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Blockade by Ouabain or Elevated Potassium Ion Concentration of the Adrenergic and Adenosine Cyclic 3′,5′-Monophosphate-Induced Stimulation of Pineal Serotonin *N*-Acetyltransferase Activity

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SUMMARY

PARFITT, ANDREW, WELLER, JOAN L., SAKAI, KAKUICHI K., MARKS, BERNARD H. & KLEIN, DAVID C. (1975) Blockade by ouabain or elevated potassium ion concentration of the adrenergic and adenosine cyclic 3′,5′-monophosphate-induced stimulation of pineal serotonin *N*-acetyltransferase activity. *Mol. Pharmacol.*, **11**, 241-255.

Rat pineal glands were cultured for 48 hr and then treated with 10 μM norepinephrine. This resulted in the predictable 50-100-fold increase in acetyl-CoA:serotonin *N*-acetyltransferase) (EC 2.3.1.5) activity at 6 hr. This increase failed to occur in glands simultaneously treated with 1 μM ouabain or 80 mM K^+. Prior treatment of pineal glands with ouabain or K^+ did not, however, depress the 100-200-fold norepinephrine-stimulated increase in the pineal content of adenosine cylic 3′,5′-monophosphate (cAMP) observed at 15-20 min; neither was there any alteration in the rate of disappearance of cAMP after this time. Ouabain or K^+ alone failed to depress the conversion of L-[^{3}H]tryptophan to either [^{3}H]5-hydroxytryptophol or [^{3}H]5-hydroxyindoleacetic acid. The conversion of L-[^{3}H]tryptophan to [^{3}H]serotonin was increased in the presence of 80 mM K^+ but was not affected by the presence of 1 μM ouabain. This suggests that ouabain or K^+ does not depress either the activity of the process responsible for the uptake and hydroxylation of L-[^{3}H]tryptophan or the general indole metabolism in the pineal gland. Treatment with N^6,$O^{2'}$-dibutyryladenosine cyclic 3′,5′-monophosphate (DBcAMP) or theophylline, compounds which are known to mimic the effects of norepinephrine on pineal *N*-acetyltransferase activity, did not stimulate *N*-acetyltransferase activity in the presence of 80 mM K^+ or 1 μM ouabain. Ouabain given *in vivo* also reduced by 55-80% the effect of an injection of 20 mg/kg of isoproterenol on pineal *N*-acetyltransferase activity. Intracellular microelectrode recordings from acutely explanted pineal glands indicated that both 40 mM and 80 mM K^+ caused a modest depolarization of the pineal cell membrane. In contrast, treatment with 1 μM ouabain for several minutes did not significantly change the resting membrane potential. However, both 80 mM K^+ and 1

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μM ouabain attenuated or reversed the hyperpolarization associated with exposure to norepinephrine. A small but significant hyperpolarizing effect was also elicited by treating the pineal gland with cAMP or DBcAMP. These findings indicate that ouabain or K^+ blocks the effect of norepinephrine on *N*-acetyltransferase activity; it appears that the blockade is at some point in the sequence of events leading to the increase in enzyme activity which follows the generation of cAMP.

INTRODUCTION

The pineal enzyme acetyl-CoA:serotonin *N*-acetyltransferase (EC 2.3.1.5) regulates daily changes in melatonin synthesis by undergoing large changes in activity which are coordinated with environmental lighting conditions (1–3). A dark-induced increase in enzyme activity appears to be normally initiated by the release of norepinephrine from sympathetic nerves entering the pineal gland (4–6). An increase in enzyme activity is also caused by injection of norepinephrine or isoproterenol (7) or by the addition of either of these compounds to the medium of pineal organ cultures (8,9). In both cases this drug-induced increase in enzyme activity is preceded by a dramatic rise in the concentration of pineal cAMP[3] (10,11). It seems highly probable that cAMP is involved in the adrenergically induced increase in the activity of this enzyme, because DBcAMP and theophylline, both inhibitors of pineal cyclic nucleotide phosphodiesterase (12), also cause an increase in the activity of *N*-acetyltransferase.

It has been shown previously that norepinephrine treatment *in vitro* causes hyperpolarization of the pinealocyte membrane (13). This finding raised the question whether hyperpolarization, or the change in ion physiology associated with it, is necessary either for the effects of norepinephrine on *N*-acetyltransferase activity in the rat pineal gland or for the norepinephrine-induced increase in pineal cAMP. To this end we determined in organ culture whether increasing the medium K^+ concentration or adding ouabain to the medium, treatments which are known to cause depolarization of membranes in neural tissue, could block the biochemical and electrophysiological effects of norepinephrine in the pineal gland.

[3] The abbreviations used are: cAMP, adenosine cyclic 3',5'-monophosphate; DBcAMP, N^6,$O^{2'}$-dibutyryladenosine cyclic 3',5'-monophosphate; *N*-acetyltransferase, acetyl-CoA:serotonin *N*-acetyltransferase (EC 2.3.1.5).

MATERIALS AND METHODS

Biochemical Studies

Organ culture technique. The rat pineal[4] organ culture technique described elsewhere (14,15) was modified as follows. Throughout the culture period glands were supported on 6-mm discs of glass-fiber filter paper (Whatman GF/A). The incubation period prior to drug treatment was 48 hr, and glands were transferred to fresh medium every 24 hr.

After the 48-hr incubation period, glands were transferred to fresh medium containing, where appropriate, either the compound under investigation or an elevated K^+ concentration. Norepinephrine was added in a 1.2 mM solution 30 min later to give a final concentration in the culture medium of 10 μM. The time of this addition is referred to as zero time.

Experiments described in Fig. 6–8 and Tables 4–6 used a top-loading incubator for the last 4 hr of culture. This incubator allows access to each gland through small ports with minimal disturbance to the culture environment (temperature, humidity, and gas composition). All points represent the means ± standard errors of the parameter values determined on four to six separate glands.

N-Acetyltransferase activity. Pineal glands were removed from organ culture and immediately frozen by placing each

[4] Each rat pineal gland used weighed approximately 1 mg.

gland in a separate polyethylene microtest tube (Brinkmann Instruments) cooled in solid CO_2. Glands were then stored at −75° for 1–3 days before assay by a modification of the method of Deguchi and Axelrod (16). Glands were individually ultrasonicated (Biosonik IV, Bronwill Scientific Company, Rochester, N. Y.; dial setting 40 "Lo" for 6 sec at 0°; 4 mm probe) in 100 μl of 0.1 M sodium phosphate buffer, pH 6.80, which contained [1-^{14}C]acetyl-CoA (0.5 mM; specific activity, 1 μCi/μmole) and tryptamine (10 mM). In one experiment (Table 7) pineal glands were individually sonicated in 65 μl of 0.1 M sodium phosphate buffer containing 1.0 mM [1-^{14}C]acetyl-CoA. Portions of 25 μl each of different sonic extracts were then added to a solution of [1-^{14}C]acetyl-CoA and tryptamine. The final reaction volume and substrate concentrations were as stated above. Incubations were begun immediately thereafter and continued for 20 to 30 min at 37°. The reaction was terminated by introducing 1.0 ml of water-saturated chloroform and mixing for 5 min. Under these conditions the recovery of the reaction product, *N*-[1-^{14}C]acetyltryptamine, was 98% as determined by using synthetic *N*-[1-^{14}C]acetyltryptamine. After centrifugation (Brinkmann centrifuge) at 20,000 × *g* for 0.5 min, the aqueous phase and protein interface were removed by aspiration, and the organic phase was washed once with 200 μl of the sodium phosphate buffer. A 500-μl sample of each chloroform extract was then dispensed into a scintillation vial and taken to dryness under a stream of air. The residual material was resuspended in 1.0 ml of 99% ethanol, and radioactivity was measured as described previously (3). Blank values were 60–100 dpm/ml of chloroform.

In some cases glands were sonicated at 0° in 100 μl of 0.01 N acetic acid, and a 25–50-μl sample was then immediately added to an assay tube. The volume of the assay and final concentration of the reagents were as described above. Enzyme activity was not affected by the preparation of samples using these methods.

cAMP assay. cAMP was assayed by a modification of the method of Gilman (17), which has been described elsewhere (18). Frozen pineal glands were ultrasonicated at 0° in 50 μl of 0.01 N acetic acid. A 25-μl sample of the sonic extract was then used to assay for *N*-acetyltransferase activity. The remainder was adjusted with 75% acetic acid to a final acetic acid concentration of 50%, and then diluted with 50% acetic acid so that a 20-μl sample of the diluted sample contained 3.3% or 33% of a single pineal gland. Two 20-μl samples of this preparation were taken to dryness overnight at 80° and used to measure cAMP. Under these conditions recovery of authentic cAMP added to the gland preparation was more than 93%.

Treatment of rats in vivo with isoproterenol and ouabain. Male Sprague-Dawley rats (Zivic-Miller Company, Allison Park, Pa.) were injected subcutaneously at 2-hr intervals with L-isoproterenol, ouabain, isoproterenol plus ouabain, or NaCl (0.1 ml). The animals were killed 4 hr after the first injection, and the pineal glands were immediately frozen as described above for the *N*-acetyltransferase assay.

In one experiment *N*-acetyltransferase activity was measured in pineal glands taken from a group of superior cervical ganglionectomized rats (Zivic-Miller) which had been similarly injected with isoproterenol, NaCl, ouabain, or ouabain plus isoproterenol.

Estimation of "protein synthesis." "Protein synthesis" was estimated by measuring the incorporation of L-[^{3}H]leucine into trichloracetic acid-precipitable material as previously described (19).

Electrophysiological Studies

Male Wistar rats (150 g) were killed, and their pineal glands were quickly removed. The capsule of the gland was split, and the gland was pinned by means of the capsule to the wax floor of a temperature-controlled (37°) superfusion chamber having a volume of approximately 0.5 ml. Warmed Krebs-Henseleit medium flowed through the chamber at 2 ml/min. The membrane potential of pineal parenchymal cells located superficially in the superfused pineal was measured with 3 M KCl-filled micro

electrodes with resistance greater than 50 Mohms. These microelectrodes were used to penetrate pineal parenchymal cells and to record the resting membrane potentials of these cells through a impedance-matched amplifier. The potentials were recorded on tape and by an ink-writing potentiometric recorder. While individual cell membrane potentials were being continuously recorded, the superfusion fluid was changed to Krebs-Henseleit medium containing various concentrations of drugs, either singly or in combination. Experiments on each gland were completed within 30 min. The general techniques have been described previously (13).

Thin-Layer Chromatographic Separation of L-[^3H]Tryptophan Metabolites

This was done according to a modification (15) of the method of Klein and Notides (20).

Source of Materials

The following chemicals were purchased: L-norepinephrine 1-bitartrate, Regis Chemical Company; theophylline, L-isoproterenol HCl, acetyl-CoA, and L-[^{3}H]tryptophan (1.1 Ci/mmole), Schwarz/Mann; N^6,$O^{2'}$-dibutyryladenosine cyclic 3',5'-monophosphate, Boehringer/Mannheim; [1-^{14}C]acetyl-CoA (50 mCi/mmole) and l-[4,5-^{3}H]leucine (41.2 Ci/mmole), New England Nuclear Corporation.

Ouabain (Calbiochem) was a gift of Dr. John Daly, National Institute of Arthritis, Metabolic, and Digestive Diseases.

RESULTS

Elevated K^+ concentration or ouabain blocks norepinephrine-induced increase in N-acetyltransferase activity and membrane hyperpolarization. Concentrations of KCl below 40 mM were without significant effect on the norepinephrine-induced increase in *N*-acetyltransferase activity (Fig. 1). At a KCl concentration of 80 mM, however, *N*-acetyltransferase activity in glands treated with 10 μM norepinephrine was reduced from about 7 nmoles/gland/hr to 0.26 nmole/gland/hr. Complete inhibition of the increase in enzyme activity was observed at 160 mM KCl. No significant inhibition of the norepinephrine-induced increase of *N*-acetyltransferase activity was observed in the presence of 80 mM choline chloride, indicating that the K^+ ion, not the chloride ion, was the active principle.

Ouabain (0.5–5 μm) abolished the norepinephrine-induced increase in *N*-acetyltransferase activity (Fig. 2).

The resting membrane potential of pineal parenchymal cells was found to be −32.4 mV. When these cells were exposed to norepinephrine in the superfusion fluid, there was an immediate hyperpolarization lasting for the duration of the norepinephrine exposure. Washing out norepinephrine caused a prompt recovery to the original resting potential (Fig. 3A). The addition of 1 μM ouabain to the superfusion fluid of pineal glands being treated with norepinephrine caused a slow return toward the original resting potential despite the continued presence of norepinephrine (Fig. 3B). Complete reversal of the norepinephrine-induced hyperpolarization required approximately 3 min. By contrast, when 1 μM ouabain was added to the superfu-

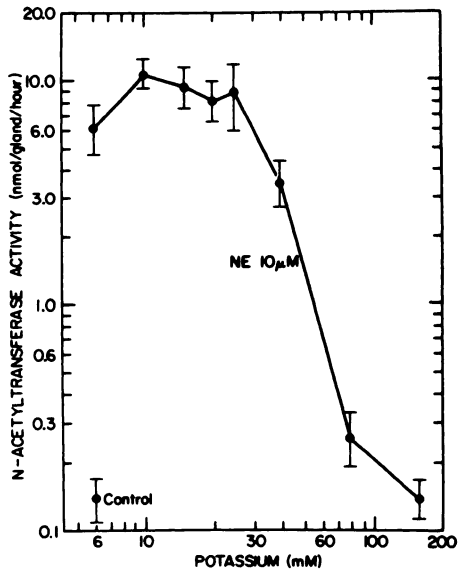

FIG. 1. *Effect of K^+ on norepinephrine (NE)-induced increase of pineal N-acetyltransferase activity*

The treatment period was 6 hr. Each point represents the mean and standard error of four to six determinations.

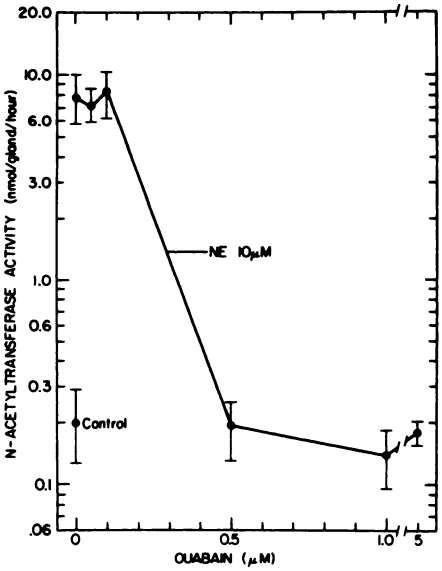

FIG. 2. *Effect of ouabain on norepinephrine (NE)-induced increase of pineal N-acetyltransferase activity*

The treatment period was 6 hr. Each point represents the mean and standard error of four to six determinations.

sion fluid in the absence of norepinephrine, no significant change in resting membrane potential was observed (Fig. 3E). Thus 1 μM ouabain, while not altering the resting potential itself, was able to reverse the hyperpolarization produced by norepinephrine. Several minutes after ouabain washout, residual effects of ouabian were apparent; norepinephrine responses at this time were still distinctly attenuated (Fig. 3D).

In other experiments it was found that a higher concentration of ouabain (10 μM) not only reversed the hyperpolarization produced by norepinephrine; but acutally shifted the membrane potential in the depolarizing direction at a rate of approximately 7.3 mV/min.

In studies in which superfusion was carried out with Krebs-Henseleit medium enriched in potassium (KCl replacing equimolar NaCl), it was observed that exposure of pineal glands to 40 and 80 mM K^+ resulted in a progressive decrease of the resting membrane potential (Table 1 and Fig. 4B). In the presence of a high K^+ concentration the effects of norepineph-

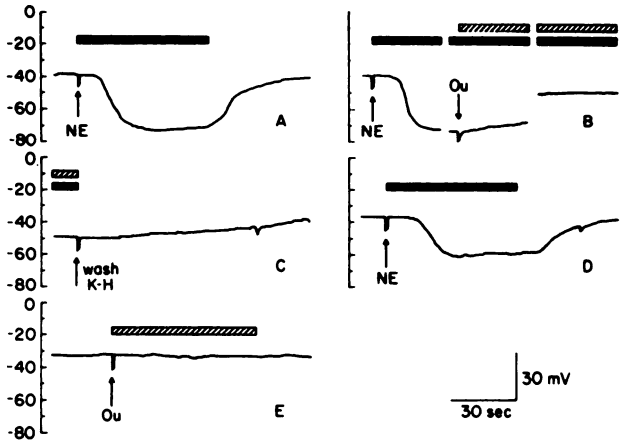

FIG. 3. *Continuous record of membrane potential recorded from a single pineal cell in the presence of norepinephrine (NE) (10 μM) and ouabain (Ou) (1 μM)*

A. Resting potential was −39 mV; at arrow norepinephrine superfusion was started, producing hyperpolarization of −33 mV. Duration of norepineprhine superfusion is indicated by heavy line above tracing. B. During norepinephrine superfusion (left panel), ouabain was introduced into the medium (center panel), producing an immediate change of membrane potential to less negative values. Three minutes later, with continued norepinephrine and ouabain superfusion (right panel), the membrane potential was stabilized at −50 mV in place of −73 mV with norepinephrine alone. C. Washout of norepinephrine and ouabain with normal Krebs-Henseleit (K-H) medium, showing return to approximately the original membrane potential. D. Retest with norepinephrine superfusion 4 min after washing out ouabain, showing residual attenuation of norepinephrine response (−24 mV). E. In another pineal cell, not previously treated with ouabain or norepinephrine, exposure to ouabain did not alter membrane potential.

rine were considerably altered. Not only was the magnitude of the hyperpolarization produced by norepinephrine diminished (Table 1), but the hyperpolarization was not maintained during the continued superfusion of norepinephrine for 1 min; the membrane potential rapidly reverted to values approaching or above the previous resting potential (Fig. 4C). In related studies we found that this reversal of the effect of norepinephrine was more pronounced in 80 mM K^+ than in 40 mM K^+. In addition, when norepinephrine was washed out, the resultant resting potential was always reduced (less negative) in comparison to its value prior to norepinephrine exposure (Fig. 4C).

Changes in norepinephrine responsiveness due to exposure to high K^+, in contrast to changes produced by ouabain exposure, were readily reversed by returning to normal K^+. Return to the initial resting membrane potential could be accelerated by an additional exposure to norepinephrine. Thus it appeared that norepinephrine facilitated the attainment of transmembrane voltage equilibrium, whether the change was from normal to high K^+ or in the reverse direction (Fig. 4E). These studies provide evidence that the norepinephrine-initiated hyperpolarization of the pinealocyte membrane can be antagonized by the same concentrations of drugs that block the norepinephrine-induced increase in the activity of pineal *N*-acetyltransferase.

TABLE 1

Effect of K^+ on resting membrane potential and on change in membrane potential due to norepinephrine treatment

Pineal glands were fixed in the chambers and were superfused with incubation medium containing the indicated concentrations of K^+. The resting membrane potential in pineal parenchymal cells was determined, and the superfusion medium was then switched to medium containing 10 μM norepinephrine. The K^+ concentration was kept constant. Data are the means and standard errors of four to nine determinations.

K^+ in medium	Resting membrane potential	Change in membrane potential due to 10 μM norepinephrine[a]	Membrane potential after treatment with 10 μM norepinephrine
mM	*mV*	*mV*	*mV*
5.8 (control)	−34.8 ± 1.6	−26.3 ± 1.7	−61.1 ± 3.0
40	−21.3 ± 2.7[b]	−11.0 ± 3.6[b]	−32.3 ± 5.1[b]
80	−19.6 ± 2.3[b]	−7.6 ± 1.6[b]	−27.3 ± 3.7[b]

[a] The change in membrane potential refers to the difference between the membrane potential recorded before norepinephrine treatment and that recorded during the period of maximum hyperpolarization after switching to a norepinephrine-containing medium.

[b] Significantly different from control value ($p < 0.01$).

Elevated K^+ concentration or ouabain inhibits DBcAMP- or theophylline-induced increases in N-acetyltransferase activity. Treatment with DBcAMP (1.0 mM) resulted in an increase in *N*-acetyltransferase activity to 6.0 nmoles/gland/hr (Fig. 5). Treatment with 80 mM K^+ reduced this increase to 0.5 nmole/gland/hr. A similar reduction in the increase in activity was observed in the presence of 1 μM ouabain. Treatment with 5 μM ouabain completely abolished the DBcAMP-induced increase in *N*-acetyltransferase activity.

Theophylline (10 mM) increased *N*-acetyltransferase activity to 1.9 nmoles/gland/hr. It was far less effective than norepinephrine (10 μM), a result which is consistent with earlier findings (9). The theophylline-induced increase was abolished by 80 mM K^+ and was strongly inhibited by 1 μM ouabain, which reduced *N*-acetyltransferase activity in the theophylline-treated glands to 0.15 nmoles/gland/hr (Fig. 6).

Norepinephrine-induced increase in cAMP concentration is not blocked by depolarizing agents. The inclusion of 80 mM K^+ or 1 μM ouabain in the culture medium reduced the norepinephrine-induced 6-hr increase in *N*-acetyltransferase activity from 5.8 nmoles/gland/hr to 0.5 and 0.58 nmoles/gland/hr, respectively. In contrast, neither K^+ nor ouabain inhibited the norepinephrine-stimulated increase in the amount of cAMP in the glands. This was measured after 20 min of treatment, a time when the norepineph-

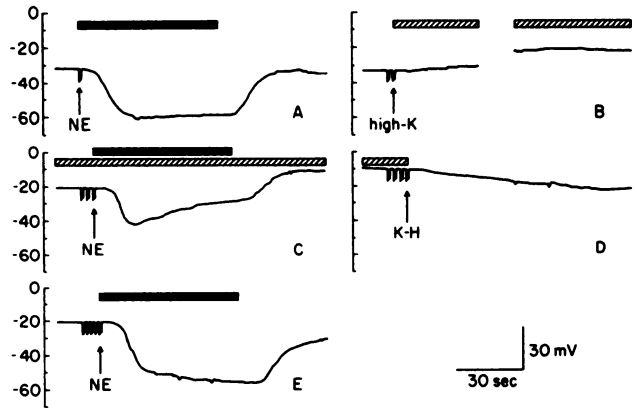

FIG. 4. *Continuous record of membrane potential recorded from a single pineal cell in the presence of 10 μM norepinephrine (NE) and 40 mM K^+.*

A. Resting potential was −31.5 mV. Hyperpolarization was produced by superfusion with norepinephrine. Duration of superfusion is indicated by heavy line above tracing. B. Introduction of 40 mM K^+ (high-K) medium, left panel; resting potential (−20.5 mV) at equilibrium 3 min later, right panel. C. Superfusion with norepinephrine in 40 mM K^+ medium, showing reduced hyperpolarization response followed by immediate reversal. When norepinephrine was washed out, the membrane potential shifted to a value approximately 10 mV less negative than the previous equilibrium value. D. Washout of 40 mM K^+ with Krebs-Henseleit (K-H) medium did not restore membrane potential to the original level seen in A. E. Re-exposure to norepinephrine, showing normal rapid hyperpolarization followed by a slow phase of increasing negativity. Subsequent washout of norepinephrine then restored the original equilibrium membrane potential as seen in A.

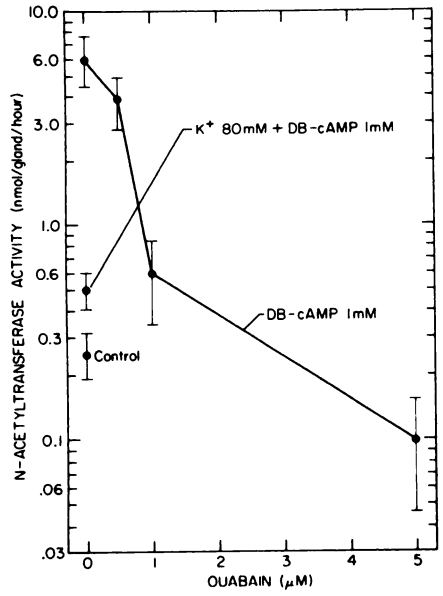

FIG. 5. *Effect of K^+ or ouabain on DBcAMP-induced increased in pineal N-acetyltransferase activity*

The treatment period was 6 hr. Each point represents the mean and standard error of six determinations.

rine-stimulated increase in cAMP concentration is maximal (10). At a concentration of 160 mM, K^+ completely blocked *N*-acetyltransferase activity but did not decrease the amount of cAMP in the norepinephrine-treated glands.

In a separate experiment, cAMP content was examined 15, 120, and 180 min after the addition of norepinephrine (10 μM). Ouabain (1 μM) again strongly inhibited the norepinephrine-induced rise in *N*-acetyltransferase activity, reducing the 3-hr value from 3.3 nmoles/gland/hr to 0.5 nmoles/gland/hr. The preceding rise in cAMP, however, remained unchanged. In the presence of norepinephrine alone or with ouabain, the cAMP content reached a mean value of about 275 pmoles/gland after 15 min (Fig. 7a and b). The rate of disappearance of cAMP was also unaffected by ouabain. Cyclic AMP content (50 pmoles/gland) was similar in both cases after 2 hr, and close to control values after 3 hr. Ouabain alone was without effect on either *N*-acetyltransferase activity or cAMP content.

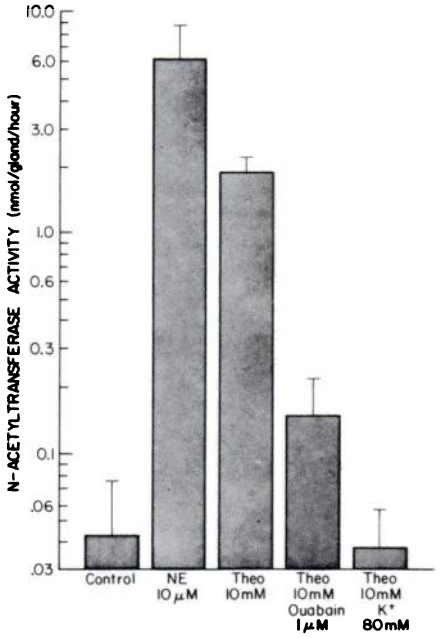

FIG. 6. *Effect of K^+ or ouabain on theophylline (Theo)-induced increase in pineal N-acetyltransferase activity*

The treatment period was 6 hr. Each point represents the mean and standard error of four determinations. NE, norepinephrine.

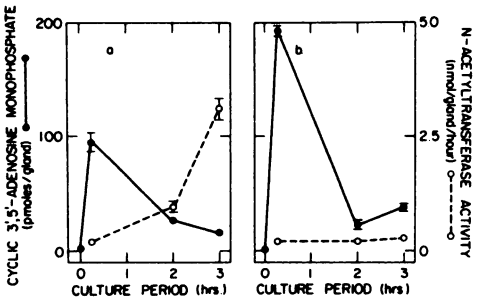

FIG. 7. *Pineal cAMP and N-acetyltransferase activity*

a. Time course of the effects of norepinephrine on pineal cAMP and *N*-acetyltransferase activity. b. Effect of ouabain on the time course of the norepinephrine-stimulated increase of pineal cAMP and *N*-acetyltransferase activity. Each point represents the mean and standard error of three determinations.

Although the norepinephrine-induced increase in *N*-acetyltransferase activity was abolished by 80 mM K^+, the norepinephrine-initiated increase in cAMP content after 15 min was doubled (Fig. 8a and b). K^+ alone was without effect on either *N*-acetyltransferase activity or cAMP content.

Pineal glands were also treated with K^+ for up to 2 hr before the addition of norepinephrine. In no case did the effect of norepinephrine treatment differ from that seen after only a 30-min prior treatment.

Cyclic AMP and DBcAMP cause hyperpolarization of pinealocyte membrane. Pineal glands were exposed to superfusion fluid containing either cAMP (0.5 mM) or DBcAMP (0.5 mM). Both compounds elicited a similar small but significant hyperpolarization of the pinealocyte membrane (Table 2).

Ouabain blocks isoproterenol-induced increase in N-acetyltransferase activity in vivo. Rats were treated with isoproterenol (20 mg/kg), a more potent adrenergic protagonist *in vivo* than is norepinephrine (7), or with isoproterenol plus ouabain (0.1–5.0 mg/kg) (Table 3). Ouabain strongly inhibited the isoproterenol-induced increase in *N*-acetyltransferase activity in normal rats and in rats which had denervated pineal glands due to superior cervical ganglionectomy.

Effects of K^+ and ouabain on "protein synthesis." The norepinephrine- and DBcAMP-induced increase in *N*-acetyltransferase activity in pineal organ cultures is blocked (8, 9) if protein synthesis is

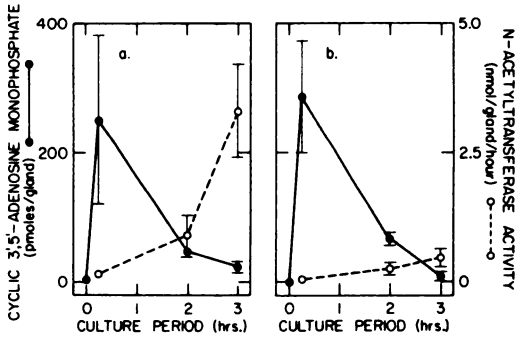

FIG. 8. *Pineal cAMP and N-acetyltransferase activity*

a. Time course of the effects of norepinephrine on pineal cAMP and *N*-acetyltransferase activity. b. Effect of 80 mM K^+ on the time course of the norepinephrine-stimulated increase of pineal cAMP and *N*-acetyltransferase activity. Each point represents the mean and standard error of three determinations.

TABLE 2

Effect of cAMP and DBcAMP on pineal cell membrane potential in explanted pineal glands

Pineal glands were fixed in perfusion chambers and were superfused with Krebs-Henseleit medium. The resting membrane potential in pineal parenchymal cells was determined, and the superfusion medium was then switched to one containing either DBcAMP (0.5 mM) or cAMP (0.5 mM). Membrane hyperpolarization occurred gradually over a period of 30–50 sec. The recording was terminated after 1 min. Data are the means and standard errors of four determinations.

Resting membrane potential	Membrane potential after treatment for 1 min with	
	cAMP	DBcAMP
mV	*mV*	*mV*
−36.1 ± 1.4		−42.5 ± 1.6[a]
−35.0 ± 1.1	−40.2 ± 1.7[a]	

[a] Significantly different from resting membrane potential ($p < 0.01$).

inhibited completely. At the concentrations which completely inhibited the norepinephrine- or DBcAMP-induced increase in *N*-acetyltransferase activity, however, "protein synthesis" was reduced no more than 25–40% by either K^+ or ouabain (Table 4). This suggests that a prime action of these blocking agents is not a rapid and nonspecific general inhibition of protein synthesis. This apparent inhibition of "protein synthesis" may have been due to a reduced rate of uptake of amino acids or might reflect specific inhibition of the production of only some proteins. The experiments *in vivo* strongly suggest, however, that ouabain can block the increase in *N*-acetyltransferase activity without causing massive nonspecific cell death, because the animals did survive the treatment in apparent good health.

Effects of ouabain or elevated K^+ concentration on conversion of L-[^3H]tryptophan to [^3H]serotonin and metabolies of [^3H]serotonin. The metabolism of L-[^{3}H]tryptophan by the pineal gland was examined to assess the specificity of the effect of ouabain or K^+ (Table 5).

As previously reported (13), treatment with norepinephrine caused an increase in the amount of [^{3}H]*N*-acetylserotonin and [^{3}H]melatonin in the culture medium and in the glands incubated with L-[^{3}H]tryptophan. The effects of norepinephrine were blocked by the inclusion of 1 μM ouabain or 80 mM K^+. The amount of [^{3}H]serotonin present in the media from cultures of glands treated with K^+ alone was substantially increased, but in no other case was it significantly different from the control value. Treatment with norepinephrine reduced by more than 50% the amount of

TABLE 3

Effect of ouabain on increase in N-acetyltransferase activity in vivo induced by isoproterenol in normal and superior cervical ganglionectomized rats

Each group in experiments 1, 2, and 3 consisted of four animals. Groups in experiment 4 consisted of three animals. Animals in experiments 1 and 3 weighed 150–180 g, and those in experiments 2 and 4 weighed 80–120 g. Drugs were injected in 0.1 ml of NaCl at 2-hr intervals. Animals were killed 4 hr after the first injection.

Treatment group	*N*-Acetyltransferase activity
	nmoles/gland/hr
A. Normal rats	
Experiment 1	
NaCl	0.10 ± 0.005[a]
Isoproterenol (20 mg/kg)	13.8 ± 1.02
Isoproterenol (20 mg/kg) + ouabain (0.1 mg/kg)	9.6 ± 1.0[a]
Experiment 2	
NaCl	0.35 ± 0.03[a]
Isoproterenol (20 mg/kg)	31.8 ± 4.6
Isoproterenol (20 mg/kg) + ouabain (1.0 mg/kg)	11.1 ± 0.68[a]
Ouabain (1.0 mg/kg)	0.35 ± 0.03[a]
Experiment 3	
NaCl	0.36 ± 0.01[a]
Isoproterenol (20 mg/kg)	13.7 ± 0.7
Isoproterenol (20 mg/kg) + ouabain (5.0 mg/kg)	4.7 ± 0.36[a]
Ouabain (5.0 mg/kg)	0.5 ± 0.02[a]
B. Superior cervical ganglionectomized rats	
Experiment 4	
NaCl	0.75 ± 0.25[a]
Isoproterenol (20 mg/kg)	34.9 ± 6.5
Isoproterenol (20 mg/kg) + ouabain (5.0 mg/kg)	10.2 ± 3.3[a]
Ouabain (5.0 mg/kg)	0.68 ± 0.14[a]

[a] Significantly less than the isoproterenol group ($p < 0.05$).

TABLE 4

Comparative effects of ouabain and K^+ on norepinephrine- and DBcAMP-induced increase of N-acetyltransferase activity and on "protein synthesis"

Pairs of pineal glands were incubated under control conditions for 48 hr and were then transfered for a 6-hr treatment period to media containing L-[3,4-^{3}H]leucine (5 μCi/ml, 3.1 μCi/μmole) and the indicated drugs. Norepinephrine (NE) and DBcAMP were added 30 min later in 1.2 mM or 0.12 mM concentrated solutions. Each value is based on six glands. "Protein synthesis" refers to the amount of radioactivity incorporated into trichloracetic acid-precipitable material by pineal glands incubated with L[3,4-^{3}H]leucine.

Expt.	Treatment (6 hr) Inducer	Blocker	*N*-Acetyltransferase activity		"Protein synthesis"	
			nmoles/gland/hr	%	*nCi/gland/6 hr*	%
1	NE (10 μM)	None	5.89 ± 1.57	100	13.7 ± 1.98	100
	NE (10 μM)	Ouabain (1 μM)	0.14 ± 0.54	2.4[a]	10.27 ± 2.31	75
	NE (10 μM)	K^+ (80 mM)	0.277 ± 0.63[a]	4.7[a]	9.67 ± 0.47	70
	None	None	0.196 ± 0.87[a]	3.3[a]	15.6 ± 2.6	114
2	DBcAMP (1 mM)	None	5.96 ± 1.57	100	19.7 ± 1.98	100
	DBcAMP (1 mM)	Ouabain (1 μM)	0.63 ± 0.25[a]	11[a]	11.3 ± 0.78[a]	58[a]
	DBcAMP (1 mM)	K^+ (80 mM)	0.50 ± 0.12[a]	8.4[a]	12.7 ± 0.63[a]	64[a]
	None	None	0.25 ± 0.65[a]	4.2[a]	16.0 ± 1.98	82

[a] Significantly lower than the induced group value ($p < 0.01$).

[^{3}H]serotonin in the pineal glands. The effect was partially blocked when norepinephrine was added in the presence of K^+, but was not affected by a simultaneous treatment with ouabain. Neither ouabain nor K^+ when added alone had any significant effect on the amount of [^{3}H]serotonin in the pineal glands.

Treatment of the glands with norepinephrine, either alone or in combination with K^+, significantly reduced the amount of [^{3}H]5-hydroxyindoleacetic acid in the culture media, but was without any significant effect on the amount in the gland. No other treatment had any significant effect on the amount of [^{3}H]5-hydroxyindoleacetic acid either in the glands or in the media.

In the first experiment the amount of [^{3}H]5-hydroxytryptophol in the pineal glands was reduced by treatment with norepinephrine, a reduction which was blocked by the simultaneous presence of K^+. This effect of norepinephrine was not seen in the second experiment, although treatment with ouabain alone increased the amount of [^{3}H]hydroxytryptophol in the culture media.

When the metabolism of L-[^{3}H]tryptophan was expressed as the sum of all the [^{3}H]metabolites present either in the glands or in the culture medium, it was found that the total conversion of L-[^{3}H]tryptophan was not reduced when pineal glands were treated with ouabain alone or K^+ alone, although it was reduced when glands were treated with either ouabain or K^+ in combination with norepinephrine. This may reflect a decreased uptake of L-[^{3}H]tryptophan in the combined presence of these agents with norepinephrine, resulting from probable ion shifts associated with the membrane potential changes which were produced only when ouabain or K^+ was added in combination with norepinephrine.

Sonic extracts of pineal glands treated with ouabain or K^+ do not reduce N-acetyltransferase activity in sonic extracts of pineal glands treated with norepinephrine alone. The blockade in the norepinephrine-induced increase in *N*-acetyltransferase activity caused by K^+ or ouabain might be due to ouabain- or K^+-stimulated production of an endogenous inhibitor of *N*-acetyltransferase activity.

However, when assayed in admixture with sonic extracts of pineal glands treated

TABLE 5

Quantitative analysis of metabolism of L-[^{3}H]tryptophan in cultured pineal glands treated with 1 μM ouabain or 80 mM K^+, either singly or in combination with 10 μM norepinephrine

Pineal glands (two per culture vessel) which had been cultured for 48 hr under control conditions were exposed to L-[^{3}H]tryptophan (0.2 mM, 144 μCi/μmole) for 4.5-hr test period. Ouabain (1 μM) and K^+ (80 mM) were present where indicated for the entire period; norepinephrine (NE) (10 μM) was present for the last 4 hr. At the end of the treatment period pineal glands were individually sonicated in 100 μl of carrier solution comprising 0.2 μmole each of *N*-acetylserotonin, melatonin, serotonin, 5-hydroxyindoleacetic acid, hydroxytryptophol, methyoxytryptophol, and methoxyindoleacetic acid. Portions of 20 μl (0.2 gland) of the sonic extracts were subjected to chromatographic analysis. A portion of each culture medium was mixed with an equal volume of the carrier solution, and 20 μl were chromatographed (15, 20). Results are the means and standard errors of six separate glands and three separate culture media in experiment 1 or five separate culture media in experiment 2. [^{3}H]Metabolites coincident with added standards but not shown in the table were not present in significant amounts.

Expt.	Treatment	Source of metabolites	L-[^{3}H]Tryptophan converted into						*N*-Acetyltransferase activity
			N-Acetylserotonin	Melatonin	Serotonin	5-Hydroxyindoleacetic acid	Hydroxytryptophol	Total [^{3}H]-metabolites	
			nCi	*nCi*	*nCi*	*nCi*	*nCi*	*nCi*	*nmoles/gland/hr*
1	None	Culture medium	0	2.76 ± 0.10	28.13 ± 2.58	18.39 ± 1.87	4.00 ± 0.37	53.28 ± 3.21	
	NE		4.17 ± 0.79[a]	11.49 ± 2.00[a]	21.87 ± 1.87	16.27 ± 2.44	5.03 ± 0.73	58.83 ± 3.82	
	NE + ouabain		0	5.50 ± 0.90[a]	22.66 ± 0.78	15.14 ± 1.50	4.72 ± 0.51	48.02 ± 1.98	
	Ouabain		0	4.37 ± 0.81	29.00 ± 5.98	23.42 ± 4.10	7.11 ± 1.32[a]	63.90 ± 7.41	
	None	Pineal glands	0.09 ± 0.02	0.16 ± 0.02	10.22 ± 2.33	3.77 ± 0.52	0.65 ± 0.12	14.89 ± 2.39	0.10 – 0.01
	NE		1.57 ± 0.25[b]	2.62 ± 0.94[b]	4.11 ± 0.57[b]	3.22 ± 0.36	0.49 ± 0.04	12.01 ± 1.18	8.66 ± 0.50[a]
	NE + ouabain		0.13 ± 0.03	0.25 ± 0.11	2.83 ± 0.82[b]	3.18 ± 1.02	0.52 ± 0.22	6.91 ± 1.33[b]	1.49 ± 0.50[a]
	Ouabain		0.07 ± 0.01	0.38 ± 0.09	7.91 ± 1.86	3.27 ± 0.38	0.58 ± 0.07	12.21 ± 1.90	0.25 ± 0.03
2	None	Culture medium	0	0.56 ± 0.13	12.94 ± 3.87	10.57 ± 1.16	4.20 ± 1.17	28.27 ± 4.21	
	NE		2.78 ± 1.01[a]	5.91 ± 0.65[a]	17.80 ± 1.26	7.88 ± 0.26[a]	2.42 ± 0.16	36.79 ± 1.77	
	NE + K^+		0	0.22 ± 0.08	9.43 ± 1.85	6.24 ± 0.48[a]	2.64 ± 0.47	18.53 ± 1.97[a]	
	K^+		0	0.80 ± 0.11	23.31 ± 4.06[a]	9.76 ± 2.48	3.27 ± 0.41	37.14 ± 4.78	
	None	Pineal glands	0.02 ± 0.01	0.05 ± 0.01	6.07 ± 0.78	2.06 ± 0.17	0.68 ± 0.08	8.88 ± 0.80	0.08 ± 0.01
	NE		1.37 ± 0.15	3.13 ± 0.23[b]	2.83 ± 0.77[b]	1.76 ± 0.25	0.31 ± 0.11[b]	9.40 ± 0.86	8.81 ± 0.29[a]
	NE + K^+		0	0.13 ± 0.06	3.72 ± 1.19	2.74 ± 0.40	0.68 ± 0.14	7.27 ± 1.26	0.32 ± 0.08[a]
	K^+		0.03 ± 0.02	0.08 ± 0.02	4.83 ± 0.61	2.14 ± 0.43	0.52 ± 0.14	7.60 ± 0.76	0.12 ± 0.02

[a] Significantly different from medium supporting untreated control glands ($p < 0.05$).

[b] Significantly different from untreated control glands ($p < 0.05$).

with norepinephrine (10 μM) alone, sonic extracts of glands which had been exposed to the combination norepinephrine plus ouabain (1 μM) or K^+ (80 mM) for 4 hr either failed to elicit any change in the resultant activity of the *N*-acetyltransferase [norepinephrine + (norepinephrine + ouabain)] or elicited only a very small decrease [norepinephrine + (norepinephrine + K^+)] (Table 6). Mixing the sonic extract of pineal glands treated with norepinephrine alone with the sonic extract of control glands which had received no treatment caused a 28.7% decrease in the resultant *N*-acetyltransferase activity. This degree of inhibition was not exceeded by substituting sonic extracts of the ouabain- or K^+ treated glands for the control glands.

The possibility of a direct effect of K^+ or ouabain on the *N*-acetyltransferase molecule was also examined. Neither of these agents, however, when added to a sonic extract of pineal glands previously cultured with norepinephrine, caused any decrease in *N*-acetyltransferase activity (Table 6).

These results suggest that the blockade of the norepinephrine-induced increase in *N*-acetyltransferase activity caused by K^+ or ouabain is not due either to the production of an endogenous inhibitor of the *N*-acetyltransferase activity or to a direct effect of these agents on the *N*-acetyltransferase molecule.

TABLE 6

Effect on N-acetyltransferase activity of mixing sonic extracts of pineal glands cultured with ouabain, K^+, or norepinephrine

Experiment 1: Cultured pineal glands which had been treated for 4 hr with norepinephrine (NE) (10 μM), ouabain (1 μM), K^+ (80 mM), norepinephrine + ouabain, norepinephrine + K^+, or nothing were individually sonicated in 65 μl of ice-cold 0.1 M sodium phosphate buffer, pH 6.8, containing 1.0 mM [1-^{14}C]acetyl-CoA. Sonic extracts of the glands from the same treatment groups were pooled, and duplicate 25-μl portions were assayed for *N*-acetyltransferase activity. Portions of 25 μl from each treatment group were then mixed with portions of 25 μl from the norepinephrine-treated group, and the combination was assayed for *N*-acetyltransferase activity. All assays were done in duplicate; duplicate values were within 8% of this mean.

Experiment 2: Pineal glands which had been cultured for 48 hr were treated for 4 hr with norepinephrine (10 μM). The glands were then individually sonicated in 40 μl of ice-cold 0.1 M sodium phosphate buffer, pH 6.8, containing 1.0 mM [1-^{14}C]acetyl-CoA. The sonic extracts were pooled, and 25-μl portions were assayed for *N*-acetyltransferase activity. Additions to the assay mixture were ouabain (1 μM) or K^+ (80 mM). All assays were done in duplicate.

Expt.	Group	Treatment	Mixture	*N*-Acetyltransferase activity
				nmoles/gland/hr
1	A	NE		13.63
	B	Ouabain		0.07
	C	K^+		0.08
	D	NE + ouabain		2.50
	E	NE + K^+		0.34
	F	None		0.07
			A + B	9.79
			A + C	11.84
			A + D	13.60
			A + E	12.70
			A + F	9.73
2			A	9.53
			A + ouabain	9.37
			A + K^+	11.95

DISCUSSION

These studies have shown that treatment with ouabain or with an elevated K^+ concentration can block the adrenergic stimulation of pineal *N*-acetyltransferase activity. There is little doubt that this effect is entirely a postsynaptic phenomenon, because the glands in the enzyme studies had been cultured for 48 hr prior to drug treatment. At this time the nerve endings, which are separated from their cell bodies located in the superior cervical ganglion (4), have lost the majority of their dense-cored vesicles (21) and the uptake of [^{3}H]norepinephrine by the pineal gland is reduced by 70%.[5] This suggests that the nerve endings, although morphologically preserved, are no longer functional, which precludes any presynaptic effects of the drugs.

Even under the conditions used for the study of the acutely explanted pineal gland, namely, those used for the measurement of membrane potential, it is clear

[5] G. R. Berg and D. C. Klein, unpublished observations.

that the observed effects of K^+ and of ouabain are essentially post synaptic. A presynaptic effect, leading to the release of norepinephrine, would be expected to produce hyperpolarization (13), whereas either no change in membrane potential (1 μM ouabain) or actual depolarization (80 mM K^+) was invariably observed. Even in the presence of added norepinephrine in the perfusion medium, both ouabain and a high K^+ concentration effectively reduced or reversed the postsynaptic effect of this amine.

While it is not possible to conclude from our results that the reversal of the norepinephrine-initiated hyperpolarization of the pineal cell membrane by K^+ or ouabain is involved in the way in which these agents block the norepinephrine-induced increase in *N*-acetyltransferase activity, this depolarizing action is a factor which is common to both and as such it must be considered as a possible mechanism of their action.

Treatment of pineal glands with 1 μM ouabain or 80 mM K^+, in combination with norepinephrine, did not result in a gross inhibition of protein synthesis. Treatment with K^+ alone almost doubled the conversion of L-[^{3}H]tryptophan to [^{3}H]serotonin, though treatment with ouabain alone was without effect. This suggests that the process of uptake and hydroxylation of L-[^{3}H]tryptophan, which in the culture pineal gland possesses a comparatively short half-life but does not show changes in activity in response to treatment of the gland with norepinephrine (22), is not depressed in the presence of ouabain or K^+ alone. The reduction of pineal gland [^{3}H]serotonin seen after treatment with norepinephrine is probably due to an enhanced conversion of [^{3}H]serotonin to [^{3}H]melatonin (9, 19). It is important to note the absence of any nonspecific depressant effects of either ouabain or K^+ on the general indole metabolism of the pineal gland. Implicit in this observation is the fact of the maintenance of numerous other biochemical pathways, such as those required for cofactor regeneration, in the presence of each of these agents. This suggests that ouabain and K^+ act relatively specifically in blocking the norepinephrine-induced increase in *N*-acetyltransferase activity, and are not generally toxic treatments.

The generation of cAMP in response to norepinephrine treatment was not affected by incubation of pineal glands with ouabain for periods of up to 2 hr. It was at all times, however, significantly increased following incubation with K^+, although K^+ itself did not stimulate the production of cAMP. It would appear that any alteration in the intracellular ionic composition or in the membrane fluxes of ions which are associated with norepinephrine-initiated hyperpolarization are not necessary for the norepinephrine-stimulated increase in cAMP in intact cells. This is in agreement with the finding of Weiss and Costa (23, 24) that pineal adenylate cyclase activity can be stimulated by norepinephrine treatment of broken cell preparations of pineal glands.

Several reports have appeared showing that treatment of neural tissue with depolarizing agents results in an increase in the concentration of cyclic nucleotides (25, 26). In our study no such increase was observed. This difference may be accounted for by the fact that the 48-hr cultured pineal gland responds as a purely post synaptic structure, whereas brain slices represent a functional collection of both presynaptic and postsynaptic cells. The effects of depolarizing agents seen in such heterogeneous preparations may thus reflect a complex result of the interaction of these agents with presynaptic and postsynaptic elements, a conclusion which is supported by the recent work of Holz, Deguchi, and Axelrod (27) using the acutely explanted pineal gland.

The observation that treatment with ouabain or with a high K^+ concentration also blocks the effects of theophylline and DBcAMP suggests to us that the blockade of adrenergic stimulation of *N*-acetyltransferase activity by ouabin or K^+ occurs because the effects of cAMP are blocked. One possibility is that the immediate next step in the sequence of events leading to the increase in *N*-acetyltransferase activity is simply a cAMP-caused hyperpolarization, and that this alone leads to an in-

crease in the activity of *N*-acetyltransferase. In support of the possibility that cAMP causes hyperpolarization is our finding that a brief treatment of the acutely explanted pineal gland with either cAMP or DBcAMP causes a small but significant hyperpolarization of the pineal cell membrane. This effect on membrane potential was smaller than that due to norepinephrine treatment. The quantitative disparity between these effects does not, however, necessarily eliminate the possibility that the norepinephrine-initiated hyperpolarization is mediated via cAMP. In our experiments cAMP was applied to the outer surface of the pineal cell membrane, whereas cAMP generated in response to norepinephrine stimulation might be expected to act at the inner membrane surface. The fact that DBcAMP produces such a small potential change, yet is as effective as norepinephrine with respect to its ability to increase the activity of the *N*-acetyltransferase in the intact pineal gland, suggests that hyperpolarization per se is not sufficient to cause the increase in enzyme activity. That some change in membrane potential or in the flux or distribution of ions is a necessary prerequisite for DBcAMP to induce an increase in the activity of the *N*-acetyltransferase is suggested by the observation that both ouabain and K^+ can prevent the increase in enzyme activity. It is also quite possible that the degree of hyperpolarization seen after treatment of the pineal gland with 10 μM norepinephrine is considerably in excess of that needed to bring about an increase in *N*-acetyltransferase activity.

Hyperpolarization of the pineal cell membrane may serve to establish a new ionic environment which is necessary for the mechanism(s) involved in this specific cAMP-induced gene expression. Alternatively, it appears possible that adrenergic stimulation results simultaneously in initiating two independent events, hyperpolarization and an increase in cAMP content. Both these events could be necessary for the increase in *N*-acetyltransferase activity to occur. Hyperpolarization or the associated change in ion concentrations could also result in an increased uptake or formation of a specific compound which is necessary for the stabilization of active *N*-acetyltransferase molecules; K^+ or ouabain could act on this process. The latter suggestion gains interest when one considers the findings that *N*-acetyltransferase activity rapidly disappears *in vivo* when animals kept in darkness for 4–6 hr are exposed to light ($t_{1/2}$ = 3 min) (28), in organ culture when induced pineal glands are treated with a high concentration of a *beta*-blocking agent (11), and in broken cell preparations of induced glands incubated at 37°. In the last case the disappearance can be prevented if the incubation system includes acetyl-CoA, cysteamine, or one of a number of cysteamine analogues[6] One of these compounds or a related compound may conceivably serve as an endogenous stabilizer of the *N*-acetyltransferase.

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