Insect Physiology and Biochemistry

M.F. B. Chaudhury

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INSECT PHYSIOLOGY AND BIOCHEMISTRY

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"Acquiring the capacity for the generation of scientific knowledge and the development of effective technology is a central issue for the sustainable development of Africa and other tropical developing regions of the world, and we are intimately engaged in this process in the area of insect science ..."

THOMAS R. ODHIAMBO
Molecular Entomology, page 16

PREFACE

The Manual for INSECT PHYSIOLOGY AND BIOCHEMISTRY has been expressly prepared for the insect physiology course given by the African Regional Postgraduate Programme in Insect Science (ARPPIS) at the International Centre of Insect Physiology and Ecology (ICIPE), Nairobi, Kenya. Although the manual is specifically written for the ARPPIS course, it will be useful for similar courses given at other institutes and universities in Africa and elsewhere.

The objectives of the insect physiology course are to give the students an understanding of the principles of insect physiology through discussions on classic and current research on structures and functions and to familiarize the students with various research methodology which are applied in the research of insect physiological problems.

The manual is divided into two sections: Lectures and Practicals. The lecture section presents synopses of 30 lectures of 15 general topics of insect physiology and biochemistry. These synopses are for the purpose of guidance only. On each lecture topic, the students will receive additional materials from the instructor, including handouts on figures, data, graphs, etc., references for additional reading, current research activities in the field and relevant research methodology. The practical section includes 40 exercises for both laboratory and field. During any one teaching term, the students will work on only 10 or 12 of these exercises. In addition, there will be opportunity to take up independent individual research projects by the students, which may last for the entire teaching term.

A major aim of this course will be to use, wherever possible, examples of tropical insects and the students will work, both in the field and the laboratory, on insects which are important in the tropics.

Because a number of areas in insect physiology and biochemistry are important and interesting from the point of active research in many laboratories around the world, knowledge in these areas are accumulating quickly. This will necessitate frequent revision of this manual, particularly in terms of additional references on new research findings. In spite of this fact, this manual will no doubt serve the students and the instructors effectively for four or five years.

M. F. B. CHAUDHURY
Nairobi, Kenya
December 1991

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Finally, I wish to thank the Pew Charitable Trusts for funding this worthy cause to move science education in Africa a few steps further.

M.F.B.C.

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GROWTH AND METAMORPHOSIS

Introduction

Since the publication of the classic paper by Stefan Kopec (1922) on the metamorphosis of the gypsy moth, *Lymantria dispar*, science has evolved from the elegant simplicity of Kopec's experiments to the technological sophistication of present-day biochemistry, immunology and molecular biology. Consequently, our knowledge of insect growth and development has increased tremendously over the past several decades.

The development of insects involves the integration and coordination of growth with shedding of the exoskeleton, the hard integument. The process is known as *metamorphosis*.

Lectures in this section will discuss the various types of metamorphosis in insects, the classical experiments as the basis of fundamental knowledge of insect growth and development, hormonal control of metamorphosis as seen from the classical scheme and as it is understood from the recent research findings. Lectures also will cover the chemistry and mode of action of various developmental hormones. References to be consulted for details on these topics are mainly those of Oberlander (1985), Riddiford (1985 and 1986) and those mentioned later on under each sub-topic.

Types of metamorphosis

The structure, form and appearance of the newly hatched insect differs extensively from that of the adult insect. Its development involves changes of form which may be the loss of juvenile structures as well as the differentiation of distinctively adult organs. The extent of these changes varies from group to group. Accordingly, there are mainly two types of metamorphosis, (1) incomplete metamorphosis or *hemimetabolous* development and (2) complete metamorphosis or *holometabolous* development.

Hemimetabolous development occurs in almost all Exopterigota. In this case, the immature stages, sometimes referred to as *nymphs*, usually resemble the adults in structural features and habits, and metamorphosis consists mainly of adult characters. The wings and genitalia appear at an early stage as external rudiments which increase in size and complexity with each successive instar. The simple gonad rudiments of the young stage gradually differentiate and grow and the efferent ducts of the reproductive system develop progressively. The changes occurring at the molt into the adult are sometimes rather more striking than those at earlier molt. This is particularly obvious in the Ephimeroptera, Odonata and Plecoptera where the nymphs (sometimes called nyads) are aquatic, with gills and other structures which are lost during transformation to the terrestrial adult forms.

Holometabolous development occurs in virtually all the Endopterygota. In the holometabolous insects there are distinct larval, pupal and adult forms. The larva does not resemble the pupa or adult. The holometabolous insects show a marked suppression of adult characters in the larval stages and a strong tendency for evolution of larval characters which are lost at the change into the pupa. Before the pupal molt but while the pupa is developing, it is called "pharate pupa", and while the adult is developing inside the puparium, the animal is referred to as "pharate adult." In Diptera, the metamorphic changes are extreme in that most of the larval cells die and the adult is reconstructed from the small groups of undifferentiated cells which are known as "imaginal disks" and the histoblasts. In the larva they are present but nonfunctional.

The group of primitive insects, the wingless Apterygota show slight or no metamorphosis. In these cases there is a maturational molt when the insects become sexually active.

Imaginal disks

Holometabolous insects possess a developmental system in which latent adult structures are carried as undifferentiated primordia within the feeding larvae. During embryonic life imaginal (adult) cells are set aside from those that will differentiate into larval structure, whereas the larval cells become polyploid, the imaginal cells remain diploid and divide during the larval stadia. For many adult structures these cells are organized into packets called *imaginal disks* that are enclosed within a cellular epithelium (peripodial membrane). Imaginal disks that are destined to differentiate into particular adult structures such as antennae, legs, wings and genitalia may be identified on the basis of size, shape and location within the insect. The imaginal disks grow during the larval period and acquire progressively more specific determination for adult structures as the cells become separated into developmental compartments. Late in the last larval stage the imaginal disks come under hormonal control, grow markedly, evaginate and synthesize a pupal cuticle. The ability of imaginal disks to respond to developmental hormones has been examined in several insects (see Oberlander, 1983).

Hormonal control of metamorphosis — classical experiments

In 1912 it was reported that amphibian metamorphosis was influenced by a substance from the thyroid and it was noted that the brain (pituitary gland) was necessary for amphibian metamorphosis. These findings interested Kopec who attempted to determine the basis of regulation of metamorphosis in insects. Kopec used 3 basic procedures, which are still valid, to examine the control of metamorphosis in the gypsy moth. These are ligation, extirpation and transplantation. He

found that the head ligation of caterpillars 7 days after the last larval molt, prevented pupation posterior to the ligation. However, 10 days after the last molt, ligation did not prevent the onset of metamorphosis. Kopec thought that the brain was involved. He demonstrated the role of the brain by extirpating the brain of larvae 7 days after the previous molt. Only 20% of the debrained larvae pupated compared to 92% of the operated controls. Conclusive evidence for the role of the brain in pupation was obtained in an experiment in which Kopec restored the ability of debrained larvae to molt by transplanting a larval brain into the operated host. During the 1930s Fraenkel and Wigglesworth confirmed and extended Kopec's findings on the endocrine role of the brain in larval molting and metamorphosis.

Wigglesworth used another basic endocrinological method called *parabiosis* to show that a blood-borne factor controlled molting in the nymph of the blood sucking bug *Rhodnius prolixus*. *Rhodnius* nymphs that were decapitated one day after feeding did not molt, while nymphs decapitated 7 days after the blood meal molted normally. When the decapitated nymphs of both ages were joined together in parabiosis, both the nymphs molted. This experiment clearly demonstrated that a substance was passed from one insect to the other which resulted in molting in both insects.

In all these early experimental studies one thing was clear: that the source of the factor (hormone) was required for a specific portion of an instar. The source-dependent period has been called "the critical period". Consequently, ligation or extirpation after this period is of no effect because sufficient hormone to stimulate molting has already been released into the hemolymph.

Using the classic methods of endocrinology — ligation, extirpation and transplantation of organs, parabiosis, blood transfusion — several authors showed that a hormone inducing molting and metamorphosis was released from a source in the head region at a critical period during post embryonic development. Although Kopec suggested the source to be the brain, Fukuda discovered the function of the prothoracic glands (PG). His experiments were confirmed and extended by Williams in his classic studies on pupal diapause in the giant silk moth *Hyalophora cecropia*.

The physiological role of *corpora allata* (CA) was elucidated by Wigglesworth in *Rhodnius*. He initially used the term "inhibitory hormone" because the secretion of the CA inhibited metamorphosis. Later the term "juvenile hormone" was introduced.

Classical scheme of hormonal control

From these early studies emerged the classical scheme of hormonal control of molting and metamorphosis. According to this scheme, the neurosecretory cells of the brain release a hormone which acts on the

prothoracic gland and stimulates the production of the prothoracic hormone, the *ecdysone*. This was assumed to act on the target tissue, the epidermis inducing deposition of a new cuticle and eventually molting. The conversion of ecdysone into *20-hydroxyecdysone* was not yet known. The character of the molt is determined by the presence or absence of the larval hormone of the corpora allata, the *juvenile hormone*. The corpora allata are assumed to be activated (or inactivated) by the nervous control from the brain. When the molting hormone and juvenile hormone are acting together, juvenile characteristics are expressed in the newly deposited cuticle. If juvenile hormone is lacking, the larval epidermis will secrete a pupal cuticle and pupal epidermis an imaginal cuticle.

Hormones at cellular level

Presently it is known that at least 3 hormones, *prothoracicotropic* hormone (PTTH), ecdysone and juvenile hormone (JH) are involved in the initiation of the molting process. The PTTH is produced by the neurosecretory cells (NSC) of the brain and released from the neurohemal organ for these cells, the corpora cardiaca (CC). The hormone stimulates the prothoracic gland (PG) to secrete ecdysone. Ecdysone then acts on the epidermis to initiate the steps in the production of new cuticle. The type of new cuticle formed depends upon the amount of JH secreted from the corpora allata (CA). When it is present in high concentrations, the new cuticle produced is larval. When the CA becomes inactive and the JH titer declines, then metamorphosis occurs.

The phenomenon of molting and metamorphosis have now been examined at molecular level using insect epidermis (Riddiford, 1985, 1987). The hormonal regulation of four larval cuticle genes of the tobacco hornworm *Manduca sexta* has been studied both *in vivo* and *in vitro*. Three class I genes are expressed both in 4th and 5th larval instars but not during the molting period. The Class II cuticle genes are first expressed just before the onset of metamorphosis causing the formation of a harder cuticle. The expression of these genes are initiated by low concentrations of 20-hydroxyecdysone (HCD) in the absence of JH and suppressed by high ecdysteroid (ECD). Thus ecdysteroid serves both to turn on and off larval cuticular gene expression depending on its concentration (see Fig. 1).

The role of JH in the maintenance of larval condition is twofold: (1) It prevents the permanent cessation of the expression of larval specific genes until the time of metamorphosis; (2) JH prevents the ecdysteroid-induced expression of certain larval cuticle genes that apparently have their primary function at the initiation of metamorphosis. For a detailed treatment of this topic see Riddiford (1985 and 1986). With a few exceptions, the change from larval to pupal cuticle secretion by

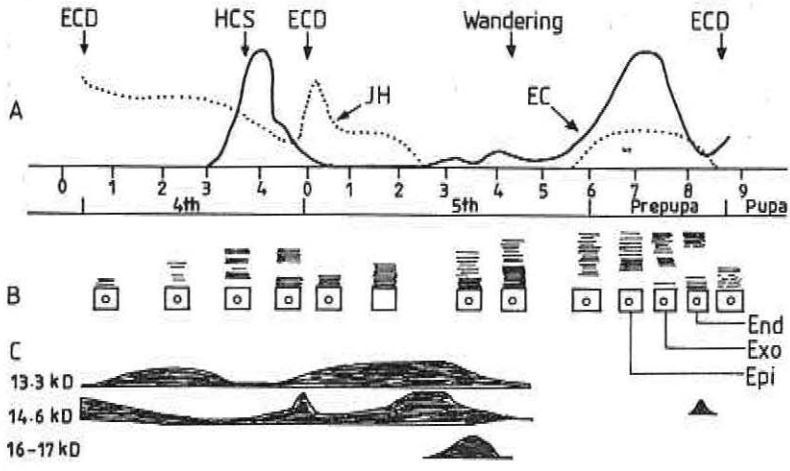


Fig. 1. Schematic summary of (A) the ecdysteroid and JH titers of *Manduca*, (B) the cuticular events that they control, and (C) the expression of Class I (13.3 and 14.6 kd) and Class II (16–17 kd) cuticle genes (from Riddiford, 1987)

epidermal cells appears to be irreversible. Treatment of pupal epidermal cells with JH does not result in the secretion of a cuticle with larval characteristics at the next molt. Such a treatment, on the other hand, results in the secretion of a second pupal cuticle rather than an adult cuticle. For this reason the effect of JH on epidermal cells has been referred to as the maintenance of the *status quo*.

Additional hormones

The terminal phases of the molting process are regulated by two additional hormones, the *eclosion hormone* and *bursicon*. The ecdysis, at least in some holometabolous insects is triggered by *eclosion hormone* (EH) from the brain. Bursicon, which is released from the perivisceral organs in most insects, regulates the postecdysial hardening and darkening of the new cuticle. (This will be discussed in detail during the discussion on insect integument.)

Chemistry and mode of action of developmental hormones

The cerebral neuropeptide, prothoracictropic hormone (PTTH) has been the subject of research for many years in the silkmoth, *Bombyx mori*, for the purpose of understanding the chemistry of the hormone. It is now known that the *Bombyx* brain contains two molecular forms of PTTH. They are 4K-PTTH and 22K-PTTH. Complete amino acid

sequence of 4K-PTTH has been determined (see Nagasawa et al., 1984 and Ishizaki et al., 1987). Substantial progress has also been made on the purification of PTTH from *Manduca* (see Bollenbacher, 1985). Again, *Manduca* appears to have a small PTTH (7K) and a big PTTH (22K). These peptide hormones can also be localized in the cephalic endocrine organs using immunohistochemistry. In *Bombyx*, using PTTH antibody, 4 pairs of dorso-medial neurosecretory cells of pars intercerebralis were found to be immunoreactive (for photographs and details, see Ishizaki et al., 1987).

The chemistry of the neuropeptide, eclosion hormone (EH) has been determined but not that of bursicon. Ecdysteroids were first isolated from *Bombyx mori* pupae. Butenandt and Karlson, in 1954, isolated the molting hormone and named it *ecdysone* (α-ecdysone). A second isolated compound 20-hydroxyecdysone, also known as β-ecdysone was also found to be an active molting agent. All ecdysone analogues are now referred to as ecdysteroids (see Fig. 2). It was not clear from the early work which of the ecdysteroids were synthesized and secreted by the prothoracic glands. In recent years, it has been shown that the prothoracic glands synthesize and release ecdysone

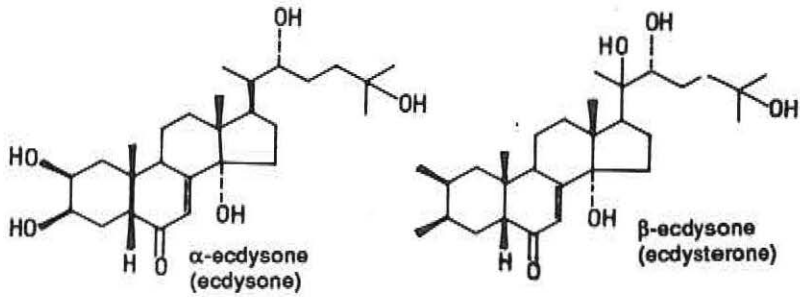


Fig. 2. The term molting hormone or ecdysteroid refers to any steroid which shows molt-promoting activity. Ecdysteroids produced by the prothoracic glands of insects control both apolysis and ecdysis. The two most important ecdysteroids in insects are α-ecdysone (or simply ecdysone) and β-ecdysone (or ecdysterone)

and not 20-hydroxyecdysone.

Purification of extracts from lepidopterous insects led to the identification of juvenile hormone (JH I) as methyl-10,11-epoxy-7-ethyl-3, 11-dimethyl-*trans*-2, 6-tridecadienoate. Some homologues JH II (17-carbon), JH III (16-carbon) and JH 0 have also been identified. In most cases JH III is found in the adult insects. In the cockroach, *Nauphoeta*, all three (JH I, II and III) are present in larvae and adult

females. The homologue JH 0 has been identified from the embryo of *Manduca sexta* (Fig. 3). Investigation on the biosynthesis of JHs has been rewarding because of *in vitro* culture of CA. For chemistry, biosynthesis and inactivation of these hormones see Oberlander (1985)

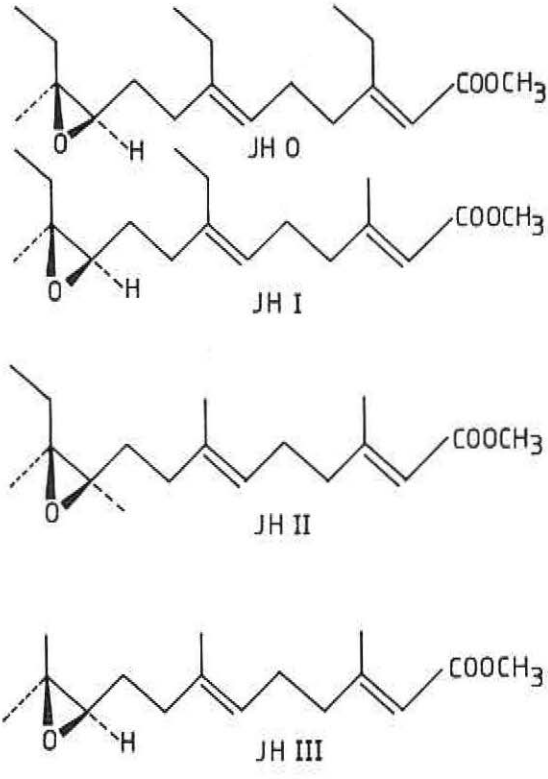


Fig. 3. Insect juvenile hormones. The hormone is one or more of a mixture of three terpenoids. The JH 0 has been reported from the embryo of *Manduca sexta*

and various chapters of Kerkut and Gilbert (Eds), vol. 7 (1985).

The mode of action of insect hormones can be analysed in terms of the cyclic nucleotide "second messenger" and intracellular protein "receptor" models of hormonal action. The evidence is fairly consistent with the hypothesis that ecdysteroids work via an intracellular protein receptor and that the prothoracic hormone and other peptide hormones such as bursicon and eclosion hormone work via the second messenger. Furthermore, juvenile hormone may act according to the steroid receptor model. For a detailed discussion of the mode of action of various hormones see various chapters of Kerkut and Gilbert (1985). See Truman and Morton (1987) specifically for eclosion hormone mode of action.

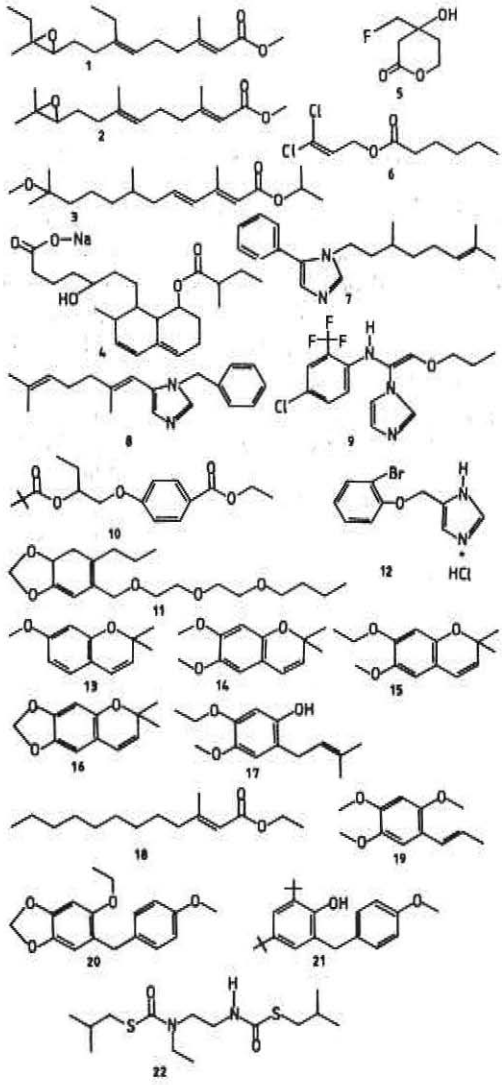


Fig. 4. Juvenile hormones (JHs) and insect growth regulators. 1, JHI; 2, JH(III); 3, methoprene; 4, compactin analogue MI-236B; 5, FMev; 6, 3,3-dichloro-2-propenyl hexanoate; 7, KK-22; 8, KK-42; 9, (*E*)-4-chloro-α, α,α-trifluoro-*N*-{1(1*H*-imidazol-1-yl)-2-propoxyethylidene}-0-toluidine; 10, ETB; 11, piperonyl butoxide; 12, 5-*O*-bromophenoxymethyl) imidazole hydrochloride; 13, precocene 1; 14, precocene 2; 15, 7-ethoxy analogue of precocene 2 (precocene 3); 16, methylene-dioxy analogue of precocene; 17, 3-ethoxy-4-methoxy-6-isopentenylphenol; 18, EMD; 19, β-asarone; 20, 5-ethoxy-6-(4-methoxyphenyl) methyl-1, 3-benzodioxole; 21, 2-4-bis (1, 1-dimethylethyl)-6-(4-methoxyphenyl-methyl) phenol; 22, *N*-ethyl-1, 2-bis (*S*-isobutylthiocarbamoyl) ethane (see Staal, 1986)

Insect growth regulators

As a result of problems with various chemical insecticides such as chlorinated hydrocarbons, alternative methods of insect control were sought. One of the alternative methods included use of insecticides developed as a result of rational leads from basic entomological research on metabolic disruptors, molt inhibitors and behavior modifiers of insects. Because the mode of action of these chemicals is species specific, stage specific and in most cases biodegradable, it is assumed that these chemicals will have less deleterious effects on non-target species. One class of such insecticides are known as *insect growth regulators* (IGRs) (Fig. 4). All compounds belonging to this group adversely interfere with the normal growth and development of insects. There are two major groups of insect growth regulators: The juvenile hormone analogues (JHAs) interfere with the normal metamorphic changes in the insect; the benzoylphenyl ureas inhibit chitin synthesis resulting in molt disruption. For a detailed discussion of various types of insect growth regulators see Retnakaran et al. (1985).

THE INTEGUMENT
(3 lectures)

Introduction
Structure and function of layers
Sclerotization and tanning
Molting, apolysis and ecdysis

THE INTEGUMENT

Introduction

The success of insects as terrestrial animals is in part due to their *integument*. The integument is the outer layer of the insect body comprising the *epidermis* and the *cuticle*. The cuticle is secreted by the epidermal cells.

The integument is the interface between the living insect body and the environment and serves many purposes. It restricts water loss from the body surface. For soft-bodied insects, the cuticle provides protection from the harsh environment. It also helps insects maintain their body shape. Within the insect, it provides a point of attachment and leverage for muscles. The integument possesses all sensory hairs which perceive stimuli in many different forms.

Structure

Since the cuticle is hard, it does not allow continuous growth. Therefore, insects often shed their old cuticle and replace it with a new one. The cuticle consists of two main fractions, protein and α-chitin, which form a composite material. The cuticle is divisible into a very thin set of chemically complex layers, the *epicuticle* under which there is a relatively gross set of layers comprising the *procuticle*.

The *epicuticle* is complex and a very thin composite structure situated on the top of the chitin-bearing procuticle. Functions of epicuticle are many and varied. The most important contribution is in water homeostasis where it contributes to both water uptake through integument or to resistance to water loss. The epicuticle may also be responsible for setting the limits of exoskeletal dimensions during intrastadial growth in some holometabolous larvae. The mechanical properties of epicuticle probably dictate the extent of distension of cuticle during engagement of blood sucking or plant sucking insects.

A generalized epicuticle consists of four distinct regions. Starting from the outside of the insect inward, these are *cement* and *wax layers*, an *outer epicuticle* and an *inner epicuticle*. However, these layers are formed almost in reverse sequence in which they exist, that is the outer epicuticle is formed first followed in turn by the inner epicuticle, wax layer and cement layer.

The *cement layer* is believed to be the product of dermal glands which are specialized epidermal cells and thought to consist of proteins and lipids that have been stabilized by polyphenols. The composition, thickness and extent of the cement layer varies from species to species. The cement layer is completely absent in honey bees. The cement layer protects the underlying wax layer against possible abrasion or impact damage.

The *wax layer* is situated just below the cement layer. The composition of this layer is dominated by hydrocarbon molecules

varying in length from 12 to 31 carbons and by esters of even-numbered fatty acids and alcohols in the C_{24}–C_{34} saturated alcohols esterified with acids. The wax layer lipids are probably secreted by the epidermis just prior to ecdysis and transported via pore canals to even finer wax canals and from there to the surface.

The physiological significance of the insect wax layer has been best documented in terms of its relationship to waterproofing. The relationship between cuticular transpiration and temperature change has been studied in *Pieris* butterfly. In *Pieris* there is an initial critical transition temperature at which there is a sudden increase in cuticular water permeability followed by a plateau region of little change and again another transition temperature at which permeability further increases. These results have been interpreted as related to the dissolution of a mobile lipid phase followed by the dissolution of saturated hardened waxes.

Although the wax layer ultimately provides the basis for water proofing in most insects, it is probably not true for maggots because water proofing in these insects is not disrupted by steeping in chloroform. These insects probably use other means for water proofing.

It has been observed that the diapausing pupae of *Manduca* produce three times more epicuticular wax than the non diapausing pupae. It has been suggested that the changes in wax synthesis could be induced by the neuroendocrine system.

The *outer epicuticle* is often designated as the cuticulin layer and is the only layer which is universal among insects. In addition to the part of exoskeleton, it also lines tracheoles and serves as an insertion point for the tonofibrillae of muscle. It is absent from the sensory receptor areas of the cuticle. The outer epicuticle can be described as a trilaminar membrane of about 12–18 nm thick. Its chemistry and composition are not known but its resistance to degradation indicates properties similar to polythene-like polymers and to quinone tanned protein.

The outer epicuticle is the first formed layer of the pharate cuticle. It is derived from the specialized region of the epical membrane of the epidermal cells. Shortly after the formation of outer epicuticle, pores of 3 nm diameter appear in the layer. These pores are believed to allow the resorption of lysed endocuticle and to prevent the inward flow of molting fluid enzymes, thus protecting the presumptive exocuticle of the pharate insect underneath. It is of interest that the pharate outer epicuticle is itself resistant to dissolution by molting fluid enzymes.

The *inner epicuticle* is an optically isotropic layer secreted by epidermis with possible contribution from oenocytes. This layer is a polyphenol-protein complex. It has been postulated that the inner epicuticle serves as the reservoir for extracellular enzymes associated with wound repair.

The *procuticle* constitutes the bulk of the integument, and it is the *chitin* bearing part of the cuticle proper. Chitin is a high-molecular

weight unbranched helical homopolymer consisting of β(1–4)-linked N-acetyl-D-glucosamine units (Cohen, 1987). In insect cuticle (and in peritropic membrane) chitin microfibrils are intimately associated with various proteins. The process of chitin formation involves an orderly sequence of complicated cellular events (see Cohen, 1987). It has been suggested that the molting hormone is directly involved in chitin synthesis and degradation. The use of insect integumental organ cultures facilitated research on cuticle biochemistry, including research on chitin and effects of insect growth hormones (Cohen, 1986).

The membrane bound *chitin synthetase* (CS) is the key enzyme in chitin formation. All currently known inhibitors of chitin synthesis act on the CS or the polymerization step. Inhibitors of chitin formation include unrelated, diverse groups of compounds. Compounds of great potential for disrupting chitin synthesis (such as the benzoylphenyl ureas) act on polymerization or on events associated with it.

The other major constituents of the procuticle are various proteins. Numerous protein bands have been noted from a single species. One of the cuticular proteins is very specialized and is known as *resilin*. Functionally resilin is an insect rubber with diverse mechanical roles. It serves as a tension spring in insect tendons (wasps), a compression spring in the jumping of fleas and proboscis oiling of certain Lepidoptera. There are other specialized proteins, like proteins of ootheca and silks are also part of the procuticle. Other constituents of procuticle include pigments, lipids, inorganic salts and water.

The properties of procuticle differ widely from one part of the animal to another and most probably depend on the particular combination of chitin and proteins present. The exo- meso- and endocuticular layers were described on the basis of their staining properties. The exocuticle is secreted before ecdysis and appears in the electron microscope to have chitin fibrils; the proteins are tanned and this layer is not dissolved at ecdysis. The mesocuticle is less organized and is not tanned. The endocuticle, which is next to the epidermal cells, appears striated in electron micrographs. The endocuticle is resorbed from one instar to the next and in starvation can be used as a nutrient source (Neville, 1975).

The epidermis forms a continuous sheet of single-layered cells beneath the cuticle as well as covering skeletal invaginations such as apodems, fore- and hind-guts and the inner surfaces of evaginated structures (wings, gills, spurs, etc.). In some insects, the number of epidermal cells appear to remain constant whereas in some others they divide mitotically. In both cases they, however, increase in size.

Though a specialized tissue, epidermis performs many functions in common with other kinds of cells: the elaboration of organelles, the

uptake of chemicals for synthesis and secretion, general self-maintenance and metabolism as well as the production of cuticle. Epidermal cells are capable of amaeboid movement in the repair of wounds, tracheole-pulling behavior under conditions of low oxygen tension (*Rhodnius*).

Sclerotization and tanning

Since cuticle of insect is hard, it does not allow continuous growth, hence insects shed their old cuticle and replace with a new one. The cuticle which is freshly synthesized is soft and flexible, but subsequently it becomes hard. The process of hardening is known as *sclerotization* and *tanning*. Sclerotization also occurs in the pupal cuticle as well as in egg capsules (oothecae) of some insects. Thus the sclerotization process is an essential biological mechanism for the successful survival of insects.

As a rule this tanning process occurs after ecdysis but some cuticular structures are sclerotized before ecdysis. The dipteran puparium is the last instar larval cuticle which is sclerotized to form a protective covering within which pupal and adult development occur.

The chemical pathways responsible for sclerotization have been studied extensively. Most cuticles are tanned either by *β-sclerotization* or *quinone tanning* or by both. Both processes use N-acetyldopamine as a substrate (see Fig. 5).

In *quinone tanning*, which occurs in *Calliphora, Ephestia, Schistocerca* and many others, tyrosine is metabolized to 3,4-dihyroyphenylalanine, which is in turn, converted to N-acetyldopamine the presumed precursor molecule in this pathway. N-acetyldopamine is secreted by the epidermal cells into the cuticle. In the epicuticle a diphenoloxidase will oxidize the diphenol to the corresponding quinone. The quinone molecule reacts spontaneously with free amino groups in the neighboring protein molecules. After further oxidation it can react with more proteins and link them together in a huge stable network.

The term *β-sclerotization* was proposed for some pathways as in *Apis, Schistocerca, Tenebrio*, etc., after it was demonstrated that the cuticular enzyme activates the position in the side chain adjacent to the aromatic ring, the β-position and it is from this position that bonds to the proteins are formed.

Results of additional experiments were interpreted to indicate that the colorless cuticles preferably use the β-sclerotization pathway whereas the brown cuticles use a combination of quinone and β-pathway. Quinone tanning and β-sclerotization occur at the same time and use the same primary substrate. Quinone tanning can occur without simultaneous β-sclerotization, whereas β-sclerotization cannot occur without some quinone tanning going on at the same time.

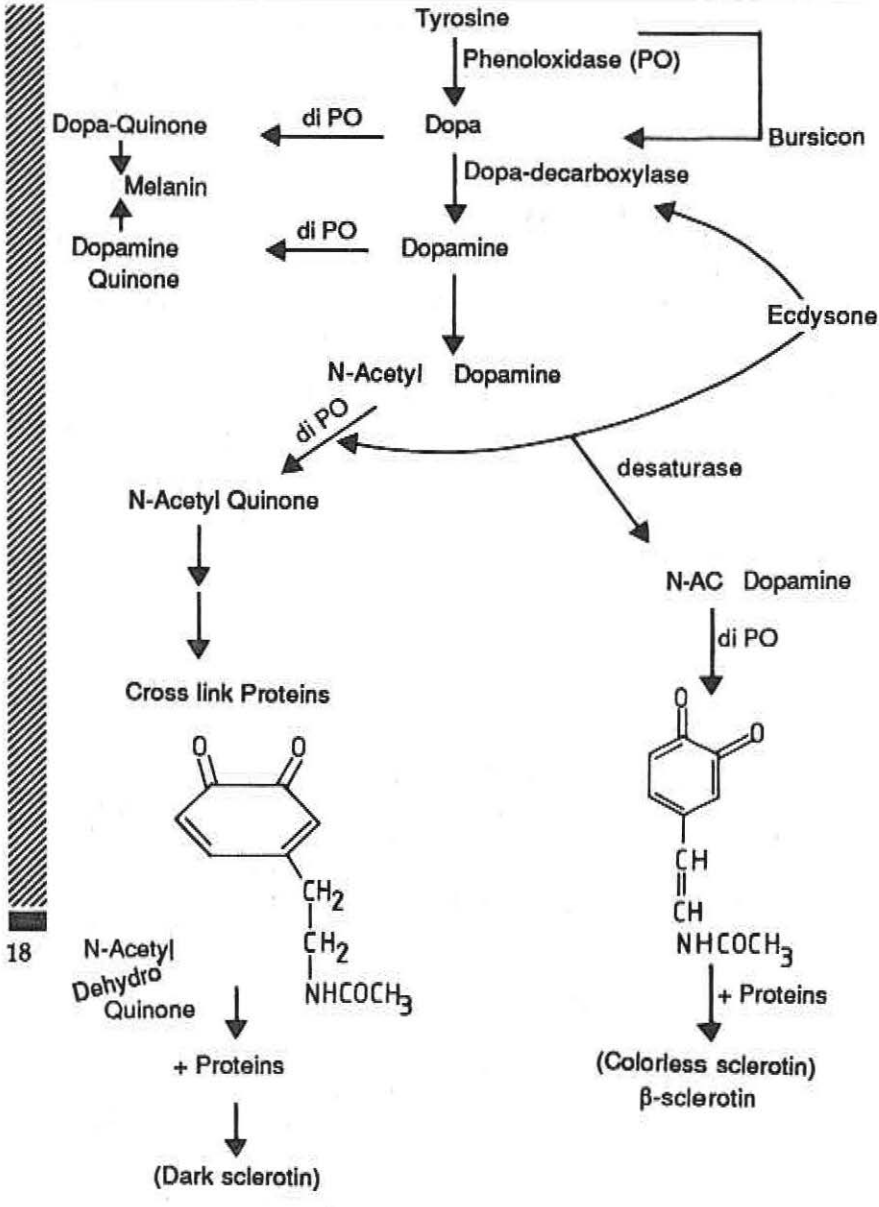


Fig. 5. Tyrosine metabolism showing pathways for quinone tanning and β-sclerotization. Ecdysone may activate Phenoloxidase and dopa decarboxylase. Bursicon stimulates the formation of Dopa from hydroxylation of tyrosine

Hormonal control of sclerotization

How does the insect determine as to how much, at which place, and at what time the sclerotization should occur?

Ecdysteroids induce sclerotization in ligated abdomens of blowfly larvae and one of the direct effects of 2-hydroxyecdysone in this system is the induction of *de novo* synthesis of the enzyme dopa-decarboxylase in the epidermal cells. This enzyme is involved in puparium formation in dipteran species.

Epidermis of some insects appears to be equipped for synthesizing the tanning agent and can be activated by appropriate hormone treatment. The cockroach epidermis can produce cuticle *in vitro*, when stimulated by 20-hydroxyecdysone, although the cuticle is not sclerotized, it was suggested that the controlling factor such as *bursicon* is missing in the *in vitro* system.

Bursicon, *a neurohormone*, was described as a hormone necessary for cuticle tanning and endocuticle formation of flies and other insects. The hormone can stimulate uptake of tyrosine into the hemocytes and thereby initiate its conversion to N-acetyldopamine as well as it can stimulate the epidermal cells directly. From the available information it can be concluded that the synthesis of tanning agent is under hormonal control and both 20-hydroxyecdysone and bursicon may be involved.

Molting, apolysis and ecdysis

According to the current usage, "molting" refers to all of the events and processes leading up to a shedding of the old cuticle, whereas "apolysis" means separation of the epidermis from the old cuticle. Therefore, apolysis is just one aspect of molting. "Ecdysis" refers to the shedding of the cuticle, again just another aspect of molting. Ecdysis is mediated by Eclosion hormone currently believed to be of universal occurrence which controls ecdysis in all insects. Additional terms are "molt" referring to the time period and events, occurring from apolysis to ecdysis; "post-molt" from ecdysis to completion of tanning and "inter-molt" from the end of post-molt to the next apolysis.

Hepburn (1985) presented a general outline of the events associated with molting and ecdysis in insects:

1. apolysis along an antero-posterior gradient;
2. mitotic division of epidermal cells as well as the expansion of epidermal cell surface area and volume;
3. secretion of molting fluids;
4. formation of pharate outer epicuticle at the surface of the apolysed and crenulated epidermis, resulting in the definition of the surface pattern of the pharate cuticle;
5. secretion and formation of the pharate inner epicuticle;
6. activation of molting fluid enzymes and lysis and resorption of old endocuticle;
7. deposition of presumptive pharate exocuticle;
8. ecdysis;

9. expansion of the new cuticle;
10. onset of tanning (which may sometimes occur before ecdysis);
11. secretion of endocuticle;
12. secretion of wax;
13. continuous deposition and tanning of endocuticle;
14. formation of an apolysial membrane for the next molt.

For details of apolysis and ecdysis consult Hepburn (1985).

MUSCLE SYSTEMS AND MOVEMENT

(2 lectures)

Introduction
Structure of muscular system
Innervation
Functioning
Locomotion — walking
Locomotion — flight

MUSCLE SYSTEMS AND MOVEMENT

Introduction

Muscles are like biological machines which convert chemical energy into mechanical activity and into heat energy. The muscles of insects represent an array of variation. They are used in flight, swimming, walking, in producing gut movement, heartbeat, in sexual activities, oviposition activities and so on.

Skeletal muscles are attached to the cuticle at both ends and usually extend across a flexible region or a joint. The skeletal muscles normally occur in antagonistic pairs (or groups), one of which reverses the action of the other. The circular and longitudinal sections of the visceral muscle of the soft organs also act as antagonists.

Structure

The machinery for the contractile property of the muscle consists of mainly fibrous proteins, specifically *actin* and *myosin*. They are powered by ATP and are controlled mainly by mechanisms regulating free internal calcium ion concentration.

Muscles are composed of cellular units called muscle *fibres*, each derived from several cells. The fibre is made up from rods of protein, the *fibrils*. Each fibrile is divided longitudinally into many segments, *sarcomeres* by partitions, the Z discs, which may be aligned on adjacent fibrils so that the whole fibre has a striated look. Within each fibril there are interdigitating thick and thin filaments, composed of the proteins myosin and actin, respectively. The "sliding filament" theory postulates that the contraction results from the myosin and actin filaments, sliding past each other, without themselves decreasing in length and bringing successive Z discs closer together with a resulting overall shortening of the muscle (for basic structure of muscles see Chapman, 1982).

Innervation

The nervous supply to a muscle consists of a small number of large axons. Each unit is innervated by a fast axon and a slow axon. Fast and slow do not refer to the speed of conduction of the impulse but to the size of postsynaptic potential, and hence muscle twitch, that is produced. It is probable that the difference in fast and slow is due to the amount of neurotransmitter released at the nerve/muscle junction following the arrival of the impulse. In addition to the normal excitatory innervation some fibres of some muscles have an inhibitory nerve supply (e.g., leg muscles of locust).

Functioning

How does the muscle function? *Neuromuscular synapses* work the same way as the interneuronal synapses. Each *action potential* causes the

release of *transmitter* which opens *ion gates* in the muscle membrane and the resultant current flow is either *depolarizing* (at *excitatory* synapses) or *hyperpolarizing* (at *inhibitory* synapses). Depolarizing currents affect the permeability of adjacent regions, so that the effects spread and reach the interior of the muscle and activates the contraction mechanism. Hyperpolarizing currents reduce the depolarizing effect of the excitatory synapses and inhibit contraction. It is probable that *α- glutamate* acts as the excitatory transmitter in many insect somatic muscles and it is generally agreed that *gamma-aminobutyric acid* (GABA) is the main inhibitory transmitter.

The ionic basis for excitatory (depolarizing) end-plate potentials is not understood well. It depends on the hemolymph composition of the species. In more primitive orders, hemolymph Na^+ and sarcoplasmic K^+ tend to be high (like vertebrates). Excitation makes the postjunctional membrane more permeable to these ions. In higher orders, the hemolymph composition is quite different. It is lower in Na^+ but higher in K^+ (and Ca^{2+} and Mg^{2+}) and organic ions. The membrane potential and contraction of many insect muscles shows less of a K^+ dependence. In some cases bathing with a high K^+ saline evokes a sustained depolarization and contraction.

As for the action of GABA, it appears to open Cl^- selective channels. The effect of the inhibitory transmitter is to hold the membrane potential firmly at the resting potential, making it more difficult to depolarize.

In addition to the above two types of neurons, many skeletal muscles receive branches of dorsal unpaired medial neurons. These release a transmitter which modulates the effect of the slow excitors, which increase the speed and vigor of contraction and also accelerating relaxation. This transmitter possibly acts by stimulating the enzyme adenyl cyclase in the muscle membrane increasing the production of cyclic adenosine monophosphate (cAMP), which in turn stimulates metabolism increasing the supply of ATP.

In all muscle fibres (except the smaller visceral fibres) the level of excitation is communicated throughout the anterior by a transverse tubule system (TTS) consisting of invaginations of the sarcolemma. In most insect muscles the TTS has a precise location relative to the sarcomere pattern. The TTS is in direct contact with the sarcoplasmic reticulum which serves as the storage reservoir for Ca^{2+}. When excitation is communicated by the TTS to the SR, it releases the stored Ca^{2+} into the sarcoplasm. The Ca^{2+} then combines with the protein chains which activate myosin ATPase and tension development (see figure in Pringle, 1975).

Locomotion — walking

The walking gait of most insects consists of a tripod type support where the first and third legs of one side and the second leg of the other

side are in contact with the substratum at any one time, and legs on opposite sides of each segment step alternately. This may vary with the speed of walking, and the topography of the walking surface. In some cases a sensory feedback is probably important in controlling each step. At a higher stepping frequencies the control is more dependent on a central programme.

Locomotion — flight

Movement of the wings during flight is dependent on the action of muscles. Insect flight mechanisms have been studied extensively in several species (see review articles by Hughes and Mill, 1974 and Pringle, 1975). Muscles may act directly by pulling on the wing bases or indirectly by altering the shape of the thorax. The upward beat is caused by the indirect, dorso-ventral muscles which pull down the top of the thorax which in turn forces the wings upward. In dragon flies the downward beat is caused by direct pulling by the muscles which are inserted onto the base of the wing. In houseflies, the indirect muscles in the thorax raise the thorax up, which forces the wing downwards. Other insects use a combination of direct and indirect muscles on the downward beat. Because the thorax is quite stiff, it resists lateral pressure so that in the middle of the wingstroke the wing articulation is unstable. Therefore, when the wings pass to this point, they flick rapidly into the up and down position. This is known as a *click mechanism*, and the sudden changes in muscle tension which it produces are important in the operation of the wing muscles.

Synchronous flight muscles are found in Odonata (dragon-flies), Orthoptera (locusts) and Lepidoptera, whereas *asynchronous* flight muscles are found in Diptera, Hymenoptera, Coleoptera and most Hemiptera. Highest wingbeat frequencies are observed in insects with asynchronous muscles. Depending on these two types of flight muscles, there are two patterns of neural control of flight muscles. For details of these see Smyth (1985).

PHYSIOLOGY OF NERVOUS AND SENSORY SYSTEMS
(3 lectures)

Introduction
Structure and organization
- Nerve cells — neurons
- Axons
- Dendrites
- Arborization
- Synapse

Central nervous system
- Brain
- Ventral nerve cord — ganglia

Visceral nervous system
Peripheral nervous system
Neurophysiological techniques
Functions
Sensory System
- Mechanoreceptors
- Auditory organs
- Chemoreceptors
- Thermo- and hygroreceptors
- Photoreceptors

PHYSIOLOGY OF NERVOUS AND SENSORY SYSTEMS

Introduction

The survival of animals depends on, among other things, their receiving and responding to information about their environment. An insect depends on its *electrically excitable cells* to regulate its relationships with its internal and external environment. These cells occur in sense organs, the nervous system and the muscle. They perceive stimuli, process information and coordinate responses by their mutual electrical interactions.

Structure and organization

The nerve cells are known as *neurons*. Most insect neurons are monopolar, that is, the cell body or soma has a single process that produces filamentous *axon* with *dendrites* at one end and *terminal arborization* at the other end. Each axon is enclosed in a nucleated coat—the *neurilemma*, and the cytoplasm of the axon is surrounded by a thin layer of fat-like material. There are usually three types of neurons — *sensory*, *motor* and *association* neuron. A fourth type is represented by *neurosecretory* cells which have specialized endocrine function. The sensory cells (neurons) are associated with the sense organs and lie near the integument. Each sensory neuron is usually bipolar. The distal process responds to stimulus while the long axon ends in an arborization within a central ganglion. Fibres from neighbouring sensory neurons may be grouped together to form a sensory (afferent) nerve. The motor neurons always lie within the ganglia; these are mostly unipolar and their axons may be grouped together to form motor nerves (efferent). They pass mostly to muscles where the axons terminate in minute conical *endplates* or in fine branches on or within the muscle fibres. The association neurons form links between the sensory and motor neurons (Fig. 6). The resulting junctions between the arborizations of adjacent nerve cells are known as *synapses*. The terminal branches of the two neurons are not usually in actual contact at a synapse. Each nerve impulse which arrives there causes the temporary release of a transmitter such as acetylcholine, which then activates the adjacent neuron so that the impulse is transmitted across the synapse.

Divisions of nervous systems

The nervous system comprises

1. the central nervous system,
2. the visceral nervous system, and
3. the peripheral nervous system.

The *central nervous system* (CNS) is composed of a double series of *ganglia* joined together by longitudinal and transverse tracts of nerve

fibres. The longitudinal tracts are *connectives* and the transverse fibres are *commissures*. Usually there is one pair of ganglia in almost every segment. However, a varying degree of fusion occurs in some higher groups. For example, the CNS of water bug (*Belostoma*) shows fusion between suboesophageal and first thoracic ganglia and again between second thoracic and the rest of the posterior ganglia from thorax and abdomen. Similarly in higher Diptera like tsetse (*Glossina*) all thoracic and abdominal ganglia are fused into one *thoracic ganglionic mass*. The *brain* which is the part of CNS lies just above the oesophagus. The *protocerebrum* forms the greater part of the brain with smaller parts *deutocerebrum* and *tritocerebrum*. Brain contains a few motor neurons concerned with antennal movements, but its main functions are those of coordination. It is responsible for maintaining the general tonus of the skeletal muscles, it controls the local reflexes which are mediated by the thoracic and abdominal ganglia, and it exerts an inhibitory action on centres in the suboesophageal ganglion.

The *visceral nervous system* or *sympathetic nervous system* is the *stomatogastric* system which is formed by ingrowth of the dorsal part of the *stomodaeum*. It includes firstly, the median *frontal* ganglion lying just anterior to the brain. This ganglion appears to exert an effect on the ionic concentrations of the blood; it is joined by bilateral connectives to the tritocerebrum and gives off a *recurrent nerve* that ends in the *hypocerebral ganglion*. The stomatogastric system contains both motor and sensory fibres and innervates the heart and fore intestine.

The *ventral sympathetic system* consists of transverse nerves associated with each ganglion of the ventral cord. They supply the spiracles of the segment. Arising from the last abdominal ganglion are

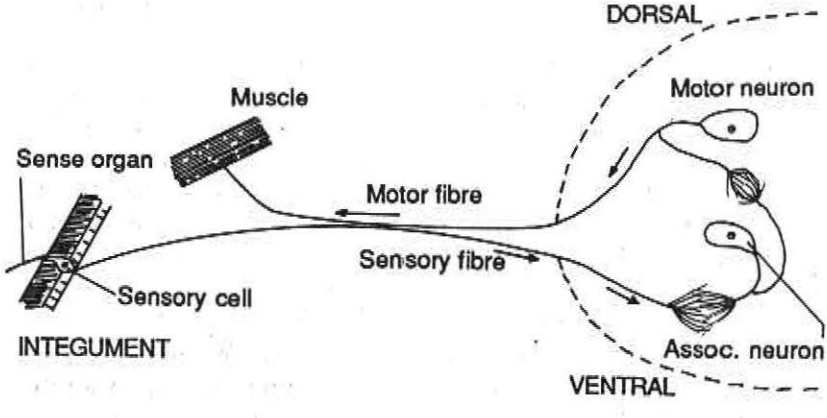


Fig. 6. Reflex mechanism of an insect. Half of a ganglion of the ventral nerve cord is shown. Arrow shows the path traversed by the nerve impulse resulting from stimulation of the sense organ

splanchnic nerves that innervate the reproductive organs and the hind intestine.

The *peripheral sensory nervous system* is composed of a fine network of axons and sensory cells lying beneath the integument. This network is best developed in soft-skinned larvae.

On the structure of nervous system there are several recent reviews which give details of structure of each of the systems (Weevers, 1985 on ganglia, Mobbs, 1985 on brain and Penzlin, 1985 on stomatogastric nervous system). For a variety of techniques used to study nervous system in insects the book on neuroanatomical techniques by Strausfeld and Miller (1980) should be consulted. For electrophysiological techniques, *Insect Neurophysiological Techniques* by Miller (1979) is most important. This book gives all the practical details and examples of most of the techniques that are in use at present.

Functions

The function of an insect nervous system is to receive and integrate information through various stimuli from internal and external environment and to coordinate and control the internal systems in response to the stimuli. Inputs to the nervous system are via the sensory receptors and outputs are via skeletal and visceral muscles and the endocrine system. The relationship of the nervous system to the endocrine system is complex, and the role of sensory input is not clear. Particularly, it is not clear how hormones influence the activity of the nervous system and thereby change the insects' behavior. For a detailed discussion of this subject see Eaton (1985).

Sensory system

Sensory perception is achieved by means of structures known as *receptors* or *sensilla*. These take various forms and are situated at the peripheral ending of the sensory nerves. Three basic types of insect sensilla are recognized as elements in different receptor systems. These sensilla, which are without pores, uniporous and multiporous are components of mechanoreceptor, photoreceptor and chemoreceptor systems. In their least modified form receptors closely resemble ordinary body hairs and only differ in being connected with the nervous system. The components of a simple type of receptor are the cuticular or external part with its *trichogen* or formative cell together with a bipolar sense cell. The cell lies just beneath the epidermis and its distal process penetrates the trichogen cell to enter the cavity of the cuticular part of the receptor. In many cases a *tormogen* or membrane cell is also present.

With use of the transmission electron microscope and the scanning electron microscope, together with the techniques for recording nerve impulses from the sensory cells associated with a single sensillum

(electrophysiology and single-cell recording) many new features of structures and function have been discovered. Recently, a new simpler terminology has been proposed to name various sensilla. For a detailed discussion see Frazier (1985).

In terms of their functions the receptors are classified as follows:

(a) mechanoreceptors (touch, tension and balance)
(b) auditory organs (perceiving sound)
(c) chemoreceptors (odours and tastes)
(d) thermo- and hygroreceptors (temperature and humidity)
(e) photoreceptors (visual organs).

Mechanoreceptors

Mechanoreception in insects serves to detect mechanical distortions resulting from contacting an object, vibrations of air, water or substrate, or distortion of internal structures resulting from muscular activity. There are three main types of mechanoreceptors.

Auditory organs

Insect sensilla that respond to long-range vibrations by the air or water at frequencies greater than 50 Hz are considered to be *auditory receptors*. There are two main types, movement receiver or pressure receiver. Acoustical sensilla of insects, consisting of hairs, *chordotonal organs* and *tympanal organs*, may respond to vibrations of the air at frequencies above 50 Hz and are acoustical receptors. The chordotonal organ at the junction of the antennal flagellum and the pedicel is called *Johnston's organ* and is found in the adult of almost all orders and is particularly well developed in male mosquitoes and midges.

Chemoreceptors

Chemoreceptors are the receptors for *smell* and *taste*. In terrestrial insects the *olfactory sense* is stimulated by low concentrations of the vapors of volatile substance while the *gustatory sense* perceives relatively high concentrations of the stimulant in aqueous solution. The source of chemicals must actually be contacted by the gustatory sensilla, so that it is also known as contact chemoreception.

Thermo- and hygroreceptors

There are several insects for which electrophysiological proof of temperature receptor functioning has been determined. The majority of the species studied possess cold receptors that increase their rate of firing to decreasing temperature. Location of these sensilla is antenna. A number of electrophysiological studies have confirmed the presence of hygroreceptors on the antennae of caterpillars, honey bees, mosquitoes, locusts, bugs and cockroaches. For details see Altner and Loftus (1985).

Photoreceptors

Photoreceptors in insects are of two kinds: *ocelli* and *compound eyes*. Typically both kinds are found in the same insects but either or both may be absent. Extraocular reception is also known. Ocelli are simple eyes that occur in adults of all orders and in endopterygote larvae. They fall in two classes: (1) *dorsal ocelli* of adults and nymphs and (2) *lateral ocelli* of larvae. The dorsal ocelli are innervated from the ocellar lobes of the brain which are located in the protocerebrum. The function of dorsal ocelli is not well known. They probably perceive changes in light intensity. Lateral ocelli or stemmata may occur singly or in groups, on either side of the head. They are innervated from the optic lobes of the brain. They are replaced by the compound eyes during metamorphosis. These ocelli are capable of limited vision of both color and form.

The major photoreceptor of insects are the compound eyes. They each consist of a few to several thousand units called *ommatidia*. Compound eyes are absent in Protura and Diplura. Each ommatidium consists of a transparent *cornea* formed of transparent cuticle and is shed at each molt. See detailed structure of a compound eye in Chapman (1982). In general, the compound eyes can perceive something of the form, movement and location of external objects and detect some differences in the intensity and color of light falling on them. How images are formed within the compound eye is still not totally understood. See Carlson and Chi (1979) for details.

ALIMENTARY CANAL, DIGESTION AND NUTRITION
(2 lectures)

Introduction
The alimentary canal
- Fore, mid and hind intestine
- Crop
- Proventriculus
- Peritrophic membrane
- Pyloric sphincter
- Rectal papillae

Digestive enzymes and their functions
- Carbohydrases
- Lipases
- Proteases

Nutritional requirements
- Carbohydrates
- Proteins — amino acids — proline
- Fats
- Sterols
- Vitamins

ALIMENTARY CANAL, DIGESTION AND NUTRITION

Introduction

Materials taken as food by insects must be processed to a form that can be absorbed into their body for subsequent assimilation. The process, digestion, renders food absorbable, by breaking it down into simpler component compounds and dissolving them chiefly through the action of enzymes secreted by specialized cells.

The lectures in this section will discuss the structure and function of the digestive system, digestive enzymes and their functions and nutritional requirements of insects.

The alimentary canal

The site of digestion, the alimentary canal, is basically a tube which encloses a part of the body separated from the coelom by the gut wall.

The alimentary canal is divided into three regions, the *fore intestine* (fore gut) that arises in the embryo as an anterior ectodermal ingrowth (stomodaeum), the *hind intestine* (hind gut) that arises as a similar posterior ingrowth (proctodaeum), and the *mid intestine* (mid gut), *stomach* or *ventriculus* formed as an endodermal sac (mesenteron) connecting the two. The fore and hind gut, being ingrowths of the integument, resemble the latter histologically and are lined with cuticle (Fig. 7).

The region of food reception, the *buccal cavity*, is not a part of the alimentary canal. Associated to this are specialized mouthparts and mechanisms for feeding, some of which may be outside the mouth, and differ for piercing and sucking, lapping or biting and chewing.

In insects with single biting mouthparts and a hypognathus head the cavity is bounded in front by the inner surface of the *labrum*, behind by the *labium* and laterally by the *mandibles* and *maxillae*. The *hypopharynx* arises near the base of the labium. The common *salivary duct* opens near its base. These are the ducts of the *salivary glands*.

The *fore intestine* begins with the *pharynx*, a relatively narrow tube which leads back from the buccal cavity and is equipped with dialator muscles arising from the frons. In Lepidoptera and Hymenoptera the pharynx participate in the formation of a sucking pump. The pharynx passes, posteriorly, into the *oesophagus*. This may either be a simple tube or it may be expanded at some point to form a *crop*. A good example is tsetse fly.

The fore intestine is separated from the mid intestine by the *cardiac sphincter*, and at this region it is often modified to form a muscular *proventriculus*. Its main function is that of a sieve occluding the passage of food if not in sufficiently divided state.

The *mid intestine* is composed of a layer of large epithelial cells bounded externally by a basement membrane. On the inner side these

cells show a *striated border* composed of large numbers of *microvilli*. A layer of minute circular muscle fibres and external longitudinal muscles is present. To increase the surface, some insects develop outgrowths or *caeca*. Another way of achieving the same result is by folding epithelial layer to form *crypts*. All methods may occur in the same species. The method of secretion by epithelial cells may be *merocrine* (cells discharge their products through the striated border without undergoing any change) or *holocrine* as in Orthoptera (cells disintegrate during the process).

In most insects and their larvae, the food is separated from the epithelial lining by a *peritrophic membrane* which forms a thin, colorless tube projecting backwards into the hind intestine. It is composed partly of chitin and is supposed to protect epithelial cells from abrasion. In some insects the membrane is absent or at least present in reduced form. These are usually fluid feeding insects. The peritrophic membrane is permeable to digestive enzymes and the product of digestion.

The *hind intestine* is sometimes divided into a narrow anterior tube, *ileum, a colon*, and a wider end region or *rectum* which opens exteriorly at the *anus*. In many insects the cellular layer of the rectum becomes greatly thickened to form six longitudinal pads or *rectal papillae*. An important function of these papillae is the maintenance of the proper salt and water balance by absorption from the contents of the rectum. A *pyloric sphincter* separates the stomach from the hind intestine.

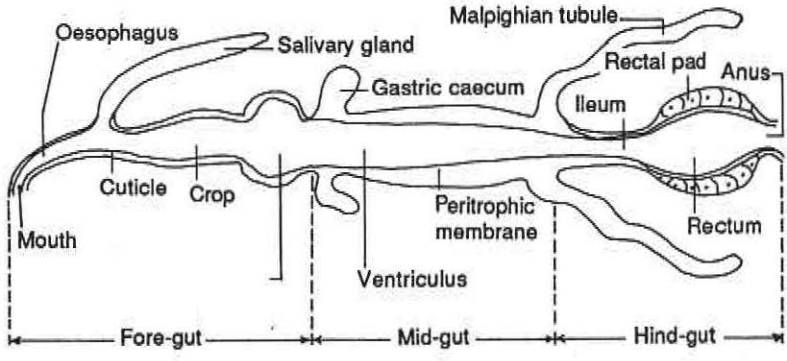


Fig. 7. A typical insect intestine. However, many variations occur, depending on the habits and mode of regulatory processes

The enzymes and their functions

Insect food has to be broken down before it can be absorbed by the midgut. Breaking down to soluble substances is accomplished by digestive enzymes. The bulk of food material available to insects

belongs to the three main classes of foodstuff — carbohydrate, protein and fat, in proportion depending on the diet of each species. A corresponding diversity of digestive enzymes capable of hydrolyzing these material to their constituent units, is therefore needed. Three main groups of enzymes have been found in insects:

a. the *carbohydrases* (catalyse the breakdown of complex carbohydrates to simple sugar) — *amylases* and *glycosidases*;
b. the *lipases* (catalyse the breakdown of fats);
c. the *proteases* (catalyse breakdown of various proteins) — endopeptidases, exopeptidases.

In *Glossina* and some other bloodsucking insects the proteases are abundant but the carbohydrases are almost absent; whereas, in butterflies and moths (nectar feeder) almost the only enzymes present are the *Invertases* which hydrolyze cane-sugar. Of these insects which can utilize cellulose a few species secrete cellulose-splitting enzymes (e.g., Cerambycid beetle larvae). The breakdown of cellulose, however, is more commonly accomplished by symbionts living in the gut. These may be bacteria or protozoa (e.g., some Scarabaeid larvae, wood feeding cockroach, *Cryptocercus* and termites). These termites can live for a long time on pure cellulose if the symbionts are present, but they soon die if the protozoans from the gut are removed experimentally.

Studies of digestion indicate that the production of digestive enzymes may be under control of the neuro-endocrine system in many insects, particularly in those whose feeding is markedly discontinuous. The release of hormones which stimulate the production of enzymes may be to some extent associated with the normal process of development.

Nutritional requirements

The nutritional requirements of insects have been largely determined by rearing insects on artificial diets. It is important to rear the insects axenically, that is, in absence of other organisms that might influence its nutrition. The nutritional requirements of a number of insects are known in biochemical terms. *Carbohydrates* are the main source of energy. Most insects require an exogenous source of carbohydrate for flight, the most energy-demanding of all life activities. However, carbohydrate is not the only fuel for flight. For example, in locust, carbohydrate is the fuel used to initiate flight, whereas *fatty acids* are metabolized during sustained flight. In tsetse, the food is low in carbohydrate (blood) and therefore, *proline*, an amino acid is used by them as their main energy source. Out of 20 amino acids, at least 10 of these are required for growth in insects. The insect diet must also include certain *vitamins* in addition to the dietary proteins. Many insects require polyunsaturated fatty acids. Lineoleate and linolenate are important for growth and wing development in several

lepidopteran species studied. Unlike vertebrates, insects (and other arthropods) cannot synthesize sterols. Almost all insects require sterol in the diet (for further details on nutrition see McFarlane, 1985).

RESPIRATORY SYSTEMS
(2 lectures)

Introduction
Tracheal system
Movement of gases
Respiration in aquatic insects
Respiration in endoparasitic insects
Respiration in eggs
Respiratory pigments

RESPIRATORY SYSTEMS

Introduction

The respiratory system of insects consists of a series of tubes known as *tracheae*, which convey oxygen to the tissues. In the early larval stages of some insects the tubes contain liquid but in most insects they are filled with air. In terrestrial and many aquatic insects the tracheae open to the exterior via *spiracles*. In some aquatic insects there are no spiracles. Here, the tracheae divide at the periphery to form a plexus, either over the general body surface, allowing cutaneous respiration, or within specialized gills (such as tracheal gills).

This section will review the general organization and structure of tracheal systems, various types of respiration including cutaneous, respiration by ventilation, the neural control mechanisms which operate various muscles related to respiration and respiration in aquatic insects. For details see Mill (1985) and Nation (1985).

Tracheal system

There are many variations in arrangement of the tracheal system. However, basically it consists of a meshwork of ramifying tubes, known as the *tracheae* and the *tracheoles*, opening externally through the *spiracles* (Fig. 8). Some aquatic and endoparasitic species have a closed tracheal system. In these cases oxygen either diffuses into the tracheae over the cuticle or passes in mainly through the integument of special respiratory outgrowths known as *gills* or *branchiae*.

There are primary, secondary and tertiary tracheae and these ramify in the various parts of the body according to the demand of oxygen supply for a particular tissue. The tertiary tracheae eventually narrow and branch into tracheoles. In large flying insects such as dragon fly, each tertiary trachea may produce 20 to 30 tracheoles. In some insects, tracheoles can move or can be pulled into tissue regions where oxygen tension is low (see Wigglesworth, 1977). In many insects, dilations of both primary and secondary tracheae, known as *air sacs*, occur. An air sac frequently provides the primary air supply to flight muscles, particularly those of small fast-flying insects like *Drosophila*. Since these air sacs provide a large surface in contact with the muscle, diffusion exchange of gases is very effective.

The spiracles are usually provided with valves and/or dust-catching setae or hairs or covered by a sieve plate containing many small pores. The number of spiracles vary from insect to insect. The usual number is 8 to 10 (polyneustic). There are a few insects with only one or two functional spiracles (oligoneustic). Spiracles may or may not have a closing mechanism. Spiracles are open in Apterygota, many aquatic larvae and some aquatic adults. There are muscles which operate the valves for opening or closing spiracles. The muscles are

innervated by a branch of the median nerve from the ganglion in the same segment or from the ganglion in the anterior segment. There has been some research to show the relationships of nervous connection to the spiracular muscles and the closure of spiracles.

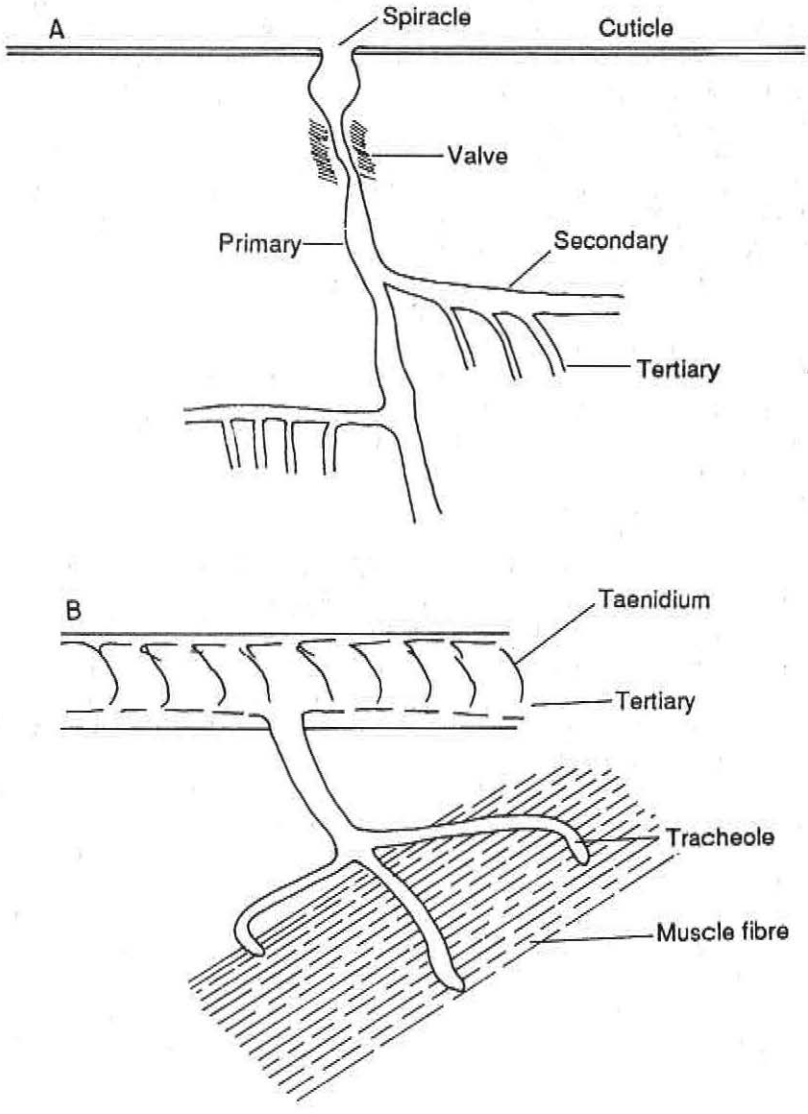


Fig. 8. Diagrammatic representation of the insect tracheal system. A, main tracheal components. B, tracheoles from tertiary tracheae enter into tissue systems

Movement of gases

Gases move through the tracheal tubes by diffusion and by active ventilation. The rate of diffusion is influenced by (1) the molecular weight of the diffusing molecules, (2) the partial pressure difference along the diffusion pathway, and (3) the permeability of the medium through which molecule must pass. In small insects diffusion can provide sufficient O_2 to the flight muscles to support flight metabolism, but in larger insects diffusion alone is insufficient to meet the energy requirements even during rest. In these insects, the diffusion pathways are shortened by the primary tracheae being forcibly ventilated. During rest, pumping movement of the abdominal musculature provide this ventilation. Two mechanisms of active ventilation have been described: (1) Abdominal pumping, either by dorso-ventral compression of the abdomen or by telescoping abdominal segments, and (2) Thoracic pumping due to wing movement.

Abdominal pumping is sufficient for some flying insects. In many other larger insects, such as locusts, thoracic pumping is important for ventilation of wing muscles. Abdominal pumping in the desert locust at rest can move 40L of air per kg per hour in through the thoracic spiracles and out through the abdominal ones. During the first 5 minutes of flight the abdomen can pump 180 L/kg/hr which later falls to 150 L/kg/hr.

Flying insects lose large amounts of water due to evaporation from the tracheal surfaces because large volumes of air are ventilated through the system. These insects are adapted to compensate this water loss by utilizing fat. For example, in desert locust about 7 g fat is burned per kg per hr to produce 8 g metabolic water per kg per hr.

Another adaptive mechanism for conserving water is the process of discontinuous release of CO_2. This process is known to occur in *Schistocerca* and several diapausing pupae of Lepidoptera. It has been most thoroughly studied in the pupae of *Hyalophora cecropia*.

Although CO_2 release is in bursts, the O_2 consumption remains constant throughout. During release the spiracles are wide open and during the interburst period they close initially but subsequently open slightly and flutter. After the valves close at the end of a burst of CO_2-release, O_2 consumption reduces the intratracheal pressure because the CO_2 produced by respiration remains largely in solution (as bicarbonate). These conditions cause the spiracles to open slightly when fresh air enters and replenishes the O_2 and causes the spiracles to close again.

Respiration in aquatic insects

Aquatic insects utilize many adaptations for respiration. Cutaneous respiration is common in these insects. Larvae of Trichoptera, Plecoptera, Odonata and others have large surface areas due to specialized organs

like tracheal gills. However, most aquatic insects breathe atmospheric air without any structural modification. Some insects carry an air bubble or a film of air on their bodies when they go down in the water. The bubble needs frequent renewing by coming to the surface for some insects, but others can hold a nonwetted air film so effectively with a plastron network which can continue to extract O_2 from the water indefinitely. The *plastron* type of respiration is one of the most common adaptations of aquatic insects for respiration. For a detailed discussion of this topic see Nation (1985).

Respiration in endoparasitic insects

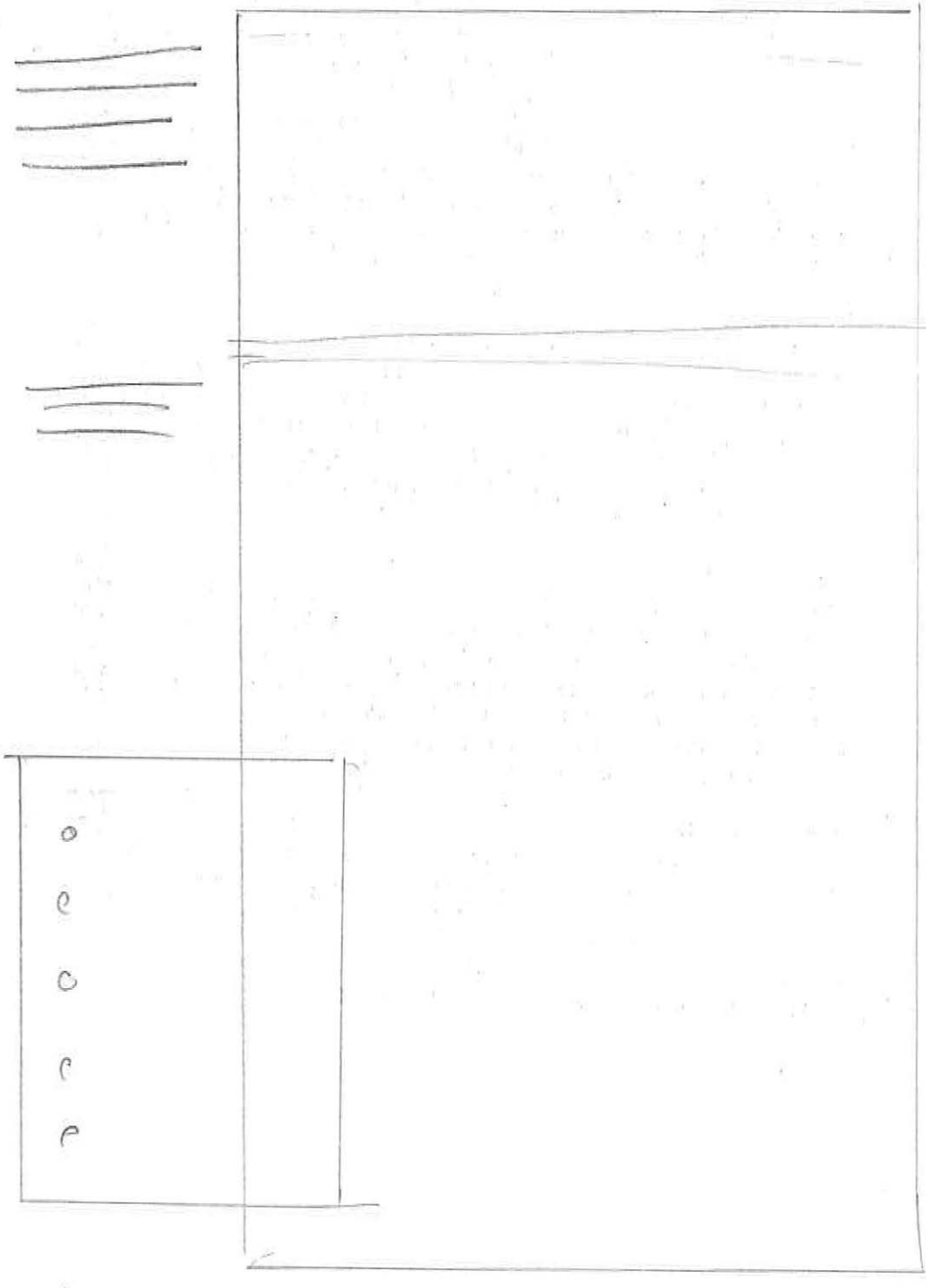
Parasitic Hymenoptera and Diptera show various types of adaptations in their respiratory process. Some rely on cutaneous respiration and others, including some chalcids and tachinid flies, use their posterior spiracles to breathe air directly by orienting them at the body surface of the host. The larvae of the bot fly *Hypoderma* migrates to the skin of the vertebrate host where it bores a tiny opening to the surface through which gas exchange occurs.

Respiration in eggs

In aquatic and semiaquatic insect eggs, the gaseous exchange occurs by simple diffusion through interstices in the egg shell. The majority of terrestrial insects' eggs contain special structures for respiration. They have a chorionic meshwork which can function as a plastron when they are submerged in water. *Aeropyles*, tubes of very small diameter connect the meshwork with the outside atmosphere. For a review of this topic see Hinton (1969).

Respiratory pigments

Hemoglobin occurs only in a small number of insects, presumably because the body fluids are not involved to any great extent in the transport of the respiratory gases. It is found in the larvae of some chironomids and in the larvae of *Gastrophilus* and in the adults and larvae of the notonectids *Anisops* and *Buenoa*. What is the function of hemoglobin in these insects? For details see Mill (1985).



CIRCULATORY SYSTEM AND HEMOLYMPH
(2 lectures)

Introduction
Structure of the circulatory system
- Dorsal vessel
- Heart
- Aorta

Hemolymph
- Plasma
- Hemocytes

Storage and transport of materials
Phagocytosis
Immune response

CIRCULATORY SYSTEM AND HEMOLYMPH

Introduction

Insect body cavity is a *hemocoel* which contains the circulating blood or *hemolymph*. That is why the system is an open system. The main functions of this system are transport and protection. Insect blood is not involved in oxygen transport. Compared to vertebrates, the insect circulatory system is very simple. However, the insect system is very efficient and works extremely well considering the size and the organization of the insect body. In addition insect blood is a complex and rich constituent. The blood composition is influenced by the developmental stage, sex, and age of the insect. Its composition changes with the molt and during the reproductive cycle. The cycle of hemolymph hormone titers during larval and adult development regulate insect growth and development.

Structure of the circulatory system

The *dorsal vessel* is the main conducting organ of the circulatory system. This vessel is divided into a *heart* and an *aorta*. The tubular heart is wider than the aorta, contains lateral *ostia*, is usually supported by the *dorsal diaphragm*. The aorta is the anterior prolongation of the dorsal vessel which carries the blood into the head. The wall of the heart is muscular and is composed of flattened cells whose outer cytoplasm is differentiated into striated muscle fibrils.

The blood or hemolymph is the only extracellular fluid in the insects. It may be clear, colorless or very pale yellow or green. It consists of a liquid plasma in which are suspended numerous colorless blood cells or hemocytes. The plasma consists of 85% water and has a pH of 6.4 to 6.8. It contains amino acids, proteins, fats, sugars (mainly trehalose) and inorganic salts (mainly sodium, potassium and chloride ions). The hemocytes are very variable in form and many different types have been distinguished. The circulating blood contains 1000–100,000 cells per mm^3. There are 7 types of cells (according to Gupta 1985): prohemocyte, plasmatocyte, granulocyte, spherulocyte, adepohemocyte, oenocytoid and coagulocyte (consult Gupta 1985 and 1979, and Woodring 1985 for morphology and character of hemocytes).

The circulation of the blood begins in an anterior stream that is maintained by waves of contraction, passing from behind forward, over the heart. During *diastole* blood is drawn into the heart through the ostia, under a negative pressure. During *systole* a positive pressure is set up and the blood is driven forward in the heart cavity and eventually leaves the open anterior end of the aorta in the head. Ultimately, the blood enters the visceral sinus after a proportion has circulated through the legs and wing veins. The ventral diaphragm

also takes part in the circulation process in the perineural sinus.

The property of rhythmicity of heart lies in the muscle fibrils of its walls. This is evident in the isolated heart or even in several portions of it since they continue to beat in the usual rhythmic manner. The pulsation rate may be influenced by many external, pharmacological or hormonal factors.

Cellular immunity

Cellular immune reactions in insects have been classified as phagocytosis, encapsulation, nodule formation and coagulation. Small doses of single-celled pathogens, such as bacteria, viruses and protozoans are phagocytized and larger doses usually are coalesced into nodules. Metazoan parasites or inert objects are enclosed in capsules.

Plasmocytes are the main hemocytes which are phagocytic. There are three steps in phagocytosis:
(1) attachment and recognition, (2) ingestion, and (3) killing-digestion.

Cellular digestion in plasmocytes seems to be similar to that in vertebrate neutrophils. The discharge of specific granules, containing alkaline phosphalages and lysozymes, into the phagosome occurs before the discharge of granules containing acid hydrolases.

A review of cellular immunity, humoral immunity and functions of blood plasma as the storage organs will be found in Woodring (1985). Both cellular and humoral immunity are promising areas of research, particularly in terms of applied research.

EXCRETORY SYSTEM AND WATER BALANCE
(2 lectures)

Introduction
Structure and function of Malpighian tubes
Structure and function of the rectum
Water and ion balance (osmoregulation)
Storage excretion

EXCRETORY SYSTEM AND WATER BALANCE

Introduction

The function of the excretory system of animals is to maintain a relatively constant internal environment for the tissue of the body by removing the nitrogenous products of protein breakdown and regulating the ionic composition of the hemolymph.

The insect excretory system is not a hydrostatic pressure filtering system as in many vertebrates. Excretion is carried out in insects by a *Malpighian tubule-rectum complex*, which is a very efficient system and is present throughout the class Insecta with a few exceptions. It also plays a vital role in *osmoregulation* and *water balance*. The selective reabsorption of water, ion and certain other chemicals is carried out by the rectum according to the physiological needs of the insects.

Structure and function of Malpighian tubules

The Malpighian tubules of insects are small blind tubes that arise by evagination of the gut usually at the junction of mid and hind gut. They are variable in number, size and shape. For example, *Drosophila* larvae have 4, and *Periplaneta* adults have about 150. These tubules remove excretory materials from the blood in the form of urine. This is secreted into the lumen of the tubule and ultimately discharged into the hind gut. In the hind gut, its composition may be modified by resorption before it passes out with the feces. The urine may be clear liquid or a thick suspension. The principal nitrogenous material is *uric acid*. However, uric acid is not the only nitrogenous compound found in the urine of insects (and in some cases uric acid may even be absent). For some species uric acid may be converted to *allantoin* or *allantoic acid* (or both) prior to voiding. *Ammonia* seems to be the principal nitrogenous excretory product in aquatic immature insects, dipteran larvae and in certain cockroaches. Other compounds such as amino acids, pteridines, tryptophan derivatives, and sometimes proteins are also present in insect urines. The urine may also contain carbohydrates of various types in some insects.

The Malpighian tubules of many species function at very different rates, which seems to be under hormonal control. In *Glossina*, *Anopheles* and *Rhodnius*, the blood-sucking species *diuresis* occurs. It appears to be a response to nervous stimulation as a result of abdominal stretching. The result is a release of a *diuretic hormone* into the blood within 15 seconds after feeding begins (in *Rhodnius*). The hormone, present in the mesothoracic ganglionic mass, is released into the hemolymph at neurosecretory axon endings located outside this mass. In other species the brain, CC and CA may be involved. The Malpighian tubules respond rapidly to the hormone release and produce urine at a high rate. Some efforts have been made to isolate, purify, and

identify the diuretic hormones. They appear to be peptides in nature and in *Glossina* it is heat stable, nondialysable, alcohol soluble and contains amino acids, glucose and sialic acid residues.

Structure and function of the rectum

The hind gut receives the primary urine from the Malpighian tubules. Modifications of the urine is then carried out in the rectum. Rectum also plays a major role in water balance and osmoregulation. For morphology and fine structure of rectum in insects in relation to their excretory function refer to review papers by Cochran (1975 and 1985). The rectum assists in reabsorbing specific components from the primary urine from the Malpighian tubules.

Water and ion balance (osmoregulation)

There are two major problems that the insect faces in relation to the process of excretion. One problem is, in the excretory process, not to lose physiologically useful products. The other problem is of water conservation, in relation to excretion, that is, the function of rectum removing water from the primary urine and transferring back to the hemolymph. Osmoregulation is primarily concerned with water and the ion balance. The rectum is involved in this function.

The relationship between excretion and osmoregulation is that the same processes, that is, ion and water movement, are used in both cases.

For terrestrial insects, the major concerns are conserving water and keeping the hemolymph ion concentration balanced. Most terrestrial insects studied are well-equipped to carry out these functions. But the situation is different for insects living in aquatic environments (or terrestrial insects intaking a large amount of liquid diet). The problem here is to void excessive liquid and at the same time retain ions. Examples are blood sucking insects and aquatic insect larvae. In both cases, large amounts of water pass through the system because of their feeding habits. The rectum produces a dilute urine by removing ions from the lumen. Successful osmoregulation is accomplished in brakish water by many species. Here, the function is to retain water and remove excessive ions. These insects void a hyperosmotic urine through rectal action. A few species of insects, however, are capable of osmoregulating in salt water. They have some special capabilities. In *Anopheles campestris*, the posterior rectum has highly developed cells. It has been shown that these cells actively secrete Na^+, K^+, Mg^{2+} and Cl^- into the lumen. This results in production of extremely hyperosmotic urine.

Is there any hormonal control of rectal function? Very little is known. Cochran (1985) indicated that some information regarding the diuretic factors from the CA of the desert locust is available. Apparently

these factors help decrease reabsorption of water by rectum. But it is not known for sure whether the diuretic factor which affects the Malpighian tubules also affects the rectum or it is an entirely different hormone.

In addition to the above accounts of the excretory system of insects, there has been some work on the topic of *storage excretion* which deals with the concept of insects being capable of storing substances present in their bodies in excess of requirement and excreting later on. See Cochran (1985) for a detailed discussion on storage excretion.

INTERMEDIARY METABOLISM AND FAT BODY

(3 lectures)

Introduction
Major metabolic pathways
Fat body and metabolism
- a. Carbohydrates
- b. Proteins and amino acids
- c. Lipids

Hormonal control of metabolism
- a. Hypertrehalosemic hormone
- b. Adipokinetic hormone

INTERMEDIARY METABOLISM AND FAT BODY

Introduction

Although every cell in the insect is responsible for the synthesis and utilization of most metabolically significant compounds, only two tissues have been studied somewhat in detail. These are fat body and flight muscle. These are the tissues which make up the major portion of the total tissue mass contained in the hemolymph. Moreover, the fat body of insect is the major synthetic and storage organ and the flight muscles are probably the most actively metabolizing tissue of the body.

This section will discuss the fat body organization, both structural and biochemical and its role in carbohydrate, protein and lipid metabolism. There are several review articles on this topic. Consult most recent reviews by Dean et al. (1985), Friedman (1985a), and Keeley (1985). Also, the class will discuss insect metabolic processes in general (see Candy, 1985, Friedman, 1985b, Downer, 1985, and Chino, 1985).

Major metabolic pathways

The major metabolic pathways in insects are comparable to those of the vertebrates, with few exceptions. Major pathways are glycolysis (tissues involve: muscle, fat body, nerves, etc.), glycerol phosphate shuttle (muscle), TCA cycle (most tissues), β-oxidation of fatty acids (muscle, fat body), fatty acid synthesis (fat body), amino acid metabolism (fat body, Malpighian tubules, muscles, gut), pentose phosphate pathway (fat body) and nucleotide metabolism (fat body).

Although the metabolism of insects is similar to other organisms, there are a number of special features of insect metabolism. Because of adaptation to various environments and life styles, insects evolved characteristic specialization. For example, ecdysis and metamorphosis result in alternate period of synthesis and breakdown of proteins, chitins, etc., restructuring of most tissues and hormonal regulation. Insect flight causes rapid oxidation of substrate by muscle and transport of substrate from storage sites.

Insects possess a wide variety of different tissues, each of which performs different functions. For example, the insect epidermis, which synthesizes components of the insect exoskeleton. The fat body is responsible for the metabolism of the products of digestion, synthesis of hemolymph proteins, lipids and carbohydrates, and storage of fats and carbohydrates. The flight muscle is capable of rapid ATP synthesis by complete oxidation of fats, carbohydrates and amino acids.

The metabolic pathways can be classified in two functional groups: one group concerned with degradation and the other group with synthesis. But a particular pathway may be involved in both functions

at times. Glycolysis, β-oxidation, TCA cycle and amino acid oxidation can all synthesize ATP which is then used for synthetic reactions to provide energy. Some of the main biosynthetic pathways in insects are gluconeogenesis, glycoside, fatty acid, amino acid, protein and purine and pyrimidine syntheses. The prerequisites for the synthetic processes are a substrate supply, a supply of energy (ATP) and for a number of biosynthetic processes, a supply of reducing agent such as NADPH. There are a number of synthetic reactions that insects are unable to perform. For example, conversion of fatty acids to carbohydrates, synthesis of certain essential amino acids and the synthesis of sterols. See details of major metabolic pathways in Candy (1985).

Fat body and metabolism

The fat body is the principal tissue for intermediary metabolism in insects. Although, fat body appears superficially to be an adipose storage tissue, it is in fact a tissue of considerable metabolic activity and it is also the main source for the hemolymph proteins, lipids and carbohydrates which serve as precursor for metabolism in other tissue.

Insect fat body is heterogeneous in its structure and widely distributed in the insect body. By being widely distributed, the fat body reduces the diffusion distances between itself, as the source tissue for the metabolites and the peripheral tissues where the metabolites are used.

The fat body is composed of either 2 or 3 cell types. The predominant metabolic-storage cells are the *adipocytes*. The second common cell types is the *urocyte* that sequesters uric acid for storage-excretion. In some insects a third type of cell is found which are known as *micetocyte* which contains various types of symbiotes especially bacteria-like organisms.

In a typical lobe of fat body, cells are arranged in layers. The metabolically active adipocytes are the outermost cells in the lobe with urocytes and micetocytes, if present, are more central. The position of adipocytes is ideal for maximal exposure to the hemolymph for metabolite exchange.

Trehalose is the major circulatory carbohydrate of most insects and is synthesized solely by the fat body. Trehalose synthesis and its regulation is discussed in Keeley (1985).

Fat body synthesizes a large number of proteins. Synthetic activities vary depending on the stage and sex of the insect. For example, a number of storage hexamer proteins are synthesized by the fat body of the feeding larvae and nymphs which reach high concentrations in the hemolymph just prior to metamorphosis (see review by Telfer and Kunkel, 1991).

A major function of the adult fat body in females is the synthesis and release of vitellogenic proteins for yolk formation during oocyte

maturation (see Kunkel and Nordin, 1985). Vitellogenin synthesis accounts for 70–90% of the protein secreted by the mature fat body in female insects. Vitellogenin synthesis is endocrine regulated, usually by JH (will be discussed in connection with the physiology of reproduction). During diapause, synthesis pattern of fat body changes. In Colorado potato beetle, three diapause proteins are produced under short-day photoperiod.

The various amino acids in insect hemolymph derive from endogenous synthesis as well as ingested and digested protein. Highly regulated amino acids such as proline and tyrosine, appear to have their major sources of synthesis located in the fat body. For example, tyrosine, which is produced by the hydroxylation of phenylalanine, is sequestered with other amino acids in the fat body of calliphorid fly larvae and built into a storage protein which is released and stored in the hemolymph throughout larval life. Later on it is moved into the cuticle where it takes part in the hardening and darkening of the adult cuticle. Proline, in those insects that utilize it for the energy source, is synthesized in the fat body from alanine through a series of reactions. Tsetse flies, *Glossina* sp use proline as the major fuel for flight. A high activity of proline dehydrogenase is characteristic of the flight muscles of tsetse. See Mordue et al. (1980) for proline oxidation pathways.

Triglycerides constitute the main storage form for fat body lipids. The most common fat body fatty acids are palmitic, palmitoleic, stearic, oleic, linoleic and linolenic. Only saturated and monoenoic fatty acids are synthesized from acetate by the fat body. Insects cannot synthesize polyenoic fatty acids. The fat body forms diglyceride and releases it as the major circulatory lipid (for details see Chino, 1985). Although exceptions exist, in general, triglycerides are the storage form for fat body lipids, while diglycerides are the mobile hemolymph lipids.

Hormonal control of metabolism

Major developmental hormones such as ecdysteroids and juvenile hormones are responsible for changes in the level of various storage compounds in the hemolymph. However, there are also a number of hormones which exert their control of specific compounds' level in the hemolymph. These hormones are hypertrehalosemic, hypotrehalosemic and adipokinetic hormones. The hypertrehalosemic hormone seems to activate the fat body glycogen phosphorylase which results in increased glucose phosphate level in that tissue. The hormone is a low molecular weight peptide the action of which can be duplicated by cAMP using fat body preparation. The adipokinetic hormone is a better known hormone which has been isolated and purified from the CC of the locust, *Schistocerca gregaria*. In locusts, it promotes the release of diacyl-glycerol from the fat body into the hemolymph, and at the same time stimulate the production of a specific lipoprotein system for transporting the lipid to the muscle. In other insects, the action of this hormone is still not very clear.

PHYSIOLOGY OF REPRODUCTION
(4 lectures)

Introduction
Types of reproduction
Male and female reproductive systems: structure and function
- Male: General plan
  - Testes and spermatogenesis
  - Accessory glands
  - Spermatophores
  - Semen
  - Sperm motility
- Female: General plan
  - Ovary, oviduct and oocytes
  - Spermatheca
  - Accessory glands
  - Vitellogenesis — hormonal control
  - Chorion formation

Ovulation
Oviposition
Fertilization
Embryonic development
Hatching

PHYSIOLOGY OF REPRODUCTION

Introduction

Processes of reproduction are most diverse in insects. The reproductive structures and strategies in insects are numerous. It would be logical to believe that the largest and the most successful groups of animals, the insects, would evolve a most diversified and extraordinary array of reproductive mechanisms. This chapter will summarize the most generalized accounts of reproduction in insects and discuss some areas of active research in this field at present.

Types of reproduction

The most common mode of reproduction is *oviparity*, where the females lay eggs which contain substantial nutrients for the developing embryo. The eggs hatch outside the female reproductive tract. *Ovoviviparity*, which is less common, involves the retention of eggs within the reproductive tract until hatching or just before hatching. *Viviparity*, on the other hand, involves not only the retention of the egg within the reproductive tract, but the provision of substantial nutrient supplies for the developing young. These three types of mechanisms are represented in cockroaches. The oriental cockroach, *Blattella orientalis* deposits an egg case soon after it is formed. The German cockroach, *Blattella germanica*, carries the egg case internally until just before the eggs hatch. The viviparous *Diploptera punctata* nourishes the developing larvae in a brood sac. The flesh flies, *Sarcophaga* spp exhibit viviparous habit where they deposit newly formed larvae instead of eggs. The tsetse flies, *Glossina* spp, exhibit a specialized viviparity, called adenotrophic viviparity, where embryos develop to maturity within the mother by receiving nourishment from the specialized uterine glands of the mother.

Sexual reproduction is the general rule in insects although many exceptions to this are known. Parthenogenesis is common in some species of flies, and in aphids, wasps and Lepidoptera.

In some social insects (termites, bees) reproduction is limited to a small number of individuals, often one queen and a small number of males (such as drones among bees). Differentiation of primary and secondary characters is suppressed among the remaining population. Suppression is maintained by pheromones secreted by the reproductives. In some cases, loss of reproductive individuals will result in sexual differentiation in workers by removing the inhibition for developing secondary sexual characters.

Male and female reproductive systems

In sexual reproduction, the function of the male reproductive system is to manufacture sperm and deliver them to the female — along with

certain chemical and/or physical stimuli to signal the female neuroendocrine system the fact that mating has occurred.

The basic plan for the internal organs of reproduction of the *male* insect is simple. The reproductive system of a typical male insect consists of a paired testes which consists of a series of testicular follicles bound together by a mesodermal sheath. Each follicle opens via a vas efferens into the mesodermal vas deferens and the paired vasa deferentia unite to connect ecdodermally derived ejaculatory duct which terminates in the gonopore. The vas deferens is expanded over part of its length to form a seminal vesicle (sperm storage organ). In many cases accessory glands are formed as diverticula from the vas deferens. The glands commonly supply the secretions which form the spermatophore. The organization and number of accessory glands vary according to insects. For example in cockroach, *Periplaneta americana,* accessory glands are represented by a structure called "mushroom body" which consists of at least two types of tubules and seminal vesicles. In this species spermatogenesis is completed in the larva and the testes and vasa deferentia are degenerated or much reduced.

The external genitalia of male insects are highly variable in anatomy. The male genitalia of insects which are ectodermal in origin, have been adopted by the taxonomists prime features for distinguishing between related species which are reproductively isolate.

The mesodermal tubules that form the vas deferens attach to the ninth abdominal segment where normally a single ejaculatory duct forms, which in turn connects to the external structure the intromittent organ, the penis. The major segment of the penis is the aedeagus, which is often withdrawn into the body cavity but is everted during copulation. Other modifications of structures on the ninth segment may include clasping organs that assist in initiating and maintaining copulation.

Spermatophores are the structures produced by the male which enclose the semen while it is transferred to the female. Spermatophores vary widely in form. They are most common among the lower orders in which the structure is usually flask-shaped (see Davey, 1985). In tsetse, the spermatophore is more or less round and possesses distinct layers of gelatinous matrix.

Retention of spermatophore to form a *mating plug* is a feature to be found in many insects (mosquitoes and other higher Diptera).

What is the composition of insect semen? The amounts of semen are small and the density of sperm is very high. For this reason the separation of sperm from seminal plasma becomes very difficult. Seminal vesicles of most insects probably do not produce any secretion.

Sperm motility has been studied in a few insects (see Davey, 1985). In certain saturniids it has been shown that a peptide of molecular weight between 1600 and 4500 was responsible for sperm activation. Very little is known regarding metabolism of sperm. There are some descriptions

of enzymatic activity where glutamate dehydrogenase is involved (Shepherd et al., 1981).

Spermatogenesis is complete in most insects studied by the time the adult emerges. However, many male insects undergo a period of sexual maturation process even after emergence because their other reproductive organs and processes have not developed fully, such as the accessory reproductive gland secretions which are essential for successful copulation.

Regulation of spermatogenesis receives far less attention than the similar event in the female (such as vitellogenesis). The formation of a mature spermatozoa is probably no less complex a developmental process than the formation of a mature egg (see reviews by Dumser, 1980 and Raabe, 1986).

Female reproductive system and its function

The functions of the female reproductive system are:

- to produce eggs
- to deposit them at an appropriate time and in an appropriate place
- to receive sperm during mating and to transport them to the spermatheca
- to keep the sperm viable
- to facilitate egg fertilization
- to provide protection to fertilized eggs (in some insects, such as in the form of an ootheca)
- ovulate and deposit (egg or larvae)

The internal organs of reproduction in female insects vary in structure from species to species but most conform to the basic plan, which has the following structures:

- paired *ovaries* with a number of *ovarioles* in each ovary
- developing oocytes are enclosed in the ovariole
- ovarioles unite anteriorly in the *terminal filament* and posteriorly in the *calyx*
- the ovaries possess lateral oviducts which unite to form the *common oviduct*
- the common oviduct opens into the *genital chamber*
- the opening of the genital chamber to the outside is the *vulva*
- *spermatheca* opens into the genital chamber via *spermathecal duct*
- *accessory glands* may also open into the genital chamber.

The function of spermatheca is to store and nourish the sperm until they are used for fertilization of eggs. Honeybees can store sperm for as long as 3 years. The structure of spermatheca varies. There is a storage portion and a glandular portion; sometimes the storage portion contains glandular cells. Secretion which is found in the lumen of the spermatheca is a mucoprotein or mucopolysaccharide. The secretion is used by sperm and is essential for their survival.

The secretory activities of spermatheca are probably under hormonal control. More work is necessary to establish exact mechanism.

Mechanism of sperm release from spermatheca is not clearly understood. It appears that the spermathecal duct is supplied with nerves which are involved (*Schistocerca*).

Species which are known to mate more than once (multiple insemination) usually use the sperm of the most recent matings for fertilization.

Female accessory gland

Functions:

(a) The gland produces a proteinaceous secretion which coats the egg and fastens it to the substrate (*Rhodnius*).

(b) In most orthopteroid insects the product of the colleterial gland is used to form egg cases (e.g., ootheca of cockroaches or foamy egg case of desert locust).

(c) The uterine gland of the adenotrophic vivaparous insect tsetse is a modified female accessory gland which produces nutrient for developing larva in the uterus of the female fly.

Oviducts

The lateral and common oviducts leading to gonopore are involved in transportation of egg during oviposition and copulation.

Most female Lepidoptera have two reproductive openings. One for discharge of eggs and is known as the oviporus. The other is the copulatory opening, the vulva. The latter leads to the bursa copulatrix which is connected to the main oviduct by a sperm duct.

In viviparous Diptera part of the common oviduct is enlarged to form the *uterus*, in which larval development occurs.

Ovaries

Each ovariole consists of a distal *germarium* in which oocytes are produced from oogonia, and a proximal part the *vitellarium* in which oocytes grow as yolk is deposited in them.

Oocytes pass back down the ovariole, enlarging as they do so, and as each oocyte leaves the germarium it is clothed by the prefollicular tissue which forms the *follicular epithelium*.

There are three types of ovariole:

1. *Panoistic:* There are no special *nurse cells*. This is found in more primitive orders of insects (Thysanura, Odonata, Plecoptera, Orthoptera and Isoptera). Also Siphonaptera among Holometabola shows this type of ovariole.
2. *Telotrophic:* This is characterized by the presence of trophic tissue as well as oogonia and oocytes in the germarium. This arrangement is found in Heteroptera and many Coleoptera Polyphaga. *Nutritive cords* are formed to connect developing oocyte to the germarium.

3. *Polytrophic:* In this arrangement, the nurse cells, or *trophocytes* are enclosed in the follicle along with the growing oocyte. They occur in Dermaptera, lice and throughout the holometabolous insects (except Siphonaptera).

The main function of the trophic tissue is to supply RNA to the oocyte in the large quantities necessary for rapid growth. In the panoistic ovarioles, however, the oocyte nucleus is the only source of RNA. This has been an area of active research in recent years. (See review by King and Buning, 1985.)

Vitellogenesis and its control

Vitellogenesis is the deposition of yolk in the oocyte, which occurs in the most proximal part of the ovariole.

Vitellogenin (Vg) the predominant yolk protein precursor is produced (in most species studied) by the fat bodies of the vitellogenic females and then transported through the hemolymph before it is taken up by the growing oocytes.

The major protein (80–90%) of the egg is referred to as *vitellin* (Vt).

The most convenient and also most reliable isolation and identification of the native vitellogenins (Vg) and vitellins (Vt) has been by immunoprecipitation techniques.

Characterization of vitellogenin and vitellin has also been made by polyacrylamide gel electrophoresis (PAGE) and SDS-PAGE.

The differences between Vg and Vt are subtle. For majority of insects they appear to be largely identical molecules. Analysis of polypeptide chains of Vts of several insects (*Manduca, Drosophila, Leucophaea*) are available. (See review by Kunkel and Nordin).

Vitellogenic oocytes of *Hyalophora cecropia* concentrate four extracellular proteins into yolk spheres. Three of these vitellogenin, lipophorin and microvitellogenin originate from the hemolymph. The fourth, paravitellogenin is a product of the follicular epithelium.

Other integral constituents present in the yolk protein are the Vg oligosaccharide, Vg lipids, esterified phosphate.

Other egg specific proteins (ESP) are also known which usually originate from the follicular epithelium (e.g., in *Bombyx mori*, Sato and Yamashita, 1991). This ESP is a glycophospholipoprotein quite different from the other yolk proteins.

Hormonal control of vitellogenesis has been studied extensively in several insect species. Each species differs in detail (for a general scheme see Bownes, 1986). (see Figs. 9–13).

Roles of cerebral neurohormones, JH and ecdysteroids in vitellogenesis have been reported. For example, the control of egg development and vitellogenesis in mosquito, *Aedes aegypti* involves at least three hormones—JH, EDNH (egg development neurosecretory hormone) and ecdysone (see Hagedorn, 1983 and Lea & Brown, 1990).

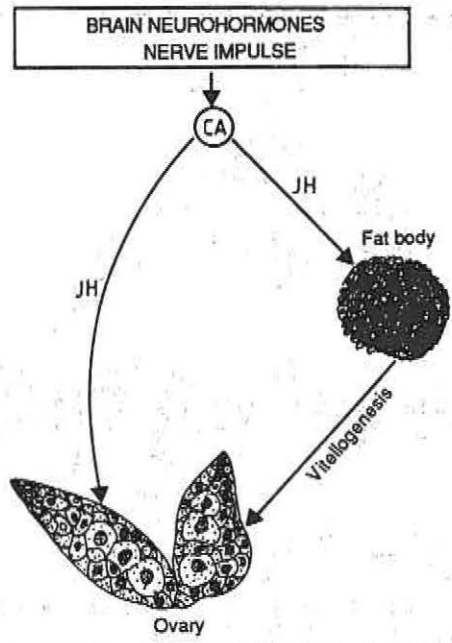


Fig. 9. Diversity of vitellogenesis. *Rhodnius* model (see Raabe, 1986)

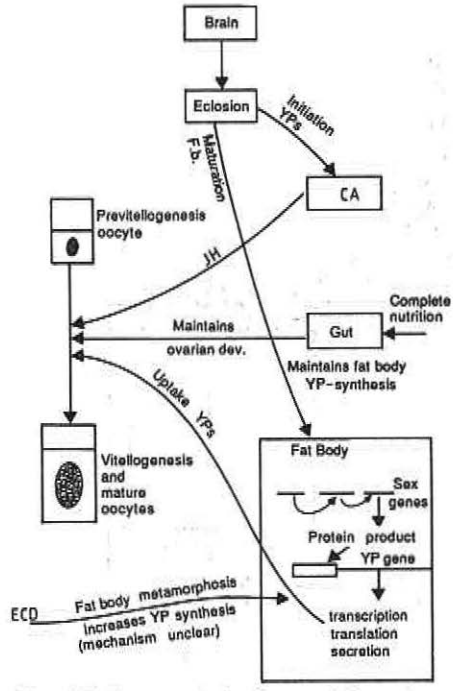


Fig. 10. Vitellogenesis in *Drosophila melanogaster*. JH, juvenile hormone; ECD, ecdysone; YP, yolk protein

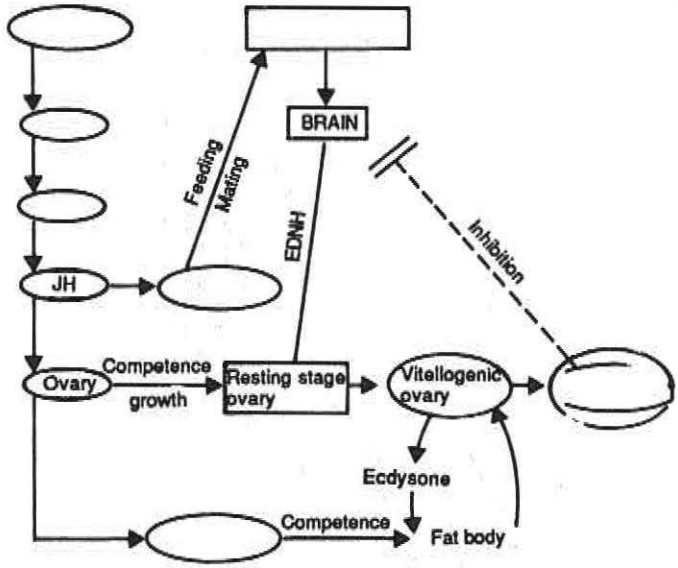


Fig. 11. Diversity of vitellogenesis. *Aedes aegypti* model (see Hagedorn in Downer and Laufer, 1983, p. 284)

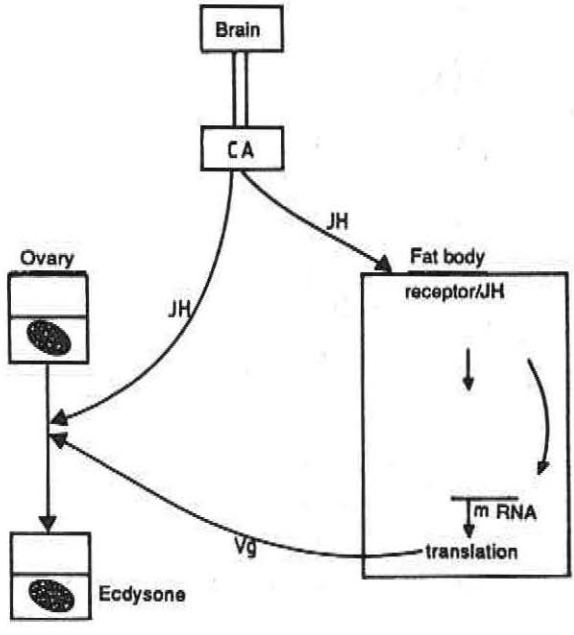


Fig. 12. Regulation of vitellogenesis in *Leucophaea maderai* and *Blatella germanica*

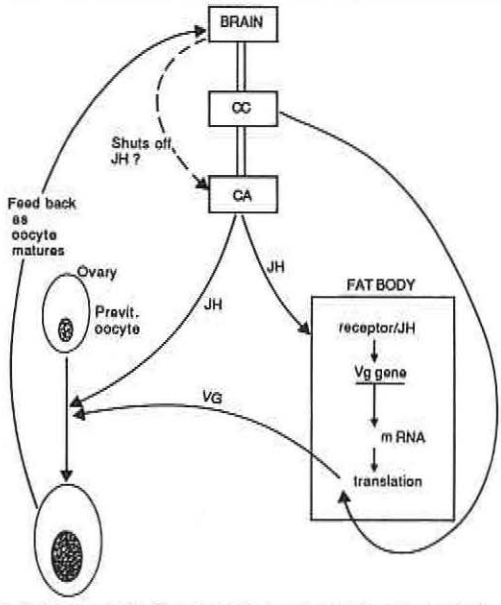


Fig. 13. Regulation of vitellogenesis in *Locusta migratoria*

It has been reported from some insects that there is an ovarian feedback control which inhibits vitellogenesis in penultimate oocyte. For postulated feedback pathways see Huebner (1983).

Chorion formation

During the last period of follicle maturation the follicular epithelium cells, which surround the single oocyte, synthesize and secrete a set of structural proteins that assemble to form the chorion.

At the physiological level the chorion functions in protecting the oocyte from mechanical stress (from predators) as well as from environmental stress (desiccation and drowning). At the same time the chorion must permit gaseous exchange and sperm penetration through the micropyle (see Hinton, 1981).

Studies on the molecular aspects of chorion formation and the structural and evolutionary studies on specific gene and gene product have been quite extensive during recent years (for a review see Regier and Kafatos, 1985).

The morphogenesis of eggshell layers (choriogenesis) has been studied in detail in *Drosophila melanogaster* (see Margaritis, 1985).

To understand how any of the functions is performed we must have the knowledge of the details of fine structures and the macromolecular arrangement of the layers and regions. Other experimental approaches including water permeability measurements and oxygen consumption, provide additional information leading to comprehensive knowledge of chorion physiology.

Ovulation

Ovulation is the expulsion of egg from the ovary into the oviducts. Ovulation is a prerequisite for oviposition. It is not clear whether these two events (ovulation and oviposition) are separately controlled. In most insects ovulation is followed by oviposition immediately. In a few insects oviposition may occur long after ovulation.

In tsetse, *Glossina* sp, the ovulated egg is retained in the uterus until larval development is complete. In desert locust, *Schistocerca gregaria*, eggs leave the ovaries and accumulate in the lateral oviducts over a period of about a week before being oviposited (Okelo, 1971).

Ovulation is under hormonal control from the brain. This has been shown from the studies on *Rhodnius* and *Glossina* (see review by Davey, 1985a).

Oviposition

The processes of oviposition and ovulation may be under the same control. However, the oviposition process is much more complex and involves integration of sensory and motor nervous systems in the process.

Oviposition is a complex behavioral event involving highly coordinated activities such as digging in soil, boring in the wood, or penetrating host epidermis.

Hormonal and neural control mechanisms of oviposition are not well understood and much more information is needed.

Fertilization

Fertilization of the oocytes occurs after the oocyte moves from the ovary into the common oviduct. The egg must be precisely orientated in the oviduct for the penetration of the sperm through the micropyle. That the sperm enter the egg through the micropyle has been demonstrated amply in the literature (see review by Retnakaran and Percy, 1985). Fertilization in the housefly *Musca domestica* and *Drosophila melanogaster* has been studied in detail.

Physiology of insect embryogenesis

Embryogenesis is initiated by egg activation and goes through several steps starting with intravitelline cleavage to blastoderm formation to gastrulation and finally to histogenesis and organogenesis (see review by Sander et al., 1985).

Towards the end of the embryogenesis hatching occurs. Muscular contractions set in and gas is secreted into the tracheae. Hatching is initiated by various ways ranging from enzymatic digestion of egg shell (in honeybee) to mechanical disruption of the chorion.

Metabolism during embryogenesis has not been studied extensively. A few authors have attempted to distinguish between yolk respiration

(before embryogenesis) and the respiration of the embryo proper. The energy requirements for embryogenesis are met by the stock of lipids, carbohydrates and to a lesser extent, proteins, all of which are accumulated during oogenesis. Stored lipids are the main source of energy during embryogenesis in most insect species studied.

Role of yolk proteins is also important because they are the major constituents in insect eggs. The protein moiety of the vitellin polypeptides appears to be used mainly as a store of raw material (amino acids) readily available for peptide synthesis.

Hormonal involvement in embryonic development have been studied (reviewed by Hoffmann and Lagueux, 1985). Ecdysteroids are known to exist in many insect eggs. One of the functions in the control of embryonic molting. Presence of juvenile hormone has also been reported from a few insects. Role of JH in embryonic development remain speculative. When *Manduca* eggs were treated 0–24h postoviposition by topical application of fluoromevalonate, a substance interfering with JH-biosynthesis, substantially reduced JH titers were found in 72–96h old eggs when compared to acetone treated control (Bergot, 1981). When the treated specimens were allowed to hatch, the larvae showed typical JH deficient symptoms (appearance of pupal cuticle, melanization, etc.).

Recently, developing embryo of cotton stainer bug, *Dysdercus fasciatus* was shown to have makisterone A and makisterone C but no ecdysone or 20-hydroxyecdysone. Embryonic sterols were highly reflective of the sterols found in the cotton seed diet upon which previous generations of the bugs had fed (Feldlaufer et al., 1991).

Hatching

When ready to emerge from the egg, an insect has to force its way through the chorion in order to reach the outer environment. In many cases the chorion is torn by means of structures known as *hatching spines* or *egg bursters*. These are located on the head, or other parts of the body, where they may remain until the insect has undergone its first molt. In some other species, the chorion is provided with an operculum which is pushed open during hatching. The force used in hatching is a chiefly muscular activity but initially an insect may swallow air which results in the increase in bulk which, in turn, can apply pressure to cause force against the chorion.

PHYSIOLOGY OF DIAPAUSE

(2 lectures)

Introduction
Diapause and environment
Diapause during embryonic stage
Larval diapause
Pupal diapause
Adult diapause

PHYSIOLOGY OF DIAPAUSE

Introduction

Diapause, defined as a spontaneous developmental arrest, may occur at any stage in the life cycle of insects. An insect may undergo diapause as an embryo, a larvae, a pupa or an adult, depending on the species. Many insects enter diapause to withstand various diverse environmental conditions. True diapause is characterized by an extremely low metabolic rate and relative inactivity as well as certain biochemical changes in the body.

There are environmental factors which induce, maintain or terminate diapause. These are usually photoperiod, temperature and availability of food. In addition, there are hormonal factors which are involved in diapause. A survey of the studies on the endocrinological basis of diapause in insects gives an idea about the varying mechanisms and ways insects adapt to the available environmental situation. For details of various kinds of diapause in insects consult Denlinger (1985).

Diapause and environment

Most insects are affected by seasonal changes in the environment. The onset of cold in winter or drought in summer has to be anticipated and its danger avoided. The insects do it by entering into the state of diapause. For some species this is an *obligatory* event in their life history, for others diapause intervenes during development only under certain conditions where this is *facultative*. Whether diapause is obligatory or facultative, the insects respond to environmental cues which is indicative of the onset of unfavorable conditions. These cues include temperature, lack of moisture, population density and especially photoperiod. In fact, a great majority of insects use day length as a signal for developmental switching into or out of diapause. There is usually a *critical photoperiod* below which the physiological response occurs only in a few individuals, but above which the response occurs in most or all of them. Either day-length or night-length may be measured, but in insects it is commonly the latter.

Diapause during embryonic stage

Many insects have an egg diapause which may occur at any of several stages of embryonic development. This may be after the germ band formation, around the time of blastokinesis or at a stage just before hatching. Diapause induction and termination at any stage of egg development is under the control of the environmental stimuli received at the preceding generation. The silkworm egg (*Bombyx mori*) enters diapause at an early phase of embryonic development. High temperature (25°C) and long-day photoperiod (16L:8D) during the

embryonic stages induces the production of diapause eggs by the moths, but low temperature (15°C) and short-day photoperiod (12L:12D) lead to non-diapause eggs. When reared on an artificial diet, the larvae maintain the ability to receive environmental stimuli and to modify the diapause nature predetermined at embryonic stages. The termination of diapause is brought about by chilling the eggs for three or more months or by soaking the eggs in concentrated HCl solution.

The females, which produce the diapausing eggs (under the influence of long-day conditions), are found to secrete a peptide hormone from the subesophageal ganglion which has been named *diapause hormone* (DH). The DH acts on the egg by arresting embryonic development at the germ band formation. The hormone also acts on the developing ovary to increase its permeability to 3-hydroxykynurenine (a precursor to the ommochrome pigment found in the serosa of the diapausing eggs), to increase trehalase activity and thus enhance glycogen synthesis and storage in an inactive form of a nonspecific esterase (esterase A). Prolonged chilling to terminate diapause activates esterase A when the eggs are brought again to warm temperature. The esterase then causes lysis of the yolk/cells which allow continuation of embryonic development. The chemistry and the mode of action of DH has been investigated (Yamashita, 1983). For examples of egg diapause in other insect species see Yamashita and Hasegawa (1985).

Larval diapause

Larvae may enter into diapause in any instar. However, most commonly diapause occurs in the last larval instar. Most of the experimental studies have been carried out in lepidopterous species. It has been shown that juvenile hormone is responsible for the maintenance of the larval diapause. In southwestern corn borer, *Diatraea grandiosella* temperature acts to determine whether or not a larva will enter diapause. At 30°C larvae progress through 6 instars and pupate after 16 to 18 days. At 23°C, the larval stages are prolonged and the 6th instar larva molts into an "immaculate" form which enters diapause. Larvae remain in this state for 20 weeks or more and may undergo a series of stationary molts during this period. It has been shown that the larval diapause is initiated and maintained by the continued activity of the CA which results in a relatively high titer of juvenile hormone during this period. For details on hormonal control of larval diapause see Chippendale (1983) and Denlinger (1985). Further studies on *Diatraea* diapausing larvae showed that high JH titers in prediapause larvae induce synthesis of high and low molecular weight proteins by fat body tissue. The low molecular weight "diapause-associated" protein has been isolated and characterized. Specific role of this protein is unknown but it has been suggested that it could be a latent JH binding protein. Recently Osir et al. (1989) reported a diapause associated protein from the stem borer *Busseola fusca*.

Pupal diapause

The endocrine control of pupal diapause is best known because it was the basis for many discoveries by Williams which revealed the interaction between the brain and the prothoracic glands. The cecropia silkmoth, *Hyalophora cecropia* is a univoltine species which undergoes obligatory diapause in pupal stage. Diapausing pupae are characterized by a high glycerol content as a protection from low temperature and very low metabolic rate. The diapause condition is broken by prolonged exposure to cold. Williams demonstrated that pupal diapause was brought about by the failure of the brain to secrete PTTH. The low temperature apparently acted on the brain to reactivate PTTH production when pupa was brought back to the room temperature. Ultrastructural evidence suggests that the cerebral neurosecretory cells, presumed to produce PTTH, of diapausing pupae of *Pieris rapae* resume their secretory activity after pupae are subjected to 5°C for 35 days (Kono, 1977).

In some other species of insects, the onset of diapause may be facultative. This is dependent upon the conditions experienced by the developing larva or even the embryo. The termination of pupal diapause in some facultative species is accomplished by chilling. In a few species diapause can also be terminated by exposing pupae to long-day photoperiods. In Chinese oak silkmoth, *Antheraea pernyi* it has been shown that the long-day photoperiod acts directly on the brain to promote the release of PTTH. Review the section on pupal diapause by Denlinger (1981 and 1985).

Adult diapause

Diapause in adult insects can manifest itself in many ways. There are behavioral, developmental and physiological features in combinations that may vary greatly between species. The adult diapause is partly a developmental diapause. Sometimes it has been named "reproductive diapause". In all cases this condition is due to a lack of juvenile hormone. In females, there is a cessation of oogenesis, whereas in males the accessory glands usually regress.

The best known example of adult diapause is that of the Colorado potato beetle, *Leptinotarsa decimlineata*. Under conditions of warm temperature, adequate food supply, and long photoperiod, newly emerged beetles come out of the soil and begin to feed. In female, this is followed by the production of vitellogenin, onset of oogenesis and the development of flight muscles. Beetles that are exposed to cool temperatures, poor food supply and short photoperiod, emerge from the soil but soon they burrow back into the soil. These beetles do not show muscle development and oogenesis does not progress. Moreover, they show specific short-day proteins in their hemolymph.

The role of the corpora allata in the adult diapause was demonstrated by removing the glands from long-day females. The allatectomized

females showed all the physiological symptoms of diapause. It has also been shown that in long-day beetles the JH titer begins to rise at adult emergence and reaches a high level by the onset of oogenesis. In short-day individuals, the JH titer shows an initial rise, but then declines to undetectable levels by the time the insects burrow into the soil. The termination of diapause is coincident with a rise in JH titer and can be induced by exposure to long-day conditions. There are neurosecretory factors which are also important for the regulation of adult diapause. For a detailed account and for other examples see deWilde (1983) and Denlinger (1985).

The term *aestivation* has frequently been used to designate dormancy occurring in the dry season in the tropics. Some insects in the tropics enter into aestivation in response to various environmental factors and food quality. One good example is the aestivation of *Chilo partellus* in Kenya (see review by Denlinger, 1985). One example from a recent report is the fungus beetle, *Stenotarsus rotundus* from the rain forest of Panama, which should be mentioned here. This beetle has a peculiar habit of aggregating in huge numbers at the base of the palm tree for 8–10 months. During this time the beetles are in diapause; flight muscles degenerate, reproductive activity is halted, no feeding is observed and the beetles are totally dependent on fat reserves of the insects. It was noted that as the group size increases, the metabolic rate decreases. The minimum rate observed in the field, 22 µl O_2 g^{-1} h^{-1}, is estimated to occur when group size reaches about 300 individuals. It appears that one function of this aggregation could be to increase humidity within the group and thereby decrease metabolic rate (Tanaka et al., 1988).

PHYSIOLOGY OF POLYMORPHISM
(1 lecture)

Introduction
Polymorphism in aphids
Polymorphism in locusts
Polymorphism in termites
Hormonal control of polymorphism

PHYSIOLOGY OF POLYMORPHISM

Introduction

Polymorphism is defined as the occurrence in the same population of two or more discontinuous phenotypes in the same life-stage in proportion in which the frequency of the rarest morph cannot be maintained only by recurrent mutation. Polymorphism can extend from the simple color morphs of an adult species to the most complex type of phenomenon such as in the social insects.

Polymorphism in aphids

Several types of polymorphic individuals are found in aphids. Some are sexual and others are parthenogenetic. There are also winged (alate) and wingless (apterous) forms. The fundatrix is the parthenogenetic female developing from the fertilized egg which gives rise to fundatrigeniae. The parthenogenetic females produced from these fundatrigeniae are the virginoparae and they can be alate or apterous. The females that lay eggs are wingless but the males are winged form.

How are these morphs determined? This has been well studied by Lees (1961). The mechanism to initiate the production of a morph is triggered in the maternal interval timer and after the elapse of a discrete period the new form is produced. Factors such as short day (12L:12D), long day (16L:8D), low or high temperatures (15 or 25°C) and isolation or crowding determine the morph. There is a maternal switching mechanism which has to be activated. The translation of environmental cues in the aphid by the switching mechanisms is through the neuroendocrine system. The production of virginoparae or oviparae is maternally determined by day-length and is controlled by the anterior neurosecretory cells. When the neurosecretory cells are destroyed only oviparae are produced and therefore, the factor from these cells promotes the production of virginoparae (see Steel, 1976). The role of juvenile hormone in the production of apterous virginoparae has been suggested but experiments are not conclusive.

Polymorphism in locusts

Almost all locust species exhibit what is known as "phase polymorphism". Locusts exist in two discrete forms, a solitary phase or *solitaria* and a gregarious phase or *gregaria*.

Phase transformation is density-dependent and is reversible between generations. Gregarization results in swarming and migration while solitarization results in disappearance of swarms. The transient stage in between the transformation is the *transiens*. While changing from *solitaria* to *gregaria* the transiens is called *congregans* and when changing from *gregaria* to *solitaria, dissocians*.

The solitary and gregarious forms are recognizable from their color and often from other morphological characters (see Uvarov, 1966).

How is phase polymorphism in locust controlled? Eggs from a single pod when reared in isolation develop into *solitaria*, and when reared under crowded conditions become *gregaria*. Neuroendocrine involvement in the transformation of *gregaria* to *solitaria* was demonstrated in *Locusta migratoria* (Joly, 1970). Also, there seems to be a pheromone "locustol" released in crowded conditions that is responsible for aggregation. It is possible that the neuroendocrine and pheromone systems are interrelated. Current research at ICIPE will definitely throw more light on this problem in the near future.

Polymorphism in termites

Termites show a remarkable type of polymorphism which is intimately related to the social organization comprising a caste system and based on division of labour. Termites can be divided into two groups: the "lower" and "higher" termites based on the caste system. In lower termites there are no workers and the labor is divided among the nymphs and the pseudergates (for example, *Kalotermes flavicollis*). The eggs hatch into larvae that develop into pseudergates and can continue to develop into nymphs which later become primary reproductives. The larvae and nymphs can become soldiers or supplementary reproductives. The caste system in higher termites is more complex with the introduction of the workers (for example, *Amitermes hastatus* and *Macrotermes michaelseni*). See details of caste differentiation in the latter species in Okot-Kotber (1981).

How is caste determined in termites? Several theories have been put forward. These are the theories of genetic, pheromonal, sensory and endocrine control. See details of these theories in Retnakaran and Percy (1985). Also, consult the same review for caste system in honeybees and alternation of generations for various species.

RELATIONSHIP WITH THE ENVIRONMENT
(2 lectures)

Introduction
Effects of temperature
Circadian rhythms
Seasonality and photoperiod
Migration

RELATIONSHIP WITH THE ENVIRONMENT

Introduction

All animals living on this earth have a particular relationship with the environment, both biotic and abiotic. This section will discuss interaction of insects with the abiotic factors of the environment. Insects have some features which are particularly important when considering their interaction with the abiotic factors of the environment. One factor is their hard integument and its rigidity and impermeability. Other factors are their poikilothermy, their small size and their ability to fly and enter into diapause.

Effects of temperature

Most physiological processes are dependent on temperature. The relationship of a physiological process with temperature is defined by the *temperature coefficient*, Q_{10}, which is the ratio by which the rate of a given process increases for a 10°C increase in temperature. In insects, Q_{10} of 2 or 3 are typical over the normal biological range, which means that their rates almost double for each 10°C rise. For practical purposes, however, a Q_{10} is not constant across whole temperature range. They are usually high at low temperatures when physiological processes become very slow. But they are low at high temperatures which fall to less than 1 near the upper lethal limit around 40°C due to enzyme inactivation. For example, for the developmental rate of *Tenebrio* pupae, Q_{10} is 6 at 10°, 3 at 20° and 2 at 30°C. Respiratory rate of pupae increases with the increase of temperature at similar rate.

Because of their small size and poor body insulation, insects' body temperature tends to be similar to environmental temperature (poikilothermy). Some insects have mechanisms to maintain a temperature which is above low ambient temperatures. For example, in the bumble-bee, *Bombus affinis*, the enzyme F-1, 6-DP phosphatase has very high activity which is about 40 times higher than that in the honey-bee. Thus, bumble-bees can generate heat by ATP hydrolysis and can maintain high thoracic temperature. Because of this, it can fly and forage in relatively cold weather which the honey-bee is unable to do.

Overheating in high ambient temperatures may also be a problem, particularly in flying insects where heat production may exceed tremendously. In air temperatures above 38°C, prolonged flight by locust is impossible, because their thorax rapidly reaches a lethal 45°C. Usually in such circumstances, insects fly with periodic rest.

Another behavioral adaptation by locust is its orientation process to maintain homeostasis. On cold mornings, the locusts stand at right angles to the sun rays. At midday, they adopt the converse posture, hanging in the vegetation with their long axis parallel to the sun rays.

Insects of temperate zone face a hazard during freezing winter. Formation of ice crystals in the cells may damage cellular structures, and high concentration of salt may occur which may denature the body proteins. Insects avoid this in part by *supercooling*, dropping tissue fluid temperature to 0°C without crystallizing, in part by pre-winter concentration of the body fluids to lower their freezing point, and in part by the development of high levels of blood glycerol.

Circadian rhythms

Insects in their activity, may be diurnal (day active), nocturnal (night active) or crepuscular (dawn or dusk active). The timing of this daily rhythmicity is due to: (1) behavioral responses to daily changes in the environment (temperature, humidity, and light); and (2) internal clock. For example, the recording of the locomotor activity of a cockroach kept under 12-hour light and 12-hour dark conditions shows that majority of its locomotor activity is performed during the first hour or two of darkness everyday. This daily rhythm of locomotory activity is either due to a photokinetic response to the start of scotophase or due to control from the internal clock. If the cockroach is kept in constant darkness, it continues the same rhythm of activity but it gradually drifts successively later and later (the so-called free-running drift). It appears that such rhythms are the result of endogenous timing from within the animal. This is known as the internal physiological "clock". This intrinsic clock somehow estimates the time of the day and is re-set every 24 hours. That is how the name circadian (about a day) was given to this rhythm.

For example, if a female silkworm moth is kept in constant darkness it will still "call" at approximately the "correct" time of the day, but the time of calling will gradually drift without any timing signal.

The timing of the release of eclosion hormone from the CC of the cecropia moth is under the control of circadian timer, probably in the brain. When brains were transplanted from pupae to isolated abdomens, the abdomen initiated ecdysial behavior on a schedule characteristic of the treatments received by the brains. It appears that the brain contains a photoreceptor and clock that time the release of eclosion hormone.

Eclosion of *Drosophila* occurs at random if the cultures are kept at constant darkness. However, their emergence can be synchronized at a particular phase every 24 hours, if pupae were exposed to a very short flash of light.

Sometimes, circadian control is superimposed over some developing capacity (such as the ability to emerge as to lay eggs). This phenomenon is known as "gating". If the capacity develops to the threshold for release after a certain time of day, the behavior or hormonal release is prevented until the proper time on the following

day. Eclosion and prepupation wandering behavior are examples of such "gated" behaviors.

Light is not the only circadian timing cue of importance. For example, in crickets it was found that temperature fluctuation under constant light could serve as an entraining cue to singing behavior in the males.

Seasonality and photoperiod

Growth and reproduction are favorable only during particular seasons. Because growth and reproduction are hormonally controlled, a wide array of species seem to have evolved seasonal control over their endocrine systems. In most cases the token signal of season is photoperiod. Because photoperiod and its pattern of change vary with season, they are better indicators of season than is temperature or any other cue. Temperature often interacts with photoperiod (day-length) as a seasonal indicator in photoperiodically controlled systems. Except near the equator (e.g. Nairobi and surroundings) the most reliable environmental cue as to the time of the year or season is day-length, which is used by majority of the species as a signal for developmental switching, say, in or out of diapause, or migration or polymorphic changes. The determination of physiological state by measurement of day-length is *photoperiodism*. This involves two processes: (1) an internal physiological clock to measure the length of the day, and (2) a mechanism to switch on the developmental pathway when the clock gives the signal.

There is usually a *critical photoperiod* below which the physiological response occurs only in few individuals, whereas, above which the response occurs in most, or all of them. The critical photoperiod for a particular response is quite precise in many insect species. For examples consult Mordue et al. (1980). See also the chapters on diapause.

Migration

Many insects evolved migratory responses which moves them from an unfavorable area to a more favorable one. This phenomenon can be stimulated by seasonal cues. Many butterfly species migrate south during fall. The best known of these is the monarch which overwinters in central Mexican highlands. In the tropics, the best known examples are the locusts.

Migratory behavior is characterized by three features: (1) it involves prolonged locomotor activity so that the insects keep moving for days; (2) the locomotor activity is relatively straightened out so that the flight takes the insect rapidly away from its starting point; and (3) it involves inhibition of those responses that the insect normally makes to stimuli from the habitat keeping it there (such as food, oviposition sites), with the inhibition being intense at the beginning of the

migratory flight, declining during it and practically diminishing at the end, when the insect lands and reproduces in a new habitat.

Migration is characterized by two features physiologically: (1) it occurs in young, pre-reproductive adults which involves delayed reproductive development and increased muscle growth and fat deposition; and (2) it is controlled, at least in part by the juvenile hormone. For example, in migratory plant bug, *Oncopeltus* the low post-metamorphic levels of JH in the blood are first replaced by intermediate titers leading to migration, provided the day-length and temperature are appropriately spring-like. Then, after about 10 days, high JH titers are reached, reproduction is switched on which induces the non-migratory, settling behavior. Because of the involvement of JH in reproduction and at the same time reproduction-migration states, some authors have suggested that JH may control both.

While it is clear that JH stimulates flight, how this relates to the seasonal control of migratory flight is less clear. See discussion by Tschinkel (1985).

BEHAVIOR: ROLES OF NERVES AND HORMONES
(2 lectures)

Introduction
Control of motor activities
Excitation and inhibition in integration of behavior
Behavior — hormonal causation
- Hormonal releasers
- Hormonal switchers of behavior
- Hormonal developmental agents

Extrinsic controlling factors
- Circadian control
- Seasonal control

Effects of behavior on physiology

BEHAVIOR: ROLES OF NERVES AND HORMONES

Introduction

Various behavioral manifestations by an insect is a *response* to a *stimulus*. All behavioral responses have three elements: (1) transduction of the stimulus by sense organ into an afferent electrical signal sent to the central nervous system (CNS); (2) integration of this signal with other relevant sensory inputs, by interneurons within the CNS and (3) transmission of an efferent command to the motor system (muscles) to produce an overt behavioural act. Responses occur both due to external stimuli and to internal stimuli from within the insect (e.g. circadian rhythms). Response can be independent of stimulus-strength or it may depend on the kind of stimulus. An insect's eclosion behavior can be independent of the strength of stimulus it receives (in form of photoperiod or internal circadian clock), but the feeding behavior of a blowfly feeding on sugar solution depends on the sugar concentration.

Control of motor activities

The basic pattern of motor output often results from the activity of endogenous pacemaker, groups of interneurons, within the central nervous system. This basic pattern is modified by inputs from peripheral sensory receptors. Major regulators of the daily periodicity of motor activities lie in the endogenous circadian pacemakers. The clock controlling locomotor rhythms in cockroaches (*Leucophaea* and *Periplaneta*) appears to be in the optic lobes. The eclosion clock of the pupae of *Hyalophora* and *Antheraea* is in the brain. The major factors modulating the periodicity of circadian pacemakers are light and temperature. There are other factors which also influence the insect's readiness to respond to stimuli during those periods of activity. The causes of readiness to respond to stimuli are not well understood in insects. In mating behavior, signals such as mating calls and pheromones may stimulate increased motor activity or changes in motor activity. Changes in motor activity may be hormonally controlled (for example, mating behavior in *Schistocerca*). The relationship of the nervous system to the endocrine system is very complex.

Excitation and inhibition in integration of behavior

These are probably the most important CNS elements for the integration of behavior. Where there is a coordination of antagonistic muscles acting, such as flight or walk, the importance of this phenomenon is seen. The landing response for a flying insect cannot be elicited without the flight motor first being inhibited. Inversely, the insect cannot take off without first inhibiting its tarsal grip on the substrate. Many responses occur more readily because of the excitatory effect of preceding responses. An example is the response of a water-satiated

blowfly. The fly, after being allowed to drink to satiation with water, will not extend its proboscis when stimulated on the labellum with water. However, it will extend its proboscis if a sucrose solution is briefly touched to the labellum before water stimulation. The brief sugar stimulation creates a heightened level of excitation in the central nervous system so that the threshold for the response to water is lowered. The increased responsiveness is proportional to the intensity of the excitatory stimulus, sugar, and it outlasts it, as an after-discharge by many seconds. The heightened level of excitation in the central nervous system is called *central excitatory state.* There is also *central inhibitory state,* which is hard to demonstrate because it is negative in effect. Many responses involve central programs which are blocked by central inhibition for most of the time. Another element of excitation is known as *post-inhibitory rebound.* This occurs where there is a pair of competing responses and one of them has been inhibited by the performance of the other. When the first response ceases to perform, the competing response, which is now released from inhibition, reappears with higher intensity. For example, the speed of a walking locust can be momentarily raised if it is allowed a brief rest. This phenomenon is opposite of *habituation* where continuous excitation of a response results in a period of inexcitability. All these nervous elements play their roles between the reception of a stimulus and the motor command output. Review integration of these phenomena in various examples given by Eaton (1985) and Mordue et al. (1980).

Behaviour—hormonal causation

There are three different ways that hormones can bring changes in the insect behavior: (1) They influence growth of neurons (for example, effecting behavior of larvae during metamorphosis to switch on adult behavior); (2) the hormones affect the growth of other tissues resulting in change in related sensory input and relevant responses (for example, JH stimulating egg-development, releasing oviposition behavior); (3) hormones act directly on nerves to change both spontaneous neural activity and behavioral responsiveness (for example, eclosion hormone, as a releaser type and JH altering sexual responsiveness in male locust, as a modifier).

Tschinkel (1985) divided the hormones into 3 groups according to their type of action: (1) behavioral releases which act directly on the nervous system to cause the appearance of programmed behaviors; (2) switches of behavioral state, which unmask the capacity for certain behaviors whose release then depends on subsequent stimuli; and (3) developmental hormones which cause the development of new capacities in the insect's nervous system, usually paralleling morphological development (these are mainly ecdysteroids and JHs).

There are a number of examples of hormonal releasers of behavior.

For example, the pupa-adult eclosion behavior of *Hyalophora cecropia* is under the control of eclosion hormone; the calling behavior of polyphemus moth is under the control of a neurohormone from the CC; the egg-laying behavior may also be controlled by a releaser hormone from the CC.

The second type, the hormonal switchers of behavior state, is one in which the hormone causes the capacity to carry out a certain behavior (change in behavioural state), but the actual release of the behavior depends on factors such as environmental conditions or other sensory input. For example, the behavioral changes associated with endopterigote insects are related to this type. Another behavior switching is the development of sexual receptivity (or activity) of adults. Hormonal control of sexual behavior has been studied in several insects and is quite heterogeneous. See examples in Tschinkel (1985).

The third type, the hormonal developmental agents, are those that bring about the development of new behavioral states or capacities. No specific examples exist of development of a behavior under hormonal influence, mainly because so many events come between the release of such a hormone and the final behavior and so many other factors are involved. (See discussion in Tschinkel 1985).

Besides the internal causes of behavior or the physiological cues that release, change or bring about the development of a new behavior pattern, there are also extrinsic controlling factors. There are circadian controls over hormones. Similarly there are seasonal controls over hormones (in relation to diapause, migration, etc.). Tschinkel (1985) has suggested a summarized diagram to consider all the components of the hormonal actions causing behavior of insects.

PHYSIOLOGY AND BIOCHEMISTRY OF PHEROMONES
(1 lecture)

Introduction
Chemistry and biosynthesis
Hormonal regulation of production
Perception

PHYSIOLOGY AND BIOCHEMISTRY OF PHEROMONES

Introduction

The term pheromone was first proposed and defined by Karlson and Lœscher to refer to a group of biologically active substances which are secreted to the outside by an individual and received by a second individual of the same species in which they release a specific reaction such as a definite behavior or developmental process. Pheromones are generally active in minute quantities. They may evoke a behavioral response with a single compound or a mixture. According to its effect, a pheromone can be a *releaser* or a *primer* pheromone. The releaser pheromone acts directly on the central nervous system to evoke an immediate and reversible change in behavior (e.g. sex pheromones). Primer pheromones, on the other hand trigger permanent physiological changes in the receiver. There may not be an immediate change in behavior, but the organism develops new response potentials which can be evoked by new stimuli (e.g. pheromones stimulating sexual maturation or controlling caste differentiation). Pheromones are further classified according to the function they perform, or their effects on the target organism (e.g. sex pheromones, aggregation pheromones, alarm pheromones, etc.)

Chemistry and biosynthesis

The site of pheromone production is usually the glandular modification of the epidermal cells. These glands can be located in almost any part of the body.

A number of pheromones have been chemically identified (see Blum 1985; Prestwich and Blomquist 1987). Chemically, many of them are terpene alcohols (bark beetles, boll weevils), alcohol acetates (Lepidoptera) and long-chain alkenes (Diptera). Biosynthesis of these compounds have been studied extensively. See Prestwich and Blomquist (1987) and Jurenka et al. (1991).

Regulation of pheromone production

The production and/or release of pheromones is influenced by a variety of environmental and physiological factors. Sex pheromone production is usually age-related and coincides with the maturation of ovaries or testes. In insects with repeated reproductive cycles, pheromone is released during periods of mating activity, and successful mating usually turns off the production of pheromone. Dietary factors also influence the release of sex pheromones in some insects. For example, the stable fly, *Stomoxys calcitrans* requires a blood meal before it can begin synthesizing the pheromone.

Hormonal regulation of pheromone production has been studied extensively in a few species recently. In a few cases it has been shown

that the juvenile hormone (JH) is directly involved in controlling pheromone production. This has been mainly investigated in cockroaches. It was shown that allatectomy prevented the production of sex pheromone in cockroaches, *Byrsotria fumigata* and *Pycnoscelus indicus* and *Periplaneta americana*. Topical application of precocene II to virgin female *P. americana* terminated pheromone production in five days.

In *Tenebrio molitor*, allatectomy of female insects prevented pheromone production (determined by bioassays). Removal of the brain or decapitation gave results similar to those of allatectomy. Reimplantation of the brain or the CA could not restore pheromone production. However, injection of JH analogues restored pheromone production. Ovariectomy had no effect on pheromone production.

Juvenile hormone has also been implicated in the control of pheromone synthesis in the male bark beetle, *Ips paraconfusus*. Here, JH is involved in controlling pheromone synthesis by causing the release of brain hormone from the CC. The brain hormone then acts to stimulate pheromone synthesis directly. The brain hormone presumably acts by increasing the activity of enzymes involved in the conversion of myrcene to the pheromone components.

Allatectomy of the female moths, *Antheraea polyphemus* and *Hyalophora cecropia* had no effect on pheromone release, the so-called "calling behavior." However, removal of the CC prevented calling behavior. Denervating the CC also prevented calling behavior. These observations led to the conclusion that a hormone produced by the CC was controlling calling behavior in these moths.

Some studies have shown that pheromone biosynthesis in the housefly, *Musca domestica* is also under hormonal control. However, in this case the ovary seems to play a key role in controlling pheromone synthesis. Removal of the CA or the CA-CC complex did not prevent pheromone production. When the female was ovariectomized shortly after emergence, pheromone production did not occur. Implanting previtellogenic ovaries into ovariectomized females and allowing the ovaries to mature to at least early vitellogenic stage restored pheromone synthesis. Removal of ovary after the onset of pheromone production did not prevent the subsequent biosynthesis of the pheromone. This indicated that a product of the ovary is required to maintain synthesis. To determine if ecdysteroids can restore sex pheromone production in ovariectomized flies, a single dose of 20-hydroxyecdysone (20-HE) was injected into insects that had been ovariectomized within 6 hours of emergence. At 16 and 24 hours after 20-HE treatment, flies produced all of the pheromone components (see Blomquist and Schal 1990).

Raina and Klun (1984) demonstrated that females of *Heliothis zea*, ligated between the head and thorax, did not produce pheromone during the calling period. They showed that injection of homogenates

of the brains and suboesophageal ganglia of females into the abdomens of ligated females resulted in production of pheromone. They isolated a compound, subsequently shown to be a neuropeptide (see Raina et al. 1987) that induced pheromone biosynthesis. Detailed studies have shown that the neuropeptide termed pheromone biosynthesis activating neuropeptide (PBAN), is present in the suboesophageal ganglion (see Tumlinson and Teal, 1990). Recent findings show that PBAN produced by the suboesophageal ganglion does not act on the pheromone gland but rather on the terminal abdominal ganglion which innervates the pheromone gland area of the moth. This ganglion then sends a second message, different from PBAN, to the gland thus inducing pheromone production.

Perception

Pheromones are generally perceived by multiporous sensilla located on the antennae. The perception of pheromones involves six steps: (1) adsorption onto the antennal surface; (2) diffusion to acceptor sites; (3) binding; (4) activation of the acceptors; (5) change of membrane conductance; and (6) inactivation of the pheromone molecule.

Insects detect their pheromones along diffusive concentration gradients descending from the emissive source. The active space, near the point of emission, has the concentration of the pheromone molecule high enough to elicit behavioral response. The shape of the active space varies with the physical environment in which the signal is generated.

The sensory physiology and the behavioral mechanisms involved in the responses have been mostly studied in moths. This is partly because the male moth's antennae are extremely sensitive to the female's sex pheromone. For example, the male *Bombyx mori* responds to as little as 40 molecules (s^{-1}) of the pheromone "bombykol".

Practical Lab Exercises

NEUROANATOMICAL TECHNIQUES — COBALT FILLING

Introduction

Most of the insect endocrine system is comprised of neurosecretory cells (NSC) that are scattered through the nervous system. Various neuroanatomical techniques have been useful in the study of these NSCs. One of the most versatile techniques in modern neurobiology is filling nerve cells with various stains. These can be injected directly into the neuron in question or, in many cases, the neuron can be "back-filled" by dipping its axon in a pool of stain. In practice, this is accomplished by placing the end of a cut nerve in the stain droplet. Moving in through the end of the cut axons, the stain then fills the cells whose axons are in that particular nerve. It is a technique that is applicable to all neurons including NSCs. The specificity of the technique is an anatomical specificity in that only the cells that use a particular nerve are stained. Hence, backfilling of a motor root will result in stained motorneurous whereas backfilling the nerve to a neurohomal organ (e.g. the *corpora cardiaca*) will result in stained NSCs.

A readily available and useful set of stains are heavy metal ions such as cobalt (Co^{++}) and nickel (Ni^{++}). Neurons are filled using dilute solutions of these ions. The ions are then precipitated as the sulphite by exposing the preparation to H_2S or $(NH_4)_2S$. In this case the resulting precipitate is brown to black and may be visualized in the cleared ganglion. An alternate precipitating agent is rubeanic acid ($C_2S_2N_2H_4$, dithiooxamide). The advantage of the latter is that the precipitates of the different ions are different colors (ranging from red to blue).

Whole mounts of filled cells can also be subjected to an intensification procedure. During this procedure, the Co or Ni serves to nucleate silver precipitation. The result is a strongly enhanced fill of the neurons in question. This exercise will deal both with the backfilling technique and the intensification procedure. The color differences seen after precipitation with rubeanic acid are lost during intensification.

Materials

Solutions:

Backfilling: Insect saline
1% $Co\,Cl_2$ or $Co\,(NO_3)_2$

Precipitation: $(NH_4)_2S$
10% formalin in saline

Intensification: Timm's solution
(4 gm Gum Acacia; 1.2g citric acid; 0.15 gm Hydroquinone in 100 ml dH_2O pH 2.7)
1% Silver nitrate

Methods

As an introduction to the technique, you will backfill some of the lateral nerve roots of the abdominal ganglia of locusts. Sever the abdomen from a last instar or adult locust. Open the abdomen by a mid dorsal incision and pin it flat. Remove the gut and gonads and then locate the ventral nerve cord (VNC). Excise a length of the VNC including 2 adjacent ganglion with a long length of lateral nerve projecting.from the more posterior ganglion of the pair. The lateral nerve will be used as a route for backfilling.

Various techniques can be used to isolate the stump of the nerve in a pool of cobalt. As a simple procedure we will use a silgard coated slide. Place a thin ridge of silicone grease transversely across the slide. Place a droplet of saline on either side of the ridge, touching the barrier but not each other. Place the ganglion in one droplet. Lift the end of the nerve out of the saline and drop it over the ridge into the other pool. Replace the saline in this pool with distilled water for about 20 sec (to swell the end of the nerve) and then replace the dH_2O with a droplet of cobalt. Place the slide in a humified chamber at 4°C overnight.

After filling, the ganglion should be carefully removed from the filling chamber and rinsed in a number of changes of saline for 15–30 min. After rinsing, a small droplet of concentrated $(NH)S$ is added to the saline (one drop in 5 ml is more than sufficient). Ganglia should be left in this solution for a maximum of 5 min. The precipitated cobalt appear as a black stain which reveals cell bodies and major axon paths but does not show details of finer dendritic branching patterns. After 2 washes in saline, the ganglia are then fixed in 10% formalin. After fixation, the ganglia can be dehydrated, cleared, and mounted for examination or the cobalt can be intensified using the Timm's method to reveal further neuronal structure.

Intensification

The intensification procedure is carried out at 60°C in the dark (usually in an oven). Heat the Timm's solution to 60°C and keep it at this temperature.

(1) Place ganglia in depression culture wells in warm dH_2O for 10 min.
(2) Add an equal amount of Timm's solution (1:1 dilution) 15 min.
(3) Draw off half of the diluted Timm's and add an equal amount (3:1 dilution) 15 min.
(4) Replace diluted Timm's with straight stuff. Leave 90 min.

Before starting final reaction make up 5 µl of 1% silver nitrate. Also measure out 10 ml of warm Timm's into each of 2 vials.

(5) Add 1 ml of 1% silver nitrate to a 10 ml vial of Timm's (stir gently, and do under dim illumination). Quickly draw off old Timm's and add Timm's + 1% $AgNO_3$. Place in the dark!

(6) Make a first check after 10 minutes, using low power illumination, then at 3 minute intervals, returning the specimen to the oven each time.
(If the solution begins to turn yellow or brown make up a new Timm's $AgNO_3$ solution and replace the old solution)

(7) To stop intensification, replace solution with 2 changes of dH_2O.

(8) Dehydrate through an alcohol series (30%; 50%; 70%; 95%; 100%; 1:1 100% : xylene; 2 xylene) then mount for examination.

Reference

Strausfeld, N.J. and Miller, T. A. eds. 1980. *Neuroanatomical Techniques*. Springer-Verlag, New York.

IMMUNOCYTOCHEMISTRY

General background

The advent of immunocytochemistry brought an extremely powerful technique for localizing specific molecules in tissue slices. There are numerous variations to this approach that are now in use. All involve immobilizing the antigen in the tissue (usually by fixation) followed by exposing the tissue to an antibody that recognize the antigen of interest (the primary antibody). Variations are in the methods used to identify the location of the primary antibody. The simplest technique is to use a second antibody that will bind to the primary antibody (for example, if the primary antibody was raised in a rabbit, and, hence, is a rabbit IgG, the secondary antibody would be raised in a different species (e.g. goat) and would recognize any rabbit immunoglobin). The second antibody (which is usually purchased from a commercial source) is then conjugated to a molecule which will aid its localization. These can be fluorescent molecules such as Fluorescence (FITC) or rhodamine. Their presence can then be determined using fluorescence microscopy. Alternatively, enzymes (such as peroxidase) or gold particles (for EM) conjugates are used. For this particular exercise we will use a peroxidase conjugated secondary antibody.

Tissue preparation

The choice of fixative is often crucial in obtaining good staining. Formalin, paraformaldehyde, and Bovin's fixative are often used. In the case of antibodies raised against small molecules, such as biogenic amines or small peptides, the small molecule (called a haptene) is typically conjugated to a large molecule (thyroglobulin, hemocyanin, etc.) in order to make it antigenic. Consequently, the resultant antibody may not recognize the native haptene by only the conjugated form. In these cases the fixation paradigm usually matches the conjugation method (e.g. if the conjugation was done using gluteraldehyde, then tissue preparation will often require gluteraldehyde fixation). In any event, the choice of fixative is often arrived at empirically.

After fixation the tissues may either be sectioned or treated as a whole mount preparation. Both methods have their benefits and short-comings. We will use the whole mount preparation. Using this method, you save the time required for sectioning but penetration of the antibody into the tissue can be a problem. Consequently, small amounts of detergent are included with the antibodies to aid penetration, long incubation times are usually required, and for the nervous system, it is sometimes necessary to remove the outer neural sheath.

Solutions: PBS : Phosphate buffered saline : (0.25g NaH_2PO_4; 1.15g Na_2HPO_4; 9gm NaCl in 1 litre H_2O)

PBS-TX : PBS plus 0.3% Triton-X100

Primary antiserum — in PBS-TX, Dilutions depend upon antibody titre, which must be determined empirically. In general, dilutions of 1/100 to 1/2000 are usual. The antiserum we will use is a monoclonal Ab and hence is a mouse immunoglobin.

Secondary antiserum. A sheep (or goat) antimouse IgG. Dilute 1/100 to 1/500 in PBS-TX.

Secondary antibody is conjugated to peroxidase.

DAB: diaminobenzadine. 10 mg/ml in dH_2O (stock)
Hydrogen peroxide.

Procedure

Day 1

1. Dissect tissue. Fix at room temperature for 1 hr;
2. Wash with 3 changes of PBS-TX
3. Incubate for 1 hr at 4°C in 10% goat serum in PBS-TX
4. Remove most of goat serum and add appropriate dilution of primary antiserum in PBS-TX. Keep at 4°C with agitation overnight.

Day 2

1. Wash tissue with 6 changes of PBS-TX; total wash time should be about 1.5 hr. Between washes keep in cold with agitation.
2. Add secondary antiserum at appropriate dilution in PBS-TX: Keep at 4°C with agitation overnight.

Day 3

1. Wash tissue with 6 changes of PBS-TX; total wash time about 1.5 hr. Keep cold with agitation between washes.
2. Give a final wash in PBS.
3. Prepare DAB solution. Add 0.5 ml DAB stock to 10 ml PBS. (Caution DAB is a carcinogen, be cautious). Add 3 µl hydrogen peroxide to DAB solution.
4. Remove PBS from tissue and replace with DAB solution. Check the tissue periodically under the dissecting microscope. (The reaction should only take a few minutes).
5. Terminate reaction by two washes with PBS.
6. Dehydrate in an alcohol series; clear in xylene, and mount preparation.

Reference

Schooneveld, H. and Veenstra, J. A. 1988. Immunocytochemistry. In *Immunological Techniques in Insect Biology*. Edited by L.I. Gilbert and T.A. Miller, pp. 93–133. Springer-Verlag, New York.

RADIOIMMUNOASSAY OF ECDYSTEROIDS

Introduction

A radioimmunoassay is a very sensitive method for the quantitative detection of hormones in blood or tissue samples. For peptide hormones one can generate a specific antibody to the pure peptide hormone. For lipid hormones such as ecdysone and the juvenile hormones, one must first conjugate the hormone to a protein such as thyroglobulin which serves as the antigen against which the antibody is produced. Thus, usually the antibody cross-reacts to some degree with molecules chemically very similar to the hormone and therefore these cross-reactions must be quantified with each new batch of antiserum.

The principle of radioimmunoassay is that one adds a known amount of labelled hormone to a sample containing an unknown amount of hormone, then adds a limited amount of antibody. The labelled and the unlabelled antigens then compete for the available antibody; after all the antibody is bound, the antibody-antigen complex is precipitated to separate it from the free hormone and the amount of labelled hormone bound determined. To quantitate the amount of unlabelled hormone present, you make a standard curve in which you add increasing amounts of unlabelled hormone and determine how much labelled hormone is bound at each concentration.

Following is the standard procedure for doing the ecdysteroid RIA with two alternative methods for separation of bound from free ecdysteroid. Also, extraction methods for both tissue samples and from hemolymph are given although in the laboratory we will only do hemolymph samples which is the preferred method to obtain a hormone titer curve.

Ecdysteroid extractions

A. Tissue samples

1. Homogenize the whole insect or tissue sample in ice-cold 75% methanol containing 1mM phenylthiourea (PTU). The weight/volume ratio should be kept at 70 mg/ml or less, depending on convenience or the required RIA (the RIA is accurate between 0.1 and 4.0ng/sample). (Twenty mosquito ovaries or 5 milkweed bug ovaries per 100 µl is typical).
2. Place homogenate at –20°C, overnight or longer, then vortex and centrifuge at 3000g for at least 10 minutes at 4°C (if possible; can be done at room temperature).
3. Transfer supernatant to 6 x 50 mm borosilicate tube and evaporate off solvent with air or nitrogen while kept in a hot (40–45°C) water bath.

B. Hemolymph samples

1. Anesthetize the insect and cut off the thoracic leg or carefully slit the thorax of the pupa. Collect drops of hemolymph into a small microfuge tube containing a crystal of phenylthiourea (PTU; to prevent darkening of the hemolymph) keeping in an ice bath. At this point the hemolymph can be frozen at –20°C until all samples are collected.
2. With the tubes on ice (we use 6 x 50mm borosilicate glass culture tubes), numbered 1 through *n*, aliquot 10 µl of blood. If the titer is extremely low, such as below 100 ng/ml 20-OH-Ecdysone equivalents use 20 µl of blood. Then add 40 µl of absolute EtOH to the 10 µl of blood or 80 µl to 20 µl of blood. Smaller volumes can be used if titers are high.
3. Vortex. Cover with foil and allow to stand on ice for 20 minutes.
4. Spin at 2000 rpm in swinging bucket rotor for 10 minutes at 4°C.
5. Aliquot out 3 10 µl units of the supernatant, each into a clean tube. Thus, you now have tube #s 1a, 1b, 1c, each with 10 µl of the supernatant from tube #1. You are merely doing triplicates of each blood sample.
6. Evaporate the alcohol solvent either with a gentle stream of nitrogen gas or in a vacuum oven without heat.

Standard curve for ecdysone

1. Make a stock solution of 1mg/ml ecdysone in absolute ethanol. The concentration is measured on the spectrophotometer at 241 nm.

   conc. = $A_2$41 x (464) x (dilution factor)/12300

2. Then dilute to make up a 4000pg/0.1ml abs. EtOH solution. If you are sure that you are only going to need two standard curves, then you only need to make about 2 mls of this solution. This solution is "1st alpha".

   A "2nd alpha" solution is also needed. It is a 1:16 dilution of "1st alpha". Dilute with absolute EtOH. This equals 250pg/0.1ml abs. EtOH.

Set the standard curve up in the following way:

tube #	pg	µl		
1	4000	100	)	
2			)	
3			)	
4	2000	50	)	
5			)	
6			)	1st α
7	1000	25	)	
8			)	
9			)	
10	500	12.5	)	
11			)	
12			)	
13	250	100	)	
14			)	
15			)	
16	125	50	)	
17			)	
18			)	2nd α
19	50	20	)	
20			)	
21			)	
22	25	10	)	
23			)	
24			)	
25	0	0		
26				
27				
28	0	0		
29				
30				

With the tubes on ice, add the indicated amount of "1st" and "2nd alpha" to the appropriate tubes. The alcohol then needs to be evaporated either by applying a gentle stream of nitrogen gas to each of the tubes or in a vacuum oven with no heat turned on. When done, place the tubes on ice covered with foil.

Day 1 of the ecdysone RIA

Day 1 is the same for both the Protein A procedure and the ammonium sulfate procedure.

1. Add 100 µl of ^{3}H ecdysone solution to all the sample tubes and all the tubes in the standard curve (tubes 1–30) after the EtOH has been blown off each tube. This is to be done on ice.
2. Add 100 µl of the antibody solution to all the sample tubes and tube #s 1–27 of the standard curve.
3. Add 100 µl of the control serum to tube #s 28–30 of the standard curve.

   Tube #s 25, 26, 27 of the standard curve are to determine the maximum of ^{3}H-ecdysone bound. These values should be around 2000 cpm or 40–50% total cpm.

   Tubes 28, 29, 30 of the standard curve are to determine non-specific binding. These values should be around 100 or less. If it is higher, it suggests that the ^{3}H-ecdysone is not as clean as it should be. This can be solved best by running the hot ecdysone on HPLC. Playing with the amount of antibody and BSA can also help.
4. Vortex tubes well.
5. Place the tubes on lots of ice and incubate in a cold room (4°C) overnight.

Day 2: Separation of bound from free

I. Protein A method

1. Add 20 µl of Protein A to each and every tube. You can add it to all the tubes before vortexing and it is not critical to do it in the cold room although we usually keep the tubes on ice.
2. Vortex the tubes and incubate for 30 minutes at 4°C.
3. Centrifuge tubes for 15 minutes at 5000 rpm at 4°C in a swinging bucket rotor.
4. Aspirate supernatant but be careful because the pellet is slippery.
5. Add 50 µl of water to each tube. Vortex.
6. Add 450 µl scintillation fluid. Count with external standard for 10 minutes.

II. Ammonium sulfate method

1. Add 200 µl of saturated ammonium sulfate to each tube. The ammonium sulfate should be kept at room temperature or as close to it as possible while pipetting which needs to be done in the cold room. Vortex each tube immediately after adding the ammonium sulfate.
2. Incubate 20 minutes in the cold room.
3. Centrifuge at 4°C, 15 minutes, 4800g or 5000 rpm in swinging bucket rotor.
4. Aspirate and discard supernatant. The pellet is a very light pink color due to the merthiolate in the antiserum and very thin.

5. Rinse pellet with 40 µl of 50% ammonium pellet in the cold room. Vortex. You can add to all tubes before vortexing but we have always vortexed immediately after adding the ammonium sulfate.
6. Incubate 20 minutes in cold room.
7. Centrifuge as last time.
8. Aspirate and discard supernatant. The pellet should be easier to see this time.
9. Add 25 µl dH_2O to each tube and vortex.
10. Add 500 µl Liquisicint (or other scintillation fluid that accepts aqueous solutions) and vortex.
11. Allow vials to set 5–6 hrs so pellet stabilizes. Count 10 minutes with external standard.

General comments

1. We use the 6 x 50 mm borosilicate glass culture tubes. I usually number all the tubes before I start by using a sharpie and then covering the number with scotch tape so the number won't come off if the tube gets wet. Also, the tubes can be recycled by soaking in acetone for 30 minutes, rinsing with dH_2O, then 3 cycles through the dishwasher. Bake 100°C for 24 hrs.
2. When vortexing, be careful not to transfer any fluid from one tube to the next on your fingertip. Therefore wipe your fingers dry between tubes.
3. To count the vials, I place the 6 x 50 mm tubes in a Bio vial with cap, then place this in a scintillation vial.

Solutions

Borate buffer

6. 184g Boric acid (0.1M)
9. 536g Borax (sodium tetraborate decahydrate) (0.1M)
4. 384g NaCl (0.075M)

Dissolve in 1 litre distilled water, pH 8.4

H-Ecdysone (Need 3600–4000 cpm/assay tube (100 µl))

Your stock ecdysone is in EtOH so the alcohol should be blown off with nitrogen, then brought up with an appropriate amount of borate buffer. For 2 standard curves and 30 samples you will need 11.7 mls of the H-ecdysone solution.

Control serum

Bleed control rabbit, making sure to take venous blood clot at 37°C for 1 hour.
Chill overnight at 4°C.
Spin 20 minutes 10,000 G (or 2500 rpm) in table top centrifuge.

Remove serum, spin again 20 minutes. Discard clot.
Aliquot in 3 mls volumes with 1:10000 merthiolate and freeze.
When ready to do the RIA, dilute 0.3 ml control serum with 4.7 ml borate buffer. If you are doing the Protein A procedure, the final borate buffer must contain 0.05g bovine serum albumin (Fraction V, Sigma) per 100 ml.

Ecdysone antiserum in borate buffer

(a) Protein A procedure
Add 8–10 µl antibody (depends on age and activity of antibody) per 100 mls borate buffer. Also, add 0.05 g bovine serum albumin (Fraction V, Sigma) per 100 mls of the borate buffer to cut down on non-specific binding which should be about 100 cpm.

(b) Ammonium sulfate procedure
Add 5 µl antibody and 3 ml control serum to 47 ml borate buffer to make enough for 500 assay tubes. You sometimes have to titrate the volumes of the antibody and the control serum to give 40–50% binding.

Protein A solution

This is usually purchased but it can be prepared from a culture of *Staphylococcus aureus* cells. We use Pansorbin (CalBiochem #507861). It is stored in the refrigerator and must be resuspended by vortexing for 2 min. or by placing in a sonication bath for 10 min. followed by a brief vortexing.

Ammonium sulfate solutions

(a) Saturated:

Add ammonium sulfate to borate buffer until no more will go into solution. There should be some ammonium sulfate at the bottom of the bottle.

(b) 50% Ammonium sulfate

Dilute above solution 1:1 with borate buffer.

Reference

Warren, J.T. and Gilbert, L. I. 1988. Radioimmunoassay: Ecdysteroids. In *Immunological Techniques in Insect Biology*. Edited by L.I. Gilbert and T.A. Miller, pp. 181–214. Springer-Verlag, New York.

ASSAY OF SPECIFIC MESSENGER RNA BY DOT HYBRIDIZATION

Introduction

Once one has a cloned DNA probe — either cDNA or genomic — for a gene, one can use this for detection of the mRNA transcribed from that gene. Electrophoresis of the RNA isolated from a tissue along with size standards (usually DNA fragments of known size) and *northern blotting* can establish the size of the mRNA and help to confirm its identity. For quantitative assay of mRNA, a relatively simple, accurate technique is *dot hybridization*. Total tissue RNA is denatured, diluted appropriately, applied in dots to nitrocellulose filter membrane, and bound by baking. The membrane is then incubated under hybridization conditions with a specific DNA probe labelled with radioactivity (32P), washed and exposed to X-ray film. Upon developing the film, spots appear over the RNA dots, with intensities proportional to the amount of mRNA homologous to the probe. These can be measured either by photometric scanning of the exposed film, or by using the film as a guide for cutting out the dots from the nitrocellulose membrane and counting them in the scintillation counter.

Objective

We shall use dot hybridization to assay vitellogenin mRNA in locust fat bodies after induction with methoprene. An individual fat body provides more than sufficient RNA for this very sensitive technique. The fat bodies will be frozen in liquid nitrogen and stored at –70°C before use for RNA preparation. Under these conditions RNA remain intact. A preparation of RNA from fat body of vitellogenic mature female locusts will be used for reference. The probe is pA4.6, a cloned payment from the coding sequence of *Locusta migratoria* vitellogenin gene A, which has been used for study of expression of this gene.

Preparation of locust fat bodies

After collecting hemolymph samples from the locusts that were treated with methoprene (and the controls), cut open the abdomens, collect the fat bodies, rinse them quickly in locust Ringer solution, and drop each into a flask of liquid nitrogen. Recover each frozen fat body and put it for storage in a pre-chilled labelled Eppendorf tube on dry ice. Store at –70°C until use.

Preparation of RNA

To prevent breakdown of RNA, glassware and aqueous solutions must be sterilized, the preparation must be kept on ice, and gloves must be worn at all times. Each frozen fat body is treated as follows. Two samples can be processed at the same time.

Into a glass homogenizer (on ice) put 0.4 ml homogenization buffer,*0.4 ml water-saturated phenol and 62.5 µl 10% SDS. CAUTION: PHENOL IS TOXIC AND IRRITATING TO THE SKIN! Add the frozen tissue and immediately grind well. Add 0.4 ml chloroform and mix with about 8 strokes of the pestle. Transfer to a 1.5 ml Eppendorf tube and spin in the microfuge in the cold for 1 minute. With a Pasteur pipette carefully remove the (lower) organic phase and discard it. The upper (water) phase contains RNA and the interface gel contains denatured protein. Add 0.4 ml chloroform, cap the tube, vortex, centrifuge, and remove the lower phase as before. Repeat these steps once or twice more, until the interface gel is only a thin layer. Then, from the upper phase, which contains the RNA, transfer 300 µl to a clean tube. To precipitate the RNA, add 2.5 volumes (750 µl) 95% ethanol, cover with Parafilm, mix and leave at –20°C overnight or –70°C 30 min.

Sediment the precipitated crude RNA by centrifuging in the microfuge for 2 min, and pour off the supernatant, draining well. Add 500 µl cold 80% ethanol, vortex to suspend the precipitate, centrifuge 1 min. pour off and drain well. Cover with Parafilm, make holes in it with a pin and dry in a vacuum. Add 75 µl sterile distilled water and allow to dissolve. One adult female locust fat body yields about 100 µg of RNA, so your solution (which represents 3/4 of the total) should be about 1 µg/µl. Some of this solution can be used for electrophoresis and northern blotting (see Maniatis et al. 1982, pp. 202–203).

Denaturation and dotting of RNA

Measure into a sterile Eppendorf tube:

RNA preparation (from above)	25 µl
Phosphate buffer, 0.25 M, pH 6.0	20 µl
Formaldehyde, 37%	25 µl

Close the tube and heat to 65°C for 10 min; chill on ice; then add:

20 x SSC**	80 µl

to make a total volume of 150 µl.

Take a piece of nitrocellulose filter membrane (Schleicher & Schuell BA 85) measuring 4 x 10 cm and mark it lightly with a fine, soft pencil to provide positions for 2 rows of 8 dots. Be careful since the membrane is fragile, and touch it only at the edges.

*Homogenization buffer: 0.1M Sodium acetate (pH 5.0) 25mM NaCl, 5mM MgCl. 25 µg/ml PVS, 35 µg/ml Spermidine 0.5% SDS

**1 x SSC = 0.15M NaCl, 0.15M Na Citrate (pH 7)

1	3	5	7	9	11	13	15
2	4	6	8	10	12	14	16

Number the positions with pencil and decide what will go in each. The two rows should be duplicates. They should include:

Blank (mRNA)

Non-vitellogenic fat body RNA (male locust)

Your two preparations

Reference vitellogenic fat body RNA at several dilutions, to give 1 µg, 100 ng, and 1 ng of RNA

Place the filter membrane on clean aluminum foil and measure 5 µl of the different samples into the marked squares. They should be absorbed as spots.

Put the entire filter sheet in a dish of 6 x SSC and let it soak for 5 min. Take it out and blot it briefly on filter paper. Then clip it between two pieces of thick filter paper (Whatman 3MM) and bake it in an oven at 80°C for 2 hr.

Hybridization

Insert two sheets into a sealable plastic bag, add 3 ml of prehybridization mixture, expel air bubbles, seal and leave at 42°C with gentle shaking for 4 hr. Then cut and open a corner of the bag, add probe which has been labelled with 32P by "nick-translations" (Maniatis et al. 1982, p. 109; Rigby et al. 1977). (CAUTION: RADIOACTIVE), re-seal and leave at 42°C overnight for hybridization.

Washing

Cut open a corner of the bag and drain out the solution into a radioactivity disposal container. Working in a tray to contain radioactivity, cut open the bag and, with forceps, transfer the filter membrane to a plastic box. Wash with the following sequence:

2 x SSC at room temperature, 2 times
0.2 x SSC—0.5% SDS at 65°C, 2 times

For each wash, add enough solution to cover the membrane well, place on a shaker at the stated temperature, shake for 20 minutes, and pour off the solution to radioactive disposal.

Autoradiography

Blot the washed membrane on filter paper and put in a clean plastic bag. Use a little adhesive tape to fix the membrane to one side of the bag. In the darkroom, put a sheet of X-ray film in a cassette, place the bag containing the filter membrane on it and fix with some pieces of adhesive tape. With a dissecting needle, punch several holes through the plastic bag and the X-ray film, to serve as guide for its position after the film is developed. Close the cassette and leave to expose a few hours or overnight.

Open the cassette, remove the plastic bag from the film, and develop the film. If the spots are too faint or too dark, re-expose for a greater or lesser time. Relocate the bag on the film so as to identify the spots.

Results

By comparing the experimental spots with the reference standards, estimate the amount of Vg mRNA in each. (For quantitative results, the spots are cut from the filter membrane and counted by scintillation). Assume that Vg mRNA is 1% of the total vitellogenic female fat body RNA used as a reference standard. Relate the mRNA levels found to the vitellogenin levels found in the hemolymph of the same animals. This should be done by assembling the class results in a Table.

References

Dhadialla, T.S., Cook, K. E. and Wyatt, G. R. 1987. Vitellogenin mRNA in locust fat body: coordinate induction of two genes by a juvenile hormone analog. *Devel. Biol.* 123:108–114.

Maniatis, T., Fritsch, E. F. and Sambrook, J. 1982. *Molecular Cloning, a Laboratory Manual.* Cold Spring Harbor Laboratory, NY.

Rigby, P.W.J., Dieckmann, M., Rhodes, C. and Berg, P. 1977. Labelling deoxy-ribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase. *I. J. Mol. Biol.* 113:237.

Thomas, P.S. 1983. Hybridization of denatured RNA transferred or dotted to nitrocellulose paper. *Methods in Enzymology* 100:255–266.

BIOASSAY PROCEDURES FOR JUVENILE HORMONES AND ANTIJUVENILE HORMONES

Introduction

A bioassay is the easiest way to determine whether a particular chemical compound or an extract of a natural product has hormonal activity. To develop a bioassay on a particular insect, one needs to determine the stage(s) in the life history that is most sensitive to the type of hormone to be assayed, using a hormone or hormone analog of known potency on carefully staged animals. Once the period of peak sensitivity is ascertained, a dose-response curve should be done at that time to determine the range of effects that the hormone causes. Based on these effects a scoring system can be devised, usually ranging from 0 (no effect) to 3 or 5 (full effect), depending on the number of distinct morphological types seen. Once these characteristics of a bioassay are set, then unknown compounds can be screened.

Juvenile hormone bioassays

Juvenile hormone bioassays can take advantage of either the morphogenetic or the gonadotropic action of the hormone. The morphogenetic assays depend on the action of JH to prevent the switchover to new patterns of gene expression and thus to a new form at metamorphosis. Therefore, it is critical that the compound to be assayed is present at the beginning of the ecdysteroid rise to initiate a metamorphoric molt since this is the stage at which the critical switching events occur. By contrast, the gonadotropic assays depend on the action of JH to promote egg maturation and thus are usually done on young adult females that have had their corpora allata removed. In this case, one has to determine the normal action of JH in the particular bioassay insect to be used. Consequently, morphogenetic assays are usually used for routine screening of potential JH analogs.

Juvenile hormones and their analogs are lipid-soluble compounds which can therefore penetrate the cuticle after topical application in an organic solvent such as acetone or cyclohexane. Acetone allows the most rapid penetration so that the compound equilibrates in the hemolymph within 1–2 hr. The disadvantages of acetone are its rapid rate of evaporation and its tendency to inactivate the natural JHs and some JH analogs after prolonged storage. Cyclohexane is an inert solvent for JHs and JH analogs which has a low rate of evaporation. However, cyclohexane does not penetrate some types of insect cuticle as well as acetone and may even damage the surface lipids of the cuticle in some cases. Therefore, acetone is the preferable solvent for application if one can afford to prepare new solutions frequently (weekly, if possible) from either the neat compound or concentrated stock solutions in cyclohexane or hexane (which should always be stored at –20°C).

Topical application basically gives a pulsed dose of hormone; the duration of its effectiveness then depends on its rate of catabolism in and/or excretion by the bioassay animal. Therefore, it is critical for the optimal bioassay that it be applied at the time of peak sensitivity. If the critical period for JH action is long or unknown, then it is often preferable to inject the JH or JH analog in light mineral (paraffin) oil (or olive oil). This mode of application permits a slow release of the compound from the oil reservoir into the hemolymph over a period of several days. For hemimetabolous larvae an alternative approach is to saturate a filter paper with a known amount of compound in acetone, evaporate the solvent, and place the treated filter paper in the rearing container so that the insect receives the hormone through topical contact over a period of time. This method, however, requires usually more hormone and of course can be only semi-quantitative since each insect may not contact the treated paper equally.

I. Hemimetabolous insects

Since hemimetabolous insects molt directly to the adult after the final larval instar, the corpora allata normally become inactive during the final larval molt so that JH is absent during the last larval instar. Application of JH to early final instar larvae will then cause a supernumerary larval molt. We have two different hemimetabolous insects on which to perform the JH bioassay, *Dysdercus fasciatus* (the cotton stainer) and *Locusta migratoria*.

A. Dysdercus

(1) Prepare Petri dishes with filter paper, a water vial, and cotton seeds.

(2) You will use 5th (final) instar larvae within 24 hours of ecdysis as this is their period of maximal sensitivity. These larvae have been held without food during this time to ensure that they remain maximally sensitive. Anesthetize the larvae in CO_2 for 5–15 min.

(3) Apply the JH analog (we are using methoprene) in 1 ul acetone using a graduated micropipette to the dorsum of the larva. Provided that we have sufficient larvae, you should do 5 larvae per dose. Doses to be used are: 5, 1, 0.1, 0.01 µg. Also, be sure to treat at least 5 with acetone as a control. Do the acetone control first, then the increasing doses of hormone so that you can use the same pipette.

(4) After application put the treated insect into the labelled Petri dish and place at 25°C.

(5) Molting should occur within 5–7 days.

(6) When the insects have molted (or attempted ecdysis), score as follows:

0 = perfect adult

1 = adultoid wings short; some larval pigmentation

2 = juvenoid; wings only slightly developed; mainly larval type pigmentation

3 = supernumerary nymph (larvae)

Plot average score per dose on semilog paper.

B. Locusta

(1) Prepare containers as directed

(2) Use 3 0–2 day old 5th instar locusts per dose. Since locusts are large, they can be injected with the JH analog dissolved in paraffin oil. Anesthetize the 5th instar locusts in CO_2, then inject into the abdomen 10 µl paraffin oil containing the following doses of methoprene: 50 µg, 5 µg, 0.5 µg, 0 µg (control)

(3) Place in labelled container, place at 25° and check after molting in 6–8 days. Score as for *Dysdercus*.

II. Holometabolous insects

Juvenile hormone can prevent metamorphosis of holometabolous insects, either at the larval-pupal transformation or at the pupal-adult transformation. The two assays that we will do on the armyworm (*Spodoptera exempta*) will illustrate the effect in each.

A. Final instar larval assay

(1) Topically apply 2 µl of the JH analog in acetone along the dorsal midline to lightly anesthetized (or unanesthetized, if possible) day 0–1 5th instar armyworm larvae. Use 10, 1 and 0.1 µg methoprene and acetone control.

(2) Replace on maize leaves and check daily for cessation of feeding, then later onset of pupation or head capsule slippage, signifying a larval type molt, and finally ecdysis or attempted ecdysis. At this point the old cuticle should be carefully removed (if not normally shed), and the resultant new cuticle scored:

0 = normal pupa

1 = pupa with traces of larval cuticle on dorsum (site of application)

2 = pupa with large patches of larval cuticle: about 50:50 larval:pupal

3 = larvae with some patches of pupal cuticle on abdomen

4 = supernumerary larvae (check carefully for traces of pupal cuticle on head and thorax)

B. Pupal assay

(1) Inject armyworm pupae in the dorsal mesothorax within 24 hrs of ecdysis with 2 µl paraffin oil containing 50, 10, 1, 0.1 and 0.0 µg methoprene. Seal with melted paraffin wax.

(2) Place in marked containers at 25°C, for 5–7 days. When old cuticle is resorbed (becomes soft), carefully remove and score resultant animal as follows:

0 = normal adult

1 = adult with patch of pupal cuticle at site of injection

2 = adultoid with large patches of pupal cuticle on head, thorax, and abdomen

3 = pupa-adult intermediate

4 = pupa with traces of adult characteristics

5 = second pupa

Antijuvenile hormone bioassays

Antijuvenile hormone agents (AJHA) are compounds which cause precocious metamorphosis and interfere with gonad development in those insects in which JH is the gonadotrophic hormone. Thus, these compounds can either interfere with JH action on the target tissue or with JH production by the corpora allata. To date, most of the AJHA found have been those of the latter category (for recent review, see G.B. Staal (1986)).

To assay for an AJHA, one usually uses the precocious metamorphosis assay although in some insects an antigonadotrophic assay may also be used. To determine whether a compound can cause precocious metamorphosis, it must be given to the prepenultimate or the penultimate instar larva just after ecdysis, then the onset of metamorphosis instead of the final larval molt assessed. In the antigonadotrophic assay the compound must be given immediately

after adult ecdysis to determine if it can prevent subsequent gonad maturation. If a compound proves positive in either assay, one then needs to assess whether one can restore the normal condition by the simultaneous or subsequent administration of JH or its analog.

This lab exercise will demonstrate the action of a known AJHA or hemimetabolus insects, precocene. Precocene is taken up by the corpora allata of sensitive insects and converted by the epoxidase sent to a highly reactive epoxide which is cytotoxic and thus destroys the corpora allata.

I. Precocious metamorphosis assay

A. Dysdercus

(1) Apply 0, 0.1, 1, 10 and 100 µg precocene II in 0.5 ml acetone solution to 9 cm discs of filter paper in glass dishes. Let dry. Then place paper in plastic Petri dishes with water and cotton seeds.

(2) Put 5–10 freshly ecdysed 3rd or 4th instar larvae in each dish.

(3) Put at 25°C and observe 5–10 days later when they molt to 5th instars or precocious adults (normally each instar takes 4–5 days although precocene may slow them down)

(4) Score as follows:

0 = normal 5th instar larvae

1 = 5th instar larvae with elongate wings and some adult pigment characteristics

2 = adultoid with some larval characteristics

3 = adult

(5) Keep those that ecdyse normally to determine if they molt subsequently. Score 0 animals should molt to adults after 7 days.

B. Locusta

(1) Topically apply 10, 100, and 300 µg precocene II in 10 µl acetone to each freshly ecdysed 4th instar larvae. Remember to do an acetone control.

(2) Score after the subsequent molt (5 to 7 days) as outlined above for *Dysdercus*.

II. Antigonadotrophic assay on **Dysdercus**

(1) Apply 0.2 and 2.0 µg precocene II in 2 µl acetone to three day 0 females.

(2) Place in Petri dishes with cotton seeds and a water vial and put at 25°C.
(3) Dissect after 9 days and count number of mature eggs (chorionated).

References

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Staal, G.B. 1986. Antijuvenile hormone agents. *Annu. Rev. Entomol.* 31:391–429.

GROWTH AND DEVELOPMENT OF INSECT EGGS

Objective

To determine the percentages of water and fats in an insect egg. Since almost all of the remainder is protein an approximation to the percent of protein can be obtained simultaneously.

Materials

Locust eggs (200 to 500 depending on how large quantities you want)
Micro-Soxhlet extractor
Sintered glass thimble for above
Hard paper extracted with ether
Small stirring rod (to go in thimble of Soxhlet)
Several small beakers (clean)
Ether and acetone
Analytical balance

Methods

Obtain about 200 egg pods. Wash carefully in distilled water (several changes) to *remove all adhering dirt and sand*. Dry on filter paper (several changes) until the eggs roll around freely (15–20 minutes).

1. Clean the Soxhlet, thimble and stirring rod in cleaning solution. Rinse out thoroughly and dry. Can dry Soxhlet by using blast of air through an air filter. Cut a piece of paper of good quality to fit the extraction thimble (or be slightly larger) and extract it with several changes of anhydrous ether.
2. Put the paper in the extractor thimble along with the stirring rod and weigh all three *together* accurately on an analytical balance. Record the weight.
3. Remove paper and stirring rod, and put the eggs in the thimble. Weigh. Put the paper on top of the eggs. Using the stirring rod crush *all* of the eggs. When you are *sure* all of the eggs are crushed, put the thimble in a drying oven and cover with a cone of filter paper. Leave overnight to dry. Weigh again (including paper and stirring rod).
4. Subtracting the combined weights of thimble, paper and rod, what are the wet and dry weights of the sample? The wet and dry weights of a single egg? What is the percent of water in the grasshopper egg?
5. Put thimble in the middle chamber of the Soxhlet extractor (including paper and rod). Put approximately 100 ml of anhydrous ether plus a few ml of absolute ethyl alcohol in the flask and assemble with water-cooled condenser. Place a small electric heater below the flask and adjust to give continuous boiling of ether. The ether vapor will rise from the flask, through a side arm to the second vessel and thence up to the condenser; here it will condense on the walls and drip back down onto the sample; when the middle chamber is about half full the siphon on the side will

overflow and draw all of the fluid back into the flask; the sample, then, is being continually extracted with successive changes of fresh solvent and the solute is being concentrated in the lower flask. After continuous extraction for two or more hours turn the heat off. Disconnect the three parts of the extractor and by tipping the middle piece set the siphon into action to drain the ether off into the lower flask (disconnected but held immediately below). Remove the thimble from the middle piece carefully and set on clean filter paper to drain.

6. Transfer fluid from lower flask to beaker of appropriate size. Wash flask with small amount of fresh ether and add it to the beaker. Why do you add the rinsing to the extract? Evaporate both beaker *and* thimble to dryness. REMEMBER ETHER CAN AND SOMETIMES DOES EXPLODE! DO NOT accelerate drying by heating over an open flame; do NOT dry in a closed oven; do NOT even dry near an open flame. The thimble will dry with sufficient rapidity sitting on the table (with dust cover made of cone of filter paper). The beaker of ether should be dried on the water-bath, but see next paragraph.
7. Take a small clean beaker (50 ml capacity) and weigh accurately on the analytical balance. When the extract has dried down to 20–30 ml volume, transfer to this smaller beaker quantitatively, rinsing the larger beaker several times with a few ml of fresh ether and adding the rinsings to the extract. Complete the drying.
8. Weigh both the beaker and the thimble (with paper and rod) on the analytical balance.
9. Does the total of extract plus residue equal the original dry weight? If not there is something wrong with your technique.
10. Calculate the percentage of total fat relative to (a) wet weight of eggs, and (b) dry weight of eggs.
11. What are the two chief components of the residue left in the thimble? Is there more protein or more fat in an egg? What else might be expected besides protein and fat?
12. Discard the residue in the thimble unless you wish to attempt fractionating it.
13. Add 15–20 ml of pure acetone to the dried fats in the beaker. Break them up with a stirring rod and stir for approximately 20 minutes. Filter quantitatively and wash the filtrate with fresh acetone. The beaker should be of known weight.
14. Dry the filter paper. Dissolve the residue with ether or petroleum ether in order to get it into another beaker (also of known weight).
15. Dry both beakers on the water-bath. Weigh each to determine weight of contents.
16. The material soluble in acetone will include mostly neutral fats but also the sterols and fatty acids that were present. The material

insoluble in acetone will contain the phospholipids (cephalins, lecithins and sphinogomyelins). Calculate the percentage of each group.

17. Further separation, identification and estimation of the lipid fractions is both time-consuming and difficult. If interested see references below, or in text.
18. Slifer, using grasshopper eggs, obtained values of 17–22% of dry weight for the *fatty acids alone*. You were extracting and weighing all types of lipids. Why did she get such high values? And why are her figures not comparable with those you obtained? (Hint: examine her technique; what did she do that you did not do?).

Report

Write an essay of approximately 500 words covering (a) the chemical composition of an insect egg, and (b) the differences between the technique as you did it as opposed to how it should be done.

References

See general references on insect eggs.

MOLTING AND PUPATION IN DIPTEROUS LARVAE

Introduction

It is now well known that the growth, molting and metamorphosis in insects are under the control of a balanced system of hormones produced by the brain and various endocrine glands. In the larvae of a fly, the endocrine glands unite to form what is known as "ring gland". This gland forms around the aorta and comprises the gland CA, CC and PG. Fly larvae are excellent materials for ligation experiments to show the activities of the endocrine glands.

Objective

To demonstrate that pupation is controlled by a hormone.

Materials

1. Fly larvae: housefly larvae, 3–5 days old (Batch 1)
   6 days or older (Batch 2)
   Tsetse larvae, freshly deposited (Batch 1)
   half hour after dep. (Batch 2)
2. Thread (unwaxed dental floss)
3. Petri dishes, filter papers
4. Dissecting dishes, kits
5. Blu-tack
6. Stereo microscope
7. 1% Methylene blue solution

Methods

A. Dissection
   1. Immobilize larvae by chilling.
   2. Fix the larvae with ventral side up on the dissecting dish using Blu-tack.
   3. Dissect to expose the entire nervous system.
   4. Use a drop of methylene blue to locate the tissue.
   5. Locate the ring gland and the brain in relation to the body segment (check carefully segment 4).

B. Ligation experiment
   1. Select several larvae of various age groups from both species.
   2. Ligate each one with the fine thread at points anterior and posterior to the ring gland.
   3. Place the ligated larvae in covered Petri dish lined with filter paper and slightly moistened.
   4. Leave a few controls (unligated but of similar age group) in separate dishes.

5. Observe the result twice a day for several days or until the controls have pupated.
6. Record your data on a table showing date of observation, time, type of ligation, age of the larvae at ligation, etc. Precisely record what you see in terms of pupation as shown by the anterior to ligation and/or posterior to ligation. Also record any mortality or other peculiarities if any. Compare the results with those of the controls.

Questions

1. What do your results indicate in regard to the hormonal control of pupation in housefly, in tsetse?
2. Why do some larvae pupate both anterior and posterior to the ligation? Why others only at one end?
3. How would you demonstrate whether pupation was controlled by hormones or by stimulation from the nervous system only?
4. What is a "critical period"? What is the critical period for pupation of tsetse larvae?

EFFECT OF JUVENILE HORMONE ON METAMORPHOSIS — I

Introduction

The function of juvenile hormone (JH) in metamorphosis is to allow the larval characteristics to be retained until the time of metamorphosis. When the titer of JH is very low the larva is ready to pupate. It would be interesting to see what happens if larvae are treated with JH or JHA before or after the critical period of pupation.

Objective

To demonstrate the role of juvenile hormone in the metamorphosis of a holometabolous insect.

Materials

1. Fifth and sixth (last) instars larvae of maize stem borer, *Chilo partellus*, (non diapausing)
2. Petri dishes
3. Microapplicator and syringes
4. JH I and a JHA
5. Solvent for diluting the hormones
6. Vials

Methods

1. Prepare dilution series for both JH I and JHA.
2. Apply topically one µl of the solvent containing 0, 1, 2, 5, 10, 20, and 50 µg of the hormone (or the analogue).
3. Treat about 10 insects per age group with each of the dilution.
4. Leave the treated larvae in the covered Petri dish for observation. Provide *Chilo* diet in the dish.
5. Record your observations for several days (until molt or pupation in control groups).

Questions

1. Why do you see a difference in the results from JH I and JHA?
2. How would you set up a bioassay to see whether a compound has JH activity?

Reference

Gilbert, L. I. (1976). *The Juvenile Hormones*. Plenum Press, New York and London.

EFFECT OF JUVENILE HORMONE ON METAMORPHOSIS — II

Objective

To test the effect of juvenile hormone on larvae and pupae.

Materials

Dysdercus, day 1 of final instar
Chilo or *Spodoptera* pupae
Petri dishes and filter paper
JH analogue
Microapplicator and syringe or calibrated glass capillaries
Dissecting kit with forceps and needle

Methods

1. *Galleria* wax-wound assay: Remove a 1–2 mm² piece of cuticle from the dorsal thorax (mesonotum) of the pupae (within 24 hours of pupation). Melt 10 mg wax on a glass slide and thoroughly mix 10 µl of the JH sample. Cool and divide into 10 equal parts. Apply 1 portion to the wound and melt the wax to seal the wound using a warm needle. After a week examine the preparation (when adult emerges the spot will be of pupal characters if the compound is active). Try various dilutions.
2. Select day-old final instar nymphs. Apply various dilutions of JH on the thorax using microapplicator or calibrated micro capillary tubes (topical application). Also run some control using the solvent you are using (usually acetone). Keep them in Petri dishes until they are ecdysed. Score for adult or larval characters.
3. Using *Galleria* test, determine the dose-response curve for minimal level of activity.
4. Set up a second experiment, using the reading from the dose-response curve to determine the peak sensitivity by putting maximal dose at various times.
5. Report your result in tabulated form and analyze statistically.

References

Gilbert, L.I. 1964. Physiology of growth and development: Endocrine aspects. In *Physiology of Insecta*. Edited by M. Rockstein. Vol. 1, pp. 149–225. Academic Press, New York & London.

Slama, K., Romanuk, M. and Sorm, F. 1974. *Insect Hormones and Bioanalogues*. Springer-Verlag, New York, Wien.

BIOASSAY FOR ECDYSTEROIDS

Introduction

Bioassays that determine the hormone action of compounds which stimulate molting were initiated by Wigglesworth. Later on many tests were developed by different workers. Well-known tests were *Calliphora* and *Musca* tests. In certain cases, indirect topical application method was used quite successfully, such as the *Chilo* "dipping" test. However, at present most of the assays for ecdysteroids use radioimmunoassay which is much more accurate and can detect extremely small quantities of ecdysteroids in the system.

Objective

The objective of the present experiment is to conduct a bioassay using *Chilo* dipping procedure. This test is based on indirect topical treatment of ligated abdomens of some lepidopteran larvae.

Materials

Last instar *Chilo partellus* larvae
Thread
Ecdysteroid
Methanol
Glasswares, Petri dishes, etc.

Methods

1. The last larvae of *Chilo* are ligated behind the metathorax, before critical period of ecdysone release.
2. The undeveloping ligated abdomens are dipped into methanolic solutions containing graded amounts of ecdysteroid to be tested (5 seconds).
3. Methanol is allowed to evaporate.
4. Abdomens are incubated at 25–28°C in a humid atmosphere.
5. Abdomens are observed for the formation of a characteristic pupal cuticle.

Questions

1. What is ED50? What concentration of the ecdysteroid was required to obtain ED50 for pupal cuticle?
2. If the ecdysteroid is injected instead of dipping, will you get a different result? Why?
3. What result will you get if you topically apply the ecdysteroid solution?

Reference

Slama, K., Romanuk, M. and Sorm, F. 1974. *Insect Hormones and Bioanalogues*. Springer-Verlag, New York, Vienna.

THE PRINCIPAL CONSTITUENTS OF THE CUTICLE

Objective

To demonstrate the principal constituents of the cuticle of the desert locust *Schistocerca gregaria.*

Materials

Three-inch test tubes
Oil bath
Spot plate
Iodine reagents
1% sulfuric acid
3% acetic acid
Alpha naphthol solution
Concentrated sulfuric acid
70% & 95% ethyl alcohol
Saturated potassium hydroxide solution
10% sodium hydroxide
5% cupric sulfate
Insects
Empty cockroach oothecae

Methods

A. Chitin

1. Place a fragment of tissue-free integument in a three-inch test tube.
2. Add 1 ml of saturated KOH.
3. Heat in an oil bath for thirty minutes at 160°C.
4. Examine the contents of the tube after they have cooled somewhat. If the cuticular material has dissolved completely, what can be said about its constitution? If chitin was present, has this treatment changed it in any way?
5. Transfer the fragments to a spot plate. Divide into two parts and place in separate depressions.
6. Rinse the fragments with 95% alcohol, 70% alcohol, and with distilled water.
7. To one fragment add one or two drops of iodine solution. Draw the iodine solution off and add a drop or two of 1% sulfuric acid. If chitin were present in the fragment, a red to purple color should be obtained. If the fragment is black, rinse it in 70% alcohol to obtain the purple color.
8. To the second fragment, add a few drops of 3% acetic acid. If chitin were present in the original cuticle, the fragment should now dissolve. Save this solution for use in the test for carbohydrates.

B. Carbohydrates (the Molisch test)

1. Macerate an ootheca (empty) in a mortar with some water and white sand.
2. Transfer a small amount of the mixture to a spot plate depression.

3. Add two drops of alpha naphthol solution. Mix. Transfer a few drops of the supernatant to a small test tube containing 1/2 ml of concentrated sulfuric acid. *Do this carefully* by allowing the supernatant to run down the side of the tube. *Do not shake or mix.* A red interface indicates the presence of carbohydrate. Does this test indicate that no carbohydrate other than chitin is present? Does it exclude the possibility that others are present?
4. Repeat steps 1 to 3, using tissue-free integument instead of an ootheca.
5. Repeat step 3 using the chitosan in acetic acid solutions obtained in the tests for chitin. Also run a blank using a few drops of 3% acetic acid without dissolved chitosan.

C. *Protein (the biuret test)*

1. Macerate a fragment of tissue-free integument in a mortar with a few drops of water and some white sand.
2. Transfer the macerated material to a spot plate depression.
3. Add a drop of 10% NaOH.
4. Mix and add a drop of copper sulfate solution. Continue to add copper sulfate drop by drop (slowly) until a pinkish color forms. This color indicates the presence of biuret, which is formed from protein or dipeptides.
5. Repeat steps 1 to 4 using an empty ootheca instead of integument.

References

Campbell, F.L. 1929. The detection and estimation of insect chitin. *Ann. Ent. Soc. Amer.* 22:401–426.

Hackman, R.H. 1964. Chemistry of the insect cuticle. In *Physiology of Insecta.* Vol. 3, pp. 471–506. Academic Press, New York and London.

Wigglesworth, V.B. 1949. The insect cuticle. *Biol. Rev.* 23:408–451.

PROTEIN CONTENT OF THE TSETSE CUTICLE

Objective

To determine the total protein content of the tsetse abdominal cuticle during pregnancy cycle and compare it with that of virgin females.

Materials

Virgin and pregnant tsetse of various age groups (up to two larviposition)
Dissecting tools
Microbalance
Oven (100°C)
Reagents (2.5N NaOH, 1N HCl-ethanol, diethyl ether)

Methods

1. Dissect out entire dorsal and ventral cuticles of the abdomen and carefully clean adhering tissue and epidermal cells.
2. Dry each cuticle individually at 100°C to a constant weight.
3. Heat the cuticle at 110°C in sealed tubes with 1 ml of 2.5N NaOH for 2 hrs.
4. Wash successively in water, 1N HCl ethanol and diethyl ether. The remaining material is assumed to be chitin.
5. Dry the remaining material to a constant weight.
6. Total protein will be the difference between the dry weight of the cuticle and the dry weight of the chitin.
7. Determine the total protein on alternate days 24 hrs after feeding throughout the first two cycles of the pregnancy.
8. Present the data graphically for both protein and chitin content for both dorsal and ventral cuticles of virgin and mated tsetse.

References

Carruthers, C.B. and Davey, K. G. 1983. Does cuticular elasticity regulate the size of the blood meal by female *Glossina austeni*. *Canad. J. Zool.* 61:1888.

Hackman, R.H. and Goldberg, M. 1971. Studies on hardening and darkening of insect cuticles. *J. Insect Physiol.* 17:335.

EPICUTICLE

Objective
To isolate and identify the epicuticle.

Materials
Test tube
Nitric acid
Sudan solution (lipid dye)
NH_4OH
Pieces of hard cuticle (locust pronotum recommended)
Pieces of soft cuticle (mosquito larvae recommended)

Methods
1. Clean the pronota in distilled water by wiping the inner surface with something soft. *Be careful not to scratch the outer surface* during this manipulation. Cut off edges to use only central area (if epicuticle extends over edge of specimen it will not separate readily). Place one pronotum in a test tube and add a few ml of 50% nitric acid. Different authors do not all recommend the same concentration of nitric acid. If you do not have success at first this is one of the several things you can vary. Heat *gently* (80–90°C). The chitin and protein of the inner cuticle (endocuticle and exocuticle) will dissolve. A sheet will remain which is only 2–3 microns thick. By gentle shaking and continued gentle heating it is sometimes possible to have this sheet separate into two sheets, a relatively thick, colored inner part, and an extremely thin (less than 0.1 micron) transparent outer sheet. If heating is continued or the temperature raised first the thick inner layer and then the thin outer layer will dissolve. If or when you get these sheets to separate pour the contents into a dish and transfer the pieces (they are friable, lift only with support) to distilled water. Once they get cold they break very easily. Repeat until you have good samples.
2. Put small pieces of each on a slide and examine with the microscope. What can you see?
3. Transfer small pieces to 70% alcohol and try to stain with Sudan dyes (in 70% alcohol). Wash the excess stain off with 70% alcohol. (Sudan dyes as used here are simple solution dyes and are as easy to wash out as to put into a tissue). A definite staining is an excellent histochemical qualitative demonstration of the presence of lipid. What is the significance of no demonstrable staining?
4. Transfer pieces (outer and inner sheet) to separate depressions on spot plates. Add concentrated nitric acid and after a few minutes neutralize with NH_4OH. An orangish brown color, if obtained, is evidence of protein. It is known as the xanthoproteic test.
5. Prepare sections, either free hand or with freezing microtome, and place on a microscope slide in water. After examining with

compound microscope to be thoroughly familiar with the parts, remove the water and *while observing continuously* replace with concentrated nitric acid. If the reaction does not seem to you to go to completion at room temperature you may warm the slide. What would you expect? What did you obtain? (See Kühnelt)

6. Take a living wax moth or meal worm larva and heat it to about 90°C or slightly higher for several minutes. This is best done in air but can be done under water. Examine under a dissecting binocular using the highest power. If the experiment has been successful the outer part of the epicuticle will have melted and run together as droplets. What does this suggest? Test these droplets with Sudan dye. Do they stain? What can you conclude from this?
7. Repeat some or all of this using mosquito larvae. The mosquito larvae has a single-layered epicuticle that is very thin (less than 0.1 microns thick). It is not as resistant to the action of concentrated nitric acid as the epicuticle of the cockroach. On slightly overheating it contracts into a gummy orangish brown mass. The addition of NH_4OH darkens this mass. It cannot be stained with Sudan dyes either before or after heating. What can you deduce about the mosquito epicuticle?
8. If you have more time you can try wax extractions along the line of Beamont's work. Consult your instructor.

References

Consult your references for insect integument.

THE PRESENCE OF RESILIN IN INSECTS

Introduction

Resilin is an elastic protein which is found in insects as an insoluble gel-like component which is present in certain patches of cuticle. Resilin is the protein which is responsible for the presence of rubber-like cuticle in insects. Since resilin in water stains with toluidine blue or methylene blue, it can be easily detected by examining stained pieces of cuticle under ultraviolet light. Resilin stained with the alkaline dyes mentioned above, fluoresces with a brilliant blue color when examined under ultraviolet light. This property will be utilized in order to test for the presence of this protein in adult honey bees.

Methods

1. Four living honey bees are killed by immersion in hot buffered water for a few minutes (pH 6.7, 95–100°C).
2. After opening the bodies, the soft parts are removed and the cuticle freed from the epidermis (hypoderm) with a jet of water.
3. The rinsed cuticle is placed for 24–48 hours at room temperature in dilute phosphate buffer (M/20, pH about 7) to which is added 5 mg toluidine blue and 5 mg light green per 100 ml buffer. A few crystals of thymol are sufficient to prevent bacterial growth.
4. Rinse in pure buffer (pH about 7) for several hours and examine the various parts of the bee under ultraviolet light (add thymol). Examine the head, thorax (including wings and legs) and abdomen separately. Long-range ultraviolet light is preferred. Typical rubber-like cuticle appears as translucent, brilliant blue patches.

Results

Draw the areas of the cuticle which contain resilin. From its location, what could you conclude about its possible role in the physiology of the honey bee? What percentage of the cuticle would you estimate is rubber-like?

References

Weis-Fogh, T. 1960. A rubber-like protein in insect cuticle. *J. Exp. Biol.* 37:889–907.

Weis-Fogh, T. 1961. Thermodynamic properties of resilin, a rubber-like protein. *J. Mol. Biol.* 3:520–531.

THE TANNING OF THE CUTICLE

Introduction

It has been recognized for over a century that the hardness and color of the cuticle is not dependent upon the chitin content, but upon something else. Recent work has shown that the process depends upon a change in the proteins of the cuticle analogous to the tanning of leather. More recently it has become apparent that the tanning process in the case of insect cuticles requires the presence of phenolic compounds. In the present exercise, we shall test several such compounds. In addition, we shall inactivate the enzymes of the cuticle with heat, and test the effect of two poisons.

The poisons which we shall test are cyanide whose effect is mediated by the fact that it forms unreactive compounds with nearly all heavy metals, and another reagent which forms a very strong coordination complex with copper.

Methods

1. Prepare cuticles of about sixty tsetse larvae by removing, as far as possible, all adherent tissue. *Do not heat the larvae.*
2. Place fifteen of the cuticles in a vial and heat the vial in a boiling water bath for 3–4 minutes to inactivate enzymes present.
3. Number and letter three spot plates in the fashion diagrammed below.

	A	B	C	D
	Fresh Cuticle	**Heated Cuticle**	**Fresh + 1% KCN**	**Fresh + Copper Reagent**
1. Catechol	0	0	0	0
2. Hydroquinone	0	0	0	0
3. Resorcinol	0	0	0	0
4. Benzoquinone	0	0	0	0
5. Tyrosine	0	0	0	0
6. Phenol	0	0	0	0
7. Dopa	0	0	0	0
8. Phenylalanine	0	0	0	0
9. Water	0	0	0	0

4. In each of the depressions in the spot plates, place the cuticles from two larvae. In the "B" depressions, use the heat-inactivated cuticles.
5. Place 8–10 drops of the indicated reagents on top of the cuticles.
6. To all "C" depressions, add 1 drop of 1% KCN; to all "D" depressions, add 1 drop of copper reagent.

7. Seal the preparation to prevent loss of moisture by placing a thin layer of grease around the outside edge and covering it with a glass plate.
8. Set the plate aside (being careful not to mix the contents of one depression with another) until the next laboratory period. Note at this time any change of color which has occurred in the cuticle.
9. Forty-eight hours later, examine your spot plates and record the amount of darkening in each depression, then answer the following questions:

Results

1. Have all of the preparations darkened equally?
2. What does this experiment show about the components necessary for the darkening of the cuticle?
3. What is the minimum number of components necessary? What is the proof for the existence of each?

References

Consult references for insect integument.

ARTHROPOD INTEGUMENT AND WATER BALANCE

Objective

(1) To measure the rates of water gain and loss at different humidities; (2) To estimate the critical equilibrium humidity for this tick; (3) To determine the site of active water vapor uptake; and (4) To determine the effect of cuticular abrasion on water balance.

Materials

Rhipicephalus appendiculatus
Mettler balance H54AR
Desiccators
Saturated salt solutions (see Winston & Bates, *Ecology* [1960] 41:232–237)
Beeswax & rosin (6 g:2 g)
Foil tick containers with holes
Silicon abrasive

Methods

Experiment I: Measure rates of water gain and loss at different water vapor activities

1. Weigh the tick and its container, place in appropriate desiccator at 26°C. The following water vapor activities will be used:

   0.0 —Drierite
   0.23—Potassium acetate saturated solution
   0.75—Sodium chloride saturated solution
   0.85—Potassium chloride saturated solution
   0.93—Potassium nitrate saturated solution

   Ticks should be pre-desiccated at 0.23 av for 24 hours prior to incubation at these activities to standardize specimens. Use at least 5 ticks per water activity.

2. Weigh ticks in their foil boats at 24-hour intervals. Weigh as quickly as possible to minimize exposure to ambient air. If ticks are to be held at a low av for the experimental period and a dramatic loss is seen, check the viability of your specimen. Plot per cent original weight (24 hours post-desiccation) versus time (days).

Experiment II: Determine the site of active water vapor uptake

Using predesiccated males (0.23 av, 24 hours, 5 males per treatment) cover the anus, mouth (not including the palps) or Haller's organ (tarsus of leg I) with the beeswax-rosin. Use 5 males with no treatment

as controls. Weigh the tick and foil container after applying the blocking material and incubate at 0.93 av. Determine weight changes at 24, 48, and 72 hour intervals after incubation at 0.93 av.

Experiment III: Effect of cuticular abrasion on water flux

Using male ticks, place 5 specimens in a plastic Petri dish containing silicic acid dust covering the sides. Allow the ticks to walk around for 3–5 minutes. Remove excess dust from the specimens using a stream of air (hose connected to building air supply). Using another group of 5, scratch the dorsum a single time with forceps. Use 5 males as controls. Place in foil containers, weigh and transfer to 0.75 av. Check at 24-hour intervals until significant weight loss is detected. Plot as per cent change from original weight against time (days).

References

Arlian, L.G. and Veselica, M. M. 1979. Water balance in insects and mites. *Comp. Biochem. Physiol.* 64: 191–200.

Edney, E.B. 1977. Water balance in land arthropods. In *Zoophysiology and Ecology*. Vol. 9. Springer, New York.

Gilby, A.R. 1980. Transpiration, temperature and lipids in insect cuticle. *Adv. Insect Physiol.* 15: 1–33.

Machin, J. 1979. Atmospheric water absorption in arthropods. *Adv. Insect Physiol.* 14: 1–48.

Maddrell, S.H.P. 1982. Insects: Small size and osmoregulation. In *A Companion to Animal Physiology*. Chapter 14. Edited by Taylor et al. Cambridge University Press, Cambridge pp. 289–305.

Needham, G.R. and Teel, P. D. 1986. Water balance by ticks between bloodmeals. In *Morphology, Physiology, and Behavioral Biology of Ticks*. Edited by J.R. Sauer and J.A. Hair, Ellis Horwood, Ltd., Chichester (in press).

Shaw, J. and Stobbart, R.H. 1963. Osmotic and ionic regulation in insects. *Adv. Insect Physiol.* 1: 315–399.

Wharton, G.W. 1985. Water balance of insects. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology*. Vol. 4, Chapter 14. Edited by G.A. Kerkut and L.I. Gilbert, Pergamon Press, New York.

TRANSPIRATION THROUGH THE CUTICLE

Objective

To determine natural transpiration and the effects of heat and scratching on the insect cuticle.

Materials

Thermometer
Thread (any of small diameter)
Large pyrex beaker with cover made from piece of cardboard
Cyanide jar or other killing agency
Ground sand
Emery powder or other fine abrasive
Vacuum desiccator (with drying agent)
Balance reading to 10 mg or less

Note — Since weighings at several successive periods are required in this it will be advantageous to get all of the following set up at the first period even if this involves overtime. Only a short time will then be required in the remaining days.

Methods

Use at least two species of large insects (e.g. armyworm and *Chilo* larvae). Label each individual specimen in some manner. Weigh each specimen individually as accurately as the available balance will permit. Record the weights. Be careful not to rub or abrade the specimens any more than can be avoided. Divide the specimens of each species into six groups (minimum of two or three specimens in each group), and treat as follows:

1. One group of each species will be placed in a dry atmosphere without any special treatment. These may be called normals or controls. A dry atmosphere is most easily obtained by keeping in a closed chamber or desiccator over anhydrous $CaCl_2$. Weigh at known intervals and calculate the water loss per day assuming that weight loss equals water loss (is the assumption absolutely accurate?). Plot weight loss against time on graph paper.
2. A second group should be killed by some standard toxin. Cyanide is good. Handle and keep records as under above.
3. A third group should be suspended in an air bath at approximately 40°C for 10 minutes. Such a bath is easily made by having a bunsen burner beneath a large beaker which is covered with a piece of cardboard; through a hole in the cardboard suspend a thermometer, and by strings suspend your specimens in the center of the space; by manipulation of the burner raise the temperature to 40° and hold it there. Remove the specimens, handle and keep records as under above.
4. A fourth group should be handled similarly but treated at 50°C, for 10 minutes.

5. A fifth group should be handled similarly but treated at 60°C, for 10 minutes.
6. A sixth group should be gently but thoroughly rubbed with an abrasive powder to scratch through the epicuticle of a large area. Handle and keep records as above.

Report — Give results in tabular or graph form. State conclusions. What hypothesis seems most reasonable to explain these data? If you were using the weighing method in a research problem, how would you proceed if you desired only moderate accuracy? If you desired as great accuracy as could be obtained?

Reference

Wigglesworth, V.B. 1945. *J. Exp. Biol.* 21:97–114.

CUTICLE PERMEABILITY

Objective

To study the permeability of the wall of the insect oesophagus.

Materials

Various living insects
Salt solutions
Acid solutions
$AgNO_3$ solution
Acetone
Thread
Distilled water
Indicator solution
Syringe
Dichlorofluorescein solution

Methods

1. The fore gut of insects is lined with a layer of cuticle (commonly called "intima") which is continuous with the cuticle on the outer surface of the body and which apparently has the same type of structure as certain parts of the exoskeleton. Usually the oesophagus is dilated posteriorly to form a pouch called the crop. In some insects this dilation extends outward from one side of the oesophagus and in extreme cases (Diptera) may form a sac connected to the oesophagus by a relatively long tube. Choose a large species and extirpate the crop from two specimens. Place them in distilled water and by gentle massage with a small brush clean away the surrounding tissues. With some species the muscle, connective and tracheal tissues form such a tough mat they have to be picked or cut away. Flush out the interior repeatedly; a small syringe is convenient for this purpose.

   Tie one end tightly with thread (what materials make better ligatures?); fill one specimen with a weak solution of acid, e.g. 1% HCl (what amount of concentrated HCl has to be added to water to make 100 ml of a 1% solution?), tie the other end tightly and immerse in distilled water colored with an indicator that will change color on acidifying. Label and set aside. Fill the other specimen with the indicator solution and immerse in weak acid. Label and set aside. Observe both of these at intervals and record any observed color changes. Cover the dishes to prevent evaporation and examine at the next laboratory period.
2. Prepare another crop, fill with distilled water, ligature both ends tightly and leave exposed to air. How long does it take for the contents to evaporate through the cuticular casing? Repeat this with a number of different species of insects. Are the results similar for all of the species?
3. Prepare several more crops. Fill one series with a strong salt solution; fill the other series with distilled water. Immerse those filled with saline solution in distilled water, and those filled with

distilled water in saline solution. Label, cover to prevent evaporation, and observe at subsequent periods. Have the contents of the crops increased, decreased or remained approximately the same? If the crops have remained the same size there are three obvious possible explanations. What are these possibilities? Plan and perform tests to determine which seems to be correct.

Since the common salt solutions are colorless, a test is needed to determine their distribution. Perhaps the easiest method is to test for the presence of Cl^- ions. Put a drop of the solution to be tested in the depression on a white spot plate, add a drop of $AgNO_3$ solution, a drop of dichlorofluorescein solution and several drops of acetone. If Cl^- ions are present a brick-red precipitate will develop.

4. Additional tests should follow the line of most interest to the student. Other species may be used for paragraph 3. Crops may be dissected out in balanced saline, the surrounding tissues left intact, the tube filled with distilled water, and the preparation left standing in aerated saline. What might happen? The penetration of other substances may be tried: sugars, preferably monosaccharides, starch, gelatin, dyes, lipids, etc. Or if the student wishes a more complex program he may plan and execute a Donnan equilibrium experiment. If electrolytes are used it is fairly simple to add electrodes and set up an electrodialysis experiment. Or membranes may be extracted in various manners (petroleum ether, KOH, etc.).

Report

Critically evaluate the results you obtained. What would you have expected from the parts you did not perform? How would one have to proceed in such experiments in order to obtain valid quantitative data?

References

Consult references for insect integument.

BLOOD CIRCULATION

Objective

To study the circulation of blood in an appendage (locust wing).

Materials

Adult locusts
Dissecting binocular with set of high power objectives
Microscope lamp
Strip of tin foil or other thin reflecting material (see below)
Millimetre rule
Depression block for holding insect
Pins and paper strips
Stop watch

Methods

1. Take a block of soft wood, paraffin, beeswax or other material that is easily carved. Gouge out a depression that will approximately fit the species of insect being used and which will leave the extended wings nearly flush with the surface of the block yet free from tension. Place the insect in this depression and arrange to hold it gently in place without introducing excess pressure or occlusions that might affect circulation in the wing. One way of doing this is to pin a narrow strip of paper across the insect behind the head and another across the top of the abdomen near the thorax; neither should be tight enough to introduce pressure complications. Nooses made of thread held in place with pins is another method. Spread the wings out to the side in a normal position; a pin into the block at the posterior edge of the wing near the base will serve to hold them there. Slip the tin foil or other strip under the wing to reflect light. Direct a strong beam of light onto the part of the wing to be examined and observe with the transmitted light reflected from the foil. Identify hemocytes (blood cells) and note their movement.

   Instead of using tin foil, a wax cell can be built on top of a piece of glass and arrangements made for supporting the wing in a parallel extended position. Light reflected from a mirror through a glass stage can then be used. If this type of cell is employed, the reflecting foil will still be needed when studying circulation in wings folded over the abdomen (see below).

2. Prepare a fairly accurate diagram of the wing including its veins. Plot the paths of blood movement as shown by blood cell movements. Check the results obtained by comparison with movements in the wing on the other side of the body. Subsequently check it for constancy on at least three (3) other specimens.

   After you have plotted the paths in one set of wings, plot it in the other pair. Again check with additional specimens.

3. Using a millimetre ruler and a watch time the velocity of blood cell movement in at least a half dozen widely separated areas of the wing, recording the rate in each and marking your diagram in some manner to show the spots and distances used for the rate determination.

   Make such measurements in both fore and hind wings.

   What would you expect to be the relationship between blood plasma velocity and blood cell velocity? Describe critically an experiment whereby this might be determined.
4. After determining the normal paths and normal velocities on some of the specimens try the effects of (a) pressure on the body, (b) pressure or tension at the wing base, and (c) occlusion by pressure of some of the normal blood paths. Present results obtained giving particular attention to interpretation.
5. Repeat your determinations of paths and velocities when the wings are in the normal resting position folded over the abdomen. Compare with the above.

Report

Present findings. Include a discussion of the significance of these data.

Reference

Beard, R.L. 1953. Circulation. In *Insect Physiology*. Edited by K.D. Roeder, pp. 232–272. John Wiley & Sons, New York.

CHEMISTRY OF BLOOD

Objective

To determine by quantitative micro-method the amount of reducing substance in an insect's blood as a selected example of studies in blood chemistry.

Materials

Large insects
Precision micro burette calibrated to 0.02 ml
Burette stand
Precision 2 ml delivery pipette
Hypodermic syringe (without needle) calibrated to 0.01 ml

Solutions:		
	0.1 normal NaOH	0.45 per cent $ZnSO_4$
	0.005 normal alkaline ferricyanide	5 per cent $ZnSO_4$
	0.005 normal sodium thiosulfate	plus
	0.005 normal potassium iodate	25 per cent NaCl
	potassium iodide	(mixed solution)
	distilled water	1 per cent starch
	3 percent glacial acetic acid	solution

Also needed: test tubes, small beakers, cotton, and larger vessels for heating and cooling small funnels

Methods

1. All glassware must be chemically clean. Start by getting necessary bottles, test tubes, burette, funnels, syringe and beakers and immerse them in cleaning solution (H_2SO_4+ $K_2Cr_2O_7$). How long glassware needs to remain in this solution depends on how dirty it is and what the dirt is. If the glassware is already what is ordinarily called clean, it may be washed with soap and water, rinsed clear of soap and treated with the cleaning solution for only an hour. After removal from the cleaning solution, it is rinsed first with tap water and then three times with distilled water. It must be allowed to dry; *NOT* wiped dry.
2. The next step is to prepare the reagent solutions. In a research problem one should check the purity of the chemicals, but to simplify the problem for the purposes of this laboratory exercise we will assume that the reagent grade chemicals are all of sufficient purity.

   0.1 normal NaOH — this is already available (would need a pH meter to prepare).

   (By definition, "A normal solution contains one gram molecular weight of the dissolved substance divided by the

hydrogen equivalent of the substance per litre of solution.")

0.005 normal alkaline ferricyanide — 0.165g. $K_3Fe(CN)_6$ plus 1.06 g. Na_2CO_3/100 ml

0.005 normal Na thiosulfate — 0.70 g in 500 ml

0.45 per cent $ZnSO_4$ — 0.45 g in 100 ml

$ZnSO_4$ + NaCl solution — 5.0 g. $ZnSO_4$ plus 25 g. NaCl in 100 ml. This is called the "iodide-zinc solution" but the iodide is not added until just before use. When ready to use add 0.25 g of KI to 10 ml of above solution.

Acetic acid solution — 3 ml glacial acetic acid + 97 ml distilled water.

1 per cent starch — 1 gram of starch made into paste in cold water and stirred into 100 ml of vigorously boiling distilled water (high accuracy not needed).

3. Collect blood from large insects. This is done by making an incision or severing a leg and collecting the blood by just sucking it up into a hypodermic syringe as it exudes. Do not include any tissues (if they also extrude from the wound). Have several specimens ready so that you can pool the blood of several individuals, *if necessary*, in order to get enough into the syringe to be able to deliver an *accurately known amount* into each tube. Inasmuch as the determinations can all be run along together, it is recommended that you collect enough blood to deliver from 0.02 to 0.05 ml of blood into each of three tubes. Also that you do this with both species. Also one *must* run an equal number of blanks. It is suggested, then, that you set up nine clean tubes prepared as follows:

4. Blood is to be collected into small test tubes containing 1 ml of 0.1 N NaOH plus 5 ml of 0.45 per cent $ZnSO_4$. Blood is to be introduced into these tubes as follows: when enough has been accumulated in the syringe (must be rapid or you will get into trouble with coagulation), force a drop out at the tip and wipe clean with a piece of *clean* filter paper, read the meniscus carefully, and then deliver the desired amount into the tube, the tip of the syringe being dipped for an instant into the solution to take off the fraction of a drop that may be extruding from the tip of the syringe. The amount used is not so important; what is important is to know *exactly how much was delivered*.

5. In this type of work "blanks" *must always* be run on the reagents. A blank is a tube that contains all the reagents, and receives all the treatments, including the final determination, but does not contain the unknown (in this case blood).

6. When the series of tubes are all ready (as described in number 4), they are placed in a bath of boiling water for four minutes, then cooled to room temperature, and filtered through washed cotton

(about 20 milligrams) into another set of test tubes. The cotton is washed *three times* with 3 ml of distilled water, the washings being allowed to add to the filtrate (will make a total of 14–15 ml in each test tube). At this stage the tubes may be stoppered and set aside in the refrigerator until the next period if desired. Be sure all tubes are clearly labelled.

7. The solutions will now be free of proteins. Add *exactly* 2 ml of the alkaline ferricyanide to each tube using a precision delivery pipette. Immerse the tubes in boiling water for exactly 15 minutes. Cool rapidly by immersing tubes in cold water for 3 minutes. The tubes may be held for some hours at this stage if desired.
8. To each tube add approximately 3 ml of freshly prepared "iodide-zinc solution" (see number 2) and 2 ml of 3 percent acetic acid. The solution is then titrated for free iodine with the standard thiosulfate solution using a micro-burette.
9. It is convenient at this stage to transfer the solutions to small beakers. Add standard thiosulfate solution (keeping an accurate record of the exact amount) until the yellow color is *almost* all gone. Then add one drop of the starch solution as an indicator (do *NOT* add the starch before beginning the titration, add it only when the end point is near), and continue titrating *slowly with continued stirring* until the red-blue color just vanishes. Record the exact amount of thiosulfate used correct to at least 0.02 ml. Same titration with all tubes, *including blanks*.
10. The titration data are then used to calculate the glucose-equivalents (why say "glucose equivalents" instead of "glucose"). First the average value of the blanks (i.e., ml of thiosulfate needed for titrating the samples which had no blood) is subtracted from each of the unknown values. Then the glucose-equivalents are calculated from the equation:

$$G = 0.1735M + \frac{0.005M}{2.27-M},$$

where G = milligrams of glucose-equivalents, and M = millilitres of thiosulfate used (after correction).

Theory

"The method depends on the quantitative reduction of potassium ferricyanide by sugar in an alkaline solution, and the iodometric titration of the *excess* ferric salt."

$$2\,K_3Fe(CN)_6 + 2\,KI = 2K_4Fe(CN)_6 + I_2$$

$$2\,Na_2S_2O_3 + I_2 = 2\,NaI + Na_2S_4O_6$$

Note that the titration is for the *excess* $K_3Fe(CN)_6$. The ferricyanide that has already been reduced by sugar is not available for the reaction, and so does not release iodine. This is why it is necessary to use a precisely known amount of ferricyanide (i.e., you are determining the amount used by subtracting the amount not used from the total).

References

Consult references on chemistry of insect hemolymph (see physiology of circulation).

CHANGES IN HEMOCYTES IN RESPONSE TO VARIOUS FACTORS

Introduction

Hemocytes vary histologically among various groups of terrestrial arthropods as does the relative frequency of each type. Additionally, cell counts vary with the physiological condition of the individual, which is correlated with the molting cycle and with the condition of food reserves.

Objective

To use a simple method to study blood cells and determine changes in them in response to molt cycle.

Materials

1. Locusts at various stages, tsetse.
2. Sarkaria's stain (to make 100 ml: dissolve 0.5g crystal violet in 95 ml dist. water and add 1.090g NaCl, 0.157g KCl, 0.085g CaCl and 0.157g $MgCl_2$. When the salts are completely dissolved, acidify with acetic acid (0.125 ml glacial acetic acid) and bring the total volume to 200 ml with distilled water. Filter. This solution will remain stable for 30 days.
3. Microscope slides and cover slips.
4. Micropipettes; thin glass rod.
5. Compound microscope (preferably with phase contrast).

Note: Alternative to No. 2. You can use saline-versene (NaCl, 0.9g; KCl, 0.942g; $CaCl_2$, 0.082; $NaHCO_3$, 0.002g; Dist. H_2O, 100 ml + 2% versene).

Methods

1. Place a drop of stain on a cover slip. Obtain a minute amount of hemolymph from the insect using a micro pipette and introduce the hemolymph in the stain (clip antenna of the insect or the femur of a leg to get the hemolymph).
2. Stir with a thin glass rod and mount by inverting the cover slip over a microscope slide.
3. Petroleum jelly (Vaseline) may be used to seal the edges and will preserve the preparation for a few days.
4. Examine the slide under the microscope using suitable magnification and type of hemocytes per field of vision. Examine 5 or 6 different fields per slide.
5. Classify hemocytes into: prohemocyte, plasmatocyte, granulocyte, spherulocyte, adepohemocyte, oenocytoid and coagulocyte according to Gupta (1985).
6. Compare your results from adult locust and adult tsetse. Compare changes in locust molt cycles.

7. Use the key of Gupta (1979) to identify types (given on the next page).
8. Present data in tabular form.

References

Gupta, A.P. 1985. Cellular elements in the hemolymph. In *Comprehensive Insect Physiology Biochemistry, and Pharmacology*. Edited by G.A. Kerkut and L.I. Gilbert, vol. 3: 401–451. Pergamon, Oxford.

Gupta, A.P. 1979. (Ed). *Insect Hemocytes*. Cambridge University Press, Cambridge, UK.

IDENTIFICATION KEY FOR INSECT HEMOCYTE TYPES

1. Nucleus compact, large in relation to cell size; central, almost filling the cell; cells may be round, oval, or elliptical but always small ..... Prohemocyte (PR)

1a. Nucleus not compact, generally small in relation to cell size 2

2. Nucleus with chromatin arranged in cartwheel-like fashion, cytoplasm hyaline, generally scant ..... Coagulocyte (CO)

2a. No cartwheel like chromatin cytoplasm not hyaline 3

3. Cytoplasm agranular or slightly granular; nucleus round or elongate, may not appear punctate ..... Plasmatocyte (PL)

3a. Cytoplasm agranular, thick and homogeneous; nucleus small, round or elongate generally eccentric; cells variable in size, shape ..... Oenocytoid (OE)

3b. Cytoplasm distinctly granular 4

4. Granules may or may not be numerous. Nucleus smaller than that of PL, generally central ..... Granulocyte (GR)

4a. Granules enlarged, appear as distinct spherules or droplets 5

5. Spherule droplets refringent owing to presence of lipid; nucleus relatively small (cf sperhulocytes), round or slightly elongate, central or eccentric ..... Adipohemocyte (AD)

5a. Spherules non-refringent, generally obscuring the nucleus, number of spheres varying from few to many; nucleus rather small, central or eccentric; cells usually larger than GR .... Spherulocyte (SP)

HEMOLYMPH COAGULATION

Objective

To study the process of blood coagulation in insects.

Materials

Compound microscope
Neutral mineral oil ("Marcol", "Nujol", etc.)
Various insects
Microscope slides
Fine pipette (made by drawing out an ordinary medicine dropper)

Methods

1. The technique involved is extremely simple and the validity of results depends primarily on the fidelity of microscopic observations. A drop of any neutral non-toxic mineral oil is placed on a clean microscope slide; a living insect is punctured or a leg or antenna severed; a drop of blood is drawn quickly but gently into the tip of a fine pipette; the blood is then transferred immediately to beneath the surface of the drop of oil and allowed to settle on the slide; microscopic observation is begun immediately and continued until no further changes are to be noted. Written records illustrated with quick sketches serve as data.

   The above method involves at least momentary exposure of the blood to air. This may be avoided by inserting, for instance, a leg into the oil drop, cutting or puncturing it and allowing a drop of blood to exude beneath the oil. Prepare slides of at least one species in groups "b" and "c" by each method and compare the results.

   With certain species coagulation processes begin very quickly. An absolute minimum of time should elapse between collecting a sample and beginning observations.
2. One individual can be used as source for a number of successive blood samples. It is usually better to make a new incision for each sample. Repeat your observations with 3–4 different samples from each individual used. Use of successive samples will also facilitate getting a complete set of written notes and sketches.
3. Repeat with several other species. If material is available use at least one species from each of the three groups listed above. These examples are selected from the much longer listing published by Yeager & Knight.
4. In vertebrates blood clotting requires the presence of the calcium ion and accordingly can be prevented by the addition of oxalate. How does the oxalate ion function in preventing such clotting? It is generally considered that this is not true for insect blood clotting. Test to see what results you obtain after mixing a drop of

blood with a small amount of oxalate solution. It is preferable to use a species from group "c" for this test. Why?

5. Try a few experiments. Suggestions include:
   (a) Agitation of the blood drop with a fine glass rod.
   (b) Diluting the blood with saline, using (1) a small amount of saline, (2) an equal amount of saline, and (3) a much larger amount of saline than blood. In doing this put the drops of saline in the oil first, then collect and add the blood sample, stirring with a fine glass rod to mix.
   (c) Blackening of certain bloods on exposure to air is an enzyme reaction involving the formation of melanins. Compare the events in a drop of *Drosophila* larvae blood exposed to air with the events in another drop under oil.
   (d) Heat-kill an insect (80°C for 5 minutes), collect a sample and check to see if coagulation will still occur after this treatment.
   (e) Mix with various toxins or lysins. Try mixing with a detergent solution.
   (f) Other experiments that are of interest to you.

Report

Give a written description of your observations illustrated with sketches. Compare your results with those of Yeager & Knight. Consider the case of plasma coagulation carefully; discuss how one should proceed to elucidate the phenomena involved.

References

Consult references for circulatory system.

PHAGOCYTOSIS BY HEMOCYTES

Objective

To demonstrate phagocytosis of foreign matter by the hemocytes of the desert locust.

Materials

Physiological saline plus 2% carmine
Physiological saline plus 0.2% acetic acid
Physiological saline
Hypodermic syringe and needle
Vaseline
Mineral oil
Glass slides
Two eight inch test tubes

Methods

Working in groups of two:

1. Obtain and anesthetize two locusts.
2. Inject 0.05 ml of physiological saline into one and same amount of saline plus nitrate and carmine into the other. Make the injection at the coxa-trochanteral joint, carefully pushing the needle through the thin conjunctival membrane and deep into the coxa along the longitudinal axis. Deliver the liquid slowly, and slowly withdraw the needle. If possible, seal the wound with a very small amount of Vaseline.
3. Place a small drop of mineral oil on a clean glass slide. Put a drop of saline plus acetic acid on the oil drop. Clip an antenna of the control insect (the one without carmine) at a point about one-third of the distance from the base. Collect a small drop of the exuding hemolymph in the acid saline on the slide. Cover the saline-hemolymph mixture with oil by stirring gently with a needle. Seal the wounded antenna with a small amount of Vaseline.
4. Examine the preparation under low and high power objectives of your microscope. Note the appearance of the cytoplasm of the cells. This step is important in that it will acquaint you with the appearance of unstained blood cell, and thus it will be easier to separate those containing carmine granules from those that do not in subsequent steps in the exercise.
5. Repeat step 3 using the locust which had been injected with the carmine suspension. Examine the slide under low and then high power. Selecting fields at random, determine the percentage of hemocytes containing carmine particles. Count a total of 200 cells. Make a rough estimate of the average number of particles per phagocytizing cell.
6. Repeat step 5 at 30 minute intervals for 3 hours.
7. In your report of this exercise present your data as tables and also graphically, showing the relationship between time and the

phagocytic index (percent cells showing phagocytized particles). Also include any observations you were able to make concerning the cell types involved in phagocytosis. The number of particles per phagocyte at the various times should also be considered.

References

See references for circulation and hemocytes.

FEEDING: EGESTION TIME

Objective

To measure the time required for ingested material to pass through the digestive tract of *Schistocerca gregaria*.

Materials

Food containing test dyes
Test tubes
Dissecting equipment

Note: Locusts are starved for 48 hours prior to the beginning of the experiment. They are then allowed access to a food that has been colored with carmine. The time of the first ingestion is noted. They are then allowed to feed at will until examined. At fixed intervals after feeding, the locust is dissected and the position of the most posterior dyed food is recorded. For purposes of recording, the digestive tract is divided into arbitrary units as follows:

FOREGUT
- Crop
- Proventribulus

MIDGUT
- Anterior ventriculus
- Mid ventriculus
- Posterior ventriculus

HINDGUT
- Anterior intestine
- Mid intestine
- Posterior intestine
- Anterior rectum
- Posterior rectum

Snipes and Tauber (1937), working with the American roach, found appreciable variations among different individuals in respect to the rate of food movement. They could detect no differences that could be attributable to differences in sex and age. Using a method involving examination of faeces, as reported by Snipes, Tauber and Gunderson (1937), they found that the egestion time of an individual roach varied appreciably from trial to trial.

Methods

1. Obtain the locusts and make the examination assigned to your group.
2. In dissecting, pin the insect dorsum downward in a dissecting dish and flood the dish with water. After the insect is inactivated

by the water, cut off the metathoracic legs. Cut away the ventral abdominal body wall by making lateral incisions and removing the sternites. Remove the fat and gonads to expose the digestive tract. Grasping the lower oesophagus with a pair of forceps, tear the tract loose anteriorly, and extend the gut tract carefully to expose all of its parts. Note and record the position of the colored food material.

3. Record your data and in making your report of this exercise, consider all the data obtained by the class.

Group Assignments

Group	(hours after ingestion)	Locusts to be examined
I	0.50, 1.00, 2.0	
II	3.0, 4.0, 5.0	
III	6.0, 7.0, 8.0	
IV	10.0, 12.0, 16.0	
V	18.0, 20.0 and 24.0	

References

Snipes, B.T. and Tauber, O. E. 1937. Time required for food passage through the alimentary tract of the cockroach *Periplaneta americana* Linn. *Ann. Ent. Soc. Amer.* 30:277–284.

Snipes, T.T. and Gunderson, H. 1937. Two methods for measuring egestion time from large insects. *Iowa State College J. Sci.* 11:253–257.

DIGESTIVE ENZYMES — I

Objective

To demonstrate the presence or absence of different digestive enzymes in the digestive system of the desert locust.

Materials

Dissecting equipment
Watch glasses
Spot plate
3 inch test tubes
Large test tubes
Glass rods
Microburner
Starch
Sucrose
Gelatin
Ethyl butyrate
Ninhydrin reagent
Benedicts solution
Potassium iodide-iodine solution
pH paper
Locusts which haven't fed for 48 hours

Methods

Working in groups of two:

1. Obtain 6 locusts which have not been fed for 48 or more hours, and have been chilled (3°C) for 3 hours.
2. Dissect the locusts one at a time removing the digestive tract and salivary glands (be careful not to rupture the salivary reservoir).
3. Separate the digestive systems into their component parts (salivary glands, foregut, midgut, hindgut) and place the different sections in four labelled watch glasses. Add a drop or two of distilled water to each watch glass to prevent desiccation while completing the manipulations.
4. Using a small glass rod, roll out the contents of the different portions of the digestive tract. Wash the sections with a few drops of distilled water. Tilt the watch glass so the fluid collects at one side of the dish. Keep the volume of liquid small so that not more than two ml is used in washing all six of the sections in each dish. After washing, discard the tissue remaining, and retain the washings in the watch glass.
5. Obtain 20 small test tubes. Divide the respective washings from each of the four gut sections into four equal parts and place in separate test tubes. Set up four control tubes containing similar amounts of distilled water. Number the tubes 1, 2, 3, and 4 for each series and the control series. Add the compounds listed below to the tubes, agitating as necessary to put the materials in solution.

Tube		Added Substrate
1	5–10 mg soluble starch	+ 2 drops .05M Buffer (pH 6.0)
2	5–10 mg sucrose	+ 2 drops .05M Buffer (pH 6.0)
3	5–10 mg gelatin	+ 2 drops .05M Buffer (pH 6.0)
4	2 drops ethyl butyrate (record approximate pH)	

6. Place the tubes in a rack in the water bath for 2 hours at 37°C.
7. Make the following tests at the end of the incubation period:

A. Amylase
   1. Place 1 drop of the liquid from tube no. 1 in a spot plate depression.
   2. Add 1 drop of KI–I_2 solution. Mix. A purple to black color indicates the presence of unhydrolyzed starch. Little or no color indicates that most or all of the starch has been hydrolyzed, and amylase is considered present.
   3. Repeat this test for the no. 1 tube in each series including the control.

B. Invertase (Sucrase)
   1. Place 2 drops of the supernatant solution of tube no. 2 in a pyrex test tube.
   2. Add 3 drops of Benedicts reagent, and boil gently for 1 or 2 minutes. A yellow to red precipitate indicates the presence of reducing sugars and means that sucrose (non-reducing) has been hydrolyzed. If no colored precipitate forms, the sucrose is unhydrolyzed and sucrase is considered to be absent.
   3. Repeat this test for the no. 2 tube in each series including the control.

C. Protease
   1. Place 2 drops of the supernatant of tube no. 3 in a pyrex test tube.
   2. Add 3 drops of ninhydrin reagent and place in hot water for a few minutes. Unhydrolyzed gelatin will give no color. If a proteinase was present and the gelatin was hydrolyzed to amino acids, a blue color reaction will occur.
   3. Repeat test for the no. 3 tube in each series including the control.

D. Esterase
   1. Test 1 drop of the liquid in tube no. 4 with pH paper. If hydrolysis of the ester has occurred, the pH of the solution will be lower than originally, and an esterase is present.

8. In writing up your report on this work, include a chart showing the presence or absence of the various enzymes in the different parts of the digestive system.

Reagents for digestive enzymes exercise

1. 0.05M Phosphate buffer pH 6.0.
   1 part Na_2HPO_4 (11.876g/L)
   9 parts KH_2PO_4 (9.078g/L)
   Make final adjustment with pH meter.
   Dilute 1:1 with water.
2. Benedicts solution.

Copper sulfate	17.3g
Sodium citrate	173.0g
Sodium carbonate	100.0g

   Dist. H_2O to 1 Liter.
3. $KI–I_2$
   2% KI, add crystalline iodine to give yellow to red color.
4. Ninhydrin
   0.5% in H_2O.

Reference

Hawk, P.B., Oser, B. L. and Summerson, W. H. 1947. *Practical Physiological Chemistry*. Blakiston Co., Philadelphia, 1323 pp.

DIGESTIVE ENZYMES — II

Objective

To examine qualitatively some of the digestive enzymes of insects.

Materials

Insect of your choice
Test tubes
Spot plate (white)
Mortar and pestle (very small)
Evaporating dishes
Chemically clean sand
Lactose or maltose sol. (3–5%)
Saccharose (=Sucrose) sol. (3–5%)
Fehling or Benedict solution
Phenylhydrazine reagent*
Phenolphthalein solution (0.5%)
50% glycerine
Thymol or toluene
Starch sol. (1–2%)
Iodine sol. (I plus KI)
Gelatin sol. (3–5%)
Buffer sol., pH 6.8
Buffer sol., pH 8.0
Buffer sol., pH 2–4
NaOH (0.1N)
Neutral formalin containing about 20% formaldehyde

Introductory note

The exact procedural details can be varied widely to suit the species of insect chosen and the interests of the student. With smaller species the entire digestive tract must be used together to get sufficient material unless the student is willing to dissect large numbers of specimens. With larger species it is feasible to dissect several dozens of specimens, isolate the various subdivisions, combine these into groups, and then test each subdivision of the digestive tract separately.

There are numerous alternative techniques and enzymes, especially in qualitative work. The interested student may substitute from e.g. Sumner and Sommers, Moore and Sandstrom, etc. All students taking this problem should at least glance through such works to gain an idea of the diversity of available methods.

Using crude gut brei one sometimes finds a positive reaction even before the addition of substrate. This is due to digestion products already present in the lumen of the gut. Starved animals or animals fed known diets can be used to circumvent this difficulty when it is encountered. An initial test at the start of an experiment is a necessary check on this possibility.

Methods

1. Grind up the regions chosen (volumetrically equal to the stomachs of 25–50 houseflies or more) in a small mortar and pestle with a

*This is essentially phenylhydrazine in dilute acetic acid. It is made by dissolving 2.2 grams of phenylhydrazine hydrochloride and 3.3 grams of sodium acetate in 22 ml of distilled water at 60°C. Cool before using. For good results the mixture should be fresh.

little clean sand and 1–2 ml of glycerine to which a little thymol or toluene has been added as preservative. The brei (tissue ground to rupture cells into a solution) may be washed out into 10 ml of 50% glycerine and stored in the cold, portions (aliquots) being used as needed for the following:

Amylase: Mix about 0.5 ml of the glycerine-brei with 5 ml of 1–2% starch solution. At intervals test by removing a drop to a spot plate and adding a trace of iodine. Decrease in color intensity (from drops of same size) and eventual absence of the blue iodine starch test demonstrate the presence of amylase. What is formed?

Saccharase (=sucrose, =invertase): Mix 1–2 ml of the glycerine-brei with 10–15 ml of 3–5% saccharose (=sucrose) solution. Test at intervals of the hydrolysis of the disaccharide by using the Fehling or Benedict test for reducing sugars. For the Fehling test the solution should be neutral or slightly alkaline. Mix Fehling solutions A and B immediately before use. Add 1 ml of brei-substrate mixture to 8–10 ml of Fehling solution and heat by immersing the tube in boiling water for 10 minutes. A precipitate of cuprous oxide shows the presence of reducing sugars. For the Benedict test add 1 ml of the brei-substrate mixture to 5 ml of Benedict's solution and heat on a boiling water bath for 3–5 minutes; a precipitate demonstrates the presence of reducing sugars (red, yellow or green depending on amount of such sugars present). What is a "reducing sugar"? Not all insects possess saccharase. Houseflies, the salivary glands of honey bees, etc. should be good sources if your species is negative.

Trypsin (=tryptase): Mix several ml of the glycerine-brei with 10–15 ml of 5% gelatin or other protein in the presence of a phosphate buffer at pH 8.0. Immediately and at intervals later test for amino nitrogen by the Sorensen formol titration. An increase in titration value demonstrates tryptic activity. How far is gelatin hydrolyzed by trypsin?

The formol titration is variously modified depending on the type of information and the precision desired. For a simple titration remove a small sample (several ml) to an evaporating dish and carefully adjust to pH 7.0. To adjust pH add small quantities of HCl and check pH at intervals with indicator or electrometer. If using indicator test small drops on a spot plate since you do not want the indicator at present to interfere with phenolphthalein titration later. (Some workers add phenolphthalein and titrate to colorless with acid; and then add formol and titrate with alkali to pink). Add one-half volume of neutral formalin containing about 20% formaldehyde (if in doubt test and neutralize to phenolphthalein by titrating with 0.1N NaOH), add two drops of phenolphthalein solution, and titrate to

a faint pink with 0.1N NaOH. The beginner is likely to titrate too rapidly and pass the endpoint at the very beginning; until somewhat experienced add alkali very slowly drop by drop with stirring. One ml of the standard base equals 1.4 mg of amino nitrogen. For more elaborate titrations see references.

Pepsin (=peptase): Identical with the trypsin test except that run in an acid buffer (pH range 2.0–5.0). Negative results are to be expected and the student should omit this unless especially interested in the point.

2. Grind tissues in clean sand plus a drop of toluene. After thorough grinding allow to air dry. Extract with a few ml of phosphate buffer at pH 6.8. To test for lactase mix several ml of the extract with 10–15 ml of a lactose solution and at intervals apply the osazone test. For this, mix equal amounts of the extract-substrate mixture and fresh phenylhydrazine reagent in a test tube, shake, loosely stopper and heat in boiling water for 10–30 minutes. Hexoses and pentoses give osazones which form a yellowish precipitate.

   To be sure of your results, remove and test a sample immediately after mixing the extract and substrate; this should be negative for hexosazones; subsequent positive tests may then be assumed to be valid. The stomach contents of some species of insects will be found positive prior to the addition of any substrate. What does such signify? (Lactose itself also forms an osazone under these conditions but the lactosazone precipitates only on cooling).

3. After the above preliminary tests, select the enzyme with which you would prefer to work and try to learn something about its properties. Perhaps the simplest instructive thing would be to determine the pH range and optimum. Proceed as under the appropriate paragraphs above but prepare and use buffers to hold the pH at units not more than 0.5 apart.

4. Alternative to paragraph no. 3, students may test temperature effects (time needed to produce detectable end product by your method at various temperatures), or enzyme poisons, or heat inactivation or such other routine simple enzyme studies as they wish.

Report

Present your data with particular emphasis on discussion of work done under paragraphs 3 or 4. In what ways are qualitative studies on enzymes in insects useful?

Reference

House, H.L. 1964. Digestion. In *Physiology of Insecta.* Edited by M. Rockstein, pp. 815–858. Academic Press, New York.

MEASUREMENT OF NUTRITIONAL INDICES

Introduction

Nutritional requirements of a number of insects are well known. Carbohydrates are the main source of energy, although proteins and fats may also be oxidized for this purpose. To determine the overall efficiency with which insects utilize their food, food intake and growth must be measured. This involves calculation of one or more coefficients.

Objective

To measure nutritional parameters in relation to feeding of larvae on different diets (in this case azadirachtin-treated). Instead, experiments can be conducted to compare 2 or more diets.

Materials

1. Fifth instar larvae of *Chilo partellus* and/or third with fourth instar nymph of *Schistocerca gregaria.*
2. Diet for *Chilo*/diet for locust (both normal and azadirachtin-treated).
3. Balance.
4. Vials and jars for holding experimental insects.

Methods

1. The objective is to measure several nutritional parameters in order to see whether azadirachtin treatment somehow affects the feeding and nutrition.
2. First treat the diet with various doses of azadirachtin (try 0.01 ppm to 10 ppm doses).
3. Put individual larvae/nymph in vials/jars with a known weight of diet.
4. On the 3rd or 4th day following introduction to the diet, the insect, the feces and the remaining uneaten diet should be separated and the remaining diet dried.
5. Weigh the individual larvae/nymph, the feces produced and the dried diet.
6. Calculate the following parameters:

   (a) Consumption (amount ingested)

   $$= \text{\% Initial dry weight of diet} \times (\text{Initial fresh wt of diet} - \text{final dry weight of diet})$$

   (b) Approximate digestibility (AD)

   $$= \frac{\text{amount ingested (mg)} - \text{feces (mg)}}{\text{amount ingested (mg)}} \times 100$$

(c) Efficiency of conversion of digested food (ECD)

$$= \frac{\text{weight gain}}{\text{Amount ingested (mg)} - \text{feces (mg)}} \times 100$$

(d) Efficiency of conversion of ingested food (ECI)

$$= \frac{\text{weight gain}}{\text{Amount ingested (mg)}} \times 100$$

7. Tabulate the data and compare control vs. azadirachtin fed diet particularly the AD, ECD, and ECI.
8. Comment: These experiments can be done to compare diets for insects as well and can be carried through to see pupal development and adult emergence.

Questions

1. Did the incorporation of azadirachtin reduce the food ingestion? At what concentration level? Can you compare *control* of *Chilo* and that of *Schistocerca*? (The ECD and ECI of lepidopterous larvae are about double those of orthopterous larvae, AD being about the same.)
2. Were the insects able to digest azadirachtin treated diet as well as the control diet? Was it statistically significant?
3. Coefficients of food utilization also vary with age (both within and between instars), sex, temperature, humidity and degree of crowding. Can you comment on any of these from your results?

Reference

Reese, J.C. and Beck, S. D. 1976. Effect of allelochemics on p-benzoquinone, hydroquinone and duroquinone on larval growth development and utilization of food. *Ann. Entomol. Soc. Amer.* 69: 59–67.

DISTRIBUTION OF OXIDATIVE ENZYMES

Introduction

All animal tissues contain a series of enzymes which catalyze the oxidation of a variety of biochemical intermediates. The oxidative enzymes fall into several classes, one of which is the dehydrogenases. These enzymes are sometimes called oxidases because they oxidize by the removal of hydrogen ions (protons). The removed hydrogen must then be transferred to an appropriate acceptor such as cytochrome or one of the coenzymes (DPN or TPN). These dehydrogenases are very important in the metabolism of fats, amino acids and sugars and are involved in the Krebs (citric acid) cycle and glycolysis. These two cycles are extremely important in the biochemistry of cells since glycolysis involves the degradation of sugars and the Krebs cycle is an important energy pathway. These two cycles overlap as do many other metabolic pathways. We are going to determine the presence of four dehydrogenases in different insect tissues utilizing hydrogen acceptors that can be visually studied. The enzymes to be studied are lactic dehydrogenase, α-glycerophosphate dehydrogenase, succinic dehydrogenase and glutamic dehydrogenase. The reactions of these enzymes are described below. Bear in mind that these reactions are *reversible.*

Lactic dehydrogenase—This enzyme converts lactic acid to pyruvic acid in the presence of the hydrogen acceptor DPN (Co I).

$$CH_3CHOH\text{–}COOH + DPN \rightleftharpoons CH_3\text{–}CO\text{–}COOH + DPN.2H$$

The left hand reaction is favored, but by adding a trapping reagent, such as cyanide (reacts with pyruvate), the reaction can be pushed to the right.

α-Glycerophosphate dehydrogenase — There are two of these dehydrogenases in animal tissues, only one of which is soluble. The insoluble enzymes react with α-glycerophosphoric acid to produce 3-phosphoglyceraldehyde, an important product in fat metabolism. It does not require a coenzyme but utilizes one of the cytochromes.

$$CH_2OPO_3 - CHOH - CH_2OH + Cy \rightleftharpoons CH_2OPO_3\text{–}CHOH\text{–}CHO + Cy + 2H$$

The soluble enzyme acts on the same substrate to produce phosphodihydroxyacetone. This reaction requires DPN for the acceptance of hydrogen.

$$CH_2OPO_3 - CHOH - CH_2OH + DPN \rightleftharpoons CH_2OPO_3 - CO - CH_2OH + DPN.2H$$

Succinic dehydrogenase — This enzyme catalyzes the oxidation of succinic to fumaric acid.

This enzyme does not require a coenzyme hydrogen acceptor and is inhibited by other dibasic acids which are structurally related to succinic.

$$COOH-CH_2CH_2-COOH \rightleftharpoons COOH-CH=CH-COOH + 2H$$

Glutamic dehydrogenase — This enzyme catalyzes the conversion of glutamic acid to α-ketoglutaric acid. Initially, the enzyme reaction removes two hydrogen ions to form the imino acid (=NH) which is hydrolyzed by water to form the keto acid and ammonia. This enzyme utilizes either DPN or TPN as hydrogen acceptors.

$$COOH-CH_2-CH_2-CHNH_2-COOH + DPN \rightleftharpoons$$

$$COOH-CH_2\ CH_2-C=NH\ COOH + DPN.2H \xrightleftharpoons{H_2O}$$

$$COOH-CH_2\ -CH_2\ -CO-COOH + NH_3$$

Tetrazolium hydrogen acceptors — Tetrazolium compounds will accept hydrogen from many compounds. These tetrazolium compounds are colorless and water soluble when they are oxidized and red and water-insoluble when they are reduced. Thus by incubating enzyme-containing tissues in solutions containing tetrazolium salts and the proper substrates, the reduction of various compounds can be followed by the deposition of red crystals of reduced tetrazolium in the tissues. We will utilize two of these tetrazolium compounds, neotetrazolium chloride and triphenyl tetrazolium chloride.

Experimental

1. Utilizing an appropriate insect (locust, tsetse) dissect out the following tissues from 4 adult specimens:
   1. Brain
   2. Central nerve cord
   3. Flight muscle
   4. Leg muscle
   5. Fat body
   6. Testes or ovary
   7. Midgut

   Dissect as quickly as possible and transfer the parts to labelled test tubes (keep cold) containing buffer (pH 6.9–7.0). Freeze until the next period. This procedure removes most of the endogenous substrate and makes it possible to determine the presence of specific enzymes. Endogenous substrate refers to substrate

normally found in the tissues; freezing for some reason makes this substrate unavailable for enzyme reactions.

2. Remove the same tissues from one or two insects and place a small piece of tissue in a spot plate depression and add to it a few drops of a substrate "cocktail". These cocktails contain 4 parts buffer, 2 parts of tetrazolium, 2 parts of distilled water or an inhibitor if one is used and 2 parts of substrate. Under a dissecting microscope, determine the rate of reddening of the different tissues in the presence of the 4 substrates. Which tissue darkens most rapidly? Are all 4 enzymes present?
3. Repeat the above procedure but instead of adding a "cocktail", add only a few drops of buffer and a few drops of tetrazolium compound. Any reddening of the tissues will reflect the presence of endogenous substrate only. Do all the tissues darken equally? At the same rate? How do these results compare to part 2?
4. At the start of the next period, remove the frozen tissues and after they have thawed, set up a series of the various tissues in a few drops of buffer plus tetrazolium. These are your controls. Set up a second series in "cocktails" and compare the rates of reddening for the tissues. How do they compare to the controls? Which tissues darken most rapidly? With what substrates? Are all the tissues uniformly red or do they appear to have red areas dispersed throughout?

   Prepare some tissues employing succinic acid in a cocktail containing malonic acid or iodoacetic acid. Do the tissues redden as rapidly as they did without these inhibitors? Why are these compounds inhibitors? Repeat with a lactic acid cocktail and add KCN (8 drops of cocktail to 2 drops of KCN). How do the results compare to the control without KCN?

   Employing the α-glycerophosphoric acid cocktail, set up some tissues in a spot plate and add a small drop of DPN solution to one tissue preparation and a drop of buffer to another (control). Do the tissues darken equally fast? If not, why not? Repeat with glutamic acid.

   Repeat this procedure employing cytochrome c instead of DPN. How do the reaction rates compare?

   Summarize your results regarding the presence or absence of the various enzymes, their relative concentrations in the tissues, the effect of inhibitors or hydrogen acceptors other than tetrazoliums. Could this method be used for quantitative work?

Reference

Evans, W.A.L. 1957. Dehydrogenase activity in the gut and associated tissues of the larvae of the blowfly *Calliphora erythrocephala* Meig. *J. Insect Physiol.* 1:150.

INSECT FAT BODY AND TRANSAMINATION

Objective

To demonstrate one role of the fat body in the intermediary metabolism of an insect by means of transamination reaction.

Materials

Locusts or tsetse
Pipettes
Test tubes
Homogenizer
L. Alanine 0.05M
α-keto glutarate 0.05M
Pyridoxal phosphate 7×10^{-7}M
Glutamic acid 0.05M
Pyruvate 0.05M
Thin layer chromatographic equipment
Phosphate buffer pH 7.3 (0.05M)

Methods

1. Isolate fat body from the insect and transfer in cold buffer and wash well.
2. Homogenize the fat body in small quantity of buffer (0.3 ml). Make up the volume to 1 ml and keep it cold on ice.
3. Set up the incubation tube as shown below.
4. In the second assay use glutamic acid instead of L. alanine and pyruvic acid instead of α-keto glutarate.
5. Incubate the experimental and control tubes of both assays at 37°C for 2 hours.
6. Stop the reaction by immersing the tubes in a hot water bath for 2 minutes after the incubation period.
7. Spot 5 to 10 µl of each of the eight samples on a single 20 x 20 cm TLC plate along a straight line 2 cm above the edge. Leave a space of 2 cm between spots.
8. Break the silica gel coating 10 cm above the spots (draw a sharp pencil line). Note the detail of the spots above this line.

Tube:	1	2	3	4
Reagents (Reactants)	Expt (ml)	Cont. (ml)	Cont. (ml)	Cont. (ml)
Phosphate buffer pH 7.3	0.20	0.20	0.20	0.20
Enzyme (fat body)	0.20	–	0.20	0.20
Pyridoxal phosphate	0.10	0.10	0.10	0.10
L. Alanine	0.30	0.30	–	0.30
α-keto glutarate	0.20	0.20	0.20	–
Distilled water	–	0.20	0.30	0.20
Total	1	1	1	1

9. Develop the plate in 75:25 phenol water-ninhydrin system (2 hours).
10. Dry the plate, heat at 85°C to develop the spots (few minutes).
11. Identify the amino acids and interpret the results of this reaction.
12. Discuss the significance of this and the importance of such transamination of amino acids.

References

Consult references for intermediary metabolism lectures.

FLIGHT MUSCLES AND WING BEAT FREQUENCY

Introduction

Insect flight muscles are specialized muscles to provide force during flight. Two types of neural control of flight muscles are known. The fast muscles with their contractions synchronized with the pattern of neural activation (low wing beat frequencies) and the slow muscles whose movement are not phase-related to motor impulses (high wing beat frequencies).

Objective

1. To compare the rate of wing beat in an insect having synchronous flight muscles with that of one having asynchronous flight muscles.
2. To observe the plane of wing vibration in still air and moving air.
3. To observe the effect of tarsal contact on flight.

Materials

1. Stroboscope — variable speed
2. Electric fan
3. Contact cement (hi-bond type)
4. Wooden applicator sticks
5. Cotton wool
6. Insects: synchronous—moths, locusts; asynchronous—blowflies, honey bee

Methods and observation

1. Calibrate the stroboscope so that the speed of an object—reciprocating (fan) or vibrating (insect wing) — may be determined.
2. Fix the applicator stick on the thorax of the insect. Make sure the wings vibrate freely by holding the insect by the applicator and blowing gently on the insect (this should cause it to fly).
3. Place the flying insect about a foot away from the stroboscope in a dark room. Make the insect fly (by blowing on it). Make sure that the insect's feet are free and not in contact with anything.
4. Adjust the speed of the stroboscope so that a single image of the wing is visible.
5. Using the calibration procedure, determine the wing beat frequency.
6. Examine whether blowing on the insect changes the frequency or not.
7. Determine the wing beat frequency for both synchronous and the asynchronous fliers.
8. Determine whether they differ or not.
9. Keeping the air as still as possible, trace the path taken by the wing tips. You may have to slightly adjust the stroboscope speed.

10. Determine what pattern is made by the wing tips.
11. Is the plane of the wing exactly parallel with the longitudinal axis of the insect during all phases of wing tip travel?
12. Now turn on the electric fan (a gentle breeze should blow on the head of the insect in a horizontal orientation).
13. Do the wing tips travel in the same plane?
14. How would the plane of travel differ if the insect were in free flight in the following modes?
    (a) flying forward
    (b) turning
    (c) hovering
    (d) flying in reverse
15. Take a small ball of cotton and place it under the feet of a flying insect (touching the feet).
16. Observe the changes in flying.
17. What are the factors involved in initiation and maintenance of flying?
18. Tabulate all your data on wing beat frequency and draw diagrams of the plane of flight. Write conclusions.

Reference

Pringle, J.W.S. 1965. Locomotion: Flight. In *The Physiology of Insecta.* Edited by M. Rockstein. 2:283–329. Academic Press, New York.

EFFECT OF CARBON DIOXIDE ON THE INSECT SPIRACLES

Introduction

The large tracheal trunks open to the outside through the spiracles. Activity of the spiracles has a direct relationship to respiratory mechanisms and water conservation in insects. In locusts, spiracles are provided with mechanisms which allows them to open and close. The mechanisms involve a muscle-nerve action on the spiracular valves.

Objective

To observe the effect of CO_2 on the opening and closing of the spiracles of the desert locust, *Schistocerca gregaria.*

Materials

1. Petri dish
2. Plasticene or Blu-tack
3. Dissecting microscope
4. Source of CO_2 (a cylinder with regulator)
5. A light source
6. Locusts (try fledgling and mature insects)

Methods

1. Position the locust on the Petri dish using the plasticene/Blu-tack to see the spiracles.
2. Arrange the light source in such a way that the insect is not overheated.
3. Note the spiracle under the microscope and pass a gentle stream of CO_2 onto it.
4. Observe the effect of CO_2. What happens?
5. Remove the CO_2 source from the spiracle. What happens now? Does the spiracle stay the same? If so, for how long?
6. This time maintain the CO_2 stream on the insect for a longer period. What happens?
7. Are the opening and closing of spiracles synchronized in two spiracles?
8. Use CO_2 only on one spiracle and not on the other. Are the results the same?

Questions

1. Is the effect of CO_2 on spiracle a local phenomenon or does it involve the central nervous system? How would you prove it?
2. How is spiracular activity related to water loss in the insect?
3. How would you investigate whether there is any respiratory flow in the tracheal system?

4. How would a pupa retain water and get rid of excess CO_2 within the body?

Reference

Hoyle, G. 1960. Control of spiracle muscles in the *Schistocerca*. *J. Insect Physiol.* 4:63–79.

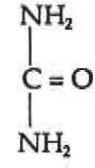
EXCRETION

Introduction

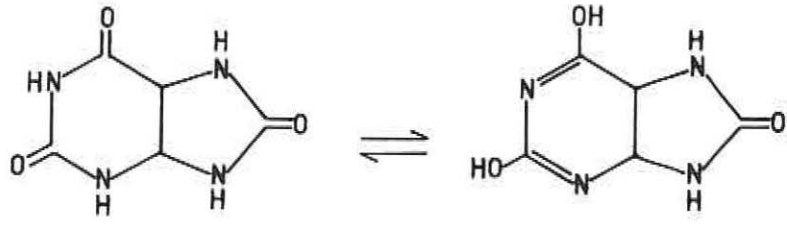
In order to survive, animals must dispose of dangerous or potentially dangerous products of metabolism before they become toxic. The two substances which are most dangerous are carbon dioxide and nitrogen. Insects dispose of the former quite easily through the respiratory system. The nitrogenous by-products of the metabolism of proteins, however, require more complex adaptations.

Nitrogenous wastes are dangerous primarily because they may give rise to the strong base, ammonia ($NH_3 + H_2O \rightleftharpoons NH_4OH \rightleftharpoons NH_4^+ OH^-$). Three principal methods are used to avoid this danger. If the animal has a large excess of water available, and can easily pump this water out, it may excrete ammonia. Most insects, however, have only a limited supply of water, and must therefore conserve it. Exceptions to this generalization are some dipterous larvae.

A second excretory product is urea. This compound has the advantage of being neutral, but the disadvantage of requiring a large amount of water for its successful excretion. For this reason, it is excreted by relatively few insects.



The third nitrogenous waste is uric acid,



Keto Dienoi

This compound has two advantages, first, it is relatively neutral and does not disturb the acid-base balance of the tissues, and, second, it is only slightly soluble in water. This latter property ensures that it does not have any great osmotic effect. For these reasons it is widely used as the main excretory product of egg-laying terrestrial animals, insects, birds, and reptiles.

Apparatus and reagents

We shall test for the presence of these excretory products by several standard qualitative methods, first on pure solutions, and then on the "urine" and feces of several species of insects (desert locust and tsetse)

A. Pure solutions

1. Uric acid

   a. *Murexide test*

   Place a little uric acid on a spot plate. Add two or three drops of strong nitric acid + 2 drops H_2O. Heat on the hot plate (lowest setting) until all nitric acid and water have evaporated. Treat the remaining deposit with dilute ammonia solution. Note the color. Now treat with a few drops of 0.1 N-NaOH, again note the color.

   b. *Benedict's uric acid test*

   To a *very* small amount of uric acid in a test tube, add some saturated Li_2CO_3, stir until completely dissolved. Heat to dissolve if necessary. Add a knife point of anhydrous Na_2CO_3 and 5 drops of Benedict's uric acid reagent. Observe the resulting color.

2. Urea:

   a. *Xanthydrol*

   Xanthydrol condenses with urea to form the insoluble compound, dixanthylhydryl urea. To 2 drops of a 1% solution of urea, add 7 drops of glacial acetic acid, then 2 drops of a 10% solution of xanthydrol in methanol. Repeat with 0.1% and 0.01% urea. Save these tubes.

3. Ammonia

   Nessler's Test — To 8 ml of water add a few drops of ammonium hydroxide. Add 1 ml of Nessler's reagent and 1 ml of water, swirl, and observe the color.

B. Tests of feces

Perform the above tests with the dried feces provided as follows:

1. Uric acid

   a. *Murexide test*

   Treat feces with concentrated nitric acid and proceed as with pure sample.

   b. *Benedict's reagent*

   Boil excreta with 2% Li_2CO_3. Cool, add uric acid reagent and Na_2CO_3. Mix.

2. Urea
   Boil sample in water. To a little of the solution add 2 drops glacial acetic acid and 10 drops 10% xanthydrol reagent.

3. Ammonia
   Boil excreta with water and add Nessler's reagent.

Report

Write a detailed report on the findings regarding excretory products of the desert locust and tsetse.

References

See references for the physiology of excretion.

WATER BALANCE PROPERTIES OF TSETSE PUPAE

Introduction

Water regulation in insects is always related to the prevailing relative humidity and temperature of the air. This is particularly evident in the flying locust. It has been clearly illustrated in *Schistocerca gregaria*, the combinations of temperature and relative humidity at which there is a balance in flying locust between water production and water loss. Pupae, although stationary, lose or gain water depending on the ambient conditions they are in.

Objective

To study water balance properly in two tsetse pupae.

Materials

1. Tsetse pupae (*Glossina* sp) of various age groups.
2. Electrobalance.
3. Thermometer.
4. Petri dishes.
5. Thermohygrograph.
6. Desiccators
7. Anhydrous copper sulphate for zero humidity.
8. Glycerol-water mixture for high humidity.
9. Steel wire mesh grids.

Methods

1. Weigh individual pupae on the electrobalance. Note weight and age of the pupae (initial weight).
2. Put one batch of pupae under room conditions (25° ± 1°C 55–65% rh) (Group A).
3. Put a second batch of pupae in a desiccator maintained at high humidity condition (Group B).
4. Put a third batch of pupae in a low humidity desiccator (Group C).
5. After 24-hour period weigh each pupa again and record the weight (second weight).
6. After weighing, divide the Group C pupae in 2 sub groups representing all ages in each sub group (Group C1 and Group C2).
7. Put Group C1 under room conditions and Group C2 in the high humidity desiccator.
8. Similarly, weigh and divide Group A and Group B pupae into sub groups and place Group A1 in high humidity and Group A2 in low humidity, Group B1 in room temperature and Group B2 in low humidity.

9. After 24 hours exposure to the new humidity regime, weigh each pupa again (third weight).
10. Calculate percentage change in weight (gain or loss) for each pupa for each 24 hour period (difference between the weight at time *t* and previous weight divided by previous weight x 100).

Questions

1. How would you determine the optimum wet weight of a pupa?
2. Describe from your findings the property of water balance as it relates to the age of the pupa.
3. What in your opinion is the effect of inundation or desiccation of pupae on the emergence of the adult?

References

Bursell, E. 1958. Water balance of tsetse pupae. *Philosophical Transactions.* 241B: 179–210.

Wharton, G.W. 1985. Water balance of insects. In *Comprehensive Insect Physiology Biochemistry and Pharmacology.* Vol. 14. (Edited by G.A. Kerkut and L.I. Gilbert), pp. 565–601, Pergamon, Oxford.

ACTIVE TRANSPORT IN INSECT MALPIGHIAN TUBULES

Introduction

Active transport is a means of transporting substances across a membrane against a concentration gradient. In insects, both the gut and Malpighian tubules have active transport mechanisms to excrete unwanted substances. In plant feeding insects the gut helps to get rid of excess K^+ from the hemolymph by actively transporting it back into the gut via specialized goblet cells. The Malpighian tubules are the main excretory organs of insects. They produce "urine" by both passive secretion and active transport which contains all small molecules in the hemolymph and is iso-osmotic to the hemolymph. This urine is then made hyperosmotic through resorption in the tubules and rectum. K^+ is actively pumped into the tubules. In most insects both acidic and basic dyes are preferentially excreted.

Objective

The objective of this exercise is to observe active transport by Malpighian tubules using some acidic and basic dyes.

Materials

Desert locusts
Dissecting dishes
Dyes: Acidic (chlorophenol red, amaranth), basic (methylene blue, Coomassie blue)
Dissecting kit
Syringes, needles (30 gauge)
Saline (for locust)

Methods

1. Anesthetize the insect.
2. Inject 0.1 ml of mixed dye solution containing 2×10^{-2}M amaranth and saturated solution of methylene blue in saline into the anesthetized insect at the coxal joint of the hind leg.
3. Use a second insect to tie a ligature across the abdomen to separate midgut from the Malpighian tubules and hind gut. Then inject dye anterior to the ligature.
4. After 2 hours anesthetize and dissect. Check midgut, Malpighian tubules and hindgut in both insects.
5. Which dye is excreted by the midgut and which one by the tubules?
6. Are all the regions of the Malpighian tubules excreting the dye?
7. Now remove the tubules and transfer them to a small Petri dish with saline. Break the tubules open to determine when the dye was taken up.
8. Do the same for the midgut.
9. From this experiment, what can you conclude about the selectivity of the excretory mechanisms in the midgut and the Malpighian tubules?

10. Dissect another insect and isolate the tubules off the gut and place in a Petri dish in saline and rinse well.
11. Put on each of the following dyes (6×10^{-4}M) in a small Petri dish:
    - Chlorophenol red
    - Amaranth
    - Methylene blue
    - Coomassie blue
12. Every 15 minutes put in fresh saline and examine under dissecting microscope. Note dye uptake: proximal, medial or distal tubule. Examine one tubule by opening up.
13. Check again after another 15 minutes. Any change in concentration of dyes?
14. What does this experiment tell you about active transport?

References

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Maddrell, S.H.P. 1971. The mechanisms of insect excretory systems. *Adv. Insect Physiol.* 8:199–331.

Phillips, J.E. 1981. Comparative physiology of insect renal function. *Am. J. Physiol.* 241: R 241–257.

IN VITRO CULTURING OF MALPIGHIAN TUBULES

Objectives

To examine factors affecting the rate of urine production in Malpighian tubules cultured *in vitro*.

Materials

Newly emerged adult flies
Dissecting Medium (100 ml distilled water containing 980 mg NaCl, 20 mg $NaHCO_3$, 100 mg KCl, 50 mg $CaCl_2$, 12 mg NaH_2PO_4)
Functional Medium (100 ml distilled water containing 880 mg NaCl, 100 mg $NaHCO_3$, 200 mg KCl, 200 mg $MgCl_2.6H_2O$, 25 mg $CaCl_2$, 60 mg NaH_2PO_4)
Functional Medium + 40 mM galactose
Dissecting dishes
Dissecting dishes containing 4 shallow wells about 1/4 inch in diameter
Mineral oil
Dissecting scopes

Methods

1. Pin a fly through the thorax, ventral surface up, in a dissecting dish containing "Dissecting Medium". Open the abdomen and dissect out the four Malpighian tubules (yellowish in appearance). Keep a small portion of gut attached to each tubule. Be very careful not to tear the tubules!
2. Fill two wells in the dissecting dish with "Functional Medium" and two wells with "Functional Medium + galactose"
3. Clasp the portion of gut attached to the proximal end of the tubule and transfer one tubule to each well.
4. Carefully pour a thin layer of mineral oil, 1–2 mm deep, over the entire surface of the dish, but if possible, try to keep the top of the "medium bubble" exposed to the air.
5. Again clasping the end of the tubule attached to the gut, carefully pull the tip of the tubule out of the medium so that it is entirely surrounded by paraffin and mineral oil. You may need to anchor it down by inserting a fine insect pin through the gut attachment. The bulk of the bubble should remain in the medium.
6. The tubules should secrete for many hours. Urine drops should form at the base of the tubule. After 3–4 hours, assess the quantity of urine produced.

References

Berridge, M.J. 1966. Metabolic pathways of isolated Malpighian tubules of the blowfly functioning in an artificial medium. *J. Insect Physiol.* 12:1523–1538.

Gee, J.D. 1976. Active transport of sodium by the Malpighian tubules of the tsetse fly *Glossina morsitans*. *J. Exp. Biol.* 64:357–368.

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SUPERCOOLING IN INSECTS

Objective

To demonstrate supercooling in insects and to demonstrate the importance of environmental factor (in this case substrate moisture) in the overwintering survival of insects.

Materials

Pupae of armyworm or *Chilo*
Sand (dried for more than 48 hrs at 120°C)
Distilled water
Paper containers with lids (such as 1/2 litre food containers)
Temperature cabinets

Methods

1. Place 120 g of sand in each of 4 containers. Add distilled water to the sand in the following amount:

   Container 1 — No water
   Container 2 — 11.5 ml
   Container 3 — 23.0 ml
   Container 4 — 26.5 ml

2. Set up a second set of containers as above.
3. Place 5 *live* pupae of the same age on top of the sand in each container; cover the container. (A pupa is considered to be alive if it responds to slight touch by moving its abdomen).
4. Place 1 set of containers at 0°C and a second set at –10°C.
5. Set up enough replicates of the containers so that some containers can be exposed only for 4 hours the others for 12 hours and the remaining for 24 hours in both the temperatures.
6. Allow the containers to be in the room temperature for 24 hours.
7. Examine the pupae to determine the percentage mortality of the pupae in each container.
8. Pool the mortality results for each sand condition, time and temperature.
9. Allow pupae to complete development at room temperature, then determine the number of adults emerging and use these data to calculate the percentage of mortality.

Questions

1. What is supercooling in terms of biological material? Did you see this phenomenon in this experiment? Explain.
2. Did the amount of moisture in the sand have any relationship on the ability of the pupae to survive cold temperatures? What is its importance?

3. Of what importance is cold-hardiness and overwintering ability in the population dynamics of insect pests? How could these characteristics of an insect pest be used in their control?

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TASTE RESPONSES IN ADULT DIPTERA

Introduction

The taste receptors of most insects, including Diptera, are located in the pulvilli of the post-tarsal segment. Dethier (1953) has experimentally shown that in Phormia and did the histology of chemoreceptors. Dethier's simple experiment in the laboratory can be conducted on suitable dipterous species without any equipment.

Objective

To localize and study the nature of taste responses in Diptera.

Materials

1. Adult flies, suitable flies and blow fly (*Phormia, Calliphora)*, flesh fly (*Sarcophaga*), housefly (*Musca*) and fruit fly (*Drosophila*).
2. Wooden applicator stick on thin glass rod.
3. Wax or any hi-bond glue.
4. Glucose, sucrose and/or fructose in solutions of 0.005, 0.01, 0.05, 0.1, 0.5, and 1.0M.
5. Other chemicals such as solutions of salt, acids and alcohols.
6. Distilled water.
7. Watch glasses to hold the test solutions.
8. Applicator stick with cotton swabs.
9. Anesthetic — CO_2 or ice.

Methods

1. Anesthetize flies. Give ample time between anesthetization and experimentation.
2. Prepare wooden applicator sticks (or glass rods) by placing a small amount of wax on the tip of each stick. Heat the wax to tackiness and touch it to the mesonotum of one of the flies (when wax cools, the fly should be affixed to the rod).
3. Place the rod with the fly in a holder (a styrofoam block will serve the purpose) until the time of experiment.
4. Starve the flies for 24 hours before the experiment, but provide them with enough distilled water to compensate for water loss.
5. Test the flies with distilled water first.
6. A *positive response* to stimuli applied to the tarsi is defined as a lowering of the proboscis toward the source of the stimulus, here the watch glass or cotton swab.
7. A *negative response* is manifested by retracting the tarsi from the stimulus in order to avoid contact.
8. Holding a fly by its attached rod, dip the tarsi into a 0.005M sugar solution in a watch glass. Do you see a positive response? Repeat this process with 0.01, 0.05, 0.1, 0.5 and 1.0M sugar solutions and

record the first positive response. Wash the tarsi with distilled water between trials.

9. Repeat the experiment with several flies and compare the data to determine the lowest concentration that will elicit positive response, that is, the *threshold concentration* for this sugar.
10. Alternatively, a cotton swab dipped in a test solution, can be rubbed on the tarsi.
11. Are the threshold concentrations the same for all sugar tested?
12. Make a few more concentrations to obtain an accurate estimate of the threshold concentration.
13. Repeat the experiment using solutions of salts, acids and alcohols. What response did you note for these materials?
14. Find the threshold values of these materials.
15. Mix an above-threshold concentration of a sugar and add to a solution of another chemical that previously failed to elicit a positive response. Introduce this mixture to the tarsi of a fly. Is the response positive? Negative?
16. Repeat No. 15 with another chemical.

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EMERGENCE BEHAVIOR OF DESERT LOCUST *SCHISTOCERCA GREGARIA*

Introduction

Insect ecdysis and emergence have been observed and described many times. When an insect is approaching the time of molting, it shows various outward signs. Some of these behaviors have been studied either partially or in great detail in several insect species.

Objective

The purpose of this exercise is to describe and classify the behavior of the desert locust during its imaginal ecdysis.

Materials

1. Insects, *Schistocera gregaria*, fifth instar nymphs, both males and females.
2. One pair of keen eyes.
3. A note book.
4. A camera if available.

Methods

1. Collect insects from the culture. The fifth instar nymphs can be synchronized to ecdyse if all perches are removed from the culture cages during the last few days of that instar.
2. Try to keep similar temperature for observation period. Keep a record.
3. Those ready to ecdyse will be recognized by their pink colour, separation of the right and left metathoracic wingbuds at the dorsal midline, softness when handled, inability to jump, general restlessness.
4. Collect from the culture cage insects showing above symptoms and behaviour as your experimental insects. Prepare them individually in separate emergence cages or containers by providing them perches.
5. Observe their behavior and record.

Questions

1. What was the time period between the cessation of feeding (before ecdysis starts) and resumption of feeding (after ecdysis)?
2. Was there any change in their locomotory behaviour? Explain.
3. Divide the entire sequence of events into 4 or 5 stages. Record time for each stage and each behavioral event during each of the stages.
4. How long did it take for the old cuticle to split and expose head, thorax and the first pair of legs?

5. How long did it take for the wings to expand?
6. How long did it take for the new cuticle to darken?
7. Write a detailed description of all the behavior shown during all the stages.
8. Discuss ventilation and molting.

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COURTSHIP BEHAVIOR OF *DROSOPHILA*

Introduction

The courtship behaviour of most western hemisphere species of *Drosophila* has been studied extensively. However, no accounts are available in the literature of a similar study on any of the African species. It will be interesting to see whether the behavior is the same in an African species.

Typically, a male approaches the female and taps her on the body with his foreleg. If the female moves away the male may or may not pursue her. If she remains in the place, he stands facing her and extends the wing nearest to the female, vibrates it for a few seconds and then closes it again. This phase is often repeated while the male moves around the stationary female. If the female changes her position during vibration the male may vibrate the other wing if it becomes the one nearer her. Eventually, the male will position himself behind the female, extend his proboscis and lick the female genitalia. Finally he will attempt to mount the female, a procedure in which she must cooperate by spreading her wings. These steps may be repeated but the sequence always is substantially the same.

The elements of courtship differ from species to species and the sequence of events appears to be characteristic of the species which obviously serves as an isolating mechanism among closely related sympatric species.

Materials

1. Cultures of *Drosophila*. It is easy to start a *Drosophila* culture using a jar with over ripe banana inside and kept outdoors for some time to collect eggs. It is essential to have aged males and virgin females for this experiment. For this reason fruit jars for egg collection may have to be put out for a few days consecutively before the starting of the courtship experiment.
2. Clear plastic boxes, about a quarter of an inch deep and large enough to fill the field of a dissecting microscope at the lowest power serve best as observation chambers. Small plastic Petri dishes of the above description and size will also serve the purpose.
3. Dissecting microscope.
4. Culture jars.
5. Food for *Drosophila*.

Method

1. Prepare a record sheet for each set of flies to be observed.
2. Put on the record sheet name of each event of courtship to be observed. These events include:

i. Orientation (male circling the female).
ii. Tapping (male touching female with the foreleg).
iii. Vibrating (male vibrating its wings).
iv. Licking (male licking the female genitalia).
v. Mounting (male attempting copulation).

3. Observation and recording is best done by two persons working as a team, one using the microscope, the other timing and recording.
4. Prepare the observation chamber by placing a small bit of *Drosophila* food in the bottom of the chamber. Introduce two pairs of unanesthetized flies.
5. Place the chamber under the microscope for observation.
6. Particular attention should be paid to the timing and the sequence of events for each species observed.

Questions

1. What are the variations you observed between 2 or more species used?
2. How does *Drosophila* courtship behavior differ from that of housefly and that of tsetse?
3. How long does a successful mating last in *Drosophila*?

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A FIELD PROJECT ON INSECT BEHAVIOR

Introduction

It is often said that the insect behavior is best studied in the field. Once some knowledge of the particular behavior is gained from the field the scientist can come back to the laboratory and devise laboratory experiments to qualify and quantify some of the factors responsible for regulating the behavior in the field.

Selecting a project

Choosing the right subject is very important. Insects are everywhere, but it is difficult sometimes to know which one of them will be best suited to observe for a behavior or a set of behaviors.

First, you have to select an insect which will interest you. Second, the insect should be available in time and space around you to study. And third, it must stimulate your curiosity, what you see in it.

What to observe?

Once you have selected an insect for studying its behavior, you have to develop a set of questions that you can answer within the time available. Also, when you have developed a set of questions, you must decide what to study first and what next. That means you must set a priority in collecting your data. Sometimes it is possible to gather data on 2 or even 3 questions more or less simultaneously. However, it is wise not to attempt to do so. Because, many times this leads to a shallow and inconclusive report in which a little bit is said about a wide variety of issues.

Numerical data

Whenever possible, put your questions in such a way that they can be answered quantitatively. Numbers enable another person to assess the reliability of the observer's conclusions. And if it is appropriate they can be analyzed statistically. It is more satisfactory if you can present a graph based on actual counts of mating pairs at regular intervals through the day that shows a sharp peak between 3 and 6 p.m. than to say without supporting evidence, that your insects tended to mate in the late afternoon. For details see Lehner (1979) for method of data collection, statistical analysis and presentation. Also, Colgan (1978) discusses quantitative techniques for the advanced student.

All you need

There are a lot of sophisticated tools for field research but you are unlikely to need all that to carry out a project successfully. All you need is a notebook, a pencil, a wristwatch and a good supply of patience. If you have a camera, you may wish to use it for photographing the behavior of your insect.

Actual observation, description and analysis

Do not be hasty in interpreting the function of what you see your insect doing. It will bias your observation. For example, quickly concluding an activity an attack "response" may prevent you from seeing that it was really a "mating strike" directed to the female. Always keep an open mind. Give a full and detailed description of what you see. If you omit something, you may have omitted a vital link between two behavioral manifestations, which will be difficult to explain later on. Try to collect data in a way that can be analyzed statistically.

Report of your findings

Submit a written report and give a 10-minute oral report. The written report should be in detail with graphs and pictures. The oral reports should convey the major points of findings.

For oral reports, remember the following points:

1. Give the background to the audience because they are unfamiliar with your project.
2. Use slides, charts and tables.
3. Show enthusiasm in your topic. It is hard for a listener to become excited about a topic if the speaker himself appears uninterested.

For written reports, keep the following points in mind:

1. Give a short "Introduction" giving the background and problem.
2. State a clear "Objective" of the project.
3. Describe "Methodology" and procedures of the project.
4. Give details of "Results" with tables, graphs, and pictures, if any.
5. Provide a "Discussion" and conclusion of the study presented.
6. Give any "References" consulted for the project.

Possible projects

1. Locomotion, feeding and mating behavior of water striders (Gerridae).
2. Foraging behavior of honeybee or bumble bee.
3. Nest building behavior of mud daubers (Vespidae), or other wasps.

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