Biochemical Pharmacology Vol. 25 pp. 903–906. Pergamon Press. 1976. Printed in Great Britain.

DIURNAL VARIATION IN THE REQUIREMENT FOR RNA SYNTHESIS IN THE INDUCTION OF PINEAL *N*-ACETYLTRANSFERASE

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(*Received* 22 *May* 1975; *accepted* 5 *September* 1975)

Abstract– There is a variable lag period in the induction of rat pineal serotonin *N*-acetyltransferase activity by β-adrenergic stimulation, which increases with the duration of the animals' prior exposure to light. There is a similar variation in the lag period when enzyme activity is induced in culture by dibutyryl cyclic AMP. Experiments with actinomycin D indicate that RNA synthesis occurs during the lag period. If actinomycin D is added after the lag period, however, it does not reduce the level of *N*-acetyltransferase activity attained. Reinductions in glands from animals which have been in the dark for 6 hr have no lag period and do not require new RNA synthesis. The extent to which enzyme activity can be reinduced in culture in the presence of actinomycin D gradually increases during the first half of the night, when there is an increased release of norepinephrine from the nerve endings. These data suggest that RNA, presumably messenger RNA, that is necessary for increased *N*-acetyltransferase activity, is synthesized and accumulates during the first half of the night. Thereafter, there appears to be a decline in the complement of RNA available for reinduction.

The circadian rhythm in rat pineal serotonin *N*-acetyltransferase (E.C. 2.3.1.5) is modulated by environmental lighting [1, 2]. Light exerts its influence upon the pineal, ultimately, via postganglionic sympathetic fibers originating in the superior cervical ganglion [3, 4]. At night, the release of norepinephrine from the sympathetic nerve endings is increased [5, 6], and there is a 50- to 100-fold increase in *N*-acetyltransferase activity. Norepinephrine interacts with the β-adrenergic receptor on the pineal cell membrane, enhancing the production of cAMP [7] which mediates the induction of *N*-acetyltransferase [7, 8].

After the night-time rise of *N*-acetyltransferase activity, exposure of rats to light reduces nerve activity [6] and causes enzyme activity to drop precipitously [9, 10]. As long as the lights stay on, enzyme activity remains at its lowest levels [19]. In animals exposed to light, pineal *N*-acetyltransferase activity can be induced by the injection of isoproterenol [7, 9], which acts directly on the β-adrenergic receptor. Enzyme activity can also be induced in organ culture by catecholamines [8, 11] or dibutyryl cAMP [8, 11].

There is a lag period in the induction of rat pineal *N*-acetyltransferase after β-adrenergic stimulation [9]. This lag period, during which there is virtually no increase in enzyme activity, varies markedly with the length of time the rats have been exposed to light prior to induction [11]. If rats have been exposed to light (and, consequently, reduced sympathetic nerve activity) for 12 hr or more, the lag period is 1–2 hr long. In contrast, if enzyme activity is reduced by brief exposure to light, at night, when *N*-acetyltransferase activity is high, catecholamines can increase enzyme activity (reinduction) with almost no lag.

The requirement for prior synthesis of messenger RNA may account for the lag period before the increase in *N*-acetyltransferase activity [12, 13]. In animals that have been exposed to light for 12 hr or more, actinomycin D, a compound that inhibits RNA synthesis, completely blocks the increase of *N*-acetyltransferase activity by darkness, isoproterenol, or dibutyryl cAMP [13]. In contrast, actinomycin D does not prevent the reinduction of *N*-acetyltransferase by isoproterenol or dibutyryl cAMP in glands whose enzyme activity has been reduced from its high night-time level by brief exposure of the animals to light [13]. Thus the requirement for RNA synthesis varies with the previous exposure of the animals to light, and the time required for its synthesis may contribute to the length of the lag period in induction.

This communication presents further evidence in support of the above hypothesis. There is a progressively diminishing requirement for new RNA synthesis during the process of induction by catecholamines or dibutyryl cAMP in organ culture. Furthermore the data indicate that there is a diurnal variation in the complement of RNA, presumably messenger RNA, available for the synthesis of *N*-acetyltransferase.

MATERIALS AND METHODS

Chemicals. Acetyl-l-[^{14}C]coenzyme A (3.5 – 6.6 mCi/m-mole) was purchased from Amersham–Searle, Chicago, Ill. (l)-Isoproterenol-(*d*)-bitartrate was a gift from Ayerst Laboratories. Actinomycin D was purchased from Sigma, St. Louis, Mo., and dibutyryl cAMP from Calbiochem, LaJolla, Calif. Other chemicals were obtained from commercial sources.

Animals. Male Sprague–Dawley rats (150–175 g), obtained from Zivic–Miller, Allison Park, Pa., were kept under diurnal lighting conditions in our facilities

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for at least 5 days before the experiments. Lights were on from 0600–1800 hr. Groups of 6 rats were killed by decapitation, at the times indicated in each experiment.

Pineal explant culture. Pineal glands were placed in organ culture in plastic petri dishes (Falcon, 60 mm dia.) containing 2.5 ml BGJ_b Fitton–Jackson medium (Grand Island Biological Co.), supplemented with ascorbic acid (0.1 mg/ml), glutamine (2.0 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml). Six pineals were incubated in each dish at 35° under 95% O_2–5% CO_2. Isoproterenol, dibutyryl cAMP, and actinomycin D were added to the medium at the concentrations and times indicated.

Assay for N-acetyltransferase activity. After the incubation periods indicated, glands were removed from culture and immediately assayed individually for *N*-acetyltransferase activity by the method previously described [14], using 20 nmoles of [^{14}C]acetyl CoA instead of 3.4. Units of activity are pmoles N-[^{14}C]-acetyltryptamine formed per 10 min.

RESULTS

Lag period in induction of N-acetyltransferase by dibutyryl cAMP. The lag in the induction of *N*-acetyltransferase activity by isoproterenol *in vivo* and in culture increases as animals are exposed to longer periods of light [9, 11]. Since the onset of enhanced cAMP production occurs immediately after β-adrenergic stimulation [7], the lag in the induction of *N*-acetyltransferase activity might be due to a step mediated by cAMP. Fig. 1 shows the time course of induction of *N*-acetyltransferase by dibutyryl cAMP in pineals taken from rats exposed to light for different periods. The glands taken from animals that had been in the light for 24 hr showed virtually no increase in activity after 2 hr and took at least 6 hr to reach their maximally induced activity. In contrast, in glands from animals exposed briefly to light at midnight, *N*-acetyltransferase levels began to rise immediately and reached a plateau within 4 hr.

Sensitivity of induction of N-acetyltransferase to inhibition by actinomycin D: relation to lag period in culture. If the lag period reflects the time during which mRNA is being synthesized and transported to the sites of translation, then it should coincide with the period of time during which induction of *N*-acetyltransferase is most sensitive to inhibition by actinomycin D. Fig. 2 shows the effect of adding actinomycin D to the culture medium at various times after isoproterenol or dibutyryl cAMP. In glands from animals exposed to light for 24 hr, actinomycin D inhibited *N*-acetyltransferase induction by 88% or more if added with or before isoproterenol or dibutyryl cAMP. However, when the antibiotic was added 3 hr or more after the isoproterenol or dibutyryl cAMP, it had little or no effect. Presumably, during the first 3 hr of induction, there is synthesis of mRNA sufficient for the appearance of full *N*-acetyltransferase activity. As this RNA accumulates there is a decreasing requirement for new RNA synthesis and decreasing inhibition by added actinomycin D.

Nocturnal decrease in sensitivity of reinduction of N-acetyltransferase to inhibition by actinomycin D. The extent of induction of *N*-acetyltransferase activity in the presence of actinomycin D in culture appears to reflect the pool of mRNA available for the reinduction of enzyme activity. Fig. 3 shows the extent of reinduction in the presence of actinomycin D at various times during the night. In pineals from animals killed at the end of their normal light period,

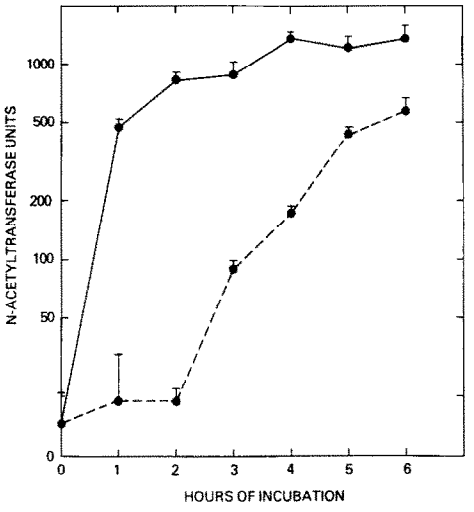

Fig. 1. Time course of induction of *N*-acetyltransferase activity by dibutyryl cAMP. Groups of six rats were exposed to light for 24 hr (●——●) or for 20 min after 6 hr of darkness (●- - - -●). Pineal glands were removed and placed in organ culture containing 10^{-3} M dibutyryl cAMP. At the times indicated, glands were assayed for *N*-acetyltransferase activity as described. Data shown represent mean ± S.E.M.

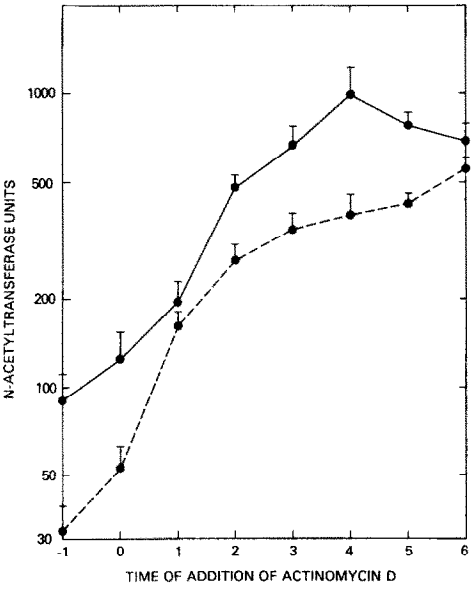

Fig. 2. Effect of time of addition of actinomycin D on inhibition of induction. Groups of 6–12 rats were exposed to light for 24 hr. Pineal glands were removed and placed in organ culture containing 10^{-7} M (1)-isoproterenol (●——) or 10^{-3} M dibutyryl cAMP (●- - - -●) at time zero. Actinomycin D (10 μg/ml) was added to the culture medium at the times indicated. Glands were assayed for *N*-acetyltransferase activity after 6 hr as described. Data shown represent mean ± S.E.M.

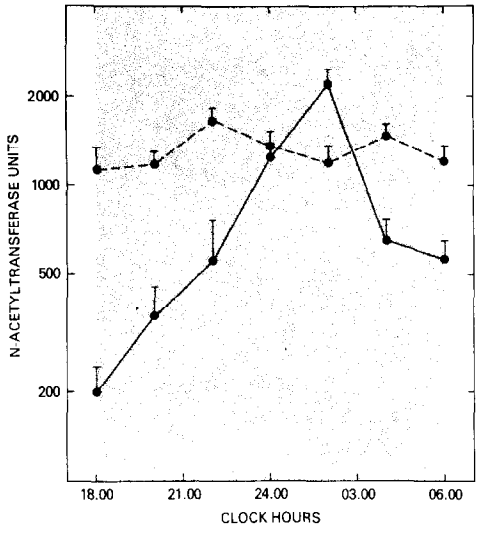

Fig. 3. Effect of time in darkness on reinduction of *N*-acetyltransferase in the presence of actinomycin D. Groups of 6–12 rats were exposed to light for 20 min after varying intervals of their normal dark period (1800–0600 hours). Pineal glands were removed and placed in organ culture containing actinomycin D (10 μg/ml) (●——●) or control medium (● - - - ●). After 1 hr incubation, (1)-isoproterenol (10^{-7} M) was added to each culture. Glands were assayed for *N*-acetyltransferase activity 6 hr thereafter as described. Data shown represent mean ± S.E.M.

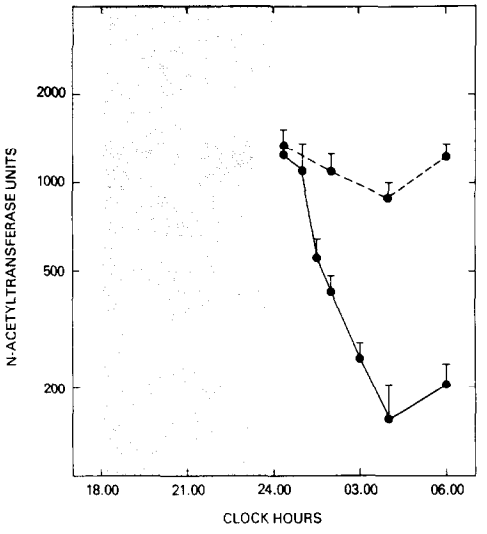

Fig. 4. Effect of time in light on reinduction of *N*-acetyltransferase in the presence of actinomycin D. Groups of 6–12 rats were exposed to light for varying times after 6 hr of their normal dark period. Pineal glands were removed and placed in organ culture containing actinomycin D (10 μg/ml) (● ● ●) or control medium (● ● ●). After 1 hr incubation, (1)-isoproterenol (10^{-7} M) was added to each culture. Glands were assayed for *N*-acetyltransferase activity 6 hr thereafter as described. Data shown represent mean ± S.E.M.

induction in culture was inhibited more than 80% by actinomycin D. In pineals obtained from animals killed after varying periods in the dark, however, reinduction becomes increasingly resistant to inhibition by actinomycin D until, after 6 or 8 hr, it is virtually unaffected by actinomycin D.*

Sensitivity to inhibition by actinomycin D of N-*acetyltransferase reinduction: effect of light.* In glands from animals exposed to light for 18 hr, induction of *N*-acetyltransferase was almost completely blocked by actinomycin D [13]. Thus, exposure of rats to light may result in a decline in the mRNA available. Fig. 4 shows the effect of varying periods of light on the extent of reinduction of *N*-acetyltransferase in the presence of actinomycin D. When animals were killed after 20 min of light at midnight, actinomycin D had virtually no effect on reinduction in culture. With increasing time in the light, however, there was a rapid decrease in the reinduction seen in the presence of actinomycin D. After 90 min in the light, reinduction in culture was inhibited more than 50% by actinomycin D, and after 3 hr in the light, more than 80%.

Sensitivity to inhibition of N-*acetyltransferase induction by actinomycin D: diurnal rhythms.* The data in Fig. 3, however, indicates that light is not the only influence which regulates the level of mRNA available for the reinduction of *N*-acetyltransferase. In pineals obtained from animals killed after 8 hr in the dark, there is a rapid decrease in the reinduction obtained in the presence of actinomycin D. Thus, in the normal diurnal cycle, it appears that the mRNA necessary for reinduction of *N*-acetyltransferase activity is synthesized and accumulates during the first half of the night until an unknown feedback mechanism shuts off further synthesis and allows the available mRNA to decline during the second half of the night.

*These experiments were performed using a concentration of actinomycin D (10 μg/ml) which inhibited the incorporation of [^{3}H]uridine into total RNA by more than 90%. When a lower dose of actinomycin D was used (1 μg/ml), induction in pineals obtained from animals killed after 18 hr in light was inhibited 45%, whereas reinduction was unaffected in glands obtained from animals exposed briefly to light after 6 hr darkness.

DISCUSSION

The lag period in the induction of *N*-acetyltransferase activity by isoproterenol, *in vivo* and in culture, suggests the synthesis and accumulation of a necessary intermediate. When dibutyryl cAMP, which bypasses the receptor-coupled adenylate cyclase, is used in culture to induce enzyme activity, the lag period is essentially the same as that seen when isoproterenol is used (Fig. 1). Thus, the intermediate whose synthesis accounts for the lag is itself induced by cAMP.

A major characteristic of the lag period is that it increases with the length of time that the animals have been exposed to light [9, 11] (and reduced release of norepinephrine from nerve endings [6]). In pineals from animals exposed to light for 12 hr or more, the lag is 1–2 hr long. In contrast, in pineals from animals exposed briefly to light after 6 hr of darkness, there is virtually no lag [13]. This suggests that, after 6 hr of darkness, an intermediate is available such that *N*-acetyltransferase activity can be increased without delay whereas after long periods of light the intermediate must be synthesized (and perhaps transported) before *N*-acetyltransferase activity

can increase. It may be supposed that the intermediate is synthesized when the animals are in darkness and declines when they are in light.*

Such an intermediate might have been a proenzyme activated by β-adrenergic stimulation. However, experiments with cycloheximide indicate that peptide synthesis is absolutely required for increased activity regardless of the animals' previous exposure to light [8, 9, 13]. Even after 6 hours' darkness, there is no evident build up of proteinacious precursor which would account for the rapid reinduction [13]. Thus, synthesis of the intermediate is likely to precede peptide synthesis.

The intermediate which accounts for the variable lag in the induction of *N*-acetyltransferase activity appears to be RNA, presumably messenger RNA. When RNA synthesis is inhibited by actinomycin D, reinductions which have no lag periods are unaffected. In contrast, inductions which have long lag periods are completely blocked [13]. However, when actinomycin D was added after the lag period (but before the rise in enzyme activity) it had a minimal effect on the increase in activity (Fig. 2), because the RNA required for induction had been synthesized during the lag period and was available during the period that new RNA synthesis was inhibited.

Thus the increase in *N*-acetyltransferase activity achieved in the presence of actinomycin D reflects the amount of the RNA, presumably messenger RNA, that is available for enzyme synthesis. When animals that have been in the light enter their normal dark period there is an increased release of norepinephrine from the sympathetic nerve endings [5, 6]. The released norepinephrine stimulates the β-adrenergic receptor and enhances the production of cAMP [7]. Cyclic AMP, in turn, induces the synthesis and gradual accumulation of the messenger RNA (Fig. 3), as well as stimulating the post-transcriptional events necessary for increased *N*-acetyltransferase activity [13]. If the animals are exposed to light, norepinephrine turnover is reduced [5] and the supply of messenger RNA falls [Fig. 4]. With increasing time in the light, a greater complement of RNA must be newly synthesized and processed before enzyme activity is increased—thus an increasing lag.

However, even in continued darkness the supply of messenger RNA falls after about 8 hr (Fig. 3). Although this could be due to a reduction of norepinephrine release during the second half of the night, attempts to demonstrate changes in norepinephrine turnover during the night have been unsuccessful.† Alternatively, there may be a reduction in mRNA synthesis without a reduction in norepinephrine release. There is evidence that continued stimulation of the β-adrenergic receptor results in reduced sensitivity of *N*-acetyltransferase activity to induction by further stimulation of the β-adrenergic receptor [11]. These changes in sensitivity take place at at least two sites—one involved in the regulation of cAMP levels and the other, perhaps secondarily, involved in the actions of cAMP [11]. Thus, unknown feedback mechanisms, resulting from the night-time increase in β-adrenergic stimulation, may come into play to turn off the synthesis of mRNA during the night. These mechanisms may be related to those which cause the fall of endogenous *N*-acetyltransferase activity during the night [15].

In conclusion, there is a diurnal variation in the requirement for RNA synthesis in the induction of *N*-acetyltransferase activity by β-adrenergic stimulation. During the normal dark period increased release of norepinephrine stimulates the β-adrenergic receptor, increasing the production of cAMP. It is proposed that cAMP, in turn, induces synthesis of RNA, presumably messenger RNA, which is necessary for the appearance of increased *N*-acetyltransferase activity. The mRNA accumulates during the first half of the night, such that reinduction under experimental conditions can be accomplished without new RNA synthesis. After a time, unknown feedback mechanisms reduce the synthesis of mRNA, its level declines, and reinduction again requires new RNA synthesis. The diurnal cycle in duration of the lag period in the experimental induction of increased *N*-acetyltransferase activity reflects the diurnal cycle in the requirement for new RNA synthesis.

*The reverse hypothesis, that an inhibitor is synthesized when the animals are in light and decays after β-adrenergic stimulation, is not supported by the data. Pineal glands from light-exposed animals do not inhibit the activity of induced glands when they are homogenized and assayed together.

†M. Brownstein and J. A. Romero, unpublished observations.

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