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SYNTHESIS OF RNA IN THE PINEAL GLAND DURING *N*-ACETYLTRANSFERASE INDUCTION

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Abstract—The biosynthesis of RNA in the cultured rat pineal was monitored during the (*l*)-isoproterenol-induced increase in *N*-acetyltransferase activity. Treatment with the β-agonist (*l*)-isoproterenol elevated pineal *N*-acetyltransferase activity 30-fold within 6 hr but had no effect on the incorporation of [^{3}H]uridine into total nuclear, total cytoplasmic or total poly(A) containing RNA. Examination of the [^{3}H]uridine-labeled total cytoplasmic RNA and poly(A)-rich messenger RNA by means of agarose gel electrophoresis revealed no significant changes in the synthesis of these RNA species by (*l*)-isoproterenol treatment of the cultured pineal gland. The lag in the appearance of newly synthesized RNA from the nucleus into the cytosol of the pineal closely corresponds to the lag in the appearance of new *N*-acetyltransferase activity. These results suggest that, although (*l*)-isoproterenol may cause a dramatic induction of *N*-acetyltransferase enzyme activity in the cultured pineal gland, there is no significant alteration in the RNA biosynthesis of the pineal gland to meet the demands of enzyme induction. The induction of *N*-acetyltransferase activity by (*l*)-isoproterenol is inhibited by actinomycin D. However, sensitivity to the antibiotic is lost 90–180 min after the initiation of induction by (*l*)-isoproterenol but 90 min before the accumulation of appreciable enzyme activity.

The rat pineal enzyme serotonin *N*-acetyltransferase (EC 2.3.1.5) undergoes a circadian rhythm which is influenced by environment lighting [1–3]. Postganglionic sympathetic fibers arising from the superior cervical ganglion innervate the pineal [4, 5] and relay the photic input from the eye. The turnover of norepinephrine at the sympathetic nerve terminal within the pineal is higher during the night than during the day [6, 7]. The released norepinephrine interacts with β-adrenergic receptors at the pineal cell membrane, causing an increase in the cellular cyclic AMP content [8, 9] which ultimately leads to a 50- to 70-fold increase in serotonin-*N*-acetyltransferase activity [9, 12]. In the transition from dark to light, the norepinephrine turnover decreases [7] and a marked drop in enzyme activity occurs [10, 11]. The induction–deinduction by dark and light may be mimicked in pineal organ culture by exogenous dibutryl cyclic AMP, β-adrenergic agonists and antagonists [11–15].

The induction of serotonin *N*-acetyltransferase in the pineal is characterized by a lag period between the β-adrenergic stimulation and the appearance of enzyme activity [10]. This lag period varies with the length of light exposure experienced by the animals prior to experimental use [15]. The lag period is longer if the exposure to light after the last dark phase is longer. This lag period has been ascribed to the need for RNA synthesis (presumed messenger RNA synthesis) during enzyme induction [16, 17]. This is based on the degree of actinomycin D sensitivity of the induction process to the length of light exposure. Actinomycin D completely blocks induction of enzyme by β-agonists or cyclic AMP in animals which have been exposed to light for around 12 hr [16]. In contrast, there is little to no actinomycin D sensitivity during *N*-acetyltransferase induction in animals which have undergone a brief exposure to light after a normal cycle of dark induction [16]. This implies that a required RNA species is not present after a long exposure to light but is present during the normal dark phase [17].

Although RNA synthesis seems to be required for *N*-acetyltransferase induction, there have been no investigations of the influence of β-agonists on the process of RNA synthesis in the pineal gland. This investigation describes RNA synthesis in the cultured rat pineal during the induction of serotonin *N*-acetyltransferase by (*l*)-isoproterenol.

MATERIALS AND METHODS

Chemicals. [1-^{14}C]acetyl-coenzyme A (55 mCi/m-mole) and [5-^{3}H]uridine (27 Ci/m-mole) were obtained from New England Nuclear, Boston, MA. (*l*)-Isoproterenol-(*d*)-bitartrate, actinomycin D and *S*-acetyl coenzyme A were purchased from Sigma Chemical, St. Louis, MO. Oligo (dT) cellulose was purchased from Collaborative Research Inc., Waltham, MA.

Animals. Male Sprague–Dawley rats (150–175 g) were purchased from Zivic-Miller, Allison Park, PA. The animals were housed under diurnal lighting conditions (12 hr light 0600–1800 hr) for at least 7 days prior to experimental use. Animals were exposed to light for 24 hr prior to sacrifice.

Pineal gland culture. Animals were decapitated, the pineals removed and cultured as described by Deguchi and Axelrod [18]. Briefly, pineals were placed in BGJb Fitton Jackson media (Grand Island Biological Co., Grand Island, NY.) containing 100 μg/ml of ascorbic acid, 2 mM glutamine and 100 μg/ml of streptomycin and 100 units/ml of penicillin. Four to eight pineals were incubated per dish in 2.5 ml media at 35° under an atmosphere of 95% O_2–5% CO_2.

Drugs and isotopes were added to the media at the times and concentrations indicated.

N-acetyltransferase assay. The incubated pineal glands were removed from culture and individually assayed for *N*-acetyltransferase activity by the method of Deguchi and Axelrod [19] except that the acetyl CoA concentration was 20 nmoles/70 μl incubation containing 0.1 μCi [1-^{14}C]acetyl-coenzyme A. Units of enzyme activity are expressed as pmoles *N*-[^{14}C] acetyltryptamine formed/pineal/10 min. Pineals which had been incubated with [^{3}H]uridine could be assayed for *N*-acetyltransferase with no interference by the tritium-labeled uridine.

RNA synthesis in the cultured pineal. Pineal glands were incubated with normal media containing 80 μCi/ml of [5-^{3}H]uridine. At the times indicated, individual pineals were removed and homogenized in 0.5 ml of 25 mM Tris–chloride (Tris–Cl), pH 7.5, 50 mM potassium chloride, 5 mM magnesium acetate and 10 μg/ml of sodium heparin with six strokes of a 1-ml glass–Teflon homogenizer. When the pineals were used for subsequent isolation of poly(A)-rich RNA, 50 mM sodium chloride (NaCl) substituted for the potassium chloride. The homogenate was centrifuged at 12,000 *g* for 10 min. The pellets were retained and are referred to as nuclear RNA while the supernatant fractions were retained for cytoplasmic RNA. A portion of the supernatant was precipitated in 10% trichloroacetic acid (TCA), filtered and washed through Millipore filters (Millipore Corp., Bedford, MA) and counted in 10 ml Instabrays (Yorktown Research, Hackensack, NJ) in a Beckman LS 355 liquid scintillation spectrophotometer to assay total cytoplasmic RNA synthesis. Nuclear pellets were dissolved in 0.5% sodium dodecylsulfate (SDS) and precipitated with 10% TCA.

Poly(A)-rich RNA was purified by the method of Krystosek *et al.* [20] by making the individual pineal supernatants 0.4 M in NaCl, 0.01 M Tris–Cl (pH 7.5), and 0.5% in SDS. Generally, 10 mg of the cellulose was added to each pineal supernatant, gently mixed, and placed onto a glass wool-plugged pasteur pipet column. The cellulose was extensively washed with 0.4 M NaCl, 0.01 M Tris–Cl (pH 7.5), and 0.5% SDS before being eluted with four 0.25-ml portions of 0.01 M Tris–Cl (pH 7.5) and 0.5% SDS. Samples were assayed for the presence of [^{3}H]uridine in 10 ml of Instabrays solution. All data are expressed as c.p.m. [^{3}H]uridine/pineal or as the percentage of [^{3}H]uridine per pineal relative to the (*l*)-isoproterenol-treated control pineal glands.

Uptake of [^{3}H]uridine into the pineal gland. Pineal glands were cultured in media $\pm$ 2 μM isoproterenol plus 80 μCi/ml of [^{3}H]uridine for 3 hr. Individual glands were blotted free of media, rinsed in phosphate-buffered saline, and homogenized in 0.5 ml of phosphate-buffered saline. The homogenate was added to 0.5 ml of 10% trichloroacetic acid and the precipitate removed by centrifugation and an aliquot of the supernatant removed for scintillation counting.

Agarose gel electrophoresis of pineal RNA. The supernatants of similarly treated pineals or the poly-A-rich RNA fractions were pooled and extracted with 50% phenol–50% chloroform containing 4% isoamyl-alcohol as described by Rozenblatt and Winocour [21]. The phenol extracts were precipitated with 2 vol. ethanol at -20° overnight. The RNA precipitates were centrifuged at 15,000 *g* for 20 min and lyophylized to remove the last trace of fluid. The RNA precipitates were dissolved in a minimal volume of 50 mM NaCl, 1 mM disodium ethylenediamine tetra acetate (Na_2EDTA), and 10% sucrose and electrophoresed on 2% agarose cylindrical gels by the procedure of Weil and Hampel [22]. After the electrophoresis the gels were sectioned into 1-mm segments and placed two successive segments/vial containing 0.5 ml of NCS solubilizer (Amersham-Searle). The vials were incubated at room temperature overnight before the addition of 10 ml Instabrays.

RESULTS

Time course of enzyme induction. Serotonin *N*-acetyltransferase activity may be induced in cultured rat pineal glands by isoproterenol. The rate of induction,

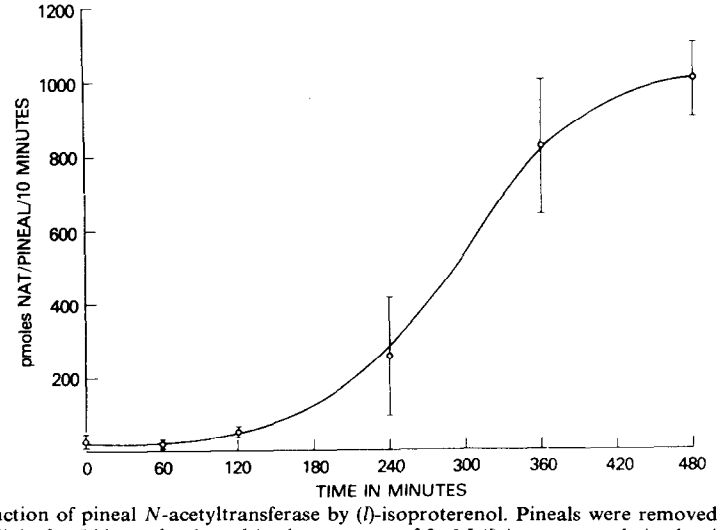

Fig. 1. Induction of pineal *N*-acetyltransferase by (*l*)-isoproterenol. Pineals were removed from animals exposed to light for 24 hr and cultured in the presence of 2 μM (*l*)-isoproterenol. At the times indicated, glands were removed and assayed for *N*-acetyltransferase activity as described. Data shown represents eight determinations of each time point $\pm$ standard error of the mean.

Table 1. Incorporation of [^{3}H]uridine into pineal RNA during *N*-acetyltransferase induction*

Treatment	NAT (pmoles/pineal/ 10 min)	[^{3}H]uridine (c.p.m./pineal/6 hr)		
		Nuclear pellet	Total cytoplasmic	Poly(A)-rich cytoplasmic
− Isoproterenol	26 ± 5	79,924 ± 8,496	47,924 ± 12,440	1,760 ± 320
+ Isoproterenol	785 ± 215	74,066 ± 11,002	47,964 ± 12,748	1,840 ± 240

* Individual pineals were incubated for 6 hr ± 2 μM isoproterenol in the presence of 80 μCi/ml of [^{3}H]uridine. Data represent the average of eight determinations ± standard error of the mean.

however, varies with the length of time that the animals have been exposed to light from the last dark period prior to culturing [15]. Under our experimental conditions the lag period between the addition of isoproterenol and the appearance of increased enzyme activity is approximately 3 hr (Fig. 1). A 30-fold increase in enzyme activity occurs by 6–8 hr. This time course of enzyme appearance was used as a base for investigating the relationship of RNA synthesis to enzyme induction.

Synthesis of RNA during N*-acetyltransferase induction.* Pineal glands were incubated in media containing [^{3}H]uridine and assayed for label incorporation into nuclear, total cytoplasmic and cytoplasmic poly(A)-rich RNA as described in Materials and Methods. Results in Table 1 indicate that the addition of 2 μM (*l*)-isoproterenol while stimulating *N*-acetyltransferase activity to increase approximately 30-fold seemed to have no real influence on labeled uridine incorporation into the various RNA fractions of the cell at the end of the 6-hr induction period. The uptake of the [^{3}H]uridine into the pineal glands was similarly not affected by (*l*)-isoproterenol treatment (Table 2). Examination of the labeled RNA by agarose gel electrophoresis (Fig. 2) revealed no real difference in the relative amount of ribosomal RNA or transfer RNA synthesis between (*l*)-isoproterenol-treated or untreated pineals. The poly(A)-rich RNA of the cytosol, i.e. messenger RNA, comprised 3.7 to 3.8 per cent of the total cytoplasmic RNA. Agarose gel electrophoresis of the poly(A)-rich RNA revealed no apparent contamination of the preparation by ribosomal or transfer RNA and no appreciable effect of (*l*)-isoproterenol on the synthesis of messenger RNA in the cultured pineal gland (Fig. 3).

Table 2. [^{3}H]uridine uptake into pineal glands during *N*-acetyltransferase induction*

Treatment	Free [^{3}H]uridine (c.p.m./pineal/3 hr)
− Isoproterenol	97,790 ± 12,810
+ Isoproterenol	95,760 ± 26,660

* Individual pineals were incubated for 3 hr ± 2 μM isoproterenol in the presence of 80 μCi/ml of [^{3}H]uridine. Data represent the average of eight determinations ± standard error of the mean.

Treatment with (*l*)-isoproterenol does not seem to affect the appearance of newly synthesized and processed nuclear RNA into the cytosol of cultured pineals. Glands were cultured for 90, 180, 270 or 360 min in media containing labeled uridine and fractionated into the nuclear and cytoplasmic constitu-

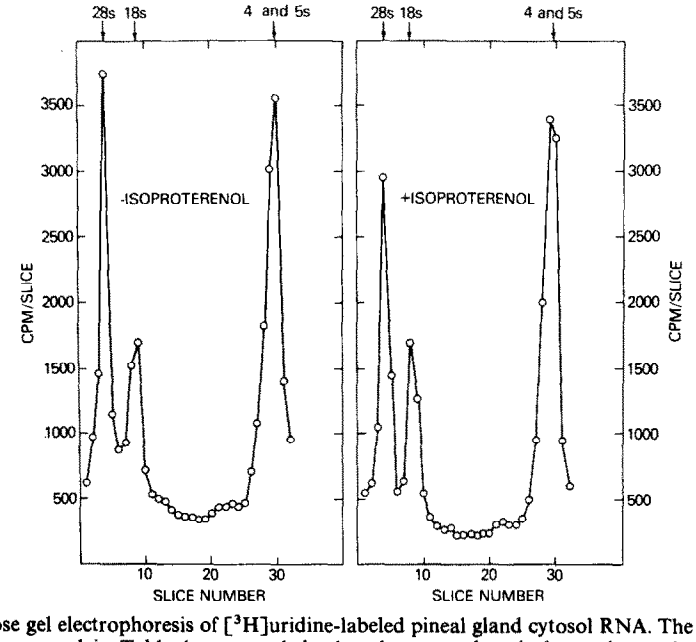

Fig. 2. Agarose gel electrophoresis of [^{3}H]uridine-labeled pineal gland cytosol RNA. The total cytoplasmic RNA represented in Table 1 was pooled, phenol extracted and electrophoresed on 2% agarose gels as described in Materials and Methods.

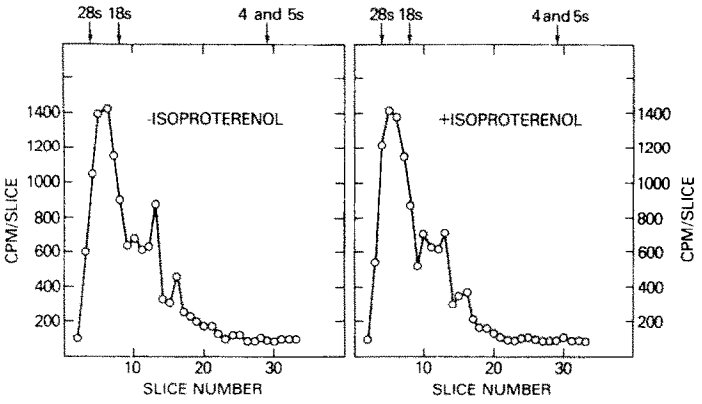

Fig. 3. Agarose gel electrophoresis of [^{3}H]uridine-labeled pineal gland poly(A)-rich RNA. The poly(A)-rich cytoplasmic RNA represented in Table 1 was pooled, phenol extracted and electrophoresed on 2% agarose gels as described in Materials and Methods.

ents. It is seen in Fig. 4 that the incorporation of [^{3}H]uridine into the nuclear pellet is essentially linear over the typical 6-hr incubation period. In contrast, there is about a 2-hr lag in the appearance of labeled RNA into the pineal cytosol. After this lag period there is an increase of labeled RNA in the cytoplasm which roughly parallels the increase in [^{3}H]uridine RNA seen in the nuclear pellet.

Effect of actinomycin D on N-acetyltransferase induction. Previous workers [16, 17, 23] have found the induction of the *N*-acetyltransferase by (*l*)-isoproterenol is inhibited by actinomycin D, suggesting that RNA synthesis is required for enzyme induction. It was decided to see at what point, if any, the induction process was insensitive to RNA synthesis inhibition by actinomycin D, that is if RNA synthesis ceased to be a factor in enzyme induction. Pineal glands were incubated in the presence of (*l*)-isoproterenol and transferred into media containing (*l*)-isoproterenol and 10 μg/ml of actinomycin D at various times through the 6-hr induction period. It is seen in Fig. 5 that the simultaneous addition of (*l*)-isoproterenol and actinomycin D inhibits enzyme induction by more than 90 per cent. If pineal glands were induced for 90 min and transferred into actinomycin D containing media, there was approximately a 55 per cent inhibition in the appearance of enzyme activity at 6 hr. Addition of actinomycin D at 3 hr after the initiation of the induction process (at a time when no

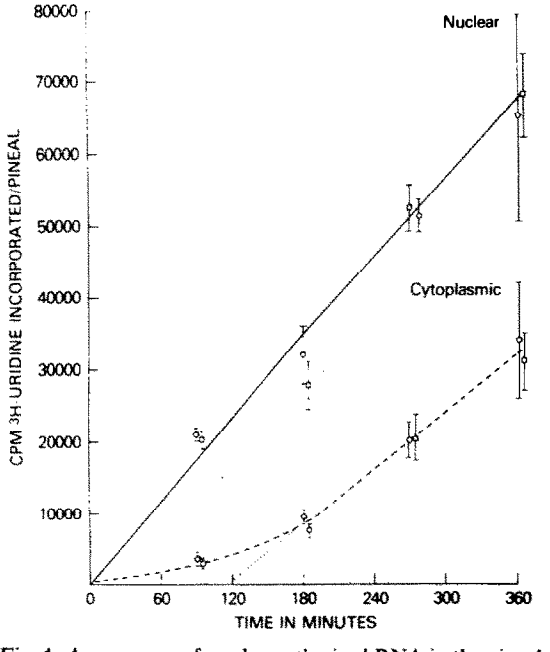

Fig. 4. Appearance of newly synthesized RNA in the pineal cytosol. Pineal glands were cultured in a medium containing 80 μCi/ml [^{3}H]uridine minus (*l*)-isoproterenol (○) or plus 2 μM (*l*)-isoproterenol (□). At the times indicated, glands were removed and processed into nuclear and cytosol fractions and assayed for labeled RNA as described. Data shown represent eight determinations at each time point ± standard error of the mean.

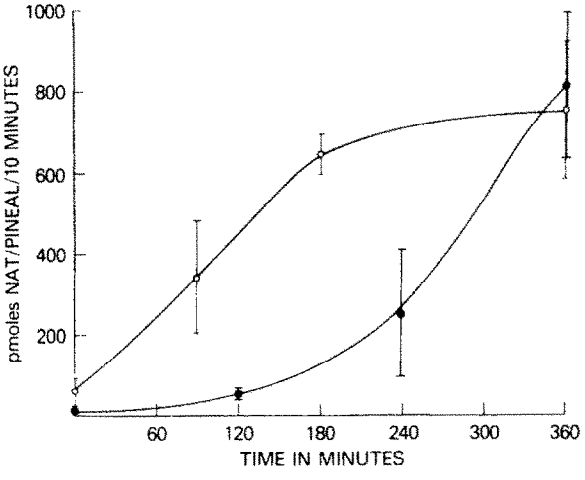

Fig. 5. Appearance of actinomycin D insensitivity to (*l*)-isoproterenol induction of pineal *N*-acetyltransferase. Pineals were cultured in a medium containing 2 μM (*l*)-isoproterenol. At the times indicated, glands were transferred into medium containing 2 μM (*l*)-isoproterenol plus 10 mg/ml of actinomycin D and assayed for *N*-acetyltransferase at 6 hr of isoproterenol treatment (○——○). Data shown represent four determinations ± standard error of the mean. The appearance of *N*-acetyltransferase enzyme activity in the cytosol of pineal glands cultured in the presence of (*l*)-isoproterenol and absence of actinomycin D (●——●) represents the data from Fig. 1.

increase in enzyme actively is evident) inhibits the appearance of *N*-acetyltransferase activity only 15 per cent when enzyme levels are measured at 6 hr. These are similar to the findings of Zatz *et al.* [17]. However, it should be noted that, when this information is compared to the appearance of increased *N*-acetyltransferase enzyme activity, it is evident that there is approximately a 3-hr lag between the initiation of the synthesis of a required RNA species and the utilization of this RNA for enzyme induction

DISCUSSION

The overall induction of *N*-acetyltransferase activity by (*l*)isoproterenol in the pineal appears to require RNA synthesis as has been suggested by others [16, 17 23]. This is primarily based on the actinomycin D sensitivity during early stages of the induction process [16, 17; this investigation].

Isoproterenol does not seem to affect general RNA synthesis in the pineal. This is evident even when the total RNA is divided into the constituent nuclear, total cytoplasmic and poly(A)-rich mRNA fractions of the cell. Separation of different size classes of RNA by agarose gel electrophoresis also indicated no difference in RNA synthesis between (*l*)-isoproterenol-treated and untreated pineal glands. This suggests that the specific induction of *N*-acetyltransferase by (*l*)-isoproterenol does not measurably perturb the RNA biosynthetic function of the pineal gland. Isoproterenol also does not influence the rate at which newly synthesized pineal RNA enters the cytosol from the nucleus (Fig. 4). A point which strengthens the hypothesis of an RNA requirement for *N*-acetyltransferase induction is the time of the lag in the emergence of newly synthesized RNA into the cytosol and the lag in the appearance of increased *N*-acetyltransferase enzyme activity. Both lag periods are essentially the same length of time, suggesting that the initiation of new RNA synthesis upon isoproterenol treatment is accomplished soon after the addition of the β-agonist. The lag in RNA transport from the nuclei into the cytosol in the pineal is appreciably longer than the observed lag in HeLa cells [24, 25], which amounts to 20–40 min.

Whatever process high concentrations of actinomycin D are affecting, the sensitivity of *N*-acetyltransferase induction to the inhibitor is abolished relatively quickly after the addition of the inducer (*l*)-isoproterenol [17]. Within 90 min of β-agonist treatment, the induction of enzyme activity has lost about 50% of its sensitivity to 10 μg/ml of actinomycin D. If an RNA species is required, then the changes in the transcriptive activity in the pineal nucleus are well under way by 90 min and have been completed by 180 min into the induction process (see Fig. 4). This is consistent with the correlation of lag periods in RNA emergence into the cytosol and the increase in *N*-acetyltransferase activity mentioned above.

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