Studies on the Energy Mobilizing Hormones in Insects.

Subrata Das
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Studies on the energy mobilizing hormones in insects

Das, Subrata, Ph.D.
The Louisiana State University and Agricultural and Mechanical Col., 1993
STUDIES ON THE ENERGY MOBILIZING HORMONES IN INSECTS

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirement for the degree of Doctor of Philosophy in

The Department of Zoology and Physiology

by

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B. Sc. (Honors), University of Calcutta, 1978
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August 1993
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ABSTRACT

Chemical factors controlling the energy mobilization in three species of insects were identified, partially characterized and their role in the process of energy mobilization were studied. High performance liquid chromatographic fractions were isolated from methanolic extracts of the corpora cardiaca (CC) from the honeybee *Apis mellifera*, the paper wasp *Polistes exclamans* and the house cricket *Acheta domestica*. Each species had a single active fraction that was able to elevate the lipid titer in *Acheta*. Pyroglutamate aminopeptidase digestion of isolated active fractions indicated that these molecules are all peptides with a terminal pyroglutamate.

Methanolic extracts of CC from *Apis*, *Polistes* and *Acheta* elevated the total blood sugar titer in *Periplaneta americana* and the blood lipid titer in *Locusta migratoria*. Injections of native CC extracts resulted in an elevation of the trehalose titer in *Polistes* and an increase in the glucose titer in *Apis*.

The adipokinetic neuropeptide from the CC of *Acheta* was sequenced and found to be identical to a previously described peptide *Gryllus bimaculatus* adipokinetic hormone (Grb-AKH). The Grb-AKH content in the CC did not change in the larval or adult stage, but its hyperlipemic effect greatly varied depending on the age of *Acheta*. 
Diel rhythms in the Grb-AKH content in the CC, fat body response, and the blood lipid and sugar levels of Acheta were described. There were two distinct peaks of total blood lipid concentration and of fat body response to Grb-AKH in Acheta. AKH increased the rate of fat body lipid secretion, and decreased the rate of fat body lipid synthesis in crickets. An inhibitory effect of Grb-AKH on the fat body protein synthesis in male crickets and on the protein uptake by the ovary in female crickets was also observed.

The presence of a heat-labile factor in the mid-gut with a hypolipemic and hypotrehalosemic effect in Acheta was also demonstrated. This factor is probably an insulin-like molecule.
INTRODUCTION

The principal trend in the evolution of insects has been the acquisition of a highly efficient flying ability which has made them one of the most successful group in the terrestrial habitat. Both structurally and physiologically, all the systems in insects are geared to sustain flight for a period of time appropriate for the individual life style and specific adaptations of a particular species. Compared to all striated muscles found in the animal kingdom, insect flight muscles are the best possible structure evolved for most efficient transformation of energy into work (Pener, 1985). Flight requires a huge and continuous supply of energy which must come from the combustion of fuels in the flight muscle. To sustain longer, active flight, insects have developed a very efficient energy mobilization processes tailored to fit specific individual needs of each species.

Nutrients commonly used for insect flight are carbohydrates in the form of trehalose, rarely glucose; lipids in the form of diglycerides, proline or sometimes a combination of several of these fuels (Pener, 1985). Although insect blood may contain considerable amounts of fuel, especially trehalose, and fuel reserves may be present in the flight muscles, they are usually sufficient only for short flights. For longer flights, fuels must be
released from the insect fat body, which is analogous to the vertebrate liver, into the insect blood which will transport it to the flight muscle. The fat body, however, does not store much fuel in the same form as it is released, so some metabolic pathway must convert the fuel to a form which can be released and transported. The breakdown of lipid to diglycerides, breakdown of glycogen to trehalose, and the synthesis of proline are major examples of metabolic processes used in the fat body to mobilize energy.

Flight is not the only reason for energy mobilization in insects. There are several other physiological processes, such as molting, cuticle synthesis, water balance and egg maturation, which also require mobilization of energy. Energy mobilization is a complex process involving many biochemical reactions in the storage tissues (fat body, epidermis), in the metabolic sinks (muscle, ovary), as well as in the blood. Coordination and synchrony in energy mobilization is achieved by participation of specific energy mobilization hormones in insects, primarily peptides released from a special neurohemal organ, the corpus cardiacum.

The first evidence of lipid utilization during insect flight has been obtained from the desert locust, Schistocerca gregaria, where it has been shown that flight provokes large increase in total blood lipid (Mayer and
Candy, 1969). Mayer and Candy have shown that corpus cardiacum (CC) extracts from *S. gregaria* can elevate blood lipid in the same species. The hyperlipemic effect in *Locusta* has been shown to be due to the presence of an adipokinetic hormone (AKH), which has been isolated and characterized (Stone et al., 1976). Several other species have shown similar hyperlipemic responses to injection of corpus cardiacum extract or synthetic adipokinetic hormone (Gäde, 1992).

The short-term hyperlipemic response in insects is mediated by a biogenic amine, octopamine. When locusts are handled, the resulting stress produces a short-term hyperlipemia (Orchard et al., 1981) which peaks at 30 min, but disappears between 1-2 h. This short-term hyperlipemia is preceded by an increase in the concentration of octopamine in the blood. It seems likely that octopamine rather than AKH promotes the rise in lipid which occurs a few minutes after flight begins (Jutsum and Goldsworthy, 1976) since blood AKH does not begin to rise until 15 min later (Cheeseman and Goldsworthy, 1979).

Downer and Steele (1972) have shown that CC extracts diminishes the concentrations of triacylglycerol and diacylglycerol in the blood of the American cockroach *Periplaneta americana*. Orchard and Loughton (1980) have obtained convincing evidence to prove that the storage lobe of the CC in *Locusta* produces a hypolipemic factor that
opposes the action of AKH. Insulin injection also decreases the lipid level in this species, indicating for the first time that the hypolipemic factor may be structurally similar to vertebrate insulin. This is interesting, since the storage lobe axons have their origin in the medial neurosecretory cells which contain an insulin like material that cross-reacts with antibodies to bovine insulin in the blowfly Calliphora vomitoria (Duve et al., 1979). Insulin has also been shown to decrease in vivo lipid output by the fat body of cecropia moth Hyalophora cecropia (Bhakthan and Gilbert, 1968).

Treatment with CC extracts elevates trehalose levels in the blood of many species belonging to different orders (Steele, 1985). The active fractions in the CC extracts responsible for hypertrehalosemia have been purified from several insects and subsequently sequenced; all of them are members of the adipokinetic hormone/red pigment concentration hormone (AKH/RPCH) family of neuropeptides (Gåde, 1990). Octopamine can also induce short term hypertrehalosemia in some species.

Unequivocal evidence has now been obtained that hypotrehalosemic and hypoglucosemic factors having insulin like properties are present in the neuroendocrine system of various insects. Blood trehalose levels in one species of blowfly Calliphora erythrocephala increase after decapitation, suggesting that the trehalose level is
restrained by a factor located in the median neurosecretory cells (Norman, 1975). Removal of the corpora cardiaca-corpora allata complex causes hypotrehalosemia in the blowfly Phormia regina (Chen and Friedman, 1977) and in the housefly Musca domestica (Liu, 1973).

At least one CC factor, AKH, has been shown to influence amino acid metabolism in two ways. Protein synthesis in L. migratoria fat body in vitro is inhibited by AKH treatment (Carlisle and Loughton, 1979). A second effect of AKH has been noted in the Colorado potato beetle Leptinotarsa decemlineata, where it has been shown to stimulate the synthesis of proline and the release of glucose from the fat body (Weeda, 1981). This is accompanied by a reduction in the concentration of alanine in the blood.

The amino acid sequence of adipokinetic hormones from 21 different insect species have been described so far (Gäde, 1992), and all of them are members of the AKH/RPCH family of neuropeptides. They are either an octa- or a decapeptide with a conserved sequence of pGlu¹, Phe⁴ and Trp⁸ showing a definite role in lipid or carbohydrate mobilization in different species (Keeley et al., 1991). Different lines of evidence suggest that cyclic 3'-5'-adenosine monophosphate (cAMP) is a natural mediator of the adipokinetic response in locusts, particularly since flight, which is known to initiate the release of AKH in
the CC (Cheeseman and Goldsworthy, 1979), also causes the cAMP level in the fat body to rise (Gäde and Holwerda, 1976).

The mechanism for controlling the release of AKH from the glandular lobe of the CC in locusts is physiologically important, because it also indirectly controls the metabolism of lipid. There is reasonable evidence that a rise in the level of blood lipid turns off the secretion of AKH, as might be expected, whereas the decline in trehalose concentration promotes secretion of the hormone. There is ample evidence to suggest that the glandular lobes of the CC are the source of AKH and that release of the hormone is under neural control, since severing the Nerve corpora cardiaca-I and Nerve corpora cardiaca-II (the nerve bundles that innervate the CC) blocks the flight-induced rise in blood lipid following flight, while the concentration of AKH in the blood rises to its maximum level.

A key element in the hormone-stimulated adipokinetic response is the participation of cAMP. The interaction of synthetic AKH or CC extracts with the receptor on the fat body cell membrane stimulates the synthesis of cAMP by the cell according to the following scheme:

\[
\text{Adenylate cyclase} \quad \text{Phosphodiesterase} \\
\text{ATP} \rightarrow \text{cAMP} \rightarrow 5’-\text{AMP}
\]
The simplistic view that AKH modulates binding of lipophorin with diacylglycerol at the interface between the fat body and blood may be only partly correct. There is only limited evidence to suggest that insects have a hormone which acts as a positive effector of lipid synthesis. Downer and Steele (1972) have suggested that CC extract may increase the rate of neutral glyceride synthesis in the fat body. The lipid promoting activity of insulin in vertebrates is well known, and since insulin-like factors have been demonstrated in certain insects, it is possible that insect hypolipemic factors also act on lipid synthesis. An important enzyme in the conversion of carbohydrate to lipid is pyruvate dehydrogenase which catalyzes the oxidation of pyruvate to acetyl-CoA. The activity of this enzyme is regulated by a phosphorylation-dephosphorylation mechanism. The total amount of pyruvate dehydrogenase in fat body of starved hawkmoth Manduca sexta is more than doubled by injection of glucose into starved mature larvae, but an increase in the active form of the enzyme (non-phosphorylated) appears to be dependent on a neuroendocrine factor from the head (Roche et al., 1980). Injection of glucose brings about a three-fold increase in the active form of the enzyme within 2 h, but this is prevented in larvae by head-ligation. Pyruvate dehydrogenase may, therefore, be the site of action for hormones controlling lipogenesis.
Based on the studies done so far on the action of adipokinetic hormones in different insect groups, a few generalizations can be made regarding its functions. AKH mobilizes lipids in insects having the capacity for long distance flight and trehalose in insects which fly only short distances. Both the hyperlipemic and hypertrehalosemic effect of AKH are mediated by the second messenger, cAMP, which ultimately activates enzyme systems by phosphorylation/dephosphorylation.

It becomes clear from the above discussion that information regarding the occurrence, distribution and functions of energy mobilizing hormones in various groups of insects is still incomplete. To improve our understanding of the energy mobilization process in insects, the present investigation was undertaken to contribute further information in the newly emerging field of insect neuropeptides.

Order Hymenoptera, which includes bees, wasps and ants, is a highly advanced insect group with diverse adaptations and life styles. No hyperlipemic or hyperglycemic hormones have been reported so far from this group of insects. Bees and wasps are basically short distance fliers that utilize sugar as their energy source during flight. Interestingly, honeybees (Apis mellifera) have almost no stored nutrients in the fat body and they must remain within a short distance of the hive or a food
source (Woodring et al., 1993). Though paper wasps (Polistes exclamans) also utilize sugars as the main energy source during flight, they can sustain flight longer, as they have a considerable amount of stored nutrients in their fat body.

The house cricket *Acheta domestica* belongs to the Order Orthoptera and is very closely related to the locusts, but it differs from locusts in not having long distance flight ability. Instead, the preferred modes of locomotion in this species are walking and jumping. Treatment with CC extract and locust AKH induces hyperlipemia in house crickets (Woodring et al., 1989). This observation points out to the fact that the physiological adaptations of house crickets are still very much similar to those of their close relatives, the locusts, in spite of the vast differences in between individual adaptations of these two species.

These three species describe above, the honeybee, the paper wasp and the house cricket, with their uniquely different physiological and behavioral adaptations, have been chosen to study the mechanism and control of energy mobilization in insects. The experiments described in this dissertation attempt to answer the following questions:

1) Are there any energy mobilizing hormones in the corpora cardiaca of the honeybee, the paper wasp and the house
cricket? If so, what is their chemical nature? Do they belong to the AKH/RPCH family of neuropeptides?

2) What are the characteristics and functions of corpora cardiaca hormones in these insects?

3) Do the amounts of corpora cardiaca hormones and their specific actions in insects vary depending on the age or time of the day, or do they remain constant?

4) Are there other functions of these corpora cardiaca hormones except energy mobilization? If so what are they?

5) And finally, is there any specific antagonistic hormone which counteracts the functions of the corpora cardiaca hormones? If so, which gland/organ does secrete it? What are its functions, and what is the nature of this molecule?
CHAPTER 1

ENERGY MOBILIZING PEPTIDES FROM THE CORPORA CARDIACA OF

THE HONEYBEE (APIS MELLIFERA) AND

THE PAPER WASP (POLISTES EXCLAMANS)
INTRODUCTION

In insects, the corpora cardiaca are the site for storage and release of intrinsically produced hormones as well as for hormones produced in the pars intercerebralis of the brain and transported to the corpora cardiaca (Orchard, 1987). The possible role of corpora cardiaca hormones in energy mobilization was first indicated by the elevation of blood trehalose by corpora cardiaca extracts in Periplaneta americana (Steele, 1961) and in Blaberus discoidalis cockroaches (Bowers and Friedman, 1963). Locusts were the first insects in which corpora cardiaca extracts were shown to elevate blood lipids titer (Beenakkers, 1969a; Mayer and Candy, 1969). All the energy mobilizing hormones thus far identified in insects belong to a group of hormones termed the adipokinetic hormone/red pigment concentrating hormone (AKH/RPCH) family. First isolated and sequenced from Locusta corpora cardiaca in 1976 (Stone et al.), there are now at least twenty-one identified members of this group of peptide hormones from the Orders Odonata, Orthoptera, Dictyoptera, Lepidoptera, Coleoptera and Diptera (Gäde, 1991, 1992; Keeley et al., 1991a). They are all octa-, nona- or decapeptides blocked at the N-terminus by pGlu and at the C-terminus by an amide and with few exceptions all contain a Phe at position 4, and Trp at position 8 (Gäde, 1992).
The Order Hymenoptera contains the largest number of social and parasitic insects, such as ants, hornets, bees and wasps, with varied physiological adaptations and needs. Carbohydrate and lipid metabolism in Hymenoptera have been reported to change under various physiological conditions, such as stress, starvation, foraging and age (Ishay, 1975; Arslan et al., 1986; Crailsheim, 1988; Woodring et al., 1993). Von Norstrand et al. (1980) found an indication of hyperglycemic action of whole head extracts of *Apis mellifera* when injected into *Periplaneta*. Recently, the hyperlipemic and hyperglycemic activity of corpus cardiacum extracts of honeybee *Apis mellifera* has been reported from our laboratory (Woodring et al., 1993). The purpose of the present investigation was to identify and characterize the energy mobilizing hormones from the corpus cardiacum of two species of the Order Hymenoptera, viz., *Apis mellifera* and *Polistes exclamans*.

**MATERIALS AND METHOD**

**Insects:**

Honeybees (*Apis mellifera*) were reared in the laboratory in queen-right observation colonies and only the foraging workers (over 20 days old) were used. The small, open faced nests of the colonial wasp, *Polistes exclamans*, were collected locally, brought into the laboratory, placed in 1 cubic foot cages and provided sucrose solution as the
food source. The three assay insects, *Acheta domesticus*, *Locusta migratoria* and *Periplaneta americana* were reared as previously described (Clifford and Woodring, 1990; Gäde, 1991, 1992).

**Peptide separation and detection:**

Corpora cardiaca were dissected under cold cricket Ringers (160 mM NaCl, 6 mM KCl, 8 mM CaCl₂·2H₂O and 4 mM MgCl₂·6H₂O) and extracted with methanol as previously described (Gade et al., 1984). Samples were vacuum dried and stored at -20°C. Resuspended in HPLC solvent, active fractions were separated with a C₁₈ reversed phase column (4.5 mm X 150 mm) using an isocratic running buffer of 26% acetonitrile and 0.1% trifluoroacetic acid with a flow rate of 1 ml/min. Peptides were detected with a UV detector set at 210 nm.

**Pyroglutamate aminopeptidase digestion:**

The active fractions from 40 corpora cardiaca of each species were separated by HPLC, collected in Eppendorf microcentrifuge tubes, vacuum-dried, then dissolved in 20 μl of a 50 mM tris-HCl buffer containing 0.132 mg dithiothreitol and 0.528 mg EDTA/ml of buffer. 20 μl of pyroglutamate aminopeptidase (1 unit, EC.3.4.19.3, Sigma, St. Louis, MO) was added to the buffer solutions with dissolved peptide or active fraction. Aliquots were taken at 0 min and after 20 min of enzyme digestion and injected on to the HPLC column. Synthetic *Blaberus discoidalis*
hyperglycemic hormone was digested and separated (Fig. 1.2A) to check the percent recovery of the synthetic peptide and general effectiveness of the pyroglutemate digestion.

**Blood collection:**

The body of the bee or wasp was placed in the slit end of a latex tube through which a slow stream of carbon dioxide flowed. The head was deflected ventrally, the exposed dorsal neck membrane was pricked with a pin and 0.5 μl blood collected. Extracts, isolated fractions and synthetic hormones were first dissolved in cricket Ringer’s and then injected in an 1 μl volume through the basal membrane of the metathoracic leg. Crickets, locusts and cockroaches were briefly anesthetized with CO₂, blood was collected from the basal membrane of a prothoracic leg and test material (1 μl) was injected into the abdomen.

**Assays:**

Blood samples were collected in the running buffer (75% acetonitrile and 25% water) and stored at -20°C until use. No change in the sugar concentrations were noted over a 3 week period of storage at -20°C. Blood glucose, fructose and trehalose were separated by HPLC according to the methods of Gade (1991) using a Hewlett Packard refractive index detector. Peaks were quantified by comparison to standards (Fig. 1.5). Total blood carbohydrates (anthrone-positive material; glucose standard) were determined using
the methods of Spik and Montreuil (1964) as modified by Holwerda et al. (1977).

Total blood lipids (vanillin-positive material; cholesterol standard) were determined using the method of Zöllner and Kirsch (1962). The blood proline and alanine titers were determined with an amino acid (ninhydrin) analyzer. For the amino acid analysis the blood proteins were precipitated with 10% TCA and a norleucine internal standard was added. The sample was centrifuged, the supernatant removed and vacuum dried, redissolved in sodium citrate buffer and injected onto the column.

**Statistical analysis:**

All data were statistically analyzed by two-tailed Student’s t-test with a 95% confidence level (p<0.05).

**RESULTS**

**Separation of active fractions:**

One minute fractions from Apis and Polistes corpora cardiaca extracts were collected and tested for hyperlipemic activity in Acheta (Fig. 1.1) to be certain there was no activity in fractions that did not show a peak in UV absorbance. In one instance two identifiable UV peaks on the chromatogram were collected in a single 1 minute fraction (see Fig. 1.1C: Polistes). In another HPLC separation of Polistes corpora cardiaca extracts, these two
Fig. 1.1. HPLC separation of peptides (A) and the methanolic extracts of 40 corpora cardiaca of Apis (B) and Polistes (C). A. represents a composite of retention times of a mixture of synthetic adipokinetic hormones: (a) Blaberus discoidalis hypertrehalosemic hormone, (b) Periplaneta americana cardioacceleratory hormone I, (c) Gryllus bimaculatus adipokinetic hormone, (d) Locusta migratoria adipokinetic hormone I, (e) Tabanus atratus adipokinetic hormone and (f) Tabanus atratus hypotrehalosemic hormone. Separation of the extracts was achieved with a C-18 reversed phase column (4.5 mm x 150 mm) using an isocratic eluent of 26% acetonitrile and 0.1% trifluoroacetic acid run at a flow rate of 1 ml.min. 1 ml fractions were collected, vacuum-dried, resuspended in cricket Ringer's and injected into 4-day old female Acheta. Each bar represents the average change in lipid titer ±SE (n=6). Significant elevations (*) (p<0.01, Student's t-test) only occurred in the fractions containing active substance(s).
peaks were collected separately and only the first peak (at 8.2 min) was found to contain the hyperlipemic activity. We use the term active fraction to mean the collected peak which when injected into Acheta produced a response. In the HPLC separation of the corpus cardiacum extracts of these two hymenopterans, significant (p<0.01) hyperlipemic activity was always limited to a single peak (*). This indicated the presence of one active molecule in each species examined. The different retention times indicates a different molecule for each species. The retention times for the active fractions of Apis (Fig. 1.1B) and Polistes (Fig. 1.1C) were different from each other and they were also different from the retention times of synthetic Gryllus bimaculatus adipokinetic hormone, Periplaneta americana cardioacceleratory hormone I, Locusta migratoria adipokinetic hormone I, Blaberus discoidalis hyperglycemic hormone and Tabanus atratus adipokinetic hormone and hypotrehalosemic hormone (Fig. 1.1A).

Assuming the active fractions in the hymenopteran corpora cardiaca to be peptides, then by comparison of peak areas with that of synthetic Blaberus discoidalis hypertrehalosemic hormone, and assuming some degree of similarity of UV absorbance characteristics, we estimated that one pair of corpora cardiaca from Apis contains about 1.9 ng and from Polistes about 2.7 ng of active peptide molecules.
Relationship to other adipokinetic/hypertrehalosemic hormones:

The enzyme pyroglutamate aminopeptidase is very specific for the hydrolysis of a terminal pGlu from a peptide. We tested the enzyme on the synthetic *Gryllus bimaculatus* adipokinetic hormone, *Periplaneta americana* cardioacceleratory hormone, *Locusta migratoria* adipokinetic hormone I, and *Blaberus discoidalis* hyperglycemic hormone (only the latter is shown in Fig. 1.2A). These are all members of the adipokinetic hormone/red pigment concentrating hormone (AKH/RPCH) family of peptides (Gäde, 1990a). In each case the retention time of digested product (peptide minus pGlu) is shifted to the right. A similar shift of the digested product of the active fraction from the two hymenopteran species (Fig. 1.2B and 1.2C) indicates that the molecules in the active fractions are likewise members of the AKH/RPCH family of peptides.

Hyperglycemic effects of corpus cardiacum extracts:

Corpus cardiacum extracts of *Apis* (0.5 pair CC equivalents) and *Polistes* (0.5 pair CC equivalents) elevated the total sugar titer (anthrone positive material) when injected into *Periplaneta* (p<0.05, Students t-test) (Fig. 1.3). Injections of *Polistes* corpus cardiacum extracts (1.0 equivalents) into *Polistes* resulted in a statistically significant elevation of trehalose titer.
Fig. 1.2. HPLC chromatograms of isolated active fractions and the shifted peak following 15 minutes digestion with pyroglutamate aminopeptidase. The shifted peak probably represents the resultant peptide hormone with the pGLU removed. The solid lines represent injection of active fractions (10 pairs of corpora cardiaca) and the dashed lines represent injections of a digest of another active fraction (also from 10 pairs of corpora cardiaca). The active fraction for each species was always run just prior to the digest. Typical retention times are given in minutes. A. 50 pmol synthetic Blaberus hyperglycemic hormone was used to calibrate the system. B. Apis and C. Polistes. Separation was achieved with a C18 reversed phase column (4.5 mm x 150 mm) using an isocratic eluent of 26% acetonitrile and 0.1% trifluoroacetic acid run at a flow rate of 1 ml/min. AUFS- absorbance units, full scale.
after 30 min (p<0.01) and 60 min (p<0.05), and a slight
decline in both glucose and fructose titers (neither
decrease alone was statistically significant) (Table 1.1).
Injections of *Apis* corpus cardiacum extracts (1.0
equivalents) into *Apis* resulted in a significant increase
after 15, 30 and 60 min in the glucose titer, and a
significant decrease in fructose (p<0.01) and trehalose
titers (p<0.05, Students t-test).

**Hyperlipemic effects of corpus cardiacum extracts:**

Corpus cardiacum extracts of *Polistes* (0.5 equivalents)
and *Apis* (0.8 equivalents) elevated the blood lipid titer
when injected into *Locusta* (p<0.05, Students t-test) (Fig.
1.3). Both extracts increased the lipid titer in a dose-
dependent manner when injected into *Acheta*, with an ED₉₀
between 0.1 and 1.0 equivalents (Fig. 1.4). Injections of
*Polistes* corpus cardiacum extracts into *Polistes* and
injections of *Apis* corpus cardiacum extracts into *Apis*
resulted in no elevation of the lipid titer (Table 1.2).
Neither species showed any hyperlipemic response to
injections of synthetic adipokinetic hormone from *Locusta*.

**Effects of corpus cardiacum extracts on amino acids:**

Corpus cardiacum extracts (1.0 equivalents) of *Polistes*
had no effect after 30 or 60 min on the proline or alanine
titer when injected into *Polistes* nor did corpus cardiacum
extracts (1.0 equivalents) of *Apis* when injected into *Apis*. 
Fig. 1.3. Hyperglycemic and hyperlipemic effects of *Apis* and *Polistes* corpus cardiacum extracts. The response of *Locusta migratoria* (left axis, dotted bars) and *Periplaneta americana* (right axis, solid bars) 60 minutes after injections of cricket Ringers (SAL), 0.8 pair equivalents of *Apis mellifera* (APIS) corpus cardiacum extract, 0.5 pair equivalents of *Polistes exclamans* (POL) corpus cardiacum extract, and 10 pmol of synthetic adipokinetic hormone (synthetic *Locusta* hormone) (AKH) was injected into *Locusta* and synthetic *Periplaneta* hormone was injected into *Periplaneta*. The response to the test substances is given as the increase over the control sugar titers of *Periplaneta* (20 mg/ml) and the increase over the control lipid titers of *Locusta* (10 mg/ml). Each bar represents the average ±SE of 6-11 samples.
Fig. 1.4. Dose response curves (increase in mg lipids/ml blood) 60 minutes following injections of the active fractions of corpus cardiacum (CC) extracts from Acheta (▲), Apis (●) and Polistes (○) into Acheta. Each point represents the average ±SE. The average control lipid titer of Acheta was 20 mg/ml.
Fig. 1.5. Change in hemolymph sugar titer in Polistes after injection with an methanolic extract of 1 corpus cardiacum equivalent (see Table 1 for average changes). Standards (A) of 5 ng of fructose (F), glucose (G), sucrose (S) and trehalose (T). Sugars were separated with a C-18 column using an isocratic running buffer of 75% acetylnitrile and 25% water at a flow rate of 1 ml/min. Detection with H/P refractive index detector (RIU/FS- refractive index units/full scale). Typical chromatograph showing change in fructose, glucose and trehalose titers after 15 minutes (B), 30 minutes (C) and 60 minutes (D).
Table 1.1. Change in the blood concentration of fructose (FR), glucose (GL) and trehalose (TR) in *Polistes* and *Apis* in response to injections of methanolic extracts of their own corpora cardiaca (CC).

<table>
<thead>
<tr>
<th>Assay</th>
<th>Assayed</th>
<th>Animals</th>
<th>Sugars</th>
<th>Ringers</th>
<th>15 min</th>
<th>15 min</th>
<th>30 min</th>
<th>30 min</th>
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<td></td>
<td></td>
</tr>
<tr>
<td>FR</td>
<td>nd</td>
<td>Polistes</td>
<td>nd</td>
<td>-3.4±0.4(4)</td>
<td>-3.9±0.6(4)</td>
<td>-4.0±0.6(8)</td>
<td></td>
<td></td>
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<tr>
<td>GL</td>
<td>nd</td>
<td>Polistes</td>
<td>nd</td>
<td>-1.9±0.2(4)</td>
<td>-1.3±0.3(4)</td>
<td>-1.9±0.3(8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TR</td>
<td>nd</td>
<td>Polistes</td>
<td>nd</td>
<td>3.4±0.6(4)</td>
<td>6.0±0.7(8)**</td>
<td>7.4±0.5(4)</td>
<td>9.7±0.8(8)*</td>
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<td></td>
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<tr>
<td>FR</td>
<td>-1.9±0.4(5)</td>
<td>Apis</td>
<td>-0.2±0.1(7)**</td>
<td>-2.9±0.6(5)</td>
<td>-0.5±0.2(7)**</td>
<td>-2.9±0.6(7)</td>
<td>-0.8±0.1(7)**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GL</td>
<td>-1.4±0.8(5)</td>
<td>Apis</td>
<td>4.6±1.6(7)**</td>
<td>-3.9±1.5(5)</td>
<td>5.5±1.6(7)**</td>
<td>-1.6±0.9(7)</td>
<td>4.9±1.1(7)**</td>
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<tr>
<td>TR</td>
<td>-7.5±2.0(5)</td>
<td>Apis</td>
<td>-2.1±0.5(7)*</td>
<td>9.3±1.7(5)</td>
<td>-2.1±0.7(7)**</td>
<td>-11.6±2.2(7)</td>
<td>-5.4±1.3(7)*</td>
<td></td>
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</table>

The number of animals (assays) used are given in parentheses and data represents average ± S.E. * indicates a significant difference (Student's t-test) of p<0.05 and ** indicates a significant difference of p<0.01 between the control injection (Ringers) and the injected corpus cardiacum extracts. nd indicates not done. Control titers of sugars in *Polistes*: FR-5.2 0.4, GL-3.0 0.2, TR-15.2 0.3 mg/ml (n=12); Control titers of sugar in *Apis*: FR-4.0 0.2, GL-7.8 0.8, TR-14.3 1.5 mg/ml (n=12).
Table 1.2. Change in total lipid concentration in *Polistes* and *Apis* in response to injections of synthetic 100 pmol locust adipokinetic hormone (AKH-I) and methanolic extracts of 1.0 corpus cardiacum equivalents.

<table>
<thead>
<tr>
<th>Test Material</th>
<th>Assay</th>
<th>Time after injection of test material</th>
</tr>
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<tbody>
<tr>
<td>Injected</td>
<td>Animal</td>
<td>30 min</td>
</tr>
<tr>
<td>Ringers</td>
<td><em>Polistes</em></td>
<td>1.2±0.9(6)</td>
</tr>
<tr>
<td>Synthetic AKH</td>
<td><em>Polistes</em></td>
<td>nd</td>
</tr>
<tr>
<td><em>Polistes</em> CCs</td>
<td><em>Polistes</em></td>
<td>1.6±0.7(7)</td>
</tr>
<tr>
<td>Ringers</td>
<td><em>Apis</em></td>
<td>nd</td>
</tr>
<tr>
<td>Synthetic AKH</td>
<td><em>Apis</em></td>
<td>nd</td>
</tr>
<tr>
<td><em>Apis</em> CCs</td>
<td><em>Apis</em></td>
<td>nd</td>
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</table>

The number of animals (assays) are given in parentheses. There was no significant difference between injections of synthetic peptides or the corpora cardiaca of the bee/wasp and the control injections.
DISCUSSION

The usual physiological role of insect adipokinetic hormone/hyperglycemic hormone is to mobilize lipids or carbohydrates from fat body reserves for use as energy substrates by peripheral tissues. These hormones are synthesized in and secreted from corpus cardiacum and are shown to have, apart from energy mobilizing effects, other activities upon injection into the same species or into a different species: cardio-acceleration (Scarborough et al., 1984), myoactivity (O’Shea et al., 1984), and inhibition of synthesis of protein (Carlisle and Loughton, 1979) and fatty acids (Gokuldas et al., 1988).

The induction of a dose-dependent hyperlipemia in crickets indicates the presence of a receptor saturable hyperlipemic peptide (as measured in an Acheta bioassay) in the active fraction of the methanolic extracts of the corpora cardiaca of Apis and Polistes. A peptide hormone must bind to the receptor and then induce a molecular change in the receptor, which initiates events inside the target cells. The amount of hormone needed for a response is thought to depend on how well a peptide is bound to the receptor, while differences in the maximal response are thought to depend on the ability of the peptide to induce the changes in receptor. In the bioassay for hyperlipemic factors (Locusta) and in the bioassay for hyperglycemic
factors (*Periplaneta*), it has been found that extrinsic hormones often do not elicit the full magnitude of response exerted by the native peptides, specifically *Locusta migratoria* adipokinetic hormone I and *Periplaneta americana* cardioacceleratory hormone I. The extent of hyperlipemic and hyperglycemic response of *Locusta* and *Periplaneta* to corpora cardiaca extracts of *Apis* and *Polistes* varied from 47-77% of the response shown by native peptides. Unlike *Locusta* or *Periplaneta*, the Acheta receptor system was saturable by the bee and wasp hyperlipemic factors, though the ED$_{50}$ and full saturation were achieved at lower doses with native *Acheta* corpora cardiaca extracts.

Our estimate of the amount of hyperlipemic or hyperglycemic factors (assuming them to be peptides) present in the corpora cardiaca of the hymenopteran species are low when compared to the corpora cardiaca content of adipokinetic hormone in the *Locusta* and *Romalea*, but are comparable with *Acheta* and *Tabanus* when the size of the corpora cardiaca in the hymenopteran species are taken into consideration (Orchard, 1987; Spring and Gäde, 1987; Woodring et al., 1990; Woodring and Leprince, 1992).

As pyroglutamate aminopeptidase is very specific in removing only the N-terminal pGlu, digestion of the specific compounds isolated from the corpora cardiaca of *Apis* and *Polistes* by this enzyme indicates that these molecules are peptides blocked at the N-terminus by a
pyroglutemate residue. Since all members of the adipokinetic hormone/red pigment concentrating hormone (AKH/RPCH) family of peptides so far identified contain a blocked glutamate at the N-terminus, we think that the molecules in the active fractions of corpora cardiaca extracts of these two hymenopteran species are also members of this family of hormones. The evidence indicates that the corpus cardiacum of Apis mellifera and Polistes exclamans, each contain a single, apparently unique peptide which like other members of the family elicit hyperlipemic/hypertrehalosemic response in several orthopteroid species.

Adult Apis mellifera (honeybees) have very limited reserves stored in the muscle or fat body and they utilize sugar stored in the crop for energy (John, 1958; Neukirch, 1982). We dissected many honeybees and observed that the volume of fat body in Apis was very small, and the clear translucent color of the fat body indicated little glycogen or fat content. We also dissected Polistes exclamans (colonial wasps) and observed a large volume of fat body in both the thorax and abdomen, and the opaque, chalky white color of the fat body indicated large glycogen reserves. When the fat body was homogenized in a test tube containing saline, a thick layer of lipid accumulated on the surface. The blood sugar level in Apis, which have a limited tissue reserves, decreased significantly when these insects were exposed to a brief starvation period (Woodring et al.,
After 60 min without feeding, the sugar in the gut of *Apis* was consumed and the blood sugar titer decreased. With ample fat body reserve, the total blood sugar titer of *Polistes* did not decrease in response to 60 min starvation.

There was no hyperlipemic response by *Apis* or *Polistes* to injections of corpus cardiacum extracts. This is an expected response, because bees and wasps utilize sugar and not lipids for energy. Those insects that use lipids for energy, for example locusts, respond to injection of corpus cardiacum peptides with mobilization of lipids and those that use sugars for energy, such as cockroaches, do not mobilize lipids in response to injections of these peptides (Keeley et al., 1991a). In a previous study we found no increase in total blood sugar titer (anthrone method) of *Apis* in response to injections of *Apis* corpus cardiacum extracts, synthetic *Locusta* adipokinetic hormone and to octopamine (Woodring et al., 1993). Using the anthrone method, we also found no change in the total blood sugar in *Polistes* after injection with corpus cardiacum extracts (unpublished).

It is reasonable that honeybees might not mobilize sugars from bodily reserves, because though they have essentially no tissue reserve, they have readily available stored reserves in the hive (Woodring et al., 1993). A honeybee could meet its energy requirements by using sugars carried in the crop (Crailsheim, 1988). However, we found
it difficult to believe that Polistes would not mobilize sugar from stored reserves, because they obviously had large glycogen reserves and were active during warm days in the winter when no nectar was available.

In the current study we found that the corpus cardiacum extracts caused a hypertrehalosemic response in Polistes, and no significant change in glucose or fructose titers. However, the combined glucose and fructose titer showed enough of a decline (Table 1.1) so that when combined with the slight (but statistically significant) increase in trehalose the net result was no significant change in total sugar titer (as determined with the anthrone assay). In the case of honeybees, injections of corpus cardiacum extracts resulted in significant decreases in fructose and trehalose, which was essentially offset by the significant increase in glucose. Therefore, an anthrone method would show no increase in total blood sugar in response to the extracts (Woodring et al., 1993).

Instead of the typical hypertrehalosemic response found in other insects that use sugar for energy (Gâde, 1991; Keeley et al., 1991b), injections of corpus cardiacum extracts into Apis produced a hyperglucosemic response. This is reasonable since that honeybees have insufficient tissue reserves to be able to mobilize and release trehalose into the blood, instead glucose and fructose are absorbed from the crop. We suggest that perhaps the
metabolic peptides in the honeybee corpus cardiacum facilitate or enhance the transport of glucose from the midgut into the blood. Since there is an equal amount of fructose present, it is possible that the peptide might also favor the conversion of fructose to glucose.

In some insects oxidation of proline serves as an energy source for flight (Wheeler, 1989), and the resultant alanine is returned to the fat body and made into proline using the energy stored in lipid (Bursell, 1981). There was no evidence in either Polistes or Apis for mobilization of proline as a transport mechanism for energy.

Factors other than metabolic peptides from the corpus cardiacum may be involved in sugar utilization in hymenopterans. An insulin-like molecule isolated from midgut cells in Apis was reported to have a hypoglycemic action (Moreau et al., 1981) when injected into Apis. Glucagon-like peptides may also be present in bees and wasps. An intestinal factor induced hyperglycemia in the hornet, Vespa orientalis, the wasp Paravespula germanica and the ant Polyrachis simplex (Ishay, 1975).
CHAPTER 2

IDENTIFICATION, CHARACTERIZATION AND PHYSIOLOGICAL ACTIONS
OF THE ACHETA ADIPOKINETIC HORMONE
The corpora cardiaca (CC) of insects are neurohemal organs that store and release neurosecretory products synthesized in the brain. In addition, the CC also contain intrinsic glandular cells producing a variety of peptide hormones affecting physiologic, metabolic and developmental processes (Wheeler et al., 1988). Those that regulate sugar and lipid metabolism comprise a group of related octa-, nona- or decapeptides, a number of which have been sequenced in the last 5 years (Gåde, 1990). In cockroaches, stick insects and some beetles (Tenebrio) the CC peptides stimulate the release of trehalose from the fat body when injected back into the same insect (Gåde, 1990). These are termed hypertrehalosemic hormones (HrTH). In locusts, crickets, and some Lepidoptera the intrinsic CC peptides stimulate the release of diacylglycerides from the fat body when injected back into the same insect (Gåde, 1990a). These are termed adipokinetic hormones (AKH) or hyperlipemic hormones. However, CC extracts or the peptides isolated from the CC from most insect species have a hypertrehalosemic effect when injected into Periplaneta and a hyperlipemic effect when injected into Locusta (Gåde, 1990b), indicating that the receptor system is as important as the peptide sequence. Acheta fits this pattern, because CC extracts cause a hypertrehalosemic response in
periplaneta and a hyperlipemic response in Locusta (Woodring et al., 1989). When the CC extracts from Acheta were injected back into Acheta there was a hyperlipemic response but no glycemic response (Woodring et al., 1989).

The adipokinetic response of Locusta was low in young adults, then increased to a plateau, and then gradually declined again to low levels (Mwangi and Goldsworthy, 1977). The responses of adult Acheta over 8 days old was half that of younger adults when injected with synthetic locust AKH (Woodring, 1989). In general, it was known that the hyperlipemic response of larval insects to CC extracts was less than that of the adult forms (Mwangi and Goldsworthy, 1977; Gade, 1980; Gade, 1988). It has been suggested that the reduced responsiveness may involve a less efficient lipophorin in the larvae (Mwangi and Goldsworthy, 1977) or reduced levels of juvenile hormone (JH) (Pener et al., 1989). Reversed-phase high pressure liquid chromatography (RP-HPLC) chromatograms have been used to quantitate the AKH content of the CC in Locusta, where it was shown that the AKH content increased almost 3-fold during the adult stage (Siegert and Mordue, 1986).

The first goal of this research was to separate and sequence the AKH (or AKHs) that occur in the CC of Acheta. The function of the peptide, time and age responses to the adipokinetic hormone treatment, and also the change in AKH content of the CC in relation to age were examined. The AKH
content of the CC and the response of the fat body to a constant dose of synthetic Acheta specific AKH were compared during cricket development. The sequence information would also be of phylogenetic interest since the sequence is known for the AKH’s in several other Orthoptera, including another cricket species (Gäde, 1990).

**MATERIALS AND METHODS**

**Rearing and handling:**

Crickets were cultured on Purina cricket chow at 30°C on a 14:10 LD cycle as previously described (Clifford et al., 1977). Newly molted last instar females were collected daily, separated from males and were designated 0-day-old when the adult emerged. Crickets were anaesthetized by a 5 sec exposure to CO₂, which has no effect on lipid titers (Woodring et al., 1978). Blood was collected from the base of the foreleg as outlined previously (Woodring et al., 1989).

**Separation:**

Corpora cardiaca from adult Acheta were collected in cold (4°C) distilled water (DW), and extracts made as described previously (Gäde et al., 1984). After vacuum drying, the pellet was suspended in a mixture of 25% solution B (0.11% trifluoroacetic acie (TFA) in DW): 75% solution A (60% acetonitrile + 0.10% TFA) (corresponding to 15% acetonitrile) and injected onto a Nucleosil C 18 column.
for reversed-phase high performance liquid chromatography (RP-HPLC) in batches of 50 to 100 glands using equipments described earlier (Gäde, 1985). A gradient from 43% solution B (57% solution A) to a 53% solution B over a period of 20 min. at a flow rate of 1 ml/min was used. The active (adipokinetic) fraction was collected manually, and the combined material from a total of 215 corpora cardiaca was used for the determination of the primary structure.

**Enzymatic deblocking and pulsed-liquid phase sequencing:**

The dried active material after RP-HPLC was enzymatically deblocked using L-pyroglutamate aminopeptidase as described previously (Gäde et al., 1988). After an incubation of 50 min the solution was chromatographed on RP-HPLC using the same solvents as above. The gradient was from 25% B to 60% B over 30 min at a flow rate of 1 ml/min. Several fractions were manually collected, dried by vacuum centrifugation and used for pulsed-liquid sequencing.

Automated Edman degradation was performed with a pulsed-liquid phase sequencer (Model 477A, Applied Biosystems, Foster City, U.S.A.) connected to an on-line phenylthiohydantoin (PTH) amino acid analyzer (Model 120A, Applied Biosystems). Sequencing reagents and solvents were from Applied Biosystems. Sequencing and PTH analysis was carried out with standard programs.
AKH contents of corpora cardiaca:

Each sample consisted of the corpora cardiaca from 10 crickets collected in 500 μl of 4°C DW, homogenized and lyophilized. For each age group three samples were collected and processed. The lyophilized homogenates were redissolved in 80% methanol and centrifuged. The supernatant was injected onto a C₁₈ reverse phase column and an isocratic mobile phase of 30% methanol (70% water containing 0.1% TFA) was employed. Peak heights of the CC extracts and synthetic Grb-AKH standards were compared to estimate the CC content during larval and adult development.

Phosphorylase activity:

The fat body extraction method and the assay for phosphorylase activity were essentially as previously described (Gade, 1981). Fifteen min after injection of the synthetic Grb-AKH, 2-3 day old females were briefly immersed in liquid nitrogen to halt all enzyme activity, then the fat body was scraped from the abdomen as it thawed. The fat body was homogenized in triethanolamine acetate buffer (pH 7, 50 mM) containing 5 mM EDTA and 20 mM NaF and centrifuged for 5 min at 10,000 × g. The layer below the upper fat layer was used for the phosphorylase assay in the direction of glycogen breakdown. All data were analyzed statistically using student’s t-test at a significance level of P<0.05.
RESULTS

Hyperlipemic and hyperglycemic response of Acheta CC homogenate:

The blood lipid level of locusts Locusta migratoria and house crickets Acheta domesticus and the total blood sugar level of cockroaches Periplaneta americana were elevated by injection of a crude extract of Acheta corpora cardiaca extract (Table 2.1). The hyperlipemic response in Acheta was found to be dose-dependent with the least responsive dose being 0.005 pr CC equivalent and a saturation dose being 0.5 pair CC equivalent.

Separation:

Crude methanolic extracts of 90 A. domesticus corpora cardiaca was fractionated on an analytical RP-HPLC column in a gradient mode using trifluoracetic acid as the ion-pairing reagent (Fig. 2.2). This method proved successful in isolating a fraction with a retention time of 9.5 min, which elevated lipids in crickets and corresponded to a distinct absorbance peak (Fig. 2.2, top). The retention time of the active fraction was different from that of several other known peptides (Fig. 2.2, bottom), but was identical to that of the adipokinetic hormone of the cricket Gryllus bimaculatus (Grb-AKH) as previously reported (Woodring et al., 1989). Another batch of 150 corpora cardiaca of A. domesticus, showed a double peak at 9 to 10 min (Fig. 2.3). It was not possible to separate
these peaks further. It was decided, therefore, to keep this material separated. Each of these 2 separate samples: one designated A, collected from 90 corpora cardiaca, showing only one peak (example Fig. 2.2, top) and a second, designated B, collected from about 150 corpora cardiaca, showing a double peak (as in Fig. 2.3), were subjected to sequence analysis.

Table 2.1. The hyperlipemic response (mg/ml) of Locusta migratoria and Acheta domesticus and the hyperglycemic response (mg/ml) of Periplaneta americana to injections of Acheta CC homogenate.

<table>
<thead>
<tr>
<th>Species</th>
<th>Pr CC</th>
<th>0 min</th>
<th>90 min</th>
<th>Change</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. migratoria</td>
<td>0.1</td>
<td>9.3±1.3</td>
<td>13.7±2.5</td>
<td>4.4±2.4</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>10.5±1.4</td>
<td>46.7±9.1</td>
<td>36.2±9.4</td>
<td>7</td>
</tr>
<tr>
<td>P. americana</td>
<td>0.1</td>
<td>20.2±3.5</td>
<td>24.6±3.9</td>
<td>4.4±1.3</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>19.1±0.9</td>
<td>38.3±5.5</td>
<td>19.8±4.8</td>
<td>4</td>
</tr>
<tr>
<td>A. domesticus</td>
<td>0.1</td>
<td>21.6±1.1</td>
<td>31.1±2.9</td>
<td>10.6±2.4</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>18.5±1.2</td>
<td>31.7±2.6</td>
<td>13.3±2.1</td>
<td>7</td>
</tr>
</tbody>
</table>

The corpus cardiacum is a paired structure and one half is designated as 0.5 pr CC. Values are means±SE.
Fig. 2.1. Dose-response curve of 4-6 day-old adult Acheta to 1 μl injections of $5 \times 10^{-8}$ to $5 \times 10^{-1}$ equivalent concentrations of extracts of Acheta CC. The response was measured as the change in plasma lipids (mg/ml) 30 min after injection. Each point represents the mean±SE (n=12).
Fig. 2.2. Top: Separation of *Acheta* adipokinetic hormone (sample A) from a water/methanolic extract of 90 CC's with a gradient from 43% solution B (57% solution A) to a 53% solution B over a period of 20 min at a flow rate of 1 ml/min on a reverse phase C18 column. Solution A was dist. water + 0.11% TFA and solution B was 60% acetonitrile + 0.1% TFA. The peak (a) is homogenous. Bottom: Retention times of Pea-CAH-I (a), Pea-CAH-II (b) (300 pmol each), Lom-AKH-I (c), and Lom-AKH-II (d) (100 pmol each) run with the same gradient.
Fig. 2.3. Separation of *Acheta* adipokinetic hormone from a water/methanolic extract of 150 CC's (sample B) with the same gradient as described in Fig.1. Note that peak (a) is not homogenous, but contains a double peak.
Sequence data

Both samples were incubated with pyroglutamate aminopeptidase and chromatographed on RP-HPLC (Figs. 2.4 and 2.5). Sample A showed a new absorbance peak at 5 min (Fig. 2.4, top) after incubation with the enzyme, and almost complete digestion was achieved after 30 min (Fig. 2.4, bottom). The new absorbance peak had a retention time about 3 min less than the intact molecule. This peak yielded the sequence given in Table 2.2. The last amino acid was detected in cycle 7 (tryptophan), and the next two gave no degradation product. The tryptophan peak was barely above background, and its identity based on the degradation procedure is not certain. Sample B was chromatographed with a shallower gradient and a 30 min digestion resulted in 3 major peaks labelled a, b, and c. These were manually collected and sequenced. Peak a yielded no data, apparently not being a peptide; peak b had the same sequence as the peptide from sample A (Table 2.2) and peak c was found to be a longer peptide and did not belong to the AKH/RPCH-peptide family (Table 2.3).

Together with the N-terminal pyroglutamate residue, which was removed by enzymatic digestion, the sequence of the adipokinetic hormone from *A. domesticus* turns out to be an octapeptide, and the other substance a hexadecapeptide. The structure of the octapeptide is identical to the peptides separated from the corpora cardiaca of
Fig. 2.4. The enzymatic deblocking of *Acheta* adipokinetic hormone (sample A, from Fig. 2.2) by means of incubation with pyroglutamate aminopeptidase and the separation of the product. The gradient was from 25% B to 60% B over 30 min at flow rate of 1 ml/min. After 5 min of digestion the chromatogram (top) shows mostly the presence of the substrate Grb-AKH (a), but after 30 min the chromatogram of the same digestion mixture shows the predominance of the product Grb-AKH minus the pGlu (b).
Fig. 2.5. The enzymatic deblocking of sample B (from Fig. 2.2) by means of incubation with pyroglutamate aminopeptidase and the RP-HPLC separation of the digestion products after 30 min. The gradient was from 25% B to 60% B over 50 min at a flow rate of 1 ml/min. Peak a: unknown, not a peptide. Peak b: same as b in Fig. 2.3 (Grb-AKH). Peak c: a hexadecapeptide (probably diuretic hormone, see Results).
Table 2.2. Amino acid sequence of adipokinetic hormone of *Acheta domesticus* by automated Edman degradation.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Sample A recovery (pmol)</th>
<th>Sample B, peak b recovery (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VAL¹</td>
<td>24</td>
<td>34</td>
</tr>
<tr>
<td>ASN²</td>
<td>40</td>
<td>36</td>
</tr>
<tr>
<td>PHE³</td>
<td>31</td>
<td>34</td>
</tr>
<tr>
<td>SER⁴</td>
<td>16</td>
<td>10</td>
</tr>
<tr>
<td>THR⁵</td>
<td>19</td>
<td>15</td>
</tr>
<tr>
<td>GLY⁶</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>(TRP⁷)*</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>_⁸</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>_⁹</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Peak barely above background (see Results)
Table 2.3. Amino acid sequence of peak c from sample B.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Recovery (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARG\textsuperscript{1}</td>
<td>116</td>
</tr>
<tr>
<td>ASP\textsuperscript{2}</td>
<td>136</td>
</tr>
<tr>
<td>ILE\textsuperscript{3}</td>
<td>241</td>
</tr>
<tr>
<td>PHE\textsuperscript{4}</td>
<td>211</td>
</tr>
<tr>
<td>HIS\textsuperscript{5}</td>
<td>46</td>
</tr>
<tr>
<td>ALA\textsuperscript{6}</td>
<td>139</td>
</tr>
<tr>
<td>GLU\textsuperscript{7}</td>
<td>90</td>
</tr>
<tr>
<td>THR\textsuperscript{8}</td>
<td>72</td>
</tr>
<tr>
<td>ASP\textsuperscript{9}</td>
<td>99</td>
</tr>
<tr>
<td>ILE\textsuperscript{10}</td>
<td>64</td>
</tr>
<tr>
<td>PHE\textsuperscript{11}</td>
<td>48</td>
</tr>
<tr>
<td>GLU\textsuperscript{12}</td>
<td>35</td>
</tr>
<tr>
<td>VAL\textsuperscript{13}</td>
<td>26</td>
</tr>
<tr>
<td>PRO\textsuperscript{14}</td>
<td>21</td>
</tr>
<tr>
<td>LYS\textsuperscript{15}</td>
<td>3</td>
</tr>
<tr>
<td>_\textsuperscript{16}</td>
<td>_</td>
</tr>
<tr>
<td>_\textsuperscript{17}</td>
<td>_</td>
</tr>
</tbody>
</table>
G. bimaculatus (Grb-AKH; Gäde and Rinehart, 1987) and the grasshopper Romalea microptera (formally called Ro II, Gäde et al., 1988). The other peptide has previously been sequenced from A. domesticus corpora cardiaca and was identified as a putative diuretic hormone (Coast et al., 1989).

**Time- and dose-response in hyperlipemia to AKH injection:**

5-6 consecutive samples were collected from each cricket for the time-response study to minimize error. 3-5 day old adult virgins were used for the time- and dose-response curves because these animals produced a consistently high response (Fig.2.6). The base line of the lipid titer in 5 day old female crickets was 20 ± 2 mg/ml and the maximal increase in response to the hormone was 20 ± 2 mg/ml, or about 100%. The ED$_{50}$ of Grb-AKH was about 0.8 pmol and for Lom-AKH-I (Locusta) it was about 1.2 pmol (Fig. 2.6). The maximum hyperlipemic response was achieved with 2 pmol for Grb-AKH and with about 5 pmol for Lom-AKH-I. The peak response to both peptides occurred about 2 h after injection (Fig.2.7); the hyperlipemic response of Grb-AKH was about 25% higher.

**The variation of fat body response with age and starvation:**

The response of the fat body during days 6 and 7 of the last larval instar to Grb-AKH was as high as in the older adults (Fig. 2.8). There was an increase in blood lipid in response to AKH during the larval instar,
Fig. 2.6. Dose-response curves for 3-5 day old virgin females. The increase in blood lipids was measured 60 min after injection of the hormone. For each Grb-AKH point (closed circles) n = 5 and for each Lom-AKH-I point (open circles) n = 8.
Fig. 2.7. Time-response curves for 3-5 day old virgin females. For Grb-AKH (closed circles) 5 pmol was injected and each point represents \( n = 5 \). For Lom-AKH-I (open circles) 50 pmol was injected and each point represents \( n = 8 \). The closed triangles represent water controls (\( n = 5 \)).
declining on the last day. Likewise there was an increasing AKH response during the first 3-4 days of the adult stage, after which the response gradually declined over the next 10 days. The blood lipid level in crickets starved for 48 hr at any age through day 12 did not change in fed crickets. For example, when 6-day old females were starved for 2 days the lipid level was 17.1±1.2 mg/ml (n=19) compared to unstarved controls 21.1±1.6 mg/ml (n=30). However, with 4 days of starvation the lipid level declined (P<0.01, Student’s t-test) to 12.0±0.09 mg/ml (n=24). In crickets starved for 2 days the hyperlipemic response 30 min after injection with 50 pmol of Locusta migratoria adipokinetic hormone I was 9 mg/ml on day 2 which declined steadily with age to 3 mg/ml in day 12 insects (Fig. 2.9)

**AKH content of corpus cardiacum:**

The content of the CC in Acheta did not vary significantly from an average of about 15 pmol throughout the last larval instar and through the first 9 days of life of the virgin adult female (Fig. 2.10). The AKH content of 16 day old virgin females was also varied little, 11.6 ± 0.78 (n=4, pooled samples), indicating no significant change through day 16.

**Stimulation of the phosphorylase a system:**

Acheta adipokinetic hormone (Grb-AKH) activates the fat body glycogen phosphorylase a system as evidenced by the increased percent of phosphorylase a (Table 2.4).
Fig. 2.8. Age-response curves for *Acheta*. 1 μl of 5 pmol of synthetic Grb-AKH was injected and the increase in blood lipids measured after 60 min. Each point for adults (filled circles) n = 5, for larvae (open circles) n = 6. The closed triangles represent water-injected adult controls (n = 5).
Fig. 2.9. Age-response curve of plasma lipids by starved adult *Acheta* to injections of 50 pmol locust adipokinetic hormone I (solid line) at 2-day intervals. Each group of crickets were starved for 48 hr prior to injection. Saline was injected as a control (dashed line). The response was measured as the change in plasma lipids (mg/ml) 30 min after injection. Each point represents the mean±SE (n=8).
Fig. 2.10. Grb-AKH content of corpora cardiaca. For each sample the CC's from 10 crickets were homogenized in cold water and lyophilized. Open circles are larvae and closed circles are adults. For each age, three samples were collected, n = 3. Methanolic extracts were injected onto a reverse phase C 18 column and an isocratic mobile phase of 30% acetonitrile (0.1% TFA) run. The AKH concentrations were derived by comparison of peak heights of CC extracts compared to that of synthetic Grb-AKH standards.
Table 2.4. The effect of synthetic Grb-AKH on the phosphorylase a system on the fat body of anesthetized Acheta.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>% Phosphorylase a</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injected 3 μl dist. water</td>
<td>42.2 ± 9.5%</td>
<td>10</td>
</tr>
<tr>
<td>Injected with 4 pmol Grb-AKH</td>
<td>70.9 ± 4.9%</td>
<td>5</td>
</tr>
</tbody>
</table>

Animals were sacrificed 15 min after injection.
Handling crickets also appeared to stimulate the system, as indicated by the relatively high percent phosphorylase a in the controls (40%).

**DISCUSSION**

**Primary sequence:**

RP-HPLC of the methanolic extract of *A. domesticus* corpora cardiaca showed in some samples one absorbance peak associated with adipokinetic activity in *A. domesticus*, in other samples two absorbance peaks which ran close to each other. Brief enzymatic digestion of these fractions with pyroglutamatic aminopeptidase yielded deblocked peptides which were accessible for sequence analyses by Edman-degradation. Sequential degradation revealed an octapeptide as the adipokinetic hormone of *A. domesticus* and a hexadecapeptide for the coeluting peptide. Although not proven in this study it is assumed that the octapeptide is blocked at its C-terminus, as all other members of the AKH/RPCH-family have a primary amide at the C-terminus. It was only indirectly shown here, because the natural peptide from *A. domesticus* CC extract had the same retention time as the synthetic peptide Grb-AKH on RP-HPLC. Thus, we have shown that *A. domesticus* contains the same peptide as the other cricket analyzed so far, *G. bimaculatus* (Gåde and Rinehart, 1987). An identical peptide was also identified
as one of the two members of the AKH/RPCH-family in the CC of the grasshopper, *Romalea microptera* (Gäde et al., 1988).

There are several instances of the same peptide occurring in two or even three different species (in two different genera) (Gäde, 1990b), but this is the first instance of the same AKH peptide occurring in three different genera. It has been suggested (Raina and Gäde, 1988) that the nomenclature of a peptide should be based on the first report, which for this hormone was for *Gryllus bimaculatus* and thus the name of the peptide in *Acheta* should be Grb-AKH. As more peptides from the CC’s of more insects are sequenced their relationship will undoubtedly contribute to understanding phylogenetic concepts in the genera or families involved. In cockroaches it was found that there was a good agreement of the phylogenetic tree constructed by morphological and physiological data and the existence of specific hypertrehalosemic hormones (Gäde, 1989). The occurrence of an identical AKH molecule in two cricket genera, *Acheta* and *Gryllus*, can be rationalized by their close relationship. The occurrence of the same peptide in *Romalea* could indicate common ancestry, but more work needs to be done and many more peptides identified.

**AKH characteristics:**

The dose response curve for *Acheta* indicate that the receptor sites have a higher affinity, that is, a better receptor fit, for its own AKH than for that of the locust.
In studies of naturally occurring members of the AKH/RPCH-family and assessing their hyperlipemic activity in Locusta, it was shown that the locust’s own AKH (Lom-AKH-I) had the greater affinity (lowest ED_{50}) and the higher ED_{\text{max}} (Gäde, 1990; Goldsworthy et al., 1986; Gäde, 1990). Even comparing the two AKH’s in Locusta, Lom-AKH-I had the greater affinity (lowest ED_{50}) and the higher ED_{\text{max}} (Goldsworthy et al., 1986; Gäde, 1990). The time to maximum response and time to recovery after injection of Grb-AKH and Lom-AKH-I was typical of that observed in Carausius (Gäde and Lohr, 1982), Tenebrio (Rosinsky and Gäde, 1988), Manduca (Ziegler and Schulz, 1986) and adult Locusta (Goldsworthy et al., 1972), a maximum response in about 90-120 min with recovery to baseline levels in about 6 h.

**Effect of age on fat body response:**

The response of adult Acheta to synthetic cricket AKH was from 2 to 3 times greater than that of the last larval stadium (this study). In the last instar the response doubled by day 6. In the adult stage of Acheta the response tripled from day 0 to day 4 then gradually declined over the next 12 days. A previous study had indicated that the response of adult Acheta to synthetic locust AKH (Lom-AKH-I) decreased after about 6-8 days (Woodring et al., 1989). Similar changes in response to AKH with age have been reported for other insects. The hyperlipemic response of Locusta to injections of CC extracts of adult Tenebrio
increased 3-fold over the age period from day 0 to day 15 of the donor beetle (Gäde, 1988). Likewise the response of Locusta to CC extracts of Carausius doubled with older donors (Gäde, 1980). During the last instar the response of Locusta to extracts of its own CC was much less than during the adult stage, but there was a peak of responsiveness in the middle of the larval instar (Mwangi and Goldsworthy, 1977). During the first few days of the adult stage of Locusta there was essentially no response to CC extracts; the response then increased dramatically (5-times resting titres) to reach a plateau by day 8 and then declined back to a very low (almost control titers) after day 35 (Mwangi and Goldsworthy, 1977). This response of adult Locusta to its own AKH (CC extract) is similar to that of Acheta, which also shows an initial increase in responsiveness followed by gradual decline.

The reason for the incomplete AKH response in young adult Locusta was not due to a shortage of available lipids, since ample dietary and stored lipids were present (Hill and Goldsworthy, 1968). Nor was the lack of response due to a shortage of the hormone, since AKH elicits in both larvae and adults an elevation of cAMP concentration in the fat body (Gäde and Beenakkers, 1977). It was suggested (Mwangi and Goldsworthy, 1977) that the reason for the lower AKH response in young locusts might involve the reduced ability to form lipoprotein A+ in the blood, which
is essential for the binding of the increased amounts of diacylglycerols released after AKH injection. However, injections of adult lipophorin (A^t and C_2 proteins) into larvae did not result in adult levels of response to AKH by larval fat body, therefore larval lipophorin composition is not the cause of reduced response to AKH (Van der Horst et al., 1987). However, adult fat body incubated in larval blood showed a reduced adipokinetic response, indicating that other larval blood factors might restrict AKH action (Van der Horst et al., 1987).

Allatectomy has essentially no effect on AKH-induced hyperlipaemia in Locusta (Pener et al., 1989; Goldsworthy et al., 1972), indicating no role of JH in response to AKH. However, in fifth-instar adultiform larval Locusta, induced by precocene treatment, the hyperlipemic response to synthetic AKH was equal to that of the adult stage (Pener et al., 1989). This prompted the suggestion that JH deficiency in adults may be responsible for high imaginal competence for the response to AKH (Pener et al., 1989). This seems unlikely in adult Acheta because the JH titer rises shortly after the final moult (Renucci and Strambi, 1983) which is at the same time the response to synthetic AKH increases.

If neither JH nor the composition of the lipophorin explain age dependent responsiveness of the fat body to AKH, then another unidentified factor must be involved.
Larval lipophorin functions in the transport of lipids to fat body storage depots and growing tissues (Van der Horst et al., 1987). The increasing AKH response in larval Acheta is related to the rapid accumulation (storage) of lipids in the fat body which peaks on days 5-6 (Clifford and Woodring, 1986). The increased response in the adult stage is related to the rapid transport of lipids from the fat body into the oocytes (Clifford and Woodring, 1986).

However, in Locusta the AKH responsiveness increased in both males and females, indicating that ovarial growth was not a critical factor (Mwangi and Goldsworthy, 1977). The conclusion is that more work needs to be done to explain the fluctuations in AKH response in insects.

**Effect of age on AKH content in CC:**

There was no significant change in AKH content found throughout the last larval instar, between the last instar larvae and adult stage or through the first 9 days of the adult female life in Acheta. In Locusta there was little change between early and late last instar female larvae (99 to 104 pmol/CC), and little change from larvae to young adult (Siegert and Mordue, 1986). However, in the adult females there was a doubling from day 0 to day 10 (127 to 271 pmol/CC) and an almost 6-fold increase from day 1 to day 42 (752 pmol/CC) (Siegert and Mordue, 1986). It was suggested that the gradual increase in AKH pool in the locust CC might be related to the time required to develop
full flight potential (Siegert and Mordue, 1986). In this regard it is interesting that adult Acheta lose flight capacity within 3-4 days following the last molt (Clifford and Woodring, 1986). The content of AKH in Locusta was almost an order of magnitude greater than what is reported here for Acheta. Two factors that might contribute to a larger AKH pool in Locusta CC would be their larger size and the capacity of the animal for long distance flight. It should also be remembered that the size of the AKH pool in the CC does not measure the production or the release rates of AKH. As far as Acheta is concerned, this means that more AKH could be produced and released at certain times during the life cycle even though the content of the CC remained constant. The regulation of the adipokinetic response would likely be based on both AKH release rate and the responsiveness of the target cells.

**Activation of fat body phosphorylase a:**

The hyperlipemic action of Grb-AKH is associated with an activation of the fat body glycogen phosphorylase a system. In the present study, about 30% of the fat body glycogen phosphorylase of control, anaesthetized adult Acheta is in the active form, which is within the range (20%) for control Acheta previously reported (Ziegler et al., 1979). This is about twice the values reported for control adult Locusta (Gäde, 1981; Van Marrewijk et al., 1984). Acheta are much more active and excitable than
Locusta and the high percentage phosphorylase a in control Acheta may represent a response to agitation. Further handling did elevate the percent phosphorylase a to 40%. Locusta phosphorylase a could also be elevated to 30% by rotating or shaking the insects in a bottle (Gäde, 1981). A basic unanswered question is what does the breakdown of glycogen to glucose-1-phosphate, which is the function of the phosphorylase a system, have to do with the primary function of AKH which is to release diacylglycerols into the blood. Perhaps the glycolytic pathway would be used to make glycerol-3-P via glyceraldehyde-3-P from the glucose-6-P formed from increased phosphorylase a activity. In the fat body of insects that utilize lipids for energy, fat body triacylglycerol is degraded first to 2-monoacylglycerol (plus two fatty acids), followed by reacylation to 1,2 diacylglycerol (Beenakkers et al, 1985). The glycerol-3-P from AKH activation of phosphorylase a activation would be used for synthesize additional diacylglycerols from the resultant extra fatty acids.
CHAPTER 3

DIEL RHYTHMS OF ADIPOKINETIC HORMONE, FAT BODY RESPONSE, AND BLOOD LIPID AND SUGAR LEVELS IN *ACHETA DOMESTICUS*
INTRODUCTION

The majority of insects have daily and annual cycles of activities and development (Saunders, 1982), the most important amongst them are the behavioral and developmental circadian rhythms (Beck, 1980). It is to be expected that the behavioral and developmental circadian rhythms in insects are overt expressions of underlying physiological processes that govern them (Beck, 1980). Though little work has been done to determine the effect of photoperiod on the concentration of biomolecules in insects, several workers have described diel or circadian changes in the levels of glycogen, trehalose, and other sugars in blood or tissues (Nowosielski and Patton, 1964; Gersch, 1976), blood protein (Hayes et al., 1970), and brain and blood biogenic amine concentration (Muszynska-Pytel and Cymborowski, 1978; Woodring et al., 1988).

Insects have a relatively high total blood lipid content ranging from 1.5 to 5.5 percent, which fluctuates under a variety of conditions such as developmental stages, locomotor activity, starvation, disease, etc. (Mullins, 1985). In all insects investigated thus far, the major neutral lipid component in the blood is diacylglycerol, and its release from the fat body is mediated by adipokinetic hormone (AKH) (Beenakkers et al., 1981). Three adipokinetic hormones, vis., Lom-AKH I, Lom-AKH II and Lom-AKH III have been isolated from the glandular lobe of the corpora
cardiaca of locusts, and injections of these hormones into insects causes hyperlipemia or hyperglycemia, depending on the recipient species (Goldsworthy et al., 1986; Gade, 1990; Oudejans et al., 1991). Synthetic Lom-AKH I as well as the native peptide (Grb-AKH) isolated from Acheta corpora cardiaca (Woodring et al., 1990) increase the blood lipid level in the adult house cricket, Acheta domesticus (Woodring et al., 1989).

Circadian rhythms in locomotor activity, feeding, drinking, oxygen consumption, acetylcholinesterase activity in the brain, level of blood carbohydrate, and in the level of serotonin and octopamine in blood and brain have been demonstrated in the house cricket (Cymborowski, 1970; Cymborowski et al., 1970; Woodring and Clifford, 1986; Woodring et al., 1988). The responsiveness to adipokinetic hormone in eliciting increases in blood lipid levels in Acheta significantly varies between crickets from photophase and scotophase (Cusinato et al., 1991). The present work was undertaken in order to identify and characterize some of the control mechanisms for variation, most specifically adipokinetic hormone. The first step was to identify the diel rhythms of adipokinetic hormone in the corpora cardiaca. The second step was to relate this rhythm to the rhythms of blood lipids and carbohydrates and to the responsiveness of the fat body. We also wanted to determine the effects of photoperiod, temperature cycle and
starvation on the entrainment or modulation of the rhythms of blood sugars.

**MATERIALS AND METHODS**

**Rearing condition:**

Crickets were reared according to the methods of Clifford and Woodring (1990) at 30±1°C under a L:D 14:10 photoregime. Only unmated female crickets were used. For the study on rhythms of adipokinetic hormone sensitivity, day 4 adult females were used because during that time of their life cycle they have a peak sensitivity to adipokinetic hormone (Woodring et al., 1989).

For the study on rhythms of blood sugars, newly ecdysed last instar female cricket larvae were separated and placed under various light conditions or total darkness for 24 days and were assayed as 15 day old adults. The effect of a temperature cycle was carried out by comparing control crickets raised in continuous darkness and constant temperature (30±1°C) for 24 days to an experimental group raised in continuous darkness for 24 day but exposed to a thermocycle of 14:10 30°C:25°C for their last 14 days.

**HPLC separation and quantification:**

Each test extract consisted of ten pooled corpora cardiaca that were sonicated for 30 sec in distilled water and centrifuged at 8000 g for 2 min at room temperature. The supernatant was freeze-dried, the peptides
resolubilized in 200 μl of 80% methanol and again centrifuged at 8000 g for 2 min. The methanolic supernatant was injected directly onto the HPLC column.

An isocratic solvent system with 26% acetonitrile in water containing 0.1% trifluoroacetic acid was used on a reverse phase C₁₈ column with a solvent flow rate of 1 ml/min to identify and quantify cricket adipokinetic hormone (Grb-AKH). Synthetic Grb-AKH was used as the standard and the corresponding peaks in samples were identified and quantified by comparing them to the peak heights of the standards (Fig. 3.1).

Bioassay:

Crickets were anesthetized lightly with CO₂, and 2 μl of blood was collected from the foreleg coxa. Following injection with 50 pmol of synthetic locust adipokinetic hormone (Lom-AKH I) (in 1 μl distilled water), blood samples were collected after 1 h for the lipid assay and after 30 min for the carbohydrate assay. Total blood lipid content was measured by the colorimetric sulphophosphovanillin assay method (Barnes and Blackstock, 1973) and total blood carbohydrate was determined by the phenol-sulfuric acid method (Montgomery, 1957). The initial levels (at each time interval) before the injection of Lom-AKH I were used to determine the normal daily cycle of the blood lipid or carbohydrate. The increased blood lipid or carbohydrate titer at each time interval after injection of
synthetic Lom-AKH I was a measure of the hyperlipemic or hyperglycemic response, hence, a measure of the sensitivity of the fat body to the hormone. 50 pmol Lom-AKH I was chosen because it induced the maximum rise in blood lipid (Woodring et al., 1989).

**Statistical analysis:**

The data were statistically analyzed with ANOVA and Student’s t-test with 95% confidence level (p<0.05). Duncan's multiple range test and Dunnett's multiple comparison test were performed post ANOVA with a critical \( \alpha = 0.05 \) to statistically define regions of significant difference (peaks).

**RESULTS**

**Identification of Acheta adipokinetic hormone peak:**

HPLC separation of Acheta corpora cardiaca (CC) extracts yielded several peaks (Fig. 3.1B), but one fraction was found to contain the hyperlipemic activity. This fraction was identified as cricket Grb-AKH, and was further confirmed by coelution with synthetic cricket Grb-AKH (Fig. 3.1C).

**Diel variation in adipokinetic hormone (Grb-AKH) in the corpora cardiaca:**

A distinct rhythmic pattern (\( P < 0.05 \), ANOVA) with three significant peaks (\( P < 0.05 \), Dunnett) in the adipokinetic hormone (Grb-AKH) content of the corpora cardiaca was found
in 4-day-old females raised in a LD 14:10h photoregime (Fig. 3.2A). The first peak occurred just before the photophase (0400-0600 h), the second peak occurred during the mid-day (1200 h) and the third and the highest peak occurred at the beginning of the scotophase (2200 h).

**Diel variation in the blood lipid level and fat body sensitivity to adipokinetic hormone:**

A diel rhythm (P<0.05, ANOVA) with two peaks (P<0.05, Dunnett) in the levels of blood lipid was observed in 4-day-old adult females raised on a LD 14:10h photoregime (Fig. 3.2B). The basal lipid level remained relatively stable (19-23 mg/ml) during the scotophase with one peak occurring during the first half of the photophase (1100 h) and a second occurring at the beginning of the scotophase (2200 h). There was nearly an 80% elevation over the lowest level at 0500 and 1300 h.

Treatment with Lom-AKH I invariably caused an increase (P<0.05, Student’s t-test) of the lipid level at any time of the day. There were two peaks of responsiveness to Lom-AKH-I, a sharp peak occurring in the early half of the photophase (1100 h), and a broad night-time peak with a slight dip just after lights-off. In adult females raised on a LD 14:10h photoregime (Fig. 3.2B), the basal lipid level remained relatively stable (19-23 mg/ml) during the scotophase with one peak occurring during the first half of the photophase (1100 h) and a second occurring at the
Fig. 3.1. Separation of adipokinetic hormone (Grb-AKH) from Acheta corpora cardiaca (CC) extracts run on a reverse phase C$_{18}$ HPLC column. (A) 50 pmol synthetic Grb-AKH (retention time 7.4 min). (B) 2.5 CC equivalent (retention time of the Grb-AKH peak 7.39 min). (C) 2.5 CC equivalent spiked with 50 pmol synthetic Grb-AKH (retention time 7.44 min). The Grb-AKH area (height x width at 50% height) in (C) is identical to the combined Grb-AKH areas under (A) and (B).
Fig. 3.2. (A) Diel changes in the Grb-AKH content of the corpora cardiaca (CC) from 4-day-old adult female crickets raised in LD 14:10h. Each point, the mean ± SE, was determined from 3 separate runs of pooled CC samples each containing 10 pairs of CCs. All three peaks (*) are statistically higher (P<0.05, Dunnett) than the remaining values (P<0.05, Dunnett). (B) Diel changes in blood lipid levels (●) and the diel change of the hyperlipemic effect (= change in blood lipid titer)(○). The hyperlipemic effect was measured as the increase over control lipid levels 60 min after injection with 100 pmol Lom-AKH I. Each point represents the mean ± SE from 6 crickets. Two peaks values (*) are statistically higher (P<0.05, Dunnett) than the remaining, though the second peak of hyperlipemic effect broadly extends through most of the night. (C) Diel changes in control blood carbohydrate levels (■) in 3-day-old larvae and in 4-day-old adult females (●). The peaks values (*) were significantly higher (P<0.05, Dunnett) that the remaining values. The diel changes in the hyperglycemic effect in response to injections Lom-AKH I (○). There was no hyperglycemic effect over control sugar levels 30 min after injection. Each point represents the mean ± SE from 6 crickets.
beginning of the scotophase (2200 h). There was nearly an 80% elevation over the lowest level at 0500 and 1300 h.

Treatment with Lom-AKH I invariably caused an increase (P<0.05, Student’s t-test) of the lipid level at any time of the day. There were two peaks of responsiveness to Lom-AKH-I, a sharp peak occurring in the early half of the photophase (1100 h), and a broad night-time peak with a slight dip just after lights-off.

**Diel variation in the total blood carbohydrate level:**

A distinct daily variation (P<0.05, ANOVA) with two separate peaks (P<0.05, Dunnett) in the blood carbohydrate concentration was observed in 4 day old females of LD 14:10h photoperiod (Fig. 3.2C). A broad and sustained peak occurred during most of the scotophase with the highest value occurring at 0600 h and a second peak was observed during the late photophase. However, in 3-day-old last instar nymphs, only a single broad peak (P<0.05, Duncan) was observed at the end of scotophase (0700 h). Injection of 50 pmol of Lom-AKH-I had no apparent effect on the blood carbohydrate level in adult Acheta.

**Effect of photoperiod on diel rhythm in blood carbohydrate level:**

Though the blood sugar rhythms under the conditions of different photoregimes appear bimodal, only the second and more distinct peak occurring in the early scotophase is statistically significant (p<0.05, Duncan) (Fig. 3.3). In
the regime with the longest photophase (LD 16:8h), there was no significant rhythm. The blood sugar rhythm is clearly endogenous, because it free ran for 24 days in crickets maintained in D:D (Fig. 3.3). The period of the free running rhythm was approximately 23 h.

**Effect of a temperature cycle on the diel rhythm of blood carbohydrate levels:**

An imposed rhythmic temperature regime (30°:25°C 14:10) had no effect on the basic pattern of blood sugars in crickets maintained in D:D when compared to a control group at a constant 30°C (Fig. 3.4). The peak blood sugar in control and temperature cycled groups were very similar with a single significant peak at 1900 h (p<0.05, Duncan).

**Effect of starvation on diel rhythm of blood carbohydrate level:**

Though starvation caused an overall decrease in the blood sugar level, a carbohydrate rhythm persisted in starved crickets, indicating that the rise in blood carbohydrates in fed crickets was not merely a response to feeding and absorption of sugars from the gut (Fig. 3.5). In contrast to the single, very broad scotophase peak starting just before lights off (p<0.05, Duncan) in fed 9 day old females, the carbohydrate peak elevation in a group of starved 9 day old females shifted from the scotophase into early photophase.
Fig. 3.3. Effect of a photoperiod on diel rhythms in blood carbohydrate levels of 15-day-old adult crickets. The scotophase for each group is represented by the solid bars in the X-axis under the respective curves, from top to bottom LD 16:8, 14:10, 12:12, 8:16h and DD. The crickets were exposed to these photoregimes for 24 days. Each point represents the mean ± SE from 5-11 crickets. There was a single peak (*) of blood carbohydrates (P<0.05, Duncan) in each photoregime except in photoregime LD 16:8h.
Fig. 3.4. Effect of a temperature cycle on diel rhythms of blood carbohydrate levels of 15-day-old adult females maintained in complete darkness for 24 days. The control group (dashed line) was exposed to a constant 30°C and the treatment group (solid line) was exposed to a daily temperature cycle. Each point represents the mean ± SE of 6-8 crickets. Peaks in both curves are statistically significant (P<0.05, Duncan), and there was no significant difference (Students t-test) in sugar level between the control and treatment group.
Fig. 3.5. Effect of starvation on diel rhythms of blood carbohydrate levels in 9-day-old adult females raised in LD 14:10h. The control groups (solid line) were always provided food and the starved group (dashed line) had the food removed on day 7 (starved for 2 days). Each point represents the mean ± SE of 6-8 crickets. The control group had a rather broad peak throughout most of the night (*) while the starved group had a more distinct peak (P<0.05, Duncan) (**) at the end of the scotophase.
DISCUSSION

In the house cricket, Acheta domesticus, distinct rhythms of adipokinetic hormone content of the corpora cardiaca, lipid and carbohydrate levels in the blood and a rhythm of sensitivity of the fat body to injected adipokinetic hormone were demonstrated. This is the first report of diel rhythms of a neuropeptide hormone and of blood lipid in insects. A previous report of a diel rhythm of blood ecdysterone titer and of prothoracic gland activity in the fleshfly, Sarcophaga bullata (Roberts, 1984), indirectly suggests a neuropeptide rhythm (prothoracicotropin hormone). The present study also demonstrates the endogenous nature of the blood carbohydrate rhythm which lasted at least 24 days in constant darkness.

Though 4-day-old adult females were used as the experimental animal for the determination of the rhythms, it was also noted that the blood lipid level was always higher in the morning than in the afternoon in 2d, 6d and 8d-old females (data not shown), which indicates the diel rhythm of blood lipids is a continuously running rhythm. Not having tested the lipid levels, adipokinetic hormone levels or the sensitivity of the hormone on crickets maintained in D:D during these days, no conclusion can be made concerning the endogenous aspects of these rhythms.
In *Locusta* the hyperlipemic effect of injected adipokinetic hormone was low in the first day or two following the final moult, increased greatly on day 4 and remained high for at least 40 days (Mwangi and Goldsworthy, 1977). In *Acheta* the hyperlipemic response doubled from day 1 to day 4 and only gradually declined in intensity up to day 16 (Woodring et al., 1990). We had previously shown that the adipokinetic hormone content of corpora cardiaca of *Acheta* remained unchanged through the first 9 days following adult ecdysis (Woodring et al., 1990). The current study, however, clearly demonstrated that during day 4 a diel rhythm of adipokinetic hormone content in the corpora cardiaca of *Acheta* is discernible. The only other insect hormone titers with a diurnal variation reported so far are the blood ecdysterone titers in the wax moth *Galleria mellonella* (Cymborowski et al., 1989; 1991) and in the fleshfly *Sarcophaga bullata* (Roberts, 1984), and the ecdysteroid synthesis rate in the prothoracic glands of *Rhodnius prolixus* (Vafopoulou and Steel, 1989).

An elevated content of adipokinetic hormone in the corpora cardiaca during the early and the late scotophase was clearly correlated with an increased blood lipid titer. However, a peak of hormone content in the corpora cardiaca could mean either a peak of synthesis and release or a peak of storage with minimal or even a constant rate of release. The rate of synthesis of adipokinetic hormone in house
cicricket was not measured in this study. Diet changes in
the content of neurosecretory cells in the brain of Acheta
were found to be correlated with the locomotor activity. A
peak of RNA and protein synthesis were observed to precede
the peak of neurosecretory material accumulation
(Cymborowski, 1983).

The two major peaks of blood lipid titer correlated in
time with the major peaks of fat body sensitivity to
injections of a constant amount of adipokinetic hormone.
This would suggest that, lacking a rhythm of hormone
release from the corpora cardiaca, the rhythm of blood
lipid titer could be attributed to the rhythm of fat body
sensitivity to the hormone. Adipokinetic hormone binds to
specific membrane receptors which induce production of
cyclic AMP. cAMP activates an inactive protein kinase to
its active form, which induces an active lipase to initiate
the conversion from triacylglycerol to diglyceride to be
released into circulation (Gادة and Beenakkers, 1977; Gادة,
1990b). Thus, the rhythm of sensitivity of the fat body to
adipokinetic hormone found in our study may indicate a
rhythm in the number and sensitivity of available
adipokinetic hormone-receptors in the membrane. If the rate
of adipokinetic hormone release from the corpora cardiaca
and the sensitivity of the fat body to adipokinetic hormone
are both rhythmic, then the regulation of blood lipid
titers is indeed complex.
A blood carbohydrate rhythm was observed in the last instar cricket larvae which corroborates an earlier report (Nowosielski and Patton, 1964). Our study showed that the cricket larvae are able to establish a distinct circadian rhythm of blood sugar in the larval or adult stage in absence of any photic cue, indicating that the circadian clock controlling the sugar rhythm in both larvae and adult crickets is intrinsic and light-entrainable. It was previously shown that the adipokinetic hormone of Acheta (Grb-AKH) regulated the mobilization of lipids but that this same peptide had no effect on the blood sugar titers (Woodring et al., 1989). Therefore, it seems unlikely that any rhythm of adipokinetic hormone in the corpora cardiaca or in the blood would be responsible for the observed blood sugar rhythm in this species. The blood sugar rhythm could be regulated by other hormones, for example, an insulin-like peptide (Moreau et al., 1982; Loughton, 1989), or the regulation could involve enzymes controlling the release of trehalose from the fat body (Steele, 1983). Octopamine is also a possible regulator of the blood sugar rhythm, because a peak in both blood and brain concentrations of octopamine was found in Acheta (Woodring et al., 1988) which roughly correlates with the broad scotophase peak of blood carbohydrate levels.

The light cycle with the longest photoperiod (LD 16:8h) in the current study dampened the blood carbohydrate
rhythm. Similar loss of rhythmicity in L:L (though long photophase and L:L are not strictly comparable) was shown by Linn et al. (1992) to abolish the rhythmicity of male responsiveness to pheromones in the gypsy moth, Lymantria dispar. In the cricket, Gryllus bimaculatus, circadian rhythmicity of hatching is lost in the eggs kept in constant light (Tomioka et al., 1991).

Temperature and thermocycles play important roles in the development and induction of circadian rhythms in insects. Increased temperature generally increases the intensity of response but not the periodicity of response (Saunders, 1982). Daily rhythms of temperature change have been associated with faster development so long as the temperature is within the optimal range of the species (Beck, 1986). In Periplaneta a pulsed 5°C change in the thermoregime during a D:D regime was sufficient to entrain a locomotor rhythm (Roberts, 1962). A thermoregime (30°C:25°C 14:10) in crickets maintained on D:D had virtually no effect on the periodicity of the blood sugar rhythm in Acheta.

Photoperiod-inducible circadian rhythm of locomotor activity, feeding and oxygen consumption rhythms develop in adult Acheta by the second or third day after the final moult, the peaks of all three rhythms occurring in the early part of the scotophase (Woodring and Clifford, 1986). However it was observed that food is always present in the
crop and it is therefore difficult to see how the feeding rhythm could directly affect the rhythms of blood carbohydrates and lipids. If food is continuously present in the gut, then there should be a steady influx of sugars and lipids from the midgut into the blood. In any case, the major blood lipid peak (early photophase) appears not to be related to the feeding rhythm because it does not correspond to the time of peak feeding. The blood carbohydrate peak is in the early scotophase and appears to be endogenous because it persists in continuous dark. However, the rhythm shifts to late scotophase in starved crickets, which indicates a possible relation of the feeding rhythm to the blood carbohydrate titer.
CHAPTER 4

EFFECT OF ADIPOKINETIC HORMONE ON LIPID AND PROTEIN SYNTHESIS IN THE HOUSE CRICKET, *ACHETA DOMESTICUS*
INTRODUCTION

In locusts, adipokinetic hormone (AKH) inhibits 90% of vitellogenin synthesis and 62% of total protein synthesis during the end of gonotropin cycle (Moshitzky and Applebaum, 1990). This observation significantly changed the scenario of the existing knowledge about the Orthopteran egg maturation process because it had already been established that the appearance of vitellogenins in insect blood is initiated by juvenile hormone (JH). In crickets, head ligation or allatectomy prevents egg maturation (Benford and Bradley, 1986). Thus, identification of two hormones (JH and AKH), which act antagonistically to control protein synthesis, is indicative of the pleotropic nature of metabolic hormones.

The family of insect adipokinetic/red pigment-concentrating hormones (AKH/RPHC) characteristically elevate blood lipids or carbohydrates by mobilizing appropriate fat body stores (Keely et al., 1991b). In most cases the AKH peptides stimulate the insect fat body to break down metabolic stores (triglycerides or glycogen) for the synthesis and release of energy compounds, either diglycerides or trehalose, into the blood. Also, under certain physiological conditions, AKH is involved in the control of synthesis of fat body lipids (Gokuldas et al., 1988).
The house cricket, *Acheta domesticus* is a frequently used experimental animal in cellular and physiological research; however, very little is known about the control of lipogenesis and lipolysis in this animal. The fat body of the cricket, which is analogous to the vertebrate liver, is the principal site of lipid storage and metabolism (Bahjou et al, 1990). The lipids synthesized by the fat body are also essential to the developing oocytes in the ovary because lipids are one of the basic storage nutrients for future embryonic development. Though it is known that AKH mediates the lipid mobilization in many insects, no specific hormone with a role in fat body or ovarian lipid synthesis or uptake has yet been identified. The purpose of the present investigation was to determine the possible role of *Gryllus bimaculatus* adipokinetic hormone (Grb-AKH) on lipid and protein synthesis in the fat body and the ovary during vitellogenic stages of female crickets and in the fat body of male crickets.

**MATERIALS AND METHODS**

**Insects and tissue preparation:**

Four d adult female and eight d adult male house cricket, *Acheta domesticus*, reared in the laboratory at 30°C and 14L:10D photoperiod and fed ad libitum with purina cricket chow, were used in these experiments. Under this condition, vitellogenesis starts on the 2nd day and reaches
maximum intensity on the 4th day. Crickets were anesthetized with CO₂, a 40-50 mg sample of fat body and ovary (only the developing ovarian follicles, all mature eggs were discarded) was quickly dissected and washed in cricket saline. The tissue was incubated in 1 ml of Schneider's Drosophila media (Gibco, Grand Island, N.Y.) in tissue-culture wells on a shaker bath for 4 h in a 95% O₂ and 5% CO₂ environment.

**In vitro rate of protein synthesis and secretion:**

In order to determine the steady-state protein synthesis by the fat body over 6 h, sufficient radiolabelled leucine was added to the medium to achieve a final specific activity of 0.2 uCi per ml. Fat body or ovary was incubated for 4 h in the ¹⁴C-leucine-media, the tissue thoroughly washed with ringer and the total TCA-precipitable protein from the tissue and the incubation media were extracted. The radioactivity of the protein samples were counted in a scintillation counter. To measure protein secretion, the total protein content of the incubation media and that of the fat body were determined by the method of Bradford with Bio-Rad protein dye using bovine serum globulin as the standard.

**Hormone treatments:**

An appropriate amount of *Gryllus bimaculatus* adipokinetic hormone (Grb-AKH) (Peninsular Laboratories, Belmont) dissolved in a stock solution containing 80%
methanol was dried and mixed with the incubation media. In most cases, the control tissue came from the same insect. Grb-AKH is the active peptide released from the corpus cardiacum of Acheta domesticus (Woodring et al, 1990).

4 d adult female crickets were CO₂-anesthetized and various doses of AKH were injected in 1 µl volume. After waiting 30 min, 2 µl of ¹⁴C-leucine (0.2 µCi specific activity) was injected in both control and treated insects. After 4 h, 10 µl of blood, the fat body, and the developing ovarian follicles were collected from each insect and the total TCA-precipitable proteins were extracted and radioactivity counted.

**In vitro rate of Lipid Secretion, Lipogenesis and Lipid uptake:**

The method used in this experiment to determine the rate of lipid synthesis utilizes high specific activity tritiated water in the incubation medium and later determines the incorporation of tritium label in tissue lipids (Jungas, 1968). Tritium from the tritiated water freely interchanges positions with the H⁺ of cellular NADPH+H⁺, thus labeling some of the cellular NADPH+H⁺. As all the hydrogens of the carbon chains of a fatty acid come from NADPH+H⁺, lipogenesis in a tissue can be monitored by measuring the incorporation of tritium in the tissue lipid.

The tissues were transferred after incubation in the Schneider’s Drosophila medium into 100% methanol. A 10 µl
aliquot of the medium was taken and the lipid content was measured by means of the phosphovanillin method (Zöllner and Kirsch, 1962). The tissue was homogenized, 4 ml chloroform and 2 ml 0.05% CaCl₂ was added and vortexed. The upper aqueous layer was discarded, 3 ml of the organic phase was added to 6 ml of scintillation cocktail and the mixture counted for tritium for 10 min in a scintillation counter.

To determine the rate of lipid uptake by the tissue, ¹⁴C-palmitate was used in the medium instead of ¹⁴C-leucine.

**Statistical analysis**

The data were statistically analyzed using Student’s t-test at a significance level of P<0.05.

**RESULTS**

**Effect of Grb-AKH on female in vitro fat body protein secretion and synthesis:**

In female crickets head-ligated for 24 h, the rate of protein secretion in an in vitro incubation system significantly decreases compared to the unligated female crickets (Fig. 4.1). Addition of Grb-AKH in the incubation media at the doses of 2, 5, 10 and 20 pmol/ml of media did not alter the rate of in vitro protein secretion by fat body from unligated or head-ligated crickets.
Fig. 4.1. Effect of various doses of Grb-AKH on the rate of protein secretion (μg protein/h/mg tissue protein) by the fat body from unligated and head-ligated 4 d old adult female crickets. The doses used were 2 (cross-hatched), 5 (blank), 10 (dotted) and 20 (solid bar) pmol/ml of media. Note that there was statistically significant difference in rate of protein secretion between unligated and head-ligated group (*, P<0.05), but there was no effect of Grb-AKH treatment when compared to the respective controls. Bars represent Mean±SE (n=6-8).
Grb-AKH was not effective in influencing the rate of \textit{in vitro} protein synthesis, into tissue protein or into the proteins secreted by the tissue into the medium (Figs 4.2 and 4.3). Likewise, there was no evidence of an \textit{in vivo} effect of Grb-AKH on the $^{14}$C-leucine incorporation into fat body, ovary, or blood protein (Fig. 4.4).

\textbf{Effect of Grb-AKH on male in vitro fat body protein synthesis:}

Compared to the 4 d old female crickets where protein synthesis remained unaffected by Grb-AKH treatment, the 8 d old male crickets showed significant inhibition of protein synthesis. Significantly less $^{14}$C-leucine was incorporated into the fat body protein as well as into the proteins secreted by the fat body in the medium (Fig. 4.5).

\textbf{Effect of Grb-AKH on female in vitro fat body and ovary lipid secretion and synthesis:}

Following the addition of Grb-AKH to the media, the effect on lipid secretion into the medium by the fat body tissue was apparent at doses of 0.5 pmol or higher (Fig. 4.6). The increase in lipid secretion was dose dependent. The control rate of lipid secretion in the ovary was lower compared to that of the fat body and there was no change in response to any doses of Grb-AKH treatment (Fig. 4.6).
Fig. 4.2. Effect of various doses of Grb-AKH on the $^{14}$C leucine incorporation in the protein secreted into the incubation media by fat body from female crickets. Dotted bars represent respective control and the solid bars represent the treated groups. Bars represent Mean±SE (n=6-8).
Fig. 4.3. Effect of various doses of Grb-AKH on the $^{14}$C leucine incorporation in tissue proteins in ovary and fat body of female crickets. The hatched bars represent respective controls of ovary tissue, the solid bars the treated ovarian tissue, the striped bar control fat body and the dotted bars represent treated fat body. Each bar represents Mean±SE (n=6-8).
Fig. 4.4. Effect of various doses of Grb-AKH on the $^{14}$C leucine incorporation in vivo in fat body, ovary and blood of 4 d old adult female crickets. The light bars represent respective controls for the Grb-AKH treated groups (solid line). Each bar represents Mean±SE (n=6-8).
Lipid synthesis in the fat body, as evident by incorporation of tritium in tissue lipids, decreased with treatment of AKH. The lowest dose used, 0.05 pmol, did not decrease the rate of lipid synthesis, but 5 and 10 pmol Grb-AKH caused a decrease in lipid synthesis to 46-48% of the rate of lipid synthesis in the control tissues (Fig. 4.7).

Lipid synthesis in the ovary was not affected by any dose of Grb-AKH (Fig. 4.7) except that of 10 pmol/ml. The apparent decrease in synthesis in response to 10 pmol Grb-AKH/ml medium may not be real because of the unusually high control value for that particular group. $^{14}$C-palmitate incorporation into fat body or ovary tissues was not affected by Grb-AKH treatment (Fig. 4.8).

**DISCUSSION**

One function attributed to the adipokinetic hormone is the mobilization of sugars or lipids from the fat body by inducing the phosphorylase enzymes (Gäde, 1990), but the experiments reported here clearly demonstrated that the adipokinetic hormone is involved in lipid and protein synthesis in crickets.

Though it is generally regarded that lipolysis is controlled by AKH (Bahjou et al, 1990), hormonal control of lipogenesis in insects has not yet been investigated.
Fig. 4.5. Effect of Grb-AKH on $^{14}$C leucine incorporation in the proteins secreted by fat body and fat body tissue proteins in 8 d old male crickets. Hatched bars represent respective controls for fat body secreted protein, solid bars the treatment, the dotted bars the control for fat body tissue protein and the dark bars the respective treatment. (*)& denotes statistically significant difference at P<0.05. Each bar represents Mean±SE (n=6-8).
Fig. 4.6. Effect of Grb-AKH on rate of lipid secretion into the media by fat body and ovary from 4 d old female adults. Solid bars represent fat body and the dotted bars ovary. (*) denotes a statistically significant difference at $P<0.05$. Each bar represents Mean±SE (n=6-8).
Fig. 4.7. Effect of Grb-AKH on $^3$H incorporation in the fat body and ovarian tissue lipid. Hatched bar represents control fat body, solid bar treated fat body, striped bar control ovary and dotted bar represents treated ovary. (*) represents a statistically significant difference at $P<0.05$. Each bar represents Mean±SE (n=6-8).
Fig. 4.8. Effect of Grb-AKH on $^{14}$C palmitate incorporation into the tissue lipid in ovary and fat body of female crickets. Hatched bar represents control ovary, solid bar treated ovary, striped bar control fat body and the dotted bar represents treated fat body. Each bar represents Mean±SE ($n=6-8$).
Because allatectomized insects show increased lipid concentrations in the fat body compared to control insects, juvenile hormone (JH) is thought to inhibit lipid synthesis (Steele, 1985). However, in Locusta, juvenile hormone induces hypertrophy of the fat body, indicating a stimulation of lipid synthesis (Butterworth and Bodenstein, 1969). Our observation of the inhibitory role of adipokinetic hormone in both lipogenesis and protein synthesis in crickets points toward a very complex control mechanism of metabolism in insects.

The fat body and ovary of 4-d adult female cricket are very active due to their important role in ovary maturation. At this time, nutrients, such as proteins and lipids, must be stored in the egg for the development of the future embryo. Insect fat body, which is analogous to the vertebrate liver, takes an important role in ovary maturation. The bulk of protein and lipid reserve stored in the ooplasm is synthesized in the fat body and taken up from the blood by pinocytosis at the surface of the oocyte (Berry, 1985). In some insects, the nurse cells of the ovary are reported to produce nutrients and store them in the developing oocyte (Berry, 1985). Thus, to understand the process of ovarian maturation in insects, it is important to study hormonal control of protein and lipid synthesis in both the fat body and ovary.
Our experiment with *Acheta* did not conclusively prove that AKH inhibits vitellogenin synthesis in crickets, as has been reported from a similar species *Locusta* (Asher *et al.*, 1984). The inhibitory role of Grb-AKH in protein synthesis *in vitro* was only evident in male crickets. It is possible that because we used fat body and ovary from a time period in females when they are primed to produce huge amounts of proteins, exogenous AKH treatment can not significantly inhibit their already acquired ability to produce proteins. It is also probable that unlike locust AKH, which has an inhibitory control over vitellogenesis (Moshitzky and Applebaum, 1990), cricket AKH does not play such role in vitellogenin synthesis, but can instead inhibit synthesis of blood proteins in general.

Insect fat body is the most important organ controlling lipid metabolism because the processes of lipogenesis from simple precursors and lipolysis for the supply of energy to the tissues takes place here. Apart from this, the fat body also stores lipids. A notable aspect of lipid synthesis and utilization in insects is the control exercised by hormones (Steele, 1985). Lipids are stored in the tissue in the form of triacylglycerol, which in the time of need, breaks down to diacylglycerol and monoacylglycerol and fatty acids. Although adipokinetic hormone has been shown to have a direct role in lipolysis and lipid mobilization in several insect species, no single
hormone has been identified to have a positive effect on lipogenesis (Steele, 1985). It has been found in several insect species that allatectomy increases lipid deposition in the fat body and other organs (Walker and Bailey, 1971; Bailey \textit{et al.}, 1975). Enhanced secretion of lipid by fat body into the medium in response to Grb-AKH treatment, which is observed in this study, corroborates to other findings on the hyperlipemic action of Grb-AKH. This hormone probably binds to specific membrane receptors to increase the intracellular cAMP level which ultimately increases lipase activity. Increased lipolysis causes an increase in the lipid secretion to the medium. Comparable results were obtained from the \textit{in vivo} system. When crickets were treated with AKH, the hemolymph lipid level increased markedly (Woodring \textit{et al.}, 1989). Lipid synthesis in the fat body was inhibited by Grb-AKH at a dose of 1 pmol/ml or higher. AKH also inhibits lipid synthesis in locusts (Gokuldas \textit{et al.}, 1988).

In contrast to the results obtained with fat body, cricket ovary did not show a change in the rate of lipid secretion after Grb-AKH treatment. The ovary used in this experiment was from 4 d old females which are at the peak of ovarian development. It is assumed that these ovaries must be metabolically very active, and if there is any effect of AKH on the ovary, this tissue should be able to show the change in lipid metabolism. Unlike the fat body,
the ovary is not regarded as a metabolic storage site for lipid, though lipids are stored in the oocyte for future embryonic development (Berry, 1985). In general, the ovary does not secrete huge amounts of macromolecules, rather it sequesters and stores them. A probable reason for not observing an AKH response in ovary is that the lipids in the oocytes are marked for exclusive storage and not for any sort of metabolic turnover. Other probable reasons may include absence of adipokinetic hormone receptors or the absence of lipases in the ovary.
CHAPTER 5

HYPOLIPEMIC AND HYPOTREHALOSEMIC FACTORS FROM THE MID GUT OF THE HOUSE CRICKET, *ACHETA DOMESTICUS*
INTRODUCTION

Possessing a highly efficient flight ability, most adult insects have a requirement for quick as well as sustained energy supply from stored reserves. Most insects store large amount of both fat and glycogen, which serve as the source of energy during flight. In general, insects capable of long distance flight utilize lipid and insects that fly only short distances utilize carbohydrates as a fuel for flight activity (Beenakkers et al., 1985). A group of peptide hormones belonging to the AKH/RPCH family of neuropeptides have been identified in various insects, and control either lipid or carbohydrate mobilization (Gäde, 1990). The hyperlipemic factor in cricket is the adipokinetic hormone, Grb-AKH, but it plays no role in carbohydrate mobilization (Woodring et al., 1989, 1990).

It has been observed in many groups of animals that hormones work in antagonistic pairs; one stimulates while the other inhibits (Steele, 1985). In the honey bee and wasps, vertebrate insulin and glucagon like polypeptides from the mid gut have been associated with hypolipemia and hypotrehalosemia (Moreau et al., 1981). The subesophageal ganglion has been shown to control lipid metabolism in the silkworm Bombyx mori (Hasegawa and Yamashita, 1967). Removal of the median neurosecretory cells in the brain inhibits carbohydrate metabolism in Locusta migratoria (Goldsworthy, 1971). Insulin-like peptides with similar
biological activities have been identified from the brain of blowfly (Duve et al., 1979; Lavenseau et al., 1984). None of these factors have been well characterized in their physiological action and are not well understood. No hypolipemic or hypotrehalosemic factor has been reported in crickets. The purpose of the present investigation was to identify and characterize the hypolipemic or hypotrehalosemic factors present in the house crickets.

MATERIALS AND METHODS

Insects:

Only 3-4 d old adult female house cricket, Acheta domesticus, reared in the laboratory (Clifford et al., 1977) on Purina cricket chow at 30°C and 14L:10D photoperiod, were used in these experiments. In order to eliminate the effect of the hyperlipemic factor, stored and secreted from the CC, all lipid bioassays were performed on crickets head-ligated 24 h prior to the assay.

Preparation of various tissue extracts:

CO₂-anesthetized Crickets were quickly dissected and the brain, subesophageal ganglion, foregut, midgut and hindgut were separated, gut contents thoroughly rinsed out, washed in cold cricket saline and transferred into a 50 mM phosphate buffer solution with 1 mM EDTA. Blood was collected in 80% methanol. Brain, subesophageal ganglion and blood were sonicated, the gut tissues homogenized in glass homogenizer at 4°C, and then centrifuged at 4°C for 30
min at 900 g. Some of the methanolic extract of the blood was vacuum-dried, and the pellet redissolved in 50 mM phosphate buffer to obtain a water-extraction of the putative hypolipemic factor. The pellet and the fat layer were discarded, supernatants were collected and recentrifuged for 30 min at 16000 g. After discarding the pellet and the fat layer, the supernatants were lyophilized and stored until use. The lyophilized materials were resupended just before use in appropriated amount of water.

**Bioassays:**

Following injection of the test materials, blood samples were collected for lipid or carbohydrate assay after 15, 30, 60 and 120 min depending on the type of experiment. Crickets were anesthetized lightly with CO₂ and 1 µl of blood was collected from the foreleg coxa. The change in blood lipid or carbohydrate titer at each time interval after injection of test materials was used as the measure of the hypo- or hyperlipemic or glycemic response. The blood lipid titer was measured by the colorimetric sulphophosphovanillin assay method (Barnes and Blackstock, 1973) and total blood carbohydrate (anthrone-positive material; glucose standard) was determined using the methods of Spik and Montreuil (1964) as modified by Holwerda et al. (1977).

For determination of blood glucose and trehalose level, blood samples were collected in the HPLC-running
buffer (75% acetonitrile and 25% water) and stored at -20°C. No change in the sugar concentrations were noted even after 3 weeks of storage under these conditions. Blood glucose and trehalose were separated by HPLC according to the methods of Gåde (1991b) with a Hewlett Packard refractive index detector. Peaks were quantified by comparison to standards. All data were statistically analyzed by two-tailed Student’s t-test with a 95% confidence level (P<0.05).

RESULTS

Effect of insulin, glucagon and extracts of the blood:

Compared to the control group (23.17±2.00 mg/ml, n=6), the blood lipid titers were 21.85±1.79 mg/ml (n=8) after insulin-treatment and 29.48±1.75 mg/ml (n=9) after glucagon-treatment. There was no effect of vertebrate insulin on the blood lipid titer of head-ligated crickets. On the other hand, glucagon induced a slight hyperlipemia (at P<0.05) in the head ligated crickets. Neither methanolic extracts nor water extracts of the blood at a dose of 20 μl equivalent of blood was able to elicit any response in the circulating blood lipid titer within 30 min after injection (Fig. 5.1).

Effect of tissue extracts on blood lipid level:

Tissue extracts were tested at a dose of 1 insect equivalent per test animal. Extracts of brain, subesophageal ganlion, fore gut and hind gut had no effect
Fig. 5.1. Effect of methanolic and water extract of cricket blood on the blood lipid level of head-ligated crickets. Each bar represents Mean±SE (n=6). Control crickets were injected with Ringer's. The open bar represents the titer before injection and the dark bars the titer 30 min after injection.
Fig. 5.2. Effect of various tissue extracts on the blood lipid level of head-ligated crickets. (*) denotes a statistically significant difference (P<0.05) from the control group. Each bar represents Mean±SE (n=6).
on blood lipid level (Fig. 5.2). Only the mid gut extract elicited a hypolipemic response after 30 min of treatment (P<0.05, student's t-test). When the crickets were injected with mid gut extract treated at 95°C for 5 min, no hypolipemic response in blood lipid level was observed (Fig. 5.3). The induced hypolipemia was totally abolished by the hyperlipemic action of injected Grb-AKH. A dose-dependent hypolipemic response was observed (Fig. 5.4). The least responsive dose was 0.5 mid gut equivalent with a saturation at 1 mid gut equivalent.

A time response relationship in the induced hypolipemia was also observed in crickets (Fig. 5.5). The maximum response was observed at 15 min after injection which gradually declined to the control level after 2 h.

**Hypotrehalosemic and hyperglycemic effects of insulin, mid gut and corpus cardiacum extracts:**

Injection of corpus cardiacum extract and insulin did not affect the glucose or trehalose levels in the blood of cricket. Mid gut extract did not decrease the total blood sugar level in crickets at 30 min after treatment; 3.46±0.28 mg/ml (n=6) in control crickets and 3.15±0.32 (n=6) in crickets injected with mid gut extracts. However, injections of mid gut extract elevated the levels of glucose and decreased the trehalose level (Fig. 5.6).
Fig. 5.3. Effect of injected heat-treated mid gut extract, mid gut extracts and Grb-AKH on the blood lipid titer of head-ligated crickets minutes after injection. Control crickets were injected with cricket Ringer's. (*) denotes statistically significant difference (P<0.05) from the control group. Each bar represents Mean±SE (n=6-8).
Fig. 5.4. Dose-dependent hypolipemic response to injection of mid gut extracts into head-ligated crickets. Each point represents the difference (Mean±SE, n=6-8) in blood lipid titer between treated group and respective control group after 30 min of treatment.
Fig. 5.5. Time-dependent responsiveness of hypolipemia in head-ligated crickets injected with 1 equivalent of mid gut extracts. Blood samples were collected from the same crickets at intervals of 0, 15, 30, 60 and 120 min. Each point represents the difference (Mean±SE, n=6) in blood lipid titre between treated and the control (injected Ringer's) group. (*) represents statistically significant differenc (P<0.05).
Fig. 5.6. The hypotrehalosemic and hyperglycemic response in the crickets injected with 1 equivalent of mid gut extract. Blood was collected from the control and treated crickets at 0, 5 and 15 min after treatment. Control crickets were injected with 1 μl Ringer's. (O) and (●) represent changes in trehalose levels; and (Δ) and (▲) represent changes in blood glucose levels in control and injected groups respectively. Each point represents the difference (Mean±SE, n=5) in trehalose or glucose titres from respective values at 0 min.
DISCUSSION

This report indicates the presence of hypolipemic and hypotrehalosemic factors in the mid gut extracts of the house cricket *Acheta domesticus*. The importance of a hyperlipemic factor (AKH) in mobilization of lipid, from the fat body into the blood, has been demonstrated (Woodring *et al.*, 1990; Cusinato *et al.*, 1993). This new evidence of hypolipemic factors in the uptake of the lipid from the blood into tissues will provide a better understanding of the overall control of lipid uptake and release in insects. Adipokinetic hormone does not induce sugar mobilization in *Acheta* (Woodring *et al.*, 1989; this study). However, the current study indicates that probably factors from the mid gut may control glucose and trehalose mobilization.

Some vertebrate hormones such as insulin and glucagon evoke similar vertebrate type responses in some insects (Ishay *et al.*, 1976; Bounias, 1979; Moreau *et al.*, 1982). When insulin is injected into insects, it produces hypoglycemia and hypolipemia. Conversely, glucagon treatment generally produces hyperlipemia and hyperglycemia. This indicates that comparable hormone-receptor systems are present in both of these distantly related animal groups, and suggests that the hypolipemic factor in insects might be an insulin-like protein.
A hypolipemic factor has been identified so far from *Locusta* and show immunological cross-reactivity with insulin (Moreau *et al.*, 1982; Lavenseau *et al.*, 1984). However, insulin did not change the blood lipid titers in head-ligated crickets, which may indicate that the hypolipemic factor is not structurally similar to vertebrate insulin as in *Locusta*.

Glucagon-induced hyperglycemia and hypertrehalosemia has been reported from *Schistocerca*, *Locusta* and *Apis* (Normann and Duve, 1969; Loughton, 1987; Bounias and Pacheco, 1979). In the house cricket, we detected a slight but statistically significant hyperlipemia induced by glucagon. Thus, like the locust, *Acheta* gives a hyperlipemic response to both insect adipokinetic hormones as well as vertebrate glucagon.

Loughton (1987) identified a water-extractable hypolipemic factor in locust blood effective at a concentration of 100 µl blood equivalents. The response was found only after induced hyperlipemia. The factor was heat labile and the hypolipemic activity was blocked by protein synthesis inhibitors such as puromycin and cyclohexamide. These observations suggest that the hypolipemic factor in *Locusta* is a protein. Neither methanolic extracts nor water-extracts of *Acheta* blood had a hypolipemic action in *Acheta*. Possibly, the amount of extract we used (20 µl blood equivalent/cricket) did not contain enough
hypolipemic factor to elicit a response. We also did not induce hyperlipemia before running the assay.

Among the various tissue extracts assayed for a hypolipemic response in crickets, only the mid gut extract induced hypolipemia. Heat treatment (95°C) abolished the hypolipemic effect suggesting that the hypolipemic factor is a protein. Another interesting observation was the change from hypolipemic to hyperlipemic response when the crickets were simultaneously treated with adipokinetic hormone (Fig. 5.3). Thus it is possible that AKH is more effective than the hypolipemic factors; however, since the concentration of the hypolipemic factor was not determined, it was not possible to compare the efficacy of these two factors.

The least effective dose to induce a hypolipemic effect was found to be 0.5 mid gut equivalent and a saturation was achieved at a dose of 1 mid gut equivalent. When the relative size of the mid gut is considered, this dose seemed to be rather high, but it also indicates that the number of molecules of the hypolipemic factor present in the mid gut is probably low. A dose-response curve with a saturable system also signifies the presence of a receptor system through which the hormone works. The time response curve indicated the strongest hypolipemic response at 15 min after injection of mid gut extract which subsequently subsides after 30 min. This observation also
reinforces the idea that these hypolipemic factors are very short-lived, probably proteinaceous, with a high turn-over rate.

There was no hypotrehalosemic or hypoglycemic factor in the corpus cardiacum of Acheta. However, injection of mid gut extracts of Acheta induced a hypotrehalosemic response, and was also associated with a hyperglucosemia response. A similar phenomenon has been reported in honey bees (Das and Woodring, unpublished).

The present work indicates the possible coupling of a pair of antagonistic hormonal factors regulating the level of blood lipid: adipokinetic hormone acting as the hyperlipemic factor and a heat-labile molecule (probably a protein) acting as the hypolipemic factor. This work is also the first report of putative factors having hyperglucosemic and hypotrehalosemic actions present in the mid gut of house cricket Acheta domesticus.
CONCLUSION

HPLC fractions isolated from methanolic extracts of the corpora cardiaca from the honeybee *Apis mellifera* and the paper wasp *Polistes exclamans* had a single active fraction that was able to elevate lipid titers in *Acheta* in a dose-dependent fashion. Pyroglutamate aminopeptidase digestion of isolated active fractions resulted in a shifted peak when rerun on the same column, indicating a peptide with a terminal pGLU.

Methanolic extracts of the corpora cardiaca of *Apis* and *Polistes* elevated the total blood sugar titer when injected into *Periplaneta americana* and the blood lipid titer when injected into *Locusta migratoria*. HPLC separation of blood sugars with a refractive index detector permitted quantitation of fructose, glucose and trehalose. Injections of *Polistes* corpus cardiacum extracts into *Polistes* resulted in a significant elevation of the trehalose titer and a slight decrease in fructose and glucose titer after 30 and 60 min. Injection of *Apis* corpus cardiacum extracts into *Apis* resulted in a significant increase after 15, 30 and 60 min in the glucose titer and a decrease in the fructose and trehalose titer.

Paper wasps utilize sugar transported in the crop for most of their energy needs, but they can also mobilize trehalose from the large glycogen reserves in the fat body.
so that they can be active during warm days in the winter when no nectar sources are available. Honeybees on the other hand lack significant fat body reserves of glycogen or lipid, but stored sugar reserves are always available in the hive and honeybees can carry up to one third of their weight as sugar in their crop. It may be suggested from the indications found during the course of this research that perhaps the metabolic peptides in the honeybee corpus cardiacum facilitate the transport of glucose across the midgut epithelium, and additionally the conversion of fructose to glucose.

The principle neuropeptide separated by reverse-phase liquid chromatography (RP-HPLC) from extracts of the corpora cardiaca of *Acheta domesticus* showed strong adipokinetic activity when injected into *Acheta*. The N-terminal pyroglutamate of the peptide was removed by enzymatic digestion, and the remaining peptide sequenced. The structure is identical to the peptide Grb-AKH previously described from the corpus cardiacum (CC) of *Gryllus bimaculatus* (pGlu-Val-Asn-Phe-Ser-Thr-Gly-Trp-NH$_2$). The ED$_{50}$ was 0.8 pmol and saturation was achieved with injection of 2 pmol of synthetic Grb-AKH. The time to achieve the maximum hyperlipaemic response was 90-120 min. The response of the fat body to injected synthetic Grb-AKH doubled in 4 days during the last stadium, but was never greater than half the maximum response of the adult stage.
The adult adipokinetic response doubled from the first to the fourth day, then gradually declined through day 16. The increased AKH response was time-correlated to fat storage in the larvae and to lipid deposits in the oocytes in the adult. Synthetic Grb-AKH activated glycogen phosphorylase in the fat body of *Acheta*. The amount of Grb-AKH present in the CC changed very little throughout the last larval stadium and through the first 9 days of the adult stage, averaging about 15 pmol/gland pair. A second peptide (a hexadecapeptide) was isolated from the CC of *Acheta* and sequenced. Its structure is identical to a putative diuretic hormone previously described in *Acheta*.

Adipokinetic hormone content in the corpora cardiaca of the house cricket, *Acheta domesticus*, varied during the day with two peaks in the scotophase and one peak in the photophase. There were two distinct peaks of total lipid concentration in the blood, one early in the photophase and the other early in the scotophase. Fat body sensitivity to adipokinetic hormone also varied in close synchrony with the lipid rhythm. It was not possible to attribute the rhythm of blood lipid titer unequivocally to either the rhythm of adipokinetic hormone content in the corpora cardiaca or to the rhythm of sensitivity of the fat body to the hormone. In adult crickets, blood carbohydrate titer underwent two characteristic peaks, one towards the end of scotophase and another in the late photophase, about 12 h
apart; whereas, in larval crickets, a single peak was observed at the end of scotophase. Adipokinetic hormone was not able to mobilize carbohydrates in Acheta. Exposure to various daily photoregimes changed the patterns of the carbohydrate rhythms. Imposition of a cyclic temperature regime in constant dark did not compensate for the lack of photic cues. Starvation for 2 d significantly changed the pattern of blood carbohydrate rhythms and indicated a significant role of feeding in the sugar rhythm.

The effect of adipokinetic hormone on lipid metabolism in fat body and ovary of the 3 day old female Acheta domestica, was investigated. The adipokinetic hormone, Grb-AKH, increased the rate of lipid secretion by fat body, whereas in the ovary, there was no change in the rate of lipid synthesis. Grb-AKH also decreased the rate of lipid synthesis in the fat body, whereas lipid synthesis did not change in the ovary.

Extracts of fore-gut, mid-gut, hind-gut, brain, corpus cardiacum, corpus allatum and sub-esophageal ganglion were assayed for possible hypolipemic and hypoglycemic effects in Acheta domestica. Only the mid-gut extract showed a statistically significant hypolipemic effect when injected with a minimal effective dose of 0.5 mid-gut equivalent. The time-dependent responsiveness of hypolipemia was evident, which showed a maximum decrease in lipid titers within 15 min after extract injection with recovery to
control titers within 1 h. The mid-gut extract increased the blood glucose titer and decreased the blood trehalose titer within 15 min after injection. Thus, this experiment gave a preliminary indication that both hypolipemic and hypoglycemic factors are present in the mid-gut.
REFERENCES


Benford H. H. and Bradley J. T. (1986) Early detection and 
juvenile hormone-dependence of cricket vitellogenin. 

Berry S. J. (1985) Reproductive systems. In Fundamentals of 
Insect Physiology (Ed. by M. S. Blum), pp. 437-466. 

Bhakthan N. M. G. and Gilbert L. I. (1968) Effects of some 
vertebrate hormones on lipid mobilization in the 

Bounias M. (1979) Relations doses/effets de l’insuline et 

Bounias M. and Pacheco H. (1979) Sensibilité de l’abeille à 
l’action de l’insulaineet du glucagon injectés in vivo. 
C.r. hebd. Séanc. Acad. Sci., Paris, Série D 289, 201- 
204.

Bowers W. S. and Friedman S. (1963) Mobilization of fat 
body glycogen by an extract of corpus cardiacum. 

In Energy Metabolism in Insects (Ed. by R. G. H. 

Butterworth F. M. and Bodenstein D. (1969) Adipose tissue 
of Drosophila melanogaster IV. The effect of the 
corpus allatum and synthetic juvenile hormone on the 
tissue of the adult male. Gen. Comp.Endocrinol. 13, 
68-74.

Carlisle J. and Loughton B. G. (1979) Adipokinetic hormone 
inhibits protein synthesis in Locusta. Nature 282, 
420-421.

Cheeseman P. and Goldsworthy G. J. (1979) The release of 
adipokinetic hormone during flight and starvation in 

Chen A. C. and Friedman S. (1977) Hormonal regulation of 
trehalose metabolism in the blowfly Phormia regina 
Meig.: Effects of cardiaectomy and allatectomy at the 
subcellular level. Comp. Biochem. Physiol. 58B, 339- 
344.


Gäde G. (1990b) Structure-function studies on hypertrehalosaemic and adipokinetic hormones: activity of naturally occurring analogues and some N- and C-
terminal modified analogues. Physiol. Entomol. 15, 299-316.


Mayer R. J. and Candy D. J. (1969) Control of haemolymph lipid concentration during locust flight: An
adipokinetic hormone from the corpora cardiaca. J. Insect Physiol. 15, 611-620.


Nowosielski J. W. and Patton R. L. (1964) Daily fluctuations in the blood sugar concentrations of the


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