*INCORPORATION OF ^{14}C-TRYPTOPHAN INTO ^{14}C-PROTEIN BY CULTURED RAT PINEALS: STIMULATION BY l-NOREPINEPHRINE**

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Abstract.—Organ cultures of individual rat pineals incorporate ^{14}C-tryptophan into proteins at a nearly constant rate for at least 48 hours. Previous studies have shown that these cultures also convert ^{14}C-tryptophan to serotonin, melatonin, and 5-hydroxyindoleacetic acid, and release these indoles into the media. The formation of ^{14}C-protein from ^{14}C-tryptophan is accelerated by the addition to the culture medium of *l*-norepinephrine or related catecholamines but is not modified by serotonin, melatonin, or 5-hydroxyindoleacetic acid.

One mechanism by which norepinephrine stimulates the synthesis of ^{14}C-protein from ^{14}C-tryptophan involves increasing the uptake of the ^{14}C-tryptophan into pineal parenchymal cells, inasmuch as (1) norepinephrine increases the intracellular content of ^{14}C-tryptophan as well as its conversion to its major products, ^{14}C-protein and ^{14}C-indoles; (2) norepinephrine does not stimulate ^{14}C-protein synthesis in pineal organs which contain a previously fixed amount of ^{14}C-tryptophan; and (3) norepinephrine does not stimulate ^{14}C-protein synthesis from ^{14}C-methionine or ^{14}C-leucine.

The finding that norepinephrine, but not serotonin, can stimulate the incorporation of ^{14}C-tryptophan into pineal proteins is consistent with the hypothesis that norepinephrine is the neurotransmitter substance utilized by pineal sympathetic nerve endings.

The parenchymal cells of the mammalian pineal organ are unusual in that they receive direct sympathetic innervation.[1, 2] This innervation controls important biochemical functions in the pineal and mediates all known pineal responses to environmental lighting.[3] If rats are housed under continuous illumination, the weight of the pineal[4] and the activity of the pineal-specific melatonin-forming enzyme, hydroxyindole-O-methyl transferase, decline;[5] darkness has opposite effects. These adaptations are not observed if the sympathetic nerves to the pineal are destroyed by removing both superior cervical ganglia.[6] The sympathetic nerve endings in the rat pineal contain relatively high concentrations of norepinephrine[7, 8] and even higher concentrations of serotonin,[9] a compound which may function as a neurotransmitter within certain regions of the central nervous system.[10] Therefore, although sympathetic nerves elsewhere in the body are thought to produce their physiological effects by liberating norepinephrine,[11] either norepinephrine or serotonin could, at least theoretically, function as the sympathetic neurotransmitter in the pineal.

Rat pineals maintained in organ culture convert isotopically labeled tryptophan to serotonin, melatonin, and 5-hydroxyindoleacetic acid and release these compounds into their culture medium.[12] We have recently shown that the rate at which these cultures form ^{14}C-melatonin is increased when norepinephrine is added to the incubation medium.[13] We now find that cultured pineals also incorporate ^{14}C-tryptophan into new proteins, and that the rate at which the ^{14}C-proteins are formed is also increased by the addition of norepinephrine. Considered in conjunction with our previous observations,[13] these data suggest that norepinephrine, and not serotonin, functions as the sympathetic neurotransmitter within the rat pineal.

Materials and Methods.—Pineal organs were removed between 11 A.M. and 1 P.M. from adult female Sprague-Dawley rats previously housed under diurnal lighting conditions; they were dissected free of adherent connective or vascular tissue under a dissecting microscope. Each pineal was clotted individually to the walls of a Wasserman tube containing 0.5 ml of nutrient medium,[12, 14] *dl*-^{14}C-tryptophan (0.5 μc in a 10^{-4} solution, except where specified), and catecholamines or other added factors. The tubes were sealed with rubber stoppers and incubated on a roller wheel at 37°C. In most experiments, the period of incubation was 48 hr. Each unincubated control group or treatment group contained six to eight pineals.

At the end of the incubation period, the nutrient medium was removed by pipetting, and each cultured pineal was washed with 1.5 ml of Hanks' balanced salt solution. The ^{14}C protein present in each organ was assayed by a modification of the method of Sokoloff and Kaufman.[15] Individual pineals were homogenized in 1 ml of ice-cold 6% trichloroacetic acid (TCA). The whole homogenate was centrifuged at 600 $\times$ *g* at room temperature; the supernatant fluid was removed, and the pellet was washed twice again with 4% TCA. It was then resuspended in 4% TCA, heated to 90–95°C for 15 min, centrifuged, and resuspended in 1 ml of 4% TCA. This suspension was filtered through a Millipore filter, and its radioactivity was counted in planchets by means of a gas-flow counter.

Total pineal protein content was measured by the method of Lowry *et al.*[16]

Results.—Synthesis of ^{14}C-protein from ^{14}C-tryptophan by rat pineals in organ culture: Pineal organs incorporated ^{14}C-tryptophan into protein at a more or less constant rate for at least 48 hours without a change of medium. In any single experiment, there was surprisingly little variation in the amount of ^{14}C protein present after incubation in control pineal glands; the standard deviation of protein counts per minute (cpm) was generally 5–10 per cent of the mean.

Effect of l-norepinephrine and other catecholamines on incorporation of ^{14}C-tryptophan into pineal ^{14}C protein: The addition of *l*-norepinephrine to the culture medium in concentrations as low as 3×10^{-5} *M* caused a significant increase in pineal ^{14}C-protein content following a 48-hour incubation with ^{14}C-tryptophan (Table 1). Higher concentrations of norepinephrine (i.e., 3×10^{-4} *M*) increased pineal ^{14}C-protein levels by 50–125 per cent in each of six separate experiments. It was not possible to determine whether norepinephrine also increased the total amount of unlabeled protein formed by cultured pineals, inasmuch as total protein content always declined by 25–30 per cent in the course of a 48-hour incubation, whether or not norepinephrine was added to the medium.

The naturally occurring catecholamines dopamine and *l*-epinephrine also

stimulated the formation of ^{14}C-protein from ^{14}C-tryptophan: *l*-epinephrine was slightly less effective than *l*-norepinephrine, while dopamine was considerably more potent (Table 2). The *d*-isomer of norepinephrine was about as effective in stimulating ^{14}C protein synthesis as the naturally occurring *l*-form.

Effect of pineal indoles on ^{14}C protein synthesis: We have previously shown that the addition of norepinephrine to rat pineals in organ culture increases the rate at which they convert ^{14}C-tryptophan to ^{14}C-melatonin.[13] In order to determine whether this compound or other characteristic pineal indoles (serotonin or 5-hydroxyindoleacetic acid) could, in turn, modify the rate of pineal protein synthesis, various concentrations of each indole were added to the culture media before incubation. None altered pineal ^{14}C-protein content (Table 3).

TABLE 1. *Effect of various concentrations of l-norepinephrine on the incorporation of ^{14}C-tryptophan into pineal ^{14}C-protein.*

Norepinephrine concentration (molarity)	^{14}C-protein content (cpm) ± s.e.m.
Control	364 ± 31
3×10^{-6}	401 ± 22
3×10^{-5}	444 ± 24*
3×10^{-4}	531 ± 20†

Pineals were incubated for 48 hr. The ^{14}C-protein content of unincubated organs (zero-time controls) was 41 ± 4 cpm.
* $P < 0.05$ differs from control.
† $P < 0.001$ differs from control.

TABLE 2. *Effect of various catecholamines on synthesis of ^{14}C-protein from ^{14}C-tryptophan.*

Catecholamine	^{14}C-protein content (cpm) ± s.e.m.
Control	134 ± 3
l-Norepinephrine	299 ± 24*
d-Norepinephrine	298 ± 24*
l-Epinephrine	233 ± 28†
Dopamine	412 ± 24‡

The ^{14}C-protein content of unincubated pineals was 12 ± 2 cpm. All catecholamines were present in a concentration of 3×10^{-4} *M*. Pineals were incubated for 48 hr.
* $P < 0.001$ differs from control.
† $P < 0.05$ differs from control.
‡ $P < 0.001$ differs from control; $P < 0.05$ differs from *l*-norepinephrine and *d*-norepinephrine.

TABLE 3. *Effect of various pineal indoles on synthesis of ^{14}C-protein from ^{14}C-tryptophan.*

Compound	Concentration (molarity)	^{14}C-protein content (cpm) ± s.e.m.
Serotonin	Control	413 ± 34
	10^{-5}	522 ± 57
	3×10^{-5}	339 ± 47
	10^{-4}	351 ± 50
Melatonin	Control	586 ± 82
	3×10^{-5}	501 ± 95
	10^{-4}	410 ± 49
	3×10^{-4}	528 ± 113
5-Hydroxyindoleacetic acid	Control	413 ± 77
	3×10^{-4}	368 ± 46

Pineals were incubated for 48 hr. The ^{14}C-protein content of unincubated organs was 45 ± 12 cpm.

Effect of l-norepinephrine on incorporation of other ^{14}C amino acids into pineal ^{14}C protein: The effect of *l*-norepinephrine (3×10^{-4} *M*) on the incorporation of ^{14}C amino acids other than tryptophan into pineal protein was studied, using media fortified with ^{14}C-methionine (0.5 μc), ^{14}C-leucine (0.2 μc), or a mixture of ^{14}C amino acids (0.05 μc, New England Nuclear Corp., NEC-445) containing ^{14}C-tyrosine, ^{14}C-phenylalanine, and 13 other amino acids, but lacking ^{14}C-tryptophan. Incubated pineals incorporated large amounts of ^{14}C-methionine or ^{14}C-leucine into protein; however, these rates of incorporation were not increased by adding *l*-norepinephrine to the culture medium. The formation of pineal ^{14}C protein from the mixture of ^{14}C amino acids was stimulated by about 15 per cent in the presence of *l*-norepinephrine (Table 4).

TABLE 4. *Effect of l-norepinephrine on the formation of pineal ^{14}C-protein from ^{14}C-methionine, ^{14}C-leucine, or a mixture of ^{14}C amino acids.*

	Zero-time Incubation		48-Hr Incubation	
^{14}C amino acids	Control	Norep.	Control	Norep.
Methionine	93 ± 11	39 ± 2	1055 ± 85	982 ± 74
Leucine	87 ± 14	65 ± 4	4199 ± 255	4008 ± 419
Mixture	115 ± 17	43 ± 4	1517 ± 66	1714 ± 32*

* $P < 0.05$ differs from control.

Effect of l-norepinephrine on uptake of ^{14}C-tryptophan or ^{14}C-α-aminoisobutyric acid by pineal organs: *l*-Norepinephrine might enhance the synthesis of ^{14}C-protein from ^{14}C-tryptophan by acting at any of several loci: (1) It might increase the rate at which the ^{14}C amino acid is taken up from the medium, either by stimulating the mechanisms which transport tryptophan into the pineal cells or by enhancing the rate at which intracellular tryptophan is utilized (i.e., in the synthesis of pineal indoles). This might increase the specific activity of the intracellular ^{14}C-tryptophan pool, thereby making more of the labeled amino acid available for conversion to ^{14}C-protein. (2) Norepinephrine might act within the pineal cell to decrease the amount of tryptophan utilized by competing biochemical pathways (i.e., those involved in the synthesis of pineal indoles); this would allow more of the amino acid to be utilized for protein synthesis. Previous studies suggest that the catecholamine does not have this effect; i.e., its presence stimulates ^{14}C-indole synthesis.[13] (3) It could act directly on the mechanisms which control protein synthesis, perhaps by increasing the synthesis of various species of RNA or the proportion of messenger RNA bound to polysomes.

To determine whether norepinephrine enhanced the rate at which ^{14}C-tryptophan is taken into pineal parenchymal cells, the intracellular ^{14}C-tryptophan content was examined at steady state in organs incubated for 48 hours with or without the catecholamine. By means of solvent extraction methods described previously,[12] it was first demonstrated that the TCA supernatant obtained by homogenizing such pineals contained negligible amounts of ^{14}C-serotonin, ^{14}C-5-hydroxyindoleacetic acid, or ^{14}C-melatonin; this indicates that essentially all of these compounds formed during the course of the incubation are released into the culture medium.[12] With a butanol–acetic acid–water

(8:2:2) paper chromatographic system, it was then demonstrated that at least 85 per cent of the radioactivity present in the pineal supernatant had the same *Rf* as authentic tryptophan. These results indicated that a reliable estimate of intracellular ^{14}C-tryptophan content could be obtained by measuring the radioactivity present in an aliquot of the pineal supernatant fluid.

Pineals incubated in the presence of *l*-norepinephrine (3×10^{-4} *M*) contained significantly more ^{14}C-tryptophan, as well as more ^{14}C-protein, than organs incubated without the catecholamine (Table 5). An additional study was then performed to determine whether pineal organs containing a predetermined amount of intracellular ^{14}C-tryptophan converted more of the labeled amino acid to ^{14}C protein in the presence of *l*-norepinephrine. Pineals were preincubated with ^{14}C-tryptophan for 24 hours and then incubated with *l*-norepinephrine (3×10^{-4} *M*) for an additional 24 hours in the presence of only unlabeled tryptophan (10^{-4} *M*). Under these conditions, no increase in pineal ^{14}C-protein synthesis was noted in tubes containing the catecholamine (Table 6). Other pineals exposed to ^{14}C-tryptophan during both the preincubation and incubation periods did produce more ^{14}C protein in the presence of *l*-norepinephrine.

The effect of norepinephrine on the initial uptake of the nonutilizable amino acid ^{14}C-α-aminoisobutyric acid was next examined. Pineals were incubated in media containing the acid, with or without added *l*-norepinephrine, for 20 minutes; control organs were unincubated. The pineals were then digested in warm sodium hydroxide, and their radioactivity was counted in a liquid scintillation spectrophotometer. Pineal organs incubated for 20 minutes with added *l*-norepinephrine (3×10^{-4} *M*) contained significantly more ^{14}C-α-aminoisobutyric acid than those whose media lacked the catecholamine (Table 5).

TABLE 5. *Effect of l-norepinephrine on uptake of ^{14}C-tryptophan and ^{14}C α-aminoisobutyric acid by cultured rat pineals.*

^{14}C amino acid	Intracellular ^{14}C Amino Acid Content (% of zero-time level)	
	Control	Norepinephrine
Tryptophan	108 ± 9	174 ± 7*
α-Aminoisobutyric acid	181 ± 21	247 ± 19†

Pineal organs were incubated with ^{14}C-tryptophan for 48 hr, or with ^{14}C α-aminoisobutyric acid for 20 min.

* $P < 0.001$ differs from control.

† $P < 0.05$ differs from control.

TABLE 6. *Effect of l-norepinephrine on synthesis of ^{14}C-protein by pineals incubated with ^{14}C-tryptophan.*

Experimental conditions	^{14}C-tryptophan	Norepinephrine	^{14}C-protein content (cpm)
Preincubated			102 ± 7
Incubated	−	−	82 ± 9
	−	+	72 ± 7
	+	−	148 ± 11
	+	+	209 ± 11*

Pineal organs were incubated for 24 hr with ^{14}C-tryptophan and nutrient medium containing unlabeled tryptophan (10^{-4} *M*). Some of the organs were then incubated for additional 24 hr with unlabeled tryptophan (10^{-4} *M*) and with or without ^{14}C-tryptophan and norepinephrine (3×10^{-4} *M*).

* $P < 0.001$ differs from control group incubated without norepinephrine.

This effect of norepinephrine was not observed among pineals incubated with the acid for one hour or longer.

Discussion.—These data demonstrate that cultured pineal glands take up ^{14}C-tryptophan from their media and incorporate it into pineal proteins at a fairly constant rate for at least 48 hours. Their synthesis of ^{14}C protein, like that of ^{14}C-melatonin,[13] is markedly stimulated by adding *l*-norepinephrine or other catecholamines to the culture media (Table 2), but not by serotonin (Table 3). This is compatible with the hypothesis that norepinephrine functions as the sympathetic neurotransmitter within the mammalian pineal.

The major mechanism through which *l*-norepinephrine stimulates the synthesis of pineal ^{14}C proteins from ^{14}C-tryptophan probably involves increases both in the rate at which the amino acid enters the pineal parenchymal cells and in its intracellular specific activity. This could result from a direct action on the transport system in the cell membrane. Alternatively, it might be the passive consequence of increased utilization of intracellular tryptophan by other metabolic pathways (i.e., for the synthesis of indoles). If norepinephrine is added to pineal organ cultures in such a manner that it is not able to enhance the cellular uptake of ^{14}C-tryptophan (i.e., by using pineals which have been preincubated with ^{14}C-tryptophan), the ability of the catecholamine to stimulate ^{14}C-protein synthesis is lost (Table 6). Pineal organs incubated with ^{14}C-tryptophan in the presence of *l*-norepinephrine contain significantly more of the ^{14}C amino acid than control pineals; they also take up the nonutilizable amino acid α-aminoisobutyric acid from their medium more rapidly (Table 5). The ability of norepinephrine to stimulate tryptophan uptake appears to be shared by several other catecholamines (Table 2). In contrast, few, if any, other amino acids share with tryptophan the capacity to have their uptake accelerated by *l*-norepinephrine, inasmuch as the catecholamine does not stimulate the synthesis of pineal ^{14}C-protein from ^{14}C-methionine or ^{14}C-leucine (Table 4). ^{14}C-protein synthesis from a ^{14}C-amino acid mixture lacking ^{14}C-tryptophan but containing ^{14}C-tyrosine and ^{14}C-phenylalanine is stimulated slightly by *l*-norepinephrine (Table 4). This suggests that norepinephrine may also facilitate the uptake of a few other amino acids besides tryptophan (perhaps the aromatic amino acids). An alternative explanation would be that the net rate of pineal protein synthesis may also, in fact, be enhanced by the catecholamine.

Total protein content of the pineal consistently decreased during the incubation period, and any protein that might have been released into the medium was present in too low a concentration for us to measure.[16] Similarly, there was too little unlabeled tryptophan in each pineal to permit its assay; hence we were unable to measure any effect that norepinephrine might have had on the specific activity of ^{14}C-tryptophan within the pineal cells. However, the data available do allow the tentative conclusion that the rate of protein synthesis within the rat pineal is relatively high: at least 0.1 μmole of ^{14}C-tryptophan per gram of tissue was incorporated into pineal protein per day of incubation; the true rate of incorporation may be considerably higher, inasmuch as our procedure did not measure any ^{14}C-protein secreted into the incubation medium.

The amount of ^{14}C-tryptophan converted to ^{14}C-serotonin and its products ^{14}C-melatonin and ^{14}C-5-hydroxyindoleacetic acid by pineal organ cultures is at least 40 times as great, however, as the amount incorporated into protein.[16]

Previous observations on daily rhythms in pineal weight and in the activity of a specific pineal protein, the enzyme hydroxyindole-O-methyl transferase,[17] have also indicated a rapid rate of protein synthesis in this organ. If rats are maintained under cyclic lighting conditions (lights on 12 hr per day), transferase activity rises and falls severalfold with a 24-hour rhythm. The stimulatory effect of darkness on transferase activity is blocked by puromycin.[17] The transferase rhythm is dependent upon signals transmitted to the pineal by its sympathetic nerves. Our present experiments suggest that this signal is ultimately mediated by norepinephrine, which is released from the sympathetic neurons as a neurotransmitter. This hypothetical function for pineal norepinephrine is supported by the demonstration that the norepinephrine concentration in the rat pineal also varies with a 24-hour rhythm, whose phase characteristics are similar to those of the transferase rhythm.[8]

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