The exact reasons for this rapid and durable "amnesia" of GS inducibility in monolayer-cultured retina cells are being explored; considering that synthesis of RNA and of enzyme protein are required for GS induction (*5, 6*) and that enzyme levels are subject to multiple types of regulation, changes at any of several control levels could be involved. A clue to the nature of this loss of inducibility is suggested by the finding that it can be largely prevented by suppressing metabolic activities in the dispersed cells. Freshly prepared cell suspensions in medium without the inducer were maintained at 4°C for 24 hours; the culture dishes were then transferred to 37°C, the inducer was added, and GS activity was determined after 24 hours. These cultures responded to the inducer by an increase in GS activity; controls maintained at 37°C in inducer-free medium did not respond to the subsequent addition of the inducer (Fig. 2).

The above finding suggests that the loss of GS inducibility in monolayered retina cells is due to durable metabolic changes that occur when the cells are maintained in a separated state. That the persistent separation of the cells from tissue-like association is a causal factor in initiating these changes is shown by their prevention when the cells are reaggregated immediately after their dissociation from the tissue and thereby enabled to restore histogenetic contacts. Other interpretations are conceivable at present; however, these experimental findings provide means for further analyzing the amnesia of GS inducibility in retina cells in the context of enzyme regulation in neural differentiation and specifically in relation to the role of cell contact in developmental processes (*1, 2, 8, 11*).

The requirement for multicellular organization in the hormonal induction of GS in the retina is consistent with other evidence that the increase of GS is a characteristic feature of differentiation in this tissue. This requirement distinguishes GS induction in the embryonic retina from inductions of adaptive enzymes in monolayer cultures of adult rat hepatoma cells which do not depend on histotypic organization (*12*). It should be recalled that the formation of various specialized intercellular junctions (synaptic and others) marks the development of the neural retina; it is therefore possible that, in the embryonic retina, the role of cell interactions in enzyme induction is especially prominent but that similar conditions apply in principle also in other developing systems to induction of enzymes associated with differentiation.

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Pineal Gland: Dibutyryl Cyclic Adenosine Monophosphate Stimulation of Labeled Melatonin Production

Abstract. *In organ cultures of intact rat pineal glands,* $N^6O^{2'}$*-dibutyryl adenosine 3′,5′-monophosphate stimulates the conversion of tritiated trytophan to tritiated melatonin, as does* L*-norepinephrine. Potential sites of stimulation of melatonin production by dibutyryl cyclic adenosine monophosphate are discussed, based on observations that the dibutyryl analog also stimulates the conversion of serotonin labeled with carbon-14 to carbon-14-labeled melatonin without altering hydroxyindole-*O*-methyl transferase activity or intracellular accumulation of serotonin labeled with carbon-14.*

Melatonin is synthesized from serotonin by *O*-methylation of the intermediate *N*-acetylserotonin (*1*). In mammals this conversion takes place only in the pineal gland, because of the presence of the unique enzyme hydroxyindole-*O*-methyl transferase (*2*). Norepinephrine stimulates the conversion of labeled tryptophan (*3*) and serotonin (*4*) to melatonin by pineal glands in two different organ culture systems. Weiss and Costa (*5*) reported that norepinephrine also stimulated the activity of adenyl cyclase in homogenates of pineal glands, implying that this monoamine may elevate the concentration of adenosine 3′,5′-monophosphate (cyclic AMP) in the gland. The role of cyclic AMP as an intermediate or "second messenger" in many hormone-target organ systems is becoming increasingly apparent (*6*), and we investigated whether the stimulatory effects of norepinephrine on melatonin synthesis in the pineal gland would be mimicked by cyclic AMP.

We tested the hypothesis that cyclic AMP may directly stimulate melatonin production using an organ culture system (*7*). Intact pineal glands were obtained from female Osborne-Mendel rats (150 to 170 g) and incubated for 24 hours in 0.5 ml of chemically defined media (*8*) supplemented with serum albumin (fraction V, Pentex, 1 mg/ml). Labeled melatonin formed from uniformly labeled [^{3}H]tryptophan or [^{14}C]serotonin (Amersham Searle) was extracted into 8.0 ml of chloroform from 0.05 to 0.10 ml of media added to 2 ml of 0.5*M* sodium borate buffer (*p*H 10). Chloroform extracts were washed once with 2 ml of this buffer and twice with 2.0 ml of 0.1*N* HCl or 1*N* HCl in the case of [^{14}C]serotonin incubations. Ninety to ninety-five percent of added melatonin was extracted by this procedure. Samples of the chloroform extracts were evaporated to dryness, and the radioactivity was measured in a liquid scintillation spectrometer. To verify the identity of the radioactive product in the chloroform extract, portions of all sample extractions within one treatment group were evaporated in an N_2

atmosphere, redissolved in a solution of alcohol and 1*N* HCl containing standard carriers (Regis Chemical), and analyzed by thin-layer chromatography (*9*). Seventy-five to ninety-five percent of the radioactivity extracted from the incubation media of pineal glands treated with dibutyryl cyclic AMP (2×10^{-4} to $8 \times 10^{-4} M$) chromatographed with authentic melatonin. The radioactivity extracted from the media of untreated and unstimulated glands was too small to allow analysis by thin-layer chromatography.

We examined the effects of L-norepinephrine (Regis Chemical Company), cyclic AMP, and dibutyryl cyclic AMP (potassium salt, Lot No. 840092; Calbiochem Corporation) on the conversion of [^{3}H]tryptophan (Table 1). Norepinephrine stimulates the conversion of [^{3}H]tryptophan to [^{3}H]melatonin (*3*). Cyclic AMP ($10^{-3} M$) had no influence on this conversion. However, dibutyryl cyclic AMP, a substance reported to have effects similar to cyclic AMP in certain tissues (*6*), produced a marked stimulation at concentrations greater than $2 \times 10^{-4} M$ (Table 1). The greater effect of dibutyryl cyclic AMP was probably a result of slower degradation by phosphodiesterase and of more rapid entry into the cell, relative to the parent compound, or a combination of these factors (*10*). The similar effects of norepinephrine and dibutyryl cyclic AMP on tryptophan conversion to melatonin, together with the known stimulatory effects of norepinephrine on pineal gland adenyl cyclase (*5*), is consistent with the idea that one mechanism by which norepinephrine may stimulate melatonin production involves elevation of tissue concentrations of cyclic AMP.

We next investigated the mechanism by which dibutyryl cyclic AMP stimulated the conversion of [^{3}H]tryptophan by studying whether stimulation in the metabolic pathway occurred before or after the synthesis of serotonin. Dibutyryl cyclic AMP stimulated the conversion of substrate concentrations ($10^{-4} M$) of [^{14}C]serotonin (Table 2), an indication that dibutyryl cyclic AMP, as L-norepinephrine (*4*), acts at a metabolic step after the synthesis of serotonin. Any mechanism which stimulated the endogenous formation of unlabeled serotonin would have been expected to decrease conversion of labeled exogenous serotonin to labeled melatonin by diluting the radioactive serotonin.

The amounts of [^{14}C]serotonin converted to [^{14}C]melatonin was less than the amount of [^{3}H]tryptophan converted to [^{3}H]melatonin. This would be anticipated from the experimental design. In studies with [^{14}C]serotonin in which the concentration of serotonin in the media was approximately $10^{-4} M$, there were two precursor sources available for acetylation. One source was endogenous serotonin synthesized continuously from tryptophan in the media and the other was [^{14}C]serotonin. Stimulation of either acetylation or *O*-methylation would have removed serotonin from both these sources, and melatonin of a lower specific activity than [^{14}C]-serotonin would be produced.

The alternate possibility was that dibutyryl cyclic AMP could have increased [^{14}C]melatonin production by increasing intracellular transport or accumulation of extracellular radiolabeled serotonin. This appeared unlikely because dibutyryl cyclic AMP did not alter [^{14}C]serotonin accumulation in glands after 24 hours of incubation (Table 2).

Hydroxyindole-*O*-methyl transferase (HIOMT) has been thought to be rate limiting in melatonin synthesis (*1*). When we assayed HIOMT activity (*11*), in glands after 24 hours of incubation, no effect of dibutyryl cyclic AMP was detected (Table 2) (*12*). In addition, neither dibutyryl cyclic AMP nor cyclic AMP altered the activity of HIOMT when added directly to homogenates of unincubated pineal glands, suggesting that these compounds did not directly activate HIOMT. These studies did not rule out the possibility that cellular HIOMT activity may be controlled by activators produced in response to dibutyryl cyclic AMP, and, in such a case, the effect of activators might be lost by the 1500-fold dilution of the crude homogenate prepared for HIOMT assay.

We found the activity of HIOMT in broken-cell homogenates of control and stimulated glands to be about 100 to 120 pmole of melatonin produced per hour. However, even in maximally stimulated glands the average approximated rate of melatonin production from [^{3}H]tryptophan or [^{14}C]serotonin did not appear to be greater than 25 pmole/hr. In unstimulated glands, this

Table 1. Effects of dibutyryl cyclic AMP, and L-norepinephrine on conversion of [^{3}H]tryptophan to labeled melatonin in rat pineal glands. The glands were incubated for 24 hours with [^{3}H]tryptophan (5.2 μc/ml, 26 μc/μm). One gland was incubated in 0.5 ml of media. Data on [^{3}H]melatonin are based on groups of three or four determinations. Statistical analysis was performed by Student's *t*-test.

Treatment	Concentration (M)	N	[^{3}H]Tryptophan converted to labeled melatonin (pmole)
Control		4	29 ± 36
L-Norepinephrine	1.0×10^{-5}	4	231 ± 40*
Cyclic AMP	1.0×10^{-3}	3	59 ± 48
Dibutyryl cyclic AMP	7.4×10^{-7}	4	73 ± 25
Dibutyryl cyclic AMP	3.1×10^{-6}	4	84 ± 28
Dibutyryl cyclic AMP	1.3×10^{-5}	3	117 ± 56
Dibutyryl cyclic AMP	5.0×10^{-5}	4	121 ± 25
Dibutyryl cyclic AMP	2.0×10^{-4}	4	544 ± 81†
Dibutyryl cyclic AMP	8.0×10^{-4}	4	620 ± 168*

* $P < .02$. † $P < .01$.

Table 2. Effects of dibutyryl cyclic AMP ($8 \times 10^{-4} M$) on conversion of [^{14}C]serotonin and [^{3}H]tryptophan to labeled melatonin, on HIOMT activity, and on content of [^{14}C]serotonin in pineal glands incubated for 24 hours. In groups 1 and 2, two glands were incubated in 0.5 ml of media with [^{3}H]tryptophan (7.8 μc/ml, 39 μc/μm). Data on [^{3}H]melatonin are based on groups of four determinations and HIOMT data on groups of eight individual determinations. In groups 3 and 4, single glands were incubated in 0.5 ml of media containing [^{14}C]serotonin (3.0 μc/ml, 56 μc/μm). The HIOMT activity is expressed as the picomoles of melatonin (-*O*-methyl-^{14}C) formed per hour per gland (*11*). Statistical analysis was performed by Student's *t*-test.

Group	N	[^{3}H]Tryptophan converted to [^{3}H]melatonin (pmole/gland)	HIOMT activity	[^{14}C]Serotonin converted to [^{14}C]melatonin (pmole/gland)	Total pineal gland [^{14}C]serotonin (ng/gland)
1. Control	8	88 ± 8.5	123 ± 18		
2. Dibutyryl cyclic AMP	8	693 ± 134.0*	104 ± 20		
3. Control	4			74 ± 20	32 ± 3.9
4. Dibutyryl cyclic AMP	4			220 ± 41*	32 ± 6.5

* $P < .01$.

rate was less than 3 pmole/hr. Although HIOMT has been thought to be the rate-limiting enzyme in melatonin synthesis (*1*), these observations suggest that dibutyryl cyclic AMP may control melatonin production in this experimental model through another mechanism. Dibutyryl cyclic AMP may primarily stimulate the acetylation of serotonin and control melatonin production through substrate availability for HIOMT.

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12. During review of this paper, Shein and Wurtman [*Science* **166**, 519 (1969)] reported that dibutyryl cyclic AMP increases the formation of [^{14}C]melatonin and [^{14}C]serotonin from [^{14}C]tryptophan. Our results agree with these because to increase synthesis of melatonin from tryptophan the synthesis of (the intermediate) serotonin may be increased. However, studies using the inhibitor of serotonin synthesis *p*-chlorophenylalanine (*p*CPA) seem to indicate that the effect of dibutyryl cyclic AMP in stimulating melatonin production is not dependent on prior elevation of serotonin production. We have found that whereas *p*CPA (1.0 m*M*) inhibits the effect of dibutyryl cyclic AMP on conversion of [^{3}H]tryptophan to [^{3}H]melatonin to 35 ± 20 percent of the normal stimulation, the effect of dibutyryl cyclic AMP on [^{14}C]serotonin conversion to melatonin is not reduced by *p*CPA, but is slightly enhanced (125 ± 20 percent). This suggests that a specific site of action of dibutyryl cyclic AMP involved in stimulating melatonin production is at a metabolic step that does not depend on the new synthesis of serotonin, and will take place when an exogenous source of serotonin is provided, as in the experiments presented here.

8 October 1969

Habituation and Dishabituation of the Gill-Withdrawal Reflex in Aplysia

Abstract. *A behavioral reflex mediated by identified motor neurons in the abdominal ganglion of* Aplysia *undergoes two simple forms of short-term modification. When the gill-withdrawal reflex was repeatedly evoked by a tactile stimulus to the siphon or mantle shelf, the amplitude of the response showed marked decrement (habituation). After a period of rest the response showed spontaneous recovery. The amplitude of a habituated response was facilitated by the presentation of a strong tactile stimulus to another part of the animal (dishabituation). Many characteristics of habituation and dishabituation in* Aplysia *are similar to those in vertebrates.*

The analysis of the neural mechanisms of learning and similar behavioral modifications requires an animal whose behavior is modifiable and whose nervous system is accessible for cellular analysis. In this and the subsequent two papers (*1*, *2*) we have applied a combined behavioral and cellular neurophysiological approach to the marine mollusk *Aplysia* in order to study a behavioral reflex that undergoes habituation and dishabituation. We have progressively simplified the neural circuit of this behavior so that the action of individual neurons could be related to the total reflex. As a result, it is possible to analyze the locus and the mechanisms of these behavioral modifications. We now describe behavioral parameters of habituation and dishabituation of the gill-withdrawal reflex in *Aplysia*.

Habituation and dishabituation are simple behavioral modifications often considered to be the most elementary forms of learning (*3–5*). Habituation is the decrement of a behavioral response that occurs when an initially novel stimulus is repeatedly presented. Spontaneous recovery of the decremented response occurs if the stimulus is withheld for a period of time. Dishabituation, the restoration of a previously decremented response, occurs following a change in the stimulus pattern, such as the presentation of another, stronger stimulus (*4*).

Parametrically similar forms of short-term habituation, which last from several minutes to several hours, have been demonstrated for a variety of behavioral responses in all animals which have clearly developed central nervous sys-

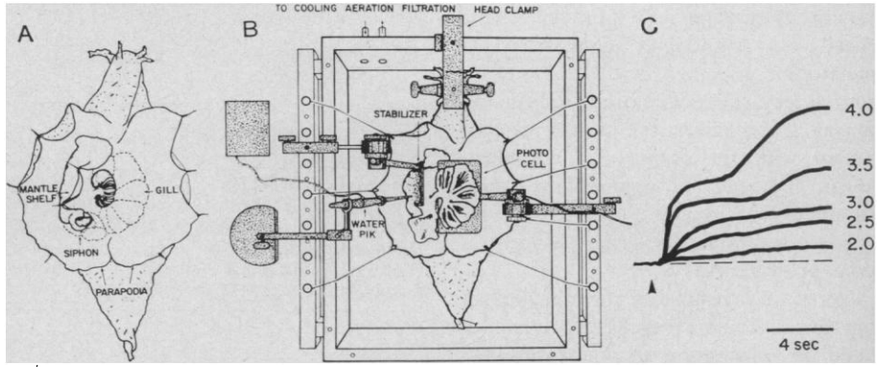

Fig. 1. (A) Dorsal view of an intact animal showing a fully contracted gill. Normally the parapodia and mantle shelf obscure the view of the gill, but they have been retracted to allow direct observation. The relaxed position of the gill is indicated by the broken lines. The tactile receptive field for the gill-withdrawal reflex includes the siphon and the edge of the mantle shelf. (B) The animal was immobilized in a small aquarium containing cooled and aerated circulating seawater. The edge of the mantle shelf was pinned to a substage, and a constant and quantifiable tactile stimulus consisting of a brief jet of seawater was delivered by a Water Pik (a commercially available oral hygiene apparatus). The stimuli were controlled by a Grass S-8 stimulator and were usually 800 msec. The gill contractions were monitored with a photocell placed under the gill. The output of the photocell was linearly related to the area uncovered as the gill contracted and was recorded on a polygraph. (C) Gill responses to individual tactile stimuli of different intensities. The stimuli were separated by very long intervals of time. The intensity of the stimulus could be adjusted anywhere from a very light touch (1.0, arbitrary units) to a very intense pressure (5.0). The weakest stimulus (2.0) evoked only a small gill contraction which consisted of a simple, short-latency withdrawal. Stronger stimuli (2.5, 3.0) evoked bigger and longer lasting gill responses of similar short latency but, if strong enough (3.5, 4.0), could bring in a second, longer latency component. The latency for this second component was quite variable, and with the strongest stimuli it sometimes merged with the first component.