MATERIALS and METHODS

A three month old, Wistar-Imamichi rat weighing 250 to 300 g was anesthesized with ether. The testes were quickly removed, decapsulated and sectioned into slices of 30 mg each. Each testis slice was put into a glass homogenizer (13 mm x 100 mm) filled with 0.5 ml of Krebs-Ringer-bicarbonate buffer (pH 7.4) containing 2 mg/ml of glucose (KRBG) and preincubated for 30 min. at 34°C. Following preincubation each testis slice was incubated at 34°C for 5 min. to 3 hours in 500 µl of KRBG containing 10 mM theophylline (KRBGT) and various concentrations of melatonin. Preincubation and incubation were performed with constant agitation (120 cycles/min.) in an atmosphere of oxgen 95 % : carbon dioxide 5 %. At the end of incubation 200 µl of the medium was taken for measurement of testosterone after which 500 µl of 10 % trichloroacetic acid was added. The tissue slice was homogenized thoroughly and centrifuged at 3000 rpm at 4°C for 15 min. and the supernatant was extracted three times with water-saturated ether. The aqueous phase was evaporated under nitrogen gas, the residue was redissolved in 500 µl of 0.05 M sodium acetate buffer (pH 6.2) and aliquots of 100 µl or 50 µl were assayed for c-GMP and c-AMP respectively.

Decapsulated testes were sectioned into slices of 250 mg. Each was separated into seminiferous tubule and Leydig cell fractions in the cooled KRBG. Separated seminiferous tubule and Leydig cell fractions were examined for testosterone and cyclic nucleotides in the same way as the testis slices.

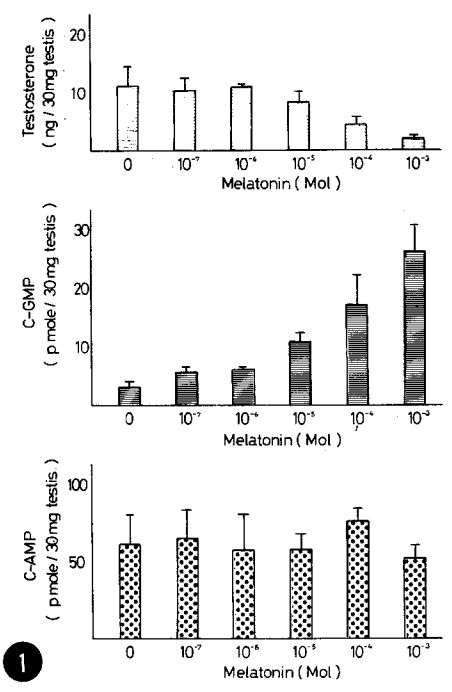
Both c-GMP and c-AMP were determined by a modified method of Steiner's radioimmunoassay (9) developed by the authors (10) and testosterone by Nieschlag's method (11).

Guanylate cyclase activity was determined by c-GMP production from GTP as described by Kimura and Murad (12). Assays used for guanylate cyclase activity were composed of 50 mM Tris-HCl buffer (pH 7.6), 10 mM theophylline, 0.1 mM dithiothreitol, 15 mM creatine phosphate, 20 µg creatine phosphokinase, 3 mM $MnCl_2$, 1 mM GTP and 50 µl of 1 % Triton X-100 solubilized homogenate of testis tissue which had been treated with 10^{-3}M melatonin, making final volume of 150 µl. Incubation was carried out at 37°C for 30 min. Reaction was terminated by adding 0.9 ml of 50 mM sodium acetate buffer (pH 4.0) and heated for 3 min. at 90°C, after which 300 µl of 25 % trichloroacetic acid was added. Each tube was agitated thoroughly and centrifuged at 3000 rpm, 4°C for 15 min. and the formed c-GMP in the supernatant was measured by radioimmunoassay method.

Protein was determined by the method of Lowry et al (13). Melatonin, GTP, c-AMP, c-GMP, $MnCl_2$, dithiothreitol, creatine phosphate, creatine phosphokinase and Triton X-100 were purchased from Sigma Chemical Co. ; Tris, EDTA, and mannitol from Daiichi Pure Chemicals Co. and theophylline from Nakarai Chemicals Co.

RESULTS

Concentrations of 10^{-4}M or more of melatonin inhibited testosterone production and increased c-GMP level, but had no effect on c-AMP level in rat testes tissue in vitro (Fig. 1). Increasing the concentrations of melatonin caused dose-responsive stimulation of c-GMP production and inhibition of testosterone production. Maximal effects of melatonin on



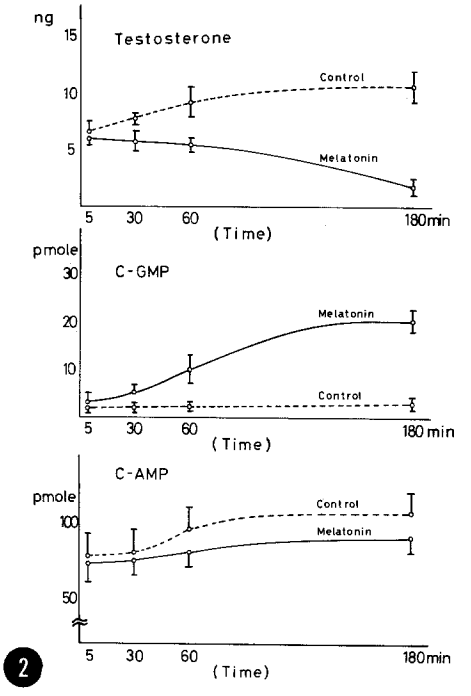


Fig. 1 : Dose-response relationships of the action of melatonin on testosterone, c-GMP and c-AMP productions in rat testis tissue slice in Krebs-Ringer-bicarbonate glucose solution containing 10 mM theophylline in vitro. Values represent the mean ± standard deviation for six testis slices.

Fig. 2 : Time courses of responses of the rat testis (30 mg) to melatonin (10^{-3}M) in KRBGT. Each point is the mean ± standard deviation for three testis slices.

both c-GMP and testosterone were observed at the concentration of 10^{-3}M, when the level of testosterone was 1.8 ± 0.5 ng/30 mg tissue or 18 % of that of the controls, and c-GMP was 26 ± 8 pmoles/30 mg tissue or 800 % of that of the controls.

The time course of the responses to melatonin (10^{-3}M) is shown in Fig. 2. Melatonin caused slight increase in c-GMP level 30 min. after incubation, after which c-GMP production gradually increased and reached its maximum level in 3 hours. The testosterone secretion was inhibited

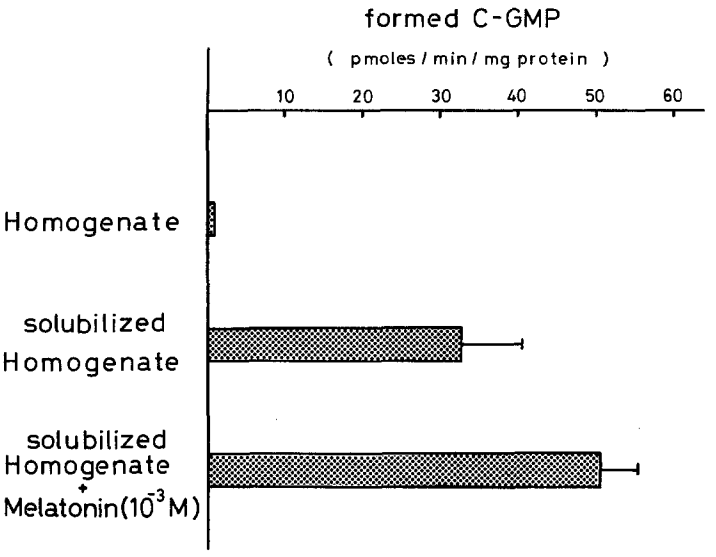


Fig. 3 : Effects of melatonin (10^{-3}M) on guanylate cyclase activity of rat testis tissue.
Guanylate cyclase activity of rat testis tissue was determined in homogenate, solubilized homogenate and solubilized homogenate of rat testis which had been treated with 10^{-3}M of melatonin.

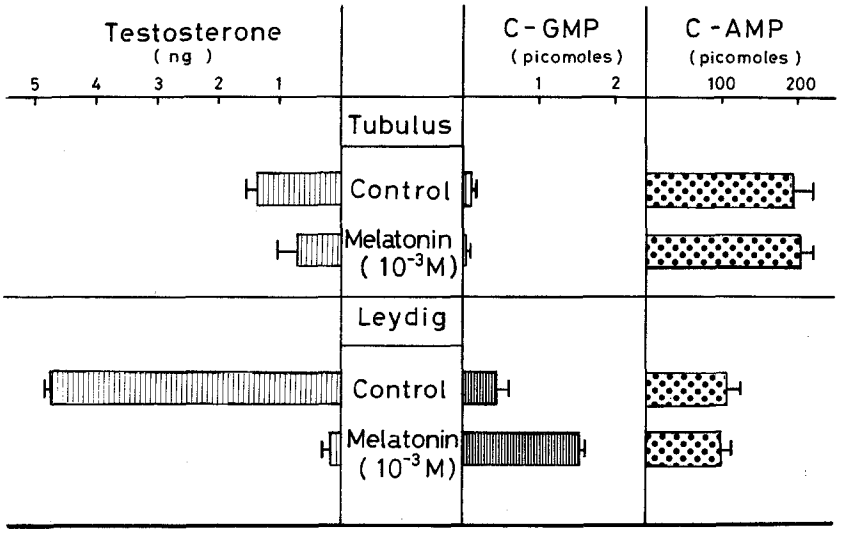


Fig. 4 : Effects of melatonin (10^{-3}M) on testosterone, c-GMP and c-AMP production in separated seminiferous tubule and Leydig cell fraction of the rat testis tissue slice (250 mg) in vitro. Values represent the mean ± standard deviation for three testis slices.

in 1 hour and gradually decreased, reaching its minimum level in 3 hours. Melatonin had no effects on c-AMP production.

Fig. 3 indicates that guanylate cyclase activity of Triton X-100 treated homogenate of rat testis which had been treated with 10^{-3}M of melatonin increased from 32.9 ± 7.7 pmoles/min./mg protein to 49.9 ± 5.2 pmoles/min./mg protein. Besides Triton X-100 treatment markedly increased the guanylate cyclase activity from 0.32 ± 0.05 pmoles/min./mg protein to 32.9 ± 7.7 pmoles/min./mg protein.

As is shown in Fig. 4, 10^{-3}M of melatonin had effects only on Leydig cell fraction, inhibiting testosterone production and stimulating c-GMP production, but had no significant effects on the seminiferous tubule fraction.

DISCUSSION

Melatonin has been shown to inhibit testosterone synthesis *in vivo* (3). Inhibitory effects of melatonin on testosterone production have at least two different loci of action, one at the hypothalamic level and the other at the testicular level.

The data shown above suggest that high concentrations of melatonin have direct inhibitory effects on testosterone production in rat testis tissue *in vitro*. The authors' data are similar to the findings of previous reports which demonstrated inhibition of testosterone synthesis by melatonin from [^{3}H] pregnenolone (7), or [^{14}C] progesterone and [^{14}C] 17α-hydroxyprogesterone (8) *in vitro*. The mechanism of direct inhibitory action of melatonin on testosterone and androstenedione production was described by L.C.Ellis (8) and D.P.Cardinali and J.M.Rosner (14), who demonstrated that high concentrations of melatonin caused the inhibition of 3β-hydroxysteroid dehydrogenase,4,5-ene isomerase, 17α-hydroxylase and 17α-hydroxypregnene-C_{17}-C_{20}-lyase.

The data shown above suggest that 10^{-4}M or more of melatonin acts

directly on Leydig cell fraction, activates its guanylate cyclase, stimulates c-GMP production and consequently inhibits testosterone production, while the c-AMP level remains unchanged.

As was suggested by Goldgerg and associates (15) such agents as acetylcholine which cause elevation of c-GMP in the perfused rat heart (16), have effects that appear to be functionally opposite to the effects of agents that cause increase in c-AMP level. In other tissues, such as the canine thyroid (17) and bovine superior cervical ganglion (18), cholinergic compounds cause increase of c-GMP, but c-AMP levels remain unchanged. Melatonin might be an agent newly found to elevate c-GMP production and consequently cause inhibitory action independent of changes in c-AMP.

The precise mechanism of melatonin action in testicular cells remains unknown, but it might be surmised as follows. Melatonin acts on Leydig cell fraction, activates its guanylate cyclase and stimulates c-GMP production resulting in activation of c-GMP dependent protein kinase, which then acts to catalyze the transfer of phosphate groups from GTP to certain proteins. Presumably these phosphorylated proteins, directly or indirectly, inhibit the key enzymes which are involve in the pathway from pregnenolone, progesterone and 17α-hydroxyprogesterone to testosterone and androstenedione.

Further data are required in order to establish precisely the mechanism of melatonin action.

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