Proc. Nat. Acad. Sci. USA
Vol. 72, No. 5, pp. 1661–1665, May 1975

Regulation of Sensitivity to *Beta*-Adrenergic Stimulation in Induction of Pineal *N*-Acetyltransferase

(adenosine 3′:5′-cyclic monophosphate/protein synthesis/postsynaptic super- and subsensitivity/catecholamine neurotransmitters/arylamine acetyltransferase)

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ABSTRACT **Stimulation by isoproterenol causes large increases in the activity of rat pineal *N*-acetyltransferase (arylamine acetyltransferase or acetyl-CoA:arylamine *N*-acetyltransferase, EC 2.3.1.5) after a variable lag period. Lengthening periods of exposure to light cause rapid increases in the sensitivity of pineal *N*-acetyltransferase to induction by isoproterenol. This supersensitivity, which develops gradually over a 12 hr period, is correlated with increasingly longer lag periods in the induction of the enzyme, and with progressively greater maximal response. Repeated administration of isoproterenol to supersensitive animals rapidly reverses the above changes and causes relative subsensitivity. The sensitivity of *N*-acetyltransferase to induction by dibutyryl-cyclic AMP added to pineals in organ culture was found to change in parallel with the sensitivity to isoproterenol. Stimulation of cyclic AMP levels in the pineal by isoproterenol was also greater in supersensitive rats than in the subsensitive animals, whether the supersensitivity had been caused by denervation or by exposure to light for 12 hr. The above experiments suggest that there are two sites for the regulation of the sensitivity of *N*-acetyltransferase to induction by catecholamines. The first site regulates the concentration of cyclic AMP achieved as a result of *beta*-adrenergic stimulation. The second site regulates the capacity of intracellular processes (i.e., induction of *N*-acetyltransferase) to respond to cyclic AMP as a second messenger.**

The pineal hormone melatonin, as well as the enzymes and intermediates involved in its biosynthesis, exhibits marked circadian rhythms under the influence of environmental lighting (1–7). Thus, the highest levels of melatonin in the rat pineal occur during the night (1), in phase with the greatest activities of serotonin-*N*-acetyltransferase (arylamine acetyltransferase or acetyl-CoA:arylamine *N*-acetyltransferase, EC 2.3.1.5) (2). As the first specific enzyme in the branch pathway for melatonin synthesis (8), *N*-acetyltransferase is in a unique position to exert control over the activity of this pathway.

N-Acetyltransferase activity (2, 9), and consequently melatonin synthesis (10), is controlled by a *beta*-adrenergic receptor mechanism. It appears likely that the circadian rhythms in *N*-acetyltransferase activity are generated by 24-hr rhythms in the turnover of noradrenaline (11) in the sympathetic terminals innervating the gland (12).

The rate of firing of these postganglionic sympathetic fibers reflects changes in environmental lighting (13); electrophysiological stimulation of the preganglionic trunk to the superior cervical ganglion elevates pineal *N*-acetyltransferase activity (14). The nocturnal increase in *N*-acetyltransferase activity is abolished by exposure of the animals to light, denervation by superior cervical ganglionectomy (9, 15), decentralization, or by treatment with reserpine, propranolol, or cycloheximide (9). These manipulations indicate that the nocturnal rise in pineal *N*-acetyltransferase activity is caused by increased release of norepinephrine onto postsynaptic *beta*-adrenergic receptors, and that the increase in enzyme activity requires new protein synthesis (9, 16).

In stimulating *N*-acetyltransferase production, the *beta*-adrenergic receptor probably acts via an adenylate cyclase cyclic AMP system (16, 17). The responsiveness of the pineal cell to *beta*-adrenergic stimulation is modified by the previous exposure of the pinealocyte to the neurotransmitter (18). There is an increase in the sensitivity of adenylate cyclase to induction by norepinephrine in pineal homogenates from rats kept in constant light or after chronic denervation of the pineal gland (20, 21). More recent studies *in vivo* and *in vitro* show a rapid, marked increase in the sensitivity of *N*-acetyltransferase and cyclic AMP to induction by isoproterenol. This supersensitivity develops as rapidly as 24 hr after denervation (22) and is thus in marked contrast with the denervation supersensitivity described by other investigators (19). Repeated administration of isoproterenol to supersensitive animals resulted in subsensitivity in the induction of *N*-acetyltransferase by catecholamines (18). These findings indicate that extended exposure of the receptor to its agonist rapidly decreases its sensitivity, whereas removal of the neurotransmitter results in supersensitivity which develops in less than 24 hr.

Diurnal variations in the sensitivity of *N*-acetyltransferase to induction by isoproterenol were reported in a preliminary communication (23). In those experiments rats were kept in alternating light and dark periods of 12 hr duration, with no prior surgical or pharmacological manipulation. Much greater doses of isoproterenol were required to produce equivalent induction in *N*-acetyltransferase activity at the beginning of the light period (0600 hr) than at the end (1800 hr). In addition, maximal response to isoproterenol is greater after 12 hr of exposure to light (23).

In this report we have examined these diurnal changes in the sensitivity of *N*-acetyltransferase to induction by catecholamines in greater detail, to determine the sites and mechanisms involved in these changes in responsiveness. We show that sensitivity changes occur both in the system that regu-

Abbreviation: cyclic AMP, adenosine 3′:5′-cyclic monophosphate.

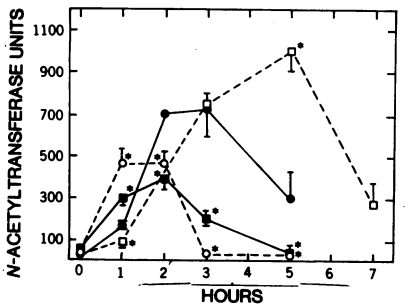

FIG. 1. Effect of light or pretreatment with isoproterenol on the time course of induction of pineal *N*-acetyltransferase *in vivo*. *l*-Isoproterenol *d*-bitartrate (2 mg/kg) was injected subcutaneously into rats, which were killed at various times after injection as indicated. *N*-Acetyltransferase activity was measured as described in the *text*. Rats were injected at various times relative to light/dark cycles: (■——■) 0630, after only 30 min light; (●——●) 1200; (□——□) 0630, lights on overnight; (○——○), 0630, lights on overnight, and pretreated with isoproterenol 21, 13, and 5 hr before the final test dose. Results are expressed as mean ± standard error ($n = 6$). $^*P < 0.01$ compared to noon group by Student's *t*-test.

lates cyclic AMP levels as well as in the capacity of cyclic AMP to stimulate induction of a specific protein.

MATERIALS AND METHODS

Chemicals. [1-^{14}C]Acetyl coenzyme A (3.5–6.6 mCi/mmol) was purchased from Amersham-Searle, Chicago. Tritiated cyclic AMP (20–30 Ci/mmol) was obtained from New England Nuclear Corp., Boston. *l*-Isoproterenol *d*-bitartrate was supplied by Winthrop Laboratories, and *l*-propranolol was a gift from Ayerst Laboratories. All other chemicals were obtained from commercial sources.

Animals. Male Sprague–Dawley rats (150–175 g) were supplied by Zivic-Miller, Allison Park, Pa. For at least 5 days before each experiment, these animals were kept in our facilities under diurnal lighting conditions with the lights on from 0600 to 1800 hr. Except as noted, drugs were injected subcutaneously in 0.154 M NaCl; drug concentrations given in milligrams refer to salt concentrations. Groups of six to eight rats were killed by decapitation at the times indicated in each experiment. Pineals were denervated by bilateral superior cervical ganglionectomy.

Pineal Explant Culture. At the specified times, pineals were removed and placed in organ explant culture in plastic petri dishes (60 mm diameter). Four to six pineals were cultured in each dish containing 2.5 ml of BGJ$_b$-Fitton Jackson medium (334 milliosmolar, Grand Island Biological Co.), supplemented with ascorbic acid (0.1 mg/ml), glutamine (2.0 mM), streptomycin (100 μg/ml) and penicillin (100 units/ml). Pineals were cultured for 10 hr at 37° under 95% O_2–5% CO_2. Isoproterenol was present in the culture medium in the concentration indicated in each experiment.

Assay of Serotonin-N-Acetyltransferase Activity. Within 30 sec after decapitation or removal from culture, pineals were homogenized and *N*-acetyltransferase assay performed as described previously (24). Twenty nanomoles of [^{14}C]acetyl-CoA were used, instead of 3.4 nmole as described in the original assay. Units of *N*-acetyltransferase activity are defined as picomoles of *N*-acetyltryptamine formed in 10 min.

Assay of Cyclic AMP. To avoid increased cyclic AMP levels after decapitation (25), pineals were removed and homogenized within 30 sec after decapitation. Homogenates were immediately heated for 5 min in a boiling-water bath to inactivate phosphodiesterases. Cyclic AMP assay was then performed by the method of Gilman (26), in a total volume of 70 μl, and under conditions which ensured the saturation of the binding protein. [^{3}H]Cyclic AMP was purified before use by Dowex chromatography.

RESULTS

Effect of Exposure to Light on the Time Course of Induction of N-Acetyltransferase by Isoproterenol In Vivo. *N*-Acetyltransferase activity was induced by subcutaneous injection of isoproterenol (2.0 mg/kg) into rats that had been exposed to light for increasing periods of time. Under these conditions, control rats (saline injections) showed no stimulation of enzyme activity. Rats injected with isoproterenol at 0630 hr (exposed to light for 30 min), showed a rapid 8-fold increase in enzyme activity 1 hr after the catecholamine was injected, with peak activity occurring between 1 and 2 hr after the injection (Fig. 1). Three hours after the injection, *N*-acetyltransferase activity was declining. In rats stimulated at 1200 hr (6 hr exposure to light), there was a slower initial rise in the activity of *N*-acetyltransferase. However, the maximum of enzyme activity attained was twice as great as that achieved in rats exposed to 30 min of light. An accentuation of this trend was evident in rats exposed to light for increasing periods of time: an increasingly slower rise followed by progressively higher levels of induction.

Effect of Pretreatment with Isoproterenol on the Time Course of Induction of N-Acetyltransferase In Vivo. As a test for the hypothesis that the faster induction of *N*-acetyltransferase in the morning (0630 hr) animals was due to the increased exposure of the pinealocytes to neurotransmitter during the preceding night, the following experiment was performed. Rats were kept in the light for a 24-hr period during which each animal received three isoproterenol injections at 21, 13, and 5 hr before a fourth test dose at 0630 hr. Pretreatment with isoproterenol shortened the time course and decreased the maximum level of *N*-acetyltransferase induction (Fig. 1).

Effect of Pretreatment with Isoproterenol on the Time Course of N-Acetyltransferase Induction In Vitro. To examine the effect of pretreatment with isoproterenol on the rate and extent of *N*-acetyltransferase induction in cultured pineals, glands from rats killed at 1200 hr were incubated in the presence of 1.0 μM isoproterenol, and assayed at different times after the beginning of the incubation. These glands were compared to pineal organs from animals killed at the same time, but which had been pretreated subcutaneously with three doses of isoproterenol (2.0 mg/kg) 21, 13, and 5 hr before being killed. The pineals from rats pretreated with isoproterenol *in vivo* responded faster than those from untreated rats (Fig. 2). However, after 8 hr the activity of the enzyme in the glands from untreated animals was significantly higher than that of the glands from the pretreated animals (Fig. 2).

Development of Subsensitivity with Repeated Stimulation with Isoproterenol at Short Intervals. To provide a more uniform and prolonged stimulus, thus more closely resembling the increased norepinephrine turnover during the night cycle, we injected rats with isoproterenol emulsion in sesame oil. Rats

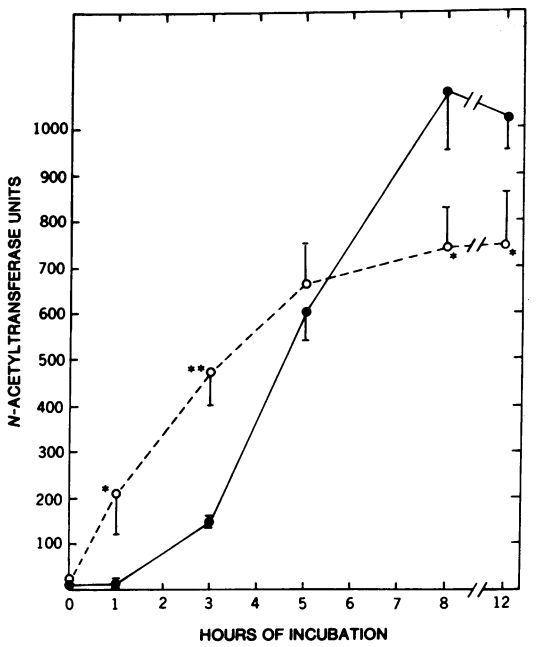

FIG. 2. Effect of pretreatment with isoproterenol on the time course of induction of pineal *N*-acetyltransferase *in vitro*. Pineal glands from rats killed at 1200 hr were incubated in medium containing 1.0 μM isoproterenol. Glands were assayed for *N*-acetyltransferase activity at the indicated times after the beginning of the incubation. (●——●) control; (○——○) rats pretreated with *l*-isoproterenol *d*-bitartrate (2.5 mg/kg) subcutaneously 21, 13, and 5 hr before being killed. Data are presented as mean ± standard error. $*P < 0.05$ compared to corresponding control point by Student's *t*-test; $**P < 0.01$.

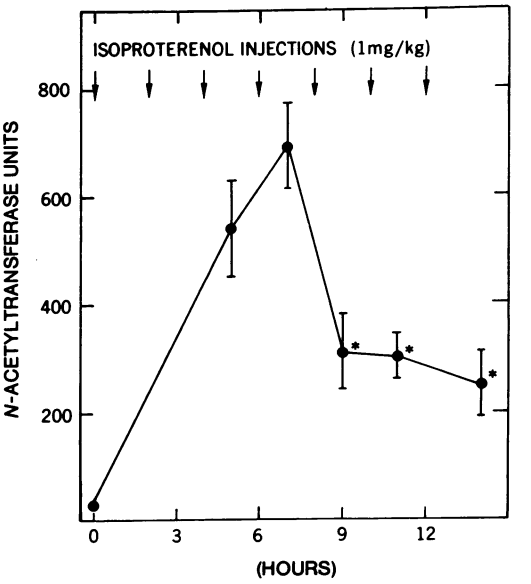

FIG. 3. *N*-Acetyltransferase activity during repeated stimulation with isoproterenol. *l*-Isoproterenol *d*-bitartrate (1 mg/kg) in sesame oil emulsion was injected subcutaneously at 0700 hr into rats that had been kept in the light for 24 hr. Injections were repeated at 2 hr intervals. During the course of the experiment, animals were killed at 0, 5, 7, 9, 11, and 14 hr after the first dose. Pineals were assayed for *N*-acetyltransferase as described in the *text*. Data are presented as mean ± standard error ($n = 6$ to 8). $*P < 0.01$ compared to peak values using Student's *t*-test.

were first made supersensitive by exposure to light for 24 hr; they were then injected with isoproterenol emulsion beginning at 0700 hr. Injections were repeated at 2-hr intervals, and groups of animals were killed periodically after the first dose. Peak stimulation occurred 7 hr after the first dose; however, after four repeated doses of isoproterenol at 2-hr intervals, the activity of *N*-acetyltransferase was markedly reduced (Fig. 3).

Progressive Development of Supersensitivity. Since exposure to light causes a decreased turnover of norepinephrine (11), and consequently an increased sensitivity in the response to *beta*-adrenergic stimulation, the course of development of this supersensitivity during a normal light period (0600–1800 hr) was examined. After varying periods of exposure to light, rat pineal glands were removed and incubated for 10 hr in the presence of increasing isoproterenol concentrations. Dose–response curves (Fig. 4) indicate that there is a gradual increase of sensitivity to induction by isoproterenol with exposure to light. With increasing time of exposure to light there is both an increase in the response to low concentrations of isoproterenol as well as an increase in the maximum response (Fig. 4).

Supersensitivity of N-Acetyltransferase to Induction by Dibutyryl Cyclic AMP. To determine whether the above changes in sensitivity reflect changes in the effectiveness of cyclic AMP as a second messenger, glands exposed to different periods of light were cultured in the presence of dibutyryl-cyclic AMP. In the pineal, propranolol does not block the stimulation of melatonin synthesis by dibutyryl-cyclic AMP (10), nor does propranolol have any effect on the induction of *N*-acetyltransferase by dibutyryl-cyclic AMP. Dibutyryl-cyclic AMP dose–response curves were done in the presence of 10^{-5} M *l*-propranolol to exclude any possible pre-synaptic effects of the nucleotide. During the first 4 hr of exposure to

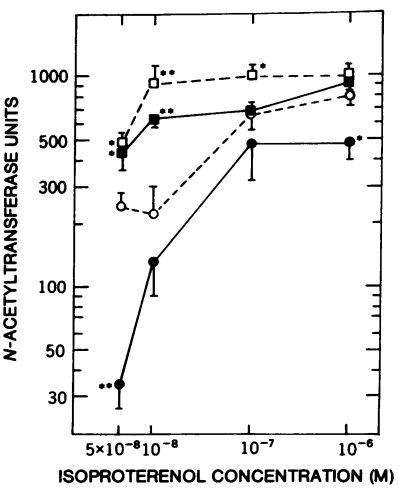

FIG. 4. Sensitivity of pineal *N*-acetyltransferase to induction by isoproterenol *in vitro* at various times during the light period. Rats kept in diurnal lighting conditions (light 0600–1800 hr, dark 1800–0600 hr) were killed at various times during the light period and the pineal glands incubated in the various concentrations of isoproterenol indicated. *N*-Acetyltransferase assay was done after 8 hr incubation. (●——●) 0630; (○——○) 1000; (■——■) 1400; (□——□) 1800. Results are expressed as mean ± standard error of the mean. $*P < 0.05$ differs from corresponding 1000 hr group; $**P < 0.01$.

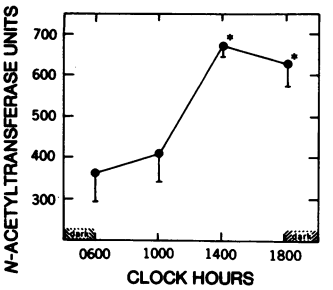

FIG. 5. Variation in sensitivity of *N*-acetyltransferase to induction by dibutyryl-cyclic AMP *in vitro*. Rats were killed at various times indicated during the light period, and the pineal glands incubated in 1.0 mM dibutyryl-cyclic AMP for 10 hr. *N*-Acetyltransferase assay was performed as described in *text*. Data are means ± standard error ($n = 6$). *$P < 0.01$ compared to morning group (0630 hr) by Student's *t*-test.

light there was no change in the response of *N*-acetyltransferase to induction by dibutyryl-cyclic AMP. However, between 4 and 8 hr, a 2-fold increase in the sensitivity to induction was found (Fig. 5). Dose–response curves at 0600 hr and at 1800 hr further document the increase in sensitivity to dibutyryl-cyclic AMP (Table 1). These results indicate that at least part of the change in sensitivity to isoproterenol does not involve the interaction of agonist with receptor or the production of cyclic AMP, but rather reflects changes in the efficiency of cyclic AMP actions intracellularly.

Variation in Cyclic AMP Stimulation by Isoproterenol. The above effects on the sensitivity to dibutyryl-cyclic AMP could partially account for the changes in sensitivity of *N*-acetyltransferase to induction by isoproterenol. We then determined whether there were changes referable directly to the system which regulates cyclic AMP levels which would also contribute to the rapid changes in sensitivity. Cyclic AMP levels were measured after stimulation with isoproterenol in animals exposed to either 30 min (0630 hr) or 12 hr (1800 hr) of light. Both intact rats and others with denervated

TABLE 1. *Diurnal variation in sensitivity of pineal N-acetyltransferase to induction by dibutyryl-cyclic AMP in cultured pineal*

Dibutyryl-cyclic AMP (M)	*N*-Acetyltransferase units (pmol/pineal per 10 min) 0600 hr	1800 hr
0	6 ± 4	ND[b]
1.0×10^{-4}	7 ± 1	18 ± 13
3.0×10^{-4}	15 ± 5	80 ± 20[a]
6.0×10^{-4}	140 ± 25	320 ± 110
1.0×10^{-3}	175 ± 25	1000 ± 155[a]
5.0×10^{-3}	1055 ± 25	—

Pineals were removed at the beginning (0600 hr) and at the end (1800 hr) of the light cycle, and were immediately placed in culture in the presence of the indicated concentrations of dibutyryl-cyclic AMP and 10^{-5} M propranolol. After 10 hr incubation, *N*-acetyltransferase activity was determined as described in the *text*. Results are expressed as mean ± standard error ($n = 6$).

[a] $P < 0.001$ differs from corresponding 0600 hr point by Student's *t*-test.

[b] ND, not detectable.

TABLE 2. *Effects of light and denervation on pineal cyclic AMP response to isoproterenol*

Treatment	Cyclic AMP (pmol/pineal) 0630 hr	1800 hr
Control (saline)	14 ± 1 (8)	8 ± 1 (8)[a]
Isoproterenol (0.5 mg/kg)	33 ± 7 (8)[b,c]	77 ± 13 (8)[a,b]
Denervated plus isoproterenol (0.5 mg/kg)	66 ± 15 (8)[b]	87 ± 14 (8)[b]

l-Isoproterenol *d*-bitartrate or saline (isotonic) was injected subcutaneously into rats at the beginning (0630 hr) or at the end of the light period (1800 hr). Pineal glands were denervated by superior cervical ganglionectomy performed 2 weeks before the experiments. Cyclic AMP levels were measured 8 min after the injection, using the method described in the *text*. Results are expressed as mean ± standard error ($n = 8$).

[a] $P < 0.05$ differs from corresponding 0630 hr group by Student's *t*-test.

[b] $P < 0.05$ differs from corresponding control group by Student's *t*-test.

[c] $P < 0.05$ differs from corresponding denervated group.

pineal glands were used. Relative to the intact animals at 0600 hr, supersensitivity was caused both by denervation and by 12 hr exposure to light (Table 2). A combination of denervation with 12 hr exposure to light did not further enhance the cyclic AMP response to isoproterenol.

DISCUSSION

The experiments described indicate that changes in the responsiveness of pinealocytes to *beta*-adrenergic stimulation occur rapidly and involve more than one site. At any given time, the sensitivity of the cell as measured by the inducibility of a specific protein (*N*-acetyltransferase), is a function of the prior exposure of the receptor to its agonist, either physiologically released from nerve terminals, or exogenously introduced.

The responsiveness of the pineal gland to catecholamines exhibits diurnal variations which may contribute to the physiologic cycles in enzyme activity and melatonin production (23). The increased responsiveness to isoproterenol as a result of reduced sympathetic nerve activity during the light period develops rapidly. Within 4 hr after the onset of light the pineal is much more sensitive to low doses of isoproterenol, and the maximum activity of *N*-acetyltransferase achievable is increased nearly 2-fold (Fig. 4).

Relative subsensitivity also appears very rapidly. Eight hours after the beginning of repeated administration of isoproterenol during the daytime there is a 50% reduction in the activity of *N*-acetyltransferase. This fall in *N*-acetyltransferase activity during the day, despite repeated stimulation with isoproterenol (Fig. 3), follows a very similar time course to the physiological fall in *N*-acetyltransferase activity during the night (23). In both cases approximately 8 hr after the onset of either physiological or pharmacological stimulation, a relative subsensitivity develops and there is a decline in enzyme activity. Repeated attempts to demonstrate that this fall may be due to changes in turnover of norepinephrine during the night have been unsuccessful.* On the basis of the above evidence, it appears that the fall in *N*-acetyltransferase

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

during the night (23) is due in part to reduced sensitivity of the cell to *beta*-adrenergic stimulation.

Pineal glands which have been exposed to high concentrations of agonist, either physiologically or pharmacologically, exhibit a rapid activation of *N*-acetyltransferase, with almost no lag period upon restimulation after enzyme activity has returned to baseline. Increasing the period of exposure to light (and, therefore, reducing the turnover of norepinephrine) produces a progressive increase in the lag period for induction and a delay in reaching maximal activity of *N*-acetyltransferase. The lag period in stimulation of *N*-acetyltransferase activity in animals exposed to light suggests that the production of a precursor is necessary before the final production of active enzyme molecules. Conversely, the immediate increase in *N*-acetyltransferase synthesis in animals which have been exposed to increased concentrations of catecholamines (by being in the dark, or by isoproterenol pretreatment) further suggests the accumulation of this probable precursor. The proposed precursor may be a specific messenger RNA, an inactive protein form, or an essential cofactor necessary for the production of active enzyme.

The slower the rate of induction of *N*-acetyltransferase, the greater the maximum achieved. This striking inverse relationship suggests that as a result of *beta*-adrenergic stimulation, and the consequent accumulation of precursor or intermediate molecules, there is also a limitation on the further synthesis of active *N*-acetyltransferase, and suggests the presence of inhibitory feedback signals or depletion of substrates.

The shift from sub- to supersensitivity that we have described is rapid, occurring gradually over a period of 12 hr. This is in sharp contrast to the usual descriptions of postsynaptic sensitivity changes (27, 28), which usually develop over a period of days or weeks, and to the results of other investigators (19–21) in the pineal gland. The changes we have described develop many times faster, and therefore, represent different phenomena.

Dose–response curves indicate that there are two components to the changes in sensitivity: a shift to the left in the curve may reflect a change in the affinity of the receptor for its agonist or in the production and stability of cyclic AMP. The increased maximum response suggests a more efficient coupling of the receptor to intracellular mechanisms, or an increase in the capacity of these intracellular mechanisms to synthesize or activate *N*-acetyltransferase.

The sensitivity of pineal *N*-acetyltransferase to induction by dibutyryl-cyclic AMP (which acts intracellularly by-passing the *beta*-adrenergic receptor) shows changes which parallel those described with isoproterenol (Fig. 5, Table 1). This suggests that the changes in the responsivity of the pineal to catecholamines reflect in part the regulation of the effectiveness of cyclic AMP as a second messenger. These changes occur concomitantly with changes in the effectiveness of catecholamines in increasing intracellular cyclic AMP levels (Table 2).

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