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Control of Hydroxyindole *O*-Methyltransferase Activity in the Rat Pineal Gland by Environmental Lighting

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The mammalian pineal gland has been shown to contain several enzymes involved in the formation and metabolism of physiologically active indoles and amines (1–3). The rat pineal gland contains the highest reported tissue levels of serotonin and 5-hydroxytryptophan decarboxylase (4, 5). In addition, the pineal gland is unique in that it is the only organ that contains hydroxyindole *O*-methyltransferase, an enzyme that converts *N*-acetylserotonin to melatonin (6). Since *N*-acetylation of serotonin has been shown to take place in several tissues (7) and since the activity of hydroxyindole *O*-methyltransferase in the rat pineal is relatively low (8), it appears that methylation is the rate-limiting step in the formation of melatonin and perhaps other methoxyindoles in this species.

The weight, morphology, and chemical composition of the rat pineal can be altered by exposing rats to continuous light or darkness; rats kept in light have small pineal glands (9), with decreased cytoplasmic basophilia, nucleolar size (10), and serotonin content (4). In preliminary reports it was shown that light also affected the hydroxyindole *O*-methyltransferase activity in the pineal gland; rats kept in constant dark had 3 to 10 times more melatonin-forming enzyme activity than rats kept in the light (8). In the hen, exposure to light increased the activity of hydroxyindole *O*-methyltransferase (11). This report will describe the time course of the effect of light and darkness on hydroxyindole *O*-methyltransferase activity in the rat pineal. It will be shown that there is a diurnal cycle in the enzymatic synthesis of melatonin and that this cycle is regulated by environmental lighting. The information about lighting reaches the pineal gland by a neural route involving the eyes and the sympathetic nervous system.

EXPERIMENTAL PROCEDURE

Preparation of Animals—Sprague-Dawley female rats weighing 160 to 180 g were used in all experiments. Some rats were kept in constant light or dark in air-conditioned rooms equipped with double door light baffles. Constant light was provided by an overhead fluorescent tube; each animal was exposed to about 100 foot-candles of light. To enable the cages in the dark room to be cleaned, a 25-watt red light bulb which provided less than 1 foot-candle of light at the level of the cages was used for about 20 minutes daily. For the diurnal studies, rats were kept in a room lighted by a fluorescent tube from 7 a.m. to 7 p.m. The room remained totally dark from 7 p.m. to 7 a.m.

Rats were blinded by bilateral orbital enucleation, under Nembutal anesthesia. In other groups of animals, both superior cervical ganglia were removed under ether anesthesia. After the operations, rats were kept in diurnally lighted rooms for at least 1 week before the enzyme studies.

Assay of Hydroxyindole O-Methyltransferase—Hydroxyindole *O*-methyltransferase was assayed in the rat pineal gland by a modification of a procedure previously described (12). This depends on the formation of ^{14}C-melatonin when the enzyme is incubated with *N*-acetylserotonin and ^{14}C-methyl-*S*-adenosylmethionine. The ^{14}C-melatonin formed enzymatically was separated from unreacted ^{14}C-*S*-adenosylmethionine by extraction into chloroform. Rats were killed by neck fracture and the head was immediately severed. A blade of a scissors was inserted into the foramen magnum and the skull was cut laterally on both sides. The roof of the skull and the adhering brain were folded back with forceps. Starting with the cerebellum, the brain was peeled away from the dura and skull. This exposed the pineal gland, a round, pearl-white structure 1 to 2 mm in diameter, which was attached to both the skull and the brain by thin filamentous strands. In most animals the pineal remained attached to the skull when the brain and skull were separated. In the small proportion of cases (5 to 10%) in which the pineal did not remain on the dural covering of the skull, it could be found in a groove anterior and cephalic to the superior colliculi, on the surface of the brain. The pineal was easily removed with a curved forceps, with a minimum of adhering connective tissue. Immediately after removal, the pineal was weighed on a 25-mg Roller-Smith balance, or stored temporarily on paper toweling impregnated with cold 0.9% NaCl solution. After weighing, the pineal was transferred to the tip of a glass pestle and homogenized in 0.5 ml of ice-cold 0.05 M phosphate buffer, pH 7.9, in a conical glass hand homogenizer. The enzyme was assayed as soon as possible after homogenization. A 200-μl aliquot of the homogenate was transferred to a 15-ml glass-stoppered centrifuge tube containing 50 μg of *N*-acetylserotonin (Regis Chemical Company, Chicago) and 50 mμc of ^{14}C-*S*-adenosylmethionine (40 μc per μmole, New England Nuclear Corporation, Boston) in a final volume of 300 μl. After 1 hour of incubation at 37°, 1 ml of 0.2 M borate buffer at pH 10 and 8 ml of chloroform were added to the reaction mixture. The tube was shaken for 5 minutes, and the aqueous phase was then removed by aspiration. Another 1-ml portion of the buffer was added to the chloroform extract, and the tube was shaken again for about 1 minute. After removal of the aqueous phase, a 5-ml aliquot of the chloroform extract was transferred to a vial, and the chloroform was then evaporated in a stream of warm air. The residue was taken up with 1 ml of ethanol, and the radioactivity was measured after the addition of 10 ml of

TABLE I

Changes in pineal hydroxyindole O-methyltransferase activity following exposure to continuous light or darkness

Exposure to	Rat	Pineal weight	Radioactivity	Hydroxyindole O-methyltransferase activity	
				Per pineal	Per mg
		mg	*c.p.m.*	*μμmoles ^{14}C-melatonin formed/hr*	
Light	1	0.70	24	0.8	1.1
	2	0.86	82	2.7	3.2
	3	1.06	55	1.8	1.7
	4	0.78	26	0.8	1.0
	5	0.84	16	0.5	0.6
	6	0.80	3	0.1	0.1
	7	0.74	79	2.6	3.5
	8	1.54	253	8.3	5.3
	9	1.04	139	4.6	4.4
	10	1.60	173	5.7	3.6
Dark	11	1.42	460	14.7	10.3
	12	1.38	425	13.8	10.0
	13	1.40	710	23.2	16.6
	14	1.24	461	15.2	12.2
	15	1.10	425	13.8	12.6
	16	0.80	208	6.9	8.6
	17	1.10	354	11.6	10.6
	18	0.94	208	6.9	7.3
Mean ± s.e.m.					
Light		0.99 ± 0.08	2.79 ± 0.84	2.45 ± 0.56	
Dark		1.23 ± 0.06	13.26 ± 1.84	11.03 ± 1.00	
p		<0.05	<0.001	<0.001	

phosphor. A control incubation in which 0.2 ml of pH 7.9 buffer was substituted for the enzyme was run concurrently to correct for the small amount of ^{14}C-*S*-adenosylmethionine (5 c.p.m. over background) that is extracted into the solvent.

The identity of the ^{14}C-melatonin formed enzymatically in the pineal gland of the rat was established by paper chromatography (2).

RESULTS

Effect of Exposure of Rats to Continuous Light or Darkness on Pineal Hydroxyindole O-Methyltransferase Activity—Groups of rats were kept in continuous darkness or light for 7 days. They were killed at 9 a.m. while still in light or darkness, and their pineal glands were immediately removed and assayed for hydroxyindole *O*-methyltransferase activity (Table I). Animals maintained in the dark had heavier pineal glands with more than 4 times as much hydroxyindole *O*-methyltransferase activity as rats kept in light. In many similar experiments, the pineal glands of rats kept in the dark were found to have 2 to 10 times as much enzyme activity.

To examine the possibility that an inhibitory substance was present in the pineals of rats exposed to light, homogenates of these glands were incubated together with those of dark-treated rats. The hydroxyindole *O*-methyltransferase activity was additive in all cases.

Rate of Change of Pineal Hydroxyindole O-Methyltransferase Activity with Illumination—To determine the time course of the effect of light or darkness on the activity of the transferase, the following experiments were performed: 50 rats were placed in constant light for 2 weeks. Groups of 10 rats were then removed from the light and put in a dark room for 0, 1, 2, 4, and 6 days. All animals were killed on the same morning, and their pineals were assayed for enzyme activity. Within 24 hours after the onset of darkness, there was a marked increase in enzyme activity, followed by a slower rise up to 6 days (Fig. 1). In a similar experiment rats were kept in constant darkness for 2 weeks and then placed in light for 0, 1, 2, 4, and 10 days. The fall in enzyme activity proceeded at a slower rate than the rise in darkness (Fig. 2). The greatest fall in enzyme activity occurred within 2 days after the rats were put in the light. Changes in the weights of the pineal glands paralleled changes in enzyme activity, but were of smaller magnitude.

Effect of Actinomycin D and Puromycin on Rise of Hydroxyindole O-Methyltransferase Activity in Darkness—Sixty rats weighing 160 g were kept in constant light for 7 days. At the end of this time, groups of 20 rats were given actinomycin D (250 μg per kg intraperitoneally in three divided doses), puromycin (30 mg per kg intraperitoneally in three divided doses), or no treatment. Half of each group were then placed in continuous darkness and the remainder were kept in constant light. Twenty-four hours later the rats were killed, and the pineals were removed, weighed, and assayed for hydroxyindole *O*-methyltransferase activity. Untreated animals, kept in darkness, showed the characteristic rise in hydroxyindole *O*-methyltransferase activity (Table II). This elevation was prevented in rats given puromycin. Actinomycin D resulted in a small and statistically insignificant rise in the activity of the enzyme in animals kept in light. In actinomycin-treated animals there was no significant difference in activity in rats kept in light and dark.

Diurnal Changes in Hydroxyindole O-Methyltransferase Activity—Rats were placed in controlled diurnal lighting for 7

days. Groups of 10 rats were then killed at noon, 6 p.m., midnight, and 6 a.m. Pineal glands were immediately removed, weighed, homogenized, and stored frozen until the following day, when all glands were assayed at the same time. Enzyme activity was found to be lowest at 6 p.m., after the animals had been in light for 11 hours (Fig. 3). By midnight (after 5 hours of darkness), enzyme activity had risen about 3-fold. Following this, there was a decrease in enzyme activity at 6 a.m., even though the animals were kept in darkness. Between 6 a.m. and 6 p.m., enzyme activity continued to fall. In numerous experiments the magnitude of the rise in activity of hydroxyindole *O*-methyltransferase between 6 p.m. and midnight has ranged between 1.6- and 3-fold. Data on the diurnal changes in pineal weight in a large number of animals were pooled. It was found that the pineal weights were minimal at 6 p.m., and rose significantly during the dark period (Fig. 4).

Control of Diurnal Changes in Hydroxyindole O-Methyltransferase Activity in Pineal Gland by Lighting—To determine whether changes in the pineal enzyme were the consequence of an intrinsic rhythm or were controlled by changes in environmental illumination, rats were subjected to various lighting regimens. These animals and control rats maintained in normal diurnal lighting (lights on from 7 a.m. to 7 p.m.) were killed at 6 p.m. and midnight, and their pineals were examined for hydroxyindole *O*-methyltransferase activity.

Groups of 20 rats were kept for 1 week in constant light or darkness or in normal diurnal lighting. Animals maintained in constant darkness had considerably more enzyme activity than rats kept in light, but there was no change in pineal hydroxyindole *O*-methyltransferase in either group between 6 p.m. and midnight (Fig. 5). The expected change was observed in rats maintained in diurnal lighting.

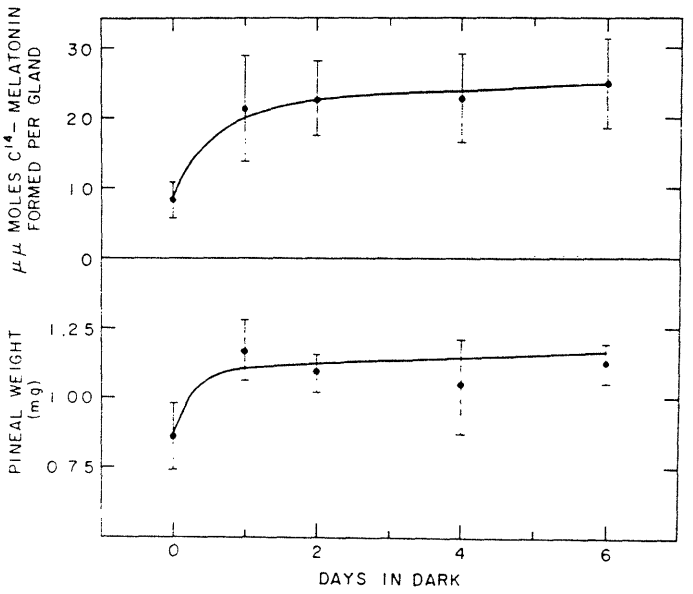

FIG. 1. Rise in pineal hydroxyindole *O*-methyltransferase and weight in darkness. The *vertical lines in brackets* represent the standard errors of the mean.

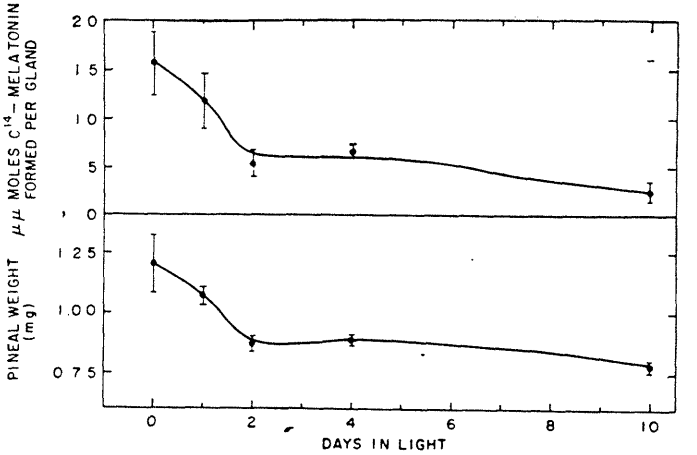

FIG. 2. Fall in pineal hydroxyindole *O*-methyltransferase and weight in light. *Vertical lines in brackets* represent the standard errors of the mean.

TABLE II

Effect of actinomycin D or puromycin on response of hydroxyindole O-methyltransferase activity to darkness

Treatment	Pineal weight	Hydroxyindole *O*-methyltransferase activity
	mg	*μμmoles melatonin formed/gland/hr ± s.e.m.*
Light	0.84 ± 0.045	11.2 ± 2.6
Dark	0.91 ± 0.092	23.8 ± 3.7*
Actinomycin, light	0.69 ± 0.059	16.4 ± 3.7
Actinomycin, dark	0.72 ± 0.055	22.8 ± 4.7
Puromycin, light	0.77 ± 0.061	11.4 ± 4.2
Puromycin, dark	0.78 ± 0.041	14.5 ± 3.2

* $p < 0.02$.

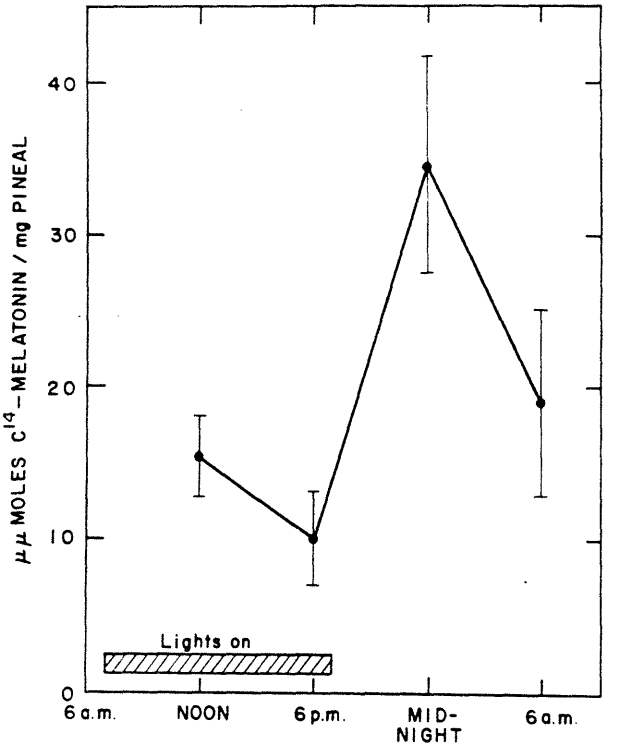

FIG. 3. Diurnal changes in pineal hydroxyindole *O*-methyltransferase activity. Results are expressed as micromicromoles of melatonin formed per mg of pineal. *Vertical lines in brackets* represent the standard errors of the mean.

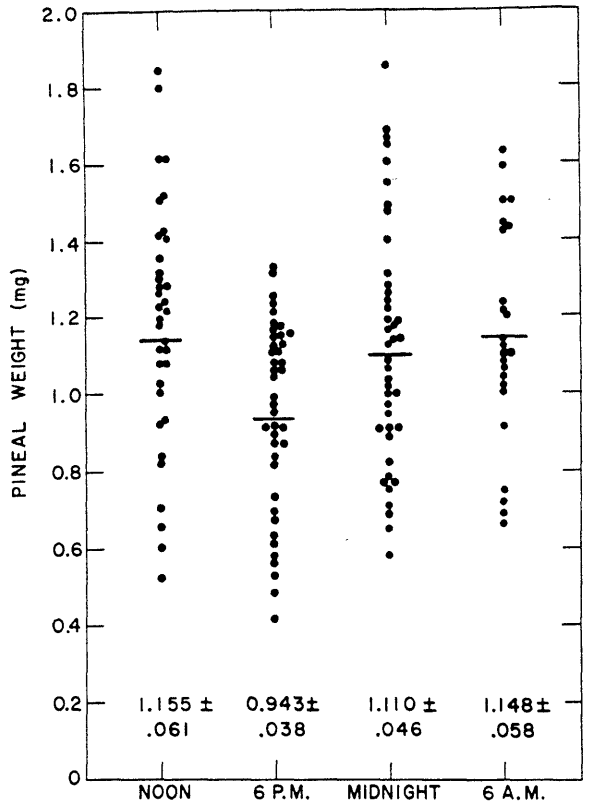

FIG. 4. Diurnal changes in pineal weight. *Horizontal lines* are mean values. The weight at 6 p.m. is significantly lower ($p < 0.01$) than at other times.

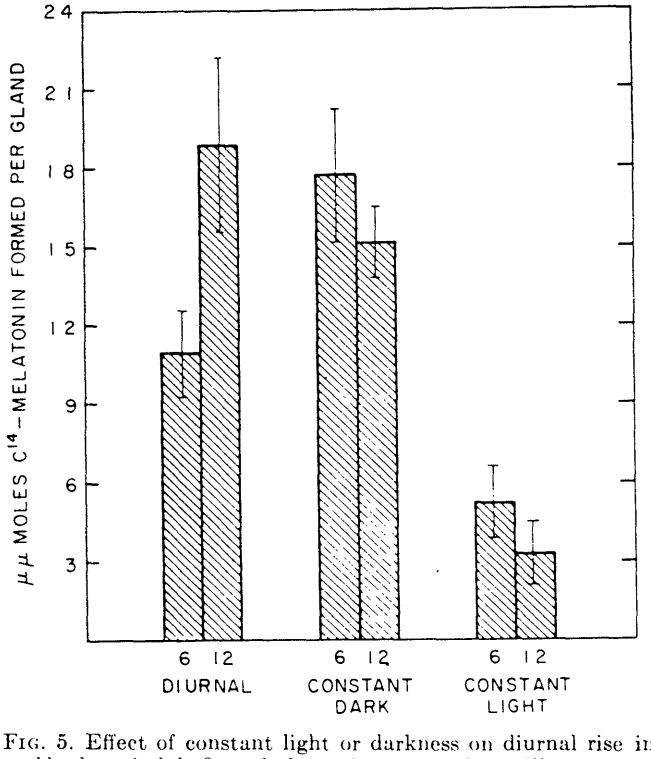

FIG. 5. Effect of constant light or darkness on diurnal rise in pineal hydroxyindole *O*-methyltransferase activity. The numbers *6* and *12* denote 6 p.m. and midnight, respectively. The *vertical lines in brackets* represent the standard errors of the mean.

Groups of 10 rats were exposed to the following lighting conditions. One group was deprived of one photoperiod of darkness by being placed in light from 7 p.m. to midnight, when they were killed. Another group was deprived of darkness for two photoperiods; these rats were kept in light from 7 p.m. until midnight the following day, at which time they were killed. Controls consisted of one group which was killed at 6 p.m. and another killed at midnight, after the usual 5 hours of darkness. The control group showed the expected rise in pineal hydroxyindole *O*-methyltransferase activity between 6 p.m. and midnight (Table III). When a single photoperiod of darkness (5 hours) was omitted, the rise in hydroxyindole *O*-methyltransferase activity was eliminated.

Pathway by Which Light Controls Diurnal Changes in Hydroxyindole O-Methyltransferase Activity—It had been previously shown that constant light and darkness influenced pineal hydroxyindole *O*-methyltransferase activity and weight by a neural pathway involving the eye and the sympathetic nerves to the pineal gland (13). To determine whether this pathway also mediated the enzyme changes which resulted from diurnal lighting, rats were subjected to various surgical procedures and pineal hydroxyindole *O*-methyltransferase activity was assayed in animals killed at 6 p.m. and midnight. All animals were kept

TABLE III

Dependence of changes in hydroxyindole O-methyltransferase activity upon diurnal lighting changes

Time	Treatment between 6 p.m. and midnight	Pineal weight	Hydroxyindole *O*-methyltransferase activity
		mg	*μμmoles melatonin formed/gland/hr ± s.e.m.*
6 p.m.	Light	1.19 ± 0.07	15.8 ± 1.3
Midnight	Dark	1.28 ± 0.07	30.2 ± 4.1*
Midnight	Light, 1 photoperiod	1.15 ± 0.08	17.1 ± 1.8
Midnight	Light, 2 photoperiods	0.90 ± 0.06†	15.0 ± 3.5

* $p < 0.01$ (differs from 6 p.m.).
† $p < 0.001$ (differs from midnight, dark).

TABLE IV

Effect of blinding and ganglionectomy on diurnal rise in hydroxyindole O-methyltransferase activity in rat pineal gland

Groups of rats were blinded or had their superior cervical ganglia removed. One week later the animals were killed at 6 p.m. or midnight, and pineals were assayed for hydroxyindole *O*-methyltransferase activity.

Treatment	Hydroxyindole *O*-methyltransferase activity	
	6 p.m.	Midnight
	μμmoles melatonin formed/gland/hr ± s.e.m.	
Control	12.3 ± 1.4	20.5 ± 1.5*
Ganglionectomy	12.6 ± 3.3	11.7 ± 2.6
Control	17.7 ± 5.1	45.9 ± 4.3†
Blind	25.5 ± 2.1	27.9 ± 2.7

* $p < 0.01$ (differs from 6 p.m.).
† $p < 0.001$ (differs from 6 p.m.).

in diurnal lighting for 7 days after the operations. In each experiment, control animals were kept under the same lighting conditions and were also killed at 6 p.m. and midnight. In the first experiment both eyes were removed from groups of six rats. In contrast to control animals, these rats showed no change in pineal transferase between 6 p.m. and midnight (Table IV). The rat pineal gland has been shown to be innervated by sympathetic nerves which originate in the superior cervical ganglia. Pineal glands of groups of 10 animals were denervated by the removal of the superior cervical ganglia from both sides of the neck. This procedure also eliminated the rise in enzyme activity which follows the onset of darkness (Table IV).

DISCUSSION

The data described here show that exposure of rats to light rapidly influences the activity of the melatonin-synthesizing enzyme in the pineal gland. In the absence of light, a marked increase in enzyme activity can be demonstrated within 24 hours. These data also show that there is a naturally occurring diurnal rhythm in hydroxyindole *O*-methyltransferase activity. Several hours after the end of the light photoperiod (7 p.m. to midnight), there is a 1.6- to 3-fold increase in enzyme activity. This rhythm could be intrinsic or controlled by an environmental stimulus. The facts that the rise in the melatonin-forming enzyme disappears when rats are placed in constant light or darkness and that it can be extinguished when the stimulus of darkness is removed for a single photoperiod suggest that this rhythm is controlled by environmental lighting. Thus changes in pineal enzyme activity which occur in prolonged light or darkness (8) may represent an exaggeration of these diurnal alterations.

The increase in enzyme activity in darkness does not appear to be the consequence of changes in the amount of inhibitory substances in the pineal gland, since mixing homogenates of glands from light- and dark-treated animals did not affect the hydroxyindole *O*-methyltransferase activity of the latter. The observation that puromycin blocks the rise in hydroxyindole *O*-methyltransferase activity that accompanies darkness suggests that the effect of illumination on this enzyme may be mediated by protein synthesis. It has been previously shown that the rate of ^{32}P incorporation into the pineal is extremely rapid (14). These observations suggest a rapid turnover of protein and other metabolically active compounds in the pineal.

A relationship between environmental lighting and melatonin-forming activity different from that found here has been reported (15). In contrast to our finding, it was claimed that melatonin-forming activity *in vitro* was elevated in rats exposed to constant light; when animals were kept in normal diurnal lighting, the enzyme activity was greatest at 6 p.m. (16). In measuring the melatonin-forming activity, serotonin was incubated with *S*-adenosylmethionine and minced pineal gland, but without an acetyl coenzyme A-generating system, and the product was measured fluorometrically after extraction into *p*-cymene. With this assay system, the enzymatic formation of melatonin was more than 10 times as great as that reported here. In a previous communication it was shown that serotonin is a poor substrate for hydroxyindole *O*-methyltransferase (12). Furthermore, no melatonin was formed when serotonin was incubated with bovine pineal gland in the absence of an acetyl coenzyme A-generating system (1). In view of these contradictory observations, melatonin synthesis in the rat pineal was measured in this laboratory under the exact conditions described by Quay, with *S*-adenosylmethionine-methyl-^{14}C (15). No evidence was found for enzymatic formation of melatonin. In a parallel experiment, *N*-acetylserotonin was used as a substrate, and melatonin synthesis proceeded at the rate reported in this paper. It would appear that the product formed enzymatically in the assay procedure described by Quay was not melatonin.

Quay has reported that the serotonin content in the rat pineal falls from 70 mμg to 10 mμg between 6 p.m. and midnight (17); during this interval, pineal melatonin levels rise 3-fold, to about 3 mμg per gland (16). These changes are consistent with the marked rise in the hydroxyindole *O*-methyltransferase activity during this time. It seems likely that not all of the serotonin that disappears from the pineal is converted to melatonin. McIsaacs, Taborsky, and Farrell have identified 5-methoxytryptophol in the pineal (18). It is possible that the synthesis of this compound, as well as of other pineal methoxyindoles, by hydroxyindole *O*-methyltransferase may also be controlled by light.

Kappers has shown that the major, if not only, innervation of the rat pineal consists of nervi conares originating in the superior cervical ganglia of the sympathetic nervous system (19). Previous studies have shown that information about constant environmental lighting is transmitted to the pineal gland via a neural route, involving the retina and these sympathetic nerves (13). The experiments described here indicate that a similar neural pathway mediates the regulation of the melatonin-synthesizing enzyme by diurnal lighting. Sympathetic nerves in the pineal gland have been shown to contain norepinephrine (20) and serotonin (21). It is possible that nerve impulses generated by light may liberate these amines and that these, in turn, influence enzyme activity.

Exposure of rats to constant light reduces the weight of the pineal gland; darkness has the opposite effect (8, 9). Our results show that the weight of the pineal gland varies diurnally. This would suggest that lighting may influence many other biochemical processes in the pineal in addition to hydroxyindole *O*-methyltransferase activity and serotonin content. It was found that exposure to constant light increases the 5-hydroxytryptophan decarboxylase activity in the rat pineal gland (22). The effect of light on this enzyme is also mediated by the retina and the sympathetic nerves. Constant exposure to light also has been found to decrease pineal succinic acid dehydrogenase activity and glycogen content (15), and to change the morphology of the pineal gland (10).

The administration of microgram amounts of melatonin (23) or 5-methoxytryptophol (18) inhibits ovarian growth and the subsequent incidence of the estrous phase of the estrous cycle in the rat. Light has been shown to increase the incidence of estrus, presumably in part by inhibiting the synthesis of melatonin, which inhibits the estrous phase of the estrous cycle (24). Since there is a diurnal rhythm in the activity of the melatonin-synthesizing enzyme, as well as the serotonin content of the pineal, it is possible that this gland may serve as a "biological clock." Information about environmental lighting is perceived by the eyes, and relayed to the pineal gland by the sympathetic nervous system. The pineal gland then converts this environmental stimulus to a hormonal message by the inhibition or activation of melatonin synthesis. Melatonin is liberated from the pineal and participates in the regulation of another cyclic event, the estrous cycle.

SUMMARY

There is a marked rise in hydroxyindole *O*-methyltransferase activity in the pineal gland when rats are kept in darkness. This rise is blocked in animals given puromycin. There is a diurnal rhythm in hydroxyindole *O*-methyltransferase, with the greatest enzyme activity occurring at midnight. This rhythm is controlled by environmental lighting. Information about lighting reaches the pineal via the eyes and its sympathetic nerves.

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