ADENYL CYCLASE AND ITS RELATIONSHIP TO INSECT DIAPAUSE IN THE EUROPEAN CORN BORER, OSTRINIA NUBILALIS (HÜBNER)

by

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APPROVAL SHEET

Title of Dissertation: Adenyl Cyclase and Its Relationship to Insect Diapause in the European Corn Borer, Ostrinia Nubilalis (Hübner)

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ABSTRACT

Title of Dissertation: Adenyl Cyclase and Its Relationship to Insect Diapause in the European Corn Borer, Ostrinia Nubilalis (Hübner)

Dale B. Gelman, Doctor of Philosophy, 1978

Dissertation directed by: Dr. J. David Lockard
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The purpose of this study was to determine if there is a link between adenyl cyclase activity and the diapause condition in the European corn borer, Ostrinia nubilalis. Insects inhabiting those latitudes where cold and warm seasons alternate with one another have evolved mechanisms which allow them to remain dormant (in a state of diapause) during the winter months of the year. Photoperiod, as well as temperature and humidity, has been shown to control the onset, maintenance and termination of insect diapause. In recent years, evidence supporting a role for the cyclic AMP system, including adenyl cyclase, as well as a role for one or more biogenic amines in the pathway between light reception and the neuroendocrine regulation of the insect life cycle and in the multitude of neuroendocrine pathways controlling insect growth and metamorphosis has been accumulating. In light of this evidence, it was decided to investigate the effects of two light regimens, short day (diapausing-inducing) and long day (pupation-inducing), on adenyl cyclase activity of various stages

of fifth instar European corn borer larval heads, and to determine the effects of the biogenic amine neurotransmitters, norepinephrine, octopamine, and dopamine on this activity. Adenyl cyclase activity was measured by a modification of the method of Krishna, et al., (1968). A summary of the results follows.

In head extracts of fifth instar European corn borer larvae reared under both long day and short day photoperiodic regimens, adenyl cyclase activity in the presence of sodium fluoride increased as the larvae progressed through early, middle and mature stages. In long day larval heads, activity decreased in late prepupae and reached a low in pharate pupae. In contrast, adenyl cyclase activity in short day larval heads peaked in early diapause and then returned to prediapause levels during late diapause. Norepinephrine significantly enhanced adenyl cyclase activity only in early diapause larval head extracts, while octopamine significantly enhanced adenyl cyclase activity in head extracts of late short day mature and early diapause larvae. Dopamine was ineffective as an activator. An analysis of the combined effect of neurotransmitter and developmental stage revealed that in general, a given neurotransmitter in combination with short day larval head extracts resulted in higher adenyl cyclase levels than that neurotransmitter in combination with long day head extracts.

Based on these results, it appears that the cyclic AMP system is in some way involved in the initiation, maintenance, and termination of diapause in the European corn borer. While the exact nature of this involvement is open to speculation, several possible mechanisms for cyclic AMP's participation in the control of larval diapause are presented.

Suggestions for future research center around the determination of the circadian variations in adenyl cyclase levels in brains and corpora allata-cardiaca complexes in insects exposed to long day and short day photoperiodic regimens, and the determination of the effects of appropriate neurotransmitters, hormones, neurohormones and other peptides on this activity. Since the cyclic AMP system in diapausing European corn borers is functioning at a relatively high level, this system might provide a vulnerable point for attack against European corn borer infestation. Further, the assay used to measure adenyl cyclase activity may be useful in determining the effects of potential insect regulators on this system.

PREFACE

For any science course to meet the needs and interests of the student, it should make him aware of the following:

1. Science is a constantly changing body of knowledge.

2. Experimentation plays a vital role in the realm of science.

3. Careful observation and an eye for the unexpected are imperative in furthering scientific knowledge.

4. The scientific method is the backbone of scientific research.

5. Current science is based on past accomplishments.

6. One of the major goals of science is to benefit mankind.

The science educator, be he the science supervisor, curriculum specialist, or classroom teacher, must be equipped to insure that the appropriate learning experiences for meeting these objectives are provided. The study described herein has not only enabled the researcher to become more familiar with a vital area of biological research, namely that of the biochemistry of insect growth and metamorphosis, but also to experience the true nature of biological research as stated above.

The methodology and results of current research endeavors often serve as models for the student laboratory

exercises of the next decade. In the field of biology, the study of biological rhythms has received attention in undergraduate as well as in graduate classes (Halberg et al. 1972; Koukkari et al. 1974). Investigation of the nature and control of insect biological rhythms is, then, a very appropriate area of investigation for the science educator.

An added contribution to the field of science education lies in the significance of the results of this study in gaining a better understanding of the role of neurohormones and neurotransmitters in learning and memory. The literature is filled with reports of links between neurohormones, neurotransmitters and many types of behavioral modification (de Wied and Gispen 1977). And as Hyden (1969) points out, it is likely that the biochemical mechanisms which operate to control innate or instinctive behavior are similar to those controlling the more complex behaviors associated with learning and memory. Therefore, the knowledge gained from the research described herein may prove to be very useful in furthering our understanding of the neuroendocrine control of learning.

ACKNOWLEDGMENTS

To Drs. J. David Lockard and Dora K. Hayes, my major advisors, I wish to express my gratitude for their direction and support. From them, I received enough guidance and supervision to insure success, but not so much as to stifle creativity and dampen the independent spirit.

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This study was conducted under the auspices of Dr. Dora K. Hayes of the Chemical and Biophysical Control Laboratory, USDA, Beltsville, Maryland. Research facilities and support for the study were provided by the Department of Agriculture's Science and Education Administration, Washington, D. C. The European corn borers used in these investigations were provided by the USDA Corn Insect Laboratory, Ankeny, Iowa.

Finally, I wish to express my deepest gratitude to my husband, Hy, my son, Jay, my mother, Ida, my father, Ben, and to Virginia, without whose devotion, cooperation, and understanding this dissertation would not have been possible.

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CHAPTER I

INTRODUCTION

The European corn borer, Ostrinia nubilalis (Hübner), is an insect pest that attacks corn and other crops. The corn borer undergoes complete metamorphosis; eggs hatch to become voraciously hungry larvae. During the larval stage the corn borer generally undergoes four molts, thus passing through five larval stages or instars. Fifteen to thirty days after hatching, depending on environmental conditions, larvae undergo metamorphosis and change into pupae. It is during the pupal stage that the larvae become transformed into adults. Adults emerge from the pupal case after one to two weeks.

Insects inhabiting latitudes where cold and warm seasons alternate with one another have evolved mechanisms which allow them to remain active during the spring, summer, and fall, and dormant during the winter months of the year. Thus, insects are able to cope with rather unfavorable winter conditions and be active when the probability for their survival is greatest. Winter diapause or "hibernation" may occur in any stage of insect development, but each insect species will always diapause at a particular stage--either egg, larva, pupa or adult. It is the fifth instar corn borer larva that will enter diapause during the fall. Diapause is broken in the spring and pupal formation ensues. This is brought about by the increase in the number of hours of daylight and the rise in the ambient temperature

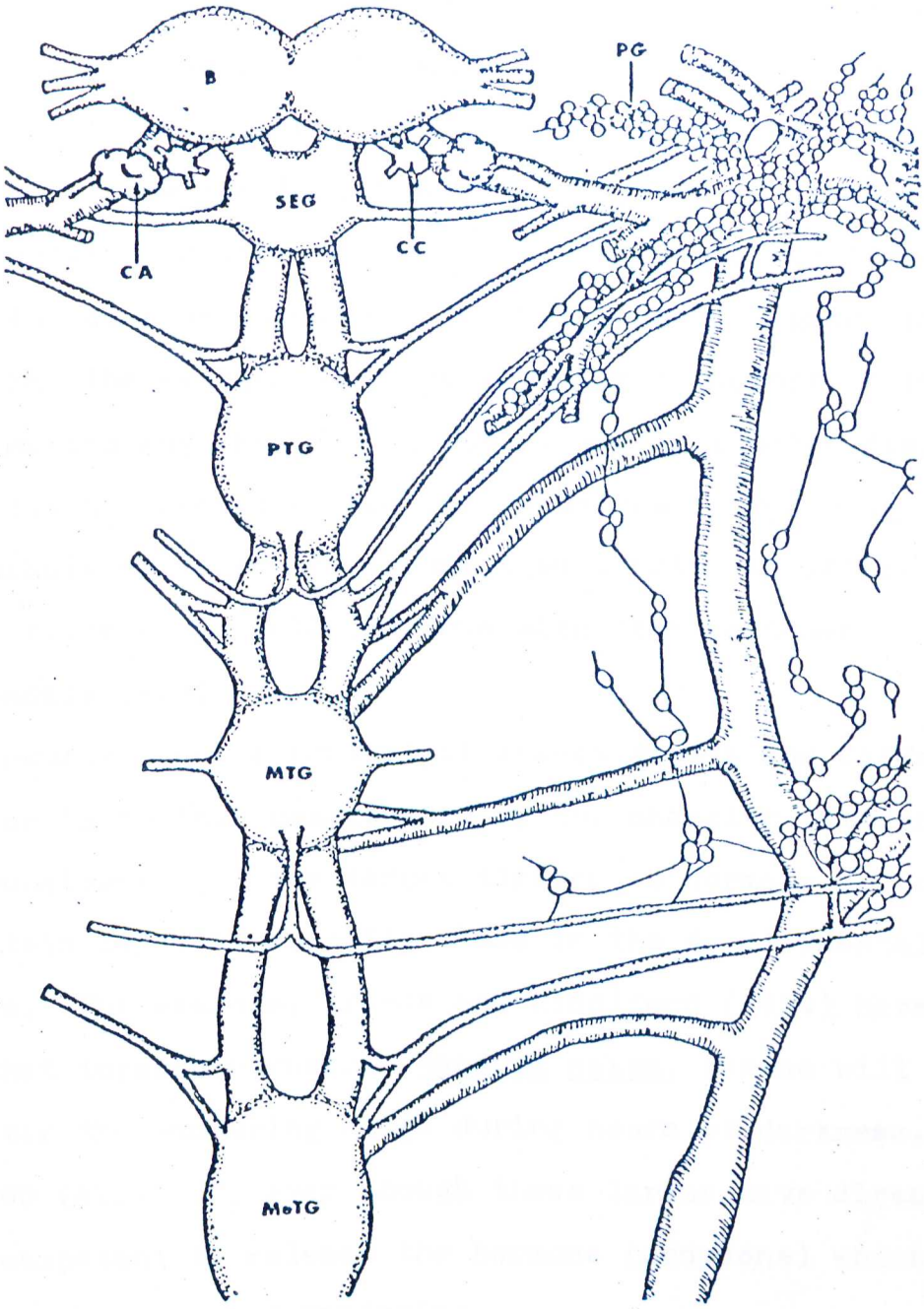
which induce the release of prothoracicotropic hormone (PTTH) from the brain. PTTH induces a chain of events which results in pupation. It is believed that a similar phenomenon must occur for the breaking of insect diapause. All the events which occur between light reception and neuroendocrine activation are not yet known. However, there is evidence to show that cyclic AMP and one or more biogenic amines may be involved.

According to Minis (1965), Kogure (1933) was the first to demonstrate that insect diapause was under the control of changes in day length, or photoperiod. Thirteen to fourteen hours or less of daylight, depending on the species, accompanied by temperatures of 25 degrees centigrade or below have been found to induce diapause in lepidopterous insects (moths and butterflies) (Lukefahr 1961; Adkisson 1965; Williams 1969). Since change in day length appears to be the environmental stimulus which triggers the onset of diapause as well as its termination (Adkisson 1965), insects must possess a photoreceptor for detecting light, a "biological clock" which keeps a record of the hours of illumination and/or darkness, and a mechanism for activating the endocrine system which controls insect growth and development accordingly. While a great deal of research has been directed towards elucidating the mechanisms involved in the initiation and termination of diapause, there are still a great many questions that remain to be answered.

These include: 1. the identity of the photoreceptor, 2. the mechanism by which the biological clock records hours of illumination, and 3. the mechanism by which the endocrine system is coupled to the "biological clock." This dissertation will concern itself with the third of these questions.

Figure 1 (from Herman and Gilbert (1966)) is a diagrammatic representation of the neuroendocrine structures which control insect diapause and metamorphosis. Williams (1969) purports that it is the brain of the oak silkworm, Antheraea pernyi, that is the receptor for photoperiodic signals. The brain interprets these signals, and when appropriate, produces PTTH which is stored in and released into the haemolymph (blood) from the corpora cardiaca (Highnam 1958; Scharrer 1952). This hormone in turn stimulates the prothoracic glands to produce molting hormone (Wigglesworth 1964; Vedeckis et al. 1974). The actual product of the prothoracic glands has been shown to be α-ecdysone (King et al. 1974; Chino et al. 1974). α-ecdysone is converted to β-ecdysone, the true molting hormone, by peripheral body tissues (Bollenbacher et al. 1975). It is β-ecdysone, then, that is believed to elicit those biochemical and morphological events commonly known as molting. In the presence of high titres of juvenile hormone, a product of the brain's corpora allata, a larval molt will occur. When titres of juvenile hormone are low, the larva will

Fig. 1. Diagrammatic representation of the anatomical interrelationships of the various structures of the insect neuroendocrine system (not to scale). B, brain; SEG, subesophageal ganglion; CA, corpus allatum; CC, corpus cardiacum; PTG, prothoracic ganglion; MTG, mesothoracic ganglion; MeTG, metathoracic ganglion; PG, prothoracic gland. (From Herman and Gilbert 1966).


B
PG
SEG
CA
CC
PTG
MTG
MoTG

metamorphosize into a pupa. In the absence of juvenile hormone, a pupal-adult molt will occur. Figure 2 (from Schneiderman and Gilbert (1959)) is a diagrammatic representation of how the insect endocrine system is involved in the growth and differentiation of lepidopterous insects.

Photoperiod appears to control insect diapause by regulating the synthesis, release and/or transport of PTTH (Williams and Adkisson 1964), for larvae will enter diapause when ecdysone production has been shut down. Recently, considerable evidence has accumulated linking an intermediate titre of juvenile hormone with this shutdown (Chippendale 1977).

Neuroendocrine control of insect growth and differentiation is further complicated by the changing pattern of responsiveness of the target tissues to hormone action (Bodenstein 1957) at specific times in the developmental schedule. For example, Truman and Riddiford (1974) have shown that tobacco hornworm, Manduca sexta, larvae will only enter the wandering stage during hours of darkness, 2400-1200 (A.Z.T.)[1], even though these larvae have already become competent to release the hormone (ecdysone) which initiates the onset of wandering.

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[1]A.Z.T. stands for arbitrary "zeitgeber" time where 'zeitgeber' refers to an environmental factor, in this case the onset of darkness, which is capable of synchronizing a circadian periodicity.

Fig. 2. Diagrammatic representation of the endocrine system's control of insect growth and metamorphosis. PTTH, prothoracicotropic hormone; MH, molting hormone; JH, juvenile hormone. (From Schneiderman and Gilbert 1964).

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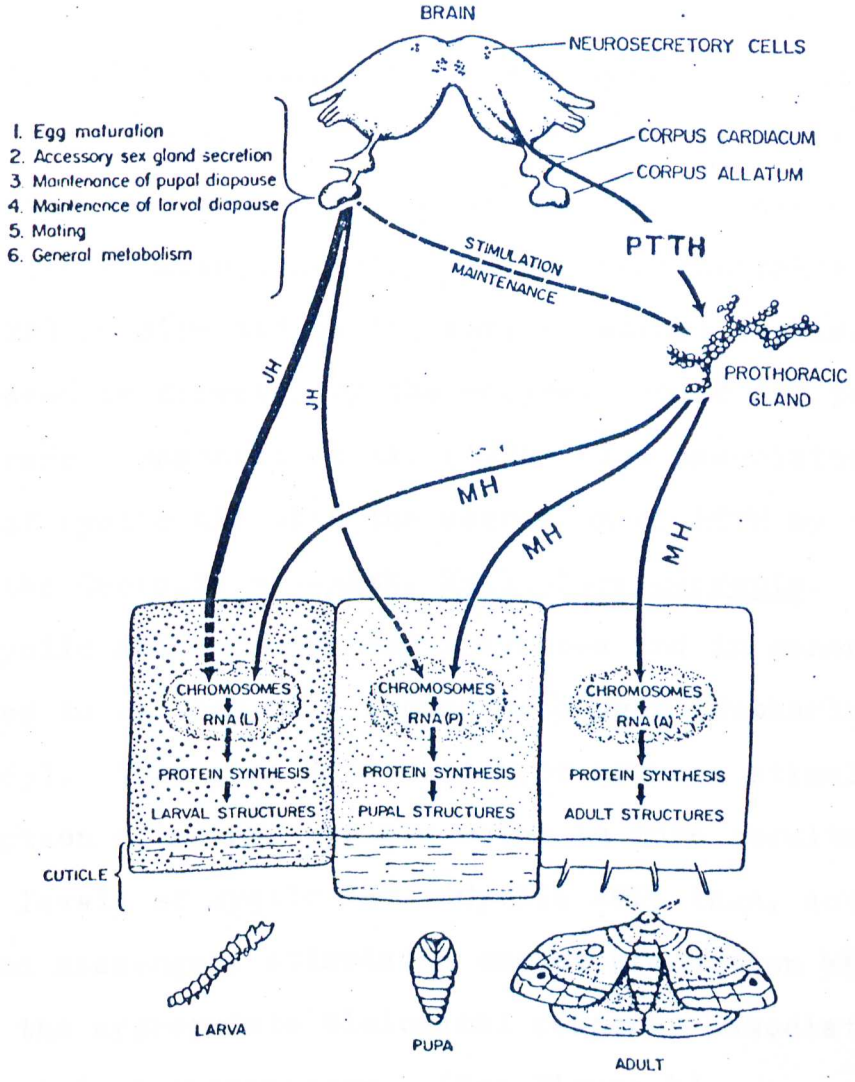
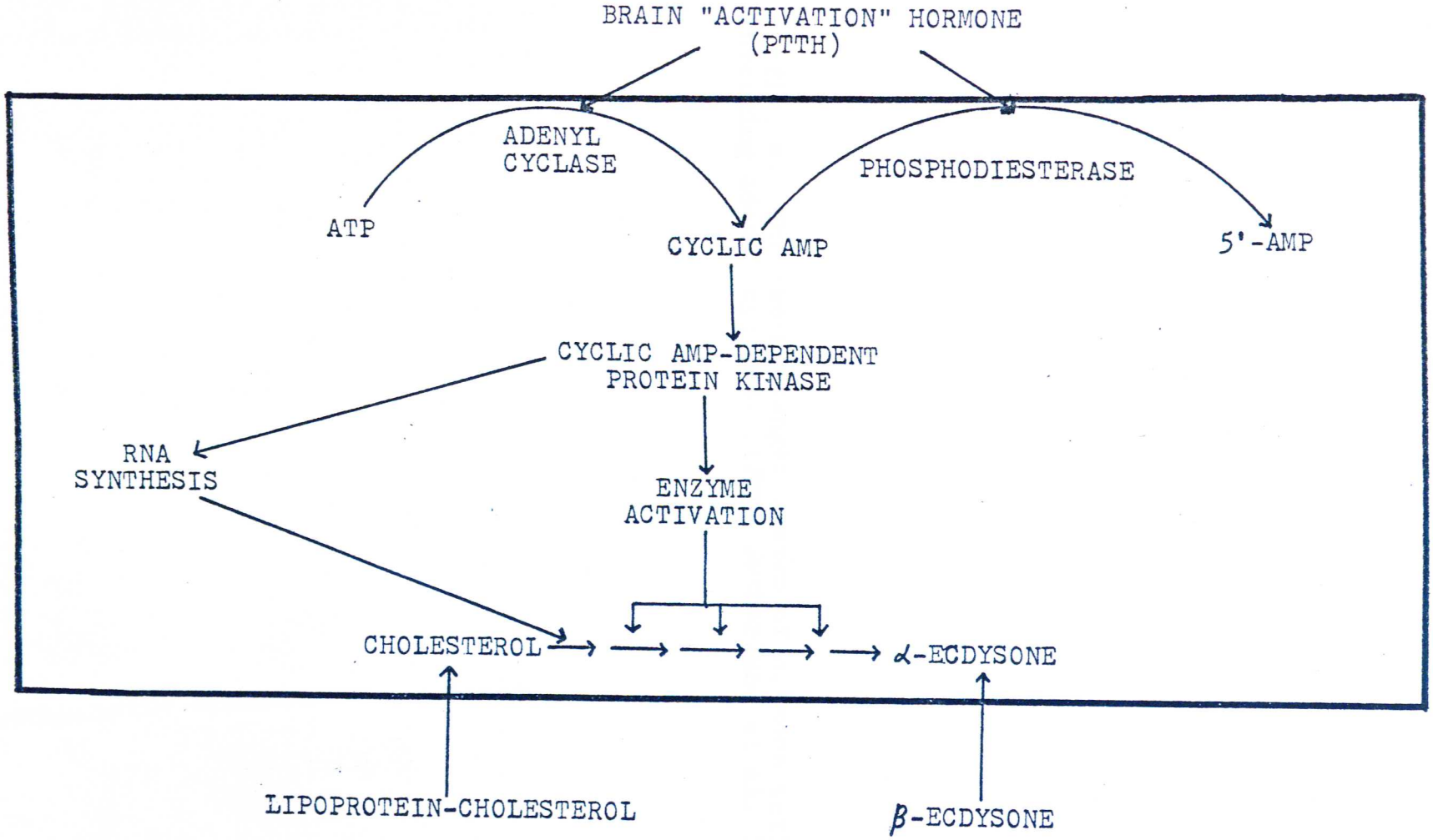

BRAIN
NEUROSECRETORY CELLS
CORPUS CARDIACUM
CORPUS ALLATUM
1. Egg maturation
2. Accessory sex gland secretion
3. Maintenance of pupal diapause
4. Maintenance of larval diapause
5. Mating
6. General metabolism
PTTH
STIMULATION
MAINTENANCE
JH
JH
PROTHORACIC GLAND
MH
MH
MH
CHROMOSOMES
RNA (L)
PROTEIN SYNTHESIS
LARVAL STRUCTURES
CHROMOSOMES
RNA (P)
PROTEIN SYNTHESIS
PUPAL STRUCTURES
CHROMOSOMES
RNA (A)
PROTEIN SYNTHESIS
ADULT STRUCTURES
CUTICLE
LARVA
PUPA
ADULT

Recently, evidence has been presented to show the involvement of the cyclic AMP system of thetobacco hornworm in PTTH's stimulation of ∝-ecdysone production. Figure 3 (from Vedeckis et al. (1974)) illustrates the currently accepted model for the production of ∝-ecdysone. The synthesis of adenosine 3',5'-cyclic monophosphate (cyclic AMP) is directed by the enzyme, adenyl cyclase; its breakdown is directed by the enzyme, cyclic AMP phosphodiesterase. Rasenick et al. (1976) have associated an increase of cyclic AMP with the secretion of PTTH by the brain of the Cecropia silkmoth, Hyalophora cecropia. The role of cyclic AMP in these two instances and in general, is believed to be that of a second messenger (Sutherland et al. 1965). Thus some transmitter or hormone stimulates the production of adenyl cyclase which in turn results in increased levels of cyclic AMP. Cyclic AMP, then, acting as a second messenger, stimulates enzyme production which initiates the appropriate biological response associated with the original transmitter. (See Figure 4).

In vertebrates, adenyl cyclase has been linked to the light-regulated circadian rhythm of serotonin and N-acetyltransferase activity (Klein and Berg 1970; Romero and Axelrod 1974). Circadian rhythms are defined as those endogenous biological rhythms whose period is an approximation to the period of the earth's rotation (Halberg 1959). Perhaps adenyl cyclase is similarly involved in

Fig. 3. Model for the production of ∠-ecdysone. (From Vedeckis et al. 1974.)


BRAIN "ACTIVATION" HORMONE
(PTTH)
ADENYL
CYCLASE
PHOSPHODIESTERASE
ATP
CYCLIC AMP
5'-AMP
CYCLIC AMP-DEPENDENT
PROTEIN KINASE
RNA
SYNTHESIS
ENZYME
ACTIVATION
CHOLESTEROL
α-ECDYSONE
LIPOPROTEIN-CHOLESTEROL
β-ECDYSONE

8a

Fig. 4. The two-messenger system of hormone action involving adenyl cyclase. (From Sutherland et al. 1965.)

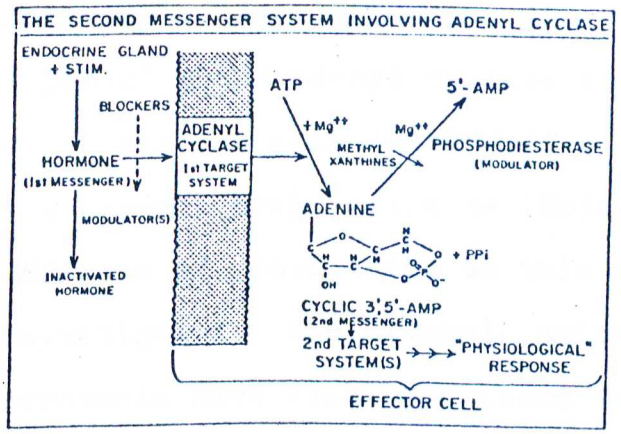
ACTION OF SEVERAL HORMONES OR NEUROHUMORS

HORMONE (FIRST MESSENGER)

(1) ENDOCRINE GLAND + STIMULATION → CELL MEMBRANE

CYCLIC NUCLEOTIDE (SECOND MESSENGER)

(2) HORMONE + CELL MEMBRANE → ENZYME(S)
PERMEABILITY BARRIERS
THIRD MESSENGER



the light-regulated mechanism which controls insect diapause. Preliminary results of Rasenick et al. (1978) support the presence of diurnal rhythms of cyclic AMP in the brains of Manduca sexta and of Cecropia. If as Weiss and Strada (1972) believe, environmental lighting is one of the prime regulators of the cyclic AMP system, these rhythms may be monitoring photoperiodic information.

A stimulatory effect of norepinephrine on adenyl cyclase activity has been demonstrated in vertebrate pineal glands (Weiss and Costa 1968a). Of great significance is the finding that time of day influences the relative sensitivity of pineal gland adenyl cyclase activity to norepinephrine. A similar stimulatory effect has been found in adult cockroach brain extracts (Rojakovick and March 1972). Effects of photoperiod on this activity have not yet been investigated. The biogenic amines, octopamine, dopamine, and serotonin have also been found to stimulate adenyl cyclase activity in nervous tissue of the American cockroach (Nathanson and Greengard 1973).

In summary, insect diapause is controlled by the photoperiodic regimen to which the insect is subjected. The brain houses the photoperiodic control mechanism. Adenyl cyclase activity has been shown to play an important role in insect metamorphosis and possibly the control of circadian rhythms. Neurotransmitters such as norepinephrine, octopamine, and dopamine appear to stimulate

adenyl cyclase activity in certain insects. In light of these findings, it was decided to investigate the effects of two light regimens, one pupa-inducing, the other diapause-inducing, on adenyl cyclase activity of various stages of fifth instar corn borer larval heads, and to determine the effects of the neurotransmitters, norepinephrine, octopamine and dopamine on this activity.

PURPOSE OF THE STUDY

The purpose of this study was to determine if there is a link between adenyl cyclase activity and the diapause condition in the European corn borer, Ostrinia nubilalis.

STATEMENT OF THE PROBLEM

The problem was to determine if adenyl cyclase is involved in the onset, potentiation, and/or termination of diapause in the European corn borer, Ostrinia nubilalis, by measuring adenyl cyclase activity of various-aged diapausing and non-diapausing fifth instar larval heads and by determining the effects of norepinephrine, octopamine, and dopamine on this activity.

STATEMENT OF THE HYPOTHESES

1. There is no difference in adenyl cyclase activity among early and late diapausing, and various-aged fifth instar LD 16:8 (LD) and non-diapausing LD 10:14 (SD) larval heads of the European corn borer.[1]

2. There is no effect of norepinephrine, dopamine or octopamine on adenyl cyclase activity in selected stages[2] of LD and SD fifth instar larvae.

3. There is no difference in the effects of norepinephrine, dopamine, and octopamine on adenyl cyclase activity within any one of the fifth instar stages selected.

4. There is no difference in the effect of any one of the neurotransmitters on adenyl cyclase activity among the fifth instar stages selected.

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[1]For LD 16:8, adenyl cyclase activity in head extracts of early, middle, mature, early prepupal, late prepupal, and pharate pupal fifth instar larvae was determined. For LD 10:14, adenyl cyclase activity in head extracts of early, middle, early mature, late mature, early diapause, late diapause, and refrigerated diapause fifth instar larvae was determined.

[2]The selection of stages was based upon the results of those experiments designed to test hypothesis one.

DEFINITION OF TERMS

1. CIRCADIAN RHYTHM: that internal, biological rhythm whose period is an approximation of the period of the earth's rotation.

2. COMPLETE METAMORPHOSIS: metamorphosis characteristic of holometabolous insects. The egg hatches into a larvae (often called a caterpillar or maggot) which undergoes several molts before changing into a pupa. It is during the pupal rather than the larval stage that there is considerable change of form in the direction of the adult. Thus, after a spectacular metamorphosis, the adult emerges from the pupal case.

3. DIAPAUSE: a period of arrested development similar to hibernation.

4. EARLY DIAPAUSE LARVA: a mature fifth instar larva (LD 10:14, maintained at a temperature of 24°C ± 1.5°C) that is between 40 and 43 days of age.

5. EARLY FIFTH INSTAR LARVA: a fifth instar larva whose body diameter at the midpoint is between 1.0 and 1.5 mm.

6. EARLY MATURE FIFTH INSTAR LARVA: a two to three week old mature fifth instar larva.

7. EARLY PREPUPA: a prepupa which has not enclosed itself in silk.

8. INSTAR: the stage of an insect between successive larval molts; the first instar being the stage between hatching and the first molt.

9. LATE DIAPAUSE LARVA: a mature fifth instar larva (LD 10:14, maintained at a temperature of 24°C ± 1.5°C) which is between 45 and 55 days of age.

10. LATE PREPUPA: a prepupa which has enclosed itself in silk.

11. LD 16:8: a light regimen characterized by sixteen hours of light and eight hours of dark.

12. LD 10:14: a light regimen characterized by ten hours of light and fourteen hours of dark.

13. MATURE FIFTH INSTAR LARVA: a fifth instar larva whose body diameter at the midpoint is 3.0 ± 0.3 mm.

14. MEASURE OF ADENYL CYCLASE ACTIVITY: amount of ATP converted to cyclic AMP as determined by a modification of the method of Krishna et al. (1968).

15. MIDDLE FIFTH INSTAR LARVA: a fifth instar larva whose body diameter at the midpoint is 2.0 ± 0.3 mm.

16. METAMORPHOSIS: a change in insect form during development.

17. MOLT: a process of shedding the exoskeleton (external skeleton); ecdysis.

18. NEUROHORMONE: a regulatory substance produced by nervous tissue at extrasynaptic sites.

19. NEUROTRANSMITTER: a substance produced by nerve cells that is responsible for synaptic transmission.

20. PHARATE PUPA: a mature fifth instar larva (LD 16:8, maintained at a temperature of 24°C ± 1.5°C) characterized by a relative quiescence, light body color, reduced body turgidity and sigmoid shape.

21. PHOTOPERIOD: the number of hours of light and dark in a given period of time, typically 24 hours.

22. PHOTOPHASE: the light portion of the photoperiod.

23. PREPUPA: a mature fifth instar larva (LD 16:8, maintained at a temperature of 24°C ± 1.5°C) whose gut contents are white in color.

24. REFRIGERATED DIAPAUSE LARVA: a mature fifth instar LD 10:14 larva that has been refrigerated under a light regimen of LD 10:14 and a temperature of 4°C ± 1.5°C for four to six weeks after having entered diapause.

25. SCOTOPHASE: the dark portion of the photoperiod.

LIMITATIONS OF THE STUDY

1. The insects used for the study are laboratory-reared. Although strains are always rejuvenated by periodic matings with field insects, differences may exist in adenyl cyclase activity of laboratory-reared and field strains.

2. Conditions for the maintenance of insect cultures are designed to simulate those of the field. Such simulation is never perfect.

3. Results cannot be generalized to all lepidopterous insects, much less to all insects. As Judy (1974) explains, it is very likely that differences exist among insect species in terms of the mechanisms which regulate the timing of a molt.

4. Differences in developmental rates exist even among insects exposed to identical environmental conditions. Thus, insects of the same age were actually at slightly different stages of development. This increased the variability of the results.

5. Approximately five percent of LD 16:8 larvae did not pupate while approximately ten percent of LD 10:14 did. This resulted in a slight decrease in the reliability of the results for non-diapausing insects.

6. The differences which exist in adenyl cyclase levels among the various groups of larval heads cannot be attributed to any particular neuroendocrine structure.

7. Effects of norepinephrine, dopamine, and octopamine are stated in terms of specific concentrations used. It is possible that other concentrations might have had different effects.

8. All larvae were decapitated between the hours of 11 A.M. and 1 P.M. Since it is plausible to expect circadian variations in adenyl cyclase levels of head homogenates, values for adenyl cyclase activity would probably differ had the insects been decapitated at a different time during the day.

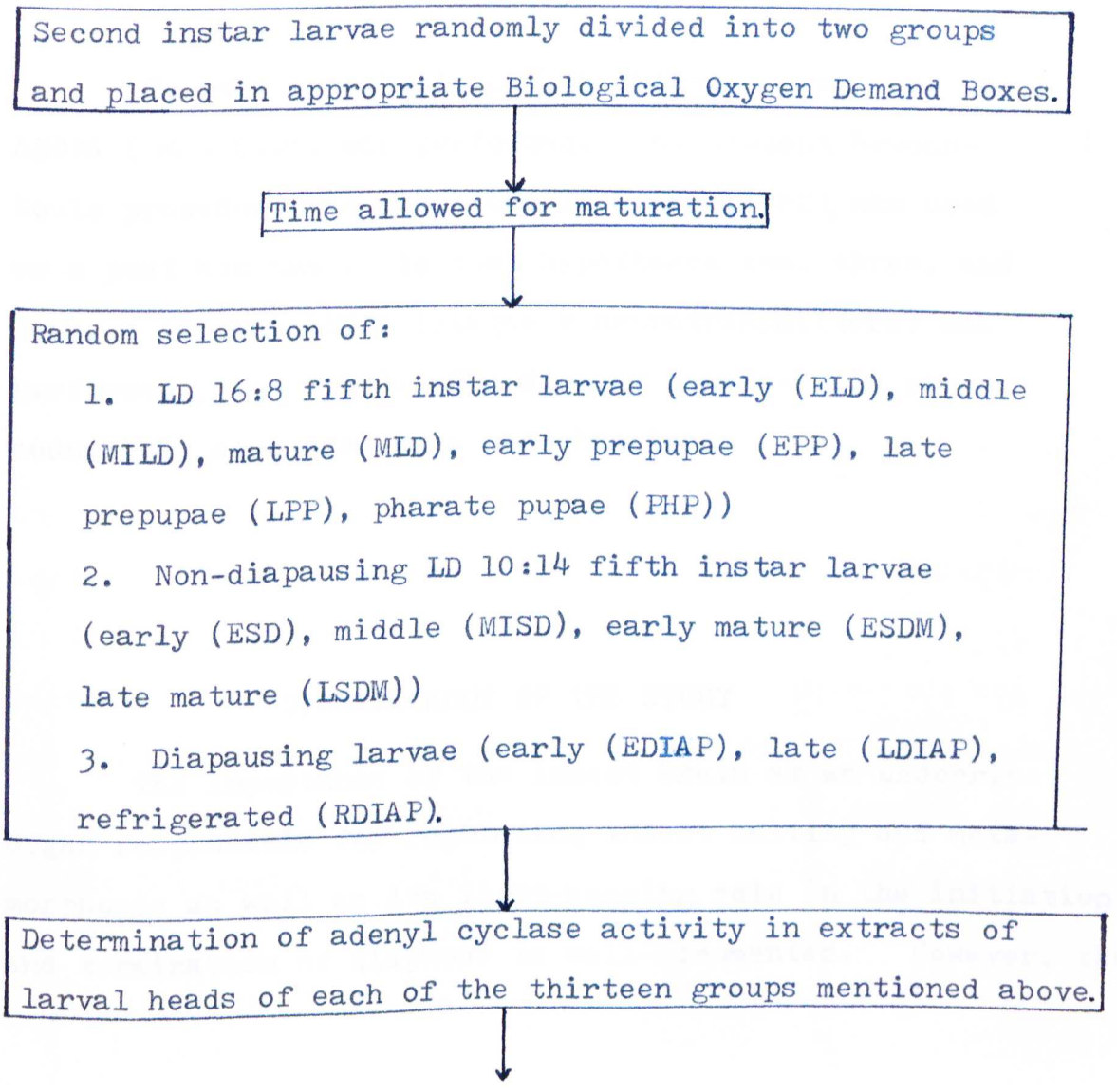
PROCEDURE

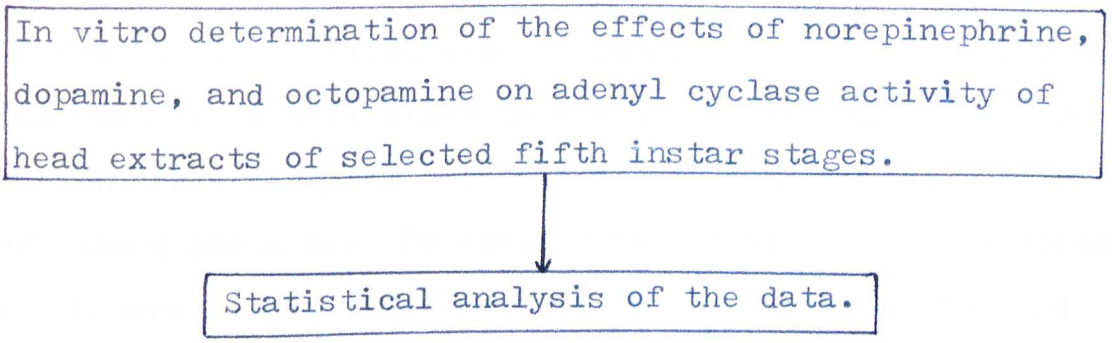
Second instar larvae received from the European Corn Borer Laboratory (USDA) in Ankeny, Iowa were randomly divided into two groups. One group was placed in a Biological Oxygen Demand Box (BOD) set to maintain a light regimen of sixteen hours of light and eight hours of dark (LD 16:8) and a temperature of 24 ± 1.5 degrees centigrade. The second group of larvae was placed in a similar box set to maintain a light regimen of ten hours of light and fourteen hours of dark (LD 10:14). These photoperiodic regimens were found to be ideal for inducing pupation and diapause respectively in LD and SD Ankeny corn borers. Fifth instar larvae were identified by their head capsule width (1.70-2.10 mm). Early, middle, and mature fifth instar larvae were identified by their body diameter, prepupae by the white color of their gut contents, and pharate pupae by their relative quiescence, light body color, reduced body turgidity and forward curvature of the head into a somewhat tucked position. LD 10:14 fifth instar larvae were assumed to be in diapause if they had not undergone pupation by the time they had reached forty days of age.

Adenyl cyclase activity of larval heads was measured by a modification of the method of Krishna et al. (1968). This assay is designed to determine the amount of radioactive cyclic AMP produced from radioactive adenosine triphosphate (ATP) due to the action of the enzyme adenyl cyclase. Breakdown of the cyclic AMP produced was prevented by the addition of a cyclic AMP phosphodiesterase inhibitor,

theophylline. The radioactive cyclic AMP was purified by chromatography on Dowex 50 H^+ ion exchange columns followed by treatment with zinc sulfate and barium hydroxide. The amount of radioactive cyclic AMP produced as a result of adenyl cyclase activity was measured in a Beckman liquid scintillation counter.

A flow chart of the research design follows:





To test hypothesis one, a thirteen group one way ANOVA ($\alpha = 0.05$) was performed. The Student Newman-Keuls procedure, as described in Winer (1962), was used as a post hoc test. To test hypotheses two, three, and four, a two way ANOVA (stages x neurotransmitters) was performed ($\alpha = 0.05$). The Student Newman-Keuls procedure was also used as a post hoc test.

SIGNIFICANCE OF THE STUDY

The importance of the insect brain as an endocrine organ responsible for regulating insect molting and metamorphosis as well as its light-sensing role in the initiation and termination of diapause is well-documented. However, the

exact mechanisms by which it accomplishes these tasks, especially its control over diapause is still a mystery. The results of this study have provided additional information concerning the biophysical and biochemical nature of insect diapause. By subjecting larvae to light regimens which were either pupa-inducing or diapause-inducing and measuring adenyl cyclase levels, the researcher was able to determine that there is a link between adenyl cyclase activity and the diapause condition in the European corn borer. By determining the effects of selected neurotransmitters on adenyl cyclase activity, the researcher was able to ascertain the relative sensitivity of adenyl cyclase receptors to these neurotransmitters during the various stages of the fifth instar studied. These findings have helped to elucidate the mechanism by which the neuroendocrine system controls insect diapause and should be useful in the consideration and design of future methods of insect control. Insects in diapause are typically more resistant to insect control procedures than those which are not in diapause. Diapausing corn borers are well concealed and are protected by a silken case. In addition, their overall metabolic rate is considerably reduced. In contrast, the cyclic AMP system in diapausing borers appears to be functioning at a relatively high level. Therefore, future control efforts might be directed at interfering with this

particular system. The assay developed to measure adenyl cyclase activity could be employed to detect the effects of potential insect regulators on this system.

ORGANIZATION OF THE STUDY

The remainder of this study is divided into four chapters. Chapter two presents a review of the literature. It includes a discussion of:

1. the life cycle of the European corn borer
2. characteristics of the diapause state
3. the control of diapause by physical factors
4. the roles of the various neuroendocrine structures (brain, corpora cardiaca, corpora allata, and prothoracic glands) in controlling insect metamorphosis and diapause
5. the involvement of the cyclic AMP system in the neuroendocrine control of insect metamorphosis and diapause.

Chapter three describes the methodology used to rear and select insects, to assay for adenyl cyclase activity, and to statistically analyze the results. Chapter four presents the experimental results as well as an interpretation of these results. Chapter five analyzes and discusses the significance of the results, and relates them to the findings of other researchers. In addition, suggestions for future research are presented.

CHAPTER II

REVIEW OF THE LITERATURE

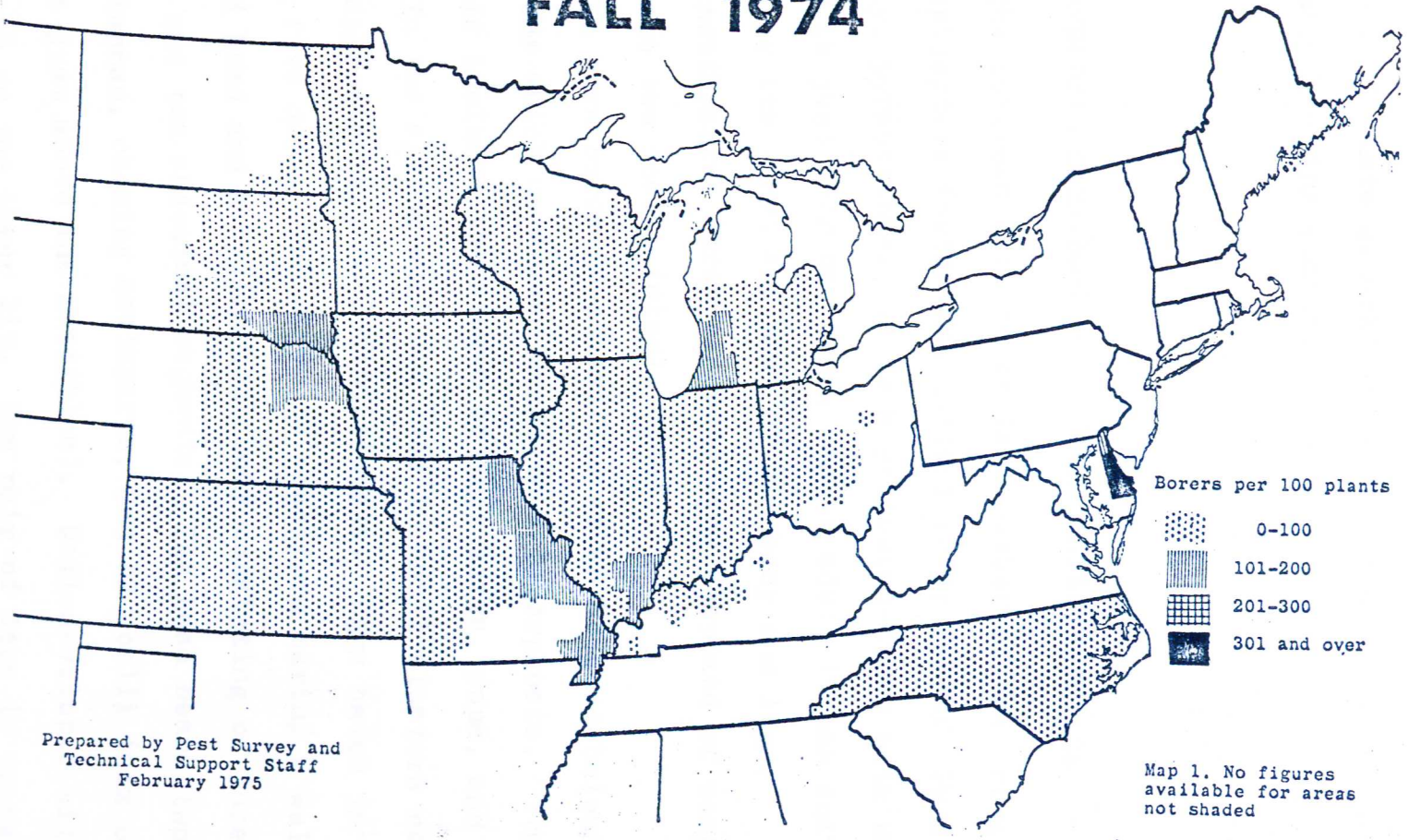
According to Shapiro and Pereverzev (1974), the European corn borer, Ostrinia nubilalis (Hübner), is one of the most widespread and destructive corn pests in the world. Ten to fifteen years ago, before the development of resistant hybrids, annual losses of corn in the United States due to European corn borer activity was estimated at 300 million dollars. In 1974 because of the use of resistant hybrids, this loss had been reduced to 140 million dollars. In the North Central United States, loss due to the larval activity of the corn borer has been estimated at between two and four percent per borer per plant. Figure 5 (from the USDA Coop. Econ. Insect Rep. 1975) shows the relative abundance of the corn borer in the North Central and Eastern United States as of 1974.

Ryder et al. (1969) have reported that the European corn borer was first discovered in the United States about 1917. This lepidopterous insect pest also occurs in other countries throughout the world including Egypt, Canada, the Soviet Union, Germany, Bulgaria, Japan, China, and the Phillipines (El-Minshawy et al. 1974).

The European corn borer attacks mainly maize (Zea mays) doing a great deal of damage to stalks, ears, and tassels. Damage done to food conducting vessels by early generation larvae results in plant weakness, reduction of

Fig. 5. European corn borer abundance, Fall 1974. (From the USDA Coop. Econ. Insect Rep. 1975.)

EUROPEAN CORN BORER ABUNDANCE
FALL 1974



Prepared by Pest Survey and
Technical Support Staff
February 1975

Map 1. No figures
available for areas
not shaded

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ear size and weight, and reduction of grain number and grain weight. Late generation larvae cause loss of ears due to ear dropping (Chiarappa 1971).

THE EUROPEAN CORN BORER--GENERAL CHARACTERISTICS

The European corn borer is a member of the insect order Lepidoptera (moths and butterflies); family, Pyralidae; subfamily, pyraustinae. It is a yellowish-brown moth whose wingspan is just over one inch. As an adult it has scales which cover its wings and most of its body and legs, sucking mouthparts, large compound eyes composed of many facets, and two ocelli (simple eyes).

The corn borer is classified as an holometabolous insect, one which undergoes complete metamorphosis. Its life cycle includes the stages of egg, larva, pupa, and adult. In the spring eggs are laid in small clusters containing approximately 20-25 individuals. Eggs hatch in three to five days. The larva is eruciform having a well-developed head and a cylindrical body consisting of three thoracic and ten abdominal segments. The head bears two short antennae, chewing mouthparts, and 12 ocelli (six on each side just above the mandibles). Well-developed silk glands open on the lower lip. One pair of legs is present

on each thoracic segment; and one pair of prolegs is found on abdominal segments three through six and segment ten. During a period of between 15 and 30 days (depending on environmental conditions) larvae generally undergo four ecdyses (molts) before metamorphosing into pupae. Thus, there are typically five larval stages each known as an instar. Particular instars are identified by head capsule width (Vinal and Caffrey 1919; Gelman and Hayes 1978). The fifth larval instar may reach a length of up to 25 mm. Towards the end of the fifth instar, larvae cease to feed and are then designated prepupae. Prepupae enclose themselves in silken cases and enter the pharate pupa stage characterized by a sigmoid shape, head bent forward into a somewhat tucked position. In less than 24 hours the cuticle is shed and the pupal stage begins. Pupae are obtect (appendages more or less glued to the body), brown in color, and generally 10 to 12 mm in length. It is during the pupal stage, which lasts for about one week, that larvae metamorphose into adult moths. Figure 6 depicts the larval, prepupal, pharate pupal, pupal and adult stages in the corn borer life cycle.

Fig. 6. The larval, late prepupal, pharate pupal, pupal, and adult stages of the European corn borer. Silken cases which enclose the late prepupa and pharate pupa have been split open so that the animal can be seen.

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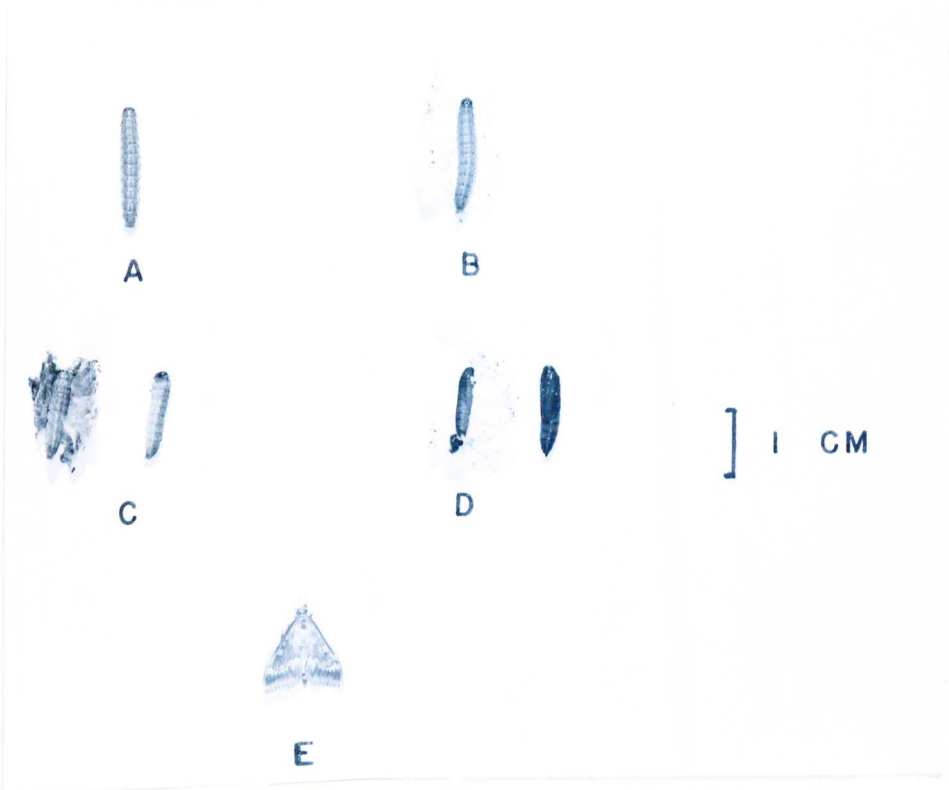


THE DIAPAUSE STATE

Insects inhabiting those latitudes where cold and warm seasons alternate with one another have evolved mechanisms which allow them to endure the low temperatures of the late fall and winter seasons. They enter a physiological state known as diapause, which is characterized by a lack of growth and morphogenesis, and which is due to profound changes in insect metabolism. The ability of an insect to enter diapause is determined genetically and may be under polygenic control (King 1974; Hong and Platt 1975). Beck (1962a) originally defined diapause as "a state of arrested development in which the arrest is enforced by a physiological mechanism rather than by unfavorable environmental conditions." However, in 1964, Beck and Alexander showed that development does occur during diapause, but at a very slow rate. Therefore, a more appropriate definition for diapause is a "state of suppressed development" (Beck 1968).

Diapause may occur in any stage of insect development (egg, larva, nymph, pupa, or adult); but in any given species, it will always occur in the same stage. Insect species may be classified according to the type of diapause they exhibit. Univoltine species will diapause in each generation at some stage during their life cycle and are therefore said to have obligatory diapause. They only produce one generation per

year. Polyvoltine species generally produce more than one generation per year. Those with facultative diapause may or may not diapause depending on environmental conditions, while polyvoltine nondiapausing insects continue their development throughout the year.

The European corn borer is a polyvoltine species with a facultative diapause. In spring and early summer, the corn borer completes its normal life cycle; but, in late summer and early fall, fifth instar larvae will enter diapause and overwinter in this state. Diapausing larvae exhibit reduced movement, reduced oxygen consumption and water content, increased fat reserves, and an overall lowering of metabolism (RNA and DNA synthesis, protein synthesis, and enzyme levels) (Beck and Hanec 1960; Schechter et al. 1971; Brown and Chippendale 1977). It is known that during diapause the corn borer can withstand prolonged periods of low temperatures (down to -30° to -40°C), temperatures which they could not tolerate in the nondiapause state (Maslennikova 1973). Anatomically, diapausing male larvae can be distinguished from nondiapausing larvae on the basis of gonadal development. As early as the fourth instar, male larvae programmed to enter a state of diapause showed underdeveloped rudimentary testes (Parker and Thompson 1927).

THE CONTROL OF DIAPAUSE BY PHYSICAL FACTORS

Since seasonal changes are accompanied by changes in the number of daylight hours per 24-hour day, and since these changes are exactly the same from year to year, insects have evolved the ability to use photoperiod (the number of hours of light and dark in a given period of time, typically 24 hours) to regulate their life cycles. Thus, insects do not enter diapause as a result of the relatively sudden onset of unfavorable conditions, but rather as a result of the changes in day length and temperature to which prediapausing stages have been exposed. As the days become shorter in late summer, the corn borer uses this signal to physiologically prepare itself for winter. Thus, larval diapause in this species, as in many others, has been shown to be in response to short days and low temperatures (Mutchmor and Beckel 1959; Beck and Hanec 1960).

Beck and Hanec (1960) have demonstrated that at a temperature of 23° C, the critical photophase (span of light) for the Northern Wisconsin European corn borer is between 15 and 15.5 hours of light for a 24 hour cycle.[1] Thus, corn borers exposed to a photophase of 15.5 hours accompanied by a scotophase (period of darkness) of 8.5

[1]Different strains of European corn borers have evolved different critical daylengths in response to the seasonal conditions of temperature accumulation and daylengths in the specific locality in which they live (Beck 1963). These critical photoperiods vary between 14 and 15.5 hours.

hours exhibited 18% diapause, while those exposed to a photophase of 15 hours accompanied by a scotophase of 9 hours exhibited 83% diapause. Figure 7 from Beck (1962a) illustrates the effect of photoperiod on the incidence of diapause among European corn borer larvae reared at a temperature of 30° C. As can be seen, at this temperature, diapause is induced by a relatively narrow range of photoperiods, those having a photophase of between 10 and 14 hours. Similar curves have been found for other insect species (Adkisson 1965; Beck 1968; Lees 1968).

That corn borer larvae are exceptionally sensitive to photoperiod is shown by the finding that diapause can be prevented in up to 70% of corn borer larvae reared in diapause-inducing photoperiods by transferring them to a non-diapause-inducing photoperiod as late in development as the fifth instar (Beck and Hanec 1960). Then too, an interruption in the scotophase of as little as 0.5 hours of light greatly reduced diapause incidence (Beck 1962a).

Beck (1962a) also found that the duration of the scotophase is actually more important than that of the photophase in inducing corn borer larvae to diapause. A scotophase of twelve hours was found to yield maximum diapause when combined with photophases of from five to eighteen hours. Even a nondiel (cycle other than 24 hours) photophase of up to 32 hours yielded significant diapause when used in conjunction with a 12 hour scotophase.

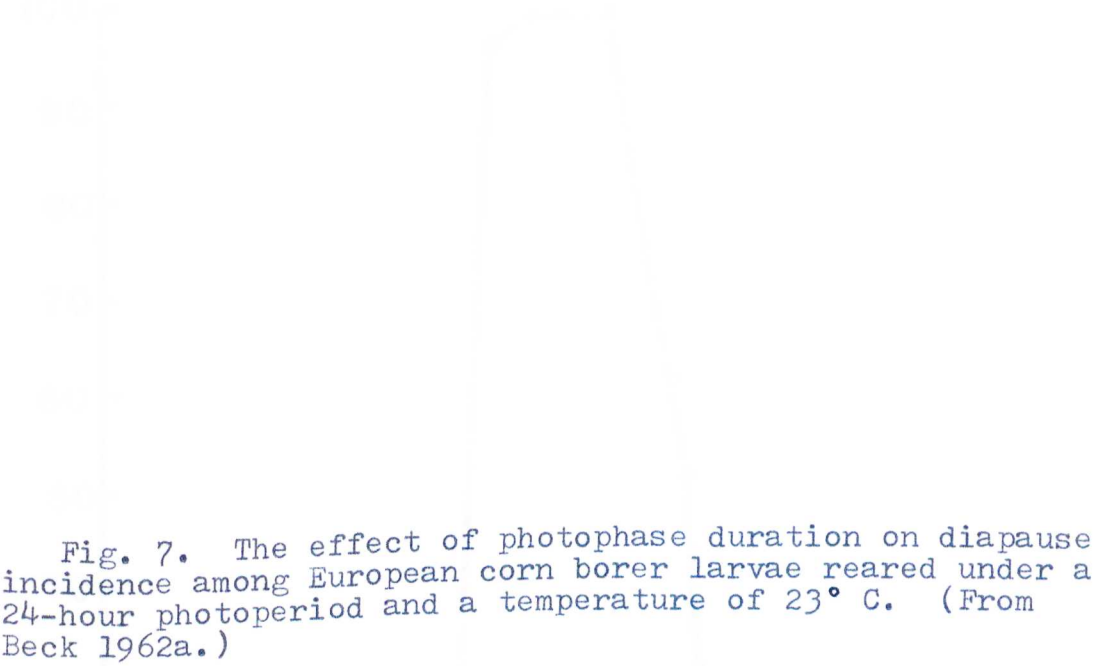
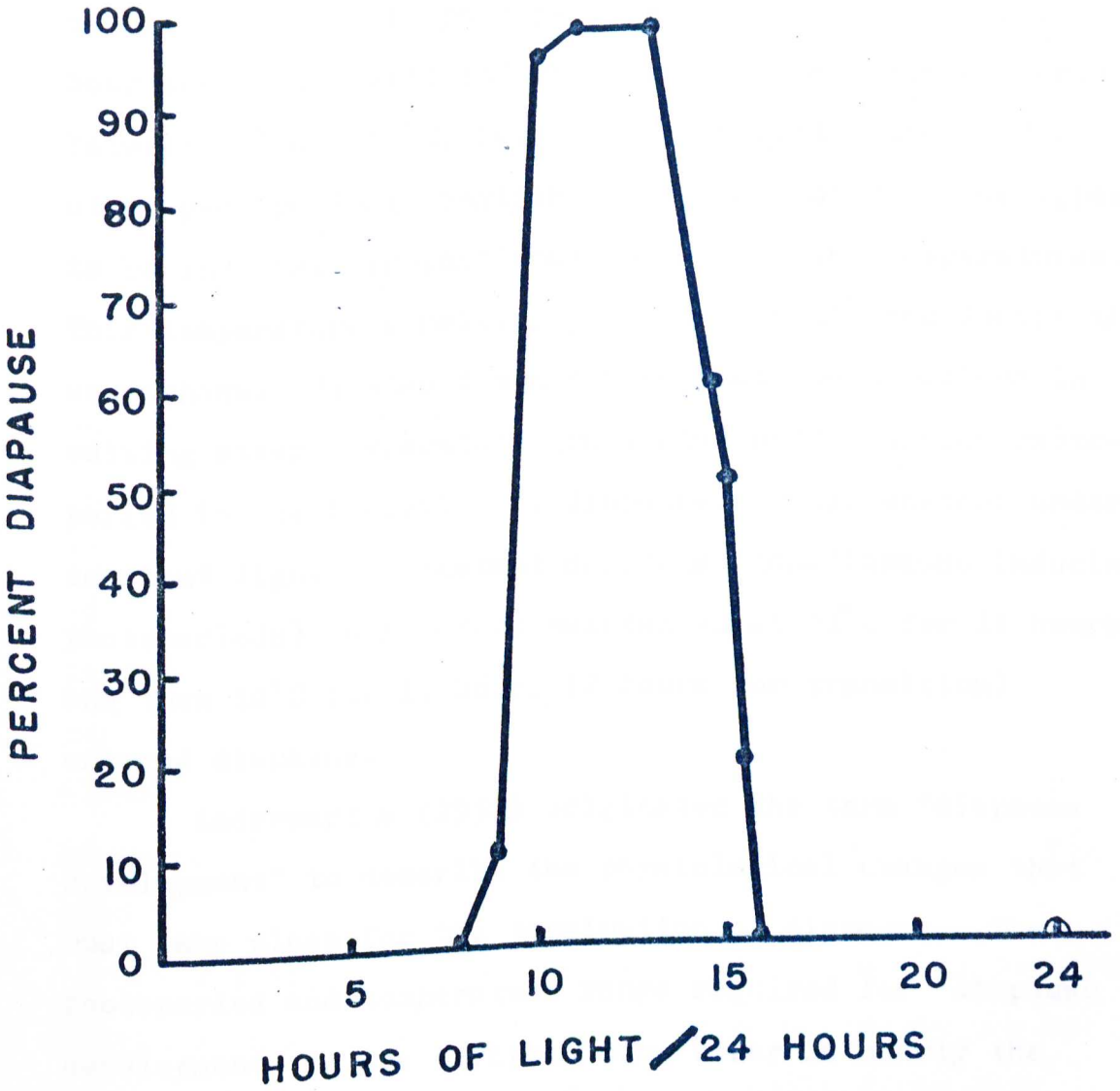


Fig. 7. The effect of photophase duration on diapause incidence among European corn borer larvae reared under a 24-hour photoperiod and a temperature of 23° C. (From Beck 1962a.)


100
90
80
70
60
50
40
30
20
10
0
PERCENT DIAPAUSE
5
10
15
20
24
HOURS OF LIGHT / 24 HOURS

Temperature, too, exerts an influence on the onset of diapause. While photoperiods having a 10.5 to 13.5 hour scotophase will induce diapause at all temperatures between 20° and 30° C, Beck (1962b) reported that under other photoperiodic regimens, the onset of diapause appeared to be inversely proportional to the rearing temperatures. This temperature sensitivity was only exhibited during the scotophase. He also demonstrated that thermoperiods involving steep temperature gradients could override photoperiod in the induction of diapause. Thus, whether under constant light or constant darkness (non-diapause-inducing photoperiods) corn borers maintained at 31° C for 11 hours and then 10° C for 11 hours (2 hours for transition) entered diapause.

Andrewartha (1952) originated the term "diapause development" to describe the physiological changes that must take place for the termination of diapause. The photoperiod and temperature range required for "diapause development" of the European corn borer is nearly the same as that for morphogenesis (McLeod and Beck 1963a). Thus, according to McLeod and Beck, diapausing larvae exposed to LD 16:8 (16 hours of light, 8 hours of dark) pupated after 30 days while those exposed to LD 8:16 pupated after 70 days.

Conditions necessary for the termination of diapause have been summarized by Beck (1963). He noted that while many species require chilling for several weeks in order to break diapause, the corn borer did not. In addition, according to Bowen and Skopik (1976), it is the duration of the scotophase rather than the duration of the photophase which appears to be important in terminating diapause. It should be noted that in the field, diapausing borers who have completed diapause development by early winter will not pupate until spring in response to increased moisture and rising temperatures. In the laboratory, the need for sufficient moisture is shown by the inability of long-day photoperiods to terminate diapause in partially dehydrated borers (Beck 1967); the need for increasing temperature is shown by the inability of long-day photoperiods to terminate diapause if the temperature is too low (Schechter et al. 1971).

The most effective wavelength for inducing the termination of diapause of European corn borer larvae has not as yet been determined. However, in the codling moth (Laspeyresia pomonella) larvae and the oak silkworm (Antheraea pernyi) pupae, light in the blue region of the spectrum was shown to be most effective in breaking diapause (Norris et al. 1969; Schechter et al. 1971). Schechter et al. suggested, therefore, that a pigment or pigment-protein

which absorbs in this region is involved in triggering the entire series of biochemical events responsible for diapause termination. Hemes, bile pigments, and carotenoids are possible candidates for this pigment.

THE "PHOTOPERIODIC CLOCK"--CIRCADIAN OSCILLATOR VERSUS AN HOURGLASS MECHANISM

Since light via photoperiod plays such an important role in the initiation and termination of diapause, insects must have evolved some mechanism for recording the hours of light and/or darkness. Such a mechanism has been dubbed a "photoperiodic" or "biological clock". Currently there exists a controversy concerning the appropriateness of using the term "biological clock" when referring to this mechanism. Those who feel that the term is a misnomer explain that "clock" connotes a single discrete organ or system, and this concept is probably incorrect. As Beck (1968) and Brady (1974) have hypothesized, the time-measuring capability is the result of an integration of numerous rhythmic functions, a combination of an unknown number of biochemical events. But, Brady has also pointed out that the term "biological clock" is a necessary term for the sake of convenience; and its use certainly is appropriate, as long as it is operationally defined as above.

Both circadian rhythms and hour glass mechanisms have been implicated in the functioning of the "biological clock" which controls the initiation and termination of insect diapause. According to Halberg (1959), a circadian rhythm is defined as a biological rhythm whose period is an approximation of the period of the earth's rotation. It is a free-running rhythm, an endogenous oscillator with a period of approximately 24 hours. Circadian rhythms can be entrained by the 24 hour light-dark cycle. When entrained, they can have a period of exactly 24 hours (Brady 1974). In circadian-based events, the underlying endogenous oscillation typically creates a period of greatest sensitivity for the production of some hypothetical metabolic product, the concentration of which controls the occurrence of a particular physiological event. In hour glass mechanisms, often referred to as interval timers, there is also an accumulation of some hypothetical metabolic product, and when this product reaches a given titre, the physiological event occurs. Photoperiod can control the occurrence of the physiological event by regulating the synthesis or lack of synthesis of the metabolic product through an alternation of light and dark reactions (Lees 1955, 1965); but, there is no underlying endogenous oscillation.

There is a whole class of circadian rhythms which involve developmental events occurring once in the life of

an individual, and which only appear as overt rhythms in synchronous populations. The term "gating" is applied to such events, implying that the event can only occur when the circadian clock opens the gate at the appropriate time (Brady 1974). Gated events can also operate via an hour-glass mechanism (Brady 1974). In either case, if an individual misses a given day's gate, it must wait 24 hours for the next one. Truman (1972) has shown that in the tobacco hornworm, Manduca sexta, the endocrine events which initiate the molt (rather than the molts themselves) are gated events. Prothoracicotropic hormone (PTTH) was produced only during a gate in the early to middle portion of the night. Thus, those larvae which had completed development (were physically ready to molt) too late to exploit the gate waited until the next night to produce PTTH and molt.

Beck (1974, 1975, 1976 a,b) has proposed the "Dual System Theory" to explain the photoperiodic determination of diapause induction in the European corn borer. According to this theory an endogenous oscillator and an hour glass mechanism interact in determining whether the corn borer will diapause or complete its life cycle and pupate. At the onset of darkness, it is proposed that a substance (S) is rapidly produced, and reaches its maximum concentration within four hours. Then it is slowly metabolized away, the rate of degradation differing during the scotophase and the

photophase portions of the 24 hour period. Thus the concentration of S will vary depending upon the length of the scotophase. Under conditions of continuous darkness, the S-system is hypothesized to act as a free-running circadian rhythm. The concentration of S during a critical time span of the 24 hour period, a time span labelled the Determination Gate, determines whether the European corn borer will diapause or pupate. This determination gate is set by a second system, the P system, which Beck believes is entrained by the S system. P, too, is synthesized and reaches a maximum concentration within four hours. After this time it is slowly degraded. The Determination Gate coincides with that period of time when the concentration of P is between two arbitrary values. These values are fairly close together making the Determination Gate quite narrow. According to Beck, the "Dual System Theory" correctly predicts the diapause incidence under a variety of photoperiods including nondiel (photoperiods other than 24 hours) and skeleton photoperiods (photoperiods in which light breaks interrupt the scotophase).

Bowen and Skopik (1976), on the other hand, have purported that their findings support an hourglass mechanism for the "photoperiodic clock" which operates in the European corn borer. According to these researchers, circadian oscillations are not involved in the photoperiodic time measurement in this species. They based their conclusions on the inability of scotophases other than eight hours

(when coupled to sixteen hour photophases) to break larval diapause. They went on to explain that if a circadian rhythm were involved, a photoperiodic schedule consisting of 32 hours of dark and 16 hours of light should result in diapause termination. Then too, in their experiments 100% of diapausing corn borers exposed to a minimum of six 8-hour dark phases accompanied by at least 4 hours of light per cycle terminated diapause. Thus the total number of hours in each cycle appeared to be unimportant, a finding which is not consistent with a circadian mechanism. Their work, however, did not eliminate the possibility of diapause termination itself being dependent on an endogenous oscillator. Thus, while an hourglass may operate in part in creating the biochemical environment necessary to break diapause, the actual termination may be gated; and this gate may be controlled by a circadian rather than an hourglass mechanism.

While at first glance the conclusions of Beck seem to be at odds with those of Bowen and Skopik, it must be remembered that Beck's model was designed to explain diapause induction, while the experiments of Bowen and Skopik were concerned with diapause termination. It is possible that different mechanisms are operating in the two cases.

THE NEUROENDOCRINE CONTROL OF INSECT METAMORPHOSIS AND DEVELOPMENT

Nerve cells communicate with each other as well as with muscles, glands, and other organs by producing chemical messengers or neurosecretions. Basically, these are of two types, neurotransmitters and neurohormones. Researchers including Scharrer (1977, 1978) and Barker (1977) have attempted to provide definitions which can be used to distinguish between them. Thus, Barker (1977) has characterized neurotransmitters as "a low-molecular-weight class of molecules synthesized by nerve cells that underlie a form of cell-cell interaction in the nervous system characterized as synaptic transmission." Transmitters, then, mediate interactions between cellular elements, interactions which cover small discrete areas and which take place in very brief periods of time (milliseconds to seconds). Synaptic transmitters include the amino acids, glycine (gly), taurine (tau), proline (pro), and γ-amino butyric acid (GABA); the biogenic amines, norepinephrine, serotonin, and dopamine; and acetylcholine. Barker defines neurohormones as "substances released by nerve cells at extrasynaptic sites (portal, general or cerebrospinal circulations) to regulate the activity of both endocrine and nonendocrine target tissues." He goes on to explain that neurohormones differ from neurotransmitters because of their longer time of action, the location of their release sites, the increased

distance between release and target sites, their peptide structure, and their ability to cause multiple actions on a variety of neural and nonneural cellular elements. However, the distinction between the two classes of neurosecretions is not as clearcut as the above definitions would suggest; for as Barker (1977) points out, peptides have been found to be released in the central nervous system and to mediate short-term events via synaptic mechanisms as well as long-term events via hormonal mechanisms. His statement, "neurohormones may or may not be released at synapses and 'putative transmitters' with actions similar to peptides may be reconsidered as neurohormones," clearly exemplifies the state of confusion that currently exists in distinguishing between neurohormones and neurotransmitters. The problem seems to be in trying to tie structure (peptide or non-peptide) to function/place of action (release into the general circulation or release at the synapse respectively). Perhaps it would be simpler to state the definitions in terms of structure or function and not both. This researcher would prefer to use function as the distinguishing characteristic and so define neurohormones as neurosecretions which exert their effects at target sites that are extrasynaptic and relatively far from their release sites. According to this definition a given molecule could act as both a neurotransmitter and a neurohormone. The underlying reason for

this duality of roles probably lies in the fact that before the evolution of endocrine glands, special neurosecretions served as regulatory hormones. With the evolution of additional endocrine apparatus (in arthropods and vertebrates), most of these hormones of neuronal origin now took on the role of mediators, integrating the functions of nervous and endocrine systems (Scharrer 1978).

Four endocrine and neuroendocrine glands (organs composed of nervous tissue which secrete neurohormones) have been shown to play important roles in controlling insect growth and metamorphosis. These are the neurosecretory cells of the brain, the corpora cardiaca and the corpora allata located in close proximity to the brain, and the prothoracic glands located in the prothorax and posterior parts of the head (Maslennikova 1973). Experimental evidence supports the following general sequence of humoral events in the regulation of growth and metamorphosis. The medial and lateral neurosecretory cells of the brain produce substances that are carried via nerves to the corpora cardiaca, one of which is believed to be PTTH (Scharrer 1952; Highnam 1958). The corpora cardiaca store and eventually release brain hormone (PTTH), a neurohormone into the blood. PTTH stimulates the release of α-ecdysone, the growth and molting hormone, from the prothoracic glands (Williams 1946; Williams and Adkisson 1964; Schneiderman and Gilbert 1964;

Yin and Chippendale 1975).[1] ∝-ecdysone is converted into β-ecdysone (MH), the true molting hormone by peripheral body tissues (Moriyama et al. 1970; King 1972a; Bollenbacher et al. 1975). β-ecdysone, then, stimulates other insect tissues such as the epidermis and gonads to initiate differentiation necessary for insect molting (Wigglesworth 1964; King 1972b; King et al. 1974). There is evidence indicating that β-ecdysone also acts as a positive feedback regulator stimulating certain neurosecretory cells of the brain's pars intercerebralis[2] to continue synthesizing and releasing PTTH (Agui and Kiyoshi 1977; Marks et al. 1972). JH[3] secreted by the corpora allata influences the nature of the molt, whether it will be larval, pupal or adult. A brain hormone (an allatropin) is believed to stimulate the corpora allata to produce JH (Maslennikova 1973).

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[1]It is widely held that the brain produces several different hormones which modify the action of a variety of glands including the prothoracic glands and the corpora allatum. Ishizaki and Ichikawa (1967) separated three active prothoracicotropic fractions from extracts of silk-worm (Bombyx mori) brains. Yamazaki and Kobayashi (1969) separated a fourth. Gersch et al. (1973) isolated two separate factors from cockroach brain and corpora cardiaca. It is significant that the effects of their two activators were different. Activator I stimulated RNA synthesis while activator II increased membrane potential.

[2]The pars intercerebralis is the central portion of the insect brain where the medial neurosecretory cells are located.

[3]Many authors believe that the various effects of JH are due to the action of two or more different hormones secreted by the corpora allata (Maslennikova 1973; Yin and Chippendale 1973).

Thus, insect growth and metamorphosis is primarily controlled by the balance between two hormonal factors, MH and JH. This balance between the two hormones is more important in controlling insect metamorphosis than is their absolute concentrations. JH has also been shown to stimulate ovogenesis in females and the development of accessory glands in males (Schneiderman and Gilbert 1959; Gilbert 1964; Wigglesworth 1966, 1970). It is interesting to note that neck-ligated tobacco hornworm larvae exhibit a delayed pupation even though PTTH cannot reach the prothoracic glands (due to the ligation) and induce them to produce α-ecdysone (Gibbs and Riddiford 1977). While "leaky" prothoracic glands have been held responsible for the larval-pupal molt in this instance, research confirming this hypothesis has not been forthcoming.

Wigglesworth (1934) was the first to suggest that hormones controlling insect growth and metamorphosis also played a role in diapause. Williams (1946) later showed that Cecropia moth (Hyalophora cecropia) diapause was initiated by the failure of the brain's neurosecretory cells to secrete a brain hormone, PTTH. A similar inactivity of the brain's neurosecretory cells was linked to the onset of diapause in the wheat stem sawfly (Cephus cinctus) (Church 1955) and the Lime hawk-moth (Mimas tiliae) (Highnam 1958). In the European corn borer, it appears that diapause is initiated by a block in the release of PTTH rather than by

its lack of production (Cloutier et al. 1962; McLeod and Beck 1963a). More recently, in insects exhibiting a larval diapause, evidence has been presented for the role of an intermediate titre of JH in arresting morphogenesis by inhibiting the transport or release of PTTH (Yagi and Akaike 1976; Chippendale and Yin 1973, 1976; Yin and Chippendale 1973, 1974, 1976). When JH titre has been reduced and PTTH is released into the blood in sufficient concentration to stimulate the prothoracic glands, diapause is terminated and pupation ensues. Takeda (1978), from electron micrograph studies, provided additional evidence for corpora allata control over PTTH production in the diapausing slug worm (Monema flavescens). He found a significant decrease in nuclear volume in neurosecretory B cells in the pars intercerebralis (associated with a build-up of neurosecretory material) with corpora allata extirpation.

Yin and Chippendale (1975) have confirmed that insect prothoracic glands (PTG) are inactive during diapause by comparing PTG ultrastructure in diapausing and non-diapausing Southwestern corn borer (Diatraea grandiosella) larvae. They found that in the former, the cells are bounded by a thin membrane, have large nuclei, and have glycogen particles evenly dispersed through the cytoplasm; while in the latter, the cells are bounded by a thick membrane, have shrunken convoluted nuclei, and have an inner cytoplasmic zone of

glycogen particles. No mention was made concerning the time of day of these observations. It is possible that there are 24 hour rhythms in these structural changes.

That the mechanism of JH control over the neurosecretion of PTTH is somewhat different from that causing pupation in last instar larvae is shown by the fact that the cessation of JH production in non-diapausing Southwestern corn borers (Yin and Chippendale 1976) and tobacco hornworms (Nijhout 1975) does not immediately initiate the production of PTTH and pupation. In the Southwestern borer, JH production ceases within 24 hours after ecdysis to the fifth instar while pupation does not occur until later in this instar. In the tobacco hornworm, premature elimination of the source of JH (by extirpation of the corpora allata) in the final larval instar was not a sufficient stimulus to initiate pupation.

While generalizations concerning the role of hormones in controlling insect growth and metamorphosis have been made across insect orders, families, genuses, and species, such generalizations should be made with caution. As Judy (1974) points out, each species fits into a certain niche; and therefore, slight differences in metabolism and its control must be expected even in closely related species. Since the European corn borer was the experimental animal used in this study, it is appropriate at this time to discuss

the specific nature of its neuroendocrine structures and their control over corn borer development and metamorphosis.

Figure 8, from McLeod and Beck (1963b), provides a lateral view of the brain and other neuroendocrine organs in the head of the European corn borer larva. Their arrangement is similar to that found in other lepidopterans except for the fact that the corpus allatum and corpus cardiacum are fused into a single structure, the corpus allatum-cardiacum complex (CAC), whereas in most other lepidopterans they are separate (Cazal 1948). The rice stem borer, Chilo suppressalis, was also found to have a CAC (Mitsuhashi and Fukaya 1960). In the European corn borer, the CAC are located one on each side of the head just posterior to the bilobed brain. Each complex contains seven large secretory cells, and is connected to the brain by two nerves, the nervus corporis cardiaca I (NCC I) and the nervus corporis cardiaca II (NCC II). While McLeod and Beck observed neurosecretory material in NCC I, they have never seen any in NCC II.

As shown in Figure 9, from McLeod and Beck (1963b), the brain is composed of 40 neurosecretory cells which can be divided into three types according to location--lateral, medial, and posterior. Upon examining these neurosecretory cells in diapausing and mature non-diapausing larvae, McLeod and Beck found no differences in either size or staining properties. Neither did they note any effect of long day

Fig. 8. Lateral view of the brain and neuroendocrine organs of the European corn borer. NCC I, nervus corporis cardiaca I; NCC II, nervus corporis cardiaca II. (From McLeod and Beck 1963b).

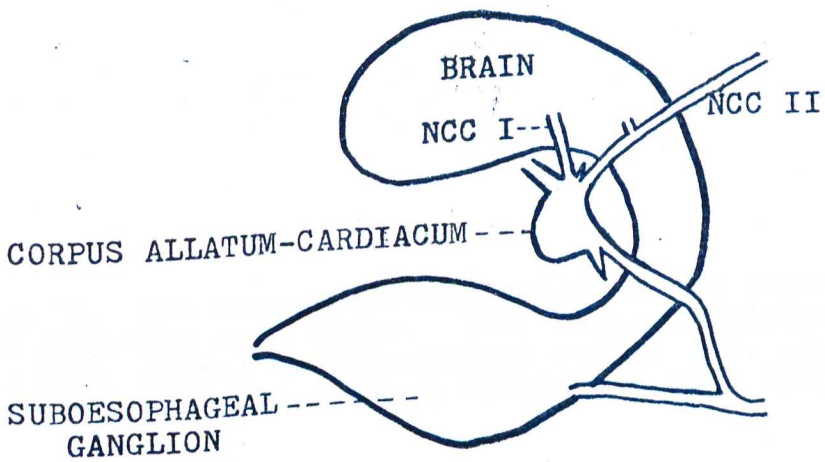
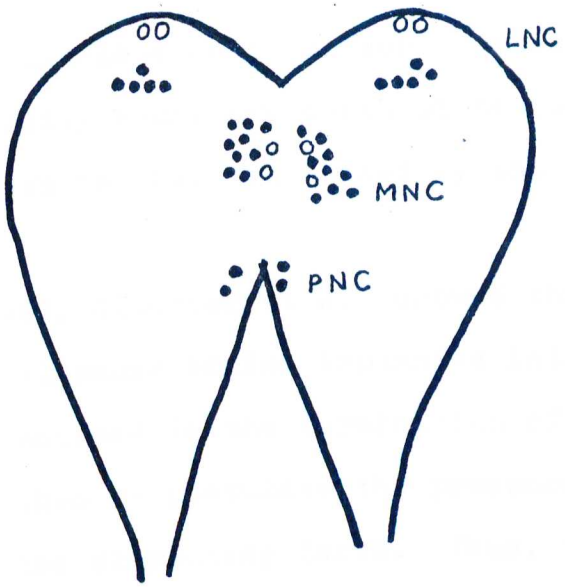

BRAIN
NCC I
NCC II
CORPUS ALLATUM-CARDIACUM
SUBOESOPHAGEAL GANGLION

Fig. 9. The neurosecretory cells of the larval brain of the European corn borer. LNC, lateral neurosecretory cells; MNC, medial neurosecretory cells; PNC, posterior neurosecretory cells. (From McLeod and Beck 1963b.)



oo NEUROSECRETORY CELLS

or short day photoperiods on neurosecretory cell structure or activity. Kono (1975), on the other hand, did find differences in the daily changes in activity of neurosecretory cells type II in short day and long day imported cabbageworm, Pieris rapae crucivora, larvae. (Pieris diapauses as a pupa.) He therefore concluded that photoperiodic time measurement of diapause induction in this insect depended upon the daily secretory cycle of neurosecretory type II cells which had been entrained by photoperiod during the larval stage.

Back in 1962, Cloutier et al. showed that in the European corn borer, diapause brains implanted into diapausing larval abdomens resulted in the termination of diapause. This finding led them to postulate the presence of a blood brain barrier in the diapausing larva. Thus, tissue damage during implantation upset the barrier and allowed the brain hormone (PTTH) to diffuse into the heamolymph. This in turn caused the termination of diapause. The results of Cloutier et al., then, offer a possible explanation for the lack of staining and size differences between the neurosecretory cells of diapausing and non-diapausing corn borer brains. Currently, it is held that the neurosecretory product necessary to stimulate the prothoracic glands and thus break diapause has already been produced before the induction of diapause, and that "a blood brain barrier" which prevents

its diffusion into the haemolymph is responsible for the onset of diapause. Houk and Beck (1975, 1976), based on an ultrastructural examination of diapause and non-diapause larval brains and horseradish peroxidase penetration studies, suggested that the perineurial type II[1] cells form the basis for the "blood-brain barrier," and that glial cells may also play an important role in the enzymatic regulation of this barrier. Their results supported those of McLaughlin (1974) and Eldefrawi et al. (1968), who carried out similar investigations on other insects.

In 1964, Beck and Alexander suggested that diapause development in the European corn borer was dependent on a physiological factor which they named proctodone. Proctodone was purported to be produced by epithelial cells of the anterior portion of the insect's hind gut. Furthermore, it was noted that the hormone's release was rhythmic, occurring only during the photophase of the photoperiod. However, Chippendale and Yin (1975) were unable to duplicate their results, and suggested instead, that the secretory activity of the ileum was due to lysosomal involvement in metamorphic reorganization. They did not, however, rule out a neural or neurosecretory role for the insect abdomen in the

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[1]The insect brain is ensheathed by a non-cellular neural lamella. Subjacent to the neural lamella is a bilayered perineurium; the outermost layer consists of perineurial type I cells and the inner layer is composed of perineurial type II cells. Glial cells underlie the inner membrane of the perineurial type II cells.

regulation of growth and metamorphosis, since there is a good deal of evidence suggesting that neural signals originating in the abdomen influence the brain's initiation of metamorphosis. (Edwards 1966; Beck 1970). And while receptors in the head are believed to receive the photoperiodic and thermal signals which regulate the onset and termination of diapause, it is possible that proprioceptive input from the abdomen might supplement this exteroceptive input.

The role of JH in initiating, maintaining, and terminating larval diapause has been well-documented (Fukaya and Mitsuhashi 1961; Yagi and Fukaya 1974; Chippendale and Yin 1976). In 1976, Yagi and Akaike showed that the larval diapause of the European corn borer too was regulated by JH. They reported prolonging the larval period with applications of JH and of accelerating pupation by allectomizing diapausing larvae. Thus, they provided an explanation for Beck and Shane's (1969) observation that diapause larvae injected with relatively high doses of α- or β-ecdysone formed larval-pupal intermediates. While at first Beck and Shane's results appeared to contradict those of Cloutier et al. (1962), it is possible that the larvae used by the latter researchers were in late diapause (low JH titre) and, therefore, pupated upon receiving implants of diapausing brains rather than forming larval-pupal intermediates.

JH titres are, then, at an intermediate level during early diapause and taper off during late diapause. Chippendale and Yin (1975), based on their work with the Southwestern corn borer, have suggested that it is the brain which interprets environmental signals and controls the levels of JH accordingly. When JH titres are sufficiently low, the brain is induced to release PTTH which stimulates the prothoracic glands to release ∝-ecdysone and initiate pupation. Therefore the brain's secretion of neurohormones not only regulates JH titres, but is also regulated by them (in activating the prothoracic glands). It is probable that a similar mechanism operates in the European corn borer and other insects which diapause as larvae. JH's role in manipulating the "blood-brain barrier" is not yet known.

A ROLE FOR CYCLIC AMP IN INSECT GROWTH AND METAMORPHOSIS

There is evidence which suggests an important role for the cyclic AMP system as part of the regulatory mechanism controlling insect growth and metamorphosis. Castillon et al. (1976 a,b) found marked changes in adenyl cyclase activity and in cyclic AMP levels in homogenized Mediterranean fruit fly, Ceratitis capitata, as the insect progressed through the larval and pupal stages. De Reggi and Cailla

(1975) found that cyclic AMP levels varied greatly during the postembryonic development of the fruit fly, Drosophila melanogaster. The formation of the puparium, larval-pupal apolysis, and pupal-adult apolysis was marked by especially rapid and drastic rises in cyclic AMP titres. Cyclic AMP also appeared to be involved in the stimulation of the prothoracic gland to produce α-ecdysone (Vedeckis et al. 1974, 1976), and in the action of bursicon in the hardening and darkening of the insect cuticle (Vandenberg and Mills 1974, 1975). Cyclic AMP phosphodiesterase activity, too, appears to vary considerably during insect development (Whitmore et al. 1973; Morishima 1973; Bielinska and Piechowska 1975; Catalan et al. 1975; Gelman and Hayes 1978). But, in most cases, the exact nature of the involvement of cyclic AMP in insect development is not known.

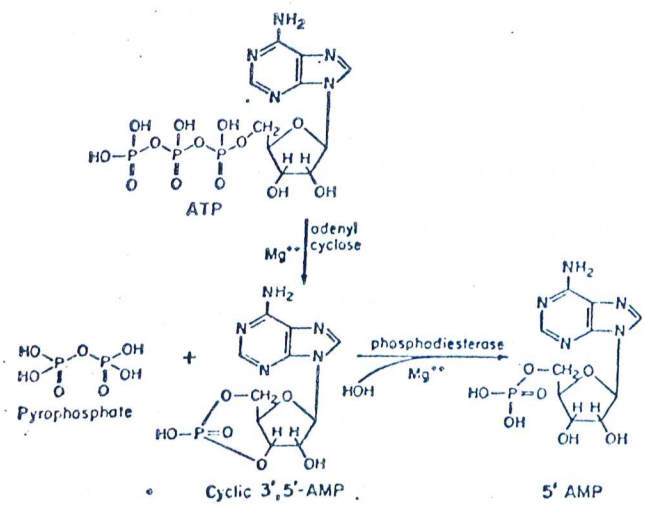
In vertebrate metabolism, however, the role of cyclic AMP in mediating hormone, neurohormone, and neurotransmitter action is fairly well understood due to the tremendous research effort in this area during the past decade. Since it has been suggested that cyclic AMP plays an important role in mediating these types of actions in insects and other invertebrates, it would be useful to discuss the mechanism of cyclic AMP action in vertebrate tissues.

Sutherland et al. (1965) originated the model in which cyclic AMP was portrayed as a second messenger in hormone

action. (See Figure 4.) Based on their earlier research, they concluded that cells of different organs contain receptor sites for a variety of hormones. As a result of hormone-receptor interaction, cyclic AMP was produced. This cyclic AMP, in turn, altered the rate of one or more cellular processes which resulted in an altered physiological response such as the production of a steroid or a second hormone.

As shown in Figure 10 from Weiss and Kidman (1969), adenyl cyclase is responsible for directing the synthesis of cyclic AMP from ATP (Sutherland et al. 1962). Cyclic AMP phosphodiesterase directs the breakdown of cyclic AMP to adenosine 5'-phosphate (5' AMP) (Butcher and Sutherland 1962). Thus the concentration of cyclic AMP at any given time is due to the relative activities of adenyl cyclase and cyclic AMP phosphodiesterase. Adenyl cyclase activity is totally particulate and localized in the cell membrane, whereas phosphodiesterase activity is largely soluble and occurs primarily in the cytoplasm (de Robertis et al. 1967; Weiss and Costa 1968b). However, Arch and Newsholme (1976) have pointed out that in invertebrates, two phosphodiesterases have been isolated, one with a low Km value and one with a high Km value. The low Km value diesterase was localized in the membrane while the high Km diesterase was found in the cytoplasm. While substances which increase the production of cyclic AMP may do so by stimulating adenyl cyclase

Fig. 10. The formation and breakdown of cyclic AMP. (From Weiss and Costa 1969.)



Synthesis and destruction of cyclic 3',5'-AMP.

activity and/or by inhibiting phosphodiesterase activity, the regulation of cyclic AMP concentration by physiological stimuli (e.g. hormones and neurotransmitters) is believed to occur mainly by influencing the activity of adenyl cyclase (Arch and Newsholme 1976).

Sutherland and his colleagues first discovered the importance of cyclic AMP as an intracellular mediator for the action of glucagon and epinephrine in the mammalian liver (Sutherland and Rall 1957; Rall et at. 1957). Since then cyclic AMP has also been implicated in the actions of adrenocorticotropic hormone (ACTH) (Haynes 1958), thyroid stimulating hormone (TSH) (Rall and Sutherland 1962), vasopressin (Handler et al. 1965), lutenizing hormone (LH) (Marsh et al. 1965), and many others. It is currently held that most, if not all, of the actions of cyclic AMP in mammalian systems are mediated in turn by the phosphorylation of proteins. Thus, the production of cyclic AMP activates a protein kinase. This activation results in the phosphorylation of a protein, which in turn, is directly responsible for the given physiological event.

In insects, too, cyclic AMP has been implicated in the action of several hormones. Vandenberg and Mills (1974, 1975) and Seligman and Doy (1972) have provided evidence which suggests a role for cyclic AMP in mediating the action of bursicon, a neurohormone necessary for cuticle

darkening and hardening. Filburn and Wyatt (1976) have reported measurable quantities of adenyl cyclase in Cecropia moth fat body, and have hypothesized that cyclic AMP is involved in enzyme regulation in a manner similar to that reported in vertebrate tissues. In Cecropia pupal wing epidermis, Applebaum and Gilbert (1972) have reported ecdysone-stimulated production of cyclic nucleotides. Berridge and Patel (1968) have linked the action of 5-hydroxytryptamine (5-HT) on insect salivary gland to cyclic AMP; and Prince et al. (1972) have purported that cyclic AMP is serving as an intracellular messenger in the action of this hormone. Cyclic AMP has also been shown to be involved in PTTH's tropic regulation of the production of α-ecdysone by the prothoracic glands of the tobacco hornworm (Vedeckis et al. 1974, 1976).

Within the last decade the cyclic AMP system has also been shown to have an important role in the functioning of the vertebrate and invertebrate nervous systems (Weiss and Kidman 1969; Greengard et al. 1973). According to Greengard (1976) cyclic AMP is involved in the mediation of the postsynaptic actions of certain neurotransmitters, in the regulation of their synthesis, and in the functioning of microtubules of the vertebrate brain.

At the synapse, Greengard (1976) suggests a role for cyclic AMP in mediating the postsynaptic actions of some neurotransmitters through the cyclic nucleotide-dependent

phosphorylation of specific membrane proteins. Thus, the neutrotransmitter released from presynaptic terminals activates a neurotransmitter-sensitive adenylate cyclase present in the postsynaptic membrane. The cyclic AMP which is produced, activates a cyclic AMP-dependent protein kinase which catalyzes the phosphorylation of the substrate protein. This phosphorylated protein changes the permeability of the postsynaptic membrane which in turn causes a change in the "post synaptic potential." The "post synaptic potential" is terminated by a phosphodiesterase and a phosphoprotein phosphatase which hydrolyze the cyclic AMP to 5'-AMP and convert the substrate protein back to the non-phosphorylated form respectively. In insects, a link between cyclic AMP and phosphorylase has been demonstrated in the cockroach nerve cord (Robertson and Steele 1972).

In presynaptic terminals, cyclic AMP-dependent kinases have also been found to regulate the synthesis of neurotransmitters. Thus cyclic AMP has been shown to activate tyrosine hydroxylase which in turn regulates the production of dopamine and norepinephrine (Goldstein et al. 1973; Harris et al. 1974).

In vertebrate brain tissue, cyclic AMP (Breckenridge and Bray 1970) and protein kinase have been implicated in the functioning of the microtubule system. Microtubules

from chick brain have been found to contain a cyclic AMP-dependent protein kinase which catalyzes the phosphorylation of a substrate protein (Sloboda et al. 1975). The relationship of this protein to microtubule function has not yet been determined.

The mechanism of action of cyclic AMP as a mediator, then, appears to be similar whether it is mediating hormone, neurohormone, or neurotransmitter action. In each case, cyclic AMP activates a protein kinase which catalyzes the phosphorylation of a substrate protein. This protein, in turn, is responsible for a given physiological response depending upon its place of action. While this mechanism cannot be labelled as universal, it is a good beginning assumption for all cyclic AMP-mediated metabolism.

Then too, whether involved in hormone, neurohormone, or neurotransmitter action, the production of cyclic AMP via adenyl cyclase activity has been found to be activator specific. For example, rat pineal gland adenyl cyclase is stimulated by l-norepinephrine but is not stimulated by polypeptide hormones such as glucagon, ACTH or lutenizing hormone which enhance the activity of adenyl cyclase in other organs. Neither was it stimulated by the biogenic amines, histamine, d-norepinephrine, serotonin or dopamine (Weiss and Costa 1968a). Usually, a given tissue receptor is only sensitive to a specific

activator as in the case of the pineal gland cited above. However, certain receptors have been found to be stimulated by more than one activator (Greengard et al. 1973), and a given activator has been shown to have an effect on several different receptors as, for example, the hormone norepinephrine on brain, pineal gland, hypophysis, salivary gland, ocular tissue, and oviduct or uterine tissue (Jost and Rickenberg 1971).

Fluoride appears to be an exceptionally potent non-specific activator of adenyl cyclase activity; but, the mechanism responsible for this activation is not understood (Sutherland 1972; Maguire et al. 1975). It has been hypothesized that adenyl cyclase is held in the membrane in an inhibited state, and that perhaps, the hormones, neurotransmitters, and/or fluoride act by counteracting this inhibition (Schramm and Naim 1970; Perkins and Moore 1971; Sutherland 1972).

Rojakovick and March (1972) were the first to demonstrate the presence of the enzyme adenyl cyclase in insect nerve tissue, specifically in the brains of the Madagascar cockroach, Gromphadorhina portentosa. Norepinephrine and epinephrine as well as fluoride ion were found to activate enzyme activity, while ecdysterone (a β-ecdysone) inhibited this activity. Since then adenyl cyclase activity has also been reported in the cockroach thoracic ganglion (Greengard et al. 1973) and in the tobacco hornworm (Manduca sexta)

larval brain (Vedeckis and Gilbert 1973). In the thoracic ganglion, three separate adenyl cyclase receptor units sensitive to dopamine, octopamine, and serotonin respectively were found. A norepinephrine-sensitive receptor was not uncovered, and it was therefore concluded that this activator partially stimulated the three receptors already identified. In the tobacco hornworm brain, β-ecdysone only behaved as an activator in the presence of the fluoride ion. It should be noted that while β-ecdysone stimulated adenyl cyclase production by tobacco hornworm brains, it inhibited enzyme production by cockroach brains. Several explanations could be offered for these seemingly contradictory results. First, Rojakovick and March's experimental animals were in the adult stage while Vedeckis and Gilbert used larvae. Since the insect brain is known to exhibit a regulatory effect on several different glands, it is plausible to expect that cyclic AMP might serve as a mediator in several different metabolic pathways. It follows that a particular hormone might act as an activator in one pathway and an inhibitor in another, especially at different stages of insect development. Another possibility to explain the difference observed, is the absence of fluoride ion in the incubation vessel used to determine the effect of β-ecdysone on adenyl cyclase activity in cockroach brains. Finally, it must be remembered that different insects have evolved somewhat different mechanisms

for the biochemical regulation of growth and metamorphosis. Insects which exhibit different types of metamorphoses such as the cockroach, a hemimetabolous insect, and the tobacco hornworm, a holometabolous insect, would be even more likely to exhibit such differences than those having similar life cycles.

Another possible role for cyclic AMP in controlling insect growth and metamorphosis is its involvement in the conversion of environmental signals into biochemical ones. Rasenick et al. (1978) based on their results which suggest an association between the activation of secretory activity in the brains of diapausing Cecropia and oak silkworm pupae and transfer to long-day photoperiods, hypothesized that the increase in cyclic AMP represents a transduction of photoperiodic signals. They have localized this cyclic AMP "spike" in the median neurosecretory cells of the brain, cells which have been implicated as the source of PTTH. Houk and Beck (1977) have suggested that dopamine or an immediate metabolite may be involved in an endogenous time-measuring system and/or diapause induction-termination of the European corn borer. In light of the numerous reports of a link between dopamine and its immediate metabolites and cyclic AMP, it is reasonable to anticipate that a role for one or more neurotransmitters and for cyclic AMP in such a time-measuring system might surface.

It should be pointed out that cyclic AMP has been implicated in the light-regulated circadian rhythms of serotonin, serotonin N-acetyltransferase, N-acetylserotonin, melatonin, and noradrenaline[1] in rat pineal gland (Axelrod 1974). During the night, levels of serotonin N-acetyltransferase, (Klein and Weller 1970), N-acetylserotonin (Brownstein et al. 1973) and melatonin (Lynch 1971) rise, while the level of serotonin falls (Quay 1963). These rhythms are generated by the increased production of the neurotransmitter noradrenaline during the scotophase portion of the 24 hour cycle (Klein and Berg 1970; Brownstein and Axelrod 1974). According to Axelrod (1974), the circadian rhythm of noradrenaline appears to arise from a "biological clock" located in the hypothalamus. This "clock" is entrained by environmental lighting (photoperiod). Noradrenaline stimulates the production of N-acetyltransferase which in turn catalyzes the conversion of serotonin to N-acetylserotonin (Weissbach et al. 1960, 1961); hence, the increase in N-acetylserotonin titres and the decrease in serotonin titres. The noradrenaline stimulated production of N-acetyltransferase is mediated by cyclic AMP (Klein and Berg 1970). Melatonin levels also increase at night because N-acetylserotonin is rapidly converted into melatonin. It is especially noteworthy that the adenyl

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[1]The names norepinephrine and noradrenaline have both been used in referring to 3,4-Dihydroxyphenyl-ethanolamine HCl.

cyclase receptor becomes supersensitive to stimulation by noradrenaline at night, and yet the basal adenyl cyclase activity does not differ from that measured during the daylight hours (Romero and Axelrod 1974). This supersensitivity has been attributed to the decreased release of noradrenaline during the daytime. Since photoperiod is an important regulator of insect life cycles, it is possible that the intermediate steps between photoreception and the release of PTTH, or in the case of diapause the inhibition of the release of PTTH, might also involve both cyclic AMP and one or more of the biogenic amines, such as norepinephrine, dopamine, and octopamine.

Inasmuch as the timing mechanism is believed to be located in the insect head, it would be of interest to examine adenyl cyclase activity in larval heads of European corn borers which have been exposed to LD 16:8 (pupa-inducing) and LD 10:14 (diapause-inducing) photoperiods. In addition, since norepinephrine appears to be involved in the light-regulated circadian rhythm controlling melatonin synthesis in the rat pineal, since dopamine and its immediate metabolites have been implicated in an endogenous time-measuring system and/or diapause induction-termination in the European corn borer, and since dopamine and octopamine have been found to stimulate adenyl cyclase activity in insect nerve tissue, it would also be of interest to determine the

effects of the neurotransmitters, norepinephrine, dopamine and octopamine on this activity.

CHAPTER III

MATERIALS AND METHODS

The procedures described herein were designed to determine adenyl cyclase levels in variously-aged fifth instar larval heads of European corn borers exposed to long day (LD 16:8) or short day (LD 10:14) photoperiods, and to determine the effects of the neurotransmitters, norepinephrine, dopamine, and octopamine on this activity. This chapter will discuss:

1. the methods used to culture the corn borer larvae
2. the methods used to identify the various stages of the fifth instar larvae
3. the adenyl cyclase assay
4. the method used to calculate adenyl cyclase activity in terms of moles of cyclic AMP produced per head per 30 minutes and in terms of moles of cyclic AMP produced per mg protein per 30 minutes
5. the methods used for the statistical analysis of the results.

MAINTAINING CORN BORER LARVAE

The European corn borers used in these investigations were obtained from the European Corn Borer Laboratory, Ankeny, Iowa. In Ankeny, on Wednesday of each week, egg masses were placed in Mason jars containing the medium of Lewis and Lynch (1969). Jars were shipped on Friday and were received at this researcher's laboratory on Wednesday or Thursday of the following week. At the time of arrival, larvae were usually in the second instar.

On the day of arrival, the jars were randomly divided into two groups. One group was placed in a biological oxygen demand box (BOD) under a light regimen of LD 16:8, pupation-inducing; the other group was placed in a similar BOD box under a light regimen of LD 10:14, diapause-inducing. The temperature was maintained at 24° ± 1.5°C in both boxes.

IDENTIFICATION OF FIFTH INSTAR STAGES

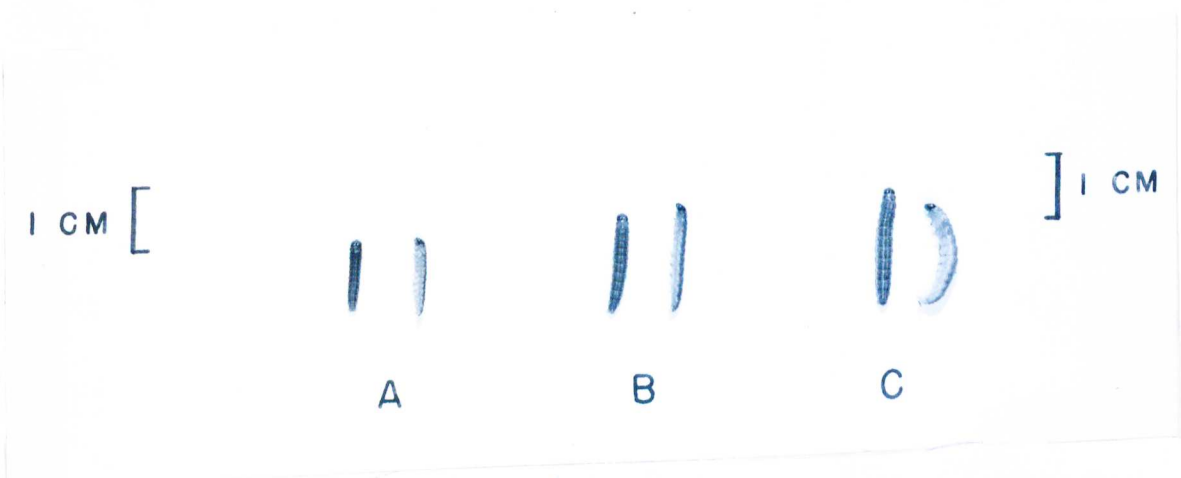
Fifth instar larvae were identified by their head capsule width of 1.7-2.1 mm (Gelman and Hayes 1978). For the purposes of this study, LD 16:8 (LD) fifth instar larvae were classified as early (ELD), middle (MILD), mature (MLD), early prepupa (EPP), late prepupa (LPP), and pharate pupa (PHP); while LD 10:14 (SD) fifth instar

larvae were classified as early (ESD), middle (MISD), early mature (EMSD), late mature (LMSD), early diapause (EDIAP), late diapause (LDIAP), and refrigerated diapause (RDIAP). Although exposure to cold is not necessary for the termination of diapause (Beck 1963), it was decided to refrigerate diapausing larvae under a photoperiod of LD 10:14 because diapause termination is facilitated in such refrigerated larvae (personal observation). Early, middle, and mature fifth instar larvae were identified by their body diameter as follows:

Early	1.0 - 1.5 mm in width
Middle	2.0 ± 0.3 mm in width
Mature	3.0 ± 0.3 mm in width

(See Figure 11.) Early mature larvae were two to three weeks old; late mature larvae were four to five weeks old. Prepupae were identified by the whitish color of their gut contents (Folsom and Wardle 1934). This color resulted from the emptying of the gut in preparation for pupation or diapause. Prepupae which had not enclosed themselves in silk were designated as early prepupae, while those which were encased in silk were designated as late prepupae. Pharate pupae were identified by their relative quiescence, light body color, reduced body turgidity, and sigmoid shape which was due to the forward curvature of the head into a somewhat tucked position (Hinton 1946).

Fig. 11. The early, middle, and mature fifth instar stages of the European corn borer.


1 CM
1 CM
A
B
C

LD 10:14 larvae which had not pupated by forty days of age and whose guts were white in color were considered to be in diapause. Larvae were considered to be in early diapause when they were 40 - 43 days of age, and in late diapause when they were 45 - 70 days of age. Early diapause larvae that had been placed in an LD 10:14 BOD box and kept at 4° ± 2°C from the age of 40 days to the age of 70 - 84 days before being sacrificed were designated refrigerated diapause larvae.

THE ADENYL CYCLASE ASSAY

Adenyl cyclase activity of the various fifth larval instar heads was measured by a modification of the method of Krishna et al. (1968). The assay is designed to determine the amount of radioactive cyclic AMP produced from radioactive adenosine triphosphate (ATP) due to the action of adenyl cyclase. The breakdown of the cyclic AMP produced is prevented by the addition of the cyclic AMP phosphodiesterase inhibitor, theophylline.

Since preliminary experiments on LD mature larval heads revealed that adenyl cyclase activity was very low in the absence of the fluoride ion, sodium fluoride (NaF) was added to the incubation mixture. Adenyl cyclase levels

were determined for homogenates of larval heads of each of the thirteen groups described above. Based on the results of these experiments, it was decided to determine the effects of norepinephrine, dopamine, and octopamine on adenyl cyclase activity in head homogenates of seven of these groups: mature LD, late prepupae, pharate pupae, late mature SD, early diapause, late diapause, and refrigerated diapause larvae. Again NaF was added to the incubation mixture. Inasmuch as it has been reported that NaF masks the stimulatory effects of certain activators (Sutherland 1972), adenyl cyclase activity of mature LD, late prepupal, pharate pupal, early mature SD,[1] late mature SD, early diapause, late diapause, and refrigerated diapause larval heads was also determined in the absence of NaF.

As an added control procedure, the adenyl cyclase assay was performed on two mature LD larval head homogenates in the absence of the phosphodiesterase inhibitor, theophylline, and on two other mature LD head homogenates in the absence of theophylline and in the presence of 10 µl of a 0.2% 3',5'-cyclic AMP phosphodiesterase solution.[2] Results were compared with those of controls containing

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[1] After examining the effects of the three neurotransmitters on adenyl cyclase activity of head homogenates of the other stages, it was decided to test their effects on head homogenates of early mature SD larvae.

[2] The phosphodiesterase was purchased from Sigma Chemical Company. It was dissolved in Tris buffer, pH 7.4.

theophylline but no phosphodiesterase. This was done to insure that the radioactive cyclic AMP formed during the 30 minute incubation period was produced as a result of adenyl cyclase activity and not as a result of some other metabolic reaction.

To prepare each larval homogenate, three larvae were decapitated.[1] Their heads were homogenized by hand in 0.6 ml of cold incubation mixture (prechilled to 0°C by placing in ice) in a 2.0 ml glass homogenizer with glass pestle. The incubation mixture contained $MgSO_4$ (6.6×10^{-3} M), NaF (2×10^{-2} M), dithiothreitol (10^{-3} M), bovine serum albumin (fraction V) (0.01%) and theophylline when desired (2×10^{-2} M) dissolved in 4.0×10^{-2} M Tris-HCl (tris (hydroxymethyl) aminomethane HCl) buffer, pH 7.4.[2,3] Two 0.1 ml aliquots of each homogenate were pipetted into 10 x 75 mm incubation tubes and allowed to come to room temperature. One of the tubes (the control) was then placed in a boiling water bath for 10 minutes. To determine the

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[1]All larvae were decapitated between the hours of 11 A.M. and 1 P.M.

[2]The results of preliminary experiments revealed that dithiothreitol and albumin were necessary for the expression of adenyl cyclase activity. These reagents appear to increase the stability of the enzyme.

[3]All the chemicals in the incubation mixture with the exception of the $MgSO_4$ and the Tris were purchased from Sigma Chemical Company. The $MgSO_4$ and the Tris were purchased from Fisher Chemical Company.

effect of norepinephrine, dopamine, or octopamine,[1] 15 µl of a 0.05 M solution of the neurotransmitter or for comparison, of Tris buffer (pH 7.4) were added to the homogenizer before homogenization.

At intervals of 20 seconds, 0.1 ml of ATP (3.0 - 4.0 x 10^{-3} M) (^{14}C - labeled ATP, specific activity 5 - 50 mc/mmol) was added to each incubation tube. The ATP was prepared by evaporating the solution containing the radioactive ^{14}C ATP (purchased from New England Nuclear Corp.) to dryness, and then adding 5.0 ml of 4 x 10^{-3} M non-radioactive carrier ATP.[2] Evaporation was carried out by blowing a gentle stream of nitrogen gas over the solution of ^{14}C ATP. The tubes were incubated for 30 minutes in a water bath at 34° ± 1°C. At the end of the 30 minute incubation period, 0.1 ml (0.5 mg) of a solution of carrier cyclic AMP[2] was added to each tube, and the reaction was stopped by placing the incubation tube in a boiling water bath for 3 minutes. The cyclic AMP was added so that the % recovery could be calculated following purification of the cyclic AMP. The cyclic AMP was isolated by chromatography on Dowex 50 - H^+ (Bio-Rad Laboratories, 50 W x 4 200 - 400 mesh) columns (0.4 by 3.3 cm) which were prepared

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[1] DL-norepinephrine HCl, DL-octopamine HCl, and dopamine were purchased from Sigma Chemical Company.

[2] The ATP and cyclic AMP were purchased from Sigma Chemical Company.

by pipetting 2.0 ml of a 50% (V/V) water suspension of the resin into columns and washing with distilled water. The 0.3 ml sample was carefully added to the column followed by a 1.7 ml distilled water wash. Elution was accomplished by carefully adding 2.0 ml portions of distilled water and collecting the second and third 2.0 ml fractions (which contained most of the cyclic AMP) in 15 ml conical centrifuge tubes. If the resin was disturbed by too forceful an addition, poor separation resulted. To the eluent were added 0.2 ml of $Ba(OH)_2$ (0.25 M) and 0.2 ml of $ZnSO_4$ (0.25 M) as described in Krishna et al. (1968). The tubes were centrifuged at 3500 rpm for 10 minutes. The $Ba(OH)_2$ - $ZnSO_4$ precipitation was repeated and the tubes were centrifuged at the same speed for 30 minutes. The supernatant was decanted and 2.0 ml of supernatant were added to 15.0 ml of Hydrofluor scintillation cocktail for counting in a Beckman liquid scintillation counter. Standards were prepared by adding 2.0 milliliters of appropriately diluted ^{14}C - labelled ATP to 15.0 ml of scintillation cocktail. Blanks were prepared by adding 2.0 ml of distilled water to 15 ml of scintillation cocktail.

Cyclic AMP recovery for each sample was calculated by comparing the moles of cyclic AMP in each supernatant with the moles of cyclic AMP in the 0.1 ml of solution added to each incubation tube just prior to boiling. The

absorption at 260 mμ of an aliquot of the desired solution or supernatant was used as a measure of the moles of cyclic AMP present.

CALCULATION OF ADENYL CYCLASE ACTIVITY

Moles of cyclic AMP produced per sample per 30 minutes were calculated as follows. The counts per minute (cpm) of each sample were corrected for % recovery by dividing total sample cpm by the % recovery for the sample. The corrected cpm for the control tube was then subtracted from that of the experimental tube. The number of moles of cyclic AMP produced was calculated from the equation:

$$\text{Moles of ATP in unknown} = \text{Moles of ATP in standard} \times \frac{\text{cpm of unknown}}{\text{cpm of standard} - \text{cpm of blank}}$$

(The moles of ATP in the standard were ascertained by measuring its absorption at 260 mμ.) Multiplying the moles of ATP in the sample by the dilution factor, 4.8, yielded the number of moles of cyclic AMP produced per head per 30 minutes.

In order to negate the effects of changes in protein content of heads on adenyl cyclase levels, adenyl cyclase activity was also expressed in terms of moles of cyclic AMP produced per mg protein per 30 minutes. To assay for

protein content of heads in any given group, one head was homogenized in 1.2 ml of 1.0 N sodium hydroxide. Duplicate 0.1 ml aliquots were pipetted, and protein concentration was determined by the method of Lowry et al. (1951). Four separate determinations were carried out for each of the thirteen larval stages. The number of moles of cyclic AMP produced per head per 30 minutes was then divided by the mg protein per head in order to determine the number of moles of cyclic AMP produced per mg protein per 30 minutes.

STATISTICAL ANALYSIS

To determine if there were any significant differences in adenyl cyclase activity in head homogenates of the thirteen stages tested, a thirteen group one way ANOVA ($\alpha = 0.05$) was performed. Since the F ratio was significant, the Student Newman-Keuls procedure, as described in Winer (1962), was used to determine wherein the significant differences lay. A one way ANOVA ($\alpha = 0.05$) was also used to ascertain if there were significant differences in the protein content of heads of the thirteen larval stages investigated. Since the F ratio was significant, the Student Newman-Keuls Procedure was used to determine wherein the significant differences lay.

A two way ANOVA ($\alpha = 0.05$) was used to determine if norepinephrine, dopamine and/or octopamine in the presence of NaF caused significant changes in adenyl cyclase activity in head homogenates of LD mature, late prepupal, pharate pupal, late SD mature, early diapause, late diapause and refrigerated diapause fifth instar larvae. A two way ANOVA ($\alpha = 0.05$) was also employed to determine if in the absence of NaF any of the three neurotransmitters caused significant changes in adenyl cyclase activity in head homogenates of LD mature, late prepupal, pharate pupal, early SD mature, late SD mature, early diapause, late diapause and refrigerated diapause fifth instar larvae. Depending upon the significance of F for main effects and for interaction, the Student Newman-Keuls procedure was used to determine wherein the significant differences lay.

CHAPTER IV

RESULTS

COMPARISON OF ADENYL CYCLASE ACTIVITY IN HEAD EXTRACTS OF VARIOUS-AGED FIFTH INSTAR EUROPEAN CORN BORER LARVAE EXPOSED TO PHOTOPERIODS OF EITHER LD 16:8 (LD) OR LD 10:14 (SD)

Adenyl cyclase levels of homogenates of heads of the thirteen groups of fifth instar larvae described in Chapter III are compared in Figure 12.[1] Adenyl cyclase activity is expressed as moles of cyclic AMP produced per head per 30 minutes. Each mean represents the average of fifteen separate determinations. Using the error mean square as the best estimate of variance, the standard error for each group is ± 1.0. The standard error for each group which has been indicated in Figure 12 has been calculated from the standard deviation of that group. As can be seen, in both LD and SD insect heads, adenyl cyclase activity in the presence of sodium fluoride increases as the larvae progress from early fifth through middle fifth to mature fifth instar. However, in LD heads, activity decreases as the larvae progress through the prepupal stage and is at its lowest level in the pharate pupa, whereas in SD heads, activity reaches a peak in early diapause and then in late diapause declines to the level exhibited by mature larval heads. The results of a one way ANOVA

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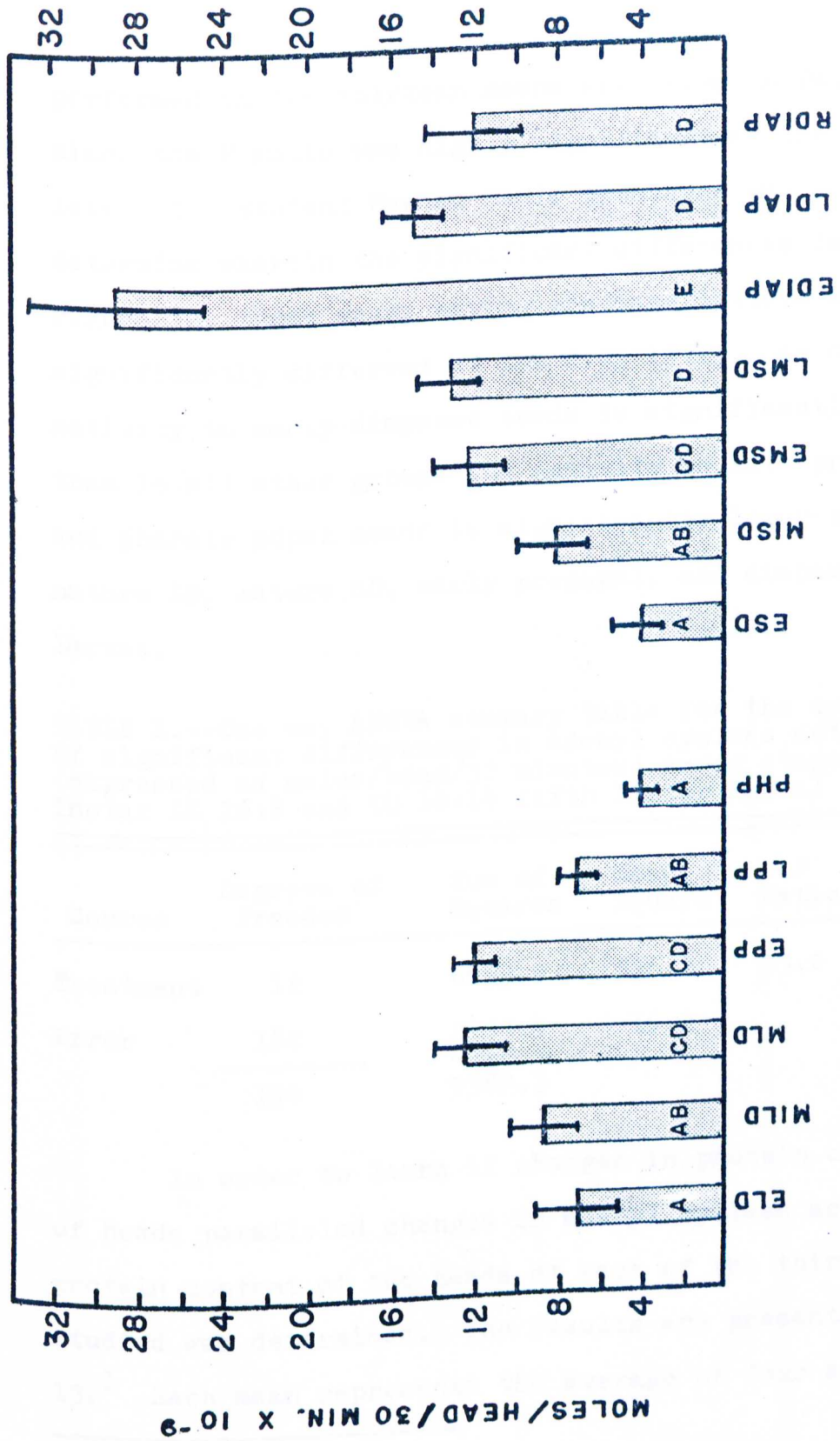
[1] In Table 9 of the Appendix, the individual values used in the determination of each group mean are listed.

Fig. 12. Adenyl cyclase activity in head extracts of LD 16:8 and LD 10:14 fifth instar European corn borer larvae expressed as moles/head/30 minutes. ELD, early long day; MILD, middle long day; MLD, mature long day; EPP, early prepupa; LPP, late prepupa; PHP, pharate pupa; ESD, early short day; MISD, middle short day; EMSD, early mature short day; LMSD, late mature short day; EDIAP, early diapause; LDIAP, late diapause; RDIAP, refrigerated diapause. Stages having the same letters (within the bars) are not significantly different at the 0.05 level.


32
28
24
20
16
12
8
4
RDIAP
LDIAP
EDIAP
LMSD
EMSD
MISD
ESD
PHP
LPP
EPP
MLD
MILD
ELD
D
D
E
D
CD
AB
A
A
AB
CD
CD
AB
A
LD 10:14
LD 16:8
STAGES
MOLES/HEAD/30 MIN. X 10⁻⁹

performed on the thirteen means are shown in Table 1. Since the F ratio was highly significant at the 0.05 level, the Student Newman-Keuls procedure was used to determine wherein the significant differences lay. In Figure 12, those means having the same letters are not significantly different at the 0.05 level. As can be seen, activity in early diapause heads is significantly higher than in all other groups and activity in late prepupal and pharate pupal heads is significantly lower than in mature LD, mature SD, early prepupal, and diapausing larvae.

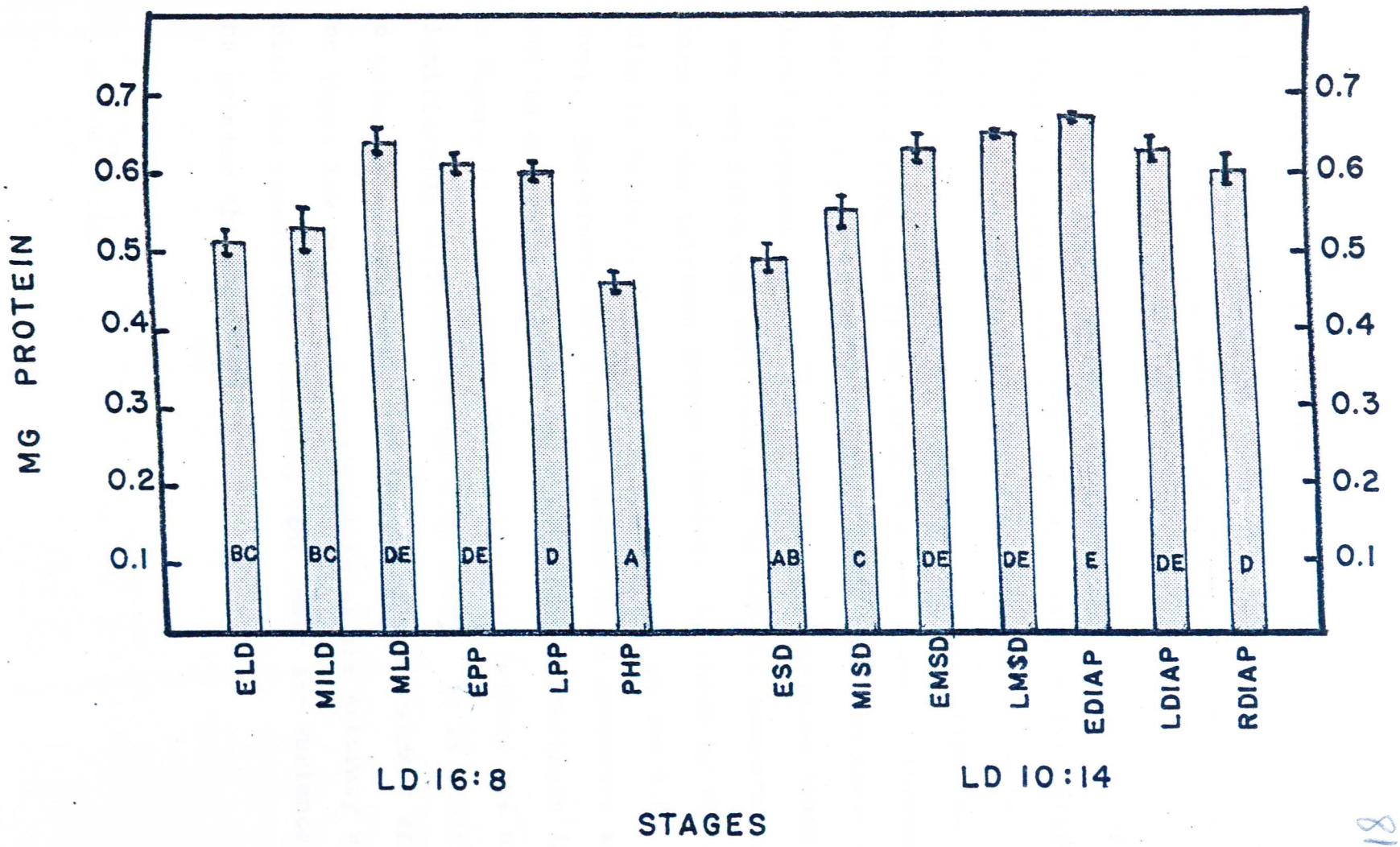
TABLE 1.--One way ANOVA summary table for the determination of significant differences in adenyl cyclase activity (expressed as moles/head/30 minutes) among stages of fifth instar LD 16:8 and LD 10:14 fifth instar larval heads

Source	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio	F Probability
Treatment	12	6433.9	536.2	33.0	0.000
Error	182	2953.4	16.2		
	194	9387.3			

In order to learn if changes in protein concentration of heads paralleled changes in adenyl cyclase activity, the protein content of the heads of each of the thirteen groups studied was determined. The results are presented in Figure 13.[1] Each mean represents the average of four separate

[1] In Table 10 of the Appendix, the individual values used in the determination of each group mean are listed.

Fig. 13. Milligrams of protein in heads of LD 16:8 and LD 10:14 fifth instar European corn borer larvae. ELD, early long day; MILD, middle long day; MLD, mature long day; EPP, early prepupa; LPP, late prepupa; PHP, pharate pupa; ESD, early short day; MISD, middle short day; EMSD, early mature short day; LMSD, late mature short day; EDIAP, early diapause; LDIAP, late diapause; RDIAP, refrigerated diapause. Stages having the same letters (within the bars) are not significantly different at the 0.05 level.


MG PROTEIN
0.7
0.6
0.5
0.4
0.3
0.2
0.1
BC
BC
DE
DE
D
A
AB
C
DE
DE
E
DE
D
ELD
MILD
MLD
EPP
LPP
PHP
ESD
MISD
EMSD
LMSD
EDIAP
LDIAP
RDIAP
LD 16:8
LD 10:14
STAGES

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determinations. The standard error for each group has been indicated. Using the error mean square as the best estimate of variance, the standard error for each group may be restated as $\pm$ 0.02.

In LD heads, protein concentration appears at first to increase during the fifth instar, peaks in the mature larvae, and then decreases again and reaches a low in pharate pupae. In SD heads, protein concentration increases during the fifth instar and continues to increase reaching a peak in early diapause heads and then shows a slight decrease in late and refrigerated diapause heads. A one way ANOVA was performed on the protein concentration means of the thirteen groups studied. As shown by the results in Table 2, F was highly significant at the 0.05 level. Therefore, the Student Newman-Keuls procedure was used to determine wherein the significant differences lay. In Figure 13, those means having the same letters are not significantly different at the 0.05 level. It is important to note the exceptionally low values of the standard errors, for these low values are a reflection of the accuracy with which the insects were beheaded (the lower the variance, the greater the accuracy).

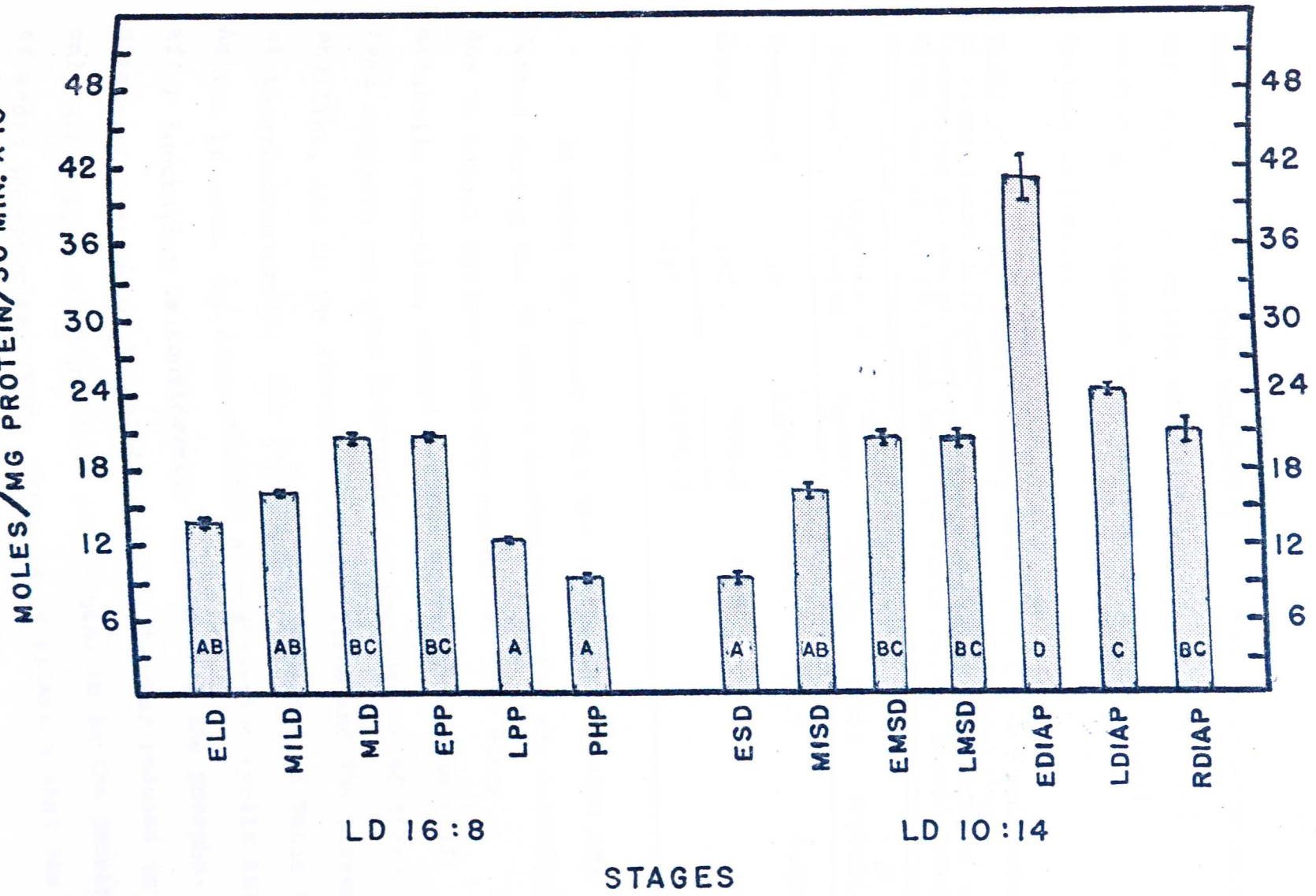
TABLE 2.--One way ANOVA summary table for the determination of significant differences in protein concentration among stages of fifth instar LD 16:8 and LD 10:14 fifth instar larval heads

Source	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio	F Probability
Treatment	12	0.224	0.019	21.136	0.000
Error	39	0.035	0.001		
	51	0.259			

In Figure 14, adenyl cyclase activity for each of the thirteen groups is expressed as number of moles of cyclic AMP produced per mg protein per 30 minutes.[1] The standard error for each group has been indicated. Using the error mean square as the best estimate of variance, the standard error for each group may be restated as $\pm$ 1.7. Since a one way ANOVA yielded a significant F value at the 0.05 level (see Table 3), the Student Newman-Keuls procedure was again utilized to determine wherein the significant differences lay. In Figure 14, those means having the same letters are not significantly different at the 0.05 level. Overall results are almost identical to those shown in Figure 12 except for the refrigerated diapause group which is no longer significantly different from the

[1] In Table 11 of the Appendix, the individual values used in the determination of each group mean are listed.

Fig. 14. Adenyl cyclase activity in head extracts of LD 16:8 and LD 10:14 fifth instar European corn borer larvae expressed as moles/milligram protein/30 minutes. ELD, early long day; MILD, middle long day; MLD, mature long day; EPP, early prepupa; LPP, late prepupa; PHP, pharate pupa; ESD, early short day; MISD, middle short day; EMSD, early mature short day; LMSD, late mature short day; EDIAP, early diapause; LDIAP, late diapause; RDIAP, refrigerated diapause. Stages having the same letters (within the bars) are not significantly different at the 0.05 level.


MOLES/MG PROTEIN/30 MIN. X 10⁻⁹
48
42
36
30
24
18
12
6
AB
AB
BC
BC
A
A
A
AB
BC
BC
D
C
BC
ELD
MILD
MLD
EPP
LPP
PHP
ESD
MISD
EMSD
LMSD
EDIAP
LDIAP
RDIAP
LD 16:8
LD 10:14
STAGES

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middle SD group. This similarity indicates that accompanying differences in protein concentration of heads are not sufficient to explain the observed changes in adenyl cyclase activity.

TABLE 3.--One way ANOVA summary table for the determination of significant differences in adenyl cyclase activity (expressed as moles/head/milligram protein) among stages of fifth instar LD 16:8 and LD 10:14 fifth instar larval heads

Source	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio	F Probability
Treatment	12	11847.4	987.3	22.7	0.000
Error	182	7900.8	43.4		
	194	19748.2			

In order to insure that the radioactive cyclic AMP formed during the 30 minute incubation period was actually due to adenyl cyclase activity and not to some other metabolic reaction, adenyl cyclase activity in mature LD head extracts was also determined in the absence of theophylline, and in the absence of theophylline and the presence of phosphodiesterase. The results are presented in Table 4. As can be seen, the concentration of radioactive cyclic AMP after incubation is considerably reduced when the phosphodiesterase inhibitor is omitted, and is further reduced to only 3.0×10^{-9} moles per head per 30 minutes in the presence of added phosphodiesterase. Thus, it is apparent that the assay is measuring adenyl cyclase activity.

TABLE 4.--Adenyl cyclase activity of head extracts of mature LD 16:8 fifth instar larvae in the absence of theophylline and in the absence of theophylline and the presence of phosphodiesterase

	(Moles/Head/30 Minutes)		
	Theophylline (Control) x 10^{-9}	No Theophylline x 10^{-9}	No Theophylline, Phosphodiesterase Added, x 10^{-9}
	14.7	5.1	2.7
	9.7	6.1	3.4
Mean	12.2	5.6	3.1

EFFECTS OF NOREPINEPHRINE, DOPAMINE, AND OCTOPAMINE ON ADENYL CYCLASE ACTIVITY IN HEADS OF MATURE LD, LATE PREPUPAE, PHARATE PUPAE, LATE MATURE SD, EARLY DIAPAUSE, LATE DIAPAUSE, AND REFRIGERATED DIAPAUSE FIFTH INSTAR LARVAE IN THE PRESENCE OF SODIUM FLUORIDE

Since adenyl cyclase activity peaked in early diapause heads and decreased markedly in late prepupal and pharate pupal heads but not in late diapause heads, it was

decided to investigate the effects of norepinephrine, dopamine, and octopamine on head extracts of the seven groups specified. The inability of these neurotransmitters to influence adenyl cyclase activity in the presence of sodium fluoride is shown by the results in Table 5.[1] The mean for each of the twenty-eight groups (seven stages x four activators)[2] and its standard error are indicated. Each mean represents the average of nine separate determinations. Using the error mean square as the best estimate of variance, the standard error for each group (cell mean) may be restated as ± 1.9. The results of a two way ANOVA are shown in Table 6. Only the main effect due to stages was significant at the 0.05 level. Based on the results shown in Figures 12 and 14, it was expected that this main effect would be significant.

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[1] In Table 12 of the Appendix, the individual values used in the determination of each group (cell mean) are listed.

[2] For convenience, the term activator refers to the addition of norepinephrine, dopamine, octopamine or in the case of the control of Tris buffer to the incubation vessel.

TABLE 5.--A summary of the effects of norepinephrine, dopamine, and octopamine on adenyl cyclase activity of seven stages of LD 16:8 and LD 10:14 fifth instar larval heads in the presence of sodium fluoride*,**

	LD Mature	Late Prepupa	Pharate Pupa	Late SD Mature	Early Diapause	Late Diapause	Refrig. Diapause
Control	13.7 ± 1.2	8.5 ± 0.9	2.5 ± 0.3	15.0 ± 1.0	35.5 ± 4.4	14.9 ± 1.5	13.2 ± 1.5
Norepinephrine	13.7 ± 1.0	8.9 ± 1.0	3.2 ± 0.5	17.3 ± 2.5	29.0 ± 3.2	15.5 ± 1.5	12.7 ± 1.2
Dopamine	10.8 ± 0.9	9.1 ± 0.7	2.7 ± 0.4	16.9 ± 2.7	31.8 ± 3.2	14.7 ± 1.1	14.0 ± 1.3
Octopamine	14.4 ± 0.7	8.4 ± 1.0	3.2 ± 0.4	19.1 ± 2.3	36.8 ± 4.8	13.9 ± 0.9	13.6 ± 1.4

*All values are multiplied by 10^{-9} and are expressed as moles of ATP converted to cyclic AMP per head per 30 minutes.

**The standard error for each group is stated after the group mean.

TABLE 6.--Two way ANOVA summary table for the determination of the presence of main effects and interaction in the analysis of the effects of norepinephrine, dopamine, and octopamine on adenyl cyclase activity of seven stages of LD 16:8 and LD 10:14 fifth instar larval heads in the presence of sodium fluoride

Source	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio	F Probability
Stage	6	19058.5	3176.4	95.6	0.000
Activator	3	74.0	24.7	0.7	0.528
Interaction	18	442.3	24.6	0.7	0.768
Error	224	7446.4	33.2		
	251	27021.1			

EFFECTS OF NOREPINEPHRINE, DOPAMINE, AND OCTOPAMINE ON ADENYL CYCLASE ACTIVITY IN HEAD EXTRACTS OF LD MATURE, LATE PREPUPAL, PHARATE PUPAL, EARLY SD, MATURE, LATE SD MATURE, EARLY DIAPAUSE, LATE DIAPAUSE, AND REFRIGERATED DIAPAUSE FIFTH INSTAR LARVAE IN THE ABSENCE OF SODIUM FLUORIDE

Inasmuch as sodium fluoride (NaF) has been known to mask the effects of certain metabolites on adenyl cyclase activity, the effects of norepinephrine, dopamine, and octopamine on adenyl cyclase levels of head extracts of the eight stages listed above were also determined in the absence of NaF. Table 7 and Figure 15 show the results of

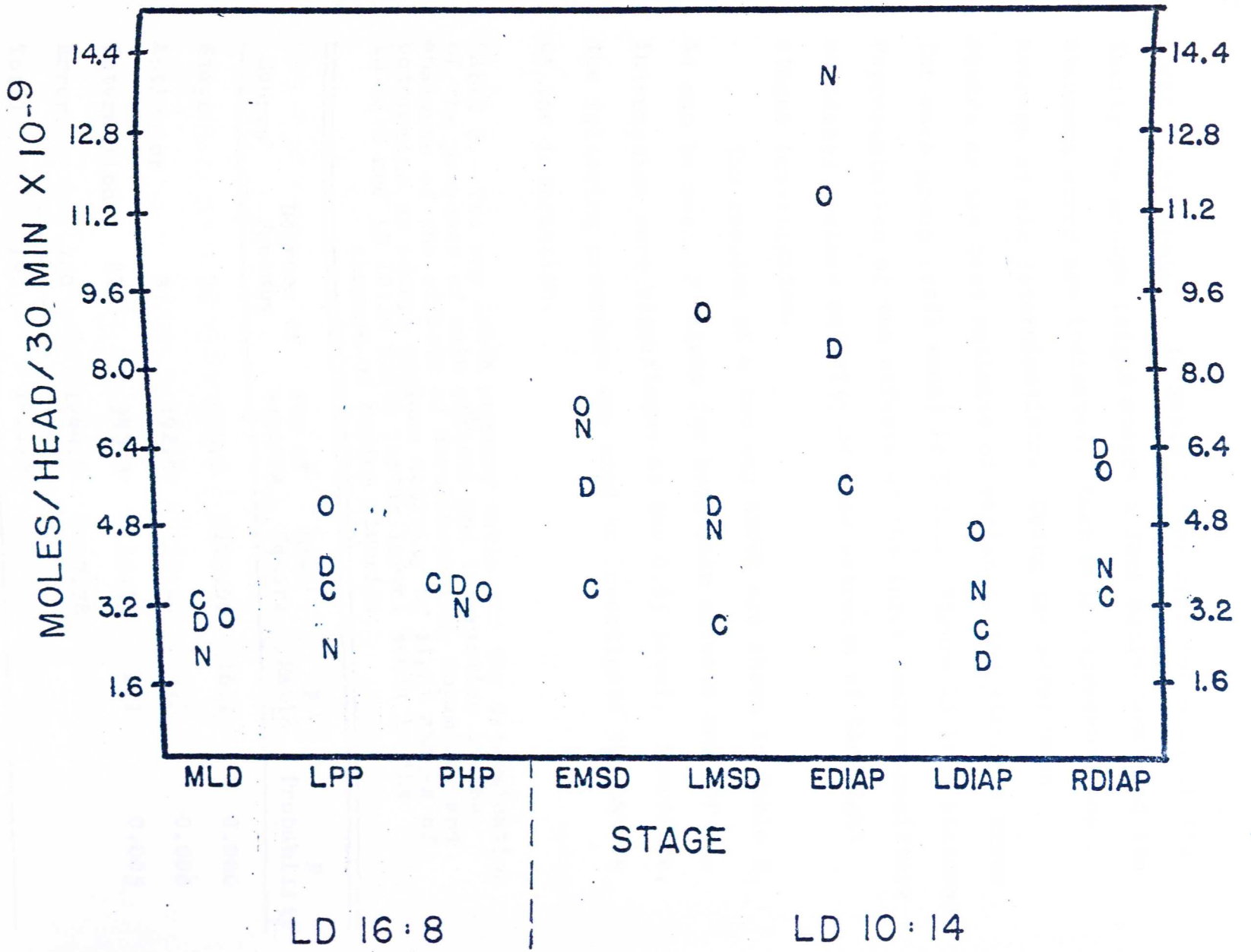
TABLE 7.--A summary of the effects of norepinephrine, dopamine, and octopamine on adenyl cyclase activity of eight stages of LD 16:8 and LD 10:14 fifth instar larval heads in the absence of sodium fluoride*,**

	LD Mature	Late Prepupa	Pharate Pupa	Early SD Mature	Late SD Mature	Early Diapause	Late Diapause	Refrig. Diapause
Control	3.2±0.6	3.4±0.5	3.3±0.7	3.4±0.4	2.8±1.1	5.5±1.6	2.9±0.6	3.6±0.6
	A a	A a	A a	A a	A a	A a	A a	A a
Norepinephrine	2.1±0.3	2.6±0.5	3.2±0.3	6.7±1.5	4.8±1.0	14.2±2.5	3.7±0.9	3.8±0.8
	A a	A a	A a	A a	A a	C b	A a	A a
Dopamine	3.0±0.6	3.9±0.7	3.6±0.5	5.5±0.7	5.2±1.2	8.4±2.0	1.9±0.7	6.5±0.6
	A a	A a	A a	A ab	A ab	AB b	A a	A ab
Octopamine	3.0±0.6	5.4±0.7	3.6±0.7	7.2±1.1	9.4±2.1	11.5±2.7	4.6±1.3	6.0±1.1
	A a	A ab	A a	A ab	B bc	BC c	A a	A ab

*All values are multiplied by 10^{-9} and are expressed as moles of ATP converted to cyclic AMP per head per 30 minutes. The standard error for each group is stated.

**Within each vertical column, those means having the same capital letter are not significantly different at the 0.05 level. Within each horizontal row, those means having the same lower case letter are not significantly different at the 0.05 level.

Fig. 15. Pictoral representation of the effects of norepinephrine, dopamine, and octopamine on adenyl cyclase activity of eight stages of LD 16:8 and LD 10:14 fifth instar larval heads in the absence of sodium fluoride. C, control; D, dopamine; N, norepinephrine; O, octopamine; MLD, mature long day; LPP, late prepupa; PHP, pharate pupa; EMSD, early mature short day; LMSD, late mature short day; EDIAP, early diapause; LDIAP, late diapause; RDIAP, refrigerated diapause.


MOLES/HEAD/30 MIN X 10-9
14.4
12.8
11.2
9.6
8.0
6.4
4.8
3.2
1.6
MLD
LPP
PHP
EMSD
LMSD
EDIAP
LDIAP
RDIAP
STAGE
LD 16:8
LD 10:14

these experiments.[1] In Table 7, the mean for each of the thirty-two groups (eight stages x four activators) and its standard error are indicated. Each mean represents the average of six determinations. Using the error mean square as the best estimate of variance, the standard error for each group (cell mean) is ± 1.1. Figure 15 is a pictoral representation of the effects of the three neurotransmitters on adenyl cyclase activity of head extracts of the eight stages investigated.

The results of a two way ANOVA are shown in Table 8. As can be seen, F values for both main effects and for interaction were significant at the 0.05 level. Therefore, the following procedure was used to investigate the nature of the interaction.

TABLE 8.--Two way ANOVA summary table for the determination of the presence of main effects and interaction in the analysis of the effects of norepinephrine, dopamine, and octopamine on adenyl cyclase activity of eight stages of LD 16:8 and LD 10:14 fifth instar larval heads in the absence of sodium fluoride

Source	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio	F Probability
Stage	7	875.6	125.08	16.1	0.000
Activator	3	192.6	64.20	8.3	0.000
Interaction	21	343.3	16.35	2.1	0.005
Error	160	1244.7	7.78		
Total	191	1411.5			

[1] In Table 13 of the Appendix, the individual values used in the determination of each group (cell mean) are listed.

To determine if the effects of the neurotransmitters were significantly different from one another within any of the eight stages, the Student Newman-Keuls procedure was carried out on the means of each column in Table 7. Within any given column, those means having the same upper case letters are not significantly different at the 0.05 level. As can be seen, only in late SD mature and early diapausing larval head extracts is adenyl cyclase activity capable of being significantly stimulated by one or more of the neurotransmitters tested.

The Student Newman-Keuls procedure was also used to determine if there were any significant differences among the control means shown in the first horizontal row of Table 7. Although adenyl cyclase activity of head homogenates of early diapause larvae appeared to be somewhat higher than that of the other seven stages studied, it was not significantly higher than the rest at the 0.05 level.

Finally, in order to further analyze the interaction and to ascertain if, for a given neurotransmitter, there were significant differences when that neurotransmitter was acting in conjunction with a given stage, the Student Newman-Keuls procedure was conducted separately on the means of each of the last three horizontal rows of Table 7. For each neurotransmitter, those means having the same lower case letter are not significantly different from one

another at the 0.05 level. As can be seen, norepinephrine in conjunction with early diapause head extracts elicits significantly higher adenyl cyclase activity than norepinephrine in conjunction with any of the other seven stages studied. Similarly, dopamine in conjunction with early diapause head extracts elicits significantly higher adenyl cyclase activity than dopamine in conjunction with LD mature, late prepupal, pharate pupal or late diapause head extracts. It should be noted that dopamine in combination with early SD mature, late SD mature, and refrigerated diapause head extracts while not yielding significantly higher adenyl cyclase levels than in combination with any of the three LD 16:8 head extracts, does yield somewhat higher enzyme levels. Octopamine, too, exhibits a differential activation of adenyl cyclase depending upon the stage investigated. Thus, when octopamine is used in combination with early diapause head extracts, adenyl cyclase levels are significantly higher than when it is used in combination with head extracts from any of the other stages studied except for those from late SD mature larvae. When octopamine is used in combination with late SD mature head extracts, adenyl cyclase levels are significantly higher than when it is used with LD mature, pharate pupal or late diapause head extracts. Finally, octopamine in combination with late prepupal, early SD mature, and refrigerated diapause head extracts while not yielding significantly higher adenyl cyclase levels

than in combination with LD mature, pharate pupal, and late diapause head extracts, does result in somewhat higher enzyme levels.

SUMMARY OF RESULTS

In head extracts of fifth instar European corn borer larvae reared under both LD 16:8 and LD 10:14 photoperiods, adenyl cyclase activity (in the presence of NaF), expressed either as moles of cyclic AMP produced per head or per mg protein, increased as the larvae progressed through early, middle, and mature stages. In LD 16:8 larval heads, activity decreased in late prepupae and reached a low in pharate pupae. In contrast, in LD 10:14 larval heads, adenyl cyclase activity peaked in early diapause and then returned to prediapause levels during late diapause. When NaF was eliminated from the incubation mixture, there were no longer any significant differences in adenyl cyclase activity among head extracts of the various stages studied.

In the absence of NaF, norepinephrine significantly enhanced adenyl cyclase activity only in early diapause larval head extracts. Octopamine significantly enhanced adenyl cyclase activity in head extracts of late SD mature and early diapause larvae, but not in head extracts of the

other six stages investigated. Dopamine did not significantly increase adenyl cyclase levels in head extracts of any of the eight stages investigated. In general, except for late diapause head extracts, a given neurotransmitter in combination with LD 10:14 larval head extracts resulted in higher adenyl cyclase levels than that neurotransmitter in combination with LD 16:8 head extracts. When sodium fluoride was present in the incubation vessel, none of the neurotransmitters enhanced adenyl cyclase activity.

CHAPTER V

DISCUSSION OF RESULTS AND RECOMMENDATIONS

In the presence of sodium fluoride (NaF), there were significant differences at the 0.05 level in the adenyl cyclase activity among early and late diapausing and various-aged fifth instar LD 16:8 (LD) and non-diapausing LD 10:14 (SD) larval heads of the European corn borer. (See Figures 12 and 14 and Tables 1 and 3.) Therefore, null hypothesis 1 which states that there is no difference in adenyl cyclase activity among early and late diapausing and various-aged fifth instar LD 16:8 (LD) and non-diapausing LD 10:14 (SD) larval heads of the European corn borer must be rejected. It should be stressed that the magnitude of the changes in adenyl cyclase activity of head extracts was not parallelled by similar magnitudes of change in the protein content of heads. This precludes the possibility that size variations alone were responsible for the observed differences in the adenyl cyclase levels of head extracts of the thirteen stages investigated.

In the presence of NaF, adenyl cyclase levels in early and middle LD larvae were significantly lower than those levels in mature LD and early prepupal larvae. Adenyl cyclase levels in early and middle SD larvae were also significantly lower than in late mature and all stages of

diapausing larvae. Thus, under both LD and SD conditions, adenyl cyclase activity was low at the beginning of the instar. In the prothoracic glands of Manduca sexta, adenyl cyclase levels were also reported to be relatively low during the early part of the fifth instar. (Vedeckis et al. 1976).

However, after the fifth instar larva has reached the mature stage, the photoperiodic regimen appeared to be quite important in influencing the levels of adenyl cyclase activity. Thus, in LD larval heads, adenyl cyclase activity was significantly lower in the late prepupa and pharate pupa than in the mature and the early prepupa. This indicates that cyclic AMP while probably having a role in the stimulation of PTTH and the initiation of pupation (Rasenick et al. 1976), may not be involved in pupation itself. In fact, it appears that the maintenance of higher levels of cyclic AMP in the mature larva may be in some way inhibitory toward the formation of the pupa. Catalan et al. (1976b) found that in whole homogenates of the fly, Ceratitis capitata, cyclic AMP levels which had reached a maximum during the third instar decreased towards the end of larval development and reached a minimum during apolysis (loosening of the exoskeleton prior to the larval-pupal molt). While there was not a similar decline in adenyl cyclase activity in Ceratitis capitata (Catalan et al. 1976a), there was a significant decrease in adenyl cyclase levels towards the

end of larval development and the onset of apolysis in European corn borer head homogenates. Catalan et al. (1976b) also found that cyclic GMP rose markedly just after apolysis and remained at a high level during the pupal stage. Therefore, they concluded that in the fly cyclic AMP plays a more important role than cyclic GMP in larval development and puparium formation while cyclic GMP rather than cyclic AMP mediates pupal development. It seems likely this is also the case in the European corn borer.

In corn borers exposed to SD photoperiods, adenyl cyclase activity peaked in early diapause heads. Since sodium fluoride was not capable of activating adenyl cyclase levels in head homogenates of stages other than early diapause insects to those levels reached by these insects, there is probably a non-NaF stimulated component of adenyl cyclase activity in early diapausing head extracts which does not exist in head extracts of the other stages. This hypothesis is supported by the somewhat higher adenyl cyclase level exhibited by early diapausing larval head extracts in the absence of NaF. (See Table 5.) However, the data was not strong enough to support a statement of significant difference in this case.

The relatively high standard error shown in Figure 12 for adenyl cyclase activity in early diapause insect heads suggests that there is a very narrow peak of adenyl cyclase

activity at this stage. In order to determine the age at which this peak occurs, the adenyl cyclase assay should be performed on head extracts of homogeneous (in regard to age) populations of early diapause larvae. Future experiments are planned in which early SD fifth instar larvae will be isolated within a few hours after ecdysis. The adenyl cyclase assay will then be performed on head homogenates of early diapause larvae for each age ranging from 20 to 28 days. (Age in this case will be determined from the time of ecdysis to the fifth instar.)

The fact that adenyl cyclase activity peaked in head extracts of early diapause larvae indicates that cyclic AMP is involved in some way in the onset of diapause. The exact nature of this involvement is open to speculation. Cyclic AMP might be involved in some way in the brain's activation of the corpora allata. Perhaps, in early diapause larval heads, the relatively high titres of adenyl cyclase result in increased levels of cyclic AMP which could be stimulatory to an as yet undetected "diapause hormone," the identity of which might be an allotropin. An allotropin is a hormone, typically secreted by the brain, which stimulates the corpora allata to produce JH. As was discussed earlier, JH levels appear to be maintained at an intermediate level in diapausing larvae, and these intermediate levels of JH are believed to be responsible for the

maintenance of diapause. Since adenyl cyclase activity in late and refrigerated diapause insect heads is significantly higher than in late prepupal and pharate pupal heads, it is possible that cyclic AMP might also play a role in maintaining this intermediate JH titre. Studies investigating adenyl cyclase levels in the corpora allatum-cardiacum complex in early and late diapausing corn borer larvae would shed more light on this possibility.

Then too, cyclic AMP could be involved in the inhibition of the release of PTTH by the brains of diapausing insects. Thus the intermediate levels of JH in diapausing larvae could be stimulating the production of adenyl cyclase. The resultant cyclic AMP produced would then be activating a peptide which is responsible for maintaining the blood brain barrier and preventing the release of PTTH. Therefore, the adenyl cyclase activity of brains of diapausing larvae should be determined and compared with activity in brains of other fifth instar stages. The effect of JH on this activity should also be investigated.

In the absence of NaF, adenyl cyclase levels of head homogenates of prepupae and pharate pupae were no longer significantly lower than those of LD mature, SD early mature, SD late mature, late diapause, and refrigerated diapause larvae. (See Table 5.) These results are not contradictory to those shown in Figure 12, if as Perkins (1973) has noted, the addition of NaF to the incubation

mixture may provide a measure of the maximum level to which it is possible to stimulate adenyl cyclase activity in the sample. Thus, in the presence of NaF, the low adenyl cyclase levels in late prepupal and pharate pupal head extracts may indicate that adenyl cyclase activity can be stimulated only slightly or not at all during the late prepupal and pharate pupal stages respectively, and further supports the idea that cyclic AMP may not play an important role in European corn borer pupation.

In the presence of NaF, null hypotheses 2, 3, and 4 which state:

2. There is no effect of norepinephrine, dopamine or octopamine on adenyl cyclase activity in selected stages of LD 16:8 (LD) and LD 10:14 (SD) fifth instar larvae

3. There is no difference in the effects of norepinephrine, dopamine and octopamine on adenyl cyclase activity within any one of the fifth instar stages selected

4. There is no difference in the effect of any one of the neurotransmitters on adenyl cyclase activity among the fifth instar stages selected

must be accepted at the 0.05 level. (See Tables 5 and 6.) However, in the absence of NaF, these null hypotheses must be rejected. (See Tables 7 and 8 and Figure 15.) Based on a comparison of the results in Tables 5 and 7, it is evident that NaF does mask the stimulatory effects of

norepinephrine and octopamine on adenyl cyclase activity in head homogenates of early diapause larvae, and the stimulatory effect of octopamine on adenyl cyclase activity of head homogenates of late SD mature larvae.

It is especially significant that norepinephrine only enhanced adenyl cyclase activity in early diapause head extracts and octopamine only enhanced adenyl cyclase activity in late SD mature and early diapause head extracts, for this specificity of activation implies a differential sensitivity of the adenyl cyclase receptor units during the different stages investigated. Since such a differential sensitivity to norepinephrine has been implicated in the light-regulated circadian rhythms of those metabolites involved in melatonin synthesis in the rat pineal, it is possible that this differential sensitivity in corn borers exposed to SD and LD photoperiods might be involved in the light-regulated mechanism responsible for the onset of diapause. Houk and Beck (1977) have implicated dopamine or one of its immediate metabolites in the maintenance of the blood brain barrier which is believed to be responsible for preventing the release of PTTH from the insect brain and thus maintaining diapause. Could the role of dopamine or one of its metabolites (i.e. norepinephrine or octopamine) be to activate an adenyl cyclase receptor unit which in turn is more sensitive to this activation in insects exposed to SD than in insects exposed to LD photoperiods?

It is also noteworthy that dopamine and octopamine in

conjunction with head extracts of early SD mature and refrigerated diapause head extracts resulted in somewhat higher adenyl cyclase levels as compared to those neurotransmitters in the presence of LD mature, pharate pupae and late diapause head extracts. While not significantly higher, these increased adenyl cyclase levels suggest that there is not only a role for cyclic AMP in diapause-associated reactions in late SD mature and early diapause larvae, as discussed previously, but that there is also a role for cyclic AMP in diapause-associated reactions in early SD mature and refrigerated diapause larvae. Rasenick et al. (1978) found that brains of diapausing Cecropia and Antheraea pernyi pupae which had been transferred to long day photoperiods had a higher cyclic AMP content than brains of similar pupae left under short day conditions. This led them to conclude that cyclic AMP mobilizes brain hormone (PTTH) and is thus involved in the diapause termination of these insects. If cyclic AMP is involved in activating PTTH, this could explain the somewhat higher adenyl cyclase activity in head extracts of late prepupae in the presence of octopamine.

It should be stressed that cyclic AMP is ubiquitous in insect tissue; therefore, a particular activator functions only in stimulating the production of cyclic AMP in a limited number of given tissues. Thus, for example, in early diapause insects, norepinephrine might stimulate the production of cyclic AMP in a particular region of the brain, and since barriers to diffusion exist, in other cephalic structures too. Octopamine might stimulate cyclic AMP production in a different

brain region and/or in a particular area of the corpora allatum-cardiacum complex. Cyclic AMP may, therefore, be involved in two or more separate but complementary metabolic reactions or pathways related to the diapause condition. This again points to the need for investigating the effects of norepinephrine, dopamine and octopamine on adenyl cyclase activity in isolated brains and parts thereof, in corpora allata-cardiaca complexes, and in other insect structures. Since NaF did stimulate adenyl cyclase activity in all stages of mature LD, prepupal, mature SD, and diapause larval head extracts, it would be desirable to test the effects of neurohormones and neurotransmitters (other than those used in these investigations) on adenyl cyclase activity in head extracts, brain extracts and corpora allata-cardiaca complexes.

Then too, it would be valuable to perform some "around the clock" studies on the European corn borer in which adenyl cyclase levels were determined at different times in the photophase and scotophase portions of the 24 hour day, for it is certainly plausible to expect that differences in activity do occur during each 24 hour cycle. Rasenick et al., (1978) based on preliminary experiments, have reported the presence of such a diurnal rhythm in brain cyclic AMP levels in Cecropia and Manduca sexta. Testing the activating effects of norepinephrine, dopamine, octopamine, and other neurotransmitters, as well as of appropriate hormones, neurohormones, and other peptides during different times of the 24 hour day might also prove to be fruitful.

Finally in the area of insect control, the findings described herein should be considered in the design of future control methods. Since European corn borers in diapause are more resistant to control procedures than other stages of European corn borers, and since one reason for this resistance is their overall low metabolic rate, the relatively active cyclic AMP system might provide a vulnerable point for attack against European corn borer infestation. And the assay described herein, which is capable of measuring adenyl cyclase levels in insect tissue in picogram quantities, would be useful in determining the effects of potential insect regulators on this system.

The results of the experiments described in this study support a link between adenyl cyclase activity and the onset, maintenance, and termination of diapause in the European corn borer. The nature of this link is open to speculation. Several possible mechanisms of action have been suggested, and the investigation of these mechanisms should provide several years of fruitful research for interested investigators.

A summary of the suggestions for future research follows:

1. a determination of the exact time in the early diapausing larval head when adenyl cyclase activity peaks

2. a comparison of adenyl cyclase levels of brains of various stages of European corn borers exposed to LD and SD photoperiods

3. a comparison of adenyl cyclase levels of corpus allata-cardiaca complexes of various stages of European corn borers exposed to LD and SD photoperiods

4. determination of the effects of appropriate neurotransmitters, hormones, neurohormones, and other peptides on adenyl cyclase activity of brains and corpora allata-cardiaca complexes

5. a performance of "around the clock" studies on adenyl cyclase activity in head homogenates, brains, and corpora allata-cardiaca complexes of the European corn borer, and a determination of the effects of appropriate neurotransmitters, hormones, neurohormones, and other peptides on this activity

6. a determination of the effects of potential insect growth regulators on adenyl cyclase activity in appropriate tissues of the European corn borer and other insect pests.

While the experiments reported in this study were directed solely at learning more about adenyl cyclase activity in various fifth instar stages of the European corn borer, it would be advantageous to learn if the cyclic AMP system is similarly linked to the diapause condition in other insects.

APPENDIX -- SUPPLEMENTARY TABLES

TABLE 9.--Adenyl cyclase activity (expressed as moles/head/30 minutes) in head extracts of LD 16:8 and LD 10:14 fifth instar larval heads,***

LD 16:8

Early LD	Middle LD	Mature LD	Early Prepupa	Late Prepupa	Pharate Pupa
2.8	9.0	10.1	11.0	5.2	4.9
1.8	9.9	7.9	8.1	4.7	3.5
2.4	7.6	10.1	12.3	7.6	2.9
3.7	6.6	7.9	12.4	5.0	3.8
6.3	9.4	14.1	11.9	7.8	2.8
11.7	11.4	15.3	10.6	6.6	3.6
11.4	3.8	18.1	15.1	5.8	3.5
14.0	16.8	15.2	11.7	6.8	3.7
13.3	8.5	19.0	12.9	9.2	3.4
8.4	4.9	15.0	16.4	11.3	4.7
4.8	7.5	12.8	9.3	7.2	4.0
10.1	7.1	8.9	11.9	8.6	5.5
3.1	7.2	12.5	11.7	10.5	7.9
4.1	6.0	14.7	11.7	5.8	6.8
6.9	8.6	10.9	12.7	8.5	3.2
Mean 7.0±1.1	8.3±0.8	12.8±0.9	12.0±0.5	7.4±0.5	4.3±0.4

TABLE 9.--Continued

LD 10:14

Early SD	Middle SD	Early Mature SD	Late Mature SD	Early Diapause	Late Diapause	Refrig. Diapause
5.2	8.5	9.6	10.8	19.7	14.7	7.6
5.3	8.6	9.6	15.2	24.5	14.2	14.7
1.5	7.9	14.5	13.3	19.0	21.4	8.2
2.0	6.4	14.8	10.6	19.7	18.1	6.0
4.3	9.9	22.4	11.2	23.6	12.2	19.8
5.6	5.0	13.5	13.3	40.0	15.6	15.6
6.6	16.5	8.2	11.2	34.3	19.1	16.0
7.5	7.6	13.7	13.9	48.4	14.5	14.9
4.4	8.0	10.4	21.1	39.6	16.4	18.6
8.0	14.3	11.4	15.2	19.5	15.2	9.5
8.1	5.6	11.9	14.7	26.3	12.7	14.1
1.8	9.4	10.9	11.9	27.9	14.9	7.5
1.4	8.8	14.6	15.3	35.4	11.8	10.5
1.3	7.1	16.4	10.8	18.2	12.2	7.6
6.0	9.9	10.9	10.6	16.1	11.4	17.9
Mean 4.6±0.6	8.9±0.8	12.9±0.9	13.2±0.7	27.5±2.5	15.0±0.7	12.6±1.2

*All values are multiplied by 10^{-9}.

**The standard error for each group is stated after the group mean.

TABLE 10.--Milligrams of protein per head of LD 16:8 and LD 10:14 fifth instar European corn borer larvae*

Stage	mg of protein	Stage	mg of protein
Early LD	0.474 0.498 0.522 0.546	Early SD	0.516 0.450 0.516 0.474
Mean	0.510±0.016	Mean	0.489±0.016
Middle LD	0.468 0.492 0.592 0.548	Middle SD	0.492 0.576 0.558 0.570
Mean	0.525±0.028	Mean	0.549±0.019
Mature LD	0.690 0.624 0.612 0.624	Early Mature SD	0.600 0.624 0.630 0.678
Mean	0.637±0.018	Mean	0.633±0.016
Early Prepupa	0.642 0.600 0.620 0.590	Late Mature SD	0.654 0.642 0.660 0.654
Mean	0.613±0.012	Mean	0.653±0.004
Late Prepupa	0.600 0.570 0.590 0.620	Early Diapause	0.670 0.670 0.670 0.670
Mean	0.600±0.010	Mean	0.67±0.000
Pharate Pupa	0.450 0.436 0.456 0.480	Late Diapause	0.642 0.642 0.612 0.612
Mean	0.456±0.009	Mean	0.627±0.009
		Refrig. Diapause	0.630 0.624 0.576 0.576
		Mean	0.601±0.015

*The standard error for each group is stated after the group mean.

TABLE 11.--Adenyl cyclase activity (expressed as moles/milligram protein/30 minutes) in LD 16:8 and LD 10:14 fifth instar larval heads,***

LD 16:8

Early LD	Middle LD	Mature LD	Early Prepupa	Late Prepupa	Pharate Pupa
5.5	17.1	15.8	17.9	8.7	10.8
3.5	18.9	12.4	13.2	7.9	7.7
4.7	14.5	15.8	20.1	12.8	6.4
7.3	12.6	12.4	20.2	8.4	8.3
12.4	17.9	22.1	19.4	13.1	6.1
22.9	21.7	24.0	17.3	11.1	7.9
22.4	7.2	28.4	24.6	9.7	7.7
27.5	32.0	23.8	19.1	11.4	8.1
26.1	16.2	29.8	21.0	15.5	7.5
16.5	9.3	23.5	26.8	19.0	10.3
19.8	14.3	20.1	15.2	12.1	8.8
6.1	13.5	14.0	19.4	14.5	12.1
8.0	13.7	19.6	19.1	17.6	17.3
13.5	11.4	23.1	19.1	9.7	14.9
9.4	16.4	17.1	20.7	14.3	7.0
Mean 13.7±2.1	15.8±1.5	20.1±1.4	19.5±3.3	12.4±3.3	9.4±3.2

TABLE 11.--Continued

LD 10:14

Early SD	Middle SD	Early Mature SD	Late Mature SD	Early Diapause	Late Diapause	Refrig. Diapause
10.6	15.5	15.2	16.5	29.4	23.4	12.6
10.8	15.7	15.2	23.3	36.6	22.6	24.4
3.1	14.4	22.9	20.4	28.4	34.1	13.6
4.1	11.7	23.4	16.2	29.4	28.9	10.0
8.8	18.0	35.4	17.2	35.2	19.5	32.9
11.5	9.1	21.3	20.4	59.7	24.9	25.9
13.5	30.1	13.0	17.2	51.2	30.5	26.6
15.3	13.8	21.6	21.3	72.2	23.1	24.8
9.0	14.6	16.4	32.3	59.1	26.2	30.9
16.4	26.0	18.0	23.3	29.1	24.2	15.8
16.6	10.2	18.8	22.5	39.3	20.3	23.4
3.7	17.1	17.2	18.2	41.6	23.8	12.5
2.9	16.0	23.1	23.4	52.8	18.8	17.5
2.7	12.9	25.9	16.6	27.2	19.5	12.6
12.3	18.0	17.2	16.2	24.0	18.2	29.8
Mean 9.4±1.3	16.2±1.4	20.3±1.4	20.3±1.1	41.0±3.8	23.9±1.2	20.9±2.0

*All values are multiplied by 10^{-9}.

**The standard error for each group is stated after the group mean.

TABLE 12.--Effects of norepinephrine, dopamine, and octopamine on adenyl cyclase activity of eight stages of LD 16:8 and LD 10:14 fifth instar larval heads in the presence of sodium fluoride*

	Control x 10^{-9}	Norepinephrine x 10^{-9}	Dopamine x 10^{-9}	Octopamine x 10^{-9}
Mature LD	15.1	12.5	7.4	11.9
	8.0	9.9	9.7	16.6
	14.7	14.3	9.5	11.3
	16.1	14.4	10.4	15.8
	16.3	12.2	9.0	16.5
	10.8	12.3	9.9	14.6
	14.4	18.5	11.5	16.0
	18.4	18.0	16.1	14.9
	9.7	11.5	13.3	12.4
Mean	13.7±1.2	13.7±1.0	10.8±0.9	14.4±0.7
Late Prepupa	10.7	8.3	10.7	11.4
	4.9	8.0	5.4	8.4
	6.6	15.0	10.9	4.9
	13.0	10.3	9.8	6.2
	5.1	8.0	8.7	8.9
	7.8	5.9	10.0	13.0
	11.0	5.1	6.0	4.6
	9.3	9.3	9.6	7.4
	8.3	9.8	10.9	10.8
Mean	8.5±0.9	8.9±1.0	9.1±0.7	8.4±1.0
Pharate Pupa	3.6	2.2	2.7	2.3
	1.5	1.4	3.6	2.4
	2.3	4.1	3.7	2.2
	1.8	4.8	2.6	3.1
	2.2	4.9	1.3	3.2
	2.6	4.9	1.4	5.1
	3.3	1.8	1.1	4.3
	1.4	3.1	3.4	2.0
	3.6	1.7	4.1	4.0
Mean	2.5±0.3	3.2±0.5	2.7±0.4	3.2±0.4

*The standard error for each group is stated after the group mean.

TABLE 12.--Continued

	Control x 10^{-9}	Norepinephrine x 10^{-9}	Dopamine x 10^{-9}	Octopamine x 10^{-9}
Late Mature SD	13.6	20.9	33.7	21.9
	19.2	13.2	24.4	21.3
	15.0	35.6	9.7	19.2
	19.9	15.9	14.0	35.3
	14.4	19.2	20.3	14.9
	16.9	15.1	10.3	12.2
	10.6	11.7	16.2	16.9
	13.8	12.9	11.9	15.8
	11.9	11.3	11.5	14.4
Mean	15.0±1.0	17.3±2.5	16.9±2.7	19.1±2.3
Early Diapause	52.5	31.1	26.9	43.0
	47.3	41.2	50.6	51.8
	34.9	37.3	28.6	58.3
	47.4	29.8	41.5	34.5
	46.4	38.7	34.4	46.9
	27.7	28.5	31.9	28.3
	25.7	24.0	30.2	32.0
	17.9	15.2	18.7	18.0
	19.7	14.8	23.3	18.1
Mean	35.5±4.4	29.0±3.2	36.8±3.2	36.8±4.8
Late Diapause	26.5	19.0	13.7	15.4
	16.0	13.0	14.3	12.4
	13.0	11.0	17.8	19.0
	11.0	25.8	20.6	13.5
	13.2	16.5	12.6	8.8
	14.0	11.1	10.1	12.5
	12.8	15.2	14.6	14.0
	12.9	14.6	17.2	13.8
	14.6	13.0	11.4	15.4
Mean	14.9±1.5	15.5±1.6	14.7±1.1	13.9±0.9
Refrig. Diapause	10.5	14.9	16.3	17.2
	19.6	12.1	8.8	17.4
	12.6	15.2	11.0	13.5
	15.8	10.7	13.2	12.7
	20.1	18.3	19.1	19.5
	12.9	13.5	16.4	13.7
	9.5	6.2	17.0	7.5
	11.2	9.5	8.4	12.5
	7.3	13.8	15.6	8.2
Mean	13.2±1.5	12.7±1.2	14±1.3	13.6±1.4

TABLE 13.--Effects of norepinephrine, dopamine, and octopamine on adenyl cyclase activity of eight stages of LD 16:8 and LD 10:14 fifth instar larval heads in the absence of sodium fluoride*

	Control x 10^{-9}	Norepinephrine x 10^{-9}	Dopamine x 10^{-9}	Octopamine x 10^{-9}
Mature LD	1.8	1.6	1.5	5.7
	1.8	0.8	1.6	3.4
	1.8	2.5	4.3	2.8
	4.0	2.5	3.4	2.2
	5.1	2.9	5.0	1.9
	4.6	2.4	2.4	1.8
Mean	3.2±0.6	2.1±0.3	3.0±0.6	3.0±0.6
Late Prepupa	3.3	1.2	3.9	5.0
	1.3	1.4	0.4	4.3
	4.4	4.3	5.5	3.9
	3.4	2.1	5.0	4.4
	4.3	2.9	4.1	6.0
	3.9	3.4	4.3	8.7
Mean	3.4±0.5	2.6±0.5	3.9±0.7	5.4±0.7
Pharate Pupa	5.6	4.4	3.9	2.6
	1.9	3.0	5.4	6.4
	4.0	2.9	3.4	2.6
	3.6	3.3	3.7	4.9
	3.7	3.3	3.9	3.4
	0.9	2.1	1.3	1.3
Mean	3.3±0.7	3.2±0.3	3.6±0.5	3.6±0.7
Early Mature SD	4.3	3.5	6.6	8.3
	3.2	7.0	6.0	4.4
	4.2	4.5	3.8	8.5
	4.2	9.7	2.8	6.0
	2.7	12.3	6.9	4.7
	2.0	3.4	7.0	11.1
Mean	3.4±0.4	6.7±1.5	5.5±0.7	7.2±1.1

TABLE 13.--Continued

	Control x 10^{-9}	Norepinephrine x 10^{-9}	Dopamine x 10^{-9}	Octopamine x 10^{-9}
Late Mature SD	0.0	3.1	2.6	5.5
	1.2	2.5	6.8	5.1
	1.7	7.4	4.9	5.2
	3.4	7.7	4.8	16.2
	3.1	2.6	2.1	15.0
	7.6	5.4	10.2	9.1
Mean	2.8±1.1	4.8±1.0	5.2±1.2	9.4±2.1
Early Diapause	3.0	18.1	11.0	20.5
	7.1	18.5	2.9	1.8
	2.9	6.2	5.3	11.5
	5.9	21.1	4.8	7.7
	1.9	11.6	15.6	11.2
	12.3	9.1	11.0	16.1
Mean	5.5±1.6	14.2±2.5	8.4±2.0	11.5±2.7
Late Diapause	1.1	4.2	4.7	10.9
	2.0	5.6	1.5	4.2
	1.7	4.3	2.8	4.5
	4.1	1.5	1.4	2.6
	4.8	0.4	0.3	2.6
	3.5	6.3	0.4	3.0
Mean	2.8±0.6	3.7±0.9	1.8±0.7	4.6±1.3
Refrig. Diapause	4.3	2.4	6.9	5.6
	5.7	3.3	7.9	4.4
	2.6	2.1	6.7	10.9
	3.4	7.6	4.7	4.7
	1.7	3.1	7.9	6.7
	3.6	4.1	5.0	3.9
Mean	3.6±0.6	3.8±0.8	6.5±0.6	6.0±1.1

*The standard error for each group is stated after the group mean.

LIST OF ABBREVIATIONS

AMP: adenosine monophosphate

ANOVA: analysis of variance

ATP: adenosine triphosphate

CAC: corpus allatum-(corpus) cardiacum complex

CPM: counts per minute

CYCLIC AMP: adenosine 3',5'-cyclic monophosphate

ELD: early long day

EDIAP: early diapause

EMSD: early mature short day

EPP: early prepupa

ESD: early short day

GMP: guanosine monophosphate

JH: juvenile hormone

LD: long day

LDIAP: late diapause

SMSD: late mature short day

LPP: late prepupa

M: molar

mc: millicurie(s)

mg: milligram

MH: molting hormone

MILD: middle long day

MISD: middle short day

ml: milliliter

MLD: mature long day

mmol: millimole

mμ: millimicron

μ: micron

μl: microliter

N: normal

NaF: sodium fluoride

PHP: pharate pupa

PTG: prothoracic glands

PTTH: prothoracicotropic hormone

RDIAP: refrigerated diapause

RPM: revolutions per minute

SD: short day

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