THE UPTAKE OF H^3-MELATONIN IN ENDOCRINE AND NERVOUS TISSUES AND THE EFFECTS OF CONSTANT LIGHT EXPOSURE

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Shortly after the discovery of melatonin (5-methoxy N-acetyl tryptamine) in bovine pineal glands (Lerner *et al.*, 1960), the radioactive compound was synthesized, and its fate in animals was studied (Kopin *et al.*, 1961). It was shown that circulating melatonin was rapidly cleared from the blood, and was almost completely metabolized, largely by 6-hydroxylation, before being excreted as a conjugate in the urine. The tissue distribution of H^3-melatonin was examined in rats. It was shown that liver, kidney, small intestine, and adrenal gland concentrated the radioactive substance, relative to plasma.

Recently, evidence has been presented which indicates that melatonin has a hormonal role in mammals: very small doses of this compound (1 μg daily) inhibit ovarian growth in maturing rats, and subsequently depress the incidence of estrus (Wurtman *et al.*, 1963a); larger doses (150 to 500 μg daily) depress the response of the thyroid gland to methylthiouracil (Baschieri *et al.*, 1963), and reduce seminal vesicle weight in mature rats (Kappers, 1962). Since several synthetic radioactive hormones have been found to be concentrated in their physiologic target organs (Jensen and Jacobson, 1962; Whitby *et al.*, 1961), it was of interest to determine the uptake of H^3-melatonin by the gonads, thyroid, and other endocrine organs. It has been demonstrated that melatonin occurs naturally in mammalian peripheral nerves (Lerner *et al.*, 1959). Since only the pineal has the enzyme hydroxyindole-O-methyl transferase (HIOMT) required for melatonin synthesis (Axelrod *et al.*, 1961), it is possible that the melatonin present in nerves is taken up from the blood. This would imply that the pineal gland normally secretes melatonin into the circulation. To test this hypothesis, the ability of peripheral nerve to concentrate circulating H^3-melatonin was studied. The subcellular distribution of H^3-melatonin was also examined, as was the *in vivo* and *in vitro* uptake of the hormone by the pineal gland.

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The amount of light to which a rat is exposed influences the weight, morphology, and chemical composition of its pineal gland: rats maintained in constant light have smaller pineals (Fiske *et al.*, 1960; Wurtman *et al.*, 1961), containing small nucleoli, decreased cytoplasmic basophilic material (Roth *et al.*, 1962), and decreased serotonin (Quay and Halevy, 1962) and lipids (Quay, 1961), compared to pineals of animals kept in darkness. A drop in pineal weight has been demonstrated after as little as 6 days of light exposure (Wurtman *et al.*, 1963b). Pineals of rats exposed to constant darkness have a striking increase in HIOMT activity, while monoamine oxidase (MAO) levels remain unchanged (Wurtman *et al.*, 1963b). Data will be presented which indicate that light exposure, in addition to regulating melatonin synthesis, also alters the physiologic disposition of the circulating substance.

METHODS. Cats weighing 2 to 4 kg were anesthetized with pentobarbital (35 mg/kg, i.p.) and were given H^3-melatonin (200 μc/μmole) into a femoral vein, in a total volume of 2 ml of saline. Four animals received 100 μc; two received 750 μc. One hour later, they were killed by cardiac ligation, and a cardiac blood sample was taken. Tissues were rapidly removed, chilled, and assayed for H^3-melatonin. Sprague-Dawley female rats, weighing 200 to 300 g, were given 50 μc of H^3-melatonin in a total volume of 0.3 ml into a tail vein, and were killed by guillotine 6 seconds to 24 hours later. Cardiac and ovarian H^3-melatonin were assayed as described.

Estimation of H^3-melatonin. Melatonin-acetyl-H^3 (200 μc/μmole) was prepared as described before (Kopin *et al.*, 1961), by acetylating methoxytryptamine with H^3-acetic anhydride. H^3-melatonin was assayed in tissue by a modification of a procedure previously described (Kopin

TABLE 1

Tissue distribution of H³-melatonin

Cats 1 and 2 were given 750 μc H³-melatonin (200 μc/μmole) and cats 3 to 6 were given 100 μc, intravenously. Animals were killed 1 hour later and tissues were assayed for H³-melatonin. Averages are expressed in terms of an administered dose of 1 mc. Each ovary was examined separately; all other paired organs weighing under 500 mg were pooled.

	1	2	3	4	5	6	Average	
	mμc/100 g						μc/100 g	μg/100 g
Plasma	—	—	72	46	39	38	0.49	0.61
Pineal	13,800	7,800	660	—	1,990	4,580	20.23	25.29
Iris-choroid	—	—	473	736	—	290	4.99	6.25
Ovary	—	2,910	450	—	—	—	4.55	5.63
		3,150	544					
Pituitary	1,950	1,270	134	110	220	510	2.34	2.92
Sympath. chain	1,940	700	249	—	—	—	2.01	2.59
Periph. nerve	—	—	178	93	132	312	1.79	2.23
Testis	1,480	—	—	166	88	—	1.50	1.88
Thyroid	1,170	863	160	—	53	215	1.40	1.75
Adrenal	863	915	—	123	100	150	1.22	1.52
Kidney	780	728	164	86	98	81	1.05	1.31
Uterus	—	548	128	—	—	—	1.00	1.25
Liver	638	773	98	90	81	62	0.86	1.08
Pancreas	600	473	99	—	76	42	0.72	0.90
Salivary glands	435	608	109	50	41	22	0.60	0.75
Spleen	413	360	69	72	—	53	0.59	0.74
Heart	375	338	49	34	61	30	0.45	0.56
Skin	390	—	37	32	—	—	0.41	0.51
Brain	240	225	49	30	38	50	0.38	0.48
Diaphragm	—	—	—	—	7	35	0.21	0.27
Adipose tissue	135	128	33	8	—	—	0.19	0.24

et al., 1961), as follows: About 0.5 g of tissue, or the entire organ when it weighed less, was homogenized in 3 volumes of cold 0.4 N perchloric acid in an all-glass homogenizer. Eight ml of chloroform were added, and the mixture was further homogenized by 10 to 12 strokes of the plunger. The mixture was transferred to a glass-stoppered tube, and centrifuged for 5 minutes. Under these conditions, 98 to 100% of the H³-melatonin is extracted into the chloroform. The upper water layer was removed by aspiration, and the chloroform extract was washed once with 2 ml of water. After centrifugation and the removal of the water, a 6-ml aliquot of the chloroform layer was transferred to a vial and evaporated to dryness under a stream of hot air. The residue was taken up in 1 ml of ethanol, and 10 ml of phosphor were added. The radioactivity was measured in a liquid scintillation spectrophotometer. The specificity of this procedure was examined by paper chromatography of the washed chloroform extract obtained from several tissues, and at several times after the administration of H³-melatonin. In all cases, there was a single radioactive peak with the same R_f as authentic melatonin.

RESULTS. *Distribution of H³-melatonin in cats.* Circulating H³-melatonin was found to be taken up by all cat tissues examined, confirming and extending the findings of Kopin *et al.* (1961) in the rat (table 1). The pineal gland concentrated H³-melatonin 40-fold over plasma; ovary and the iris-choroid layer of the eye concentrated the circulating hormone 10-fold. Other endocrine tissues, as well as peripheral nerve and the sympathetic chain, concentrated melatonin 3- to 5-fold. With the exception of kidney and liver, most other tissues contained about as much H³-melatonin as an equal weight of plasma. Adipose tissue had the lowest concentration of H³-melatonin of all tissues studied, indicating that the ability of such tissues as ovary, pineal, and adrenal to concentrate the radioactive substance was unrelated to their relatively high lipid content. The fraction of the administered dose of H³-

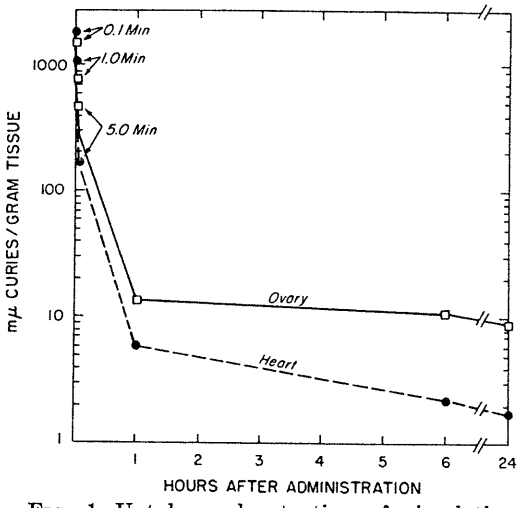

FIG. 1. Uptake and retention of circulating H^3-melatonin.

Six groups of six rats each were given 50 μc of H^3-melatonin (200 μc/μmole) intravenously, and were killed at various time intervals.

melatonin taken up by a unit weight of tissue was unrelated to the size of the dose. This suggests that the administered H^3-melatonin behaved as a true tracer substance.

Relation between tissue H^3-melatonin concentration and the distribution of the cardiac output. After the intravenous administration of substances which are rapidly cleared from the circulation, the fraction of the injected dose which is present in a tissue bears a relation to the fraction of the cardiac output delivered to that tissue (Wurtman *et al.*, 1963c). To determine whether the ability of certain tissues, such as ovary, to concentrate H^3-melatonin was the result of their receiving an exceptionally large proportion of the cardiac output per gram, ovarian and cardiac H^3-melatonin were determined in rats from 6 seconds to 24 hours after injection. Figure 1 indicates that in the first minute after injection, the heart contained more H^3-melatonin than an equivalent weight of ovary, suggesting that it received a greater proportion of the cardiac output per gram. Subsequently, the heart lost H^3-melatonin at a more rapid rate than ovary, so that after 1 hour the latter tissue contained 2.5 times as much H^3-melatonin per gram as heart, and 24 hours later more than 5 times as much.

Subcellular distribution and in vitro uptake of H^3-melatonin. The subcellular distribution of H^3-melatonin was studied in the rat ovary and adrenal using sucrose density gradients (Potter and Axelrod, 1963). One hour after the intravenous administration of 100 μc of the radioactive hormone, 90 to 95% of the radioactivity was found in the supernatant fraction. The mitochondrial fraction contained 2 to 3% of the radioactive material; little or none was found among the other particulate material. The pellet containing the densest material was not analyzed. These tissues were also studied by homogenization in isotonic KCl and centrifugal fractionation into 1000 × *g* ("nuclei and cell debris"), 10,000 × *g* ("mitochondrial"), 100,000 × *g* ("microsomal"), and supernatant fractions. Again, most of the radioactivity was present in the soluble supernatant fluid. About 10 to 20% was found in the 1000 × *g* pellet, while a small but consistent proportion of the tissue radioactivity was associated with the "mitochondrial" fraction.

Fresh bovine pineal slices weighing a total of 20 mg were incubated at 37°C with Krebs-Ringer bicarbonate buffer and 0.5 μg of H^3-melatonin, in an atmosphere of 95% oxygen–5% carbon dioxide. After 1 hour, the slices were assayed for H^3-melatonin. Pineal slices were found to concentrate H^3-melatonin 1.5- to 4-fold (four experiments), as compared to the medium. Slices kept at 0°C for the same length of time showed no concentration of H^3-melatonin. Other incubated pineal slices were homogenized in isotonic KCl and fractionated as described above. The supernatant fluid contained 97.4% of the radioactivity; the 1000 × *g*, 10,000 × *g* and 100,000 × *g* fractions contained 0.8%, 1.5%, and 0.3%, respectively. Almost all of the radioactive material present in pineals after incubation with H^3-melatonin was shown chromatographically to be the unchanged compound.

Effect of light on the distribution of H^3-melatonin. Twenty-eight day-old Sprague-Dawley rats were exposed either to continuous fluorescent light or to normal diurnal lighting for 5 weeks. At the end of this time, all animals were sexually mature; light-treated rats had an incidence of estrus of 75%, as compared to 45% among their controls. The animals were than given 50 μc of H^3-melatonin by tail vein, and killed 45 minutes later. Hearts and ovaries were assayed for H^3-melatonin. Light-treatment resulted in a highly significant decrease in the concentration of H^3-melatonin by ovary, but did not alter cardiac concentration of the hormone (table 2). Pineal

glands of light-treated rats also contained less H³-melatonin than controls; however, the total number of counts present in this small organ was too low to permit a reliable evaluation of the effects of light.

DISCUSSION. Circulating melatonin was found to be highly concentrated by the pineal, the iris-choroid, and the ovary; it was also concentrated 2- to 5-fold in other endocrine organs, and in peripheral and sympathetic nerves. The high level of H³-melatonin in the pineal could be the result of either a specific concentrating mechanism, or mixture of the tracer with a large endogenous melatonin pool. It is likely that both of these mechanisms play a role, since a temperature-dependent *in vitro* concentrating mechanism has been demonstrated, and the level of endogenous melatonin in the pineal, the only tissue which can synthesize it, is at least 1000-fold greater than any other tissue studied (Barchas and Lerner, personal communication). No particular storage site for H³-melatonin could be demonstrated in the pineal, ovary, or adrenal. H³-melatonin storage differs from that of H³-catecholamines, which are bound in most tissues in dense core vesicles (Wolfe *et al.*, 1962; Potter and Axelrod, 1963). It is possible that H³-melatonin does bind to a particle which is too fragile to withstand homogenization.

The 10-fold concentration of H³-melatonin by cat ovary, and 3- to 5-fold concentration by thyroid and pituitary, are of interest in view of the recent observations that melatonin inhibits ovary growth, the estrus cycle (Wurtman *et al.*, 1963a) and thyroidal uptake of I^{131} (Baschieri *et al.*, 1963). In order for a hormone to act on a physiologic target organ, it is not necessary that that organ selectively concentrate the hormone. However, in the few cases in which the tissue uptake of physiologic doses of hormones has been studied, target organs have been found to concentrate them. For example, estradiol is preferentially retained by uterus and vagina (Jensen and Jacobson, 1962), while catecholamines are bound in high concentrations in heart, spleen, and uterus (Whitby *et al.*, 1961; Wurtman *et al.*, 1963d). H³-melatonin is also taken up by brain; thus its physiologic effects on gonad and thyroid could result from an effect on this organ as well. The rat ovary concentrates H³-melatonin 2.5- to 5-fold more than heart after 1 hour, as compared to 10-fold in the cat. Since the ovary is a heterogeneous structure, containing germinal epithelium, corpora lutea, follicular tissue and fluids, a varying amount of hemorrhage, and other elements, it is possible that this difference in ovarian concentration of melatonin is related to a species difference in the proportion of each of these tissue components in ovary.

TABLE 2

Effect of constant light on the concentration of H³-melatonin by rat ovary and heart

	No. Animals	Lighting	Organ Wt (mg)	cpm/ Organ	cpm/g
Ovary	21	Diurnal	52	188 ± 27	3620 ± 520
Ovary	21	Constant	65	139 ± 25	2140 ± 380*
Heart	7	Diurnal	560	555 ± 80	995 ± 140
Heart	7	Constant	545	460 ± 75	850 ± 140

Rats were exposed to constant or diurnal lighting for 5 weeks. They were then given 50 μc of H³-melatonin (200 μc/μmole) intravenously, and killed 45 minutes later. Hearts and ovaries were assayed for H³-melatonin. Data are given as mean ± S.E.
* $P < .01$.

Peripheral nerve contains endogenous melatonin (Lerner *et al.*, 1959), and cannot synthesize this substance (Axelrod *et al.*, 1961), but can concentrate it from the circulation. It appears likely that the endogenous melatonin in nerve is derived from the circulation; thus melatonin is probably secreted by the pineal gland into the blood.

It has previously been demonstrated that exposure of rats to constant light inhibits melatonin synthesis in the pineal gland (Wurtman *et al.*, 1963b). This may constitute a mechanism for the effect of light upon the estrous cycle. The data presented here indicate that in rats exposed to constant light, the uptake of melatonin by the ovary is also diminished. This may serve as another means whereby light could affect the estrous cycle.

SUMMARY

The uptake of circulating H³-melatonin was examined in endocrine and other tissues, in cats and rats. It was found that the pineal gland, iris-choroid, ovary, and other endocrine and peripheral nervous structures took up and retained this compound. The high uptake by ovary was unrelated to hemodynamic factors. Exposure of rats to constant light markedly inhibited the

concentration of melatonin by ovary, but not by heart. Bovine pineal slices were found to concentrate H^3-melatonin. Subcellular distribution studies in pineal, ovary, and adrenal showed that most of the retained H^3-melatonin was confined to the soluble supernatant fraction.

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