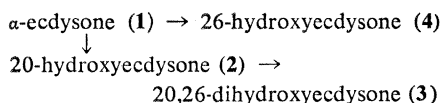ing this period of development. From the foregoing results, the biosynthetic–metabolic pathways for the molting hormones during this stage of embryonic development can best be expressed as follows:

α-ecdysone (1) $\rightarrow$ 26-hydroxyecdysone (4)
$\downarrow$
20-hydroxyecdysone (2) $\rightarrow$
                20,26-dihydroxyecdysone (3)

In the egg, α-ecdysone serves as the common precursor for both routes; but clearly the pathway to 26-hydroxyecdysone is the principal one as indicated by the strikingly large quantities of this hormone, whereas the conversion of α-ecdysone to 20-hydroxyecdysone is a minor pathway. However, during pupal-adult development α-ecdysone serves as a precursor for 20-hydroxyecdysone, which at this stage is the predominant molting hormone (4, 8). This has been substantiated in studies with the labeled ecdysone precursor 22,25-dideoxyecdysone which is efficiently converted to the three insect ecdysones (1, 2, 3) during both prepupal and pupal-adult development (5, 11). However, during larval development in the hornworm, this same ecdysone precursor is principally metabolized to a number of ecdysone analogs which lack the hydroxyl group at C-22 (6).

On the basis of the chemical and biochemical information on the molting hormones in the tobacco hornworm, then, different biosynthetic-metabolic pathways as well as quantitative and qualitative differences in the ecdysones occur in different developmental stages of this insect. Consequently, different ecdysones could function at different stages of insect development, and the qualitative nature of the molting hormones could well dictate the type of molt. If this is true, certain of the current concepts concerning the hormonal control of molting and metamorphosis in insects may require a reevaluation and revision.

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Octopamine-Sensitive Adenylate Cyclase: Evidence for a Biological Role of Octopamine in Nervous Tissue

Abstract. *An adenylate cyclase that is activated specifically by very low concentrations of octopamine has been identified both in homogenates and in intact cells of the thoracic ganglia of an insect nervous system. This enzyme appears to be distinct from two other adenylate cyclases present in the same tissue, which are activated by dopamine and by 5-hydroxytryptamine, respectively. The data raise the possibility of a role of octopamine-sensitive adenylate cyclase in the physiology of synaptic transmission.*

Recent studies have indicated that the actions of some neurotransmitters may be mediated through adenosine 3',5'-monophosphate (cyclic AMP). For example, evidence now suggests that cyclic AMP mediates dopaminergic transmission, thereby modulating cholinergic transmission, in mammalian sympathetic ganglia (1). Included in this evidence is the observation that low concentrations of dopamine increase ganglionic cyclic AMP through the

activation of a dopamine-sensitive adenylate cyclase (2). Evidence has also been presented that cyclic AMP may mediate the inhibitory effect of norepinephrine on Purkinje cells of the cerebellum (3), a tissue known to contain a norepinephrine-sensitive adenylate cyclase (4).

It has been suggested that octopamine (5), which is found in both vertebrate and invertebrate nervous systems, may, in some species, function as a
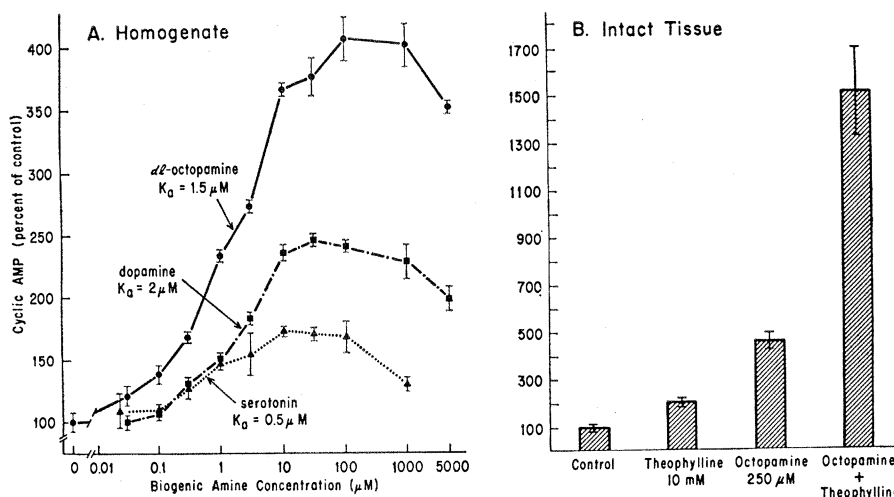

Fig. 1. Effect of octopamine on cyclic AMP accumulation in (A) homogenates and (B) intact tissue preparations of cockroach thoracic ganglia. (A) Effect of various concentrations of *dl*-octopamine, dopamine, and serotonin on adenylate cyclase activity in homogenates. The control activity, per milligram of protein, in the absence of added biogenic amine was 10.0 ± 1.5 pmole/min. (B) Effect of 250 μM *dl*-octopamine and 10 mM theophylline, alone and in combination, on the accumulation of cyclic AMP in intact hemiganglia. The control was 17.7 ± 2.7 pmole per milligram of protein. The values shown in both (A) and (B) are the means and ranges for two to three replicate samples, each assayed in duplicate.

neurotransmitter (6). Because no direct experimental evidence has been reported for such a function of octopamine, it seemed of interest to know whether an adenylate cyclase specifically sensitive to octopamine could be demonstrated in neural tissue. By analogy with the catecholamines, the existence of such an octopamine-sensitive adenylate cyclase could be considered to provide some experimental support for the possibility that octopamine could act as a neurotransmitter. In the following experiments we describe the identification of such an octopamine-sensitive adenylate cyclase in an insect nervous system. We have found that this enzyme is stimulated specifically by extremely low concentrations of octopamine, and that it appears to be distinct from two other adenylate cyclases present in the same tissue, which are activated by dopamine and by 5-hydroxytryptamine (serotonin), respectively.

For our studies, we used the thoracic ganglia of the cockroach, *Periplaneta americana*, since the enzymes necessary for octopamine synthesis are known to be present in this invertebrate (7) and the application of exogenous octopamine can lead to the activation of phosphorylase in this animal's ventral nerve cord (8). Specifically, we studied the effects of octopamine and other biogenic amines on the accumulation of cyclic AMP in tissue homogenates and intact preparations of these ganglia. Figure 1A shows the effect of *dl*-octopamine on the adenylate cyclase activity of ganglion homogenates (9). Stimulation of enzyme activity was observed at concentrations of octopamine as low as 0.03 μM. The concentration of octopamine necessary for half-maximum activation (K_a) was about 1.5 μM, and that for maximum stimulation (400 percent of control) was about 30 μM. In contrast, concentrations of octopamine as great as 250 μM had no effect on endogenous phosphodiesterase activity (10), indicating that the observed increases in cyclic AMP were due to stimulation of adenylate cyclase and not to inhibition of phosphodiesterase.

Dopamine, serotonin, and L-norepinephrine, substances known to occur in insect nervous tissue (11), were also tested for their effect on adenylate cyclase activity. Dopamine produced less stimulation than octopamine but had a low K_a (2 μM) (Fig. 1A). Serotonin also caused a comparatively small maximal stimulation of adenylate cyclase activity (170 percent of control at 25 μM), but it, too, was effective at

low concentrations (K_a, 0.5 μM). L-Norepinephrine (data not shown) was capable of stimulating enzyme activity to the same degree as octopamine, but much higher concentrations were required to produce this effect: half-maximum activation by norepinephrine occurred at about 30 μM. Neither dopamine, serotonin, nor norepinephrine affected phosphodiesterase activity (10).

The additive effects of octopamine, dopamine, and serotonin on adenylate cyclase activity are shown in Table 1. Various combinations of optimum concentrations of these three amines were fully additive in stimulating enzyme activity. These data suggest that the effects of octopamine cannot be accounted for by an activation of a dopamine or serotonin receptor, but, rather, that each of these three amines activates a separate and distinct receptor site. From additivity experiments similar to those shown in Table 1, we were unable to obtain similar evidence for a norepinephrine receptor, and it was concluded that the stimulatory effect of norepinephrine on adenylate cyclase activity was attributable to a partial activation

Table 1. Additive effects of *dl*-octopamine (OCT), dopamine (DA), and serotonin (5-HT) on the stimulation of adenylate cyclase activity in a homogenate of cockroach thoracic ganglia. For each amine, the concentration used was that which caused maximal increase in enzyme activity when added alone. The effects of the α-adrenergic antagonist, phentolamine (Phent), and the β-adrenergic antagonist, propranolol, on the adenylate cyclase activity of the same ganglion homogenate are also shown. All data have been expressed as the absolute increase in cyclic AMP formation above that (0.72 ± .01 pmole) observed in the absence of additions. The data represent the mean values and ranges for two to three replicate samples, each assayed in duplicate. The results are similar to those obtained in several other experiments.

Addition	Cyclic AMP increase (pmole)
Additivity	
OCT, 100 μM	2.24 ± .13
DA, 50 μM	1.22 ± .01
5-HT, 25 μM	0.51 ± .05
OCT, 100 μM + DA, 50 μM	3.52 ± .16
OCT, 100 μM + 5-HT, 25 μM	2.91 ± .19
DA, 50 μM + 5-HT, 25 μM	1.81 ± .16
OCT, 100 μM + DA, 50 μM + 5-HT, 25 μM	4.05 ± .09
Adrenergic blockers	
OCT, 2 μM	1.19 ± .14
OCT, 2 μM + Phent, 2 μM	0.30 ± .06
OCT, 2 μM + Phent, 10 μM	− 0.10 ± .05
OCT, 2 μM + propranolol, 10 μM	1.11 ± .05
Phent, 2 μM	− 0.08 ± .03
Phent, 10 μM	− 0.11 ± .06
Propranolol, 10 μM	− 0.04 ± .04

of the receptor sites for octopamine, dopamine, and serotonin. The fact that the K_a for norepinephrine (30 μM) was much higher than that for any of the three other amines (Fig. 1A) is consistent with this interpretation.

Phentolamine, an α-adrenergic antagonist, strongly inhibited the activation of adenylate cyclase by octopamine, whereas propranolol, a β-adrenergic antagonist, had little effect on the octopamine-stimulated increase in enzyme activity (Table 1). Phentolamine was also much more effective than propranolol in blocking the stimulation of adenylate cyclase by norepinephrine and by dopamine. Neither phentolamine nor propranolol was very effective in blocking serotonin stimulation.

Tyramine (*p*-hydroxyphenylethylamine), phenylethylamine, and phenylethanolamine, three compounds structurally related to octopamine which have been found in various tissues (12), were tested for their effect on the adenylate cyclase activity of ganglion homogenates. All three amines failed to stimulate enzyme activity, except at very high concentrations. The K_a for tyramine was greater than 40 μM, and that for phenylethylamine and phenylethanolamine, greater than 200 μM. Carbachol, γ-aminobutyric acid (GABA), L-glutamate, and histamine had no effect on adenylate cyclase activity in ganglion homogenates, when tested at concentrations as high as 250 μM.

In addition to measuring the effect of octopamine on adenylate cyclase activity in tissue homogenates, we also investigated the effect of octopamine on the accumulation of cyclic AMP in intact hemiganglia (13). In preparations incubated in insect Ringer solution, octopamine caused a 4.5-fold increase in cyclic AMP. In the presence of theophylline, a phosphodiesterase inhibitor, octopamine produced a 15-fold increase over controls incubated in insect Ringer solution and a 7-fold increase over ganglia incubated in the presence of theophylline. In other experiments with intact hemiganglia, it was found that 250 μM dopamine plus 10 mM theophylline caused a 3.2-fold increase, and 250 μM serotonin plus 10 mM theophylline caused a 2.2-fold increase in cyclic AMP, compared with controls incubated in the presence of theophylline alone.

Our results demonstrate the occurrence in nature of an octopamine-sensitive adenylate cyclase (14). It is known from other work that the conversion of tyrosine to tyramine and the

conversion of tyramine to octopamine are stimulated by extracts from cockroach ganglia and hemolymph (7), and that octopamine itself is present in high concentrations in the thoracic nerve cord of the lobster (6). Unfortunately, the absolute levels of octopamine are not known for the thoracic ganglia of the cockroach. In addition, although it has been suggested that octopamine might function as a primary neurotransmitter in those invertebrate species in which it is found in high concentrations (6), there have been as yet no reported investigations of possible electrophysiological effects of octopamine on invertebrate nerve cells. It is known, however, that norepinephrine and dopamine, both of which increased ganglionic cyclic AMP in our experiments, are excitatory when applied to neurons of the cockroach abdominal ganglia (15). Because octopamine also increased cyclic AMP in intact ganglia, it will be important to determine whether this phenolic amine has any effects on the electrical activity of nerve cells in this or similar ganglia. In this regard, it is of interest that octopamine-stimulated adenylate cyclase activity, in our experiments, was 2.2-fold higher in cockroach thoracic ganglia themselves than in the interganglionic nerve connectives (data not shown), suggesting that the enzyme may be localized more in nerve cell bodies and synaptic areas than in axons (of which the connectives are composed).

It has been shown (8) that octopamine can activate phosphorylase in cockroach nerve cord, and it was suggested that this glycogenolytic effect of octopamine in insects might be mediated through an increase of cyclic AMP. If the activation of phosphorylase by octopamine in the cockroach is, in fact, mediated through an increase in cyclic AMP, a possibility supported by our results, this would not rule out a possible role (6) of octopamine as a neurotransmitter. Indeed, our demonstration in invertebrate ganglia of an adenylate cyclase specifically sensitive to low concentrations of octopamine provides a possible mechanism by which this phenolic amine could be involved both in the regulation of carbohydrate metabolism as well as in the physiology of synaptic transmission.

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J. D. M. Albano [*Advan. Cyclic Nucleotide Res.* **2**, 25 (1972)]. Under the experimental conditions used, enzyme activity was linear with respect to time and enzyme concentration.
10. For measurement of phosphodiesterase activity, assay conditions were identical to those described above (9), except that 3 pmole of cyclic AMP was used in place of ATP, theophylline was sometimes omitted, and the incubation time varied from 1 to 15 minutes. Under these conditions, control activity (rate of disappearance of added cyclic AMP) per milligram of protein was 15 ± 1 pmole/min. Activity in the presence of either 250 μM dl-octopamine, dopamine, serotonin, or L-norepinephrine was not significantly different from control.
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13. To measure the accumulation of cyclic AMP in intact tissue, ganglia were hemisected into left and right halves and given preliminary incubation in oxygenated insect Ringer's for 20 minutes at 23°C. Then 8 to 12 half-ganglia were transferred to each incubation tube, which contained 1.0 ml of insect Ringer solution (9) and the appropriate test agent; the tubes were incubated for 10 minutes at 23°C with continuous oxygenation; the insect Ringer's was then aspirated, and 0.65 ml of cold 98 percent ethanol–0.2N HCl was added, and the tissue was immediately homogenized. The homogenate was centrifuged, the supernatant was removed and evaporated to dryness, and cyclic AMP was assayed (9). The precipitate was dissolved in 0.8 ml of 1.1N NaOH and assayed for protein [O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951)] with bovine serum albumin as the standard.
14. The additivity studies (Table 1), dose response relationships (Fig. 1A), and use of homogenates make it most unlikely that the observed effects of octopamine on adenylate cyclase activity were due to release of endogenous dopamine, norepinephrine, or serotonin from stores within the thoracic ganglia.
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Activation of Hemoglobin C Synthesis in Sheep Marrow Culture

Abstract. *Erythropoietin preferentially stimulates hemoglobin C synthesis in suspension cultures of marrow cells from sheep homozygous for hemoglobin A; the amount of synthesis is dependent on the dose of erythropoietin and is blocked by antiserum to erythropoietin. The results provide the first in vitro evidence that erythropoietin mediates the hemoglobin A → C "switch" in sheep and indicate that bone marrow cultures may be used to investigate the mechanisms involved in the preferential gene activation characteristic of the hemoglobin A → C system.*

Goats and sheep possess the unusual property of synthesizing a new hemoglobin under the influence of a variety of erythropoietic stimuli (1). Thus, when sheep homozygous for hemoglobin A (Hb A) are made anemic, their hemoglobin type switches to C (Hb C) because of the selective synthesis of βᶜ globin chains (2). No such alteration is seen in animals homozygous for hemoglobin B (Hb B). These alterations in hemoglobin synthesis, under the influence of external factors, are of particular interest in that they provide a model

for study of the mechanisms involved in differential gene activation in higher organisms. While several lines of in vivo study have provisionally identified the switching factor as erythropoietin (ESF), investigation into the mechanisms of the switch has been limited by the size of the animal and the length of time for the effect to be seen. We report that the hemoglobin A → C switch may be induced in vitro in suspension cultures of sheep marrow cells from animals with Hb A.

Five healthy and hematologically

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