on these neurons) and increase the neuronal activity in brain areas innervated by raphe neurons (*22*). Higher doses of LSD decrease activity in these areas (*23*). These findings and the results of my study suggest that the decrease in raphe neuronal activity and the consequent release of inhibition in areas innervated by raphe neurons is responsible for the increase in punished behavior produced by LSD. This interpretation supports the evidence (*1–3*) for the role of neurons that contain serotonin in mediating the suppression of behavior by aversive stimuli.

The punishment-attenuating effect of LSD occurred at extremely low doses. Since this behavioral effect is a common feature of most clinically effective antianxiety drugs (*4*), in low (possibly nonhallucinogenic) doses, LSD and mescaline may also prove effective in treating anxiety.

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24. I thank M. A. Thomas, Sr., and J. Maak, Jr., for technical assistance and Dr. D. Frost for help in preparing the manuscript.

20 October 1975; revised 16 January 1976

Cholera Toxin Induces Pineal Enzymes in Culture

Abstract. *Addition of choleragen to rat pineal organ cultures caused a long-lasting stimulation of adenylate cyclase activity, and this was followed by increases in serotonin* N*-acetyltransferase and cyclic adenosine monophosphate phosphodiesterase activities. These effects of choleragen were not blocked by the β-adrenoceptor antagonist propranolol, but the increases in cyclic adenosine monophosphate phosphodiesterase and serotonin* N*-acetyltransferase activities could be prevented by the protein synthesis inhibitor cycloheximide. The results indicate that cholera toxin can mimic the induction of pineal enzymes that normally follows β-adrenoceptor activation and suggest that increased cyclic adenosine monophosphate is a necessary and sufficient signal for such changes in enzyme activity.*

The pineal hormone melatonin (5-methoxy-*N*-acetyltryptamine) (*1*) is synthesized from 5-hydroxytryptamine by the enzymes serotonin *N*-acetyltransferase (SNAT) (*2*) and hydroxyindole *O*-methyltransferase (*3*). The activities of these enzymes are regulated by the sympathetic innervation to the pineal, and they can be induced by addition of norepinephrine or other β-adrenoceptor agonists to pineals maintained in culture (*4*). The norepinephrine-stimulated increase in the activity of SNAT appears to be the key regulatory step in the formation of melatonin (*4*), and this response seems to involve a norepinephrine-stimulated adenylate cyclase and increased intracellular cyclic adenosine monophosphate (cyclic AMP) (*5*). In agreement with this hypothesis, high concentrations of the cyclic AMP analog dibutyryl cyclic AMP, or the phosphodiesterase inhibitor theophylline (*4*), can induce SNAT in cultured pineals. However, it has otherwise not been possible to separate the effects of adenylate cyclase activation from the effects of β-receptor stimulation. In the present experiments, we describe the use of cholera toxin (choleragen) as an alternative and novel tool for investigating the regulation of enzyme activities in the rat pineal organ. The only known mechanism of action of choleragen is an irreversible activation of adenylate cyclase in intact cells (*6*) following its binding to a cell-surface receptor, the ganglioside GM_1 (*7*). We find that choleragen activates adenylate cyclase and increases SNAT activity in pineal cultures. We also show that choleragen causes an increase in cyclic AMP phosphodiesterase (E.C. 3.1.4.17) in the pineal and that this results in a decrease in cellular cyclic AMP levels toward normal values, although adenylate cyclase remains maximally activated.

Pineal bodies were rapidly removed after decapitation of male Sprague-Dawley rats (150 to 200 g) and placed in a defined culture medium (see legend to Fig. 1). The pineals were maintained in culture for 12 hours to allow degeneration of adrenergic nerve endings and to stabilize enzyme levels and β-adrenoceptor sensitivity. They were then transferred to Krebs-Ringer solution (16 pineals in 2 ml) either with or without 50 μg of choleragen per milliliter and incubated at 37°C for 15 minutes, after which they were returned to culture for further periods of up to 24 hours. Choleragen had no effect if added directly to the culture medium, presumably because the high ganglioside content of the culture medium effectively neutralized the toxin. The pineals were removed from culture at various times, and enzyme activities or cyclic AMP content were determined (see legend to Fig. 1).

Adenylate cyclase activity was increased by more than threefold 1 hour after exposure to choleragen and showed no significant decline in activity for up to 24 hours after such exposure (Fig. 1A). The magnitude of this response and the irreversible activation are typical of the response to choleragen of adenylate cyclases in whole cells of many other types (*7*). The SNAT activity also increased after exposure to choleragen. This effect was significant after 4 hours and maximal after 6 hours; SNAT activity remained significantly elevated at 24 hours (Fig. 1B). Serotonin *N*-acetyltransferase in-

creased up to 20-fold above control values, with a lag period of a few hours; this is very similar to the increase of pineal SNAT observed after β-receptor activation in cultured pineals (*8*), or to the response of pineal SNAT after the administration of a β-receptor agonist in vivo (*9*).

Decreasing the concentration of choleragen in the incubation medium led to decreased responses in both adenylate cyclase and SNAT activities with half maximal responses in each being elicited at 0.2 to 1.0 μg of toxin per milliliter. Addition of the β-receptor blocker *l*-propranolol ($10^{-5}M$) to the incubation medium had no effect on the activation of either enzyme by choleragen (Table 1). Choleragen was still able to elicit a large increase in SNAT activity in pineal organs that had already been cultured for 60 hours to allow total degeneration of adrenergic nerve endings. This finding, in conjunction with the lack of effect of propranolol, indicates that the toxin exerts its actions exclusively through postsynaptic mechanisms. Thus the activation of adenylate cyclase through a mechanism not involving β-adrenoceptor activation can give the same metabolic response as that normally seen following such receptor activation, which indicates that adenylate cyclase activation, and not β-receptor occupation, is the signal for the induction of SNAT in the pineal.

Table 1. Effect of drugs on choleragen-induced stimulation of pineal enzymes in rat pineal organ in culture. Pineal organs were cultured for 12 hours and then incubated in oxygenated Krebs-Ringer either with ("Toxin") or without ("Control") 50 μg of choleragen per milliliter for 15 minutes (see legend to Fig. 1). Pineals were then returned to culture for an additional 6 hours. *l*-Propranolol ($10^{-5}M$) was added to control and toxin media during the 15-minute period of exposure to choleragen. In other experiments cycloheximide (50 μg/ml) was added to the culture medium of control and toxin-treated pineals for the last 6-hour period of culture. Each point is the mean of four determinations ± standard error of the mean.

Treatment		Adenylate cyclase (picomoles per 10 minutes per pineal)	SNAT activity (picomoles per 10 minutes per pineal)	Cyclic AMP phosphodiesterase (picomoles per 10 minutes per pineal)
Untreated	Control	59.4 ± 16.5	5.9 ± 2.5	542 ± 49.4
	Toxin	204.6 ± 36.3*	81.1 ± 19.1*	957 ± 97.5*
l-Propranolol ($10^{-5}M$)	Control	56.1 ± 13.2	5.0 ± 1.9	530 ± 65.8
	Toxin	201.3 ± 29.7*	78.5 ± 16.3*	1029 ± 118*
Cycloheximide (50 μg/ml)	Control	66.0 ± 16.5	4.8 ± 2.6	484 ± 52.4
	Toxin	198.0 ± 26.4*	5.5 ± 3.2	530 ± 60.8

*$P < .01$ when compared with corresponding control value.

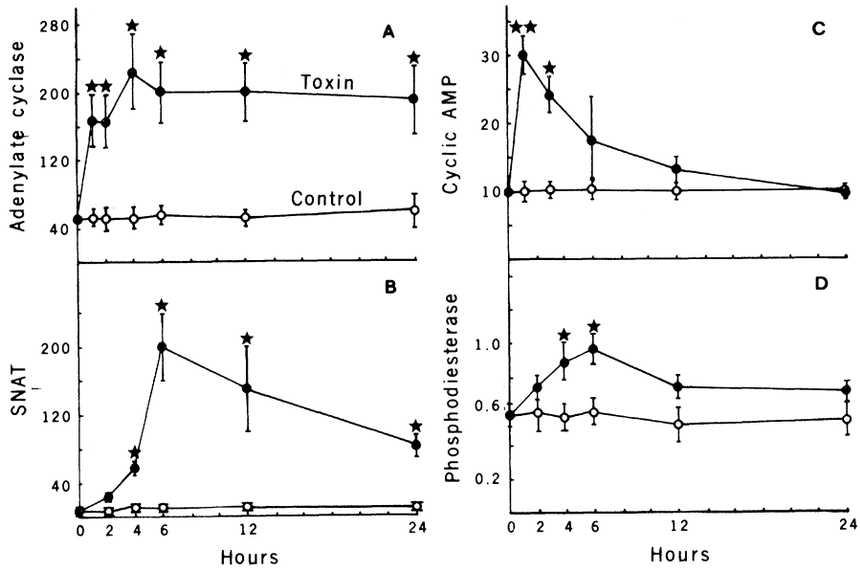

Fig. 1. Effect of choleragen on pineal enzymes and cyclic AMP content. Pineal organs were cultured on Millipore filter supports on an expanded metal grid (*4*) in 90 percent Eagle's minimal essential medium and 10 percent heat-inactivated bovine fetal serum containing 100 international units of penicillin and streptomycin per milliliter, 2 m*M* glutamine, and 25 m*M* glucose at 37°C in an atmosphere of 95 percent O_2 and 5 percent CO_2. After being cultured for 12 hours, pineals were transferred to oxygenated Krebs-Ringer bicarbonate solution either with (*Toxin*) or without (*Control*) 50 μg of choleragen per milliliter (*7*) and incubated at 37°C in a shaking water bath for 15 minutes. These organs were then returned to the culture system for up to 24 hours and were removed and frozen at various times after incubation with choleragen. Each pineal was homogenized in 100 μl of distilled water, and 20 μl of the homogenate was assayed for adenylate cyclase activity by the method of Miller *et al.* (*13*), 50 μl for SNAT activity by the method of Deguchi and Axelrod (*14*), and 3 μl for cyclic AMP phosphodiesterase activity by the method of Thompson and Appleman (*15*) using 1 μ*M* cyclic [^{3}H]AMP as substrate; values were corrected by the method of Lynch *et al.* (*15*). Pineal cyclic AMP content was determined in samples prepared by the method of Deguchi and Axelrod (*16*), and cyclic AMP was determined by the method of Brown *et al.* (*17*). (A) Adenylate cyclase activity (picomoles of cyclic AMP formed per pineal per 10 minutes); (B) SNAT activity (picomoles of *N*-acetyltryptamine formed per pineal per 10 minutes); (C) cyclic AMP content (picomoles per pineal); and (D) cyclic AMP phosphodiesterase activity (nanomoles of cyclic AMP hydrolyzed per pineal per 10 minutes). Each point is the mean of five determinations; vertical bars indicate ± standard error of the mean. ★$P < .05$, ★★$P < .01$ when compared with control values.

Addition of the protein synthesis inhibitor, cycloheximide, to the culture medium prevented the increase in SNAT after exposure to choleragen but did not affect the adenylate cyclase stimulation (Table 1). This indicates that SNAT is induced via adenylate cyclase through a mechanism dependent on protein synthesis.

Although adenylate cyclase was still maximally activated 24 hours after exposure to choleragen, SNAT activity was significantly reduced at 24 hours from its peak at 6 hours after such exposure. We, therefore, measured whole cell cyclic AMP levels and cyclic nucleotide phosphodiesterase levels after choleragen exposure. Whole cell cyclic AMP levels increased rapidly to 300 percent of control values after 1 hour but had returned to basal levels 6 hours after choleragen exposure (Fig. 1C). Cyclic AMP phosphodiesterase activity, assayed at 1 μ*M* substrate concentration, was significantly increased 4 hours after choleragen exposure and was maximally activated (170 percent of control) after 6 hours, thereafter declining to basal levels (Fig. 1D). The choleragen-induced increase in cyclic AMP phosphodiesterase was unaffected by propranolol but was dependent on protein synthesis, since it was blocked by the addition of cycloheximide to the culture medium (Table 1).

The finding that the decrease in the cellular content of cyclic AMP occurred at the same time as the increase in cyclic AMP phosphodiesterase activity, in a situation with a high but stable adenylate cyclase activity, suggests that regulation of cyclic AMP phosphodiesterase activity in the pineal body can exert an important control over cyclic AMP content. A β-receptor agonist given in vivo can elevate cyclic AMP phosphodiesterase in the pineal body with a time course and maximal response similar to that seen af-

ter addition of choleragen in culture (*10*). Since the evidence presented here and in previous studies suggests that cyclic AMP is the signal for SNAT induction in the pineal organ, it seems likely that changes in phosphodiesterase activity can play a key role in determining the magnitude of this and other cellular responses to β-receptor stimulation in the pineal body, by modifying the half-life of the intracellular mediator, cyclic AMP. Phosphodiesterase activity appears to be induced by high levels of cyclic AMP, and to decline when cyclic AMP levels decline, with a short lag period. These changes may play a part in the well-documented phenomena of sub- and supersensitivity in the pineal body (*11*). After periods of maximal receptor stimulation, phosphodiesterase activity will be increased, thereby decreasing the half-life of newly formed cyclic AMP; but after periods of receptor inactivity, phosphodiesterase activity will be low, thereby increasing the half-life of newly formed cyclic AMP. However, differences in [^{3}H]alprenolol binding to sub- and supersensitive pineals have recently been demonstrated (*12*), which suggests that there may be several mechanisms participating in these phenomena.

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17 November 1975; revised 31 December 1975

Phytohemagglutinin-Induced Lymphocyte Transformation in Humans Receiving Δ^9-Tetrahydrocannabinol

Abstract. *Eight otherwise healthy male chronic marijuana smokers were hospitalized for a period of 30 days. Initially they received placebo, then a sustained dose of 210 milligrams of Δ^9-tetrahydrocannabinol (Δ^9-THC) per day for 18 days, followed by placebo. Lymphocyte responses to phytohemagglutinin were examined during each of these periods. Neither the daily ingestion of marijuana extract containing 210 milligrams of Δ^9-THC for 18 days nor the history of chronic marijuana smoking had a depressive effect on the lymphocyte responses of these subjects to phytohemagglutinin.*

A controversy currently exists regarding the status of cell-mediated immunity in otherwise healthy marijuana smokers. Nahas *et al.* (*1*) reported that in vitro blastogenic responses to phytohemagglutinin (PHA) and allogeneic cells in chronic marijuana smokers were depressed by 40 percent, bringing them to levels seen in patients with cancer or uremia or transplant recipients with iatrogenic immunosuppression. Although this work has not been directly confirmed, Gupta *et al.* (*2*) found a 5 percent mean decrease in the rosette-forming capacity of lymphocytes from chronic marijuana smokers, supporting the concept of a T-cell defect. By contrast, Silverstein and Lessin (*3*) noted no difference between chronic marijuana users and normal controls as evaluated by in vivo response to skin sensitization with 2,4-dinitrochlorobenzene, a technique for determining the functional integrity of cell-mediated immunity. Most recently White and coworkers (*4*) have reported that they could find no significant difference between the blastogenic responses of lymphocytes from long-term marijuana smokers and matched control subjects in response to the mitogens PHA and pokeweed. This is in direct conflict with the earlier report by Nahas *et al.* (*1*), underscoring the need for further investigation of this subject. A recent report indicates that oral delta-9-tetrahydrocannabinol (Δ^9-THC) is an effective antiemetic in patients receiving cancer chemotherapy (*5*). The use of Δ^9-THC in these patients would be undesirable if it contributed significantly to immune suppression.

A major problem in interpretation of these studies has been the experimental variation introduced by the use of chronic marijuana smokers who smoke material of unknown potency at unspecified times in a nonregulated manner. The purpose of this study has been to compare the PHA-induced blastogenesis of normal human subjects and subjects receiving a known quantity of Δ^9-THC, the major psychoactive component of marijuana, at scheduled time intervals under carefully controlled hospital conditions.

Eight male volunteers between the ages of 21 and 30 were selected for this study. They were part of an extensive study of many marijuana effects. All were regular marijuana smokers (mean frequency of 13.5 marijuana cigarettes per week for an average duration of 3.5 years). They denied the regular use of drugs (other than alcohol and tobacco), particularly narcotics, barbiturates, and amphetamines. A complete medical history and physical examination confirmed their good mental and physical health. Subjects were asked to refrain from all drug usage for 1 week prior to admission to the Clinical Research Ward of Langley Porter Neuropsychiatric Institute in San Francisco, California. They were then admitted in groups of two and kept under constant observation for the 30 days of the hospitalization period.

Each subject received a capsule containing either placebo (0.2 ml of ethanol) or drug (a crude marijuana extract in 0.2 ml of ethanol, recently assayed for Δ^9-THC content) (*6*) every 4 hours during the entire hospitalization period. Placebo was given in a double blind situation to all subjects for the first 6 days, during which time baseline data were acquired. Then rapidly increasing doses of drug were given until a maximum of 210 mg of Δ^9-THC per day in divided 30-mg doses was reached. This dose level was maintained for 18 days, followed by an abrupt switch to placebo for the remaining 4 days of hospitalization. In addition to the oral doses, all subjects smoked a 1-g marijuana cigarette just before and on the last day of prolonged oral administration. Blood samples were drawn for lymphocyte cultures during the initial placebo period just before the drug was started, at the end of the period of prolonged drug administration, and at the end of the final placebo period. The control subjects were aged 21 to 50 years and were recruited from laboratory staff and friends. Most had never used cannabis and none had any in the previous 6 months. They all were in excellent health.

Blood samples were drawn with 10