

PINEAL SEROTONIN *N*-ACETYLTRANSFERASE ACTIVITY: PROTECTION OF STIMULATED ACTIVITY BY ACETYL-CoA AND RELATED COMPOUNDS

SUE BINKLEY,^{1,2} D. C. KLEIN and JOAN L. WELLER

Section on Physiological Controls, Laboratory of Biomedical Sciences, National Institute of Child Health and Human Development, National Institutes of Health, Building 6, Room 130, Bethesda, MD 20014, U.S.A.

(Received 24 March 1975. Accepted 11 June 1975)

Abstract—Rat pineal serotonin *N*-acetyltransferase activity increases 30–70-fold at night in the dark and then decreases rapidly when animals are exposed to light. Activity of the enzyme is also stimulated by L-norepinephrine in organ culture. When homogenates of glands stimulated by dark *in vivo* or NE *in vitro* are incubated at 37°C, enzyme activity will also rapidly decrease. This decrease can be prevented by one of the cosubstrates of the enzyme, acetyl-CoA. Protection can also be conferred by cysteamine (β -mercaptoethylamine, HS-CH₂-CH₂-NH₂) which is the terminal portion of the CoA molecule. This protection mechanism could be involved in the physiological control of enzyme activity.

THE ACTIVITY of rat pineal serotonin *N*-acetyltransferase (arylamine: acetyl-CoA *N*-acetyltransferase, EC 2.3.1.5) at night in the dark reaches values that are 30–70 times higher than the light-time values (KLEIN & WELLER, 1970; ELLISON *et al.*, 1972). When rats are exposed to light during their normal dark period, a rapid decrease in enzyme activity occurs ($t_{1/2}$ = 3 min; KLEIN & WELLER, 1972). Day (light-time) pineal glands can also be stimulated to have high *N*-acetyltransferase activity values by treatment in organ culture with NE (KLEIN *et al.*, 1970).

During our early efforts to purify pineal *N*-acetyltransferase from glands obtained at night in the dark, it became apparent that dark-stimulated enzyme activity was very unstable in glands or homogenates incubated in the absence of acetyl-CoA. In this report we describe (1) the thermal instability of the dark- and NE-stimulated enzyme activity, and (2) the effects of temperature and chemicals on the stability of stimulated enzyme activity.

MATERIALS AND METHODS

Male or female Osborne-Mendel rats (180–225 g) were used. Animals were killed by decapitation, and their pineal glands were immediately removed. The pineal glands were then frozen on solid CO₂ or prepared for organ culture (see below). Samples of liver taken at the time of decapitation were frozen.

Experimental protocols

A. *In vivo* pineal experiments. Rats were maintained for 3–7 days prior to killing in LD14:10 (14 h of light alternat-

ing with 10 h of dark) with the dark period ending at 11 or 5 a.m. The rats were decapitated in dim red light within the last 6 h of the dark portion of their light cycles or at precise intervals after the animals had experienced a dark-to-light transition.

B. *Organ culture of pineal glands.* Pineal glands used for organ culture (KLEIN, 1972) were obtained from rats decapitated during their normal light period. The pineal glands were incubated for 18 h after which NE was added (final concentration 10⁻⁴ or 10⁻⁵ M) to stimulate *N*-acetyltransferase activity (KLEIN *et al.*, 1970). At the end of 5½ hours, test compounds were added to some of the cultures. At that time or after 30 min, glands were removed from culture and frozen.

C. *In vitro experiments with homogenates.* Tissue homogenates were prepared at 0°C in ground glass tissue grinders.

(1) Temperature stability of *N*-acetyltransferase activity.

Pineal glands were pooled and homogenized in 0.1 M-sodium phosphate buffer, pH 6.8 (1 gland per 10 μ l buffer). Liver homogenate was made with 1 mg of tissue/30 μ l of buffer. To determine stability, samples of the homogenates (10 μ l) were incubated at 0°, 24°, or 37°C for up to 24 h and then assayed for *N*-acetyltransferase activity (KLEIN, 1972).

(2) Chemical protection of *N*-acetyltransferase activity.

Pineal homogenates containing dark- or NE-stimulated enzyme activity were made (1 gland per 15–20 μ l of the phosphate buffer). A 5 μ l sample of homogenate was added to a tube containing the test compound dissolved in 5 μ l of the phosphate buffer. To determine the stabilizing effects of test compounds, the tubes were incubated at 0°, 24° or 37°C for up to 24 h and were then assayed for *N*-acetyltransferase activity.

(3) Effects of the test compounds in the assay of *N*-acetyltransferase activity.

Samples (5 μ l) of homogenate containing dark-stimulated enzyme activity were added to tubes at 0°C containing the test solutions and the assay substrates. The mixture was then assayed for *N*-acetyltransferase activity.

Abbreviations used: NEM, *N*-ethylmaleimide; cAMP, cyclic AMP.

¹Present address Department of Biology, Temple University, Philadelphia, PA 19122, U.S.A.

²S. Binkley was supported by postdoctoral fellowship NIH FO2 HD52858.

(4) Reactivation of 37°C-inactivated *N*-acetyltransferase activity.

Samples (5 μ l) of homogenates containing dark-stimulated enzyme activity were inactivated by incubating at 37°C in the absence of acetyl-CoA for 20 min. The inactivated homogenate was incubated for up to 24 h with test preparations and was then assayed for *N*-acetyltransferase activity.

Statistics

Data are given ± 1 S.E. or as the average of duplicate determinations. Statistical analysis was by Student's *t*-test.

Materials

Cysteamine analogs were given to us by Dr. June A. Schafer and the Walter Reed Army Institute of Research.

RESULTS

The effect of temperature on the dark-stimulated enzyme activity was determined (Fig. 1). Rapid loss of activity occurred at 37°C. This loss of activity was not observed in homogenates from unstimulated pineal glands nor in homogenates of liver (Fig. 2).

Prevention of thermal loss of enzyme activity

Enzyme activity is linear at 37°C under assay conditions for 20 min (KLEIN & WELLER, 1970, 1972). In view of our observation that activity is lost in homogenates at 37°C, we suspected that the presence of the substrates in the assay was preventing loss of dark- or NE-stimulated enzyme activity. The protective ability of the substrates was tested (Fig. 3). Only low activity occurred in the presence of serotonin. However, in the presence of acetyl-CoA considerable activity during incubation was observed at 37°C. We concluded that acetyl-CoA, but not serotonin, was able to protect against the thermal inactivation of enzyme activity. This thermal instability and the pro-

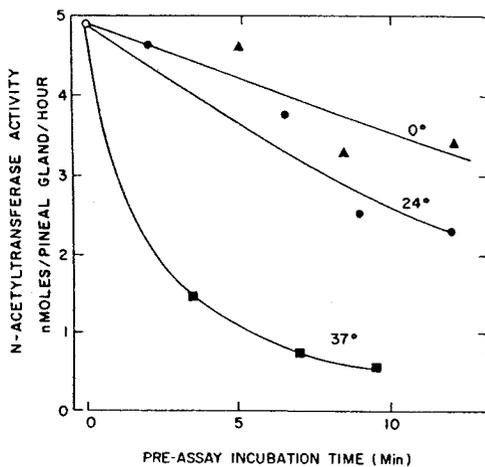


FIG. 1. Short term thermal instability of dark-time pineal *N*-acetyltransferase activity. Each point is a mean of three determinations, each determination was within 10% of the mean. Samples of homogenates were incubated in buffer for the indicated time at the indicated temperature, and then [14 C]serotonin and acetyl-CoA were added for the enzyme assay.

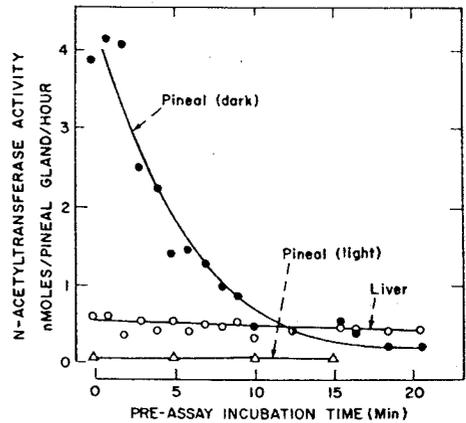


FIG. 2. Short term thermal instability of pineal gland *N*-acetyltransferase activity as compared to day pineal and liver *N*-acetyltransferase. Samples of homogenates were incubated at 37°C for the indicated time, and then [14 C]serotonin and acetyl-CoA were added for enzyme assay. Liver values in nmol/mg per h; 37°C. Each point is the mean of two determinations, each determination was within 10% of the mean. Values for unstimulated enzyme activity (pineal, light) were more than 2-fold greater than blank controls.

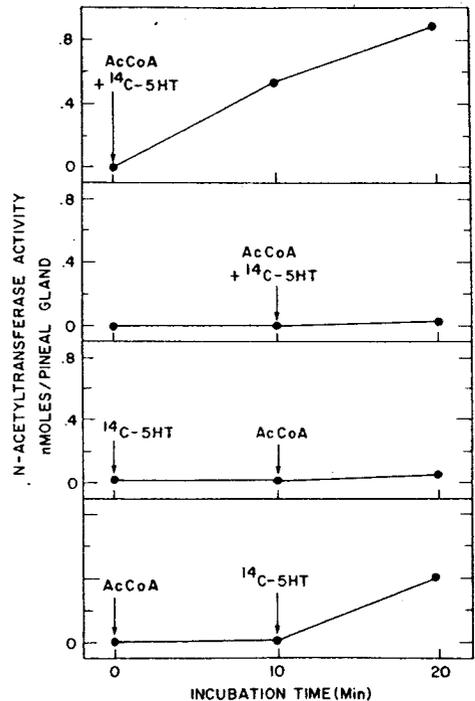


FIG. 3. Effects of the substrates 2 mM-acetyl-CoA and 1 mM [14 C]serotonin on dark-stimulated *N*-acetyltransferase activity. Dark-time pineal glands were homogenized and incubated with substrate mixtures at 37°C. When both substrates were added (AcCoA + 14 C-5HT) enzyme activity was linear as measured by amount of product produced; if the enzyme was incubated without the substrates followed by substrate addition there was no linearly increasing product production. Clearly, something in the substrate mixture made a difference to the enzyme activity. By adding the substrates in sequence (bottom two experiments) we were able to determine that acetyl-CoA was essential for maintenance of the enzyme activity but that serotonin cannot maintain the enzyme activity.

TABLE 1. THERMAL INSTABILITY OF STIMULATED *N*-ACETYLTRANSFERASE ACTIVITY AND PROTECTION BY ACETYL-CoA

Experimental material	Pre-assay incubation of homogenate (10 min)	<i>N</i> -acetyltransferase activity (nmol/pineal gland per hr)
100,000 g supernatant of pineal glands from dark-killed rats	37°C, 4 mM-acetyl-CoA	2.58 ± 0.05
	37°C	0.61 ± 0.06
	0°C	2.62 ± 0.22
Homogenates of pineal glands from organ cultures	37°C, 4 mM-acetyl-CoA	0.12
	37°C	0.09
	0°C	0.15
Homogenates of pineal glands from organ cultures, NE 10 μM	37°C, 4 mM-acetyl-CoA	1.48
	37°C	0.17
	0°C	1.93

tection by acetyl-CoA were also observed in 100,000 g supernatant fractions containing dark- or NE-stimulated enzyme activity (Table 1). Therefore, all components necessary for thermal inactivation were present in the cytosol. In addition, NE-stimulated enzyme activity exhibited thermal instability and acetyl-CoA protected against the thermal instability. This indicates a similarity between NE-stimulated and dark-stimulated enzyme activity.

We approached the question of what portion of the acetyl-CoA molecule was responsible for protecting *N*-acetyltransferase activity (Table 2). A number of compounds with structures similar to portions of acetyl-CoA were tested (Table 2a). CoA and cysteamine protected against thermal loss of *N*-acetyltrans-

ferase activity. The relative effectiveness of these compounds was examined: the concentration of acetyl-CoA that protected 50% was 0.3 mM, that of CoA was 2 mM, and that of cysteamine was 0.7 mM.

The protective ability of 4 mM concentrations of cysteamine-related compounds was examined (Table 2b). Cystamine (oxidized cysteamine) was ineffective, which indicates that the reduced sulfhydryl group in cysteamine is necessary for protection. β-Mercaptopropylamine was ineffective; this is evidence that the protective agent cannot have a 3-carbon chain between the amino and sulfhydryl groups. Mercaptoethanol and ethanolamine, alone or in combination, were ineffective, indicating that the amino group and the sulfhydryl group must be on the same molecule

TABLE 2. CAPACITY OF CHEMICAL AGENTS TO PROTECT DARK-STIMULATED *N*-ACETYLTRANSFERASE ACTIVITY AT 37°C FOR 10 min IN HOMOGENATES

Name	%	Structure
(a) Acetyl-CoA and Analogs		
Acetyl-CoA	96	
CoA	47	
Pantethine (disulfide)	3	
Pantothenic acid	<0	
5'-ADP	23	
Sodium acetate	13	
(b) Cysteamine and Analogs		
Cysteamine (β-Mercaptoethylamine)	56	HS-CH ₂ -CH ₂ -NH ₂
Cystamine	<0	S ₂ -(CH ₂ -CH ₂ -NH ₂) ₂
<i>N</i> -Acetyl- <i>S</i> -acetoacetyl-cysteamine	23	CH ₃ -CO-CH ₂ -CO-S-CH ₂ -CH ₂ -NH-CO-CH ₃
λ-Mercaptopropylamine	0	HS-CH ₂ -CH ₂ -CH ₂ -NH ₂
β-Dimethyl-β-mercaptoethylamine	113	HS-C(CH ₃) ₂ -CH ₂ -NH ₂
D,L-Penicillamine (8 mM)	77	HS-C(CH ₃) ₂ -CH(COOH)-NH ₂
<i>S</i> -Acetylcysteamine	6	CH ₃ -CO-S-CH ₂ -CH ₂ -NH ₂
<i>N,S</i> -Diacetylcysteamine	4	CH ₃ -CO-S-CH ₂ -CH ₂ -NH-CO-CH ₃
α-Methyl-β-mercaptoethylamine	5	HS-CH ₂ -CH(CH ₃)-NH ₂
<i>N</i> -Acetylcysteamine	0	HS-CH ₂ -CH ₂ -NH-CO-CH ₃
Mercaptoethanol	13	HS-CH ₂ -CH ₂ -OH
Ethanolamine	0	HO-CH ₂ -CH ₂ -NH ₂
Mercaptoethanol + Ethanolamine	13	

Data are expressed as %:

$$\frac{100 \times (\text{protected value} - \text{unprotected } 37^\circ\text{C control value})}{0^\circ\text{C control value} - \text{unprotected } 37^\circ\text{C control value}}$$

Standard errors were less than 10%. Compounds marked <0 inhibited enzyme activity by 0–20%. All concentrations of protecting agents were 4 mM unless otherwise indicated. Samples of homogenates were incubated for 10 min at 37°C in the absence or presence of the indicated compound, and then [¹⁴C]serotonin and acetyl-CoA were added for enzyme assay.

for protection. *N*-Acetylated, *S*-acetylated, and *N,S*-diacetylated derivatives of cysteamine were also ineffective. However, the *S*-acetylated derivative of cysteamine has a very short half-life ($t_{1/2} = 4$ min) in water at neutral pH due to hydrolysis or rearrangement (SCHRIER, 1967) so that the *S*-acetylated form cannot be assumed to have been present as such for the whole period of time. Two β -dimethyl derivatives of cysteamine, β -dimethyl- β -mercaptoethylamine and penicillamine, were effective protecting agents, but a single α -methyl group blocked protective ability. We investigated the long term protective ability of acetyl-CoA and β -dimethyl- β -mercaptoethylamine at 0°C. Only the homogenates treated with acetyl-CoA were fully active after 24 h.

Since sulfhydryl groups were implicated in the protection mechanism by the experiments with cysteamine analogs, we tested a number of well-known sulfhydryl protecting or inhibiting agents. Of these, only *N*-ethylmaleimide (NEM, 0.2 mM), had a substantial effect. It reduced activity to 37% of its original value in homogenates even in the presence of acetyl-CoA. In the absence of acetyl-CoA, NEM reduced *N*-acetyltransferase activity to levels below that for 37°C unprotected controls. Iodoacetamide also inhibited enzyme activity. A concentration of 16 mM, but not 4 mM, dithiothreitol protected *N*-acetyltransferase activity. Diamide (HARRIS & BIAGLOW, 1972; diazenedicarboxylic acid bis *N,N* dimethyl amide) which oxidizes GSH, GSH and GSSG were slightly inhibitory compounds. Cysteine and isethionine (which are involved in cysteamine metabolism) were without effect. Some other compounds examined in our experiments were ineffective (*S*-adenosylhomocysteine, NE, dibutyryl cAMP) or slightly inhibitory (serotonin, cAMP, ascorbic acid, *S*-adenosylmethionine).

Arginine vasotocin, which is reported to be in the pineal gland (PAVEL & PETRESCU, 1966), contains the sequence necessary to protect dark-stimulated enzyme activity (RHN-CH₂-CH₂-SR). Arginine vasotocin is an octapeptide with a disulfide bond between two cysteine residues. From our studies with other disulfide forms of cysteamine analogs, we knew that a disulfide should be ineffective. We therefore predicted that reduced arginine vasotocin would protect against thermal destruction of enzyme activity. Our prediction was verified because 2 mM-arginine vasotocin which had been reduced by treatment with 16 mM-dithiothreitol protected against loss of enzyme activity (74%) better than did either 8 mM-dithiothreitol (33%) or 2 mM-arginine vasotocin (inhibitory) alone.

Inhibition of NE-stimulated N-acetyltransferase activity in organ culture

In organ culture, treatment with NEM lowered NE-stimulated enzyme activity (1189 ± 215 pmol/pineal gland per h) to a level (170 ± 108 , NEM 10^{-3} M; 12 ± 7 pmol/pineal gland per h, NEM 5×10^{-3} M) below values for nonstimulated enzyme activity (198 ± 47 pmol/pineal gland per h). We have seen

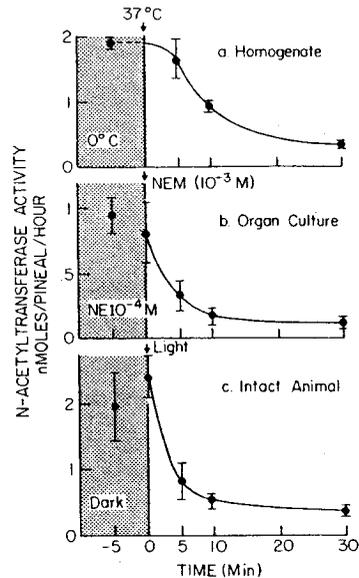


FIG. 4. Rapid decrease in pineal *N*-acetyltransferase activity in three different types of experiments: (a) Rapid decrease *in vitro* due to heating at 37°C of pineal gland homogenate which initially contained dark-stimulated *N*-acetyltransferase activity; (b) Rapid decrease *in vitro* of NE-stimulated *N*-acetyltransferase activity in pineal organ culture caused by NEM; the initial high activity was stimulated with $5\frac{1}{2}$ h of NE following a 22 h incubation with no drugs; values for control glands which received no NE or NEM were not different from the 30-min NEM values; (c) Rapid decrease *in vivo* in rats exposed to light 1 h before their normal lights-on time in an LD14:10 light cycle.

no change in a 30 min period in stimulated enzyme activity without NEM when NE is present. The time course for NEM-caused decrease in enzyme activity (Fig. 4b) was very similar to that for the rapid decline in *N*-acetyltransferase activity in response to 37°C incubation of homogenates *in vitro* or light *in vivo* (Fig. 4a,c). In similar organ culture studies we found that the sulfhydryl inhibitor, iodoacetamide (1 mM), was less effective than NEM; diamide (1 mM), cupric chloride (1 mM) or sodium oxalate (2 mM) had no effect. Addition of cysteamine or GSH to cultures did not enhance the NE-stimulated enzyme activity.

Reactivation of thermally inactivated N-acetyltransferase activity

It was not possible to reactivate thermally inactivated *N*-acetyltransferase activity by incubation with liver homogenate (0.5 mg wet wt/5 μ l, 10 min), cysteamine (2 and 10 mM, 40 min) or the KCN-*versene* system used to reactivate papain (40 min, ANDERSON & VASINI, 1970). The KCN-*versene* system was used because it reactivates papain through sulfhydryl group reactions.

DISCUSSION

The thermal destruction of dark- or NE-stimulated *N*-acetyltransferase activity in pineal homogenates

may be of physiological importance. The time course for 37°C inactivation at the gland concentrations used is remarkably similar to the *in vivo* time course for activity loss which occurs when rats are either exposed to light (Fig. 4c) or injected with the β -adrenergic blocking agent, 1-propranolol, during their normal dark periods (KLEIN & WELLER, 1972; DEGUCHI & AXELROD, 1972). This thermal inactivation of *N*-acetyltransferase activity seen in homogenates of pineal glands and the rapid loss of *N*-acetyltransferase activity seen *in vivo* may involve the same mechanism.

The significance of our findings is several fold. First, the information about protecting agents can be put to use for experimental preservation of stimulated pineal *N*-acetyltransferase activity; it may be possible to use this in purification of the enzyme. Second, the protecting agents suggest a possible means for regulation of the rhythms in *N*-acetyltransferase activity, that is, by the periodic presence of a natural protecting agent. Third, our experiment with arginine vasotocin tenuously links a putative pineal peptide with the regulation of melatonin synthesis at an *in vitro*

biochemical level via regulation of *N*-acetyltransferase activity.

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