PROTEIN SYNTHESIS IN THE CORPORA ALLATA OF THE COCKROACH *DIPLOPTERA PUNCTATA*

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Zoology
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ABSTRACT

Protein synthesis in the cockroach *Diploptera punctata* changes depending on the developmental stage. In mated females, changes in protein synthesis parallels the cycle of juvenile hormone (JH) biosynthesis, although the cycle of protein synthesis is delayed by one-two days. JH biosynthesis is high on days 4 and 5 (maximum) and then decreases on day 6. Increased protein synthesis occurs on days 4, 5 and 6 (maximum) and then decreases on day 7. This apparent relationship was investigated further by using the inhibitors of protein synthesis, cycloheximide, anisomycin and actinomycin D or inhibitors of JH biosynthesis, the allatostatins, and examining effects on both processes. Protein synthesis was measured *in vitro* using incorporation of $[^{15}\text{S}]$-methionine, and JH biosynthesis measured using the radiochemical assay. The transcription inhibitor actinomycin D did not significantly inhibit protein synthesis in mated CA. However, translation inhibitors almost completely inhibited protein synthesis (89-94%). Neither transcription nor translation inhibitors have a significant effect on JH biosynthesis by the CA of day 7 mated females. Dip-AST 7 and 13 appear to have specific effects on the synthesis of certain proteins. Dip-AST 2, the most potent inhibitor of JH biosynthesis, showed no effect on protein synthesis.
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## Abbreviations

<table>
<thead>
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<th>Definition</th>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>A-site</td>
<td>acceptor site</td>
</tr>
<tr>
<td>AST</td>
<td>allatostatin</td>
</tr>
<tr>
<td>Ca⁺²</td>
<td>calcium</td>
</tr>
<tr>
<td>CA</td>
<td>corpora allata</td>
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<tr>
<td>Cav-AST</td>
<td><em>Calliphora vomitoria</em> allatostatins</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CC</td>
<td>corpora cardiaca</td>
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<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>JH</td>
<td>juvenile hormone</td>
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<tr>
<td>JHA</td>
<td>juvenile hormone acid</td>
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<tr>
<td>kDa</td>
<td>kilodalton</td>
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<tr>
<td>Mas-AST</td>
<td><em>Manduca sexta</em> allatostatin</td>
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<tr>
<td>min</td>
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<tr>
<td>mM</td>
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<tr>
<td>M</td>
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<tr>
<td>Mᵣ</td>
<td>relative molecular mass</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
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<tr>
<td>PKC</td>
<td>protein kinase C</td>
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<tr>
<td>P-site</td>
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<td>RF</td>
<td>specific protein factor</td>
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<tr>
<td>RER</td>
<td>rough endoplasmic reticulum</td>
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<tr>
<td>DAG</td>
<td>diacyl glycerol</td>
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<tr>
<td>Da</td>
<td>dalton</td>
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<tr>
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<td><em>Diploptera punctata</em> allatostatin</td>
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<tr>
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<td>Radioimmunoassay</td>
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<td>SDS-PAGE</td>
<td>sodium doecyl sulfate-polyacrylamide gel</td>
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<tr>
<td>SER</td>
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<tr>
<td>TCA</td>
<td>smooth endoplasmic reticulum</td>
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<tr>
<td>tRNA</td>
<td>Trichloroacetic acid</td>
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<td>μl</td>
<td>transfer ribonucleic acid</td>
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INTRODUCTION

I. Structure and function of the corpora allata

The corpora allata (CA) are endocrine glands which are the site of juvenile hormone biosynthesis and release. They are located in the posterior regions of the head, or in rare instances in the thorax. The location of the CA varies depending on the order of the insect. The more primitive position is a lateroventral location of paired glands, and the more derived condition, a more dorsal location of paired glands and fusion into a single body (Tobe & Stay, 1985). As noted by Cazal (1948) in most insects, the CA receive nerves from both the brain and the subesophageal ganglion (Tobe & Stay, 1985). However, in some primitive orders of insects, nerves appear to come only from the subesophageal ganglion. In some other species, innervation arises only from the brain.

Figure 1 shows a diagrammatic representation of the principal nervous tracts and their cells of origin in the brain-retrocerbral complex of Diploptera punctata (Thompson and Tobe, 1990).

The shape of the CA most often is ovoid to round but in some cases may be elongate or polylobed (Cazal, 1948). In the cockroach D. punctata the CA are paired, spheroidal glands and are “small-cell type” (Johnson et al., 1985), not the vesicular type described by Cazal (1948). The size of the glands varies between species; even within species; size differs with age, sex, polymorphism, and activity of the glands (Tobe & Stay, 1985). Changes in JH biosynthesis, CA volume and CA cell number have been shown to be interrelated. Szibbo and Tobe (1981), found that cell number, volume and cytoplasmic-nuclear ratio all increased to a maximum on day 5 in mated females and then
decreased in the latter half of the gonotrophic cycle. This pattern parallels the cycle of JH biosynthesis. These authors proposed that increases in cell number are the result of cell division and that the corresponding decreases in the later part of the gonotrophic cycle most likely involved cellular destruction. In a review by Tobe and Stay (1985), it has been noted that the increase in volume and cell number of CA during development is generally thought to be associated with somatic growth rather than an increased activity of the CA. Whereas Johnson et al. (1993), found that the increase in cell number did not occur in virgin *D. punctata*, they nevertheless suggested that mating stimulates an increase in both cell number and in cytoplasmic volume.

Comparisons of the ultrastructure of CA between species shows much variability, but some generalizations can be made. The CA are surrounded by a continuous noncellular basal lamina, which occasionally projects between glandular cells into the interior of the gland (Tobe & Stay, 1985). Gap junctions are present, suggesting that the CA cells are electrically coupled. Lococo et al., (1985) demonstrated this by dye injection and intercellular recordings in CA cells of *D. punctata*. Intercellular spaces often occur in the outer layer of cells of the CA in *D. punctata*, whereas the interior cells are more closely packed. However, as the first vitellogenic cycle proceeds, spaces may appear among interior cells (Johnson et al., 1985).

CA are usually rich in mitochondria and possess a large amount of smooth or rough endoplasmic reticulum, or both. Other organelles in the CA include, the nucleus, golgi complexes, ribosomes, lysosomes and their presence and individual morphology varies within and between species (Sedlak, 1985). Changes in the ultrastructure of the CA are correlated with change in rates of JH biosynthesis. There are noticeable
ultrastructural changes that occur with changing rates of JH biosynthesis (Johnson et al., 1985). In this same study, these authors demonstrated that the growing ovary stimulates both the increase in JH biosynthesis and changes in cytoplasmic organelles associated with elevated JH biosynthesis. In glands with increasing rates of JH biosynthesis, the cytoplasm is less compressed and the mitochondria have a less dense matrix, greater diameter and are more irregularly shaped. The smooth endoplasmic reticulum changes from easily seen to obscure tubules, whereas the rough endoplasmic reticulum appears longer and possesses highly curved segments. In the most highly active glands, newly formed autophagic vacuoles appear. In glands with decreasing rates of activity, the CA are compact with condensed cytoplasm and the mitochondrial matrix becomes denser with a smaller width and less irregular shapes. The smooth endoplasmic reticulum appears tubular and distinct, and the rough endoplasmic reticulum appears to be in whorls. Golgi complex are more conspicuous, and autophagic vacuoles continue to be present.

II. Juvenile Hormones

Juvenile hormones (JHs) are sesquiterpenoid molecules produced by the corpora allata (CA), and to date have been found in the class Insecta and in a few plant species. The various Juvenile hormones, JH I (C_{18}JH), JH II (C_{17}JH), and JH III (C_{16}JH), have been isolated from haemolymph, whole body extracts and from in vitro incubations of the CA in various species of insects. JH O and 4-methyl JH I have been isolated from embryos of M. sexta (Tobe and Stay, 1985). In the cockroach D. punctata, the JH present in adults, penultimate and final instars is JH III (Szibbo et al., 1982; Tobe et al., 1985).
Many studies indicate that JH I and JH II (and their acids) are found exclusively in the Lepidoptera, whereas most other insect orders produce JH III (Schooley et al., 1984; Wyatt and Davey, 1996). The JH III biosynthetic pathway is shown in figure 2. JH III bisepoxide (JHB₃), a derivative of JH III has been found in several higher dipterans (Richard et al., 1989 a, b; Cusson et al., 1991; Duve et al., 1992).

Juvenile hormones play a major role in the metamorphosis and reproduction of most insect species. During larval and pupal development, JH is involved in metamorphosis, whereas in adults it governs a wide variety of functions related to reproductive maturation and function in a range of tissues in both female and male insects (Wyatt and Davey, 1996). Low rates of JH biosynthesis are correlated with metamorphosis, whereas reproductive functions such as vitellogenesis in adult females are associated with high JH biosynthetic rates (Engelmann, 1979; Wigglesworth, 1964). In most insect species, at a critical stage in the final larval stage, JH disappears, allowing the completion of metamorphosis. With respect to larval development, JH modifies the actions of 20-hydroxyecdysone and suppresses the development of adult characters (Wyatt and Davey 1996). The suggestion that the presence of JH might influence ecdysteroid titre in D. punctata has been made on the basis of the observed elevation in ecdysteroid titres only after JH release began to decline (Kikukawa and Tobe 1986b). During reproduction, JH stimulates the synthesis and uptake of vitellogenins in adult females. JH is also involved in other aspects of reproduction such as, behaviour, the control of accessory gland function and early events in the gonads (Wyatt and Davey 1996; Davey 1984).
III. Juvenile hormone biosynthesis and titre

Juvenile hormone biosynthesis has been studied in many insect species. In *D. punctata*, there is a relationship between the maturation of oocytes and the cycle of JH biosynthesis in mated female cockroaches (Tobe and Stay, 1977; Tobe, 1980; Stay *et al.*, 1983; Rankin and Stay, 1984). Specifically, it was found that in the absence of the ovary, the CA synthesized JH at lower than normal rates (Stay *et al.*, 1983). Further examination of this relationship revealed that basal oocytes are integral components involved in the increase of JH biosynthesis. Ovaries with basal oocytes of various lengths were implanted into 2-day old mated ovariecutomized females and rates of JH biosynthesis were measured. This study showed that previtellogenic ovaries and those at the end of vitellogenesis are not associated with an increase in rates of JH biosynthesis, whereas vitellogenic ovaries were. The length of the basal oocytes are indicators of the degree of ovarian stimulation of JH biosynthesis (Rankin and Stay, 1984). To further show that this relationship between the ovary and JH biosynthesis occurred in mated females, male CA were placed into allatectomized females. The male CA responded to stimulatory and inhibitory signals from the ovary and they also showed changes in cell number and volume (Stay and Woodhead, 1990) analogous to those seen in mated female CA. Although this relationship exists in *D. punctata*, the relationship between the ovary and the activation of the CA seems to be species-specific, as this relationship has not been demonstrated in all insects. For example, in the cockroach *Blattella germanica*, activation of the CA was not dependent on the presence of the ovary (Gadot, *et al.*, 1991).
Tobe (1980) and Johnson et al. (1985) described a relationship in the first gonodotrophic cycle by relating oocyte size to the rate of JH release. When female adults emerge and mate, (basal) oocytes are previtellogenic and JH biosynthesis is low. On days 2-3 the oocytes become vitellogenic and rates of JH biosynthesis begin to rise. Rates climb to a maximum on days 4-5 when oocytes are continuing to grow. On days 5 and 6, rates of JH biosynthesis decline rapidly, while the oocytes continue to grow and become chorionated. On day 7 oviposition occurs and the oocytes are retracted into the brood sac. JH biosynthesis at this time is low. The cycle of JH biosynthesis in mated females was also described by a study that examined JH titre in *D. punctata* (Tobe et al., 1985). In mated females, a low JH titre is found in the first part of the first gonotrophic cycle, with maximum titres on days 4-5 and then declining rapidly thereafter, to low titres after day 6.

Virgin *D. punctata* do not mature oocytes at a rapid rate and do not show the cycle in JH biosynthesis described in mated females (Stay and Tobe, 1977). There are four larval stages in *D. punctata* and each of these exhibit distinct patterns of JH biosynthesis (Kikukawa and Tobe, 1986a). First instars show low rates of JH biosynthesis for the first six days, then an increased rate which remains high for the next three days, but sharply decreases thereafter. In second instars, rates of JH biosynthesis increase gradually from day 0-9, followed by reduced rates. In third instars, there is a high initial rate of production but within the first two days, the rate decreases sharply; rates then increase and remain high during the later half of the stadium. Fourth instars show high rates in newly ecdysed larvae but this level decreases sharply within the first 2 days. A small peak occurs on day 4, but thereafter, JH release declines and is virtually
zero by day 10. JH production is undetectable from day 10-18 (Kikukawa and Tobe, 1986a).

In addition to JH biosynthesis, the haemolymph titre may also be regulated in part by JH binding proteins and by JH degradation. The activities of degradative enzymes and the concentration of binding proteins change during insect life cycles and these changes may be related to fluctuations in hormone titre (De Kort and Granger, 1996). For example, JH esterase (JHE), an enzyme involved in degradation of JH in mated female D. punctata, has an activity profile which is generally opposite to the profiles of JH titre and biosynthesis (Tobe et al., 1985). Since JH titre and JHE activity are inversely correlated during day 5-8 in mated females, it has been suggested that this esterase may reduce the high titre of JH observed in day 5 animals (King and Tobe, 1988).

Juvenile hormone binding proteins (JHBPs) were first described by Whitmore and Gilbert (1972) and have since been studied in detail in a variety of different species. There are very specific high-affinity and low-affinity JHBPs in insect haemolymph, although the physiological significance of the low-affinity JHBPs is questionable (Trowell, 1992; Prestwich et al., 1994). The existence of a low molecular weight and two high molecular weight classes of JHBPs is well documented (De Kort and Granger, 1996). The haemolymph of the cockroach D. punctata contains a high molecular weight, high affinity JHBP. Binding of JH III to this JHBP is saturable, reversible and enantioselective (King and Tobe, 1988). This JHBP was later described as JH-III-binding lipophorin (JHBL) (King and Tobe, 1992) and the JH III binding site on JHBL resides on apolipophorin I (apoLp-1, 234 kDa). In D. punctata, lipophorin titre increases in day 5-6 mated females and declines significantly in final instars and mated females on day 6-7
(King and Tobe, 1993). Lipophorin titre corresponds to JH titre and biosynthesis in *D. punctata* (Tobe *et al.*, 1984, 1985). The decline of JHBL titre in day 6 mated females coincides with a large increase in JH esterase activity (Rotin *et al.*, 1982; King and Tobe, 1993).

**IV. Regulation of the corpora allata**

Regulation of the CA and JH biosynthesis is not fully understood, although it is thought that the regulation involves many factors, both extrinsic and intrinsic (Stay *et al.*, 1994; Tobe *et al.*, 1994). The interaction of a number of second messenger systems seem to play a role in the regulation of the CA. It is also known that in the cockroach *D. punctata*, the activity of the CA is in part under the control of inhibitory neuropeptides (Stay *et al.*, 1994). These neuropeptides are known as allatostatins (ASTs). There are also peptides that have stimulatory effects on JH biosynthesis in the CA, and these are known as allatotropins. The only characterized allatotropin to date is a 13 residue peptide found in the Lepidopteran, *Manduca sexta* (Kataoka *et al.*, 1989).

**a) Allatostatins**

A large family of neuropeptides that inhibit JH biosynthesis *in vitro* has been isolated from brains of *D. punctata* as well as from other species (Stay *et al.*, 1994). Seven different allatostatins ranging in size from 8-18 amino acids have been isolated and purified from *D. punctata* (Dip-ASTs) (Woodhead *et al.*, 1989; 1994; Pratt 1991). Allatostatins have been described in other species of cockroaches, including, *Periplaneta americana*, and *Blattella germanica* and also in the cricket *Gryllus bimaculatus* (Weaver *et al.*, 1994; Belles *et al.*, 1994; Lorenz *et al.*, 1995; Stay *et al.*, 1996). Five ASTs have
been isolated from the brain of the blowfly, *Calliphora vomitora* (Duve et al., 1993). These peptides are known as callatostatins (Cav-ASTs) and they have sequence similarity to cockroach ASTs. Although these five peptides inhibited JH production by the CA of *D. punctata in vitro*, none of them showed allatostatic activity in mature female *C. vomitora*. One AST has also been isolated from the tobacco hornworm, *Manduca sexta* (Mas-AST), but this peptide has no sequence similarity to the cockroach ASTs and only inhibits JH biosynthesis in selected Lepidoptera at specific stages (Kramer et al., 1991; Tobe et al., 1995). For a complete review of all known AST-like sequences determined to date in insects, see Bendena et al. (1997).

An AST-encoding gene was first isolated from *D. punctata* by polymerase chain reaction (PCR) amplifications using cockroach brain cDNA (Donly et al., 1993; Bendena et al., 1994). The sequence of the AST precursor contained all signals for an exported protein which could be processed into 13 amidated AST-type peptides. These peptide sequences confirmed the structures of the seven previously isolated ASTs (Woodhead et al., 1989; Woodhead et al., 1994; Pratt et al., 1991), and revealed six new candidates (Donly et al., 1993). These 13 peptides are characterized by a common C-terminal sequence Tyr/Phe-Xaa-Phe-Gly-Leu/Ile-NH2 (X= Ser, Gly, Asp, Ala, Asn), which *in vitro* appears to be the active core capable of inhibiting JH biosynthesis (Stay et al., 1994; Hayes et al., 1994; Tobe et al., 1995). The common Phe-Gly-Leu-amide is found in all 13 peptides with the exception of peptide number 13 in which Leu is replaced with Ile. The AST polypeptide precursor has acidic domains which separate the Dip-ASTs into four regions, comprising Dip-AST 1-4, Dip-AST 5-10, Dip-AST 11, and Dip-AST 12 & 13. It is not known whether this separation of peptides into groups is a requirement
for the stability of the precursor or if there is some functional significance to this separation (Bendena et al., 1994). The AST precursor from P. americana has a similar organization to that of D. punctata. The P. americana precursor contains 14 AST peptides, whereas the D. punctata precursor has 13 AST peptides. Between these two species of cockroaches, five of the peptide sequences are completely conserved (Ding et al., 1995, Tobe et al., 1995). In contrast to the two cockroach precursors are those from Pseudaelitia unipuncta and Drosophila melanogaster; these Manduca-like precursors both contain a putative single AST peptide (Tobe et al., 1995).

Although Dip-AST-like peptides are found in many insect species, they appear to have been adapted for distinct functions. For example, Cav-ASTs inhibit JH biosynthesis by the CA in vitro in D. punctata but not in C. vomitoria, suggesting other functions in flies (Duve et al., 1993). In D. punctata, not only are the Dip-ASTs able to inhibit JH biosynthesis by the CA, but all 13 of the Dip-AST peptides are also capable of inhibiting both myogenic and proctolin-induced contractions of hindgut visceral muscle in a dose-dependent manner (Lange et al., 1995). This myoinhibitory activity has also been demonstrated in the blowfly C. vomitoria (Duve and Thorpe, 1994). Previous studies have revealed AST-like immunoreactive material in hindguts, midguts, oviducts and antennal pulsatile organs in D. punctata. However, only hindgut muscle contractility appears to be sensitive to the Dip-ASTs (Lange et al., 1993; Lange et al., 1995; Yu et al., 1995). Interestingly, Dip-ASTs that are most potent in inhibiting JH biosynthesis by the CA are relatively ineffective as inhibitors of proctolin-induced muscle contraction in the hindgut. Conversely, the most potent Dip-ASTs in the hindgut assay are relatively ineffective as inhibitors of JH biosynthesis (Bendena et al., 1997). It has been suggested
that the functional differences in each AST may reside at the level of receptors in different tissues (Bendena et al., 1997).

Dip-ASTs are found in many parts of the nervous system in adult female \textit{D. punctata} at many stages of the reproductive cycle (Rankin and Stay, 1987). In adult female \textit{D. punctata}, the quantity of AST-like material was determined in the brain, CA, and haemolymph by RIA and ELISA. The quantity of AST-like material was also measured in haemolymph of last instar females (Yu et al., 1993). This study suggests that humoral inhibition by ASTs may be one of the factors involved in inhibition of JH biosynthesis. The corpus cardiacum is rich in AST-like immunoreactive material and therefore represents one potential site of release into the haemolymph (Stay et al., 1991b; Yu et al., 1993). Yu et al. (1993) also suggested that ASTs from the brain might influence CA cells in a paracrine fashion following release from axon terminals, as well as by way of humoral pathways.

It has been clearly demonstrated that sensitivity of the CA to ASTs changes in larval and adult \textit{D. punctata} (Pratt et al., 1990; Stay et al., 1991a). The study by Pratt et al. (1990) demonstrated that the sensitivity increases slightly as rates of JH gradually decline in the penultimate stage. In final instar larvae and during pregnancy in adult females, both sensitivity and rates of JH biosynthesis declines. In adult females, there is a pronounced increase in sensitivity at the end of the vitellogenic cycle as JH biosynthesis declines abruptly. Glands from day 5 mated adult females (maximal rates of JH production) were not sensitive to AST but became highly sensitive on day 6. It is thought that the sensitivity of the CA is related to the stage of gland activity rather than to the actual rate of JH biosynthesis (Stay et al., 1991). A recent article by Stay et al. (1996)
indicates that the modulation of JH biosynthesis depends not only on the release of AST but also on the ability of the CA to respond to the AST.

Not only does sensitivity change within the CA, but as described previously, different tissues have different sensitivities to ASTs. The onset of AST activity on both JH biosynthesis and muscle contraction is very rapid, but is also rapidly reversible. For this reason, it has been suggested that mechanisms for inactivation of these peptides must exist, one of these being degradation of the peptides (Bendena et al., 1997). It has been shown that the regulation of AST activity is in part controlled by modification of their rates of degradation and that metabolism of ASTs occurs by a specific cleavage, resulting in a change in the functionality of the molecule (Garside et al., 1997). This study demonstrated that there are peptidases in the haemolymph which have the ability to degrade ASTs, and it is these enzymes which represent one of the primary vehicles for the inactivation of the ASTs in the haemolymph.

The mode of action of allatostatins remains unclear. In D. punctata CA, AST is thought to bind to specific membrane receptors (Cusson et al., 1991; Stay et al., 1994). It is likely that the ASTs exert effects on JH production, at least in part through the regulation of intracellular Ca\(^{2+}\) concentration and regulation of other ion channels (Tobe et al., 1994). It is also likely that second messengers are involved in the transduction of AST signals in the CA cells. The effects of Dip-AST treatment on changes in cyclic nucleotide levels in CA have not been successful (Cusson, et al., 1992; Tobe et al., 1994). In a study examining second messenger systems in CA of D. punctata, DAG and IP\(_3\) appear to be two of the intracellular messengers involved in signal transduction of Dip-AST 7 and Cav-AST 5 (Rachinsky et al., 1994). The isolation and cloning of allatostatin
receptors should in part address the question of what role these second messenger systems play.

b) Second Messengers

Calcium plays a major role in the regulation of JH biosynthesis in the cockroach *D. punctata*. Extracellular Ca\(^{2+}\) causes rates of JH release to increase in a dose-dependent manner, with maximum release occurring at an extracellular Ca\(^{2+}\) concentration of 3-5 mM. Accordingly, when extracellular Ca\(^{2+}\) is absent, JH release is significantly inhibited (Kikukawa et al., 1987). Organic blockers of calcium channels such as nifedipine and verapamil modulate JH release only slightly, whereas non-specific ionic Ca\(^{2+}\) channel blockers like lanthanum inhibited JH release greatly (Kikukawa et al., 1987). The effect of the calcium ionophore A23187 was also examined in this study, they were found to dramatically reduce JH release. The effects of the ionophores seem to be associated with variations in intracellular calcium concentrations. In *D. punctata*, the cell membranes of the CA contain voltage-dependent Ca\(^{2+}\) channels. McQuiston and Tobe (1991a), hypothesized that Ca\(^{2+}\) current might play an important role in the regulation of intracellular Ca\(^{2+}\) concentration and JH biosynthesis. Changes in the electrical properties of the CA may be important in the regulation of JH biosynthesis, possibly by altering Ca\(^{2+}\) influx (McQuiston and Tobe, 1991b). Aucoin *et al.* (1987) suggest that both calcium-dependent and calcium-independent (involving cAMP) pathways function to reduce the rate of JH biosynthesis in *D. punctata*.

Control of juvenile hormone biosynthesis also appears to involve cyclic nucleotides. High concentrations of octopamine were found to elevate the cAMP content
of the CA and inhibit JH biosynthesis, suggesting octopamine involvement in neural control of the CA (Thompson et al., 1990). It has been shown that experimentally elevated cAMP levels inhibit JH production (Meller et al., 1985; Aucoin et al., 1987). In the cockroach *D. punctata*, cAMP and cGMP undergo large changes during the gonotrophic cycle. In virgin CA, the levels of both cAMP and cGMP remain relatively constant during the gonotrophic cycle as does JH biosynthesis (Stay and Tobe, 1977a). However, in mated females, large changes in cAMP and cGMP can be correlated to changes in JH biosynthesis (Tobe, 1990). This same study suggests that cAMP-dependent kinases are involved in the inhibition of JH biosynthesis during pre- and post-vitellogenesis and that cGMP-dependent kinase are involved in the inhibition of JH biosynthesis upon completion of vitellogenesis.

Another second messenger system that has been studied in *D. punctata* is the 1,4,5-inositol trisphosphate (IP$_3$)/diacylglycerol (DAG) second messenger system. Feyereisen and Farnsworth (1987) demonstrated that the IP$_3$/DAG second messenger system is involved in the regulation of JH biosynthesis. A subsequent study showed that treatment of CA cells with a DAG kinase inhibitor (thereby elevating DAG), resulted in a dose-dependent inhibition of JH release. It has been suggested that DAG is an intermediate in allatostatin-induced inhibition of JH biosynthesis (Rachinsky et al., 1994; Rachinsky and Tobe, 1996).

**V. Protein Synthesis**

a) Transcription

A gene is transcribed by copying sequence information from a DNA sequence to an RNA sequence using the complementary strand of DNA as the template. The primary
transcript is a single strand of RNA which is a direct copy of the DNA sense strand sequence and is referred to as the precursor or pre-mRNA. This pre-mRNA is processed within the nucleus to form mRNA. Processing of pre-mRNA involves the addition of a 'cap' at the 5' end, removal of introns and usually the addition of a poly (A) tail at the 3' end. Upon completion of these events, the mRNA is transported to the cytoplasm where it can be translated into protein (Arnstein and Cox, 1992).

b) Translation

Moldave (1985) presents a good overview of translation in protein synthesis in eukaryotes. In order for translation of proteins to occur, the information in the triplet coding sequence of mRNA is translated and this specifies the amino acid sequence to form a growing polypeptide chain. This process occurs in three distinct phases, initiation, elongation, and termination.

Initiation decodes the initiation codon, AUG, which codes for methionine. The transfer RNA involved in this step is known as Met-tRNA$_f$ or (Met-tRNA$_{f0}$). The initiation process involves GTP, ATP, a number of initiation factors (eIFs), mRNA, and ribosomal subunits to form an 80S initiation complex. Initiation is finalized with the formation of an 80S initiation complex containing a ribosome, initiator Met-tRNA$_f$ and mRNA.

Elongation translates all of the internal codons, between the initiation and the termination triplets. Translation of proteins uses aminoacyl-tRNAs and requires GTP and elongation factors (EFs). Briefly, elongation comprises; i) binding of an appropriate aminoacyl-tRNA to the A site (acceptor site) on the ribosome, ii) peptide bond formation between the incoming aminoacyl moiety and peptidyl residue at the P site (donor site) on
the ribosome, and iii) movement of the ribosome on the mRNA. This sequence is repeated until termination occurs. Amino acids are added one at a time to the nascent peptidyl chain and this occurs from the N-terminal residue toward the C-terminal residue.

Termination of translation occurs when a termination codon UAA, UAG, or UGA, enters the A site. This process requires GTP and a specific protein factor (RF). The binding of the termination factor at the A site results in the hydrolysis of the peptidyl-tRNA ester at the P site and release of the completed polypeptide chain.

VI. Protein Synthesis Inhibitors

There are many compounds that inhibit protein synthesis. Some inhibit at the level of translation whereas others inhibit transcription. Even within the groups of inhibitors, the mode of action may differ. For example, cycloheximide and anisomycin are both translation inhibitors, but each one inhibits a different step in the elongation cycle during the translation of proteins.

Anisomycin is an antibiotic that inhibits protein synthesis in eukaryotic systems by specifically blocking peptide bond formation. It is believed that anisomycin inhibits the substrate interaction with the donor and acceptor sites of the peptidyltransferase center. This inhibitor specifically binds to the 60S ribosomal subunit and it is thought that there is a single binding site for this antibiotic per ribosome (Verner and Beers, 1995).

The antibiotic cycloheximide is also a translation inhibitor. Although it was initially thought that cycloheximide interacted with elongation factors, the ribosome was later suggested as the site of interaction. After experiments employing ribosomes from
cycloheximide-resistant strains, the ribosome is now widely accepted as the site of action. This antibiotic inhibits the peptidytransferase activity of the 60S ribosomal subunit. Cycloheximide has been observed to prevent polysome formation, suggesting inhibition of initiation and translocation. Furthermore, it has even been postulated as an inhibitor of chain termination. Caution should be taken when using this inhibitor because cycloheximide also inhibits DNA and RNA synthesis in intact cells (Verner and Beers, 1995).

In addition to translation inhibitors are the antibiotic inhibitors of transcription. Actinomycin D inhibits transcription by binding tightly and specifically to double-helical DNA and thereby prevents it from being an effective template for RNA synthesis. Actinomycin D has been used as a highly specific inhibitor of the formation of new RNA in both prokaryotic and eukaryotic cells (Stryer, 1988).

Cycloheximide has been used to study for example, insulin receptor metabolism (Knutson et al., 1985), sensitivity in regulation of Acyl Coenzyme A in hamster ovary cells (Chang et al., 1986), and endocytosis pathways in amoebae (Gonzalez and Satre, 1991). In insect systems, cycloheximide has been used in studies of eclosion hormone sensitivity in Manduca sexta (Morton and Truman, 1995). Other studies have used cycloheximide and Actinomycin D to demonstrate that β-tubulin synthesis and ecdysteroid synthesis are coregulated in the prothoracic gland of M. sexta (Rybczynski and Gilbert, 1995). Protein synthesis inhibitors were also used in a study of apoptosis in the spruce budworm, Choristoneura fumiferana (Palli et al., 1996). In this study, actinomycin D, anisomycin and cycloheximide all induced apoptosis in a continuous
midgut cell line. These studies only represent a handful of studies that have been performed using protein synthesis inhibitors. These examples demonstrate, however, that the use of these inhibitors can be valuable in many different cells and systems, and in a variety of different organisms.

VII. Protein Actions

Proteins act as receptors, enzymes, transporters, and are even involved in second messenger systems. In both vertebrates and invertebrates, peptides can bind to membrane receptors and initiate a cascade of events generally starting with an increase in intracellular cAMP and proceeding through specific changes in protein phosphorylation and protein synthesis to the eventual production and secretion of steroid hormones (Orme-Johnson, 1990; Papadopoulos, 1993; and Rybczynski & Gilbert, 1995). One of the most studied second messengers is cyclic AMP, which associates with a specific receptor protein, and in eukaryotic cells appears to serve as the regulatory subunit of a protein kinase. The cyclic AMP-dependent protein kinase phosphorylates a large number of cellular proteins, including either enzymes or structural proteins (Rosenfeld and Barrieux, 1979). In Manduca sexta, the action of PTTH on the prothoracic gland follows the events described above. PTTH appears to bind to one or more surface receptors resulting in an elevation of free Ca$^{2+}$ in the cells. This increase in Ca$^{2+}$ acts via calmodulin to activate a Ca$^{2+}$-calmodulin-stimulated adenylate cyclase (Meller et al., 1988). An increased cAMP concentration stimulates a cAMP-dependent protein kinase to mediate the increased phosphorylation of several proteins (Gilbert et al., 1988; Rybczynski and Gilbert, 1994). Although many of the same second messenger systems
have been examined in the cockroach *D. punctata*, a defined sequence of events, as described in *M. sexta*, is not known for the cockroach.

As noted previously, enzymes involved in biosynthetic pathways can be activated or inactivated by means of phosphorylation/ dephosphorylation. In *D. punctata*, there is indirect evidence that reversible phosphorylation of HMG-CoA reductase occurs in the JH III biosynthetic pathway (Feyereisen, 1985). This author explains that this reversible phosphorylation would occur by means of hormonal or neural control of the enzyme and might be caused by changes in cAMP or Ca²⁺-calmodulin levels in response to allatostatic or allatotropic stimuli, or by the level of phosphorylation of the hormone receptor itself.
Purpose Of This Study

The corpora allata of insects have been studied extensively in a variety of insect species. In the viviparous cockroach *Diploptera punctata*, the regulation of the CA and of JH biosynthesis have been the focus of numerous studies. As a result of these studies, it has been determined that regulation of the CA and JH biosynthesis appears to involve the interaction of a number of second messenger systems, including the cyclic nucleotides and calcium (Tobe, 1990). In this cockroach, it is also known that production of JH by the CA is inhibited by neuropeptides known as allatostatins (Dip-ASTs) (Stay et al., 1994). Although the mode of action of ASTs has not been studied extensively, recent studies have implicated second messenger systems in transduction of the AST signal (Rachinsky et al., 1994).

In this study, further investigation of the regulation of the CA and JH production was performed through the examination of protein synthesis. Proteins synthesized in the CA may be acting as modulators of JH biosynthesis by acting as receptors, as constituents of second messenger systems, as the enzymes that are involved in this pathway, or as Co-factors of these enzymes.

The objectives of this study were:

1) to examine profiles of proteins that are present in the CA during different developmental stages of *D. punctata*;

2) to determine if inhibition of protein synthesis by transcriptional and translational inhibitors affected JH biosynthesis by the CA;

3) to examine the effect of Dip-ASTs on protein synthesis in the CA;

4) to correlate changes in protein profiles with JH biosynthesis;

5) to determine if experimental manipulations, including AST treatment, affect specific protein synthesis.
Figure 1. Brain-retrocerbral complex of *Diploptera punctata*.

Diagrammatic representation of the principal nerves and their cells of origin in the brain-retrocerbral complex of *Diploptera punctata*. AL: antennal lobe; AN: antennal nerve; CA: corpora allata; CC: corpora cardiaca; CEC: Circumoesophageal connective; FG: frontal ganglion; HG: hypocerebral ganglion; LNSC: lateral neurosecretory cells; MNSC: medial neurosecretory cells; NCA I and II: nervi corporis allati I and II; NCC I and II: nervi corporis cardiaci I and II; ON: esophageal nerve; OPN: optic nerve; PAN: postallatal nerves; RN: recurrent nerve; SEG: subesophageal ganglion; TNSC: tritocerebral neurosecretory cells (from Thompson and Tobe, 1990).
Figure 2. Biosynthetic Pathway Of JH III.

The conversion of HMG-CoA to mevalonate (HMG-CoA reductase) and methyl farnesoate to JH III (epoxidase) occur on or within the endoplasmic reticulum and all other steps occur in the cytosol. The unique enzymes to insects are farnesoic acid O-methyltransferase, which is involved in the conversion of farnesoic acid to methyl farnesoate and methyl farnesoate epoxidase, which is involved in the conversion of methyl farnesoate to JH III (see Tobe and Stay, 1985).

* Italics refer to the enzymes that are involved in the pathway.
JH III Biosynthesis Pathway

exogenous precursors; Glucose or Acetate

\[ \downarrow \text{(thiolase)} \]

Actoacetyl CoA

\[ \downarrow \]

3-hydroxy-3-methylglutaryl-CoA

\[ \downarrow \text{(HMG-CoA reductase)} \]

Mevalonate

\[ \downarrow \]

\[ \downarrow \]

\[ \downarrow \text{(isomerase)} \]

\[ \downarrow \]

\[ \downarrow \text{(prenyl transferase)} \]

\[ \downarrow \]

Farnesyl pyrophosphate

\[ \downarrow \text{(phosphatase)} \]

Farnesol

\[ \downarrow \text{(dehydrogenase)} \]

Farnesal

\[ \downarrow \text{(dehydrogenase)} \]

Farnesoic Acid

\[ \downarrow \text{(methyl transferase)} \]

Methyl farnesoate

\[ \downarrow \text{(epoxidase)} \]

JH III
Materials and Methods

I. Animals

Newly emerged *D. punctata* females were removed from stock cultures. Virgins, mated and 4th instar animals were transferred each day from the stock culture to glass jars that were subsequently kept in an incubator. The incubator was maintained at 27°C +/- 1°C and 50% relative humidity with a 12 h light, 12 h dark cycle. Insects were reared on Purina Lab Chow and water. Mating was confirmed by the presence of a spermatophore and the oocyte length was measured to ensure the correct physiological age of the animal. Virgins were obtained by isolating the last-instar females from the colony and maintaining them in separate jars. Day 0-10 mated and virgin females and day 0-20 4th instar females were used in the developmental study. Day 7 mated females were exclusively utilized for protein inhibitor studies, Dip-allatostatin studies and the radiochemical assay. Day 7 mated females were checked for oviposition to ensure the animals were the correct physiological age.

II. Analysis of Protein Synthesis

II.1 Tissue Preparation

a) Developmental study

The cockroaches were cold-anaesthetized before dissection. Corpora allata (5 pairs) were dissected from mated or virgin females (day 0 to day 10) or 4th instar females (day 0- day 20). The tissues were placed in saline and visible fat body and corpora cardiaca were removed. The CA (5 pairs) were then placed in 20µl of SDS reducing buffer (0.5 M Tris-HCl, pH 6.8, Glycerol, 10% SDS, 2-b-mercaptoethanol, and 0.05%
bromophenol blue), vortexed, and heated at 95°C for 4 min. Samples were then vortexed for a second time, centrifuged for 10 sec at 3000 g (to draw sample off tube wall) and stored at -70 °C until sufficient material for electrophoresis was obtained. The same protocol was followed for mated female brains (day 0 to day 10) with the exception that 2 brains per 20 μl sample were used.

b) Protein inhibitor study

The protein inhibitors used in this study were cycloheximide: 0.05 and 2 mM and anisomycin, 10⁻⁸ - 10⁻⁴ M (translation inhibitors) and actinomycin D, 50 μM - 5 μM, 10 μM (transcription inhibitor). Cycloheximide and anisomycin were dissolved in TC199 (stock solution). Actinomycin D was dissolved in 0.6% ethanol (stock solution).

Corpora allata (5 pairs) from day 7 mated females were dissected directly into TC199 (GIBCO, 1.3 mM Ca²⁺, 2% Ficoll, methionine-free) and incubated for 30 min. Following this preincubation, the TC199 was removed and replaced with TC199 containing protein inhibitor and was incubated for 2 h. Upon completion of this incubation, the solution was removed and replaced with TC199 containing protein inhibitor for a second 2h incubation. After the final incubation, samples were washed with TC199 and transferred at 4°C to 20 μl of SDS reducing buffer. Samples were vortexed, and heated at 95°C for 4 min. Samples were vortexed again, centrifuged for 10 sec at 3000 g (to draw sample off tube walls) and stored at -70°C until sufficient material for electrophoresis was obtained.
c) Dip-allatostatin study

Stock solutions (20 μl, 10⁻⁴M) of Dip-allatostatin 7 and Dip-allatostatin 13 were used for dose-response studies. Concentrations ranging from 10⁻¹₀⁻¹₀⁻⁵ M were used for all the Dip-ASTs. Preparation of Dip-allatostatin 2 was performed in several steps. Stock samples of Dip-AST 2 were dried under N₂ and resuspended with 7 μl 1 N HCl and vortexed. TC199 was added, vortexed and neutralized with 1 N NaOH.

The same protocol for protein inhibitors (section II.1-b) was followed with the exception that protein inhibitors were replaced with the Dip-allatostatin 2, 7, or 13.

II.2 Gel electrophoresis (Coomassie blue stained)

The standard Laemmli (1970) SDS-polyacrylamide gel electrophoresis (PAGE) system was employed to separate proteins. The Mini-PROTEAN II apparatus (BIO RAD) was used. The gels were discontinuous polyacrylamide gels which consist of a stacking (upper) gel and a resolving or separating (lower) gel. Stacking gels were 4% acrylamide (pH 6.8) and separating gels were 7% or 12% acrylamide (pH 8.8). Gels were cast immediately prior to running the samples. The electrophoresis buffer consisted of Tris base, glycine, and SDS, pH 8.3. A 20 μl sample was added per gel lane. One lane per gel was retained for molecular weight standards, 5 μl of high range (Cat # 161-030, 200-45 kDa) or low range (Cat # 161-0304, 97-14 kDa), BioRad standard was added. After electrophoresis, gels were stained for 30 min with 0.1% Coomassie blue R-250 in methanol-acetic acid-water (40:10:50). The gel was then destained for 2 h with methanol-acetic acid-water (40:10:50) to remove background. The gel was placed in
dH$_2$O overnight and then dried (BIORAD model 583 Gel Dryer) at 60°C for 1 h onto filter paper.

**III. Analysis of Protein Synthesis using $^{35}$S-methionine Labeling**

**III.1 $^{35}$S-methionine tissue preparation**

**a) Developmental study**

Cockroaches were cold-anaesthetized before dissection. Corpora allata (5 pairs) were dissected from mated or virgin females (day 0-day 10) or 4th instar females (day 0-day 20). In addition, mated female brains (2) were dissected from day 0 to day 10 animals. The tissues were preincubated in 100 μl TC199 (GIBCO, 1.3 mM Ca$^{2+}$, 2% Ficoll, methionine-free) for 30 min. After preincubation, the TC199 was removed and replaced with TC199 containing $^{35}$S-methionine, final volume 100 μl (final specific radioactivity 37 TBq/mmol, 1000 Ci/mmol) and incubated for 2 h. Following this incubation, the TC199 medium containing $^{35}$S-methionine was removed and replaced with TC199 to wash the CA and eliminate any unbound $^{35}$S-methionine. The CA were then transferred to 25 μl of SDS reducing buffer at 4°C. The sample was vortexed, and heated at 95°C for 4 min. Samples were vortexed again, centrifuged for 10 sec at 3000 g and stored at -70°C until sufficient material was collected for protein determination and gel electrophoresis.

**b) Protein inhibitor study**

CA were dissected, and the same tissue preparation and protocol were followed as previously described (section II.1-b). Upon completion of the first 2 h incubation the solution was removed and replaced with TC199 containing [$^{35}$S]-methionine and protein
inhibitor (final specific radioactivity, 37 TBq/mmol, 1000 Ci/mmol) and incubated for 2 h. Following this second 2h incubation, CA were washed with TC199 and transferred to 25 µl of SDS reducing buffer. Samples were vortexed, and heated at 95°C for 4 min. They were vortexed again, centrifuged for 10 sec at 3000 g and stored at -70 °C until ready for protein determination and gel electrophoresis.

c) Dip-allatostatin study

The above procedure (section III.1-b) for protein inhibitors was used, with the exception that the protein inhibitors were replaced with Dip-allatostain 2, 7, or 13. Dip-allatostatins were prepared as previously described (II.1-c), except that TC199 containing [35S]-methionine replaced TC199.

III.2 Determination of [35S]-methionine incorporation into protein

Filter paper (3 mm) was cut, labeled and placed on parafilm. Duplicate samples (2 µl) were spotted onto the filter papers. The spotted filter paper was air dried in the fume hood for 10 min, then treated with 20% TCA (10 min, 4°C) to precipitate proteins, washed in 5% TCA (2 x 5 min, 4°C), then twice (1 min) in 100% ethanol and air dried (10 min). Filter papers were then immersed in scintillation fluid (cytoScint™) and counted using a Beckman LS 6500 multi-purpose scintillation counter to determine the amount of [35S]-methionine incorporated into precipitated protein (cpm).

Incorporation of [35S]-methionine into protein was performed on samples from developmental, protein inhibitor, and Dip-allatostatin studies.
III.3 Gel electrophoresis (Autoradiography)

The standard Laemmli SDS-PAGE system was used to separate proteins (see above procedures, II-2. For any given gel the number of TCA precipitable cpm was equal in each unless noted. After electrophoresis gels were fixed in TCA-acetic acid-methanol-water (20:10:30:40) for 20 min, then washed in 10% acetic acid/ethanol for 2 x 10min. Gels were dried (BIORAD model 583 gel dryer) at 60°C for 1 h onto filter paper. The dried gel was exposed to Kodak X-omat film in a cassette for approx. 15 h at room temp. The film was developed using the Kodak M35A X-OMAT Processor.

IV. Radiochemical Assay

Rates of juvenile hormone release were determined using the *in vitro* radiochemical method (Tobe and Pratt, 1974; Feyereisen and Tobe, 1981a), modified by Tobe and Clarke, (1985). Corpora allata were dissected directly into TC199. TC199 solutions containing protein inhibitors or Dip-allatostatins were made. CA were removed from TC199 medium and placed in 50 μl of TC199 containing protein inhibitor/Dip-allatostatin. Tubes were covered with parafilm and incubated for 2 h on a shaker. After this incubation, the solution was removed and 50 μl of radioactive TC199 containing (L[^14]C-S-methyl]-methionine) and protein inhibitor/Dip-allatostatin was added (final specific radioactivity, 55mCi/mmol). Tubes were then covered with parafilm and incubated for another 3 h. The incorporation of L[^14]C-S-methyl]-methionine into JH III at its penultimate step of biosynthesis by the CA was used to quantify JH biosynthesis. Following the last incubation period, corpora allata were removed from the medium. The medium was then extracted in 300 μl isoctane. 200 μl was taken from the top layer and
placed into scintillation fluid (cytoScint™) and radioactivity was determined using a Beckman LS 6500 multi-purpose scintillation counter.

V. Data Analysis

GraphPad Prism™ version 2.01 was used for all statistical analyses (t-test’s, ANOVA, Tukey’s multiple comparison test). Gels (Coomassie blue-stained and autoradiographs) were scanned with a Macintosh computer using The Microtek ScanMaker III scanner. Microtek ScanWizard™ PPC version 3.07 (scanner controller for Macintosh® 1995) was used to scan the gels. Gel scans were placed in Adobe® photoshop® version 4.0 powerPC™, to save onto diskette. After the scanning process, SigmaGel™ analysis software, version 1.0, was used to determine relative molecular mass (kDa) and quantify the intensity of protein bands. Standard curves were generated for intensity measurements (appendix I and II).

VI. Statistical Analysis

Unless otherwise noted, measurements are presented as means, (x) +/- the standard error of the mean (SEM). Where appropriate (indicated in figure legends), student’s t-test (two-tailed) or repeated measures one way analysis of variance (ANOVA) were used to evaluate statistical significance. The null hypothesis was rejected if P<0.05. Following ANOVA, the Tukey’s multiple comparison post test was applied. Percent inhibition in figures 13, 17, and 21 was calculated by division of sample values by control values multiplied by 100 to give a percentage.
RESULTS

I. Developmental studies

i. Protein Synthesis in female cockroaches

Protein synthesis by *D. punctata* corpora allata at different developmental stages was investigated by measuring the incorporation of \[^{35}\text{S}\]methionine into proteins *in vitro*. Mated and virgin female *Diploptera* show different profiles of protein synthesis (Fig. 3). Rates of protein synthesis in CA of mated cockroaches were low on day 0, eventually increasing to a maximum on days 4, 5, and 6, then decreasing after day 6. There was no statistically significant change in rates of protein synthesis during the 10 day period in virgin cockroaches. There was a significant difference in protein synthesis between mated and virgin animals (Fig. 3) over the ten day period. Protein synthesis in 4th instar animals was initially low, but increased after day 8 to a maximum at day 14 that was approximately half the maximum synthetic rate of mated adult females (Fig. 4).

As a control, mated female brains were compared to the CA. We found that there was no statistically significant change in rates of protein synthesis during days 0-10 (Anova p<0.05) (Fig. 5). Therefore the only significant increase in protein synthesis occurred in mated female CA, with maximal synthesis occurring on day 6.

ii. Protein synthesis profiles

Protein synthesis in the different developmental stages of the cockroach are represented by SDS-PAGE in Figures 6.1 - 9.2. These gels are representative of replicates that were performed for each tissue and developmental stage. Bands that consistently appeared on the gels were examined in greater detail. In mated and virgin
**Figure 3. Protein synthesis in the CA of mated and virgin cockroaches.**

Protein synthesis in the CA of different ages (days 0-10) of mated and virgin female cockroaches. Mated females are represented by (●) and virgin females by (○), with synthesized protein expressed as cpm per CA·h⁻¹. Points represent the mean of 4-5 replicates. Vertical bars indicate SEM. Mean oocyte length is included as a more accurate indicator of physiological age. Symbols indicate statistically significant differences between the means in mated females according to one-way ANOVA (p<0.05). Following this analysis, the Tukey's multiple comparison test indicates: ◆-significantly different from day 0, ●-significantly different from day 1, ★-significantly different from day 6, ■-significantly different from day 10. There was no difference between the means for virgin females as determined by one-way ANOVA (p<0.05). There was a significant difference between mated and virgin females as determined by a two-way ANOVA.
Incorporation of $[^{35}\text{S}]$ methionine into protein (cpm per CA·h$^{-1}$)

Age (days) vs. Incorporation Rate

Virgin vs. Mated Groups

Inset: Oocyte length (mm)

Graph shows changes in incorporation rate over time.
Figure 4. Protein synthesis in the CA of 4\textsuperscript{th} instar female cockroaches.

Protein synthesis in the CA of 4\textsuperscript{th} instar female cockroaches of different ages (0-18 days post-molt), n=1. Synthesized protein is expressed as cpm per CA·h\textsuperscript{−1}. 
Incorporation of $[^{35}S]$ methionine into protein (cpm per CA $\cdot$ h$^{-1}$)

Age (days)
Figure 5. Protein synthesis in the brain of mated female cockroaches.

Protein synthesis in the brain of mated female cockroaches of different ages (days 0-10). Synthesized protein is expressed as cpm per brain-h⁻¹. Points represent the mean of 2 replicates. Vertical bars indicate SEM. One-way ANOVA (p<0.05) indicates no statistical significant difference between the means.
CA
Mated females
Coomassie blue-stained gel

Figure 6.1 Profile of protein bands in the CA of mated female cockroaches.

Proteins from CA of mated females were separated on a 7.5% SDS-PAGE and stained with Coomassie blue. Each lane represents animals of a specific age. Standards are shown in kDa. A) Day 0-6 animals. B) Day 7-10 animals, and Fat body (fb). Arrows to the right of the gels represent bands that were analyzed and discussed.
CA
Mated females
Autoradiograph

Figure 6.2 Profile of protein synthesis in the CA of mated female cockroaches.

Proteins from CA of mated females were separated on a 12% SDS-PAGE. Bands were revealed by $[^{35}\text{S}]$methionine incorporation and autoradiography. The amount of protein per gel lane was adjusted to the same amount of incorporated radioactivity (cpm). Each lane represents animals of a specific age. Standards are shown in kDa. A) Day 0-5 animals. B) Day 6-10 animals. Arrows to the right of the gels represent bands that were analyzed and discussed.
Figure 7.1 Profile of protein bands in the CA of virgin female cockroaches.

Proteins from CA of virgin female adults were separated on a 7.5% SDS-PAGE and stained with Coomassie blue. Each lane represents animals of a specific age. Standards are shown in kDa. A) Day 0-6 animals. B) Day 7-10 animals, and Fatbody (fb). Arrows to the right of the gels represent bands that were analyzed and discussed.
Figure 7.2 Profile of protein synthesis in the CA of virgin female cockroaches.

Proteins from CA of adult virgin females were separated on a 12% SDS-PAGE. Bands were revealed by [35S]methionine incorporation and autoradiography. The amount of protein per gel lane was adjusted to the same amount of incorporated radioactivity (cpm). Each lane represents animals of a specific age. Standards are shown in kDa. A) Day 0-5 animals. B) Day 6-10 animals. Arrows to the right of the gels represent bands that were analyzed and discussed.
Brain
Mated females
Coomassie blue-stained gel

Figure 8.1 Profile of protein bands in the brain of mated female cockroaches.

Proteins from brains of mated female adults were separated on a 12% SDS-PAGE and stained with Coomassie blue. Each lane represents animals of a specific age. Standards are shown in kDa. A) Day 0-5 animals. B) Day 6-10 animal. Arrows to the right of the gels represent bands that were analyzed and discussed; M, 70, 54, 44, 41, 35, 32, 30, 20 kDa (top to bottom).
Brain
Mated females
Autoradiograph

Figure 8.2 Profile of protein synthesis in the brain of mated female cockroaches.

Proteins from brains of mated female adults were separated on a 12% SDS-PAGE. Bands were revealed by [35S]methionine incorporation and autoradiography. The amount of protein per gel lane was adjusted to the same amount of incorporated radioactivity (cpm). Each lane represents animals of a specific age. Standards are shown in kDa. A) Day 0-5 animals. B) Day 6-10 animals. Arrows to the right of the gels represent bands that were analyzed and discussed; M, 76, 55, 44, 42, 36, 33, 31, 20 kDa (top to bottom).
CA
4th instar females
Coomassie blue-stained gel

Figure 9.1 Profile of protein bands in the CA of 4th instar female cockroaches.

Proteins from CA of 4th instar females were separated on a 7.5% SDS-PAGE and stained with Coomassie blue. Each lane represents animals of a specific age. Standards are shown in kDa. A) Day 2-10 animals. B) Day 12-18 animals. Arrows to the right of the gels represent bands that were analyzed and discussed; M, 203, 81, 76, 55, 52, 50, 31 kDa (top to bottom).
CA

4th instar females

Autoradiograph

Figure 9.2 Profile of protein synthesis in the CA of 4th instar female cockroaches.

Proteins from CA of 4th instar females were separated on a 12% SDS-PAGE. Bands were revealed by [35S]methionine incorporation and autoradiography. The amount of protein per gel lane was adjusted to the same amount of incorporated radioactivity (cpm). Each lane represents animals of a specific age. Standards are shown in kDa. A) Day 0-12 animals. B) Day 14-18 animals. Arrows to the right of the gels represent bands that were analyzed and discussed; M, 84, 60, 55, 52, 48, 37, 35, 27 kDa (top to bottom).
animals, the intensity of protein bands on Coomassie blue-stained gels (Figure 10) did not change dramatically with age. For the purpose of this thesis Coomassie stained bands refers to the total SDS-extractable protein from the CA that was observed on the Coomassie blue-stained gels. There were, however, some significant increases in the intensities of the 50 kDa (Fig. 10d) and the 47 kDa (Fig. 10e) bands on days 4 and 5 in mated animals. A comparison of newly synthesized proteins (autoradiographs) from virgin and mated females, (Fig. 11) showed a significant difference in mated females compared to virgins females. Significant increases were noted in all bands on day 6 in mated females. These results are in general agreement with the protein synthesis (incorporation of $^{35}$S-methionine) results described earlier. In mated females, we found maximum intensities on day 6 for each of the bands (Fig.11). Examination of the first five days in mated and virgin animals, revealed that the bands with the higher $M_r$ (e.g. 82, and 72 kDa) in Coomassie blue-stained gels (Fig. 10) have higher intensities in virgins than in the mated Diploptera. Comparison of the autoradiographs in figure 11 indicate that, both mated and virgin females had protein band intensities that were similar at day 0.

Protein bands of 4th instar animals appeared to be more intense in Coomassie blue-stained gels than in the autoradiographs (newly synthesized protein see Figure 12). Newly synthesized proteins in autoradiographs showed protein bands from both 4th instar and adult virgin animals of different developmental stages had similar intensities. In mated animals, intensities of protein bands were consistently higher (compare Figures 11 and 12). In the brain, intensity of protein bands for both (Coomassie gels) and newly synthesized proteins (autoradiographs) showed no apparent increase (day 0-10), although
Figure 10. Intensity of protein bands (CA) in mated and virgin cockroaches (Coomassie blue SDS-PAGE).

Intensity of protein bands from CA of mated and virgin female cockroaches of different ages (days 0-10). Mated females are represented by (●) and virgin females by (★). Points represent the mean of 2-3 replicates. Vertical bars indicate SEM. Intensity represents relative intensity as the pixel value of a selected point on the gel in terms of that pixel's brightness. Gels were scanned at 600 dpi. Symbols indicate statistically significant differences between the means in mated females according to one-way ANOVA (p<0.05). Following this analysis the Tukey’s multiple comparison test indicates a significant difference from day 0 (●) or day 6 (★). There was no difference between the means for virgin females, as indicated by one-way ANOVA (p<0.05). A two-way ANOVA was used (p<0.05) and determined no significant difference between mated and virgin females.
a) 82 kDa

b) 72 kDa

c) 54 kDa
d) 50 kDa

e) 47 kDa

f) 38 kDa
Figure 11. Intensity of protein bands (CA) in mated and virgin cockroaches ( Autorad SDS-PAGE).

Intensity of newly synthesized proteins from CA of mated and virgin female cockroaches of different ages (day0-10). Mated females are represented by (●) and virgin females by (●). Points represent the mean of 2-3 replicates. Vertical bars indicate SEM. Intensity represents relative intensity as the pixel value of a selected point on the gel in terms of that pixels brightness. Gels were scanned at 600 dpi. Symbols indicate statistically significant differences between the means in mated females according to one-way ANOVA (p<0.05). Following this analysis the Tukey's multiple comparison test indicates, ●-significantly different from day 0, and ●-significantly different from day 6. There was no difference between the means for virgin females indicated by one-way ANOVA (p<0.05). There was a significant difference between mated and virgin animals for all bands as indicated by two-way ANOVA (p<0.05).
a) 92 kDa

Intensity

Age

M
V

b) 70 kDa

Intensity

Age

M
V

c) 54 kDa

Intensity

Age

M
V

d) 50 kDa

Intensity

Age

M
V
Figure 12. Intensity of protein bands from the CA in 4th instar cockroaches.

Intensity of protein bands from the CA of 4th instar female cockroaches of different ages (days 0-10). Intensity represents relative intensity as the pixel value of a selected point on the gel in terms of that pixels brightness. Gels were scanned at 600 dpi. A) Intensity of protein bands from Coomassie blue-stained gel. 7.5% SDS-PAGE was used to separate proteins. B) Intensity of protein bands from autoradiograph (\(^{35}\)S-methionine). 12% SDS-PAGE was used to separate proteins.
the intensity was higher than it was for CA protein bands (Fig. 13). Therefore, the results describing change in band intensity agree with the data for protein synthesis ([35S]-methionine incorporation).
Figure 13. Intensity of protein bands from the brain in mated cockroaches.

Intensity of protein bands from the brains of mated female cockroaches of different ages (days 0-10). Intensity represents relative intensity as the pixel value of a selected point on the gel in terms of that pixel's brightness. Gels were scanned at 600 dpi. A) Intensity of protein bands from Coomassie blue-stained gel. B) Intensity of protein bands from autoradiograph ($^{35}$S-methionine). 12% SDS-PAGE was used to separate proteins.
II. Protein inhibitor and allatostatin studies

1. The effect of protein synthesis inhibitors on protein synthesis in the CA of *Diploptera punctata*.

   **Cycloheximide**

   a) Protein synthesis

   We found that cycloheximide inhibited protein synthesis (incorporation of $[^{35}\text{S}]$methionine into proteins *in vitro*) by the CA by 91% relative to the control sample (Figure 14). The effect of cycloheximide on JH release was also determined, and is represented as the % JH release relative to control levels. Both stimulation and inhibition of JH biosynthesis were observed, although they were not statistically significant (Figure 15).

   b) Protein synthesis profiles

   Protein synthesis profiles from CA treated with cycloheximide are represented by SDS-PAGE. The gels that are shown are representative of replicate gels.

   Figure 16 indicates the bands of interest that were separated on SDS-PAGE. Figure 16 A, SDS-PAGE stained with Coomassie blue, showed that even in the presence of cycloheximide, many proteins were present. Newly synthesized proteins were inhibited (autoradiograph) with the exception of one band (24 kDa) that was visible in the presence of cycloheximide (Fig 16 B). Proteins with M, 55, 50, and 44 33, 32 kDa consistently appeared on gels. The 24 kDa band was present even when protein synthesis was inhibited and most other bands were not present, as seen in the autoradiograph (Fig. 16 B).
Figure 14. The effect of cycloheximide on protein synthesis in the CA of day 7 mated female cockroaches.

The effect of cycloheximide on protein synthesis in the CA of day 7 mated female cockroaches as revealed by $[^{35}\text{S}]$methionine incorporation. Control represents no treatment with cycloheximide. Treatment used were $5 \times 10^{-5}$ M and $2 \times 10^{-3}$ M. Bars represent the means of 8-9 replicates. Vertical bars indicate SEM. * indicates significant difference from control (T-test, p<0.05).
Incorporation of $[^{35}\text{S}]$ methionine into precipitated protein (cpm)
Figure 15. JH release by CA of day 7 mated female cockroaches in the presence of cycloheximide.

The effect of cycloheximide on JH release by CA of day 7 mated female cockroaches. The percentage of inhibition of JH release is shown as a percentage of control levels of JH. Negative values represent stimulation. Points represent the means of 36-39 replicates. Vertical bars indicate SEM.
CA
Day 7 mated females
Cycloheximide

Figure 16. The profile of protein bands in CA treated with cycloheximide.

The effect of cycloheximide on protein synthesis in day 7 mated female CA as revealed by Coomassie blue staining, [35S]methionine incorporation and autoradiography. Proteins were separated on a 12% SDS-PAGE. Each lane represents the condition a-control (no cycloheximide); b-5x10^{-5} M; c-2x10^{-3} M (a_{2}-c_{2} are replicate conditions) under which CA were incubated. A) Coomassie blue gel, arrows to the right of the gels represent bands that were analyzed and discussed, Mr 78, 55, 50, 44, 33, 32, 22 kDa (top to bottom). B) Autoradiograph, arrows to the right, Mr 83, 65, 55, 46, 42, 31, 27, 24 kDa (top to bottom).
Examination of the intensity of the protein bands from CA incubated in the presence of cycloheximide (Fig. 17) reveals that newly synthesized proteins in the CA can be inhibited. When examining Coomassie stained bands (Fig. 17 A), the intensity did not appear to change drastically even though cycloheximide was present. However, examination of newly synthesized proteins (Fig. 17 B) on the autoradiograph showed a significant decrease in intensity of the protein bands. These results are in agreement with those obtained from incorporation of $^{35}$S-methionine into protein (cycloheximide) results (Figure 14).

ii) Anisomycin

a) Protein synthesis

Figure 18 shows the effect of anisomycin on protein synthesis (incorporation of $^{35}$S-methionine into proteins in vitro) in the CA of D. punctata. Fifty percent inhibition occurred at $10^{-6}$ M, and 89-94% inhibition was achieved at $10^{-5}$ and $10^{-4}$ M relative to control values. At $10^{-3}$ M, no significant difference from the control was observed, which was likely attributable to the small sample size. As with cycloheximide, anisomycin did not have a significant effect on the rates of JH biosynthesis by the CA (Fig. 19).

b) Protein synthesis profiles

The effect of anisomycin on profiles of protein synthesis were observed using SDS-PAGE. The gels that are shown are representative of replicate gels.

The protein bands that were consistently observed on SDS-PAGE are the same as those noted for the cycloheximide-treated CA (Figure 20). Figure 20 A shows the
Figure 17. Intensity of protein bands at different concentrations of cycloheximide from day 7 mated female cockroaches.

Intensity of protein bands at different concentrations of cycloheximide in CA from day 7 mated female cockroaches. Intensity represents relative intensity as the pixel value of a selected point on the gel in terms of that pixels brightness. Gels were scanned at 600 dpi. A) Intensity of protein bands from Coomassie blue-stained gel. B) Intensity of protein bands from autoradiograph (35S-methionine incorporation). Points represent the mean of 2 replicates and vertical bars indicate SEM. 12% SDS-PAGE was used to separate proteins. *Indicates significant differences for all bands compared to similar bands in controls (T-test, p<0.05).
Figure 18. The effect of anisomycin on protein synthesis in the CA of day 7 mated female cockroaches.

The effect of anisomycin on protein synthesis in the CA of day 7 mated female cockroaches as revealed by \([^{35}S]\)methionine incorporation. Control represents no treatment with anisomycin. Concentration of anisomycin ranged from 10\(^{-8}\)-10\(^{4}\)M. Bars represent the means and the n=2-5. Vertical bars indicate SEM. ★ indicates statistical difference from control (T-test, p<0.05).
Figure 19. JH release by CA of day 7 mated female cockroaches in the presence of anisomycin.

The effect of anisomycin on JH release by CA of day 7 mated female cockroaches. The percentage of inhibition of JH release is shown as a percentage of control levels of JH. Negative values represent stimulation. Points represent the means of 29-32 replicates. Vertical bars indicate SEM.
Coomassie blue-stained gel of the CA from day 7 mated females in which proteins were present, even in the presence of anisomycin. Autoradiography (Fig. 20 B) demonstrates that proteins were not synthesized at $10^{-5}$ and $10^{-4}$ M anisomycin. As with the cycloheximide treatment, one band (24 kDa) persists in the presence of anisomycin, even at the highest concentration tested.

Figure 21 shows the intensity of protein bands from CA that were incubated with anisomycin (see methods). Examination of the Coomassie stained bands in CA from day 7 mated females indicate that there was no apparent difference in intensity in the presence of anisomycin (Fig. 21 A). However, newly synthesized proteins (autoradiograph, Fig. 21 B) showed a difference in band intensities at $10^{-4}$ M anisomycin. The 25 kDa protein band at $10^{-4}$ M was significantly different from all other band intensities at that concentration.

iii) Actinomycin D

a) Protein synthesis

Actinomycin D ($10^{-5}$ M) incorporation of $^{35}$S-methionine into proteins \textit{in vitro} by the CA and was the only concentration of actinomycin D compared with the control value (Fig. 22). The reason for this was that the amount of ethanol was comparable only under these two conditions (0.6%). There was no significant difference between $10^{-5}$ M and the control value. The only results that can be compared with respect to JH biosynthesis are the control and $10^{-5}$ M conditions, thus making it difficult to speculate on the effect of Actinomycin D on JH biosynthesis (Figure 23).
Figure 20. The profile of protein bands in CA treated with anisomycin.

The effect of anisomycin on protein synthesis in day 7 mated female CA as revealed by Coomassie blue staining, [$^{35}$S]methionine incorporation and autoradiography. Proteins were separated on a 12% SDS-PAGE. Each lane represents condition (control, [anisomycin]) a-control (no anisomycin); b-10$^{-8}$ M; c-10$^{-7}$ M; d-10$^{-6}$ M; e-10$^{-5}$ M; f-10$^{-4}$ M, under which CA were incubated. A) Coomassie blue gel, arrows to the right of the gels represent bands that were analyzed and discussed, M, 76, 57, 52, 45, 33, 32, 23 kDa (top to bottom). B) Autoradiograph, arrows to the right, M, 72, 44, 36, 25, 20 kDa (top to bottom).
Figure 21. Intensity of protein bands at different concentrations of anisomycin from day 7 mated female cockroaches.

Intensity of protein bands at different concentrations of anisomycin in CA from day 7 mated female cockroaches. Intensity represents relative intensity as the pixel value of a selected point on the gel in terms of that pixel's brightness. Gels were scanned at 600 dpi. A) Intensity of protein bands from Coomassie blue-stained gel. B) Intensity of protein bands from autoradiograph (\(^{35}\)S-methionine incorporation). Points represent the means of 4 replicates and vertical bars indicate SEM. 12% SDS-PAGE was used to separate proteins. * is significantly different from all other bands treated with 10^{-4}M. *(20kDa) is not significantly different from control band. All other bands at 10^{-4}M are significantly different from their respective control bands (T-test, p<0.05).
Figure 22. The effect of actinomycin D on protein synthesis in the CA of day 7 mated female cockroaches.

The effect of actinomycin D on protein synthesis in the CA of day 7 mated female cockroaches as revealed by \(^{35}\text{S}\)methionine incorporation. Control represents no treatment with actinomycin D (0.6% ethanol). The concentration of actinomycin D ranged from \(5 \times 10^{-4}-10^{-4}\) M. Bars represent the means and the number of replicates are stated. Vertical bars indicate SEM.
b) Protein synthesis profiles

The effect of Actinomycin D on protein synthesis was determined using SDS-PAGE. The gels that are shown are representative of replicate gels.

Actinomycin D did not inhibit Coomassie stained bands (Fig. 24 A) or de novo synthesis (Fig. 24 B) at any concentration tested. The protein bands that consistently appeared on SDS-PAGE were grouped as with cycloheximide- and anisomycin-treated bands (Figure 24).

Figure 25 shows the intensity of protein bands that were examined. The only conditions that were compared were control and $10^{-4}$ M. At that concentration the 30 kDa band was significantly different from control band (Fig. 25).

2. The effect of Dip-allatostatins on protein synthesis in the CA of *Diploptera punctata*

i) Dip-allatostatin 2

a) Protein synthesis

At $10^{-8}$ M, Dip-allatostatin 2 produced a small but significant inhibition of protein synthesis (incorporation of $^{35}$S-methionine into proteins *in vitro*) compared to the control (16%). No other concentration produced significant changes in protein synthesis (Fig. 26). The effect of Dip-allatostatin 2 was not dose dependent.

b) Protein synthesis profiles

Proteins were present on Coomassie blue-stained gels and autoradiographs (Fig. 27 A, 27 B). Treatment with Dip-AST 2 did not result in inhibition of protein synthesis
Figure 23. JH release by CA of day 7 mated female cockroaches in the presence of Actinomycin D.

The effect of actinomycin D on JH release in CA of day 7 mated female cockroaches. The percentage of inhibition of JH release is shown as a percentage of control levels of JH release. Negative values represent stimulation. Bars represent the means of 24 replicates. Vertical bars indicate SEM.
**CA**  
**Day 7 mated females**  
**Actinomycin D**

**Figure 24. The profile of protein bands in CA treated with actinomycin D**

The effect of actinomycin D on protein synthesis in day 7 mated female CA as revealed by Coomassie blue staining, [$^{35}$S]methionine incorporation and autoradiography. Proteins were separated on a 12% SDS-PAGE. Each lane represents condition (control, or [actinomycin D]) under which CA were incubated. a- no actinomycin D; b- no actinomycin D, 0.6% ethanol; c-5x10$^{-8}$ M; d-5x10$^{-7}$ M; e-5x10$^{-6}$ M; f-10$^{-5}$ M. A) Coomassie blue gel, arrows to the right of the gels represent bands that were analyzed and discussed, M, 73, 56, 50, 44, 33, 32, 22 kDa (top to bottom). B) Autoradiograph, arrows to the right, M, 70, 56, 46, 44, 36, 30, 24 kDa (top to bottom).
Figure 25. Intensity of protein bands at different concentrations of actinomycin D from day 7 mated female cockroaches.

Intensity of protein bands at different concentrations of actinomycin D in CA from day 7 mated female cockroaches. Intensity represents relative intensity as the pixel value of a selected point on the gel in terms of that pixel's brightness. Gels were scanned at 600 dpi. A) Intensity of protein bands from Coomassie blue-stained gel. B) Intensity of protein bands from autoradiograph (35S-methionine incorporation). Points represent the means of 4 replicates and vertical bars indicate SEM. 12% SDS-PAGE was used to separate proteins. * (30 kDa) is significantly different from control band at 10^{-5} M (T-test, p<0.05).
Figure 26. The effect of Dip-allatostatin 2 on protein synthesis in the CA of day 7 mated female cockroaches.

The effect of Dip-allatostatin 2 on protein synthesis in the CA of day 7 mated female cockroaches as revealed by [3S]methionine incorporation. Control represents no treatment with Dip-allatostatin 2. Concentration of Dip-allatostatin 2 ranged from $10^{-10}$- $10^{-6}$ M. Bars represent the means and the number of replicates are stated. Vertical bars indicate SEM. * indicates significant difference from control (T-test, p<0.05).
Incorporation of $[^{35}\text{S}]$ methionine into precipitated protein (cpm)
to the same degree as seen following treatment with inhibitors of protein synthesis. The bands that were examined were again grouped as defined previously (Figure 27).

The intensities of the bands as seen on the Coomassie blue-stained gels (Fig. 28 A) do not appear to change in the presence of Dip-AST 2. Similarly, with newly synthesized proteins on the autoradiograph (Fig. 28 B), there is no significant difference in intensity following treatment with Dip-allatostatin 2.

ii) Dip-allatostatin 7

a) Protein synthesis

Treatment with Dip-AST 7 resulted in a 51% inhibition of protein synthesis (incorporation of $^{35}$S-methionine into proteins in vitro) at $10^{-6}$ M (P<0.05). Lower doses of Dip-AST 7 failed to produce significant inhibition (Fig. 29). Inhibition of protein synthesis with Dip-AST 7 appears to occur in a dose-dependent manner.

b) Protein synthesis profiles

The results of incubation of CA with Dip-AST 7 were similar to those obtained with Dip-AST 2, and are shown in Figure 30.

Examination of the intensities of protein bands on the Coomassie blue-stained gels (Fig. 31 A), indicates that they do not appear to change in the presence of Dip-AST 7. Examination of specific newly synthesized proteins revealed that at the highest concentration used, intensities of selected bands (36, 25 and 20 kDa) were significantly different from control intensities (Figure 31 B). These data are consistent with results from the protein synthesis (incorporation of $^{35}$S-methionine into proteins) experiments (Figure 29).
CA
Day 7 mated females
Dip-AST 2

Figure 27. The profile of protein bands in CA treated with Dip-allatostatin 2.

The effect of Dip-allatostatin 2 on protein synthesis in day 7 mated female CA as revealed by Coomassie blue staining, [³⁵S]methionine incorporation and autoradiography. Proteins were separated on a 12% SDS-PAGE. Each lane represents condition (control, or [Dip-allatostatin 2]) under which CA were incubated. a-no Dip-AST 2; b-10⁻¹⁰ M; c-10⁻⁹ M; d-10⁻⁸ M; e-10⁻⁷ M; f-10⁻⁶ M. A) Coomassie blue gel; arrows to the right of the gels represent bands that were analyzed and discussed; M, 78, 54, 50, 44, 33, 32, 22 kDa (top to bottom). B) Autoradiograph; arrows to the right, M, 68, 52, 43, 35, 32, 25, 19 kDa (top to bottom).
Figure 28. Intensity of protein bands at different concentrations of Dip-allatostatin 2 from day 7 mated female cockroaches.

Intensity of protein bands at different concentrations of Dip-allatostatin 2 in CA from day 7 mated female cockroaches. Intensity represents relative intensity as the pixel value of a selected point on the gel in terms of that pixels brightness. Gels were scanned at 600 dpi. A) Intensity of protein bands from Coomassie blue-stained gel. B) Intensity of protein bands from autoradiograph (35S-methionine incorporation). Points represent the means of 3 replicates and vertical bars indicate SEM. 12% SDS-PAGE was used to separate proteins. No statistical significance between experimental and control bands (T-test, p<0.05).
Figure 29. The effect of Dip-allatostatin 7 on protein synthesis in the CA of day 7 mated female cockroaches.

The effect of Dip-allatostatin 7 on protein synthesis in the CA of day 7 mated female cockroaches as revealed by \(^{35}\)S methionine incorporation. Control represents no treatment with Dip-allatostatin 7. Concentration of Dip-allatostatin 7 ranged from $10^{-10}$ to $10^4$ M. Bars represent the mean and $n=3-7$. Vertical bars indicate SEM. * indicates significant difference from control (T-test, $p<0.05$).
Incorporation of $^{35}$S methionine into precipitated protein (cpm)
Figure 30. The profile of protein bands in CA treated with Dip-allatostatin 7.

The effect of Dip-allatostatin 7 on protein synthesis in day 7 mated female CA as revealed by Coomassie blue staining, [35S]methionine incorporation and autoradiography. Proteins were separated on a 12% SDS-PAGE. Each lane represents condition (control, or [Dip-allatostatin 7]) under which CA were incubated. a- no Dip-AST 7; b- $10^{-10}$ M; c- $10^{-9}$ M; d- $10^{-8}$ M; e- $10^{-7}$ M; f- $10^{-6}$ M. A) Coomassie blue gel; arrows to the right of the gels represent bands that were analyzed and discussed; M, 76, 54, 50, 44, 33, 32, 22 kDa (top to bottom). B) Autoradiograph; arrows to the right, M, 70, 44, 36, 34, 25, 20 kDa (top to bottom).
Figure 31. Intensity of protein bands at different concentrations of Dip-allatostatin 7 from day 7 mated female cockroaches.

Intensity of protein bands at different concentrations of Dip-allatostatin 7 in CA from day 7 mated female cockroaches. Intensity represents relative intensity as the pixel value of a selected point on the gel in terms of that pixels brightness. Gels were scanned at 600 dpi. A) Intensity of protein bands from Coomassie blue stained gel. B) Intensity of protein bands from autoradiograph (35S-methionine incorporation). Points represent the means of 4 replicates and vertical bars indicate SEM. 12% SDS-PAGE was used to separate proteins. *(36 kDa), *(25 kDa), *(20 kDa) at 10⁻⁶ M were significantly different from their respective control bands (T-test, p<0.05).
iii) Dip-allatostatin 13

a) Protein synthesis

Dip-AST 13 was able to produce small but significant changes in protein synthesis (incorporation of $^{35}$S-methionine into proteins *in vitro*) but the effect did not appear to be dose-dependent (Fig. 32). Very low ($10^{-10}$ M) doses produced a 17% inhibition of protein synthesis, whereas $10^{-8}$ M doses produced a stimulation of synthesis of approximately equal magnitude (18% stimulation). Intermediate and higher doses did not produce any significant change from control levels of protein synthesis.

b) Protein synthesis profiles

Proteins from CA incubated with Dip-AST 13 were separated on Coomassie blue-stained gels and autoradiographs (Figure 33). As with the other Dip-ASTs, the Coomassie blue-stained and newly synthesized protein gels did differ drastically from each other with respect to separation of protein bands on the gels. The bands were grouped as previously determined (Figure 33).

When examining Coomassie stained bands in the CA, intensities of protein bands (Fig. 34 A) does not appear to vary with the concentration of Dip-AST 13. However, band intensities of newly synthesized proteins (autoradiograph, Fig. 34 B) did not vary appreciably, with the exception of bands at low concentrations of Dip-AST 13 (Fig. 34 B). At $10^{-10}$ M, the intensity of all bands except 25 kDa band decreased. Interestingly, there was a difference in protein synthesis at this low concentration as well (figure 32).
Figure 32. The effect of Dip-allatostatin 13 on protein synthesis in the CA of day 7 mated female cockroaches.

The effect of Dip-allatostatin 13 on protein synthesis in the CA of day 7 mated female cockroaches as revealed by \([^{35}\text{S}]\text{methionine incorporation. Control represents no treatment with Dip-allatostatin 13. Concentration of Dip-allatostatin 13 ranged from }10^{-10}-10^{-4} \text{ M. Bars represent the means and } n=1-3. \text{ Vertical bars indicate SEM. } \star \text{ indicates significant difference from control (T-test, } p<0.05).
Incorporation of $[^{35}\text{S}]$ methionine into precipitated protein (cpm)
Figure 33. The profile of protein bands in CA treated with Dip-allatostatin 13.

The effect of Dip-allatostatin 13 on protein synthesis in day 7 mated female CA as revealed by Coomassie blue staining, [³⁵S]methionine incorporation and autoradiography. Proteins were separated on a 12% SDS-PAGE. Each lane represents condition (control, or [Dip-allatostatin 13]) under which CA were incubated. a- no Dip-AST 13; b-10⁻¹⁰ M; c-10⁻⁹ M; d-10⁻⁸ M; e-10⁻⁷ M; f-10⁻⁶ M. A) Coomassie blue gel; arrows to the right of the gels represent bands that were analyzed and discussed; M, 76, 53, 50, 45, 33, 32, 22 kDa (top to bottom). B) Autoradiograph; arrows to the right, M, 68, 53, 43, 35, 33, 25, 19 kDa (top to bottom).
Figure 34. *Intensity of protein bands at different concentrations of Dip-allatostatin 13 from day 7 mated female cockroaches.*

Intensity of protein bands at different concentrations of Dip-allatostatin 13 in CA from day 7 mated female cockroaches. Intensity represents relative intensity as the pixel value of a selected point on the gel in terms of that pixels brightness. Gels were scanned at 600 dpi. A) Intensity of protein bands from Coomassie blue-stained gel. B) Intensity of protein bands from autoradiograph (\(^{35}\)S-methionine incorporation). Points represent the means of 3 replicates and vertical bars indicate SEM. 12% SDS-PAGE was used to separate proteins. *(25 kDa) no significant difference from control band. All other bands treated with \(10^{-10}\) M were significantly different from their respective control bands (T-test, \(p<0.05\)).
DISCUSSION

I. Developmental protein synthesis

Many of the developmental changes in *D. punctata* arise from changes in the production of JH and with associated change in biosynthetic pathways in the CA (Stay and Tobe, 1977). In this study, we set out to describe changes in profiles of protein synthesis in CA of different developmental stages of *D. punctata*. We found that profiles of protein synthesis in the CA change depending on the developmental stage of *D. punctata*. In this study, when we refer to profiles of protein synthesis, it should be understood that this describes the rate of protein synthesis according to incorporation of \([^{35}S]\)-methionine into protein *in vitro*.

There is a relationship between the basal oocyte length and the cycle of JH production in mated *D. punctata* (Stay and Tobe 1977; Stay *et al*., 1983; Rankin and Stay, 1984). Mated females mature oocytes at a rapid rate and show a distinct cycle of JH biosynthesis whereas virgin females do not (Stay and Tobe 1977). Figure 35 illustrates the cycle of JH biosynthesis, oocyte length, and cell number in the CA of mated females during a ten day period. The protein synthesis profile in CA of mated cockroaches parallels the cycle of JH biosynthesis. For example, JH biosynthesis is high on days 4, 5, with a slight decline on day 6 (Fig. 35). This seems to correlate with the profile of increased protein synthesis on days 4, 5, 6 with the decline occurring on day 7 in mated animals (Fig 3). As with JH biosynthesis in virgin cockroaches, there is no apparent cycle of protein synthesis (Fig. 3). Mated and virgin female animals show different rates of protein synthesis (Fig. 3). We found a significant difference between
Figure 35. Changes in CA cell number, basal oocyte length and JH biosynthesis as a function of age during the first reproductive cycle.

Schematic representation showing the typical cycle of JH biosynthesis and changes in CA cell number and basal oocyte length (based on Szibbo and Tobe, 1981).
JH Biosynthesis —
(pmoll/hr per pair CA)

Number of Cells ——
(x100)

Age (Days)

0 25 50 75 100

0 2 4 6 8 10 12

0 1 2 3 4 5 6 7 8 9 10 11 12

Oocyte Length ——

0.5 1.0 1.5 2.0 2.5

(x100)
mated and virgin animals for newly synthesized proteins (revealed by autoradiographs) in the CA during the 10 day period (Fig. 11). However, during the ten days examined, there was no significant difference for Coomassie-stained protein bands in the CA between mated and virgin animals (Fig. 10). On the basis of these findings, it is proposed that the increase in protein synthesis may be related to the modulation of JH biosynthesis. Although it was not our objective to identify specific proteins in the CA, we have suggested some possibilities, on the basis of their likely involvement in cellular and physiological activities occurring within the CA, and their appropriate molecular weights.

**Proteins involved in cell division and the cell cycle**

In mated female *D. punctata*, CA cell number, volume and cytoplasmic-nuclear ratio all increase to a maximum on day 5 and then decrease in the latter half of the gonotrophic cycle. This pattern parallels the cycle of JH biosynthesis (Szibbo and Tobe, 1981). Proteins synthesized in mated CA also show this increase in the first part of the gonotrophic cycle, with the maximum occurring on day 6 and then decreasing thereafter (Fig. 3). Szibbo and Tobe (1981) proposed that increases in cell number are attributed to cell division, and the corresponding decreases in the later part of the gonotrophic cycle involve cellular destruction. Therefore, it is likely that the increase in protein synthesis in mated animals is involved in the growth, mitosis or cell division and destruction of cells in the CA. Cellular destruction in the CA may involve programmed cell death, known as apoptosis. The possibility of apoptosis in CA cells is interesting with respect to protein synthesis because of a family of proteins known as caspases. These caspases are cleaving enzymes and have recently been proposed to be involved in apoptosis (Barinaga, M.,
Therefore in the latter part of the gonotrophic cycle when cellular destruction or apoptosis may occur some of the proteins present in this study could include caspases.

In 4th instar cockroaches, there is a high initial rate of JH biosynthesis which decreases within two days, followed by a small increase on day 4. Thereafter, JH biosynthesis declines and is virtually zero by day 10. JH biosynthesis is undetectable in 4th instars between days 10-18 (Szibbo et al., 1982). Protein synthesis in 4th instar animals (Fig. 4) showed an increase starting around day 8 when JH declines drastically. Interestingly, in 4th instar animals, increases in cell number and volume occur in the CA at a time when the CA exhibit the lowest JH biosynthesis. In mated animals, we found that protein synthesis was highest when JH biosynthesis is highest, and remained high on day 6 when JH biosynthesis begins to decrease. Therefore as in mated animals, the increases in protein synthesis coincide with increases in cellular division and volume. This may indicate that some of the increase in overall protein synthesis is associated with the synthesis of proteins involved in cell division and the cell cycle.

Cell growth involves two well recognized events: the duplication of cellular DNA and the physical division of the cell into two daughter cells. The cell cycle consists of four distinct periods: G₁ (first gap) phase is the gap between the previous nuclear division and the beginning of DNA synthesis; S (synthetic) phase, the period of DNA synthesis; G₂ (second gap) phase, the gap between DNA replication and nuclear division; and M (mitotic) phase, the period of mitosis, during which the cell actually divides (Darnell et al., 1990).

Progress through the cell cycle is generally associated with altered patterns of translation, and changes in profiles of protein synthesis during the mammalian cell cycle.
were shown in an early study by Kolodny and Gross (1969). This study revealed that the pattern of protein synthesis in G2 is different from that for other stages of the cell cycle. Proteins such as kinases and phosphatases appear to be the 'key players' in the cell cycle (Lewin, 1990). Attention has been focused upon the role of protein phosphorylation in regulating progression through the cell cycle (Mayer-Jaekel et al., 1994). Therefore, it is possible that some of the proteins visualized in the CA of D. punctata may be the kinases and phosphatases that are implicated in cell cycle regulation. The M phase in the cell cycle is characterized by the activation of a protein kinase called M phase promoting factor (MPF). MPF consists of two subunits, of 34 kDa and 45 kDa. The 34 kDa subunit is referred to as p34^cd^2 and has been identified as a phosphoprotein. The 45 kDa protein is known as cyclin (Lewin, 1990). Homologs of this MPF have been described in virtually every eukaryotic cell examined to date (Witters 1990). In vertebrates, p34^cd^2 is just one member of a family of related protein kinases, (cdks) that form complexes with members of a family of regulatory subunits, the cyclins, to control progression through the cell cycle (Reed, 1992; Mayer-Jaekel et al., 1994). The p34 protein requires a cyclin partner for full kinase activity. Interestingly p34^cd^2 kinase is itself regulated by a phosphorylation state. It is activated by an enzyme identified in Xenopus as p40^{M015} kinase and dephosphorylated by cdc25 protein phosphatase (Mayer-Jaekel et al., 1994). In the yeast Schizosaccharomyces pombe, the timing of mitosis is thought to be connected with a protein known as p80^{cd25}. The level of this protein increases 4- to 5-fold as cells approach the G2/M boundary, and then increases further at M phase (Lewin, 1990). The components of the M phase kinase, cdc25 has a counterpart in the string gene of D. melanogaster (Edgar and O'Farrell, 1989). Proteins synthesized in D. punctata CA
during development could very well correspond to some of these proteins. For example, in both mated and virgin animals, SDS-PAGE showed bands that were 35 kDa and 32 kDa and a 31 kDa band in 4th instars (Fig 6.2, 7.2 and 9.1); one of these bands could be a homolog of the p34 kinase involved in cell cycle regulation. In addition, a 44 kDa protein was present in mated and virgin animals, which could be a cyclin protein. Cyclins accumulate during the cell cycle and are destroyed during mitosis. The destruction of cyclin inactivates the kinase and the cell is required to exit mitosis (Lewin, 1990). This may explain why there is an increase in protein synthesis in mated animals but not in virgins. Increased synthesis in mated CA (Fig. 11) may reflect the increase in mitotic rate and cell number. Accumulating cyclins would also be reflected in an increase in band intensity (Coomassie, Fig. 10). Destruction of these cyclins results in cessation of mitosis, decreasing the need for new protein synthesis and also reducing the amount of protein seen after day 6.

Another example of proteins involved in the control of cell growth are the guanosine triphosphate (GTP)-binding proteins and their involvement in the control of cellular growth may be direct or indirect. One class of GTP-binding proteins comprises the small GTP-binding proteins (smgs) which are about 21-25 kDa in size (McCormick, 1990). The functions of these smgs are not well understood, but include processes fundamental to normal cell growth. The best known smgs are the ras proteins, which control pathways intimately associated with regulation of cell growth (McCormick, 1990). In the CA, a 25 kDa protein occurs in both mated and virgin females and a 27 kDa band in 4th instars (Fig. 6.2, 7.2 and 9.2) and could correspond to a smg. There is evidence that growth-regulated p21 proteins, encoded by ras protooncogenes and
oncogenes, and nuclear envelope proteins, are covalently attached to farnesyl residues, which anchor them to cell membranes (Goldstein et al., 1990). The inhibition of mevalonate synthesis prevents farnesylation of p21^ras proteins and blocks cell growth. Insects cannot synthesize cholesterol de novo as vertebrates can. However, vertebrate sterol biosynthesis and juvenile hormone biosynthesis in insect CA share a common pathway up to farnesyl pyrophosphate synthesis (Feyereisen, 1985; Martinez-Gonzalez, et al., 1993). In D. punctata, the conversion of HMG-CoA to mevalonate by the enzyme HMG-CoA reductase occurs early in the JH biosynthetic pathway (Fig. 2) on the endoplasmic reticulum (Tobe and Stay, 1985). Thus, it may be proposed that the inhibition of mevalonate synthesis, and blocking of cell growth and protein synthesis could theoretically all be related; decreases in JH biosynthesis may be partly attributed to the inhibition of HMG CoA reductase which in turn blocks cell growth and therefore may account for decreases in protein synthesis. This is interesting because, rather than inhibition or a decrease in protein synthesis causing a decrease in JH biosynthesis directly, an unidentified factor may be blocking both, by inhibiting mevalonate synthesis.

**Proteins involved in the cytoskeleton**

The cytoskeleton plays important roles in the cell including movement of nuclei and organelles and delivery of secretory vesicles to a particular region of the cell surface (Barnes et al., 1990). The cytoskeleton consists of three types of filamentous cytoplasmic structures: microfilaments, intermediate filaments, and microtubules. Microfilaments comprise actin filaments, which in non-muscle cells, appear to be involved in both cellular motility and in the structure of the cytoplasmic matrix (Pollard, 1990). Actin filaments are helical polymers of 42 kDa subunits, connected by non-
covalent bonds (Carraway and Carraway, 1992). The 44 kDa band in *D. punctata* CA could correspond to actin (Figs. 6.2 and 7.2). Intermediate filaments (IFs) are found in the nucleus (major component of nuclear lamina) and in the cytoplasm of eukaryotic cells, although the functions of IFs remain unclear (Carraway and Carraway, 1992). Microtubules are composed of the protein tubulin, which in its native form, exists as a heterodimer comprising α- and β-tubulin. Both α- and β-tubulin are more labile than other types of filamentous structures, undergoing rapid and extensive alterations during cell division (Carraway and Carraway, 1992). It appears that almost all intracellular organelles can move along or be linked to microtubules (Kelly, 1990). Tubulin in the pig brain has a relative molecular mass of 110 kDa and is made of two filaments, consisting of α-tubulin (56 kDa) and β-tubulin (53 kDa). In insects, β-tubulin is 55 kDa in *D. melanogaster* and 50 kDa in *M. sexta* (Fasman, 1989; Rybczynski and Gilbert, 1995). Although we have not purified the proteins from *D. punctata* CA, the fact that eukaryotic cells contain cytoskeletal proteins and that proteins of appropriate molecular mass were observed, it is likely that some of the proteins are cytoskeletal. Mated and virgin cockroach CA contain proteins with a Mr of 54 and 50 kDa, and one or both of these may be α or β-tubulin (Figs. 6.1, 6.2, 7.1 and 7.2). The intensity of the 50 kDa band in mated animals changes significantly over the ten day cycle, in both Coomassie blue-stained gel, (Fig. 10) and in autoradiographs (representing newly synthesized proteins, Fig. 11). In addition to the 50 kDa band, Figure 11 illustrates that the intensity of the 54 kDa band also significantly increases in mated animals. If these proteins do correspond to tubulin,
tubulin production might be reasonably expected to increase with an increase in cell number.

Tubulin is often associated with movement of organelles and as mentioned above, can be involved in the movement of secretory vesicles to the surface (Kelly 1990; Barnes et al., 1990). Newly synthesized JH is secreted by the CA almost immediately, but there is no evidence that JH is released by exocytosis. In *D. punctata*, the endoplasmic reticulum (ER) membranes often closely approach the plasma membrane, and it has been suggested that JH release depends on a juxtaposition, or possibly fusion of the ER membranes with the plasma membrane followed by diffusion of JH molecule through the lipid layers (Johnson et al., 1985). Tubulin could thus be involved in the movement of the ER to the plasma membrane. It is possible that tubulin synthesis may increase with high rates of JH biosynthesis because at these times, there would be more movement of ER to permit more JH release. On the other hand, at the beginning and end of the vitellogenic cycle in mated animals, (time of low JH production), less tubulin would be needed as a consequence of the reduced movement of the ER (Fig. 11 c, d).

Not only could the proteins observed in the CA be cytoskeletal proteins, but they could also be proteins associated with cytoskeletal filaments. For example, there are many proteins associated with actin, IFs and tubulin. A few examples of proteins associated with actin are profilin (16 kDa) and actin-binding protein (250 kDa) (Korn, 1978). Alpha (66 kDa) and β-internexin (70 kDa) are intermediate filament-associated proteins (Pachter et al., 1985; Napolitano et al., 1985). Some examples of proteins that bind or associate with microtubulin are kinesins (120 kDa and 62 kDa). They have been
found in bovine brain, sea urchin eggs and *D. melanogaster*. Another example is the dynein family of proteins in which subunits range between 14 and 120 kDa depending on the source (Vale, 1990). Although some of these relative molecular masses correspond to bands that were observed in this study (Figs. 6.1-8.2), they may not have been analyzed in detail in this study. Therefore, the examples given here provide an indication of proteins likely to be present in the CA.

**Structural and membrane proteins**

Membrane-associated structural proteins, known collectively as the spectrin-based membrane skeleton, form a ubiquitous membrane structure capable of interacting with a variety of integral membrane proteins. Spectrins can be viewed as a major class of structural proteins and the plasma membrane equivalent of the cytoplasmic structural proteins such as actin, IFs and tubulin (Bennett, 1990). The spectrin-based membrane skeleton was first characterized in human erythrocytes, but is also known in non-erythroid cells (Bennett, 1990). Spectrin encompasses a family of proteins found in *D. melanogaster* and echinoderms (Byers *et al*., 1989; Fishkind *et al*., 1987). It is likely that some of the proteins observed in the CA are membrane and/or structural proteins.

**Cellular organelles and protein involvement**

The smooth endoplasmic reticulum (SER) is directly involved in the biosynthesis of JH, a major function of a typical CA cell (Tobe and Stay 1985; Johnson *et al*., 1985). The conversion of HMG-CoA to mevalonate (HMG-CoA reductase) and methyl farnesoate to JH III (*methyl farnesoate epoxidase*) occur on or within the endoplasmic reticulum; see Figure 2 (Tobe and Stay, 1985). Since the smooth endoplasmic reticulum is the site of these enzymes, one might expect that this organelle is a major component of
CA cells. Although Johnson et al. (1985) had difficulties visualizing the SER, changes in its form were recognizable, and the changes did correlate with rates of JH biosynthesis. This study also revealed that during certain stages, vesicles and cisternae with some ribosomes attached were continuous with smooth endoplasmic reticulum membranes. These authors suggest that the rough endoplasmic reticulum is functioning to produce enzymes needed within the SER, possibly enzymes involved in JH biosynthesis (Johnson et al., 1985). The proteins that we observed in gels of *D. punctata* CA may include some of the enzymes involved in the JH III biosynthetic pathway (Fig. 2). For example, HMG-CoA reductase in its native membrane-bound form is approximately 97 kDa and the truncated fragment that contains the catalytic domain is 60 kDa (Goldstein et al., 1990). In the cockroach *B. germanica*, a 45 kDa protein was identified and it was suggested that this protein could correspond to a subfragment of HMG-CoA reductase (Martinez-Gonzalez et al., 1993). In *D. punctata* CA, there is a band with a *M*, of 44 kDa that could represent a homolog of the *B. germanica* HMG-CoA reductase subfragment (Figs. 6.2 and 7.2). On the other hand, if these enzymes are not being synthesized de novo, there is also the possibility that the proteins observed in the CA gels may be regulatory proteins or cofactors associated with these enzymes. For example, Feyereisen (1985) found indirect evidence for the reversible phosphorylation of HMG-CoA reductase in the CA of *D. punctata*. Therefore regulatory proteins such as this may account for some of the proteins that we observed.

In the CA of *D. punctata*, rough endoplasmic reticulum (RER) is present in small amounts. However, free ribosomes are present, which may be functioning in the production of proteins required within the cell (Johnson et al., 1985). Towards the end of
vitellogenesis, cisternae of RER form long, curving and eventually circular shapes. These circular membranes seem to be indicative of the early steps in autophagic vacuole formation, suggesting that this probably reflects a turning off of protein synthesis and the beginning of decreased rates of JH biosynthesis. Our results in mated female CA at the end of vitellogenesis indicate a decrease in protein synthesis (Figs. 3, 11) and may reflect the turning off of protein synthesis in the RER suggested by Johnson et al. (1985).

Rather than the turning off of the RER, another alternative for the decrease in protein synthesis in mated animals after day 6 may be the breakdown of proteins that are no longer required. Selective protein degradation is almost always a component of regulatory mechanisms that involve timing of cellular events (Hochstrasser, 1995). Intracellular protein degradation must be very specific, as the mistargeting of essential proteins or degradation of proteins at inappropriate times could cause much devastation in the cell. In eukaryotic cells, the ubiquitin-mediated pathway seems to be a major system for selective protein degradation (Hershko and Ciechanover, 1992). Ubiquitin (76 amino acid residue polypeptide) and its associated degradation pathway have been highly conserved among diverse eukaryotes (Hochstrasser, 1995; Hershko and Ciechanover, 1992). Briefly, this selective degradation occurs through the ligation of proteins destined for degradation to ubiquitin; the complexes are then degraded by a specific protease complex that acts on ubiquitinated proteins. This type of degradation is important for modulation of levels of specific proteins. For example, regulatory proteins or enzymes usually have fast turnover rates, so that their levels can be rapidly changed in response to appropriate stimuli (Hershko and Ciechanover, 1992). This may be the case with some of the proteins synthesized in the CA of the cockroach. In mated animals, there is an
increase in protein synthesis with maximum production on day 6, followed by a decrease thereafter (Figs. 3 and 11). The ubiquitin-mediated degradation pathway may contribute in part to the decrease in proteins. This degradation may be of enzymes involved in the JH III biosynthetic pathway, or of regulatory proteins regulating these enzymes. On the other hand, degradation of cyclins or proteins involved in cell division, as discussed earlier, may be occurring. If these proteins are indeed degraded and cell division ceases, it may further explain the decrease in cell number at this time. Szibbo and Tobe, (1981) suggested that the decrease in cell number was attributed to the destruction of cells, but such destruction may also include this regulatory mechanism of cell division. It is important to note that although there is a decrease in protein synthesis after day 6 that may involve degradation, some proteins continue to be synthesized. These proteins are most probably those that are constantly produced in the cell for normal cell function. Furthermore, if the ubiquitin-mediated pathway is regulating protein degradation, there are additional enzymes associated with this pathway that must be produced in the CA.

Receptors

Developmental changes in sensitivity of CA to allatostatins have been proposed to be inversely related to rates of JH biosynthesis during the first gonadotrophic cycle (Pratt et al., 1990; Stay et al., 1991a; Stay et al., 1994). The most biosynthetically active CA (Day 4 and 5 adult mated females) show the lowest degree of inhibition by allatostatins. Conversely, on day 6-7, when JH biosynthesis is low, the CA show a high degree of inhibition by allatostatins (Pratt et al., 1990; Stay et al., 1991a; Stay et al., 1994). This generalization relates to females during the first and second vitellogenic cycles and in the first half of the penultimate stadium, but appears not to apply to pregnant females or to
late penultimate or final instars (Pratt et al., 1990; Stay et al., 1994). The sensitivity of the CA to ASTs may involve de novo protein synthesis in the CA of Diploptera. Presumably, the ASTs act on the CA by way of a membrane receptor. In this species, allatostatin-binding proteins (putative receptor) of 59 kDa and 39 kDa have been identified from CA membranes (Cusson et al., 1991). Our results show bands of similar Mₐ as these putative receptors (Figs 6.2, and 7.2). It is known that cell surface receptors are continually being synthesized, internalized, perhaps recycled, and degraded (Limbird, 1986). Therefore, a small portion of the protein synthesis observed in our study could represent AST receptors. If such is the case, the pattern of protein synthesis observed in the CA may be partly associated with increases in the number of receptors on the surface of CA cells. Protein synthesis is high on day 6; part of this increase in synthesis may be attributable to the production of new receptors, which might correspond to the period of high sensitivity to the ASTs (Figs 3 and 11). If part of the increase in protein synthesis reflects receptor production and the corresponding responsiveness of CA to AST, then the decline in protein synthesis on day 7 (when the sensitivity to AST is still high) is difficult to explain. Although this decrease in synthesis on day 7 does not differ significantly from day 6 (Figs. 3 and 11), protein synthesis does begin to decline at this time. A possible explanation for this may be that on day 6, AST receptors are newly synthesized, and then these receptors are recycled on day 7, permitting a decline in protein synthesis of AST receptors; this may contribute to a portion of the decline in protein synthesis. Recycling of receptors has been recognized in other systems from the observation that following cycloheximide treatment, cells continue to internalize receptor-bound ligands at a steady rate for many hours without depletion of their surface receptors (Brown et al.,
1983). Following binding of a ligand to a receptor, it is then internalized via a coated pit and moved through the cell in a coated vesicle. The ligand then dissociates from the receptor in an endosome. From this point, the receptor can be recycled to the plasma membrane while the ligand is degraded in a lysosome. In addition to changes in numbers or synthesis and turnover of AST receptors, upregulation and downregulation of receptors may also be occurring, as suggested by Stay et al. (1994).

**Proteins involved in second messenger systems**

The proteins bands that were observed by gel electrophoresis (SDS-PAGE) in this study may also be involved in second messenger systems that have been implicated in the regulation of the CA. If these proteins are involved in the regulation of JH biosynthesis, they may be associated with the second messenger systems previously shown to modulate CA activity, for example, cyclic AMP (Meller et al., 1985) or cyclic GMP (Tobe, 1990). Several of these studies have been discussed in detail in the Introduction. Phosphorylation events may cause an increase in protein synthesis and this may in turn activate JH biosynthesis. On the other hand, phosphorylation may block the action of AST, and therefore permit the increase in JH biosynthesis.

We have discussed here some of the many possibilities with respect to the function of proteins. The nature of the proteins has been correlated with physiological functions at the time in question. By comparing selected M, of these proteins to other known proteins, we have estimated and suggested the possible identity of these proteins in *D. punctata* CA.
Effects of protein synthesis inhibitors

Although distinctive profiles of protein synthesis were found in the CA of 4th instar (Fig. 4), virgin and mated animals (Fig. 3), only mated females showed a profile of protein synthesis that parallels the JH biosynthetic cycle that occurs in these animals. We know that this profile of protein synthesis is unique to CA of mated females because this profile was not observed in the brains of mated females (Figs. 5 and 8.1-2). Because protein synthesis in mated females appears to follow a pattern which mirrors the cycle of JH biosynthesis, we investigated this apparent relationship further by experimentally manipulating one process and looking for effects on another. We employed actinomycin D, cycloheximide, and anisomycin, which are known inhibitors of eukaryotic protein synthesis, and determined effects on changes in the rates of JH biosynthesis. ASTs, peptide inhibitors of JH biosynthesis, were also examined for their effects on protein synthesis. Day 7 mated females were used for this series of experiments. JH biosynthesis is normally low in these animals and protein synthesis is just beginning to decline from its maximal levels.

We found that inhibitors of protein synthesis had varying effects on protein synthesis in the cockroach CA, depending on whether they inhibited synthesis at the level of transcription (DNA to RNA) or translation (RNA to protein). Although translation inhibitors, cycloheximide and anisomycin almost completely inhibited protein synthesis by the CA relative to the control samples (Figs. 14 and 18), there was no significant effect on JH release (Figs. 15 and 19). In examining Coomassie-stained protein bands from the CA, the translation inhibitors did not completely inhibit protein synthesis at any of the
concentrations used (Figs. 16-A and 20-A), although at high concentrations of cycloheximide and anisomycin, we observed inhibition of newly synthesized proteins (Figs. 16-B and 20-B). These results are consistent with our analysis of protein band intensity (Figs. 17 and 21). The 24-25 kDa protein is the only newly synthesized protein band that is not inhibited by either translation inhibitor (Figs. 16 and 20). It is not clear why this band in particular is not affected by inhibitors of protein synthesis and further investigation of this protein band is necessary to provide a possible explanation.

The transcription inhibitor Actinomycin D had no significant effect on either protein synthesis or JH release (Figs. 22 and 23). We found that the profiles of Comassie-stained protein bands (Fig. 24-A) and newly synthesized proteins (Fig. 24-B) for day 7 mated female CA were similar. There was no inhibition of newly synthesized proteins at any concentration (Fig. 24-B), in contrast to the effect of the translation inhibitors. Band intensities for Coomassie blue-stained gels did not significantly change when compared to control bands (Fig. 25-A). Band intensities of newly synthesized proteins also did not change except at the highest concentration tested; at this concentration, actinomycin D did inhibit the synthesis of a 30 kDa protein by a small but significant amount (Fig. 25-B). These results suggest that transcription of mRNA at this stage in development (day 7) is essentially complete and that translation predominates at this time. Plumpness or ‘swelling’ of the nucleus is usually associated with increased transcription and may be associated with the presence of enzymes involved in hormone synthesis. In this study, transcription probably occurred earlier in the vitellogenic cycle, as suggested by Johnson et al. (1985), based on the observation that the plumpness of nuclei in D. punctata occurs very early in the cycle of CA activity. In mated females, JH
biosynthesis is low by day 7 and we know that protein synthesis is decreasing by this time. Therefore, transcription would be expected to be reduced at this time, as would the rate of translation of proteins. Protein degradation through the ubiquitin system or its insect counterpart is likely during this period. Our results suggest that at day 7, JH biosynthesis is not directly affected by newly synthesized proteins, in light of our observation that inhibition of protein synthesis did not alter JH biosynthesis. Because of the pattern of protein synthesis (increasing and decreasing rates of synthesis) observed in *D. punctata*, it will be useful to examine the effect of protein inhibition at another developmental stage. For example, if protein synthesis is inhibited at the time when protein synthesis is increasing, before day 6 (Fig. 3), the effect on JH biosynthesis might be different. During this period, the effect on JH biosynthesis might be more pronounced particularly if these newly synthesized proteins are involved in cell division or in the JH biosynthetic pathway, or the movement of organelles within the cells. Nevertheless, although the newly synthesized proteins on day 7 do not appear to directly influence JH biosynthesis, there may be already existing proteins that exert an effect on this synthesis. For example we know that Day 6 mated female CA are highly sensitive to ASTs (Pratt *et al.*, 1990; Stay *et al.*, 1991a; Stay *et al.*, 1994) and, as discussed previously, new receptors may be produced at this time. Therefore by day 7, receptor recycling may be occurring. ASTs might act to maintain low rates of JH biosynthesis, and although no newly synthesized proteins are involved, inhibition of protein synthesis may not exert an effect on JH biosynthesis at this time because the required proteins have already been synthesized and continue to function.
II. Effects of Dip-ASTs on protein synthesis

Because of the important role of ASTs in the control of JH biosynthesis, we determined if ASTs influence the synthesis of proteins in cockroach CA. The mechanisms by which ASTs inhibit JH biosynthesis are as yet unknown, although Rachinsky et al., (1994) suggested that DAG and PKC may be involved in the decline of JH biosynthesis and likely function as signal transducers for ASTs (Tobe et al., 1994). Downstream effects in second messenger systems likely involve phosphorylation and dephosphorylation of target proteins, possibly including enzymes involved in the JH biosynthetic pathway. Specifically, we determined if protein synthesis changed following incubation of day 7 mated female CA with Dip-ASTs. Such proteins could be involved in AST signal transduction or in the JH biosynthetic pathway, and would therefore be targets for further investigation.

We used Dip-allatostatins 2, 7 and 13, which are excellent, moderate, and poor inhibitors of JH biosynthesis, respectively. Dip-AST 2 is the most potent inhibitor of JH biosynthesis, whereas Dip-AST 7 has intermediate potency and Dip-AST 13 has little effect (Table 1). The review by Bendena et al., (1997) provides a complete rank order of the thirteen Dip-ASTs, in terms of inhibition of JH biosynthesis.

None of the three Dip-ASTs effected a drastic change in protein synthesis in the CA. No specific protein bands either appeared or disappeared following treatment with ASTs. However, some protein bands did decrease in intensity following treatment. Curiously, we found that Dip-AST 13 and 7, the least and the intermediate potency inhibitors of JH biosynthesis, had the greatest effects on protein synthesis. Conversely,
Table 1. *Diploptera punctata* allatostatins

<table>
<thead>
<tr>
<th>Dip-allatostatin designation *</th>
<th>Primary Structure (amino acid sequence)</th>
<th>Effectiveness as an Inhibitor in Juvenile Hormone Biosynthesis Rank order</th>
<th>ED$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dip-allatostatin 2</td>
<td>↗ AYSYVSEYKRLRVYNFGLa (18)</td>
<td>1</td>
<td>1.0 x 10$^{-11}$</td>
</tr>
<tr>
<td>Dip-allatostatin 7</td>
<td>↗ APSGAQRLYGFGLa (13)</td>
<td>3</td>
<td>4.1 x 10$^{-10}$</td>
</tr>
<tr>
<td>Dip-allatostatin 13</td>
<td>IPMYDFGla (8)</td>
<td>12</td>
<td>2.6 x 10$^{-8}$</td>
</tr>
</tbody>
</table>

* refers to designation according to cDNA

↑ Dip-AST peptides which have been isolated and sequenced

ED$_{50}$: Molar concentration of Dip-AST required for 50% inhibition of JH release in 3-hr *in vitro* radiochemical assay with pairs of 2-day virgin CA compared to groups of controls (n=7).

( Bendena *et al.*, 1997)
Dip-AST 2, the most potent inhibitor of JH biosynthesis, had no significant effect on protein synthesis. Although Dip-AST 2 significantly inhibited incorporation of S\textsuperscript{35}-methionine into protein at a concentration of 10\textsuperscript{-8} M (Fig. 26), examination of Coomassie-stained band intensity and newly synthesized protein band intensity revealed no change (Fig. 27 and 28). Therefore, Dip-AST 2 seems to have little effect on protein synthesis. It may be that endogenous ASTs have already affected the activation of receptors by this time (day 7). It is surprising that this AST, the most potent inhibitor of JH biosynthesis, had little effect on protein synthesis.

Dip-AST 7 significantly inhibited protein synthesis at the highest concentration tested, 10\textsuperscript{-4} M (Fig. 29), although there was no difference in the staining intensity of Coomassie-stained protein bands of the CA (Figs. 30 and 31-A). However, three newly synthesized low Mr protein bands did significantly decrease, relative to their respective control bands, at 10\textsuperscript{-4} M (Fig. 30 and 31-B). These data compliment the data from incorporation of [S\textsuperscript{35}]-methionine showing decreased protein synthesis at this concentration (Fig. 29). The 36 kDa, 25 kDa, and 20 kDa protein bands may decrease because of the presence of Dip-AST 7. Previously, we speculated on the identity of these proteins, but without their characterization, it is impossible to determine a precise effect. However, these proteins might be involved in the cascade of events occurring in response to AST treatment. The fact that the intensity of these protein bands seem to be decreasing following Dip-AST 7 treatment provides a rationale for the further study of these proteins.

Dip-AST 13, interestingly, significantly inhibited protein synthesis at the lowest concentration (10\textsuperscript{-10} M), and at 10\textsuperscript{-8} M, there was a small but significant stimulation (Fig.
32). This reversal of response is not uncommon. This also may imply two receptors, or two receptor states, with different affinities. It is therefore important that this response be further characterized in future studies. At the lowest concentration of Dip-AST 13, (Figs. 33 and 34-B) the intensities of bands of newly synthesized proteins decreased significantly. The only band that did not significantly change at this low concentration was the 25 kDa band. It is potentially significant that once again this protein band shows a response different from all other proteins. Corresponding to the results from the stimulation of incorporation of S\textsuperscript{35}-methionine into protein for Dip-AST 13 at 10\textsuperscript{-8} M (Fig. 32), the results from band intensities do indicate slight increases, although these changes were not significant (Fig. 34-B).

Future studies should include the identification of selected proteins from D. punctata CA. It would be very informative to pursue at least the 54, 50, 44, 32 and 25 kDa bands, since we have indicated some possible identities and activities in this study. Also the 36, 25 and 20 kDa bands did decrease following Dip-AST treatment so that their identification might be of importance in future studies on mode of action of ASTs. Western blot analysis could be performed with antibodies specific to, β tubulin, cyclins, and ras proteins, for example. We have established a pattern of protein synthesis for mated female animals in this study and in follow-up studies, it would be beneficial to examine other times during development. Inhibitors of protein synthesis should also be used at the beginning of the vitellogenic cycle or day 5. The use of Dip-ASTs on different days, for example, at times of loss sensitivity to AST (days 4 and 5) might be useful as well. Another interesting point from this study is the possible involvement of
ras proteins in the JH biosynthetic pathway and the inhibition of mevalonate production in turn inhibiting cell growth. This relationship may be very important and may warrant further examination.

**Conclusions**

This study has shown that there is a distinct profile of protein synthesis in the CA of mated female *D. punctata*. This profile of protein synthesis in mated animals appears to parallel the cycle of JH biosynthesis. Manipulation of protein synthesis through the use of inhibitors of protein synthesis did not result in a significant effect on JH biosynthesis. The transcription inhibitor actinomycin D did not inhibit protein synthesis in mated female CA. However, translation inhibitors almost completely inhibited protein synthesis in the CA from day 7 mated females. Neither transcription nor translation inhibitors have a great effect on JH biosynthesis by the CA of Day 7 mated females. The reverse manipulation, using Dip-ASTs to inhibit JH biosynthesis and assess the effect on protein synthesis, has provided some evidence for a specific effect on the synthesis of certain proteins. Dip-AST 7 and 13 in particular may exert this effect on protein synthesis. Dip-AST 2, while the most potent inhibitor of JH biosynthesis, show no major effects on protein synthesis.
Summary

The CA of *D. punctata* are influenced by many factors. This study was performed to determine: a) the developmental profile of protein synthesis in the CA; b) the effects of inhibitors of protein synthesis on JH biosynthesis; c) the effects of ASTs on protein synthesis.

1. We found a distinct profile of protein synthesis in mated female animals that paralleled the cycle of JH biosynthesis in mated female *D. punctata*. Maximal protein synthesis occurs on day 6 at which time JH biosynthesis begins to drastically decrease. This profile was not seen in the CA from virgin females or in brains of mated female animals.

2. The profile of protein synthesis in 4th instar animals showed increasing protein synthesis on days that corresponded to time of low JH biosynthesis.

3. Although the proteins were not characterized in this study, suggestions of their identities were provided on the basis of cellular and physiological activities in the CA and relative molecular mass of known proteins.

4. From studies using inhibitors of protein synthesis, synthesis of proteins in the CA of day 7 mated females did not significantly affect JH biosynthesis.

5. There is some evidence that Dip-ASTs can affect the synthesis of certain proteins in the CA of day 7 mated females.
REFERENCES


Appendix I

Standard curve of the intensity of protein bands versus protein concentration.

A standard curve was produced to show the intensity of protein bands in relation to the number of CA. This was performed to show that intensity increased in a linear fashion as the amount of tissue increased. Intensity represents relative intensity as the pixel value of a selected point on the gel in terms of that pixel's brightness. Gels were scanned at 600 dpi. The results of the standard curve were then used in the thesis as a standard upon which intensity readings could be compared. Proteins were separated on a 12% SDS-PAGE and stained with coomassie blue. Intensity was measured with SigmaGel™ gel analysis software.
Appendix II

Standard curve of the intensity of protein bands versus protein concentration.

A standard curve was produced to show the intensity of protein bands in relation to the number of CA. This was performed to show that intensity increased in a linear fashion as the amount of tissue increased. Intensity represents relative intensity as the pixel value of a selected point on the gel in terms of that pixels brightness. Gels were scanned at 600 dpi. The results of the standard curve were then used in the thesis as a standard upon which intensity readings could be compared. Proteins were separated on a 12% SDS-PAGE and ^{35}S-methionine incorporation was evaluated by autoradiography using different exposure times of (a) 2 hours, (b) 4 hours, (c) 6 hours, and (d) 14 hours. Intensity was measured with SigmaGel™ gel analysis software.
APPENDIX III

CHEMICALS:

Caledon:  Methanol
           Hydrochloric Acid

BDH:  Acetic Acid glacial
       Calcium Chloride
       Glycine
       Sodium Hydroxide
       TCA-Trichloroacetic Acid

BioRAD:  Ammonium Persulfate
         Bromophenol Blue
         SDS-Sodium Dodecyl Sulfate
         SDS-PAGE Molecular Weight Standards, Low and High Range

Gibco BRL, Research Products, Life Technologies:
   TC199 medium

Sigma:  Acrylamide
        Actinomycin D
        Anisomycin
        Bovine Serum Albumin - Protease Free
        Brilliant Blue R
        Cycloheximide
        Dip-Allatostatin 2
        Dip-Allatostatin 7
        Ficoll
        Glycerol
        N,N’ Methylene - bis - Acrylamide
        TEMED
        Trizma-Base
        2-b-Mercaptoethanol
APPENDIX IV

Equipment:

AND FY 3000 Electronic Balance
Beckman Ω32pH meter
Beckman LS 6500 multi-purpose scintillation counter
BHG Hermle Microcentrifuge
BIORAD Gel Dryer model 583
Gilson pipetman
Kodak M35A X-OMAT Processor
Mettler AE163 Balance
VWR Scientific Vortex Genie 2

Gel Electrophoresis Equipment:

BIORAD Mini-Protean® II Dual Slab Cell
- lower buffer chamber and lid
- casting stand
- inner cooling core
- sandwhich clamp assemblies
- glass plates
- spacers
- combs
Fisher Biotech voltage supply (Fisher Scientific)