjects. The protocol was approved by the human research committee of St. Marianna University School of Medicine, Kawasaki, Japan.

Melatonin determination

Ovarian tissue was homogenized in 1.0–2.0 ml of ice-cold 10 mM phosphate-buffered saline (PBS) containing 50 mM disodium EDTA (pH 7.4). After centrifugation at 10 000 *g* for 10 min at 4°C, the resulting supernatant (33.3–77.8 mg protein) was extracted with 6–10 ml of chloroform. The chloroform phase was evaporated, and the residue was stored at –80°C until analysis by high-performance liquid chromatography (HPLC). The recovery of the extraction

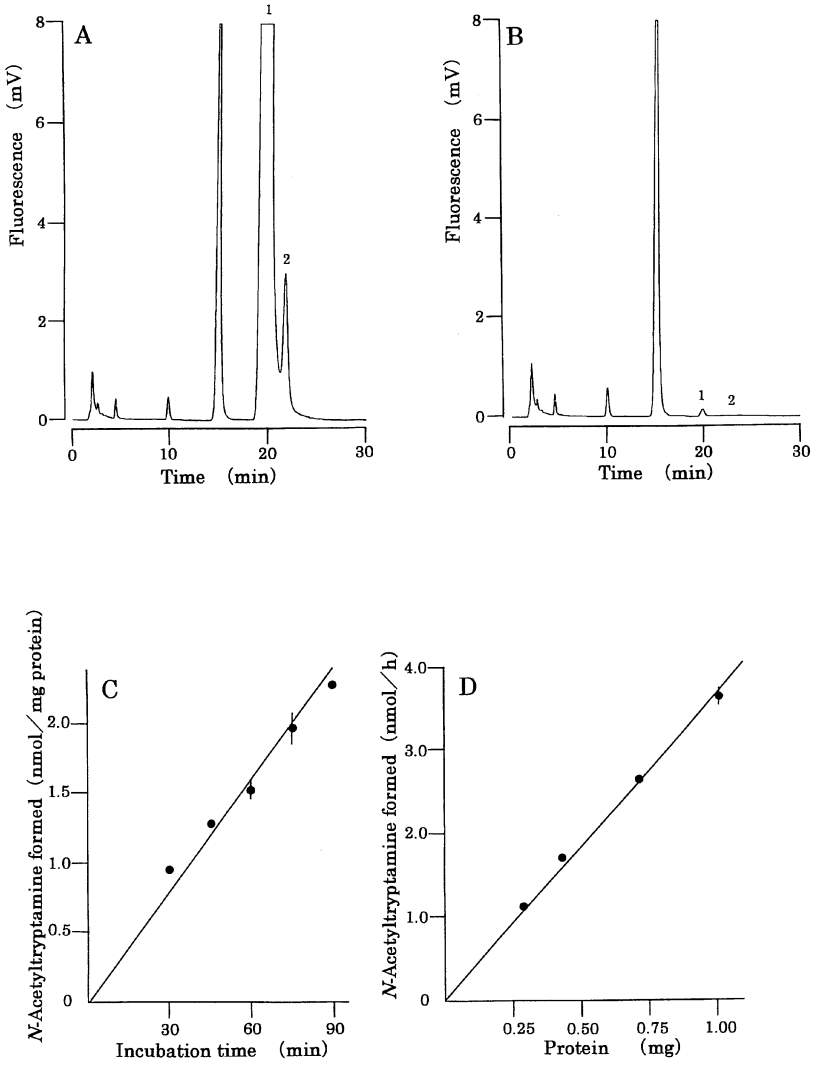

Figure 3. Arylalkylamine *N*-acetyltransferase (NAT) activity in human ovary. (**A**) and (**B**) representative chromatograms of (**A**) human ovary homogenates incubated with acetyl coenzyme A and tryptamine and (**B**) human ovary homogenates incubated with acetyl coenzyme A and without tryptamine. The numbers in the figure indicate the elution positions of (1) tryptamine and (2) *N*-acetyltryptamine. (**C**) Time course of *N*-acetylation of tryptamine using human ovary homogenate as the enzyme source. (**D**) Effects of human ovary protein concentration on the amount of enzymatically formed *N*-acetyltryptamine. In (C) and (D), each point and vertical line indicate the mean ± SEM of triplicate determinations. See text for experimental conditions.

procedure was 80.5 ± 5.1% (mean ± SEM, n = 4). The dried residue was redissolved in 100 or 150 µl of the HPLC mobile phase consisting of 50 mM ammonium acetate buffer (pH 4.3) and 18% methanol (v/v), and then filtered through a 0.45 µm filter. The filtrate (30–50 µl) was applied to a chromatographic system equipped with a Superiorex ODS S-5 µm column (4.6×150 mm, ID; Shiseido, Tokyo, Japan) and a fluorometric detector, RF-550 (Shimadzu, Kyoto, Japan). The detector was operated at an excitation wavelength of 280 nm and an emission wavelength of 340 nm. All separations were carried out isocratically at a flow rate of 1.0 ml/min using the above-mentioned HPLC mobile phase at 30°C. Peaks were identified by their retention times, and melatonin was quantified by its peak height. To identify the melatonin peak, the methanol concentration of the HPLC mobile phase was varied within the range 12–20%. In addition, the authenticity of the melatonin peak was verified by co-elution with an authentic standard. The limit of sensitivity of the assay was as low as 60 fmol for a 2:1 signal-to-noise ratio.

Tryptophan and serotonin determinations

Ovarian tissue was homogenized in 450 µl of ice-cold 10 mmol/l PBS (pH 7.4) and the homogenate was centrifuged at 20 000 *g* for 20 min at 4°C. Subsequently, 50 µl of ice-cold 1.5 mol/l perchloric acid containing 0.12% each of cysteine and disodium EDTA was added to the resulting supernatant (1.29–1.52 mg protein). After centrifugation at 20 000 *g* for 20 min at 4°C, the resulting supernatant was filtered through a 0.45 µm filter and 25–50 µl of the filtrate was loaded on a HPLC system equipped with a Capcell pak C18 UG 80 S-5 µm column (250×4.6 mm, I.D.; Shiseido, Tokyo, Japan) and the same fluorometric detector as that used for melatonin determination. The fluorometric detector was used with the excitation and emission wavelengths set at 280 and 340 nm respectively. The HPLC mobile phase consisted of 12.2 mM citric acid, 11.6 mM ammonium phosphate, 2.5 mM sodium octylsulphate, 3.3 mM dibutylamine phosphate, 1.1 mM disodium EDTA and 7.5% acetonitrile (v/v), and was pumped at a flow rate of 0.7–1.0 ml/min and 30°C. The pH of

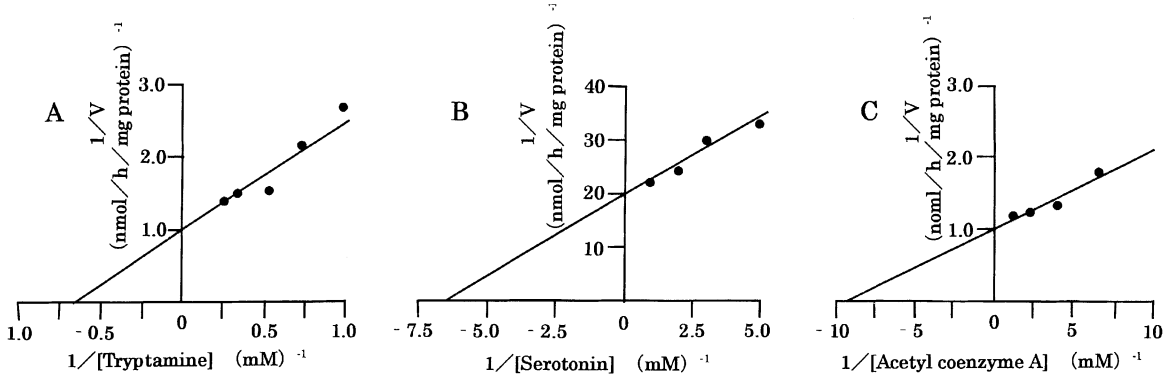

Figure 4. Lineweaver–Burk plots of human ovary NAT for (**A**) tryptamine, (**B**) serotonin and (**C**) acetyl coenzyme A. To determine the K_m values for tryptamine and serotonin, acetyl coenzyme A concentration was fixed at 1.0 mM. For acetyl coenzyme A K_m determination, the tryptamine concentration was 1.0 mM. All points represent the means of duplicate determinations, which differed by <10%. The K_m values were determined by linear regression analysis. The data shown are representative of three experiments.

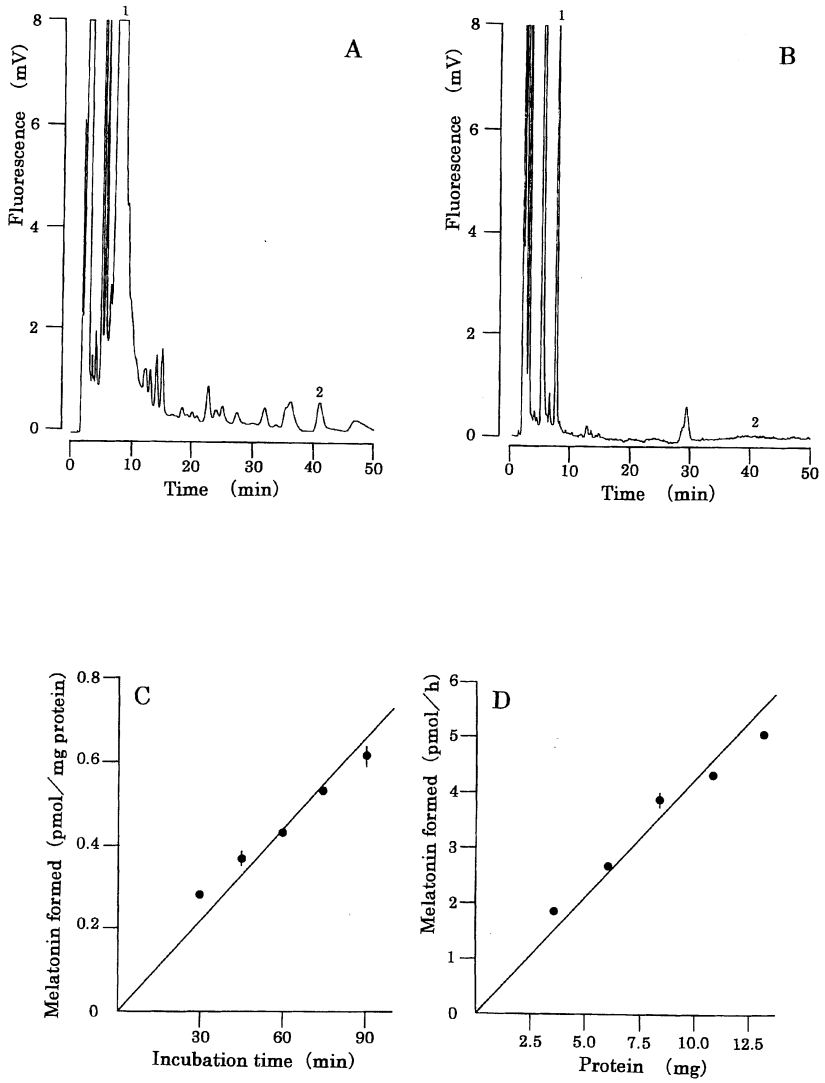

Figure 5. Hydroxyindole-*O*-methyltransferase (HIOMT) activity in human ovary. (**A**) and (**B**) Representative chromatograms of (**A**) human ovary homogenates incubated with *S*-adenosyl-L-methionine and *N*-acetylserotonin and (**B**) human ovary homogenates incubated with *S*-adenosyl-L-methionine and without *N*-acetylserotonin. The numbers in the figure indicate the elution positions of (1) *N*-acetylserotonin and (2) melatonin. See text for experimental conditions. (**C**) Time course of *O*-methylation of *N*-acetylserotonin using human ovary homogenate as the enzyme source. (**D**) Effects of human ovary protein concentration in the assay mixture on the amount of enzymatically formed melatonin. In (**C**) and (**D**), each point and vertical line indicate the mean ± SEM of triplicate determinations. See text for experimental conditions.

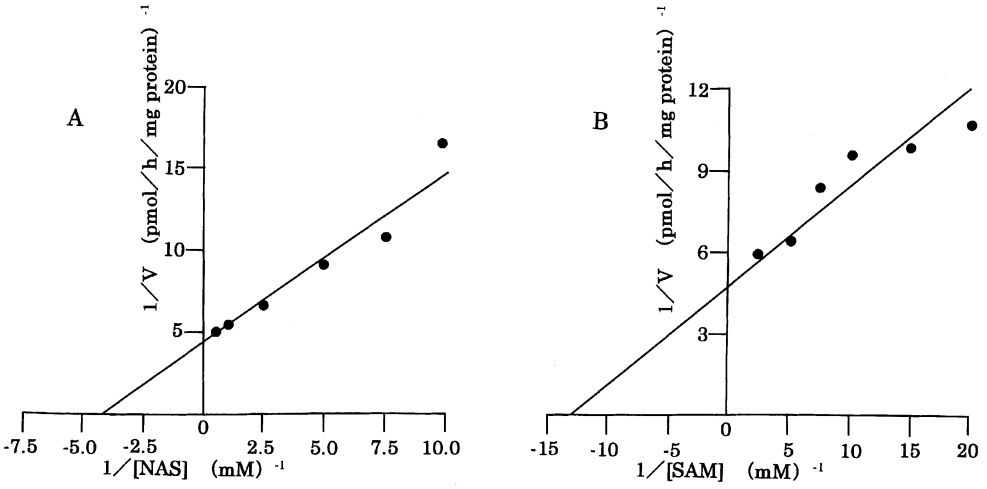

Figure 6. Lineweaver–Burk plots of human ovary hydroxyindole-*O*-methyltransferase (HIOMT) for (**A**) *N*-acetylserotonin (NAS) and (**B**) *S*-adenosyl-L-methionine (SAM). To determine the K_m values for NAS, the SAM concentration was fixed at 0.1 mM. For determination of the SAM K_m, the NAS concentration was 1.0 mM. All points represent the means of duplicate determinations, which differed by <10%. The K_m values were determined by linear regression analysis. The data shown are representative of three experiments.

the mobile phase was 3.8. Peaks were identified according to their retention times, and tryptophan and serotonin were quantified according to their peak heights. The authenticity of the tryptophan and serotonin peaks was verified by co-elution with an authentic standard.

NAT activity assay

NAT activity was assayed by measuring the amount of *N*-acetyltryptamine formed from tryptamine and acetyl coenzyme A (Itoh *et al*., 1995). Ovarian tissue was homogenized in 0.2–5.0 ml of ice-cold 0.25 M potassium phosphate buffer (pH 6.5) containing 1.4 mM acetyl coenzyme A. The homogenate was centrifuged at 20 000 *g* for 20 min at 4°C, and 75 µl of the resulting supernatant was mixed with 25 µl of 8 mM tryptamine HCl in 0.25 M potassium phosphate buffer (pH 6.5). The mixture was incubated for various times at 37°C, using final acetyl coenzyme A and tryptamine concentrations of 1.0 mM. The enzyme reaction was stopped by addition of 20 µl of 6 M perchloric acid. After centrifugation at 20 000 *g* for 20 min at 4°C, the resulting supernatant was filtered through a 0.45 µm filter and the filtrate (1.0–5.0 µl) was loaded on a HPLC system equipped with the same column and fluorometric detector as those used for melatonin determination. The detector was used with the excitation and emission wavelengths set at 285 and 360 nm respectively. The mobile phase consisted of 50 mM phosphoric acid, 30% methanol (v/v) and 0.65 mM sodium octylsulphate, adjusted to pH 3.5 with NaOH, and was pumped at a flow rate of 1.0 ml/min and 30°C. Peaks were identified by the retention times, and *N*-acetyltryptamine was quantified by its peak height. As controls, reaction mixtures were incubated without tryptamine or without an enzyme source, and analysed using HPLC. In addition, serotonin was used as the substrate, and *N*-acetylserotonin was detected by HPLC with fluorometric detection. The HPLC conditions were the same as those for melatonin determination. For kinetic analysis, tryptamine, serotonin and acetyl coenzyme A were used at various concentrations.

HIOMT activity assay

HIOMT activity was assayed by measuring the amount of melatonin formed from *N*-acetylserotonin and *S*-adenosyl-L-methionine (Itoh *et al*., 1997b,c). Ovarian tissue was homogenized in 0.2–3.0 ml of ice-cold 50 mM sodium phosphate buffer (pH 7.9), followed by centrifugation at 20 000 *g* for 20 min at 4°C. Aliquots (55 µl) of the resulting supernatant were mixed with 25 µl of 3.2 mM *N*-acetylserotonin and 0.32 mM *S*-adenosyl-L-methionine in 50 mM sodium phosphate buffer (pH 7.9), and incubated for various times at 37°C. The final concentrations of *N*-acetylserotonin and *S*-adenosyl-L-methionine were 1.0 and 0.1 mM respectively. The enzyme reaction was stopped by addition of 20 µl of 6 M perchloric acid. After centrifugation at 20 000 *g* for 20 min at 4°C, the resulting supernatant was filtered through a 0.45 µm filter, and the filtrate (40–50 µl) was subjected to HPLC analysis under the same conditions as those used for melatonin determination. As controls, reaction mixtures were incubated either without *N*-acetylserotonin or without an enzyme source, and analysed using HPLC. For kinetic analysis, *N*-acetylserotonin and *S*-adenosyl-L-methionine were used at various concentrations.

Protein assay

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

Results

On the chromatogram obtained from the human ovary extracts, a peak with a retention time identical to that of authentic melatonin was found by reverse-phase HPLC coupled with fluorometric detection (Figure 2A,B). Peaks with retention times identical to those of other indole compounds including *N*-acetylserotonin, which is the direct melatonin precursor, were also detected on the HPLC chromatogram. The melatonin concentration (mean ± SEM, $n = 3$, in women aged 28, 35 and 36 years) was 6.68 ± 3.61 fmol/mg protein.

In human ovary extracts, peaks of tryptophan and serotonin, which are melatonin precursors, were also found by HPLC with fluorometric detection (Figure 2C,D). On the HPLC chromatogram, peaks with retention times identical to those of other compounds, e.g. noradrenaline and dopamine, were also detected. The concentrations (mean ± SEM, $n = 3$, aged 27, 46 and 50 years) of tryptophan and serotonin were 42.2 ± 1.1 pmol/mg protein and 295.8 ± 11.8 fmol/mg protein respectively.

When human ovary homogenate was incubated with trypta-

mine and acetyl coenzyme A, an *N*-acetyltryptamine peak was detected using HPLC analysis (Figure 3A). No *N*-acetyltryptamine peak was detected in control incubations without tryptamine (Figure 3B) or without an enzyme source (data not shown). The rate of *N*-acetyltryptamine formation proceeded linearly for 90 min at 37°C (Figure 3C). A linear relationship was observed between the amount of human ovary protein present in the assay mixture (0.280–1.01 mg) and that of *N*-acetyltryptamine formed (Figure 3D). Ovarian NAT activity (mean ± SEM, $n = 4$, aged 35, 36, 50 and 51 years) was 1.84 ± 0.75 nmol/h/mg protein. Kinetic analysis gave apparent K_m values (mean ± SEM, $n = 3$) of 1.54 ± 0.10 mM, 0.15 ± 0.02 mM and 0.10 ± 0.01 mM respectively for tryptamine, serotonin and acetyl coenzyme A (Figure 4), as determined from Lineweaver–Burk plots.

On the HPLC chromatograms obtained from reaction mixtures of human ovary homogenate with *N*-acetylserotonin and *S*-adenosyl-L-methionine (experimental incubation, Figure 5A), or without *N*-acetylserotonin (control incubation, Figure 5B), the melatonin peak was higher in the experimental incubation than in the control incubation. In mixtures of substrates without an enzyme source, no melatonin peak was detected using HPLC (data not shown). The rate of melatonin formation proceeded linearly for 90 min at 37°C (Figure 5C). A linear relationship was observed between the amount of protein in the human ovary (3.59–13.2 mg protein) and that of melatonin formed (Figure 5D). The HIOMT activity (mean ± SEM, $n = 3$, aged 27, 28 and 50 years) in the human ovaries was 0.28 ± 0.07 pmol/h/mg protein. Kinetic analysis of human ovary HIOMT for two substrates, *N*-acetylserotonin and *S*-adenosyl-L-methionine was carried out, and the apparent K_m values (mean ± SEM, $n = 3$) determined from Lineweaver–Burk plots were 0.26 ± 0.04 mM and 0.08 ± 0.01 mM respectively (Figure 6).

Discussion

The results of the present study suggest that the human ovary may synthesize melatonin from serotonin by the sequential action of NAT and HIOMT for the following reasons: (i) melatonin and its precursors, serotonin and *N*-acetylserotonin, were detected in human ovary extracts (Figure 2); (ii) activities of NAT and HIOMT were also found in human ovary homogenates (Figures 3, 4, 5, 6); (iii) the apparent K_m values for substrates of NAT and HIOMT in the human ovary were similar to those already reported for pineal glands of humans and other mammals (Figures 3, 4 and 6; Sugden and Klein, 1983; Namboodiri *et al*., 1987; Bernard *et al*., 1995). Thus, it seems likely that human ovary NAT and HIOMT function physiologically as melatonin-synthesizing enzymes. In addition, tryptophan was detected in the human ovary extracts (Figure 2), suggesting that serotonin may be synthesized from tryptophan in the ovary, as it is in other melatonin-synthesizing tissues, e.g the pineal gland and retina (Axelrod, 1974; Cahill *et al*., 1991).

It has been reported that melatonin is present in preovulatory follicular fluid and its concentration in the follicular fluid is significantly higher than that in peripheral serum (Brzezinski *et al*., 1987; Ronnberg *et al*., 1990). Thus, it is possible that melatonin synthesized by the ovary may be released into the follicular fluid. However, we cannot exclude the possibility that the melatonin detected in the ovary and preovulatory follicular fluid is derived from the circulation, because rat and cat ovaries have been shown to take up and retain circulating [^{3}H]-melatonin (Wurtman *et al*., 1964). To obtain further evidence for local melatonin synthesis by the human ovary, it will be necessary to examine whether the melatonin-synthesizing enzymes and their substrates are found in the same cells or tissue compartments, and to determine the proportion of ovarian melatonin that is derived from local production.

cDNA encoding *NAT* and *HIOMT* has been cloned from human pineal glands (Donohue *et al*., 1993; Rodriguez *et al*., 1994; Coon *et al*., 1996). Although it has been reported that *NAT* and *HIOMT* mRNA is also expressed in extrapineal tissues, e.g. retina and brain (Rodriguez *et al*., 1994; Coon *et al*., 1996), nothing is known about the expression of these genes in the ovary.

It has been demonstrated that melatonin plays an important role in reproduction (Tamarkin *et al*., 1985; Brzezinski, 1997; Sirotkin and Schaeffer, 1997). A putative melatonin receptor is present in the human ovary (Cohen *et al*., 1978; Yie *et al*., 1995a), and melatonin administration increases the production of progesterone by human ovarian granulosa cells *in vitro* (Webley *et al*., 1988; Brzezinski *et al*., 1992; Yie *et al*., 1995b). Therefore, it is possible that melatonin synthesized in the ovary may regulate reproduction at the follicular level. The physiological role of melatonin detected in the ovary should be further investigated.

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