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SCIENCE

Melatonin Synthesis: Adenosine 3′,5′-Monophosphate and Norepinephrine Stimulate N-Acetyltransferase

Abstract. *Treatment of cultured rat pineal glands with norepinephrine or dibutyryl adenosine 3′,5′-monophosphate causes a six- to tenfold stimulation of* N-*acetyltransferase. This enzyme converts serotonin to* N-*acetylserotonin, the immediate precursor of melatonin. The increased synthesis of melatonin caused by norepinephrine treatment appears to be the result of stimulation of* N-*acetyltransferase by an adenosine 3′,5′-monophosphate mechanism.*

Melatonin, the unique pineal methoxyindole, is formed as follows: tryptophan → hydroxytryptophan → serotonin → *N*-acetylserotonin → melatonin (*1*). Studies with intact rat pineals in vitro indicate that melatonin synthesis (*1*) may be controlled by the neurotransmitter norepinephrine (NE) (*2, 3*). The stimulatory effect of this amine might be mediated in part by adenosine 3′,5′-monophosphate (cyclic AMP) (*4–6*), the "second messenger" of many hormones (*7*). These conclusions are based on the following observations. Adenyl cyclase in pineal homogenates and whole pineal glands is stimulated by NE (*4*). The addition of either NE or N^6,O^2-dibutyryl adenosine 3′,5′-monophosphate (dibutyryl cyclic AMP) to pineal gland cultures stimulates the conversion of labeled tryptophan to melatonin (*2, 3, 5, 6*). Cyclic AMP does not produce a similar effect when added to pineal cultures (*5, 6*). This is probably secondary to a slower rate of transport across cell membranes and a more rapid intracellular degradation by a phosphodiesterase (*8, 9*).

The specific metabolic site, or sites, in melatonin synthesis at which the stimulatory effect of NE may be mediated by cyclic AMP (*5, 6*) is not known. No enzyme or transport mechanism in the pineal gland has been observed to be altered after treatment with NE or dibutyryl cyclic AMP.

We report that addition of NE or dibutyryl cyclic AMP to pineal organ cultures results in a six- to tenfold stimulation of net *N*-acetyltransferase synthesis and melatonin production. This indicates that one metabolic site at which the neurotransmitter NE regulates melatonin production through a cyclic AMP mechanism is *N*-acetylation.

Rat pineal glands (*10*) were incubated for 24 hours (*3, 11*). In the first study (Table 1), the incubation medium contained [^{3}H]tryptophan (5 μc/ml, 25 μc/μmole, Schwarz BioResearch). Melatonin production was estimated by measuring the amount of [^{3}H] melatonin that appeared in the incubation medium (*2, 3, 11*). Chloroform extraction (*2, 11*) yielded a radioactive compound that was identified as melatonin by two-dimensional thin-layer chromatography (*12*).

N-Acetyltransferase activity in the homogenate of an individual rat pineal gland or in a portion of a pool of pineal homogenates (*10*) was estimated by measuring the amount of *N*-[^{14}C]acetylserotonin and [^{14}C]melatonin formed by pineal homogenates after incubation with [^{14}C]serotonin (0.5 m*M*) and acetyl coenzyme A (0.5 m*M*) for 30 minutes (*13, 14*).

After the pineal glands were incubated for 8 to 24 hours (Table 1), *N*-acetyltransferase activity in control glands (groups 2 and 3) declined to approximately 30 to 60 percent of the original activity (group 1). Pineal glands incubated in medium containing 1.0 m*M* dibutyryl cyclic AMP (group 4) or 0.1 m*M* NE (group 5) produced six- to tenfold more melatonin during the 16-hour treatment than did control glands (group 3). Homogenates of treated glands had six- to tenfold higher *N*-acetyltransferase activity at the end of culture.

The addition of either 0.1 m*M* NE, 1.0 m*M* dibutyryl cyclic AMP, or 10 m*M* cyclic AMP to enzyme assays of pooled homogenates of cultured control glands or fresh glands did not cause a stimulation of *N*-acetyltransfer-

Table 1. *N*-Acetyltransferase activity and total [^{3}H]melatonin in medium during the last 16 hours of incubation of pineal gland organ cultures. Individual pineal glands were incubated for an initial period of 8 hours, and then transferred to a second culture vessel for an additional 16 hours. Some incubations were stopped at zero time (group 1) and 8 hours (group 2). Control designates that glands were not incubated with drugs. Concentrations of 0.1 m*M* NE, 1.0 m*M* dibutyryl cyclic AMP (DB-cAMP), and 10 μg of cycloheximide (cyclo) per milliliter were present where indicated. *N*-Acetyltransferase activity in homogenates of pineal glands and total [^{3}H]melatonin in the medium from the second culture period were determined at the end of culture. [^{3}H]Melatonin was not detectable in the medium from the first 8-hour incubation, *N*-Acetyltransferase activity is expressed as the number of picomoles of [^{14}C]-serotonin *N*-acetylated per gland per hour. Total [^{3}H]melatonin is expressed as the number of picomoles produced per gland during the last 16 hours of culture. Data are given as mean ± S.E. Number of glands in each group appears in parenthesis. Not detectable, N.D.

Group	Treatment during organ culture: Period 1 (0 to 8 hr)	Treatment during organ culture: Period 2 (9 to 24 hr)	*N*-Acetyltransferase activity		[^{3}H]Melatonin in medium	
1	Not incubated	Not incubated	38.2 ± 13.4	(3)		
2	Control	Not incubated	16.6, 32.3	(2)		
3	Control	Control	14.6 ± 3.1	(6)	86 ± 13	(6)
4	Control	DB-cAMP	159.0 ± 28.8	(3)*	962 ± 21	(3)*
5	Control	NE	88.0 ± 12.2	(3)*	692 ± 11	(3)*
6	Cyclo	Cyclo	14.1 ± 3.3	(3)	N.D.	
7	Cyclo	Cyclo + DB-cAMP	34.2 ± 6.1	(3)†	267 ± 27	(3)*
8	Cyclo	Cylco + NE	42.3 ± 2.7	(3)*	69 ± 11	(3)

* Significantly different from group 3, $P \leq .01$. † Significantly different from group 3, $P \leq .05$.

Table 2. The effect of dibutyryl cyclic AMP or norepinephrine on the formation of *N*-[^{14}C]acetylserotonin and [^{14}C]melatonin. Two or three intact pineal glands were incubated for 24 hours in 0.5 ml of medium containing 0.1 m*M* [3^{1}-^{14}C]serotonin creatinine sulfate (56 μc/μmole; Amersham Searle). Norepinephrine (0.01 m*M*) or dibutyryl cyclic AMP (1.0 m*M*) was present where indicated. At the end of the experiment the media were analyzed (*11, 12*). Data are given as the mean ± standard error of the distintegrations per minute (dpm) of labeled *N*-acetylserotonin or melatonin in 0.5 ml of the medium. Numbers in parentheses indicate the size of each experimental group.

Treatment	*N*-[^{14}C]Acetylserotonin (dpm/gland)	[^{14}C]Melatonin (dpm/gland)
	Experiment 1	
Control (4)	1,800 ± 600	5,600 ± 1,200
NE (4)	11,800 ± 3,100*	34,800 ± 3,100*
	Experiment 2	
Control (3)	600 ± 600	4,300 ± 1,200
Dibutyryl cyclic AMP (3)	37,300 ± 4,400*	39,100 ± 4,300*

* Significantly greater than control value, $P \leq .05$.

ase activity. Thus, the effects observed in organ cultures could not be reproduced in broken cell preparations (*15*).

The addition of cycloheximide, an inhibitor of protein and melatonin synthesis in cultured pineal glands in vitro, had no apparent effect on *N*-acetyltransferase activity (group 6) relative to control glands (group 3). However, cycloheximide treatment of cultured pineals did partially inhibit the stimulation of *N*-acetyltransferase activity by dibutyryl cyclic AMP (group 7) and NE (group 8) relative to glands treated with dibutyryl cyclic AMP only (group 4) or with NE only (group 5). Almost no [^{3}H]melatonin was released into the medium by glands treated with NE and cycloheximide (group 8), even though *N*-acetyltransferase activity was partially stimulated. Cycloheximide treatment probably inhibited other metabolic processes necessary for formation of melatonin.

The addition of cycloheximide (10 μg/ml) to enzyme assays showed that cycloheximide does not inhibit *N*-acetyltransferase during enzyme assay. The enzyme activity in the homogenates of control and treated glands is not due to the formation and isolation of a nonacetylated derivative of [^{14}C]serotonin, nor is it the result of increased gland content of acetyl coenzyme A (*14, 15*).

These results indicate that *N*-acetyltransferase activity is elevated by NE through a cyclic AMP mechanism which is dependent on the net synthesis of new protein.

The increased production of melatonin caused by treatment with NE or dibutyryl cyclic AMP during organ culture is probably the result of the increased conversion of serotonin to *N*-acetylserotonin. This explanation was examined (Table 2). Intact pineal glands were incubated for 24 hours in medium containing substrate concentrations (0.1 m*M*) of [3^{1}-^{14}C]serotonin (*12, 13*). Dibutyryl cyclic AMP or NE was added to the medium of some organ cultures. This treatment resulted in an increase in *N*-[^{14}C]acetylserotonin and [^{14}C]melatonin. The concentration of nonradioactive *N*-acetylserotonin in individual pineal glands could not be determined with the available techniques. Presumably, the increase of *N*-[^{14}C]acetylserotonin in the medium is caused by the increased production of *N*-acetylserotonin.

Hydroxyindole - *O* - methyltransferase (HIOMT), the unique pineal enzyme, converts *N*-acetylserotonin to melatonin (*1, 16*). In experiments in which melatonin synthesis by cultured pineal glands was stimulated seven- to tenfold, only a small (10 to 20 percent) increase in the activity of HIOMT was observed (*3*). This does not seem large enough to have caused the massive stimulation of melatonin production that occurred. We found no stimulatory effect of dibutyryl cyclic AMP on HIOMT activity of cultured pineal glands. Direct effects of cyclic AMP or dibutyryl cyclic AMP have not been observed (*6*). Apparently NE does not regulate melatonin production by stimulating HIOMT through a cyclic AMP mechanism.

Our findings are consistent with the following hypothetical sequence of events. The stimulation of adenyl cyclase by norepinephrine (*4*) produces an increase in the cellular concentration of cyclic AMP. This nucleotide increases the net formation of *N*-acetyltransferase. *N*-Acetylserotonin synthesis is increased, and the amount of *N*-acetylserotonin in the cell increases. This results in the accelerated enzymic formation of melatonin by simple mass-action (*11*) and in the net efflux of *N*-acetylserotonin and melatonin from the cell.

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10. Pineal glands weighing approximately 1 mg were obtained from male Osborne-Mendel rats ("pathogen-free" NIH colony) that weighed 180 to 220 g. These animals were raised in a controlled environment of light and dark (14 hours light, 10 hours dark). Each gland was dissected free of all adhering tissue and blood and also soaked for a total of 10 to 15 minutes in two washes (20 μl) of culture medium at room temperature before being used for culture.
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12. D. C. Klein and A. Notides, *Anal. Biochem.* **30**, 480 (1969). A sample was applied to the corner of a coated thin-layer chromatography plate (silica gel, type 254 F, Brinkmann). Plates were developed in two directions. Solvents were chloroform, methanol, and acetic acid (93 : 7 : 1) in the first direction and ethyl acetate in the second direction.
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14. To estimate *N*-acetyltransferase activity, one pineal gland was homogenized in 20 μl of 0.1*M* sodium phosphate buffer (*p*H 6.8) containing 10 nmole of acetyl coenzyme A and 10 nmole of [3^{1}-^{14}C] serotonin creatinine sulfate (Amersham Searle, 56 μc/μmole, 1.23×10^{6} dpm per assay). Assays were run in the homogenizers for 30 minutes at 37°C. The reaction was stopped by addition of 20 μl of ethanol and 1*N* HCl solution (1 : 1) containing serotonin, *N*-acetylserotonin, melatonin, 5-hydroxytryptophol, 5-hydroxyindole acetic acid, 5-methoxytryptophol, and 5-methoxyindole acetic acid, all at a concentration of 1 m*M*. A 20-μl sample of this mixture was analyzed by thin-layer chromatography (see *12*); labeled *N*-acetylserotonin and melatonin were separated from the radioactive oxidation products that were formed. The total amount of *N*-[^{14}C]acetylserotonin and [^{14}C]-melatonin that was isolated was used as a measure of enzyme activity. Approximately 75 to 95 percent of [^{14}C]serotonin that was acetylated was isolated as *N*-acetylserotonin. Preliminary studies indicate that within the conditions described, the assay was linear with time and with concentration of crude gland homogenate.
15. Plastic tubes (0.4 ml) were prepared containing 10 nmole of [3^{1}-^{14}C]serotonin in 5 μl of 0.1*M* sodium phosphate buffer (*p*H 6.8). A portion (5 μl) of pooled gland homogenate, acetyl coenzyme A, drug, or buffer was added as indicated to a final volume of 20 μl. All additions were made in the sodium phosphate buffer. Assays were run as described (see *14*).
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