MOLECULAR PHARMACOLOGY, 9, 605–611

Control of Pineal *N*-Acetylserotonin by a *Beta* Adrenergic Receptor

MICHAEL BROWNSTEIN,[1] JUAN M. SAAVEDRA,[2] AND JULIUS AXELROD

Laboratory of Clinical Science, National Institute of Mental Health, Bethesda, Maryland 20014

(Received February 20, 1973)

SUMMARY

BROWNSTEIN, MICHAEL, SAAVEDRA, JUAN M., AND AXELROD, JULIUS: Control of pineal *N*-acetylserotonin by a *beta* adrenergic receptor. *Mol. Pharmacol.* **9**, 605–611 (1973).

A specific and sensitive assay for *N*-acetylserotonin, the precursor of the pineal hormone melatonin, is described. The levels of *N*-acetylserotonin in the pineal gland rise *in vivo* after the administration of *l*-isoproterenol. *l*-Propranolol, a *beta* adrenergic blocking agent, reverses the rise in *N*-acetylserotonin caused by isoproterenol within 15 min. Phentolamine, an *alpha* adrenergic blocking agent, is without effect. Turning on the lights or administering *l*-propranolol at night causes a rapid decrease in the amount of *N*-acetylserotonin in the pineal. Thus the level of *N*-acetylserotonin varies as does the level of *N*-acetyltransferase, which in turn is controlled by stimulation of a *beta* adrenergic receptor.

INTRODUCTION

Marked circadian rhythms in the levels of serotonin *N*-acetyltransferase (EC 2.3.1.5) (1) and of *N*-acetylserotonin (2), the precursor of the pineal hormone melatonin, in the rat pineal gland have been reported. During the night the activity of serotonin *N*-acetyltransferase and the amount of *N*-acetylserotonin, the product of this enzyme, are high; during the day both the enzyme and its product are low. The *N*-acetyltransferase rhythm is regulated by sympathetic nerves arising in the superior cervical ganglia (3). During the day, when the *N*-acetyltransferase activity is low, L-dopa, *l*-norepinephrine, and *l*-isoproterenol cause a marked increase in *N*-acetyltransferase (4, 5). This increase is blocked by *l*-propranolol, a β adrenergic blocking agent, but not by phenoxybenzamine, an *alpha* adrenergic blocking agent. Exposure of rats to light at night causes a rapid decrease in the activity of pineal *N*-acetyltransferase (5, 6). Propranolol also produced a precipitous decrease in the level of *N*-acetyltransferase at night (5). In this report a specific, sensitive enzyme assay for *N*-acetylserotonin is described. Using this assay it was demonstrated that the level of *N*-acetylserotonin in the pineal gland is under the control of a *beta* adrenergic receptor, stimulation of which alters the activity of *N*-acetyltransferase.

MATERIALS AND METHODS

Sprague-Dawley male rats, 160–200 g, obtained from Hormone Assay Laboratories, Chicago, were used in all experiments except in studies of the effect of light and propranolol on *N*-acetylserotonin levels during the night. In the latter experiments 180-g Osborne-Mendel (NIH strain) female rats were used. Osborne-Mendel rats show more

[1] Research Associate in the Pharmacology-Toxicology Program, National Institute of General Medical Sciences, National Institutes of Health, Bethesda, Maryland 20014.

[2] Recipient of Public Health Service International Fellowship F05 TW 1738 and Fellow *ad honorem*, Consejo de Investigaciones Cientificas y Tecnicas, Republica Argentina.

marked circadian changes in pineal indole metabolism than do Sprague-Dawley rats. All rats were housed under diurnal lighting conditions with lights on from 6:00 a.m. to 6:00 p.m. During the day rats were killed by decapitation between 12:00 noon and 2:00 p.m. Each experimental group consisted of five to seven animals. Drugs were dissolved in 0.9% NaCl and injected subcutaneously in a volume of 0.5 ml. During the night rats received injections at 10:00 p.m. and were handled with the aid of a dim red light.

N-Acetyltransferase assay. *N*-Acetyltransferase was assayed using the method of Deguchi and Axelrod (7); 20 nmoles of [1-^{14}C]acetyl coenzyme A instead of 4 nmoles were used in a 70-μl reaction mixture.

Purification of hydroxyindole O-methyltransferase. Frozen bovine pineals were obtained from Pel-Freez Biologicals, Inc., Rogers, Ark. Hydroxyindole *O*-methyltransferase from bovine pineal was partially purified by a modification of the method previously described (8). All purification procedures were carried out at 4°. Bovine pineal glands were homogenized in 5 volumes of isotonic KCl and centrifuged at 78,000 × *g* for 1 hr. To the soluble supernatant fraction, solid ammonium sulfate was added. The 36–55% precipitate was dissolved in 5 ml of 5 mM sodium phosphate buffer, pH 7.9, and subjected to three successive dialyses, 3 hr each, against 200 volumes of the same buffer. After dialysis the enzyme was divided into 0.5-ml aliquots and stored at −15° until used. The enzyme preparation had a protein concentration of 15–20 mg/ml and an activity of about 350–400 units/ml, using *N*-acetylserotonin as substrate. One unit of enzymatic activity is defined as the amount of enzyme that forms 1 nmole of product per milligram of protein per hour.

Assay of N-acetylserotonin. This assay depends on the enzymatic transfer of the [^{3}H]methyl group of *S*-adenosyl-L-[*methyl*-^{3}H]methionine to the hydroxyl group of *N*-acetylserotonin by hydroxyindole *O*-methyltransferase (EC 2.1.1.4) (9). The enzymatically formed [^{3}H]melatonin is separated by extraction into a nonpolar organic solvent. The use of *methyl*-^{3}H-labeled *S*-adenosyl-L-methionine makes it possible to measure as little as 0.20 pmole of *N*-acetylserotonin. Osborne-Mendel rats of either sex, weighing 200–250 g, were used. The animals were killed by decapitation, and the pineals were immediately removed, homogenized in 0.4 ml of ice-cold 0.1 N HCl, and centrifuged at 16,000 × *g* for 30 min. Brain, retina, and pituitary glands were also removed, frozen on Dry Ice, weighed while still frozen, and homogenized in 20 volumes of ice-cold 0.1 N HCl. Aliquots (50 μl) of the supernatant fluid were transferred to 15-ml glass-stoppered centrifuge tubes containing 5 μl of 0.1 N NaOH, 200 μl of 0.2 M sodium phosphate buffer (pH 7.9), 5 μl (0.55 nmole) of *S*-adenosyl-L-[*methyl*-^{3}H]methionine (specific activity, 4.5 mCi/μmole), and 10 μl (4 units) of partially purified hydroxyindole *O*-methyltransferase. The final volume of the reaction mixture was 270 μl. Nine picomoles of *N*-acetylserotonin were added to another aliquot as an internal standard. The mixture was incubated for 20 min at 37°, and the reaction was stopped by the addition of 0.5 ml of 0.5 M borate buffer, pH 10. The radioactive products were extracted into 6 ml of toluene by mixing for 15 sec with a Vortex mixer (Scientific Industries Inc., Springfield, Mass.) and centrifuged. A 5-ml aliquot of the organic phase was transferred to a counting vial and allowed to dry in a chromatography oven at 80° overnight. One milliliter of ethanol was added to the counting vials, followed by 10 ml of phosphor containing 40 ml of Liquifluor (New England Nuclear Corporation) per liter of toluene. The radioactivity of the samples was determined by liquid scintillation spectrometry. Blanks were obtained by replacing the tissue extract in the reaction mixture with 50 μl of 0.1 N HCl. Blank counts ranged from 150 to 250 cpm.

Several precautions should be observed when performing the enzymatic *N*-acetylserotonin assay. The sensitivity of the assay relies on the low blank values, between 150 and 250 cpm. It is essential that hydroxyindole *O*-methyltransferase be dialyzed to reduce the amount of endogenous substrates. After dialysis the enzyme is divided into aliquots and kept in a freezer at −20°. Unnecessary freezing and thawing should be avoided. An evaporation step is neces-

sary to remove [^{3}H]methanol formed enzymatically from *S*-adenosyl-L-[*methyl*-^{3}H]methionine (10).

Thin-layer chromatography. The identification of [^{3}H]melatonin, the product of the reaction, was made by thin-layer chromatography on coated Eastman chromagram sheets of silica gel, 100 μ in thickness. The solvents used were (a) chloroform–methanol–acetic acid (93:7:1), (b) methyl acetate–2-propanol–ammonium hydroxide (10%) (45:35:20), (c) acetone–ammonium hydroxide (99:1), and (d) toluene–acetic acid–ethyl acetate–water (80:40:20:1). All sheets were activated by heating for 30 min at 90° in an oven immediately prior to use. Aliquots of rat pineal supernatant were assayed as described above. Radioactive standards were prepared by incubating 90 pmoles of authentic *N*-acetylserotonin with 5 μl of *S*-adenosyl-L-[*methyl*-^{3}H]methionine (0.55 nmole), 10 μl of partially purified hydroxyindole *O*-methyltransferase (3.5–4 units), 200 μl of 0.2 M sodium phosphate buffer (pH 7.9), 5 μl of 0.1 N NaOH, and 50 μl of 0.01 N HCl for 20 min at 37°. Blanks were prepared in the same way without the addition of *N*-acetylserotonin. After the enzymatic reaction the toluene extracts were dried under vacuum at 40° for 1 hr, and the residue was taken up in 50 μl of ethanol, with the addition of nonradioactive melatonin (2 μg) as carrier, and spotted on the chromatography sheets. Other nonradioactive 5-hydroxyindoles were cochromatographed on the same sheet. After development, the sheets were stained with Ehrlich's reagent (1 g of *p*-dimethylaminobenzaldehyde in 10 ml of concentrated HCl plus 90 ml of acetone). The sheets were then marked in 1-cm sections and placed in vials containing 2 ml of ethanol and 20 ml of phosphor, and the radioactivity was measured. In the pineal at least 90% of the radioactive product extracted was found to have the same R_F values as authentic melatonin in three solvent systems (Fig. 1). The radioactive peaks were also found to be isographic with the peaks obtained from the radioactive standards in all the systems examined.

Although *N*-acetylserotonin is the best substrate for hydroxyindole *O*-methyltransferase, other hydroxyindoles, such as serotonin, *N*-methylated derivatives of serotonin, and 5-hydroxyindoleacetic acid, are also methylated (8). Compounds that are substrates for hydroxyindole *O*-methyltransferase were carried through the entire assay procedure. Serotonin, *N*-methylserotonin, bufotenine, and 5-hydroxyindoleacetic acid, in concentrations equimolar with that of *N*-acetylserotonin, produced less than 1% of the activity of *N*-acetylserotonin (Table 1).

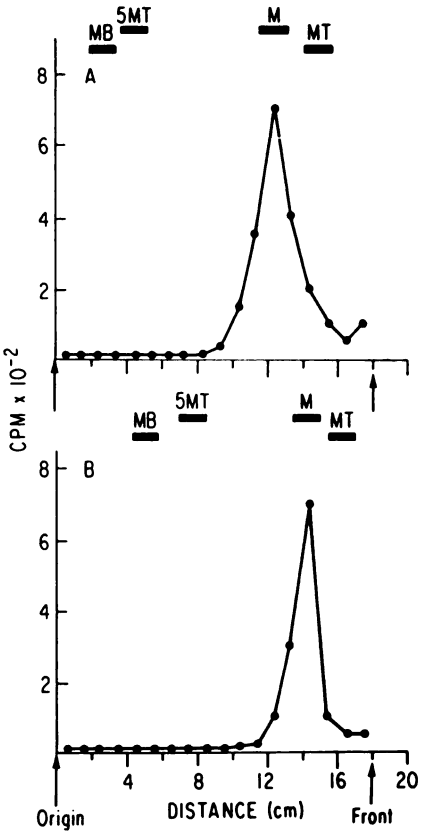

FIG. 1. *Specificity of N-acetylserotonin assay*
Rat pineal glands were treated as described under MATERIALS AND METHODS. Solvents used were toluene–acetic acid–ethyl acetate–water (80:40:20:5) (A) and acetone–ammonium hydroxide (99:1) (B). M, melatonin; MT, methoxytryptophol; MB, methylbufotenine (*O*-methyl-*N*,*N*-dimethylserotonin); 5MT, 5-methoxyserotonin.

Drugs. The following drugs were obtained from Regis Chemical Company: *N*-acetyl-

TABLE 1

Specificity of N-acetylserotonin assay

The substrates were carried through the procedure as described in the text. Blank values were 230 cpm.

Substrate	Amount	Activity obtained in assay	Percentage of activity from 4.5 pmoles (1 ng) of *N*-acetylserotonin
	ng	*cpm*	%
N-Acetyl-serotonin	1	6,300	100
Serotonin	1	0	0
	100	50	2.5
Bufotenine	1	50	0.7
	100	1,650	25
N-Methyl-serotonin	1	150	2.5
	100	180	2.7
5-Hydroxy-tryptophol	1	120	2
	100	10,000	160
5-Hydroxy-indole-acetic acid	1	0	0
	100	0	0

serotonin, serotonin creatinine phosphate, melatonin, methoxytryptamine, *N*-methylserotonin, bufotenine bioxalate hydrate, 5-methoxy-*N*,*N*-dimethyltryptamine, 5-hydroxyindoleacetic acid, methoxyindoleacetic acid, 5-hydroxytryptophol, and methoxytryptophol. *S*-Adenosyl-L-[*methyl*-^{3}H]methionine (4.5 mCi/μmole) was purchased from New England Nuclear Corporation. [1-^{14}C]Acetyl coenzyme A (5.8 Ci/mole) was purchased from Amersham/Searle. We are indebted to Ayerst Laboratories for providing *l*-propranolol, and to Ciba-Geigy Corporation for phentolamine.

RESULTS

Assay conditions. The enzymatic *O*-methylation of *N*-acetylserotonin was found to be linear with time up to 20 min, and with enzymatic concentrations up to 5 μl (100 μg of protein). A linear relationship with *S*-adenosyl-L-[*methyl*-^{3}H]methionine up to 5 μl (0.55 nmole) was also found. The amount of [^{3}H]melatonin formed in the reaction was proportional to the amount of *N*-acetylserotonin added to the reaction mixture (0.5–70 pmoles) (Fig. 2). Product formation was a linear function of the amount of *N*-acetylserotonin added either to buffer or to pineal extracts. The recoveries of *N*-acetylserotonin added to pineal gland supernatant fractions were 95–100% when compared with *N*-acetylserotonin added to the reaction mixture. In any given experiment the recovery varied with a 5% range for duplicate samples. Internal standards of *N*-acetylserotonin were used to correct for recoveries.

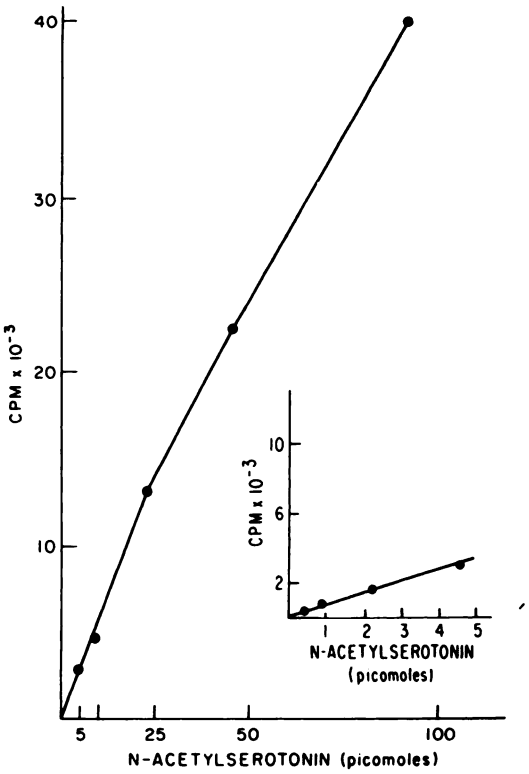

FIG. 2. *Sensitivity of N-acetylserotonin assay*
Various amonts of *N*-acetylserotonin were dissolved in 0.1 N HCl and carried through the whole procedure as described in the text. Blanks were 150 cpm.

Effect of exposure to light and of beta adrenergic blockade on pineal N-acetyltransferase. At night, when the activity of *N*-acetyltransferase is high (1), the amount of *N*-acetylserotonin in the pineal was elevated (2). When animals were transferred from darkness to light at night there was a rapid decline in *N*-acetyltransferase activity (5, 6) ($t_{1/2}$ = 3 min), and a correspondingly fast increase in serotonin (11). Propranolol also caused a precipitous fall in *N*-acetyltrans-

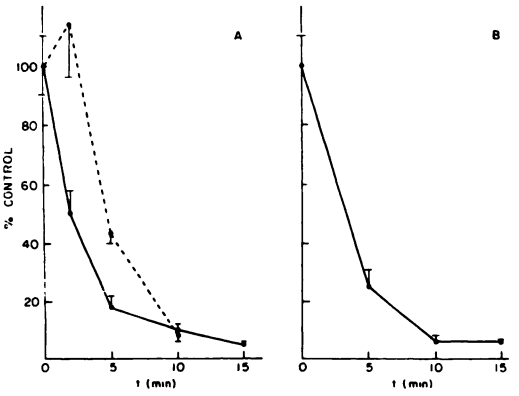

FIG. 3. *Effects of light and l-propranolol on N-acetylserotonin at night*

A. Rats were placed in a light room at 9:00 p.m. and killed after various periods of exposure to light. ——, *N*-acetylserotonin levels; - - -, *N*-acetyltransferase activity. Vertical bars give the magnitude of the standard errors. N = 5–7/group.

B. Rats received injections of *l*-propranolol (20 mg/kg) in a dark room at 9:00 p.m. The procedure was accomplished with the aid of a dim red light. *N*-Acetylserotonin was assayed at various times after injection.

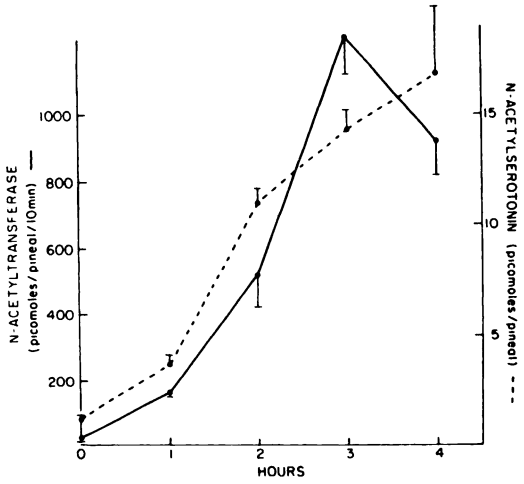

FIG. 4. *Time course of l-isoproterenol effects on N-acetylserotonin and serotonin N-acetyltransferase*

Rats were treated with *l*-isoproterenol (5 mg/kg). The enzyme and its product were assayed at various times after injection. Vertical bars represent standard errors (N = 5–7/group). - - -, *N*-acetylserotonin levels; ——, enzyme activity.

ferase activity and a rapid increase in serotonin (12). Transferring animals from darkness to light or injecting *l*-propranolol into animals in the dark resulted in a marked, speedy fall in *N*-acetylserotonin in the pineal (Fig. 3).

Effect of isoproterenol and adrenergic blocking agents on N-acetylserotonin and N-acetyltransferase levels. Isoproterenol (5 mg/kg) given during the day, when the *N*-acetyltransferase was low, caused an elevation in *N*-acetyltransferase activity (5). In the first hour there was a small increase in the level of the enzyme, followed by a more pronounced increase during the second, third, and fourth hours. The *N*-acetylserotonin gradually increased in response to the elevation of the transferase (Fig. 4). Four hours prior to death between 1:00 and 2:00 p.m., rats were given an injection of isoproterenol or 0.9% NaCl. The two sets of animals were each divided into three groups. One group received no further drug treatment; one group received *l*-propranolol (20 mg/kg) 15 min prior to death, and one group received phentolamine (25 mg/kg) 15 min prior to death. The last group of rats showed ptosis by the time they were killed. The *N*-acetylserotonin content of glands from isoproterenol-treated animals was 21 times higher than that of glands from NaCl-treated controls (Table 2). Administration of *l*-propranolol to control rats before death resulted in no change in *N*-acetylserotonin from the control figure, but within 15 min the *beta* adrenergic blocking agent completely reversed the rise in *N*-acetylserotonin caused by isoproterenol (Table 2). This finding is consistent with the observation that propranolol rapidly reduces *N*-acetyltransferase activity after the enzyme has been induced by isoproterenol (13).

Phentolamine, an *alpha* adrenergic blocking agent, produced no significant change in the *N*-acetylserotonin content of glands from isoproterenol-treated animals or NaCl-treated controls (Table 2). Phentolamine has been shown to have no effect on either the daytime level of *N*-acetyltransferase or the level of the enzyme after its induction by isoproterenol (12).

N-Acetylserotonin in tissues. *N*-Acetylserotonin levels were examined in rat brain re-

TABLE 2

Effects of propranolol and phentolamine on isoproterenol-induced increase in N-acetylserotonin

Isoproterenol (5 mg/kg) was given to rats 4 hr and propranolol (20 mg/kg) or phentolamine (25 mg/kg) 15 min prior to killing at 1:00 p.m. All drugs were given subcutaneously to five to seven animals per treatment group.

Treatment	*N*-Acetylserotonin
	pmoles/pineal
0.9% NaCl	0.75 ± 0.13
Propranolol	0.73 ± 0.19
Phentolamine	0.87 ± 0.14
Isoproterenol	15.06 ± 1.90[a]
Isoproterenol + propranolol	1.30 ± 0.27
Isoproterenol + phentolamine	19.19 ± 2.86[a]

[a] Differs from NaCl-treated controls ($p < 0.001$).

gions, retina, and pituitary. Brains were dissected according to the method of Glowinski and Iversen (14). Negligible levels (less than 4 pmoles/g) were found in these tissues during either day or night.

DISCUSSION

The development of a sensitive and specific assay for *N*-acetylserotonin has allowed us to study the regional distribution of this indole in the brain and peripheral organs of the rat. *N*-Acetylserotonin is present in significant concentrations only in the pineal gland. We have confirmed the finding of a marked circadian rhythm in *N*-acetylserotonin levels in the pineal (2): the nocturnal content is 25 times as high as that measured during the day. Turning on the light at night causes a precipitous fall in *N*-acetylserotonin, the time course of which is similar to that seen for *N*-acetyltransferase, the enzyme which synthesizes *N*-acetylserotonin. Administration of *l*-propranolol also causes a rapid fall in both *N*-acetylserotonin and *N*-acetyltransferase (5). These observations suggest that stimulation of pineal *beta* adrenergic receptors at night induces *N*-acetyltransferase, which in turn synthesizes *N*-acetylserotonin from serotonin. The fact that serotonin, which is lower at night than during the day (15), increases rapidly at night when animals are placed in a light room or treated with propranolol (11, 12) provides further evidence in favor of the above hypothesis.

Additional evidence to support this thesis comes from studies undertaken during the day, when the levels of *N*-acetylserotonin and *N*-acetyltransferase are normally low. A single dose of isoproterenol causes *N*-acetyltransferase to increase many times over the control level within 3 hr (4). The increase in activity of this enzyme appears to produce a decrease in serotonin (12) and a concomitant increase in *N*-acetylserotonin. The increase in *N*-acetyltransferase (13) and the decrease in serotonin (12) can both be rapidly reversed by propranolol, but not by phentolamine. Other *beta* adrenergic blocking agents, such as pronethalol and practolol, reverse the effect of isoproterenol on *N*-acetyltransferase (13). The isoproterenol-induced increase in *N*-acetylserotonin is also reversed by *beta* adrenergic blockade. The rapidity of the fall in *N*-acetylserotonin when *N*-acetyltransferase activity is diminished by light or by a *beta* blocking agent presumably reflects rapid metabolism of *N*-acetylserotonin by hydroxyindole *O*-methyltransferase, to produce melatonin. The pineal gland has relatively large amounts of the *O*-methylating enzyme (8). Thus the sequence of biochemical events which lead to the formation of melatonin exhibits a hierarchical system of controls. First, stimulation of the *beta* adrenergic receptors by the release of norepinephrine from sympathetic nerve terminals in the pineal produces an increase in cyclic 3′,5′-AMP and an induction of *N*-acetyltransferase. Circadian stimulation appears to result from 24-hr changes in nerve impulse traffic along sympathetic fibers innervating the pineal; there is a diurnal cycle in norepinephrine turnover in the pineal—the turnover is at least twice as high at night as it is during the day.[3] Similarly, pharmacological or physiological manipulations which cause or mimic the release of the *beta* adrenergic agonist produce a rise in *N*-acetyltransferase (4, 5, 16) and,

[3] M. Brownstein and J. Axelrod, unpublished observations.

consequently, of *N*-acetylserotonin and melatonin (17). When *N*-acetyltransferase is induced, the synthesis of melatonin is limited only by the availability of its substrate, serotonin, the level of which is in turn controlled by uptake of tryptophan from the circulation and by the activity of tryptophan hydroxylase. This model for regulation of indole metabolism might be applied to the control of indoles in regions of the central nervous system where serotonin-containing nerve cells might be innervated by adrenergic nerves.

ACKNOWLEDGMENT

The authors thank Dr. Takeo Deguchi for his helpful criticism and advice throughout the course of these studies.

REFERENCES

1. D. C. Klein and J. L. Weller, *Science* **169**, 1093–1095 (1970).
2. D. C. Klein and J. L. Weller, *Excerpta Med. Int. Congr. Ser.* **256**, 52 (1972).
3. D. C. Klein, J. L. Weller, and R. Y. Moore, *Proc. Nat. Acad. Sci. U. S. A.* **68**, 3107–3110 (1971).
4. T. Deguchi and J. Axelrod, *Proc. Nat. Acad. Sci. U. S. A.* **69**, 2208–2211 (1972).
5. T. Deguchi and J. Axelrod, *Proc. Nat. Acad. Sci. U. S. A.* **69**, 2547–2550 (1972).
6. D. C. Klein and J. L. Weller, *Science* **177**, 532–533 (1972).
7. T. Deguchi and J. Axelrod, *Anal. Biochem.* **50**, 174–179 (1972).
8. J. Axelrod and H. Weissbach, *J. Biol. Chem.* **236**, 211–213 (1961).
9. J. Axelrod and H. Weissbach, *Science* **131**, 1312 (1960).
10. J. Axelrod and J. Daly, *Science* **150**, 892–893 (1965).
11. H. Illnerová, *Life Sci.* **10**, 955–960 (1971).
12. M. Brownstein, R. Holz, and J. Axelrod, *J. Pharmacol. Exp. Ther.* In press.
13. T. Deguchi, *Mol. Pharmacol.* **9**, 184–190 (1973).
14. J. Glowinski and L. L. Iversen, *J. Neurochem.* **13**, 655–669 (1966).
15. W. B. Quay, *Gen. Comp. Endocrinol.* **3**, 473–479 (1963).
16. P. H. Volkman and A. Heller, *Science* **173**, 839–840 (1971).
17. H. J. Lynch, P. Wang, and R. J. Wurtman, *Life Sci.* In press.