

*STIMULATION OF C¹⁴-MELATONIN SYNTHESIS FROM
C¹⁴-TRYPTOPHAN BY NORADRENALINE IN RAT
PINEAL IN ORGAN CULTURE**

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Abstract.—Previous work has shown that the activity of the melatonin-forming enzyme in the rat pineal gland is elevated in rats kept in continuous darkness as compared to those kept in continuous light. Information about environmental lighting reaches the pineal gland via nerves that liberate noradrenaline. Rat pineal glands in organ culture can form C¹⁴-melatonin from C¹⁴-tryptophan as follows: tryptophan → 5-hydroxytryptophan → serotonin → melatonin.

Noradrenaline was found to stimulate the synthesis of C¹⁴-melatonin from C¹⁴-tryptophan in rat pineals in organ culture. Other compounds related in structure to noradrenaline increase melatonin and serotonin synthesis and inhibit the formation of the deaminated product of serotonin, 5-hydroxyindole acetic acid. Cycloheximide, a compound that inhibits protein synthesis, also prevents the formation of serotonin, melatonin, and 5-hydroxyindole acetic acid from tryptophan in pineal organ culture. These observations suggest that noradrenaline liberated from sympathetic nerves stimulates the formation of melatonin either by increasing the formation of new melatonin-forming enzyme, by increasing transport of tryptophan into the pineal cell, or by inhibiting the metabolism of serotonin by the alternate deaminating pathway.

The rat pineal gland contains an enzyme, hydroxyindole-O-methyltransferase, that O-methylates N-acetylserotonin to form melatonin.¹ The activity of this enzyme is more than twice as great among rats kept in continuous darkness as among animals maintained in continuous light.² When the sympathetic nerves to the pineal are cut, light or darkness no longer produce changes in the activity of the melatonin-forming enzyme.³ These observations suggest that the release of a transmitter substance from the pineal sympathetic nerves is influenced by environmental lighting and that this neurotransmitter affects the activity of hydroxyindole-O-methyltransferase.

Recently we have shown that the rat pineal gland in organ culture can form melatonin from its amino acid precursor, tryptophan.^{4, 5} The use of this experimental system has made it possible to examine the factors in the sympathetic nerves that influence hydroxyindole-O-methyltransferase. In both the innervated pineal gland⁶ and the rat pineal grown in organ culture,⁵ the synthesis of melatonin proceeds as follows: tryptophan → 5-hydroxytryptophan → serotonin → N-acetylserotonin → melatonin. Serotonin can also be metabolized by deamination and oxidation to form 5-hydroxyindoleacetic acid (HIAA).⁷

This report will show that the sympathetic nerve transmitter, noradrenaline, increases the synthesis of melatonin by pineal glands maintained in organ culture.

Materials and Methods.—Pineal glands, taken from Sprague-Dawley female rats weighing 160–180 gm, were clotted to the walls of a Wasserman tube.⁴ Nutrient media⁴ (0.5 ml) containing DL-C¹⁴-tryptophan (New England Nuclear, 0.5 μ c in a 10⁻⁴ M solution) and added factors were sealed with a rubber stopper and incubated for 2 days at 37°C. The culture tubes were rotated in a roller wheel. Each group contained from six to eight individual pineal glands. Control groups were incubated with C¹⁴-tryptophan and added factors without pineal gland. At the end of the incubation period, the tubes were kept at -10 to -70°C. The contents of the tubes were assayed for total radioactivity, C¹⁴-melatonin, C¹⁴-serotonin, and C¹⁴-5-hydroxyindoleacetic acid, as previously described.⁵

L-Noradrenaline-D-bitartrate; L-adrenaline-D-bitartrate, tyramine, and dopamine; and DL-octopamine HCl and tryptamine were obtained commercially. β -Isopropylphenylhydrazine (Castron) and D-noradrenaline were kindly supplied by Lakeside Laboratories, Milwaukee, and Winthrop Sterling Laboratory, Rensselaer, New York, respectively.

Results.—*Effect of noradrenaline on the synthesis of C¹⁴-melatonin:* Pineal glands in culture tubes were incubated in the presence of varying amounts of noradrenaline. At the end of two days of incubation, the media were assayed for C¹⁴-melatonin, C¹⁴-serotonin, and C¹⁴-HIAA. At a concentration of 3×10^{-4} M, noradrenaline caused about a threefold increase in the formation of C¹⁴-melatonin from the C¹⁴-tryptophan (Table 1). At lower concentrations, the catecholamine also stimulated melatonin synthesis to a lesser extent. The addition of noradrenaline to the incubation medium produced no change in the net amount of C¹⁴-serotonin present, but there was a slight decrease in HIAA. The effect of noradrenaline on the formation of radioactive serotonin, melatonin, and HIAA over a period of time is shown in Figure 1. The stimulating effect of noradrenaline on melatonin synthesis is apparent as early as four hours after the start of the incubation.

The identity of the C¹⁴-melatonin formed from tryptophan in the presence of noradrenaline was examined chromatographically. The medium obtained after incubating rat pineals with C¹⁴-tryptophan and 3×10^{-4} M noradrenaline for two days was mixed with an equal volume of pH 10 borate buffer (0.2 M), and the apparent C¹⁴-melatonin formed was extracted into chloroform in the same manner as in the procedure described for measuring C¹⁴-melatonin.⁵ The chloroform extract was washed with 2 ml of borate buffer; then the organic

TABLE 1. *Stimulation of melatonin formation from tryptophan in pineal glands in organ culture by noradrenaline.*

Concentration of noradrenaline	C ¹⁴ -melatonin	C ¹⁴ -serotonin	C ¹⁴ -HIAA
0	0.45 \pm 0.05	2.1 \pm 0.18	1.1 \pm 0.10
3×10^{-5} M	0.65 \pm 0.10	2.1 \pm 0.35	0.9 \pm 0.18
1×10^{-4} M	0.79 \pm 0.16	2.6 \pm 0.5	0.9 \pm 0.18
3×10^{-4} M	1.20 \pm 0.15*	2.7 \pm 0.32	0.6 \pm 0.09†

Groups of eight culture tubes containing a rat pineal were incubated with C¹⁴-tryptophan (1×10^{-4} M) for two days at 37°C, and the various indole metabolites were measured. Results are expressed as per cent formation from tryptophan.

* $P < 0.001$. † $P < 0.05$.

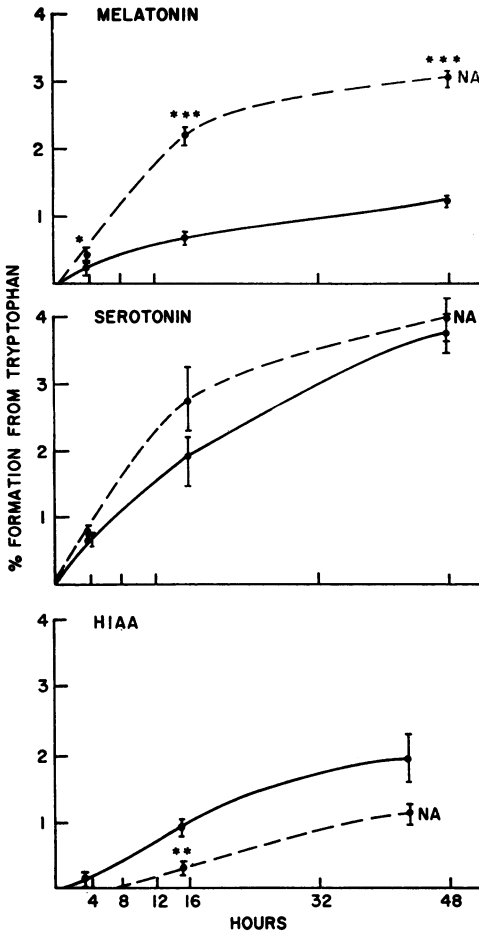


FIG. 1.—Formation of metabolites of tryptophan and the effect of noradrenaline (NA). Each group containing culture tubes of pineal glands of six rats was incubated at 37°C with C^{14} -tryptophan ($1 \times 10^{-4} M$) in the presence or absence of noradrenaline ($3 \times 10^{-4} M$). After various periods of time, the culture tubes were assayed for indole metabolites. Results are expressed as per cent formation from tryptophan. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

To examine this possibility, Catron, a potent monoamine oxidase inhibitor, was incubated with C^{14} -tryptophan. This compound caused an inhibition in the formation of C^{14} -HIAA and an elevation in the C^{14} -serotonin and C^{14} -melatonin levels.

Effect of metabolites of tryptophan on the formation of melatonin: Pineal glands in organ culture were incubated for two days with C^{14} -tryptophan together with serotonin, melatonin, or HIAA at concentrations varying from $3 \times 10^{-4} M$ to

phase was evaporated to dryness and taken up in a small volume of methanol. This extract was subjected to ascending paper chromatography with Whatman no. 1 paper and butanol:acetic acid:water 8:2:2. A single peak of radioactivity was found with the same R_f as authentic melatonin.

Effects of other phenylethylamine derivatives on melatonin formation: Compounds structurally related to noradrenaline were examined for their ability to modify the synthesis of C^{14} -melatonin in pineal organ culture (Table 2). All the phenylethylamine derivatives studied, as well as tryptamine, stimulated the synthesis of melatonin from C^{14} -tryptophan to varying degrees; the β -hydroxylated primary amines, L- and D-noradrenaline and octopamine, produced the greatest stimulation. D-Noradrenaline, L-adrenaline, dopamine, tyramine, and tryptamine all elevated the C^{14} -serotonin content and markedly inhibited the formation of its deaminated product, C^{14} -HIAA. In contrast, L-noradrenaline had no effect on C^{14} -serotonin synthesis and only slightly inhibited C^{14} -HIAA formation. This suggested that the phenylethylamine derivatives and tryptamine inhibit the deamination of C^{14} -serotonin, thus diverting its metabolism to the alternate pathway of O-methylation.

TABLE 2. *Effect of various phenylethylamine derivatives on the formation of melatonin in pineal organ culture.*

Amine	C ¹⁴ -melatonin	C ¹⁴ -serotonin	C ¹⁴ -HIAA
None	0.45 ± 0.05	1.3 ± 0.2	0.80 ± 1.6
L-Noradrenaline	1.31 ± 0.20*	1.4 ± 1.8	0.61 ± 1.2
D-Noradrenaline	1.68 ± 0.15†	2.85 ± 0.2*	0
L-Epinephrine	0.84 ± 0.16	2.21 ± 0.3‡	0.76 ± 0.2
Dopamine	0.87 ± 18‡	2.4 ± 0.3‡	0
Tyramine	0.72 ± 0.06‡	6.4 ± 0.05‡	0
DL-Octopamine	2.7 ± 0.30‡	7.4 ± 0.71‡	1.0 ± 0.11
Tryptamine	1.4 ± 0.16‡	3.4 ± 0.6‡	0.25 ± 0.05‡
Catron	1.1 ± 0.20*	3.5 ± 0.8‡	0.22 ± 0.07‡

Groups of six culture tubes containing a rat pineal gland were incubated with C¹⁴-tryptophan ($1 \times 10^{-4} M$) and amines ($3 \times 10^{-4} M$) for two days at 37°C. Results are expressed as per cent formation from tryptophan.

* $P < 0.01$. † $P < 0.001$. ‡ $P < 0.05$.

$3 \times 10^{-5} M$. None of these compounds had any effect on the amounts of isotopically labeled melatonin, serotonin, or HIAA formed.

Formation of melatonin in pineal organ culture after cycloheximide: To determine whether the formation of new enzyme protein was required for the synthesis of melatonin, rat pineal in organ cultures was incubated with C¹⁴-tryptophan and an inhibitor of protein synthesis, cycloheximide,⁸ 10 μg of cycloheximide (20 μg/ml) completely inhibited the formation of C¹⁴-serotonin as well as its O-methylated and deaminated metabolites—C¹⁴-melatonin and C¹⁴-HIAA (Table 3)—while 1 μg partially inhibited melatonin and serotonin formation.

Discussion.—Noradrenaline, the neurotransmitter of sympathetic nerves,⁹ increases the formation of C¹⁴-melatonin from C¹⁴-tryptophan by pineal glands in organ culture. The observation that sympathetic nerves influence the activity of the melatonin-forming enzyme in the rat pineal³ and that noradrenaline stimulates the synthesis of C¹⁴-melatonin suggests that the effect of sympathetic nerves on pineal hydroxyindole-O-methyltransferase activity is mediated via its neurotransmitter. The concentration of noradrenaline discharged from the nerves is in the order of $10^{-4} M$,¹⁰ or about the same level as that found to stimulate the *in vitro* formation of melatonin. Serotonin, a compound present in both pineal sympathetic nerves and parenchymal cells,¹¹ did not effect the formation of C¹⁴-melatonin.

The mechanism whereby noradrenaline increases the synthesis of C¹⁴-melatonin from its amino acid precursor appears to be complex. The formation

TABLE 3. *Effect of cycloheximide on biosynthesis of melatonin from tryptophan in pineal organ culture.*

Cycloheximide (μg)	C ¹⁴ -melatonin	C ¹⁴ -serotonin	C ¹⁴ -HIAA
0	0.43 ± 0.06	1.9 ± 0.32	1.0 ± 0.1
1.0	0.12 ± 0.02*	0.4 ± 1*	0.62 ± 0.08
10.0	0	0	0

Groups of six culture tubes containing a rat pineal gland were incubated with C¹⁴-tryptophan ($1 \times 10^{-4} M$) and cycloheximide for two days at 37°C. Results are expressed as per cent formation from tryptophan.

* $P < 0.001$.

of melatonin from tryptophan involves several steps. Tryptophan is transported into the pineal cell where it is incorporated into protein and is also metabolized via several pathways (Fig. 2). A fraction is hydroxylated to form 5-hydroxytryptophan, which is then decarboxylated to yield serotonin. Most of the serotonin is then deaminated by monoamine oxidase and then reduced to 5-hydroxytryptophol or oxidized to HIAA. A small fraction is N-acetylated to N-acetylserotonin and then O-methylated to melatonin by hydroxyindole-O-methyltransferase. Noradrenaline could increase melatonin formation by acting at one or more of these steps. Preliminary studies indicate that noradrenaline increases the intracellular transport of C^{14} -tryptophan and the incorporation of this amino acid into pineal proteins.¹² The increase in the total 5-hydroxylated indoles found when noradrenaline is present in the

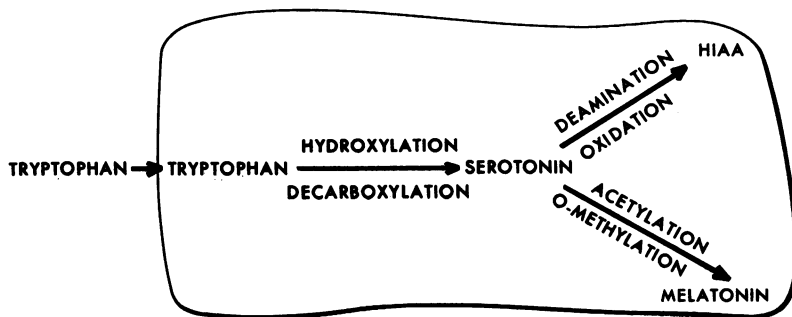


FIG. 2.—Uptake and metabolism of tryptophan in pineal organ culture.

medium would indicate that the hydroxylation step is also stimulated. The fall in the formation of C^{14} -HIAA would also suggest that noradrenaline inhibits monoamine oxidase, thus diverting the metabolism of serotonin to the alternate pathway of acetylation and O-methylation.

Phenylethylamine and monoamine oxidase inhibitors were found to increase the synthesis of both C^{14} -melatonin and C^{14} -serotonin and to inhibit the formation of C^{14} -HIAA. Thus, these compounds appear to elevate melatonin mainly by inhibiting monoamine oxidase. Noradrenaline, on the other hand, did not elevate the serotonin level; and it depressed the formation of C^{14} -HIAA only to a small extent, indicating that this catecholamine causes a rise in C^{14} -melatonin formation by other mechanisms in addition to monoamine oxidase inhibition. Melatonin is the only product of C^{14} -tryptophan whose synthesis is increased by noradrenaline, hence this amine may exert a major effect on the O-methylation step.

The formation of melatonin from tryptophan in pineal culture requires new enzyme synthesis as demonstrated by the inhibition of formation of all metabolites by a protein synthesis inhibitor, cycloheximide. Almost complete inhibition of melatonin formation occurred at concentrations of cycloheximide (1 μ g) which are not toxic in tissue culture for six hours,¹⁴ during which time melatonin formation can be measured. Previous studies have also shown that

puromycin given *in vivo* blocks the effect of light and darkness on the melatonin-forming enzyme.¹³ These findings, together with the observation that noradrenaline stimulates the incorporation of C¹⁴-tryptophan into proteins in pineal organ culture,¹¹ suggest that the catecholamine stimulates new enzyme protein formation.

Summary.—Noradrenaline stimulates the synthesis of C¹⁴-melatonin from C¹⁴-tryptophan by rat pineals in organ culture. Other phenylethylamine derivatives increase melatonin and serotonin synthesis and inhibit the formation of 5-hydroxyindole acetic acid. Cycloheximide inhibits the formation of serotonin, melatonin, and 5-hydroxyindole acetic acid from tryptophan in pineal organ culture.

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