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Insect Neuropeptides
Chemistry, Biology, and Action

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Foreword

THE ACS SYMPOSIUM SERIES was founded in 1974 to provide a medium for publishing symposia quickly in book form. The format of the Series parallels that of the continuing ADVANCES IN CHEMISTRY SERIES except that, in order to save time, the papers are not typeset, but are reproduced as they are submitted by the authors in camera-ready form. Papers are reviewed under the supervision of the editors with the assistance of the Advisory Board and are selected to maintain the integrity of the symposia. Both reviews and reports of research are acceptable, because symposia may embrace both types of presentation. However, verbatim reproductions of previously published papers are not accepted.

Preface

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FIFTEEN YEARS HAVE ELAPSED since Alvin N. Starratt and Brian E. Brown released their pioneering publication announcing the initial determination of a primary structure for an insect neuropeptide—the pentapeptide proctolin. In the early 1980s, only a few American, Japanese, and European universities, as well as the Zoecon Corporation in the United States, were engaged in insect neuropeptide research. Neuropeptide discoveries in vertebrates and molluscs—and the advent of exquisitely sensitive techniques for isolation, sequence determination, and synthesis of neuropeptides—rapidly accelerated discoveries in both the mammalian and insect arenas. More than 50 insect neuropeptides have been sequenced and reported in the literature, and that number is increasing rapidly. Concomitant with these developments have been several symposia, conferences, and workshops that address various aspects of insect neuroscience.

Three years ago, with the exponential growth of insect neuropeptide identification, we felt that the time had arrived to hold a comprehensive symposium to assess the state of the science in bioactive insect neuropeptides and to identify the most promising directions for future research. The appropriate occasion arose in conjunction with the International Chemical Congress of Pacific Basin Societies. Twenty-four leading researchers in insect neurobiology, biochemistry, chemistry, and molecular biology participated in this symposium. Recent results of research on developmental, homeostatic, behavioral, reproductive, and metabolic neuropeptides were presented and discussed. Important papers were also presented on neuropeptide synthesis, tertiary structure conformation, gene identification, and expression of foreign peptides in baculoviruses—the latter having potential as a means of introducing neuropeptide regulating genes into living insects.

This volume reports the current state of our knowledge. We hope that it will be read and perused by both the initiated and those just entering this challenging field of research.

As the senior editor (Julius J. Menn), I extend my deepest appreciation to Herbert Roller, whose foresight, knowledge, and inspiration were instrumental in introducing me to the exciting field of insect neuropeptides.

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The cover illustration is based on a model of two related insect neuropeptide active core regions in a β-turn conformation generated from molecular dynamics on a Cray supercomputer.

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Abbreviations and Nomenclature

In addition to abbreviations and acronyms which are individually identified in the following manuscripts, two systems are used throughout this volume:

I. Insect peptide nomenclature follows the rules prescribed in Raina and Gäde, *Insect Biochem.* 1988, *18*, 785-787.

II. Single-letter amino acid codes:

Ala	A	Leu	L
Arg	R	Lys	K
Asn	N	Met	M
Asp	D	Phe	F
Cys	C	Pro	P
Gln	Q	Ser	S
Glu	E	Thr	T
Gly	G	Trp	W
His	H	Tyr	Y
Ile	I	Val	V

Chapter 1

Neuropeptide Research in Historical Perspective

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The impressive advances made over the years in the identification and functional interpretation of bioactive neuropeptides are the result of a broadly based comparative and multidisciplinary approach. The insights gained from studies in insects and vertebrates show remarkable parallelisms between the two groups. What we have learned is that neuropeptides are engaged in multiple forms of intercellular communication in the control of a variety of biologically important integrative functions.

At the outset, it appeared that proteinaceous products dispatched by a special class of neurosecretory neurons, such as those of the pars intercerebralis of insects, function exclusively in a neurohormonal capacity. Following the classical discovery by Kopeć (1922) of a brain hormone controlling insect metamorphosis much information has been gained on a number of essential neurohormonal functions carried out by neuropeptides in the control of postembryonic development as well as reproductive, metabolic, muscular, and additional activities.

These blood-borne messengers may reach terminal effector sites directly in a one-step operation, or they may accomplish their task indirectly by signals to non-neuronal glands of internal secretion (corpus allatum, prothoracic gland). These two-step operations are analogous to those in vertebrates where the dispatch of adenohypophysial hormones is controlled by hypothalamic signals. The effective mode of operation of this neuroendocrine connection, i.e., the delivery of neural directives to endocrine glands proper by means of hormones provided by "nonconventional" neurons is a major concern of the discipline of neuroendocrinology.

An interesting feature which the neuroendocrine axis of vertebrates and insects has in common is the interaction of stimulatory and inhibitory neurohormonal directives to the respective glands of the endocrine system. Their existence has been demonstrated by a combination of structural and experimental studies. The sites of production (neuronal perikarya) and release (axon terminals) of the respective, selectively stained messenger substances could be ascertained by tracing their disposition throughout

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the neurosecretory neuron. The inhibitory (allatostatic) role performed by a group of these neurons became apparent from the effect of the surgical interruption of this pathway which prevents the proximo-distal transport of the messenger substance and thus abolishes its action.

A major step forward, which extended our understanding of the range of neuropeptide activities, occurred after the advent of electron microscopy. The observation of synapse-like ("synaptoid") contacts between peptidergic neuron terminals and endocrine cells (in the corpus allatum as well as the adenohypophysis) clearly indicated that close-range signals from neural to endocrine cells contribute to the process of neuroendocrine communication. Subsequently, such peptidergic contacts turned out not to be restricted to endocrine cells; they also occur on various somatic cells. More importantly, they establish connections between conventional as well as peptidergic neurons.

At this juncture, a new type of interneuronal communication, i.e., the capacity of neuropeptides to act as neurotransmitters, had become established. Moreover, the structural parameters of peptidergic innervation were seen to include junctional complexes between two peptidergic neurons that seem to enable a reciprocal exchange of signals.

Some peptidergic terminals are separated from neuronal elements by a narrow gap of intercellular space, an arrangement suitable for a paracrine form of regulation. This spatial proximity makes possible peptide-mediated neuromodulation, i.e., the fine-tuning of signals between two synaptically linked neurons by the neurochemical intervention of a third neuron. Less can be said about the possible functional significance of neuropeptides that are colocalized with conventional neurotransmitters in a great number of neurons, or of those produced by glial elements.

The presence of neuropeptides at these and many other sites has been ascertained by use of immunocytochemical techniques, made possible by the increasing availability of appropriate peptide antibodies. This development took place in conjunction with rapid advances in the chemical identification of a growing number of bioactive peptides and the production of synthetic analogs. Numerous tests carried out in insects with antibodies raised against mammalian neuropeptides revealed reaction products within and outside of the nervous system. Conversely, certain neuropeptides first identified in invertebrates were shown to occur also in mammals and other vertebrates. These commonalities are indicative of a long evolutionary history, as well as a wide distribution of active neuropeptides in neural and non-neural tissues. One of the new insights gained from the use of multiple antisera and carefully conducted specificity tests was the immunocytochemical detection in the insect brain of molecules closely resembling mammalian ACTH, prolactin, and insulin,. The localization of these substances suggests a neurotransmitterlike or neuromodulatory rather than a hormonal role.

In recent years, the improvement of appropriate techniques has facilitated the chemical identification of a number of insect neuropeptides as well as comparison with their respective counterparts in vertebrates. Among these are metabolic, myotropic, allatotropic, and allatostatic factors.

In insects as well as vertebrates, the effectiveness of regulatory neuropeptides is known to depend on stereospecific high-affinity binding sites located in the plasma membrane of the respective cells to be addressed. Such receptors have been demonstrated for several opioid peptides by means of radio-ligand techniques in the brain and digestive tract of insects. Differences in binding-site density in the brain were shown to be correlated with the cyclicity of the female's reproductive activity, those in the midgut with its autonomic nerve supply. The existence of complex decoding processes for such receptor-mediated stimuli has been carefully documented in vertebrates. Recent evidence indicated that a comparable second-messenger system, involving inositol lipids and mobilization of intracellular calcium, operates also in insects, i.e., in the peptidergic modulation of the intrinsic rhythmicity of the heart.

Among recent developments in the elucidation of neuropeptide activities in non-neuronal tissues and cells, those in the immune system are receiving the greatest attention. Fascinating results obtained in invertebrates are in line with those in mammals. Various neuroactive principles have been shown to participate in cell-mediated immune responses in insects as well as molluscs and mammals. These messenger substances, in addition to lymphokines, are instrumental in the bidirectional exchange of signals between the neuroendocrine apparatus and the immune system. Furthermore, neuropeptides, among them particularly endogenous opioids, influence autoregulatory activities of immunocompetent cells. These include adherence and migratory behavior of these cells. The stimulation of locomotory activity is preceded by distinctive conformational changes (flattening and formation of pseudopodia-like cellular processes). There is evidence for the presence, in immunocytes as well as cell-free hemolymph, of Met-enkephalin which appears to play a distinctive role in these activities, perhaps by interaction with a special subtype of delta receptor. Moreover, the inhibitory effect of the opioid antagonist naloxone on these processes indicates that they are receptor mediated.

There is ample documentation, obtained primarily in mammals, for the concept that the biosynthesis of the great majority of regulatory neuropeptides occurs by way of enzymatically cleaved large precursor molecules produced under the direction of mRNA templates. Future work will have to show to which extent these and the subsequent steps (posttranslational processing, release, and degradation of these products) also occur in insects.

The wide distribution of regulatory neuropeptides throughout the animal kingdom, and their close chemical similarity or even identity

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indicate that they have been well preserved in the course of evolution. Neuropeptides seem to have come into existence before the most primitive nervous systems. Their hormonal and pheromonal functions must have antedated those taking place at close range. A small number of ancestral proteins encoded by ancestral genes may have given rise to a multiplicity of active peptides in concert with the evolution of complementary receptor molecules. Our current knowledge of insect neuropeptides is consistent with and actually supports these views.

In summary, the advances made in the elucidation of the nature and varied functional capacities of neuropeptides have gone far beyond our expectations. In insects, as well as vertebrates, these chemical messengers perform major hormonal functions including that of bridging the gap between the two systems of integration. Their participation in chemical signalling within the nervous system makes possible the fine-tuning of interneuronal communication. Outside of the confines of the neural and endocrine apparatus peptidergic messengers, in particular opioids, perform various additional functions, e.g., those in immunoregulatory activities.

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Chapter 2

Biologically Active Insect Peptides

Prospects for Applied and Fundamental Knowledge

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Neuropeptides reveal a new approach to understanding insect life processes and the development of measures to control these processes. Peptide structural characterization and structure-activity studies allow expansion of analysis beyond the peptide itself into molecular genetics, precursor identification, biosynthesis and processing, receptor binding, and degradation. Robust bioassays and initial peptide purifications are essential. Integration of these technologies, from bioassay through gene sequencing, presents unique opportunities for the development of potent, flexible and specific control measures which will include production of antagonists and superagonists, delivery of fusion peptides to target pests by the baculovirus system, development of peptidomimetics, receptor blockers, enzyme inhibitors and poisons.

Factors which Kopec (1,2) postulated as coming from the brain and affecting the ability of the larva to develop and undergo successful pupation include the extensively studied prothoracicotropic hormone (PTTH; see 3-6 for reviews), which now is known to be only one of scores of biologically active peptides described in insects. These peptides control a vast range of critical events associated with post-embryonic development and metamorphosis, including ecdysteroid and juvenile hormone biosynthesis, behavior, muscular contraction, fat and sugar metabolism, cuticular assembly and coloration, and others. Many recent reviews discuss these peptide activities (4,7-13, other chapters this volume). The field of identified biologically active peptides continues to expand rapidly. In the past six years alone, there has been a ten-fold increase in the number of characterized insect neuropeptides (9).

Bioassays commonly provide the initial means of detecting neuropeptide activity. The factor(s) responsible for this activity are assigned names reflecting the functions detected with the bioassay (*e.g.*, adipokinetic hormone, AKH; diuretic hormone, DH; eclosion hormone, EH; pheromone biosynthesis activating neuropeptide, PBAN; prothoracicotropic hormone, PTTH; and so forth). As more of these factors are discovered and characterized, not only are biological

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homologies observed but biochemical and structural ones are revealed as well. Such structural homology is shown by the AKH/red pigment concentrating hormone (RPCH) family (8) and the myotropic neuropeptide family (8). As separation and characterization occur, the factors are assigned chemical and/or structural identity and, in the case of peptides, become identified by amino acid sequences. It is not suprising that a nomenclature standard has been proposed (14) to catalog the burgeoning number of reported sequences.

Amino acid sequence information provides an avenue to capitalizing on the powerful capabilities of molecular genetics. Nucleotide sequences, deduced from primary structures, are used in constructing oligonucleotide probes for searching out neuropeptide genes. This transition from factor-to-sequence-to-gene is becoming more common, as documented in the ICINN series (15-17) and this present volume, and suggests that gene identification and manipulation, as well as sequencing of insect neuropeptides, will undergo a logarithmic increase in the near future.

Because of the rapidly developing nature of the field, periodic symposia, such as the one on which this volume is based, will likely proliferate in the next few years in an effort to keep researchers focused on current developments. Such symposia will become more specialized, attending to areas such as sequenced peptides, insect neuropeptide molecular genetics, peptide families, insect neuropeptide processing, and so forth. In this overview, some recent developments in bioactive peptide research are considered, as are directions where such developments may lead.

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Sequences and Structure

The elucidation of the primary structures of proctolin (29) and AKH (30), signaled the beginning of the structural identification of insect neuropeptides. These relatively small peptides (pentapeptide proctolin and decapeptide AKH) remained the only known insect peptide sequences for a number of years until, in the early 1980's, accumulated technical advances in peptide isolation and microsequencing techniques facilitated the analysis of insect peptides (8-12).

As of this writing, close to 50 primary insect neuropeptide structures have been reported. At least six of these structures have been used to deduce reported nucleotide sequences for oligonucleotide probe generation and, in some cases, gene sequencing (Table 1). The first reported sequences used in molecular genetic studies came from *Bombyx mori, Drosophila* spp. and *Manduca sexta* (Table 1). However, perhaps the most exciting aspect of these reports is that, in an era when peptide synthesis and nucleic acid polymerization can be carried out chemically in many laboratories, published sequences can be used as a "springboard" to the discovery and identfication of homologous sequences in other species. This will be especially useful with pest species for which extensive physiological data and reliable bioassays may be lacking. Recently developed methods such as the polymerase chain reaction (PCR; 31,32) and its variants (mixed oligonucleotide primed amplification of cDNA, "MOPAC", 33; anchored PCR, 34) facilitate the production of nucleic acid probes from partial amino acid sequences and trace amounts of extracted DNA. Even if only partial homology exists between a published amino acid sequence and a related sequence in a target species, it is possible through the use of PCR/MOPAC (33) to construct a battery of useful probes for target DNA screening. In addition, anchored PCR methods (34) allow the generation of complementary DNA sequences using only trace amounts of extracted DNA, even from a specific tissue from a single animal. The complementarity of the partial sequence thus obtained from the target species can be tested by hybridization to a reported nucleotide sequence from the original species. Methods such as anchored PCR can be used to expand the known sequence in both directions from the anchored region leading to identification of the gene in

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Table 1. Bioactive Insect Neuropeptides for which Partial or Entire Gene Structures are Reported

Peptide[1]	Species	AA Sequence	Properties[2]	Gene Structure[3]	Reference
Mas AKH	*Manduca sexta*	$_{p}$ELTFTSSWG$_{NH_2}$	M,P,a,b	D,S	[18]
Bom Bombyxin	*Bombyx mori*	CYSLLVDVSCPRLCCED	P	D	[19,20]
Bom EH	*B. mori*	SPAIASSYDAMEIC-IENCAQCKKMFG-PWFEGSLCAESCI-KARGKDIPECESF-ASISPFLNKL	M,P[4]	D,S	[23]
Mas EH	*M. sexta*	NPAIATGYDPMEICI-ENCAQCKKMLGAWFE-GPLCAESCIKFKGKLI-PECEDFASIAPFLNKL	M[5],P[6]	D	[24]
Drv FMRFamide	*Drosophila virilis*	$_{N}$FMRF$_{NH_2}$	M,P,a	S	[28]
Drm FMRFamide	*D. melanogaster*	$_{N}$FMRF$_{NH_2}$	M,P,a	S	[25,27]
Bom PTTH	*B. mori*	[GNIQVENQAIPDPPCTCKYK-KEIEDLGENSVPRFIETRNC-NKTQQPTCRPPYICKESLYS-ITILKRRETKSQESLEIPNE-LKYRWVAESHPUSVACLCTR-DYQL(RYNNN)] X 2	7	D	[26], [Kataoka *et al.* this volume]

[1] Nomenclature per [14].
[2] M, sequence of mature peptide; P, sequence used to generate probe; a, C-terminal amidated; b, blocked at N-terminal.
[3] D, structure deduced from amino acid sequence, cDNA probe reported; S, gene sequence.
[4] Four probes were prepared relative to various selected sequences within mature sequence.
[5] Sequence reported in [21] and [22].
[6] N-terminal sequence of 24 amino acids used to design probe.
[7] Mature PTTH is a homodimer [26]; residues in parentheses may be lost during final *in vivo* processing [Kataoka *et al.*, this volume].

the target species. It is possible to determine gene sequences and produce large quantities of a gene without gene cloning.

To be sure, molecular genetics suffers from limitations. Many of its methods cannot be used without the availability of at least one known amino acid sequence. Thus, peptide isolation remains a critical need. Oligonucleotide probes can be too short, leading to useless or "false positive" hybridizations. Also, an appropriate bioassay is required to assess the authenticity of any product of molecular biology. However, the use of molecular genetic techniques will cause an explosion of information on insect neuropeptides and their sequences within the very near future.

Inducers and Inhibitors

One may arbitrarily classify all bioactive peptides in terms of their ability to cause or prevent a physiological event. In insects, the more populated category is that of the inducer. Adipokinetic, diuretic, eclosion, egg development neurosecretory (EDNH), hypertrehalosemic (HTH), pheromone biosynthesis activating, prothoracicotropic and other neuropeptides were detected because of behavioral, biochemical, morphological or physiological changes resulting from their actions. The practical implications of a knowledge of such inducers is that agents may be developed which prevent the appearance or action of the inducer. During inducer studies, and in attempts to isolate the molecules, inhibitory effects are often found. Some of the most prominent are prevention of ovarian maturation, oostasis (35-38), by the so-called oostatic hormone (OH), suppression of juvenile hormone biosynthesis by the allatostatins (39-42), and inhibition of Malpighian tubule-mediated fluid excretion by antidiuretic hormone (43). Of particular current interest is the role of the allatostatins with regard to their inhibition of the synthesis of juvenile hormone, a key effector of reproduction and development. A number of these inhibitors have been isolated and sequenced (44,45, Stay *et al.* and Pratt *et al.*, this volume), as has the inducer of JH biosynthesis, allatotropin (46). Thus, a situation is presented with JH where both the inducer and inhibitor of JH biosynthesis are characterized, although from different species. The availability of structural data should facilitate the isolation and characterization of homologous sister molecule(s) (see preceeding section) essential for the development of detailed models on regulation of this physiolgical process. Ultimately, specific agents can be designed for disruption of the process.

While it is clear that JH is essential to development and reproduction in insects, it is important to note that its impact upon these events is time- and species-related. This suggests that the control of JH titer is both subtle and complex. In larval insects, the presence of JH at continuously declining titers effects varying degrees of maturation until metamorphosis is "permitted" in its absence (49). With regard to reproduction, the timing of the appearance of JH in the female, as well as its titer, leads ultimately to mature oocytes (52). Juvenile hormone, secreted within a few hours of emergence in the mosquito, stimulates ovarian follicular development to a stage where the follicles become competent to respond to neurohormone (EDNH) and produce the ecdysteroid necessary for vitellogenesis (53,54). In addition, fat body tissue becomes responsive to ecdysone and synthesizes vitellogenin after exposure to JH (55). In adult roaches, JH regulates cyclical ovarian maturation. Corpora allata activity (*i.e.*, JH biosynthesis) falls in the presence of mature follicles and rises again after egg laying (56,60). Juvenile hormone stimulates vitellogenesis in *Danaus plexippus* (57) and vitellogenin gene expression in *Locusta migratoria* (58). Conversely, an apparent lack of JH does not prevent vitellogenesis in either *Hyalophora cecropia* or *Lymantria dispar* (59,61). Furthermore, *L. dispar* ovarian maturation occurs during late larval development and JH actually has an inhibitory effect on vitellogenesis (62).

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No doubt the involvement of JH in the regulation of reproductive physiology in these varied species is very precisely, and perhaps uniquely, regulated. Degradation of JH by specific and non-specific esterases affords one means of titer regulation (50,51). The other, of course, is synthesis (44-46). Structural knowledge of the regulating agents (*e.g.*, allatotropins and allatostatins) will be essential in sorting out the complexities of such regulation. The ability to monitor and manipulate the major factors responsible for the control of JH titer - inducer, inhibitor and degradative enzymes - will be crucial to the development of heuristic models describing, in molecular detail, the regulation of JH titer and, consequently, the regulation of developmental and reproductive events. In addition, such models can be used to design the analysis of other systems, such as EDNH-OH and DH(47,48)-ADH, as these peptide sequences become available. Furthermore, they will facilitate analysis of newly reported factors, such as allatinhibin (63).

Diversity: Biosynthesis and Processing

With the proliferation of identified sequences, identification of inducers and inhibitors of specific physiological events and the analytical power of the continuously evolving molecular biology, the regulation of insect development, metamorphosis, reproduction, metabolism and behavior will be elucidated at an accelerated pace. The impact of this knowledge most certainly will result in a "new physiology". Neurohormones as end-product effectors will no longer be the sole or primary focus of research. Research will focus on structural relationships and functional interrelation to other active peptides. As more amino acid sequences of bioactive insect peptides are reported, structural diversity among peptides of equivalent or related biological activity is increasingly evident. Multiple forms of PTTH, differing in molecular weight, have been reported for *B. mori* (64,65), L. dispar (66,67) and *M. sexta* (68), and *A. aegypti* EDNH was reported to exist in two molecular weight ranges (69). The significance of this type of diversity may lie in related but different physiological responses evoked by these different molecules. For example, in *M. sexta*, it is suggested that the two molecular weight forms of PTTH are produced and/or secreted in stage-specific ratios (70). Prothoracic glands from day-0 pupae were stimulated to produce ecdysone more efficiently by the large form of the hormone extracted from larval brains, whereas they were more efficiently stimulated by the small form when pupal brains were the source of the hormones. This type of physiological diversity will be an important consideration when developing control agents and in determining application strategies. The large and small PTTH's, however, do not seem to share any sequence homology (Suzuki *et al.*, this volume, 26,71), and thus their genetic origins may be quite different. Another level of diversity is seen among homologous primary structures which display similar biological activities. The 4K-PTTH of *B. mori* (72) exists in at least 4 forms, separated by ion-exchange methods. This suggests slight differences in primary structures. The allatostatin from *Diploptera punctata* exists as 4 sequences with some shared homology (44,45). Extensive sequence homologies exist within the AKH/RPCH/HTH family (9,11), and in the myotropin family, which includes proctolin and the leucokinins (9). Such structural diversity among molecules of related activities may function to accomodate different physiological states as, for example, in the control of JH-titers and the ecdysiotropic activation of prothoracic glands. Only an extensive cataloging of structure-activity effects will begin to clarify these relationships. Of more immediate application, and certainly a requirement for explaining how multiple forms come to appear, is a consideration of the biosynthesis and processing of peptides.

Neuropeptides are usually synthesized as large precursors from which the

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active, end forms are cleaved and modified (73-81). Diversity arises at the transcriptional, translational and post-translational levels (81,82). Those events which contribute to the diversity seen in neuropeptide sequences and structures, the processing and modification steps which yield mature peptides, offer numerous targets of attack in the design of control agents. Each of the translational and post-translational steps outlined in Figure 1 is dependent upon enzymatic activity, specific amino acid sequences, or both. Disruption of any event would certainly prevent normal bioactive peptide production. However, certain processing steps may prove to be of a more practical use than others. For example, transport of propeptides through the rough endoplasmic reticulum (RER) into the Golgi, and subsequent sorting into appropriate secretory granules, are processes dependent upon signals, amino acid sequences within the transported peptide (84,85), in a manner analogous to the signal sequence guidance of the prepropeptide during its cotranslational insertion into the RER. Disruption of the normal secretory process might be accomplished through the introduction, via the baculovirus system (see below), of defective fusion peptides which would not be sorted properly. Also, construction of fusion peptides with signal sequences to direct them to constitutive rather than regulated secretory pathways (85,86) could facilitate their secretion into the hemolymph before, and in larger quantities than, the endogenous peptide, usurping its effect. Residence in the Golgi and secretory granules subjects the propeptide to a variety of proteolytic and non-proteolytic modifications (75,79). The most common proteolytic processing site is characterized by pairs of basic residues (-K-R-,-R-R-,-K-K-,-R-K-) (74,81). Such sites delineate a bioactive sequence within the propeptide or propolypeptide, and are attacked by trypsin-like endopeptidases (78,81) and carboxypeptidase-B-like enzymes (87). There is increasing evidence that important monobasic sites exist (76,78,81,83) and that cleavages at these sites occur after dibasic cleavage (76). Monobasic cleavage may be important in contributing flexibility to the production of different bioactive peptides from a common precursor (78). In fact, the diversity in bioactive peptide sequences and tissue-specific expression of bioactive peptides may result from a combination of the co-expression of different processing enzymes, expression of processing enzymes at different levels (*i.e.* titer), time of enzyme-prohormone contact and the presence of enzyme modulators and inhibitors (81). Further, there may exist both "general" and "specific" converting enzymes (88,99). Although many of these issues are not yet clearly resolved, it is clear that proteolytic modification offers a promising approach toward manipulation of neuropeptide processing (*e.g.* modification of cleavage sites via genetic engineering; development of enzyme inhibitors; manipulation of the enzyme genes; etc.) and, ultimately, to the control of insect development and reproduction.

Another aspect of the Golgi and secretory granule components of peptide maturation is the extensive array of non-proteolytic, post-translational modifications. Included are gylcosylation, acetylation, amidation, sulfation, phosphorylation, and others (75,79,82). These modifications are mediated by enzymes, rendering them amenable to disruption or inhibition through manipulation of the enzyme, as suggested above with proteolytic enzymes. The formation of the N-terminal pyroglutamate moiety is of interest because the pG-blocked N-terminus is present in many insect neuropeptides sequenced thus far, including AKH, the myotropins and the B-chain of Bombyxin II (8,10,11,71). Many insect neuropeptides are amidated at the C-terminus (8,10,11), a common feature of vertebrate bioactive peptides (79). Because of the frequency of C-terminal amidation, it has been extensively studied (79,89,90). Amidating enzymes have been isolated from a number of species (91-94), assays for their detection have been refined, kinetics studied (89,90), and cDNA clones obtained (95-97). The extensive background on C-terminal amidation from the vertebrate literature (98), the role of

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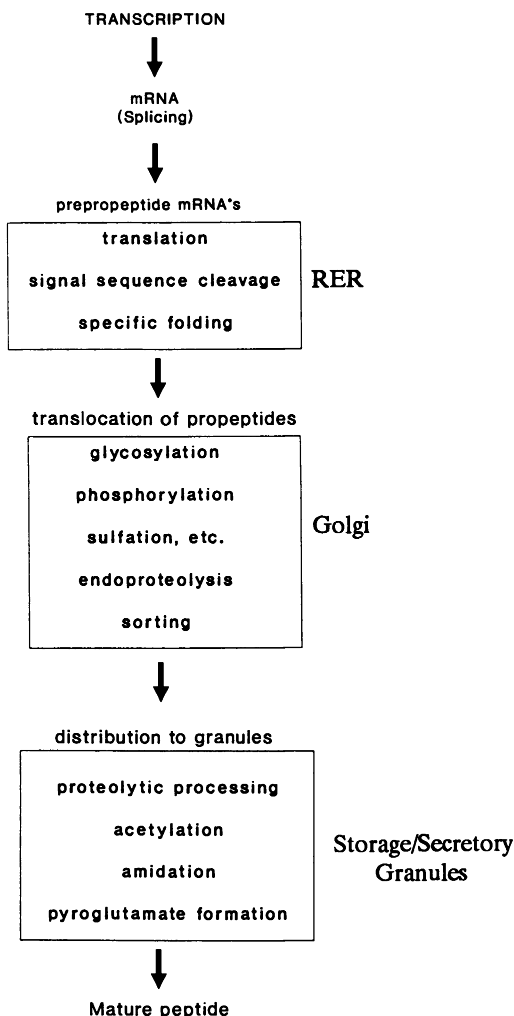

Figure 1: Post-Translational Events in Neuropeptide Maturation
Following gene activation and transcription, some mRNA's may undergo splicing events which lead to sequences not derived directly from the gene. During translation, the nascent prepropolypeptides, guided by an N-terminal hydrophobic signal sequence, insert through the wall to the lumen of the rough endoplasmic reticulum (RER). Specific folding occurs and the signal sequence is cleaved off. The propeptides may again fold, with sulfide bond formation, and translocate to the Golgi. During transit through the Golgi, propeptides can be modified by glycosylation, phosphorylation, sulfation, hydroxylation, etc., and some endoproteolysis. Intra-Golgi sorting of propeptides occurs, presumably via a signal-based (*i.e.* amino acid sequence-dependent) mechanism, leading to distribution into selected secretory/storage granules. Within the granules, propeptides undergo maturation in the form of endo- and exoproteolytic cleavages and trimming, and modifications of the C- and/or N-termini which may include acetylation, amidation or blocking through the formation of pyroglutamic acid residues. The mature peptides are stored or secreted. For references see 74,75,78,79,81,87 and the text.

amidation in imparting stability to bioactive peptides (100) and its frequent occurrence in insects make amidation a prime target in the search for inhibitors or disruptors as a means to develop control agents.

The processes of prepropolypeptide synthesis, translocation, proteolytic processing and non-proteolytic modification can be enzymatically defined. These definitions are continuing to be developed and clarified. There are limited reports on insect neuropeptide processing (101,102), but these investigations should increase rapidly with the identification of precursor sequences via molecular genetics. The identification of processing enzymes, both proteolytic and non-proteolytic, will further open whole new areas for exploration.

Binding and Metabolism

The binding of a neuropeptide to a receptor should become a critical area of research as more primary structures are elucidated. Although we have no information at present on receptors for bioactive insect peptides, tools for such studies are available, largely from the vertebrate field. Radiolabeled peptides may be synthesized for use as probes in sub-cellular studies, with photo-affinity labeling and biotinylated peptides offering promising approaches for receptor isolation (103). A prerequisite for successful labeling is a knowledge of structure-activity relationships which identify sites for binding (recognition) and signal transduction (104). Sites for modification of the peptides can be chosen so that a photo-activated linkage is made while preserving biological activity. Structure-activity studies on insect peptides have been carried out primarily with AKH- and myotropin-related families (11,104,105, Nachman *et al.*, this volume), but studies of other peptides, including the allatostatins (42,44) and PBAN (Kempe *et al.*, Pesticide Science, in press) are now being reported. With knowledge of the ligand-receptor interactions and molecular characterization of the receptor itself, it should become possible to design superagonists and antagonists which compete with the natural peptide for the same receptor.

Bound, bioactive peptides are de-activated by membrane-associated peptidases (106). Reports from work with *Schistocerca gregaria* on AKH degradation (107,108) indicate that similar mechanisms exist in insects and, indeed, may employ enzymes similar to endopeptidase-24:11 (see below), which is important in attacking blocked peptides (106,108). Extracts of various tissues of *Periplaneta americana* can degrade proctolin at both N-terminal and internal sites (109) indicating the presence of amino- and endopeptidases in the soluble fraction. The cardioacceleratory peptide (CC-2) of *P. americana* was degraded both *in vitro* and *in vivo* with a half-life of $\approx$1hr in each case (110). This was considerably longer than the half-life (as short as 1-2 minutes) indicated for proctolin (109). Assuming that the metabolic studies with tissue extracts exposed proctolin and CC-2 to similar enzyme mixtures (109,110), the differences in biological half-lives suggest that the primary, and perhaps secondary, structure of proctolin make this peptide more generally susceptible to proteolysis than CC-2. Alternatively, specific degradative enzymes may be present which recognize particular structural features. From vertebrate work, we know that numerous peptidases exist with a variety of cleavage specificities (106,111).

Neuropeptide degradation in vertebrates involves both soluble and membrane associated enzymes (112). Similar mechanisms probably exist in insects. In fact, both soluble and membrane-bound proteolytic activities directed at bioactive peptides have been demonstrated in insect systems (107-110). A vertebrate-like endopeptidase activity, the Zn-metalloendoprotease (endopeptidase-24:11), is present in the locust and is sensitive to the specific inhibitor, phosphoramidon (107-108,111).

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The compelling similarities between vertebrate and insect neuropeptide degradative mechanisms, given the limited number of studies on insects, should allow the use of vertebrate models and methods, including assays, in studying insect systems. As with neuropeptide biosynthesis and maturation, the involvement of specific enzymes in neuropeptide degradation may present exciting opportunities for the design of novel and selective control agents.

Conclusion

Peptides, enzymes, receptor proteins and related genetic constructs do not, per se, represent viable control agents. Lability alone obviates direct field application. Information encoded within the structures of these molecules, however, makes them our most potent and important resource for understanding critical physiological events in the life of the insect, and in the design of measures to control these events.

The two major routes to the exploitation of structural information in the development of insect control agents are: 1) the deduction of nucleic acid sequences from amino acid sequences, and 2) structure-activity studies which identify critical sites of interaction. With the first route, molecular genetics can be used to identify precursors and processing enzymes, prepare antagonists and superagonists through gene fusion and, in selected species, utilize baculovirus technology (113; Summers *et al.*, this volume) to deliver the fusion peptide to the target. With the second route, active-site and binding-site structures are used to design peptidomimetics, enzyme inhibitors and receptor blockers. Such design will employ discovery protocols and strategies similar to those used in drug design (114,115). Neither approach alone is a panacea. Considered together, however, their potential is immense. A shared dependence upon peptide structural information should make advances in one approach benefit the other as we strive to understand the biochemical milieu in which the "master molecules" (116) of insect life processes operate.

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Chapter 3

Bombyx Prothoracicotropic Hormone

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Prothoracicotropic hormone (Bombyx PTTH) of the silkworm, Bombyx mori, was extracted from adult heads. Bombyx PTTH is suggested to be a glycopeptide and consists of two essentially identical subunits. Amino acid sequencing and cDNA analysis revealed the whole amino acid sequence of the subunit, composed of 104-109 residues. Bombyx PTTH stimulated adult development in brainless Bombyx pupae at a dose of ca. 0.1 ng and also enhanced the release of ecdysone in vitro at a concentration of 10^{-11} M. Immunohistochemistry and in situ hybridization showed that Bombyx PTTH was transcribed and translated in two pairs of dorso-lateral neurosecretory cells in the brain.

The endocrine function of the insect brain was first documented by Kopec as early as 1922 in the gypsy moth, Lymantria dispar(1). Presently, the brain peptide, termed prothoracicotropic hormone (PTTH), is generally recognized as activating prothoracic glands to produce and release a molting hormone, ecdysone, and as playing a central role in the endocrine control of insect growth, molting and metamorphosis. Since two Japanese groups, the Kobayashi group (2) and the Ichikawa group (3), had independently succeeded in extracting PTTH activity from the silkworm, Bombyx mori by the early 1960s, many efforts have been made to isolate PTTH from various insects (4). The main difficulty in isolation of PTTH, however, was its minute content in each insect. To overcome this difficulty, we had to collect a large number of adult Bombyx heads as starting material and have recently succeeded in the isolation of PTTH. This progress in protein chemistry in combination with recombinant DNA technology finally provided us with the whole amino acid sequence of Bombyx PTTH.

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Isolation of Bombyx PTTH

We used the *Bombyx* adult heads as a source of PTTH (5) along with bombyxins (formerly 4K-PTTH, insulin-related peptides possessing PTTH activity in the brainless pupae of *Samia cynthia ricini*) (6), eclosion hormone (7), melanization and reddish coloration hormone (8) and pheromone biosynthesis activating neuropeptide (9). All these peptide hormones have been isolated and characterized.

During purification of *Bombyx* PTTH, samples were bioassayed using the debrained pupae of racial hybrid J-122 x C-115 of *Bombyx*, and their PTTH activities were expressed in terms of *Bombyx* units as described previously (10). After many trials, one molecular species of *Bombyx* PTTH was isolated through 16 steps of purifications (11). Heads were homogenized successively with cold acetone (step 1) and 80% ethanol (step 2), and the residues were extracted with 2% NaCl (step 3), and the extract was heated in boiling water to remove the resulting precipitates (step 4).

The supernatant was subjected to fractional precipitation with ammonium sulfate (step 5) and then with acetone (step 6). PTTH was recovered in the precipitates with 35-55% acetone, while bombyxins were recovered with 55-75% acetone. The 35-55% acetone precipitates were subsequently purified through five steps of conventional chromatography; gel filtration on Sephadex G-50 with 0.5M Tris-HCl (pH 8.5) (step 7), anion exchange on DEAE-Sepharose Cl-6B with 0.2M sodium acetate (pH 5.2)(step 8), cation exchange on CM-Sepharose Cl-6B 0.1-0.5M NaCl in 0.05M sodium acetate (pH 5.2) (step 9), Hydrophobic adsorption on Octyl-Sepharose Cl-4B with 4M ammonium acetate, 0.2M ammonium acetate and 40% acetonitrile in 0.2M ammonium acetate (step 10) and gel filtration on Sephadex G-75 with 0.01M phosphate buffer containing 0.2M NaCl and 2% butanol (step 11).

The resulting active fraction was further processed in five steps of high performance liquid chromatography (HPLC); reverse phase HPLC on Develosil $5C_8$ (Nomura Kagaku) with 20-25% acetonitrile in 0.08% trifluoroacetic acid (TFA) (step 12), reverse phase HPLC on Hi-Pore RP-304 (Bio-Rad) with 20-40% acetonitrile in 0.08% TFA (step 13), reverse phase HPLC on Hi-Pore RP-304 with 20-40% acetonitrile in 0.1% heptafluorobutyric acid (HFBA), cation exchange HPLC on TSKgel (Toyo Soda) with 0.02-0.5M sodium acetate (pH 5.2) in 10% acetonitrile (step 15) and reverse phase HPLC on Hi-Pore RP-304 with 20-40% acetonitrile in 0.1% HFBA (step 16).

At step 15, PTTH activity was recovered in four consecutive fractions, each of which corresponded to a UV peak at 280 nm, and the most active fraction was further purified at step 16 to afford four active fractions with nearly equal specific activities (0.1 ng/*Bombyx* unit). Finally, a fraction that gave a single amino-terminal sequence was obtained. These results indicated that *Bombyx* PTTH was highly heterogeneous. The amount of *Bombyx* PTTH isolated from the most prominent peak at step 16 was only 5.4 ug from 500,000 *Bombyx* heads.

The isolated sample was subjected to amino acid analysis and

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amino-terminal amino acid sequence analysis. The amino acid analysis showed that Bombyx PTTH characteristically contained a high proportion of glutamic acid and/or glutamine , aspartic acid and/or asparagine, and proline, and a low proportion of histidine, methionine and phenylalanine. The amino-terminal amino acid sequence proved to be G-N-I-Q-V-E-N-Q-A-I-P-D-P-.

The pure sample was exhausted by these analyses, indicating that 500,000 heads was insufficient to get more extensive sequence information. To solve the problem, we adopted two strategies: one was the extraction from more material and the other was the cloning of PTTH cDNA based on the partial information about the amino acid sequence.

Amino Acid Sequence Analysis of Bombyx PTTH

To get more sample, PTTH was extracted from 3,000,000 silkworm adult heads and purified through the same procedure as described above. At step 15, the activity was recovered in four fractions, I, II, III and IV. Fractions II and IV were further purified by the final HPLC step to afford several active fractions, II-1, II-2, ---, IV-4 and IV-5, respectively. The amount of each fraction was about 10-20 ug, and their specific activities were about 0.1 ng/Bombyx unit.

The amino-terminal amino acid sequence analysis of each fraction revealed that every fraction was comprised of three kinds of peptides with different proportions. One of them showed the same amino-terminal sequence, G-N-I-Q-V-E-N-Q-A-I-P-D-P-, as observed in the previous experiment, and the other two peptides had shortened sequences, N-Q-A-I-P-D-P- and Q-A-I-P-D-P-, in which six or seven amino acid residues were deleted from the amino-terminal of the former sequence. However, all active fractions possessed similar amino acid compositions and, in addition, after V8 protease digestion, showed almost identical peptide mapping patterns on HPLC.

These data strongly suggested that Bombyx PTTH consisted of heterogeneous molecular species, possessing essentially the same sequence. Based on this assumption, the amino acid sequence of Bombyx PTTH could be deduced from the combination of sequencing data obtained from various fractions, even though the amount of each fraction was insufficient to determine the whole sequence. Fractions II-1 and II-3 were used for amino-terminal amino acid sequence analyses and electrophoresis after reductive carboxamidomethylation. After the same derivatization as above, III-4 was subjected to chymotryptic digestion, and the combined fractions from IV-1 to IV-3 and from IV-4 to IV-5 to V8 protease digestion. As summarized in Figure 1, the amino-terminal amino acid sequence analysis on the carboxamido-methylated Bombyx PTTH clarified 35 residues (CAM-2) from the amino-terminal including cysteine residues. Sequence analyses of chymotryptic (C-1 to C-4, C-6 to C-9) and V8 protease (E-1 to E-8, E-10, E-12) peptides, and the alignment of the resultant sequences made it possible to deduce the total sequence as composed of 104 amino acid residues, expect for a residue at position 41.

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A lambda-gt 11 cDNA expression library was prepared by oligo(dT) priming poly(A) RNA from Bombyx brains taken from 5th-instar larvae. The cDNA expression library was screened with polyclonal antibodies against the amino-terminal 15 residues of Bombyx PTTH cDNA conjugated to bovine serum albumin and with an isolated positive clone as a hybridization probe, providing seven positive clones. Nucleotide sequence analysis proved that Bombyx PTTH cDNA contained an open reading frame encoding pre-pro-Bombyx PTTH of 224 amino acid residues (molecular mass 26,027). As schematically shown in Figure 2 (12), the pre-pro-Bombyx PTTH consists of a putative signal sequence (29 amino acid residues), two peptide components tentatively termed as p2K (22 amino acid residues) and p6K (57 amino acid residues), and a PTTH subunit (109 amino acid residues) in this order from 5' end. These components are bounded by proteolytic cleavage sequences, indicating that they are separated after translation. The last component was defined as the Bombyx PTTH subunit because the amino acid sequence of this component up to 104th residue completely matched with the sequence determined by amino acid sequencing of the purified Bombyx PTTH except for the residue at position 41. The carboxyl-terminal five residues, -R-Y-N-N-N, in the pre-pro-Bombyx PTTH were not detected by peptide sequencing, indicating the possibility of post-translational processing at the carboxyl-terminal end.

The residue at position 41 is deduced to be Asn, suggesting an N-glycosylation site, since a threonine residue exists at position 43, constituting a consensus sequence, -N-X-T-. Finding the glycosylation site suggests that Bombyx PTTH may be a glycoprotein as previously indicated (13). The heterogeneity of Bombyx PTTH may be partly due to the presence of a glycosidic side chain.

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Structure and Properties of Bombyx PTTH

The molecular mass of Bombyx PTTH was previously estimated to be 22 kD by gel-filtration using a crude preparation (11). Using SDS polyacrylamide electrophoresis followed by silver staining, the purified fractions of Bombyx PTTH showed rather broad band corresponding to approximate 30 kD under non-reductive conditions, whereas the band at 30 kD disappeared under reductive conditions and, instead, two main bands corresponding to 16 kD and 17 kD were observed. The relative density of the two bands detected by silver staining varied among fractions. S-carboxy-amidomethyl Bombyx PTTH also afforded two bands at 16 kD and 17 kD. These results and sequencing data suggest that Bombyx PTTH is a glycopeptide composed of two essentially identical subunits, 16 kD and/or 17 kD, which are connected by disulfide bond(s) (Kataoka, et al., in preparation), although the mode of disulfide bonding remains to be clarified.

The Bombyx PTTH was active at a dose of ca. 0.1 ng, when injected into brainless Bombyx pupae, but was completely inactive in debrained Samia pupae even at a dose of 10 ng (11). The Bombyx PTTH also enhanced the release of ecdysone by the prothoracic gland of Bombyx in vitro at a concentration of

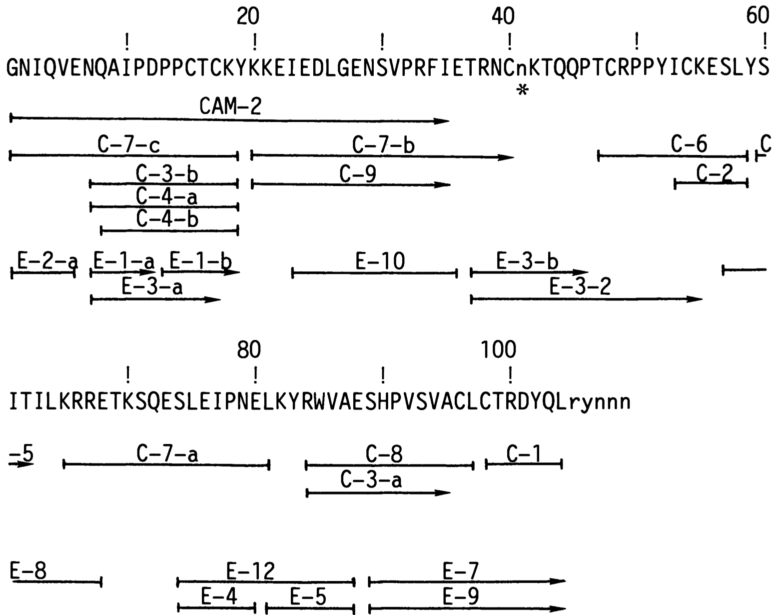

Figure 1. Summary of sequencing of Bombyx PTTH. Bars show the peptide sequenced, in which the designations and numbers correspond to the type of derivatization or enzyme used in digestion: CAM, carboxamidomethylation; C, chymotrypsin; E, V8 protease. Arrows indicate the sequence whose carboxyl-terminals are not determined. Small letters show the residues deduced solely from cDNA analysis. Asterisk indicates the site of probable glycosylation.

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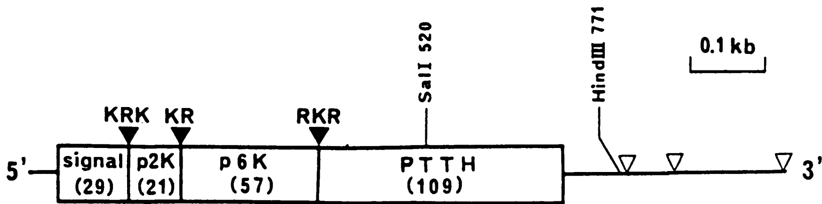

Figure 2. Schematic representation of pre-pro-Bombyx PTTH cDNA. Coding regions are indicated by boxes: numerals in parenteses are the number of amino acid residues in the respective components. Putative proteolytic cleavage sites are indicated by KRK, KR, and RKR. Triangles represent the polyadenylated tract.

ca. 10^{-11} M in the incubation medium.

By use of a synthetic pentadecapeptide corresponding to the amino-terminal region of Bombyx PTTH, a monoclonal antibody that specifically recognized Bombyx PTTH was prepared. Immunohistochemistry of Bombyx brains using this antibody revealed that two pairs of dorso-lateral neurosecretory cells were immunoreactive (Mizoguchi, et al., in preparation). Further, in situ hybridization using ^{35}S-labeled complementary RNA probe showed that the Bombyx PTTH gene was transcribed in the same cells, indicating that Bombyx PTTH is synthesized in these cells (12). In Manduca sexta two pairs of brain neurosecretory cells in a similar location have been immunohistochemically identified as the PTTH-producing cells (14).

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Chapter 4

Gypsy Moth Prothoracicotropic Hormone

Progress Toward Identification

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The processes of molting and metamorphosis in insects are regulated by a neuropeptide, prothoracicotropic hormone (PTTH) that acts through the stimulation of ecdysteroid synthesis and secretion by the prothoracic glands. PTTH exists in various insect species as a family of peptides comprised of two major classes that fall within the following mass ranges: large PTTH, 11-27 kD; small PTTH, 4-7 kD. In the gypsy moth, *Lymantria dispar*, we estimate ranges of 11-15 kD and 4-6 kD for large and small PTTH, respectively, from both larval and pupal brains. PTTH activity, with a molecular mass of <5 kD, has also been demonstrated in extracts of whole, pre-hatch *L. dispar* eggs. Small PTTH-specific nucleotide sequences have been amplified from *L. dispar* cDNA by the polymerase chain reaction (PCR), and fragments of the expected size have been obtained. The development of bioassays for *L. dispar* PTTH and recent work on PTTH in other insect species are discussed.

Neuroendocrine control of invertebrate development was first demonstrated by Kopec (1,2) with neck ligation and brain extirpation-reimplantation studies using the gypsy moth, *Lymantria dispar* (Lepidoptera: Lymantriidae). His demonstration of a "pupation factor", originating in the brain, provided the impetus for identification of numerous invertebrate physiological processes controlled by peptide neurohormones (3,4). In insects, identified processes now number over thirty (5). Knowledge of these processes has provided for the development of bioassays used in the isolation of neuropeptides. Since 1975, over forty insect peptide neurohormones have been isolated and sequenced (5-8).

The pupation factor, discovered by Kopec (1,2), was subsequently demonstrated in the commercial silkworm, *Bombyx mori*, and eventually isolated and sequenced (see review by Kataoka *et al.*, this volume). It is now known as the prothoracicotropic hormone (PTTH), and it exists as a family of neuropeptides with two molecular mass ranges in *B. mori* (small, *ca.* 4 kD and large, *ca.* 22 kD). The small forms have recently been named "bombyxins" (9). Another lepidopteran, the tobacco hornworm, *Manduca sexta*, has received considerable attention in the isolation, mode of action, and cellular localization of PTTH (10-14). Studies with *M. sexta* also revealed two molecular forms (*ca.* 7 kD and 27 kD), although their

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amino acid sequences have not been determined. Research on the PTTHs and their action in *B. mori*, *M. sexta*, and other insect species has been extensively reviewed (10-28). Since these reviews were published, new studies have appeared (29-53) that include the following interesting aspects: discovery of PTTH activity in eggs or embryos of *B. mori* (29,31), *M. sexta* (30) and *L. dispar* (47,50-53,70); cloning and expression of the bombyxin gene complex in *B. mori* (40-42); and, development of *in vivo* and *in vitro* systems for recording electrical activity in the nervi corporis cardiaci (NCC I + II) and PTTH release from the corpus allatum of *M. sexta* (45).

Our interest with the gypsy moth was to apply the basic information derived from previous studies to the development of pest control methods based on neuropeptide-regulating processes. The advantages and problems of this approach have been described (5,6,8,54,55). One mechanism for developing this approach relies on the determination of neuropeptide amino acid sequences. Neuropeptide primary structures can be obtained in two ways: 1) neuropeptide isolation and sequencing and 2) gene isolation and sequencing. With the former, an appropriate bioassay is required for detecting neuropeptide activity throughout purification. With the latter, the whole or possibly partial neuropeptide sequence must be available from a related species and must share suitable homology with the neuropeptide gene of interest. These two approaches have been used in an attempt to determine the primary structure of *L. dispar* PTTH.

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Partial Purification of Gypsy Moth PTTH

At the outset of our investigation, a few well-defined *in vivo* assays were available for the detection of PTTH activity (34,56-58). While some experiments showed that PTTH activity could be detected in extracts from heterologous species (59-61), other investigations showed that such analyses could be misleading (62,63). In particular, as illustrated by the purification of *B. mori* PTTH, bioassay of *B. mori* PTTH with *B. mori* brainless pupae revealed only a large form of the neurohormone, whereas bioassay with *Samia cynthia ricini* pupae revealed a small form that was inactive in the *B. mori* assay (62). Considering the possibility of isolating a PTTH molecule that was active only in a heterologous assay and thus would presumably provide no useful information for developing pest control methods in the species of origin, we decided to develop a homologous assay for the PTTH of *L. dispar*.

Our initial efforts were focused on the development of an *in vitro* assay for gypsy moth PTTH for reasons described previously (27,64). This assay was based on similar *in vitro* assays developed for *M. sexta* (60,65,66) and *B. mori* (67,68) and utilizes prothoracic glands (PGs) from day 5, fifth instar (L5D5) female larvae. Glands of this age were selected because they showed maximal activation as compared to glands from other days of the instar. This assay (27,64, Kelly *et al.*, in preparation) involves the incubation of individual glands in Grace's medium for 2 hours followed by incubation of one gland of a pair in Grace's-medium-prepared extracts containing PTTH activity (*i.e.* extract-activated) and incubation of the second gland in fresh Grace's medium (*i.e.* control). The amount of ecdysteroid secreted into the medium during the second 2-hour incubation is quantified by radioimmunoassay (RIA), and the activation ratio (A_r) is determined by dividing the determined amount of ecdysteroid secreted into the extract-activated medium by that secreted into the control medium. Dose-response analyses revealed saturable kinetics with an ED_{50} of *ca.* 0.02 brain equivalent/µl of medium for L5D5 brains and PGs. The initial pre-incubation period is necessary with *L. dispar* PGs to allow for deactivation following gland excision. Without this pre-incubation period, no activation by brain extracts could be demonstrated within the first hour of incubation, because the glands were apparently maximally activated (Kelly *et al.*, in

preparation). A similar *in vitro* assay has been developed with day 2 pupal glands (48, Fescemyer *et al.*, in preparation) so that larval and pupal forms of *L. dispar* PTTH can be compared and quantified using assays from stages homologous to the stage from which the PTTH activity was extracted.

To verify *in vitro* results, we have also developed an *in vivo* assay for *L. dispar* PTTH (53, Thyagaraja *et al.*, in preparation). This assay is comparable to larval assays developed for *M. sexta* (56) and *B. mori* (58) and uses last instar female larvae. Neck ligation prior to day 7, the day when a small pre-peak in hemolymph ecdysteroid titer appears, blocks further development. Injection of PTTH-containing extracts posterior to the ligation, including post-embryonic egg extract, reinitiates development and subsequent pupation. Attempts to develop an *in vivo* assay comparable to the pupal-adult *B. mori* assay (57) were unsuccessful, since brain removal as close as 15 min post-pupal ecdysis failed to block *L. dispar* female adult development (Thyagaraja *et al.*, unpublished results).

Purification of *L. dispar* PTTH was first attempted with L5D5 female brains, since PGs from larvae of this age were most responsive to PTTH-containing extracts, as indicated above. Two regions of activity with molecular masses of approximately 15,000 and 5,000 daltons (Table I) were observed by high performance size-exclusion chromatography (HP-SEC) (69). These forms were designated large (PTTH-I) and small (PTTH-II), respectively. Crude extracts of

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TABLE I. PTTH Activity and Forms in Whole Eggs and Brains from Various Stages of *Lymantria dispar*[1]

Stage[2]	Age	Form[3]	Ar_{max}[4]	ED_{50}[5]
adult	day 1	crude	19	0.03
pupae	day 1	crude	38	0.04
larvae	day 1	crude	50	0.03
	day 5	crude	60	0.02
		large[6]	69	0.08
		small[6]	20	0.02
egg	pre-hatch	crude	12	N.D.
		small[6]	11	N.D.

[1] Data taken from (27,51) and Masler *et al.*, in preparation.
[2] Fifth instar larvae, pupae and adults were all female from our New Jersey laboratory strain. Eggs were mixed-sex collected at or near Harper's Ferry, Maryland.
[3] Extracts were prepared in methanol-water-trifluoroacetic acid (90.0:9.9:0.1) according to Masler *et al.* (69) and assayed in the *L. dispar in vitro* assay with L5D5 female PGs as described in the text.
[4] Ar_{max} is the maximum activation ratio. Determination of the activation ratio (A_r) is described in the text.
[5] ED_{50} is in brain equivalents/µl.
[6] The various forms (large, *ca.* 15 kD; small, *ca.* 5 kD) were obtained by HP-SEC as described in the text. For the egg extracts, only a small form with molecular mass less than the larval small form was obtained.

L5D5 brains showed the highest activity on L5D5 PGs based on their lower ED_{50} value and higher Ar_{max}, as compared to brains from day 1 larvae, pupae and adults (Masler *et al.*, in preparation). However, the ED_{50} of L5D5 brains (0.02 brain equivalent/µl, or 0.5 brain equivalent/25 µl, the volume required for assay with each PG), which was comparable to that of *B. mori* brains (68), suggested that several thousands or possibly millions of brains might be required for final purification of *L. dispar* PTTH.

Recent discoveries of PTTH activity in extracts of embryos and whole eggs (29-31) suggested an alternative to mass dissection of larval brains. Since gypsy moth infestations occur annually throughout much of the eastern United States, millions of *L. dispar* egg masses are available in forested areas in an arrested state of development (*i.e.*, diapause) for much of the year. Our initial tests demonstrated extensive PTTH activity in extracts of pre-hatch whole eggs (70). However, this discovery was tempered by the realization that much of the activity was destroyed by boiling or extraction with organic solvents, conditions to which larval *L. dispar* PTTH is stable (27,69). Subsequent analyses revealed that the unstable activity was due to ecdysteroid ketoreductase present in *L. dispar* egg extracts (50, Kelly *et al., Arch. Insect Biochem. Physiol.*, Vol. 14, 1990, in press), similar to the activity demonstrated in *M. sexta* hemolymph (71). The activity remaining was, however, stable to boiling and organic solvent, and HP-SEC analysis revealed activity in a small molecular form for extracts from pre-hatch whole eggs (50) (Table I). We are now pursuing the purification of embryonic and post-embryonic PTTH with high performance reverse-phase and ion exchange methods (see Masler *et al.*, this volume).

Determination of Gypsy Moth PTTH Gene Sequences

Although gene sequencing methods have been available for years and used to sequence many vertebrate neuropeptide genes, only within the past two years have insect neuropeptide gene sequences become available (8). These insect sequences were determined from sequencing of neuropeptide-positive gene fragments incorporated into cDNA or genomic clones. Positive clones were identified primarily by nucleic acid probes prepared from the known amino acid sequences obtained following neuropeptide purification. Only in the cases of drosulfakinin (72) and *Drosophila melanogaster* FMRFamide-related peptide (73,74) was sequence information or a probe from another species used for identifying positive clones. For insect neuropeptides with a significant degree of homology between species, these techniques, and the newer molecular methods for amplifying specific genomic DNA, cDNA and/or mRNA sequences (8), will greatly enhance the speed with which insect neuropeptide sequences are determined.

With regard to PTTH, the amino acid sequence has been published for bombyxin (75,76). We have attempted to use one of the new molecular techniques, mixed oligonucleotide primed amplification of cDNA (MOPAC) (77) to determine the nucleotide sequence of the gypsy moth small PTTH gene. Based on the bombyxin A-chain amino acid sequence (75), nucleic acid primers were prepared for hybridization to both ends of the bombyxin A-chain gene fragment. Gypsy moth brain cDNA was amplified by the polymerase chain reaction (PCR). After fifty amplification cycles, sufficient DNA was obtained to visualize a fragment on an ethidium bromide-stained agarose gel. This fragment had the expected molecular size, equivalent to 60 base pairs (Davis *et al.*, unpublished results) (Fig. 1). For these experiments, *B. mori* amino acid sequence information was used to design mixed oligomer primers which included EcoRI (sense primers: 5'-GGAATTCTIGA(TC)GA(AG)TG(TC)TG(TC)TT-3') and SpeI (anti-sense primers: 5'-ACATGTA(AGCT)(GC)(AGCT)A(AG)(AGCT)A(AG)(AGCT)AC

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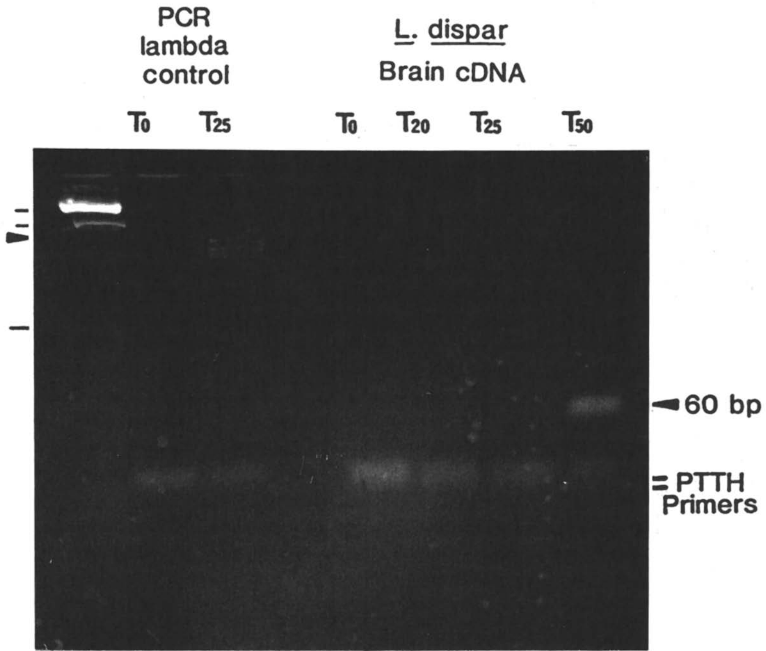

Figure 1. Ethidium bromide-stained 4% NuSieve GTG agarose (FMC Bioproducts, Rockland, ME) gel of bombyxin A peptide MOPAC fragment (*ca.* 60 b.p.) from *L. dispar* brain cDNA.

(AG)TC(AGCT)AC-3') restriction sites. Amplification reactions were performed using a Perkin Elmer Cetus DNA Thermal Cycler (1 cycle: 95° C, 80 sec and 25 cycles: 95° C, 30 sec; 50° C, 30 sec; 72° C, 30 sec) in 100-µl reactions containing 10 mM Tris-HCl (pH 8.3 at 25° C), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 200 µM each dNTP, 2.5 units Taq DNA Polymerase (Perkin Elmer Cetus, Norwalk, CT). Lanes 5 through 7 contain amplification products from *L. dispar* brain cDNA and mixed oligonucleotide primers for bombyxin A peptide after 0 (T_0), 20 (T_{20}) and 25 (T_{25}) amplification cycles. Lane 8 contains second stage reaction products after an additional 25 amplification cycles (T_{50}) in the presence of the mixed oligonucleotide bombyxin A peptide primers and a 5-µl aliquot of melted agarose excised from the *ca.* 60 b.p. region of a 4% NuSieve GTG agarose gel of electrophoresed initial amplification reaction products (*L. dispar* brain cDNA, T_{25}). Lane 1 contains HindIII digested lambda DNA molecular weight markers. Lanes 2 (T_0) and 3 (T_{25}) contain PCR control amplification products using control lambda DNA template and primers (Perkin Elmer Cetus) prior to amplification (T_0) and after 25 amplification cycles (T_{25}). Similar amplification with primers based on the B-chain sequence (76), gave a fragment equivalent to 84 base pairs.

Following the recent publication of the gene sequence for bombyxin (41), and more recently that of four clustered bombyxin genes (42), amplification with antisense A-and sense B-chain primers, expected to amplify the entire A-chain/C-peptide/B-chain region, gave a fragment equivalent to 220 base pairs, as expected (Fig. 2). Reaction conditions were as in Figure 1 except for changes in amplification cycles (1 cycle: 95° C, 7 min; 37° C, 5 min; 55° C, 2 min; 72° C, 7 min and 40 cycles: 94° C, 30 sec; 37° C, 15 sec; 50° C, 30 sec; 72° C, 2 min) and addition of Taq polymerase during the 72° C step of the 1st cycle. Lanes 3 through 5 show amplification reaction products of first stage amplification (1st PCR) after 30 (T_{30}) and 40 (T_{40}) cycles (lane 4 and lane 5, *L. dispar* egg cDNA template; lane 3, "cntr," template minus control at T_{40}). *Ca.* 220 b.p. regions were excised from control (template minus) and *L. dispar* egg cDNA template electrophoresed reaction products and 5-µl aliquots were used as template in the second stage amplification (2nd PCR) for control ("cntr," lane 6) and *L. dispar* egg cDNA template (T_{40}, lane 7) MOPAC reactions. Lanes 1 and 2 contain sense primers (B) and anti-sense primers (A) alone. Lane 8 contains HindIII digested lambda DNA. We are presently attempting to determine the nucleic acid sequences for these amplified fragments.

Summary

Future progress in developing biologically-based methods of pest control will undoubtedly involve the insertion of toxic peptides into expression vectors, such as baculoviruses. That neurohormone genes, or modified versions thereof, can be used in this manner for practical control has yet to be demonstrated, although recent construction and utilization of a vector for expression of diuretic hormone in *B. mori* larvae suggests feasibility (78). In any event, the development of molecular techniques for the study of insect neuroendocrinology holds great promise for studying both the metabolism and mode of action of insect neuropeptides (8).

Note Added in Proof

Since the submission of the original manuscript, there have been several significant publications and developments which bear on studies with PTTH. These include the publication of the gene sequence for *B. mori* large PTTH (79) and development of anchored PCR (80) and the ExoMeth sequencing method (81). We are currently

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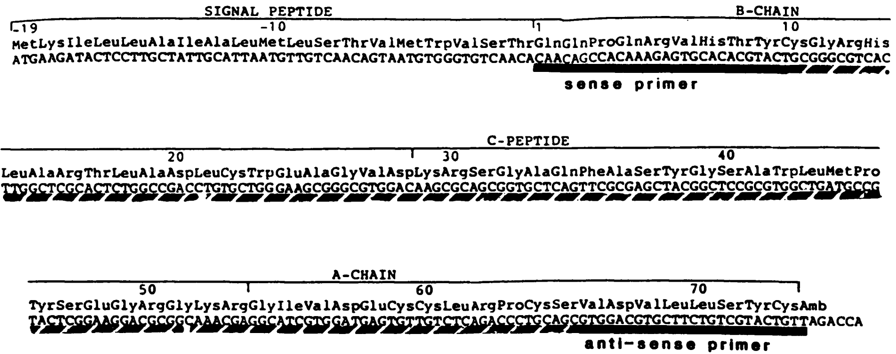
Bombyx mori 4K-PTTH (bombyxin)



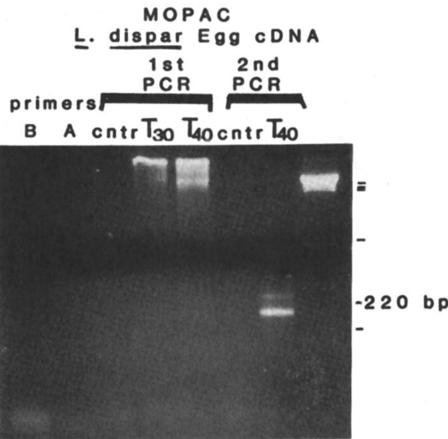


Figure 2. *B. mori* bombyxin (small PTTH) sequence showing the location of mixed oligonucleotide sense primers (5'-GA(AG)GAATTCCA(AG)GC (AGCT)GT(AGCT)CA(TC)AC(AGCT)TA(TC) T-3') and anti-sense primers (5'-ACAGTGA(AGCT)GA(AGCT)AG(AGCT)AG(AGCT)ACGTC (AGCT)AC-3') using the template from Iwami *et al.* (41). Ethidium bromide-stained 4% NuSieve GTG agarose gel of two-stage amplification of *L. dispar* pre-hatch egg cDNA with mixed oligonucleotide primers based on the *B. mori* A fragment (anti-sense primers) and B fragment (sense primers) amino acid sequences.

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using these methods and sequence information to obtain the sequence for large PTTH in the gypsy moth (Davis *et al.*, unpublished data).

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Chapter 5

Insect Myotropic Peptides

Isolation, Structural Characterization, and Biological Activities

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In the early 1980's, dramatic improvements in both peptide isolation technology and the instrumentation for structural characterization resulted in an avalanche of new insect neuropeptide structures. Of the 50-60 known structures, about half exhibit effects on the contractile activity of insect visceral muscle at physiological concentrations. This report describes the strategies and tactics that were utilized to successfully isolate, purify, and structurally characterize this group of insect neuropeptides.

The single most important requirement for a successful isolation of a biologically active natural product is a reliable bioassay system for following the compounds of interest through the purification scheme. The semi-isolated heart of _Periplaneta americana_ and the isolated hindgut of _P. americana_ or _Leucophaea maderae_ fulfill that requirement as shown by the number of successful insect peptide isolations which used one of those three preparations as a bioassay. Approximately half of the 60 or so insect neuropeptides whose structures are known were isolated based upon an effect (either stimulatory or inhibitory) upon cockroach visceral muscle. Our laboratory has used the isolated hindgut of the cockroach, _L. maderae_, as a bioassay preparation for more than 20 years (_1_). This preparation requires minimal dissection skills and the associated instruments are easy to operate. In addition, the hindgut is physically rugged and if care is taken to assure constant aeration, a single hindgut will remain viable for an entire day. After dissection and suspension of the gut in the chamber, a 30 min to 1 hr period of equilibration is allowed during which time the saline is replaced 3-5 times. At that point, a relatively constant pattern of spontaneous contractions is established and the assay of fractions for activity can begin. Alteration of the pattern of spontaneous contractile activity (either stimulatory or inhibitory)

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indicates the presence of a biologically active component in that fraction. When the test saline is replaced with fresh saline, the effects of both stimulators and inhibitors cease almost immediately and within 2-5 min the spontaneous contractile activity returns to a pre-exposure pattern and the gut is ready to accept another fraction. During our work on the isolation of the cockroach, cricket, and locust peptides, up to 80 samples/day were evaluated on a single hindgut preparation. With few exceptions (noted later), the receptors of the L. maderae hindgut as well as the heart and hindgut of P. americana were quite sensitive to many of the insect myotropins and consequently only small quantities of material were expended during purification procedures.

The remainder of this report describes how these visceral muscle bioassays were used to successfully isolate and structurally characterize more than two dozen insect neuropeptides.

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Cardioacceleratory Neuropeptides of P. americana

In late 1984, two independent research teams almost simultaneously published the structural characterization and synthesis of two myotropic neuropeptides isolated from P. americana corpora cardiaca (cc) extracts (2,3). Both teams used reverse-phase, high-performance liquid chromatography (RP-HPLC) with ion-pairing reagents to obtain pure peptides for analysis. A C-18 reverse-phase column was used by both teams and purification was accomplished with a solvent programmed run followed by an isocratic run of the active peaks. In one case, the two peptides were isolated based on their stimulatory action on a locust somatic muscle preparation (2). In the second case, stimulation of the semi-isolated P. americana heart was used to detect active HPLC peaks and fractions (3). Mass spectral analysis of the intact peptides (2) or their chymotryptic fragments (3) was used to deduce the sequences. Sequence methods utilizing Edman chemistry were precluded since both peptides exhibited a blocked amino terminus (enzymatic deblocking procedures had not been developed at this time). The structures obtained by both groups were:

M-I (2), CC-I (3) (Pea-HGH-I)	pQVNFSPNWamide
M-II (2), CC-II (3) (Pea-HGH-II)	pQLTFTPNWamide

Both of these peptides exhibited considerable sequence identity with the locust adipokinetic hormone (Lom-AKH-I) and the crustacean red pigment-concentrating hormone (RPCH), as had been suggested by chromatographic behavior, N-terminal analysis, and amino acid analysis (3,4). Indeed, both peptides were shown to be far more potent as stimulators of hyperglycaemia than as myotropins (3). Based upon similarity of structure and activity, Pea-HGH-I and II are classified as members of the AKH/RPCH family of neuropeptides.

A single pair of cc contained about 100 pmol and 40 pmol of Pea-HGH-I and II, respectively. The relatively high titers of these two peptides was indeed fortuitous as neither of the myotropic

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bioassays was particularly sensitive to these compounds. The threshold of activity concentration for the locust hindleg preparation was reported to be about 100 nM (2), while the threshold concentration on the isolated cockroach heart was determined to be about 10 nM with concentrations of 100 nM evoking a 30% increase in heartbeat rate (3).

In 1989, a third cardioacceleratory peptide was isolated from P. americana cc extracts and structurally characterized (5). Corazonin, pQTFQYSRGWTNamide, was purified with RP-HPLC on a C-18 column with two solvent systems which differed only with respect to the ion-pairing reagent. The N-terminal pyroglutamic acid (pQ) was removed enzymatically and the primary sequence of the 2-11 fragment was obtained by gas-phase sequencing. The presence of a C-terminal amide was elucidated by subjecting the C-terminal tetrapeptide (obtained from a trypsin digest of corazonin) to four cycles of manual Edman degradation followed by the identification of PTC-Asn-amide with HPLC.

Corazonin was present at a much lower titer (2 pmol/pair cc) than the cardioaccelerators Pea-HGH-I and II whose titers were reported to be 100 pmol- and 40 pmol/pair cc, respectively (3). However, corazonin was about 100-fold more potent than Pea-HGH-I and II. Concentrations of corazonin as low as 0.2 nM evoked significant increases in heartbeat rate.

Structurally, corazonin does not seem to fit into any of the known insect neuropeptide families. The presence of a polar arginine residue and the absence of a Phe residue at position 4 exclude corazonin from the AKH family, while the absence of the C-terminal pentamer, Phe-X-Pro-Arg-Leu-NH_2, excludes corazonin from the leucopyrokinin peptide family discussed later in this report.

Proctolin

In 1967, Brown (6) described a myotropin present in P. americana hindguts that stimulated the contractions of an isolated hindgut of the same species. This myotropic peptide, which we now know as proctolin (RYLPT-OH), was subsequently isolated (7) and structurally characterized (8) after a multi-year effort. A whole-body extract totaling 125 kg of P. americana was processed with contemporary purification techniques (open column ion-exchange, adsorption, and thin-layer chromatography) to obtain 180 μg of pure peptide which was sufficient for structural characterization with hand sequencing methods. During the past 14 years, proctolin has been the focus of numerous physiological, pharmacological, immunocytochemical, and biochemical studies. Proctolin is widely distributed among the arthropods but its presence in other phyla is not well established. Although initially described as a neurotransmitter, recent evidence suggests that proctolin acts as a neuromodulator and perhaps also as a neurohormone. A thorough and up-to-date review on the status of proctolin research was recently published (9).

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Hindgut-stimulating Peptides of L. maderae

Because of the complex nature of the sample matrix, a four-column HPLC purification system was developed to purify the myotropic peptides from whole head extracts of *L. maderae* (10). After initial fractionation on a phenyl reverse-phase column, active samples were sequentially passed through C-1 and C-18 reverse-phase columns. Final purification was accomplished on an I-125 protein separation column operated in a normal-phase mode. The C-1 column provided the breakaway step as more than 95% of the UV-214 absorbing material in the phenyl column fractions was retained less than 24 min but all of the myotropins were eluted after 26 min postinjection. Additionally, the C-1 column separated some of the myotropins that would have been quite difficult to resolve on C-18. For example, leucokinin I (10) and leucopyrokinin (11) were isolated in the same 2-min fraction from the phenyl column. On the C-18 column, their retention times differed by 36 sec and the two peaks were only partially resolved (Holman, unpublished observation). However, the problem of separating leucokinin I (LK-I) and leucopyrokinin (LPK) never materialized as LPK was retained 20 min longer on C-1 than LK-I. Similarly, leucokinin VIII (LK-VIII) and leucosulfakinin (LSK) were collected in the same phenyl column fraction (12,13) and exhibited very similar retention times (51.4 min and 51.0 min, respectively) on C-18. However, the problem of separation never materialized because LK-VIII and LSK were collected from C-1 in fractions that were separated by 15 min.

The I-125 protein column (operated in a normal-phase mode) was chosen as the final purification step for two reasons: 1) the small (sometimes invisible) amount of residue remaining after the previous purification steps was easily dissolved in 95% acetonitrile:5% water (the initial-conditions solvent for this system); and 2) the peaks collected from this column were in a solution (75%-85% acetonitrile) suitable for peptide stabilization and required no further manipulation (other than capping) prior to storage. The structures of the 11 myotropic cockroach peptides isolated with this method are listed, along with appropriate references, in Table I (leucokinins I-VIII), Table II (leucosulfakinins), and Table III (leucopyrokinin).

Three separate 3000-head extracts were required for the isolation and characterization of the *Leucophaea* peptides. Nine of the peptides obtained from the first 3000-head extract were submitted to analysis by mass spectrometry but no useful data were obtained from that effort. An additional 1500 head equivalents of one peptide (later named LSK-II) from the second 3000-head extract were submitted to mass spectrometry with the same result. Leucokinins I-VIII (10,12,14,15) and the two LSK's (13,16) were structurally characterized from this second extract. Because LPK was not collected during the C-1 fractionations of the second extract, a third 3000-head extraction was performed. From this extract, LPK was successfully purified, enzymatically deblocked, and structurally characterized. Mass spectrometry was not attempted (11).

Although we felt secure with our structural characterizations of the sulfated LSK's, our evidence was based primarily upon the

Table I. Structures of the Leucokinins and Achetakinins

Peptide	Structure	Ref.
LK-I (Lem-M-I)	DPAFNSWGamide	10
LK-II (Lem-M-II)	DPGFSSWGamide	10
LK-III (Lem-M-III)	DQGFNSWGamide	14
LK-IV (Lem-M-IV)	DASFHSWGamide	14
LK-V (Lem-M-V)	GSGFSSWGamide	15
LK-VI (Lem-M-VI)	pQSSFHSWGamide	15
LK-VII (Lem-M-VII)	DPAFSSWGamide	12
LK-VIII (Lem-M-VII)	GADFYSWGamide	12
AK-I (Acd-K-I)	SGADFYPWGamide	†
AK-II (Acd-K-II)	AYFSPWGamide	†
AK-III (Acd-K-III)	ALPFSSWGamide	†
AK-IV (Acd-K-IV)	NFKFNPWGamide	†
AK-V (Acd-K-V)	AFHSWGamide	†

† Holman, G. M., et al. in Chromatography and Isolation of Insect Hormones and Pheromones, in press.

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synthesis of sulfated peptides that matched the HPLC retention times and biological activities of the natural products. The LSK's isolated from the third head extract provided us with the material to obtain additional proof of the sulfated nature of these peptides. About 1 nmol of natural LSK-II and an equivalent amount of synthetic LSK-II were submitted to fast-atom bombardment mass spectrometry at the USDA Western Regional Research Center at Berkeley, CA. Both compounds showed ions at 1317.2 (MH^+) and 1237.3 ($MH^+ - SO_3$) consistent with a single sulfate group and a pyroglutamyl residue in the structure. Basic hydrolysis of the

Table II. Structures of Sulfated Insect Neuropeptides*

Peptide	Structure	Ref.
LSK (Lem-SK-I)	EQFEDY(SO_3H)GHMRFamide	13
LSK II (Lem-SK-II)	pQSDDY(SO_3H)GHMRFamide	16
DSK I (Drm-SK-I)	FDDYGHMRFamide	17
DSK II (Drm-SK-II)	GGDDQFDDYGHMRFamide	17
PSK (Pea-SK-I)	EQFDDY(SO_3H)GHMRFamide	19
LSK-II (non-sulfated)	pQSDDYGHMRFamide	19
Lom-SK	pQLASDDY(SO_3H)GHMRFamide	†

* Structures of DSK I and II were determined by molecular biological techniques. Expression of these peptides has not been demonstrated.
† Schoofs, L., et al. in Chromatography and Isolation of Insect Hormones and Pheromones, in press.

remaining LSK-II natural product and all of the LSK natural product followed by amino acid analysis demonstrated the presence of a tyrosine sulfate residue in both peptides (13,16). LSK and LSK-II were the first gastrin/cholecystokinin-like peptides to be isolated from an invertebrate source.

Table III. Structures of Leucopyrokinin (LPK) and Related Peptides

Peptide	Structure	Ref.
LPK (Lem-PK)	pQTSFTPRLamide	(11)
Lom-MT-I	GAVPAAQWFSPRLamide	(21)
Lom-MT-II	EGDFTPRLamide	†
Lom-PK	pQDSGDEWPQQPFVPRLamide	‡
Hez-PBAN	DPEQIDSRTKYFSPRLamide*	(22)
Bom-PBAN	DPEEMESRTRYFSPRLamide*	(23)
LPK-5 Ser	pQTSFSPRLamide	(20)

† Schoofs, L., et al. Insect Biochem., in press.
‡ Schoofs, L., et al. Gen. Comp. Endocrinol., in press.
* Both Hez- and Bom-PBANS' are 33-residue peptides. Only the C-terminal 16 residues are shown.

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In addition to the L. maderae myotropic peptides, a series of five myotropic peptides were isolated and structurally characterized from head extracts of the cricket, Acheta domesticus, with the same purification system and bioassay (Holman, G. M., et al. Chromatography and Isolation of Insect Hormones and Pheromones, in press.). The structures of these five myotropic peptides, the achetakinins, are shown in Table 1. Like the leucokinins, the achetakinins (Ak's) contain a C-terminal pentapeptide core which is responsible for the myotropic activity (12). In Ak's III and V, the C-terminal pentamer is the same as the leucokinin pentamer (Phe-X-Ser-Trp-Gly-NH_2, where X = Asn, His, Ser, or Tyr) but in Ak's I, II, and IV a slightly different pentamer (Phe-X-Pro-Trp-Gly-NH_2) is present.

The receptors of the L. maderae hindgut are quite sensitive to the leucokinins (10,12,14,15) and the achetakinins (Holman, G. M., et al. Chromatography and Isolation of Insect Hormones and Pheromones, in press.) as the threshold for activity concentrations range from 0.27 nM to 0.029 nM. Head titers of the leucokinins ranged from 0.48 pmol/head for LK-I (10) to a low of 0.06 pmol/head for LK-VIII (12). Head titers of the achetakinins ranged from 0.15 pmol/head (AK-III) to 0.02 pmol/head (AK-II) (Holman, G. M., et al. Chromatography and Isolation of Insect Hormones and Pheromones, in press.).

Since the publication in 1986 (13,16) of the gastrin/cholecystokinin-like leucosulfakinins (LSK's), several additional insect sulfakinins have been structurally characterized (Table II). The structures of the Drosophila sulfakinins (DSK's) were deduced from a gene sequence which was isolated from Drosophila genomic DNA and head cDNA libraries (17). Although expression of the DSK's remains to be demonstrated, sulfated synthetic replicas are biologically active on the isolated cockroach hindgut (Holman, unpublished observation) as predicted by a study (18) which demonstrated that the hexamer, Tyr(SO_3)-Gly-His-Met-Arg-Phe-NH_2, was the "core" structure required for myotropic activity.

Perisulfakinin, PSK, was isolated by chance during the investigation of cardioacceleratory-hypertrehalosaemic peptides of P. americana that resulted in the structural characterization of corazonin (5). Amino acid analysis and gas-phase sequencing of an

inactive but pure HPLC peak yielded a sequence (Table II) identical to LSK except for a residue of Asp at position 4 instead of Glu (19). Barium hydroxide hydrolysis followed by amino acid analysis revealed a sulfated Tyr residue. Subsequently, the myotropic nature of PSK was demonstrated on the isolated hindgut preparation of P. americana where the threshold of activity concentration was determined to be 0.25 nM, virtually the same as the threshold concentration (0.22 nM) of LSK on the isolated L. maderae hindgut (13). In addition to PSK, the non-sulfated form of LSK-II was isolated and structurally characterized from the P. americana cc extracts, but the biologically active sulfated form was not found (19).

The final peptide structure shown in Table 2 represents an insect sulfakinin recently isolated from br-cc/ca extracts of the locust, Locusta migratoria, and structurally characterized. A modification of the four-step HPLC purification system (10) in which all gradients were extended to a higher final acetonitrile concentration was utilized. In addition, a C-8 reverse-phase column was substituted for the C-18 column (Schoofs, L., et al. Chromatography and Isolation of Insect Hormones and Pheromones, in press.). The isolated hindgut of L. maderae was used as the bioassay. Lom-SK contains residues of Leu and Ala at the 2- and 3-positions, respectively. The remainder of the sequence is identical with the 2-10 sequence of LSK-II.

The presence of similar insect sulfakinins in species as diverse as cockroaches and Drosophila suggests this peptide family may be widely distributed among the Insecta. In addition, the C-terminal octapeptide core appears to have been conserved during evolution.

The octapeptide leucopyrokinin (LPK) was the eleventh and final myotropic peptide isolated from L. maderae (11). Unlike the leucokinins and LSK's, LPK exhibits a myotropic activity on the muscles of the cockroach foregut and oviduct (Holman, G. M. and Nachman, R. J., unpublished observation.). LPK was the highest titered of the Leucophaea myotropins (1.36 pmol/head) but was the least potent on the isolated hindgut preparation (threshold concentration = 0.6 nM). LPK shares a 50% sequence identity with Pea-HTH-II at the 1, 4, 5, and 6 positions and is amidated at the C-terminus. Yet LPK does not contain Trp, which is present at position 8 in every AKH/RPCH peptide. Furthermore, LPK is positively charged with a residue of Arg at position 8. These structural observations show that LPK is not a member of the AKH/RPCH family. A study of LPK analogs supports this view (20). LPK analogs in which Asn replaced Arg at position 7 or Trp replaced Leu at position 8 exhibited drastically reduced (1000-fold) myotropic activity. Finally, N-terminal truncation of AKH/RPCH peptides destroys biological activity whereas LPK can be truncated to the C-terminal pentapeptide core (Phe-Thr-Pro-Arg-Leu-NH_2) and still retain significant (25%) myotropic activity (20).

Although LPK was the only peptide isolated from the Leucophaea head extracts that contained the amidated C-terminal pentapeptide, Phe-X-Pro-Arg-Leu-NH_2 (X = Thr), additional neuropeptides containing that pentapeptide core have subsequently been isolated from other insect species and structurally characterized (Table

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III). Lom-MT-I (X = Ser) (21), Lom-MT-II (X = Thr) (Schoofs, L., et al. Insect Biochem., in press.), and Lom-PK (X = Val) (Schoofs, L., et al. Gen. Comp. Endocrinol., in press.) were isolated from br-cc/ca extracts of Locusta migratoria. The isolated hindgut preparation of Leucophaea was used as the bioassay. None of the three locust neuropeptides appear to stimulate contractions of the isolated locust hindgut. However, both Lom-MT's stimulate contractions of the isolated locust oviduct while Lom-PK stimulates the isolated locust foregut. Two recently characterized neuropeptides, the pheromone biosynthesis activating neurohormones (PBAN's) both contain the C-terminal pentamer Phe-Ser-Pro-Arg-Leu-NH_2. The two lepidopteran neuropeptides both contain 33 amino acid residues, of which only six differ. Hez-PBAN was isolated from the adult cotton boll worm, Heliothis zea (22) while Bom-PBAN was isolated from the silkworm, Bombyx mori (23). Like LPK, Bom-PBAN can tolerate N-terminal truncation. The amidated C-terminal decapeptide of Bom-PBAN retained a reduced but definite PBA activity (23). Based upon the observations of Nachman et al. (20), we predicted that the PBAN's would stimulate the isolated visceral muscles of Leucophaea. This prediction was correct. Hez-PBAN (Nle^5, Nle^{14}) stimulated the contractions of the isolated Leucophaea hindgut above a threshold concentration of 14.5 ± 4.2 nM (Holman, G. M. and Nachman, R. J., unpublished.). At threshold concentration, Hez-PBAN (Nle^5, $Nleu^{14}$) was about four-fold less active than LPK on the isolated Leucophaea oviduct (6.3 ± 2.5 nM vs. 1.6 ± 0.26 nM, respectively) (Holman, G. M. and Nachman, R. J., unpublished.). The studies above suggest that the LPK family of peptides may be widely distributed among the insect species and possess a number of functions. The pentapeptide core, Phe-X-Pro-Arg-Leu-NH_2, appears to have been well conserved during evolution.

Neuropeptide Inhibitors of Visceral Muscle

Only two neuropeptides that inhibit the spontaneous contractions of visceral muscle have been structurally characterized. These two peptides, which contain a C-terminal FLRFamide tetramer are almost identical as shown:

Leucomyosuppressin pQDVDHVFLRFamide (24)

SchistoFLRFamide PDVDHVFLRFamide (25)

Leucomyosuppressin (LMS) was isolated concurrently with the leucokinins, leucopyrokinins, and leucosulfakinins from Leucophaea head extracts (24). LMS reversibly inhibits the spontaneous contractions of the isolated Leucophaea hindgut in a dose-dependent fashion. The threshold of activity concentration was 0.078 nM, and each cockroach head contained 0.23 pmol LMS (24).

A similar peptide, SchistoFLRFamide, was recently isolated from extracts of the locust (Schistocerca gregaria) thoracic nervous system by gel filtration followed by RP-HPLC on a C-18 column. Samples were initially fractionated with a water:acetonitrile gradient using heptafluorobutyric acid as the ion-pairing agent.

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The appropriate fraction was collected and rechromatographed on the same column with the same gradient but a different ion-pairing reagent (10 mM triethylamine adjusted to pH 6.5 with trifluoroacetic acid). SchistoFLRFamide was isolated on the basis of cross-reactivity to antibodies raised against the molluscan peptide FMRFamide. Sequence analysis was performed with a pulsed-liquid phase sequencer using Edman chemistry (25).

Like LMS, SchistoFLRFamide is a potent inhibitor of visceral muscle contraction. When applied to the semi-isolated *Schistocerca* heart preparation, concentrations of SchistoFLRMamide between 1 nM and 0.1 nM produced an observable inhibitory effect. At 1000 nM, spontaneous heart contractions were abolished and remained so for several minutes after removal of the peptide-containing solution (24). In addition, SchistoFLRFamide had an effect upon the locust extensor-tibiae muscle preparation (somatic muscle). Low concentrations (.01 nM-10 nM) produced a potentiation of the amplitude of slow motor neuron induced twitch tension while higher concentrations (100 nM-1000 nM) produced a variable biphasic response with an amplitude of contraction decrease followed by an increase (25).

LMS was isolated on the basis of its action upon visceral muscle. However, it also effects motor neurons (26). LMS attenuated the evoked transmitter release from the presynaptic membrane of excitatory motor neurons terminating on the skeletal muscle of the mealworm, *Tenebrio molitor*, but had no postsynaptic effects on that preparation. Although the mechanisms of LMS-induced inhibition of excitatory presynaptic potentials have not been precisely identified, one preliminary experiment suggested that metabolites of arachidonic acid may function as a second messenger for LMS at that site (26).

Conclusions and Future Directions

Beginning with the initial success of Brown and Starratt (7,8) and continuing to the present, myotropic bioassays have played a major role in the isolation and structural characterization of insect neuropeptides. The success will likely continue since myotropic bioassays are sensitive, rapid, reliable, and reproducible; characteristics desirable to the isolation specialist. Insect neuropeptide isolation projects utilizing a myotropic bioassay have been responsible for the discovery of four structurally unique peptide families and the initial demonstration of the existence of natural-analog series which may be quite common in insects.

Additional myotropic/inhibitory neuropeptide structures will be characterized on the basis of immunological similarity to vertebrate and invertebrate peptide structures, and also with the techniques of molecular biology. Two initial successes with those methods are discussed in this report (17,25). In addition, antibodies raised against FMRFamide were used to isolate and structurally characterize a nonapeptide containing C-terminal FMRFamide from head extracts of *Drosophila* (26). Subsequently, the gene that codes for this nonapeptide and eight other FMRFamide-related structures was isolated and sequenced (27,28).

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As new insect neuropeptide structures are revealed, synthetic replicas will become available. Studies of these synthetic materials will advance our understanding of chemical messengers and the functions they control. Most likely, new physiological and biochemical activities influenced by multifunctional peptides will be discovered. Evolutionary relationships among insect species and between insects and other animals, both vertebrate and invertebrate, will be clarified. Immunocytochemical studies will reveal specific identifiable neurons that synthesize these peptides, and biochemical studies will demonstrate the precise mechanisms of peptide biosynthesis, processing, post-translational modification, secretion, transport, and metabolism. Structure-activity relationships and computer modeling studies will demonstrate how biological information is encoded upon the structure of these peptides and will increase our knowledge of messenger-receptor interactions. Insect neuropeptides, including the myotropins, are the master regulators of insect development, metabolism, homeostasis, behavior, and reproduction. They are the molecular messengers that provide coordination between the tissues that make up the organisms we call insects.

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Chapter 6

Myotropic Neuropeptides

Physiological and Pharmacological Actions

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Proctolin, the first peptide identified from insects, has a broad distribution among members of the class and does not appear to function as a conventional neurotransmitter. More recently, eight peptides of quite similar structure were isolated from head extracts of the cockroach Leucophaea maderae. Experiments with these peptides (Leucokinins I-VIII) on various types of visceral muscle showed a selective activation of the hindgut. Subnanomolar amounts of the leucokinins (3×10^{-10}M) caused an increase in the frequency and amplitude of hindgut contractions. The muscles of the foregut and oviduct were 100 to 1000 fold less sensitive to these peptides. The heart by comparison was either unresponsive or inconsistent. Another myotropic peptide leucopyrokinin showed a similar response profile with the same visceral muscles, but it caused a more consistent stimulation of the heart. A peptide (leucomyosuppressin) that inhibits spontaneous visceral muscle activity was also isolated from head extracts of L. maderae. This peptide suppresses muscle activity of the foregut and hindgut in the 5 to 9×10^{-11}M range. The other visceral organs were less responsive.

It has long been recognized that muscle cells provide the instrumental means for body movement in animals. The cellular basis for this activity exists in the unique organelles called myofibrils, which consist of alternating strands of two different protein molecules known as actin and myosin. These myofibrils are oriented parallel to the longitudinal axis of the elongate muscle cell and shorten in the process of contraction to generate a mechanical

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force. The stimuli that initiate these contractions can occur in three ways: 1) by nerve impulses transmitted to the subsynaptic membrane on the surface of the muscle fiber; 2) by substances that circulate in the blood and interact with the surface membrane; and 3) by spontaneous changes in the level of excitability within the muscle itself. In each of these cases, a chemical messenger is required at some point during the process of stimulation. For example, when an electrical impulse reaches the nerve terminal in vertebrate muscle acetylcholine is released into the synaptic gap. Once this messenger activates receptor proteins in the membrane of the muscle fiber, the following sequence of events takes place: 1) specific ion channels are opened; 2) an electrical signal is generated which propagates along the surface of the fiber; and 3) the release of intracellular calcium causes a shortening of the myofilaments. In recent years, a whole array of chemical messengers which control or regulate muscle function have been identified and described in insects. These substances have been variously described as neurotransmitters, neuromodulators, and neurohormones in the context of some particular neurophysiological preparation under investigation. However, it is now clear that a specific chemical effector may function as a neurotransmitter at one nerve-muscle junction and as a neuromodulator at another. Thus it has become conventional to simply refer to such neurochemicals as transmitter substances, and the neuropeptides have quickly become the largest group of such chemicals (1). Moreover, the study of the actions of these peptides has opened a new dimension to the ever widening spectrum of chemical communication that seems to regulate insect behavior. The following properties outline the distinctive functional aspects of this group of chemical messengers (2,3): 1) the events activated by peptides generally have a threshold in the nanomolar (rather than the micromolar) range; 2) neuropeptides often initiate a sustained regulation of some type of complex activity or cause a long-lasting increase in excitability; 3) many peptides are released from neurohemal regions along axons or at neurosecretomotor endings, rather than at synaptic junctions; and 4) peptides can activate receptors on cells quite remote from the point of their release.

Although many of the earlier studies on animal ethology and neurophysiology gave an inadequate account of the role that muscles have in behavior, it is now recognized that these organs are the principle avenues for the expression of nearly all variations in animal behavior (4). Moreover, recent studies have shown that even a single muscle can be the channel for expressing different patterns of behavior. These differences are reflected not so much in the geometry of innervation, as in the capacity of nerve terminals to release more than a single chemical effector. Each transmitter substance seems to evoke its own unique pattern of contractile events. Such a fact is particularly well illustrated in the extensor-tibiae of the hind leg of the locust (5). This muscle is innervated by three identified motor neurons and one identified modulatory neuron. A small group of fibers at the proximal end of the muscle exhibits a spontaneous and rhythmic pattern of contraction and relaxation. Apparently this myogenic rhythm helps to pump blood and air into the large jumping leg of the locust.

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Although these rhythmic contractions of the extensor-tibiae are initiated by spontaneous depolarizations in certain fibers within the muscle, they are regulated by a specific cell on the dorsal surface of the metathoracic ganglion. This cell is found in a cluster of neurosecretory cells called the dorsal unpaired median (DUM) neurons. Neuromuscular transmission and muscular contraction in the extensor-tibiae muscle is modulated by the biogenic amine octopamine and by the peptides, proctolin, and FMRFamide. Octopamine and proctolin are contained within neurons which innervate the muscle, while FMRFamide- and small cardioactive peptide (SCP_B)-like peptides appear to be released into the locust hemolymph as neurohormones. Proctolin speeds up the myogenic rhythm and generates an increase in basal tension, while octopamine reduces the frequency of the rhythm. In addition, the rhythm is stimulated by such peptides as the adipokinetic hormone (AKH)-related peptides M_1 and M_2 and by the small cardioactive peptide (SCP_B). The full physiological significance of such a divergent set of peptides that can activate the myogenic rhythm of this muscle is difficult to assess, other than to say that they must in some way fine-tune behavioral commands and responses in the living state.

The majority of insect neuropeptides discovered thus far seem to have the property of regulating the contractile activity of either skeletal and/or visceral muscles. Whether these are the principal or auxiliary modes of action for a given peptide must await a more comprehensive assessment of their individual profiles of activity in a variety of physiological systems. What follows is a summary of our current understanding of the physiological and pharmacological properties of the insect neuropeptides that regulate muscle activity.

Proctolin

The discovery of the neuropeptide proctolin was the fruitful consequence of Brian Brown's sustained interest in pharmacologically active agents that affect visceral muscles of the cockroach *Periplaneta americana*. In 1967, he reported (6) on the presence of an unidentified substance in extracts of the hindgut which caused a slow type graded contraction of the longitudinal muscles of that organ. Neurally evoked contractions were quite similar and this "gut-factor" was depleted from the hindgut after surgical section of the proctodeal nerve. Moreover, its specific activity (bioactive units per milligram protein) was approximately 25 times higher in the proctodeal and stomodeal nerves than in the viscera that they innervate. Although the substance was heat stable and dialyzable, it was not inactivated by chymotrypsin.

In an effort to obtain more information on the nature of this substance, Holman and Cook (7) attempted to isolate and characterize the active agent from the cockroach *Leucophaea maderae*. Three substances were found in these extracts that stimulate the isolated hindgut. Two of the compounds were identified as L-glutamic acid and L-aspartic acid. When the hindgut was exposed to these amino acids, a single slow contraction resulted which was indistinguishable from a neurally evoked response. The third substance, however, caused a complex series of changes in the

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frequency and amplitude of rhythmic contractions and the character of this response was remarkably similar to that reported by Davey (8) and Brown (9) for extracts of the corpus cardiacum (CC). Further study of this third material by Holman and Cook (10) showed that its biological activity was destroyed by pronase, but not by chymotrypsin. Moreover, this peptide was found in extracts of hindguts, terminal ganglia, proctodeal nerves, and heads of L. maderae.

Once Brown and Starratt (11,12,13) isolated and identified proctolin (Arg-Tyr-Leu-Pro-Thr) from the cockroach P. americana, it was possible for neurobiologists to commence studies on the physiological and pharmacological actions of proctolin in many insects. It was quickly recognized that not only visceral muscles, but also skeletal muscles, were stimulated by proctolin. O'Shea and Adams (14) have recently provided an excellent review of the details of this peptide's activation of skeletal muscle, along with many other important highlights. The commercial availability of synthetic proctolin also provided the occasion to compare the high performance liquid chromatographic (HPLC) profile of proctolin with previously reported peptides of undetermined structure. Holman and Cook (15) found by HPLC fractionation and quantitative bioassay that the proctolin-like activity reported earlier in extracts of the foregut and hindgut of L. maderae was the result of that peptide. However, the hindgut stimulating neurohormone (HSN) reported in head extracts of the same cockroach could not be accounted for as proctolin. Thus another myotropic neuropeptide must have been responsible for the in vitro synthesis (16) and release (17) of HSN activity from the brain-CC complex of L. maderae. Indeed, the development of a reverse-phase HPLC system eventually provided a clear separation of five active fractions from these head extracts (18). This finding resulted in the purification and primary structure determination of 11 myotropic peptides (19-25), and of one that suppresses contractile activity (26).

Evidence of the involvement of proctolin in the regulatory mechanisms of insect nerve-muscle systems has continued to grow and the remainder of this section discusses these more recent developments and their significance. Proctolin-like immunoreactive (PLI) neurons have been detected in the blowfly, Calliphora erythrocephala (27); the Colorado potato beetle, Leptinotarsa decemlineata (28); and the fruitfly larvae, Drosophila melanogaster (29). In the blow fly, 80-90 neurons in the brain-suboesophageal complex were found to be PLI. The thoracic ganglia, by comparison, contained 100-130 PLI neurons, while the abdominal section had only 60. These neurons were of different types: interneurons, motor neurons, and neurosecretory cells. A small, stereotyped population of PLI neurons was found in the Drosophila larval central nervous system (CNS). In the periphery, proctolin-immunoreactive neuromuscular endings were identified on both visceral and skeletal muscle fibers. On the hindgut, the neuropeptide is associated with endings on intrinsic circular muscle fibers. In this study, the presence of proctolin was verified in the CNS, hindgut, and segmental body wall by tissue extraction followed by reverse-phase HPLC and quantitative bioassay. Evidence for a proctolin-like substance has also been found in the adult Colorado potato beetle

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(28). Numerous PLI neurons occur in all ventral ganglia and in the frontal ganglion. Two groups of neurosecretory cells in the suboesophageal ganglion contain a PLI substance. In these cells, this substance is co-localized with FMRF-amide-like material. Nerve terminals on the musculature of the fore- and hind-gut and of the vas deferens, and on certain segmental muscles show evidence of PLI. Upon fractionation of extracts of the nervous system of Leptinotarsa decemlineata by HPLC, most of the proctolin-like bioactive material co-migrated with authentic proctolin. Obviously such detailed mapping and/or localization of neurons by immunocytochemical techniques can be of great assistance to the neurobiologist who is analyzing nerve-muscle systems that involve proctolinergic neurons.

The presence of proctolin in the reproductive tract of a variety of insects and the action of this peptide on such tissues has been a current focus for many studies. The visceral muscles of the oviducts of Locusta migratoria, for example, are remarkably sensitive to proctolin (30). Amounts of the peptide as low as 2 fmol induce a tonic contraction that is dose-dependent up to 200 fmol. A proctolin-like immunoreactive substance was found in the axons of nerves leading to the oviduct as well as in a number of cell bodies in the VIIth abdominal ganglion. Moreover, reverse-phase HPLC of tissue extracts of oviducts, oviducal nerves, and the VIIth abdominal ganglia indicate that the proctolin-like bioactive substance co-elutes with authentic proctolin. The peptide was present in areas of the oviduct that receive extensive innervation while it was ten-fold less in areas that receive little or no innervation.

The oviducts of the cockroaches L. maderae (31) and P. americana (32) also contain proctolin. In both instances, quantitative estimates of proctolin-like bioactivity were made following the separation of extracts on reverse-phase HPLC. After depolarization of the tissue in high potassium saline, the proctolin-like substance in P. americana was released from oviducts in a calcium dependent fashion. Oviducts in L. maderae (31) showed some responsiveness to proctolin in a calcium-free medium and the peptide also appeared to facilitate the re-entry of calcium into muscle after depleted preparations were returned to normal levels of external calcium.

Two recent studies have shown that proctolin has a potent stimulating effect on the visceral muscles of the oviduct in Rhodnius prolixus (33,34) and in the stable fly Stomoxys calcitrans (35). In both of these insects, reverse-phase HPLC and quantitative bioassay were used to demonstrate the presence of a proctolin-like substance in extracts of the reproductive tissues. Immunohistochemical preparations revealed intense proctolin-like immunoreactivity in terminal arborisations of nerves on the spermathecae, common oviduct and bursa of Rhodnius. The muscles of the ovarian sheath in stably fly responded to proctolin, and 0.5 to 0.7 picomoles of proctolin-like bioactivity per ovary were detected in extracts. Oviduct extracts of the stable fly by comparison contained 1.2 to 1.7 picomoles of proctolin per organ.

The accessory gland tubules of the male cricket Gryllus bimaculatus (36) contract spontaneously, but they also receive

innervation from dorsal unpaired median neurons (DUMR7) that arise from the terminal ganglion. When these DUMR7 neurons were electrically stimulated, contractions were evoked in muscles of the gland tubules. Glutamate at 10^{-4}M, and proctolin, at low concentrations of 10^{-9}M produced sustained contractions of the accessory gland. This increase in basal tonus with each substance was also dose-dependent. By use of reverse-phase HPLC and bioassay, proctolin was detected in accessory gland extracts and in the saline medium surrounding glands subjected to high potassium saline.

Proctolin appears to potentiate synaptic transmission in the central nervous system of the cockroach P. americana (37). Bursts of spike activity in the ventral nerve cord of this insect were elicited by mechanical stimulation of the cercal organs. In the presence of micromolar proctolin, the peak frequency and the duration of bursts were slowly, but significantly, increased. Carbacol, by comparison, caused an immediate enhancement of spontaneous activity, but potentiation of bursts was not observed. Thus, it was concluded that proctolin might function as a neuromodulator in the terminal ganglion.

Although convincing evidence has been presented for a spike-mediated mechanism of excitation-contraction coupling in insect visceral muscle (38-41), nanomolar amounts of the neuropeptide proctolin can cause sizeable contractions in potassium depolarized hindgut preparations (42). This observation suggested that peptides may affect the contractile elements of muscle directly without the intervention of membrane polarization. Indeed, the first serious challenge to the exclusiveness of the electromechanical theory of coupling in vertebrate intestinal smooth muscle occurred when it was discovered that oxytocin, as well as acetylcholine and histamine, could produce contractions in potassium-depolarized smooth muscle (43). More recent research has shown that at least two distinct calcium transmembrane channels exist in the visceral muscles of the hindgut of the cockroach L. maderae (44) and the oviduct of the locust L. migratoria (45): 1) a voltage dependent channel which initiates phasic contractions in these muscles through the generation of action potentials; and 2) an agonist-operated channel which may be associated with little or no change in membrane potential. Although calcium appears to enter the muscle cells by two channels in both of these preparations, different intracellular messenger systems are linked to the membrane receptors for proctolin. In the hindgut of L. maderae (46,47) cyclic nucleotides are involved, while in the locust oviduct (48) inositol phospholipid hydrolysis seems to be the mediator of the physiological action of proctolin.

An interesting discovery about the adenylate cyclase enzyme located in the hindgut of L. maderae (49) was that it responded to increasing amounts of proctolin in a biphasic manner. Initially proctolin (10 pM) caused a 50% stimulation of adenylate cyclase activity and as the peptide concentration reached 50 pM, the enzyme activity reached a value 700% above basal activity. When a peptide concentration of 100 pM was attained the enzyme activity dropped back to 50% above basal activity but as the concentration of proctolin reached 1 nM the enzyme activity again peaked at a value of 700% above the basal level. These two peaks of adenylate cyclase

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activity parallel closely the concentration of proctolin required to evoke two types of muscular-contraction in the hindgut: 1) an increase in the frequency and amplitude of phasic contractions (50 pM); and 2) large increases in tonic contractions (1 nM).

The 14 years that have elapsed since the discovery of proctolin have provided many invertebrate neurobiologists the opportunity to assess the physiological actions of this peptide; in many respects, these studies have led the way in increasing our understanding of how peptides function in a living system. The accomplishments of this period can best be summarized by the following brief statements: 1) proctolin in subnanomolar amounts can activate both visceral and skeletal muscles for extended periods of time; 2) proctolin can often coexist with other chemical effectors in a single neuron, and as a cotransmitter may impart its own unique character to muscle action; 3) many of the reported physiological actions of proctolin violate the traditional definition of a transmitter substance; 4) proctolin can activate muscle cells through voltage dependent as well as receptor operated channels; and 5) the mode of action of proctolin is not universal but diverse and multiphasic with each insect nerve-muscle responding in its own unique way.

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Leucokinins

The leucokinins (LK's) are a new class of insect myotropic neuropeptides isolated from head extracts of the cockroach L. maderae. These octapeptides all contain a similar core sequence of 5 amino acids that extend from position 4 through 8. This sequence Phe-X-Ser-Trp-Gly-NH_2 seems to be required for hindgut stimulation. The initial response of the hindgut to the LK's was characterized by an increase in the frequency and/or amplitude of phasic contractions (50,51). At higher peptide concentrations, a tonic component was generally present. All of the LK's showed a response at 3 x 10^{-10}M that was 5-10% above the mean level of spontaneous activity. The maximum response for each of the peptides was recorded at a concentration 2.1 X 10^{-7}M. Thus, the intrinsic activities for the LK's are nearly equal because the dose-response curves have about the same asymptotic limits. A comparison of the dose concentrations that gave a half maximal response (ED_{50}) for the 8 peptides is shown in Table I.

In addition to these responses, each of the LK's caused a protracted excitation of contractile events if hindgut preparations were allowed to remain in saline solutions containing the peptide (Figure 1). All eight of the LK's evoked contractions from hindguts after membrane depolarizations with 158 mM potassium. This offers clear evidence for the presence of receptor-operated channels for the LK's in the hindgut. Moreover, the fact that hindguts exposed to a calcium free medium failed to respond to the peptides suggests that these receptor-operated channels are calcium dependent.

Although the hindgut showed high sensitivity to the LK's, other types of visceral muscle were less responsive. The foregut and oviduct, for example, were 100-1000 fold less sensitive than the hindgut. A concentration of more than 10^{-8}M of each of the peptides was required to elicit a detectable response from these

Table I. Chemical properties of the leucokinins from L. maderae and their pharmacological action on the hindgut

Leucokinins	pmol/head*	Structure*	Threshold of synthetic product*	ED_{50}
LK I	0.48	DPAFNSWG-NH_2	2.0×10^{-10}M	3.6×10^{-9}M†
LK II	0.35	DPGFSSWG-NH_2	1.6×10^{-10}M	1.5×10^{-9}M†
LK III	0.22	DQGFNSWG-NH_2	7.2×10^{-11}M	4.3×10^{-9}M†
LK IV	0.23	DASFHSWG-NH_2	1.4×10^{-10}M	2.9×10^{-9}M†
LK V	0.17	GSGFSSWG-NH_2	5.2×10^{-11}M	8.6×10^{-10}M†
LK VI	0.16	pQSSFHSWG-NH_2	5.6×10^{-11}M	3.3×10^{-9}M†
LK VII	0.09	DPAFSSWG-NH_2	1.2×10^{-10}M	6.2×10^{-9}M†
LK VIII	0.06	GADFYSWG-NH_2	2.9×10^{-11}M	7.7×10^{-10}M†

* References 19, 20, 22, and 23.
† References 50 and 51.

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organs. The heart by comparison either gave inconsistent responses or failed to respond to the peptides.

An unexpected biological activity for the LK's was recently discovered in studies of isolated malpighian tubules of the yellow fever mosquito Aedes aegypti (52). Each of the LK's at 100 pmol caused a depolarization of the transepithelial voltage of these tubules. Leucokinin VIII, for example, inhibited transepithelial fluid secretion at low concentrations (10^{-11}M) and stimulated fluid secretion at high concentrations (3.5×10^{-9}M). Together, the depolarizing effects on voltage and the stimulation of fluid secretion suggest that LK's increase the chloride permeability of the tubule wall. Structure-function studies suggest that the active region of the octapeptide is largely restricted to the C-terminal pentapeptide.

Leucopyrokinin

Leucopyrokinin (pQTSFTPRL-NH_2) was the most abundant of the myotropic neuropeptides found in head extracts of L. maderae. Each head equivalent contained 1.36 pmol of the peptide, a value much less than that obtained for either M_1 or M_2. When the hindgut was exposed to leucopyrokinin (LPK), a pronounced elevation in the amplitude and frequency of phasic contractions was often evident, and only a small change in tonus was detected even at higher concentrations of the peptide. This was in marked contrast to the more evident changes in tonus evoked by proctolin and the leucokinins in a comparable concentration range. LPK caused an initial burst of potentiation in neurally evoked contractions of the hindgut but this was followed by an intermittent pattern of potentiations that was quite unlike the uniform type observed with proctolin (Wagner and Cook, unpublished information).

Although the foregut and oviduct of L. maderae were about 100 times less sensitive to LPK than the hindgut, the character of the response was similar. When these tissues were exposed to the peptide, the most evident response was an increase in the amplitude and frequency of contractions, with little indication of any change

in tonus. The response of the heart to LPK was fairly consistent. Application of the peptide in concentrations between 10^{-9}M and 5 x 10^{-8}M caused an increase in the amplitude and frequency of myocardial contractions. However, when the heart was exposed to 10^{-7}M LPK a marked suppression in amplitude was often observed.

Leucomyosuppressin

Leucomyosuppressin (pQDVDHVFLRF-NH_2) was the first structurally identified neuropeptide that inhibits muscular contractions in insects (26). The response of the isolated hindgut of *L. maderae* to leucomyosuppressin (LMS) was dose dependent and reversible. Both the frequency and amplitude of spontaneous contractions were reduced near the threshold concentration for the peptide (9 x 10^{-11}M). Neurally evoked contractions of the hindgut were also suppressed by LMS in a dose dependent manner (Cook and Wagner, unpublished information). However, repeated exposures of the hindgut to the peptide often caused a desensitization of the preparation. As is the case with tyramine, LMS produced an inhibition of the hindgut to glutamate, proctolin, and LPK. Thus, LMS is not a specific antagonist for peptides.

The foregut of *L. maderae* was as sensitive as the hindgut to LMS, with a threshold near 5 x 10^{-11}M. The oviduct and heart by comparison were much less sensitive and consistent in their responses to the peptide. Most oviduct preparations showed a detectable inhibition to LMS at 2.4 x 10^{-8}M, but in some instances a concentration of 10^{-7}M produced a stimulation. Although LMS on occasion caused an inhibition of spontaneous contractions of the heart at 10^{-9}M, most preparations that did respond required a concentration in excess of 10^{-8}M. The response of the heart to LMS was by no means consistent; many preparations failed to respond to the peptide at all, while others responded initially but failed after a second exposure.

Recent experiments have shown that LMS at concentrations higher than 100 nM can reduce the amplitude of neurally evoked EPSP in the skeletal muscles of the mealworm *Tenebrio moliter* (53). However, the peptide has no effect on the glutamate-induced depolarization of these muscles. Micromolar amounts of LMS have also been shown to inhibit both the frequency and amplitude of spontaneous heart contractions in the locust *Schistocerca gregaria* in a dose dependent (54) manner. Immunocytochemical studies (55) have demonstrated that the major source of LMS in *L. maderae* appears to be localized in the pars intercerebralis and in the region between the protocerebral and deutocerebral lobes of the brain. A pair of intensely reacting cells were found in each of the thoracic ganglia. The abdominal ganglia, by comparison, contained several cells that were only moderately active.

Concluding Remarks

The fact that the structural determination of the majority of insect neuropeptides, thus far described, has relied on some kind of muscle assay has raised several concerns. First, it has been pointed out that muscle activation may not represent the primary functional role

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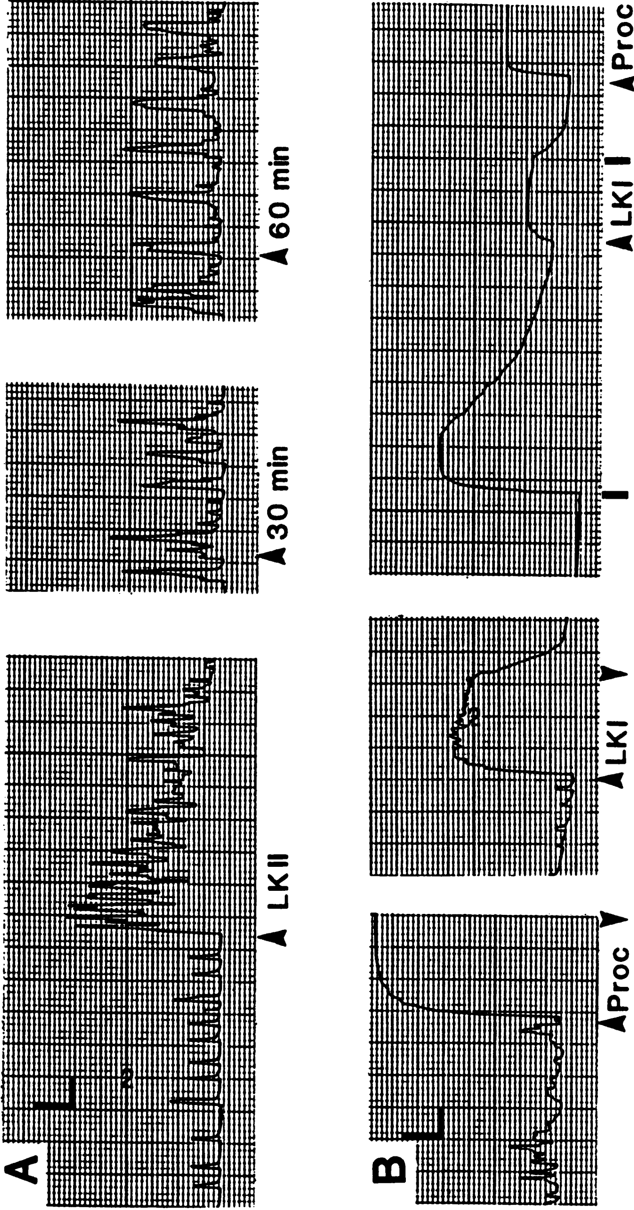

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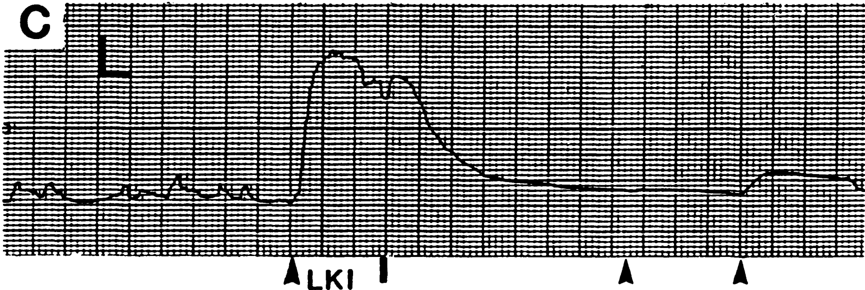

Figure 1. Protracted response of the hindgut to leucokinin II (A) and the effects of potassium depolarization (B) and a calcium free medium (C) on the action of leucokinin I and proctolin. (A) Response of the hindgut to 2.7 x 10^{-9}M LK II (first arrow). (B) Response of the hindgut to 2 x 10^{-7}M proctolin (first arrow) and 2 x 10^{-7}M LK I (second arrow). Downward arrows indicate a rinse in fresh saline solution. Addition of 158 mM potassium with 2 mM calcium at first and second bars. Response of the hindgut to 2 x 10^{-7}M LK I (third arrow) and 2 x 10^{-7}M proctolin (fourth arrow) after potassium depolarization. (C) Response of the hindgut to 2 x 10^{-7}M LK I (first arrow) followed by the addition of a calcium free medium (bar). Failure of the hindgut to respond to 2 x 10^{-7}M LK I (second arrow). Calcium contracture after the addition of 2 mM calcium to the preparation (third arrow). Displacement calibration 1 mm. Time mark 1 min. (Reproduced from reference 50.)

for these peptides. This may well prove to be true. However, the expressed concern seems to be rooted in attempts to establish insect peptide nomenclature on the basis of primary biological function (56) but this concept appears inadequate for three reasons: 1) no unambiguous guidelines have been set to determine the primary biological function for any peptide; 2) it has already been established that most peptides are multi-functional entities; and 3) when the structure of peptides are first announced knowledge on the range of their biological action is generally rather limited. A more expedient format for insect peptide nomenclature has recently been suggested by Robb et al. (57). In this instance, the peptide was named on the basis of the insect source and a few key notes of its chemistry.

The question "why are there so many myotropins?" has echoed another concern. If we accept the fact that literally hundreds of muscles constitute the basis of movement in insects and the premise that this movement is the principle avenue for expressed behavior, can we then be surprised that many different chemical messengers (i.e., neuropeptides) are involved in regulating the process? Moreover, can we rightly assume that this action has no physioloigcal significance simply because many different agents seem to induce an excititory or inhibitory response? Careful study has already shown that subtle differences do exist between these substances and their respective responses even within a single muscle (58). What we are looking at is a continuum of intercellular communication and only patient and meticulous research will unravel its complexity. Certainly the multifunctional nature of neuropeptides provides a fascinating lead in our efforts to understand the chemical mechanisms that regulate insect motor behavior sequences. However, to accomplish this end, we must have a better comprehension of the range of messages that these agents may well impart within living systems. This without doubt will require much more research on the comparative physiological and pharmacological action of the peptides.

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Chapter 7

Metabolic Neuropeptides

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Peptides in the adipokinetic/red pigment-concentrating hormone family have broader consequences on fat body metabolism than were described originally. Classically, peptides of this family mobilize fat body stores to elevate levels of circulating metabolites. In the fat body of the cockroach, *Blaberus discoidalis*, a hypertrehalosemic hormone (Bld-HrTH) stimulates not only trehalose synthesis but also the synthesis of cytochrome hemes during fat body mitochondriogenesis and the rate of synthesis of juvenile hormone-mediated proteins. Bld-HrTH appears to cause the stimulation of fat body heme synthesis through gene expression. Recent studies have elucidated the structure and organization of the adipokinetic hormone genes and their resulting preprohormones. Finally, analog studies with Bld-HrTH provide evidence about its structure-activity relationships at fat body receptors.

The present discussion concerning neuropeptides that regulate insect metabolism restricts itself to those neurohormones that affect fat body energy balance and the metabolism of lipids, proteins and carbohydrates. The first indication that neurohormones could influence insect fat body metabolism was the elevation of hemolymph trehalose by corpora cardiaca (CC) extracts in *Periplaneta americana* (1). This hypertrehalosemic response resulted from the degradation of fat body glycogen (2) and was later shown to occur in *Blaberus discoidalis* cockroaches (3). In the locusts, *Locusta migratoria* and *Schistocerca gregaria*, CC extracts elevated hemolymph lipids (4,5). As in the cockroaches, locust CC extracts also caused hyperglycemia in 6-day-old, adult male *L. migratoria* at the expense of fat body glycogen (6). Injection of *L. migratoria* CC into *P. americana* did not elevate hemolymph lipids but stimulated hyperglycemia (7); the opposite was true when *P. americana* CC were administered to *L. migratoria*. Therefore, the nature of the response was apparently a function of the target tissue and not the hormone *per se*.

Since these initial reports, numerous peptides have been described for what is now recognized as the insect adipokinetic/red pigment-concentrating hormone (AKH/RPCH) family. The first representative of this family isolated and characterized from insects was the *L. migratoria* adipokinetic hormone (Lom-AKH-I) (8). Lom-AKH-I was found to be related to the red pigment-concentrating hormone (RPCH) that had been described previously from crustaceans (9). Subsequently, two

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related octapeptides were reported from *P. americana* that are both cardioacceleratory and elevate hemolymph carbohydrates (10-12), and research in our laboratory identified a fourth peptide of the AKH/RPCH family that stimulates trehalose synthesis in the tropical cockroach *Blaberus discoidalis* (13). Since these initial reports, in addition to RPCH from crustaceans, there are presently thirteen defined peptides recognized in the insect AKH/RPCH family distributed among twenty species from the orders Orthoptera, Lepidoptera, Coleoptera and Diptera (14, Table I). The peptides are all octa-, nona- or decapeptides blocked at the N-terminus by pGlu and at the C-terminus by amide and containing $pGlu^1$, Phe^4 and Trp^8 as conserved residues. From these conserved structural properties and their wide species distribution, it can be concluded that the adipokinetic/hyperglycemic hormones (AKH/HGH) probably exert critical regulatory actions within most, if not all, insects.

Table I. The adipokinetic/red pigment-concentrating hormone family of peptides

†Abbreviation	Spp. of Origin	Sequence (1 2 3 4 5 6 7 8 9 10)
Bld-HrTH	*Blaberus discoidalis*	pGlu-Val-Asn-Phe-Ser-Pro-Gly-Trp-Gly-ThrNH_2
Lom-AKH-I	*Locusta migratoria*	pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-ThrNH_2
Lom-AKH-II	*Locusta migratoria*	pGlu-Leu-Asn-Phe-Ser-Ala-Gly-TrpNH_2
Scg-AKH-II	*Schistocerca gregaria*	pGlu-Leu-Asn-Phe-Ser-Thr-Gly-TrpNH_2
Pea-CAH-I	*Periplaneta americana*	pGlu-Val-Asn-Phe-Ser-Pro-Asn-TrpNH_2
Pea-CAH-II	*Periplaneta americana*	pGlu-Leu-Thr-Phe-Thr-Pro-Asn-TrpNH_2
Mas-AKH	*Manduca sexta*	pGlu-Leu-Thr-Phe-Thr-Ser-Ser-Trp-GlyNH_2
Hez-AKH-II	*Heliothis zea*	pGlu-Leu-Thr-Phe-Ser-Ser-Gly-Trp-Gly-AsnNH_2
Cam-HrTH-II	*Carausius morosus*	pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly-ThrNH_2
Taa-AKH	*Tabanus atratus*	pGlu-Leu-Thr-Phe-Thr-Pro-Gly-TrpNH_2
Taa-HoTH	*Tabanus atratus*	pGlu-Leu-Thr-Phe-Thr-Pro-Gly-Trp-Gly-TyrNH_2
Grb-AKH	*Gryllus bimaculatus*	pGlu-Val-Asn-Phe-Ser-Thr-Gly-TrpNH_2
Rom-AKH-I	*Romalea microptera*	pGlu-Val-Asn-Phe-Thr-Pro-Asn-Trp-Gly-ThrNH_2
RPCH	*Pandalus borealis*	pGlu-Leu-Asn-Phe-Ser-Pro-Gly-TrpNH_2

† Neurohormone nomenclature used throughout is according to Raina and Gade (15).

The most widely recognized physiological action of the insect AKH/HGHs is to mobilize lipids or carbohydrates from fat body reserves for use as energy substrates by peripheral tissues. In *Leptinotarsa decemlineata*, proline is the main substrate for flight muscle metabolism (16), and Lom-AKH-I promotes fat body proline synthesis (17). Pea-CAH-I and -II were isolated based upon their stimulation of the heartbeat rate in *P. americana*, but they also cause hyperglycemia (11).

In addition to the AKH/RPCH family, neuroparsins A and B constitute a recently described class of neuropeptides that stimulate fat body metabolism. The neuroparsins are 14 kDa proteins produced in PAF-positive, medial neurosecretory cells and transported to the storage lobes of *L. migratoria* CC for storage-secretion (18). Neuroparsins have several putative physiological actions that include inhibition of the corpora allata-juvenile hormone system (19), antidiuresis (20) and both hyperlipemia and hypertrehalosemia (21). However, unlike CC extracts or the hyperglycemic peptides, the neuroparsin-related hypertrehalosemic action is expressed without decreases in fat body glycogen. Furthermore, neuroparsin elevates trehalose within 15 min and its effect disappears after 1 hr; whereas, CC glandular lobe extracts continue to elevate hemolymph trehalose beyond 1 hr (6). For the purposes of this review, the significance of neuroparsins as regulators for insect fat body metabolism remains unclear since they were described only recently, they have a broad spectrum

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of diverse physiological actions and the mechanisms by which they exert hypertrehalosemia remain uncertain.

The remainder of this review will concern principally the insect AKH/HGH neuropeptides and their actions on fat body intermediary metabolism. Although we will review the AKH/RPCHs in general, we will focus, in particular, on the Bld-HrTH of *B. discoidalis* cockroaches, and what my coworkers and I have found recently concerning multiple physiological actions by this peptide.

The Role of AKH/HGH in Fat Body Carbohydrate Metabolism

Evidence for the presence of hyperglycemic factors in the CC were reported first in *P. americana* (1) and supported subsequently in *B. discoidalis* (3). The HGHs act on the fat body, the synthetic source for trehalose in insects (22), to elevate phosphorylase activity and the conversion of glycogen stores to the precursors for trehalose synthesis (2,3). Initially, it was believed that HGHs activated phosphorylase via the synthesis of adenosine 3'5'-cyclic monophosphate (cAMP) in the same manner that glucagon or epinephrine activate liver phosphorylase in vertebrate animals (23). Injections of intact adult *P. americana* with synthetic Pea-CAH-I and -II result in a 50% net increase in fat body cAMP over water-injected controls accompanied by a more than 3-fold increase in fat body phosphorylase activity (24). However, the CAHs fail to elevate cAMP levels of fat bodies from *P. americana in vitro* even though both phosphorylase activity and trehalose synthesis increase (25). In the latter case, Ca^{2+} is essential for the action of the CAHs, and its omission from the incubation medium inhibits the hypertrehalosemic response.

Bld-HrTH administration to *B. discoidalis in vivo* or to isolated fat body fails to stimulate either fat body cAMP levels or adenylate cyclase activity and supports the previous findings (25). Nevertheless, for *B. discoidalis*, fat body phosphorylase activity is elevated and trehalose levels increase both *in vivo* and *in vitro*, and calcium is essential *in vitro* in addition to Bld-HrTH. No stimulation of trehalose synthesis is noted with agents that elevate adenylate cyclase, such as forskolin, or by inhibitors of phosphodiesterase such as theophylline or isobutylmethylxanthine (IBMX). Additions of cAMP, dibutyryl cAMP or 8-bromo-cAMP are not stimulatory to trehalose synthesis either *in vivo* or *in vitro*. This same result was observed for *P. americana* in that neither cAMP nor dbcAMP stimulated trehalose production by fat body *in vitro*, and xanthine inhibitors of phosphodiesterase that should cause accumulation of intracellular cAMP were inhibitory, except for isobutylmethylxanthine (IBMX) which was stimulatory for unknown reasons (26). We have not observed a stimulatory effect by IBMX with *B. discoidalis* fat body *in vitro*.

From our studies and those of others (25,26), we must conclude that cAMP is not a second messenger for the hypertrehalosemic hormones to stimulate glycogen mobilization and trehalose synthesis in the cockroach fat body. Possibly, injection of Pea-CAH-I or -II *in vivo* (24) activated another cAMP-dependent regulatory system for trehalose synthesis within *P. americana*. One such system might be the octopamine-dependent, excitatory-induced hypertrehalosemic (EXIT)-response (27). We find that octopamine elevates cAMP in *B. discoidalis* fat body *in vitro*; however, the absence of elevated fat body cAMP after Bld-HrTH treatment *in vivo* indicates that Bld-HrTH is not acting through this secondary mediator.

The exact mechanism by which the AKH/RPCHs activate fat body phosphorylase and trehalose synthesis remains uncertain. In *P. americana*, CC extracts with hypertrehalosemic activity neither stimulate trehalose-6-P synthase for trehalose synthesis nor increase fat body trehalose permeability (28). In all insect species tested, phosphorylase activation and glycogen degradation occur in response to CC extracts or AKH/HGH peptides (2,3,29-32). Therefore, all the data suggest

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that hemolymph trehalose elevation is a response to trehalose synthesis following glycogenolysis.

Ca^{2+} is required for phosphorylase activation in fat bodies of both *P. americana* (25) and *B. discoidalis* (personal observation). Addition of Ca^{2+} elevates fat body phosphorylase kinase activity in *P. americana* (33), and calmodulin inhibitors suppress CC-stimulated trehalose production by the fat body *in vitro*. However, direct addition of calmodulin to fat body phosphorylase kinase also suppresses the kinase activity. It is proposed that Ca^{2+} interacts directly with a calmodulin-like subunit of phosphorylase kinase to activate the enzyme, and the presence of exogenous calmodulin competes with the enzymic subunit for available Ca^{2+} (33). These results suggest that the HGHs may influence adipocyte Ca^{2+} levels related to phosphorylase activation to promote glycogenolysis for trehalose synthesis. Possibly, HGH-mediated fat body Ca^{2+} levels may interact with polyphosphoinositides, diacyl glycerol and protein kinase C as second messengers for endocrine message transduction and phosphorylase activation.

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The Role of AKH in Fat Body Lipid Metabolism

The existence of adipokinetic neuropeptides was identified in locusts based upon the elevation of hemolymph lipids by CC extracts (4,5). Subsequent isolation and structural definition of Lom-AKH-I (8) provided the basis for defined studies on fat body lipid mobilization using synthetic peptide.

Early investigations indicated that stored lipids (triacylglycerides) are mobilized from the fat body and secreted as diacylglycerides in response to injections of CC extracts (5). The exact nature of the hormone-dependent lipolytic activity within the fat body has remained elusive since no definitive enzyme is identified as AKH-responsive.

Unlike the hyperglycemic response, where cAMP does not appear to act as a fat body second messenger, cAMP mediates the AKH-dependent degradation of fat body lipid stores (34-38). Fat body protein kinase activity increases in locusts treated with either cAMP, RPCH or extracts of CC glandular lobes (37). RPCH stimulates a 73% increase in fat body lipase activity *in vivo* and increases by 2.3-fold the rate of release of diacylglycerol by fat body *in vitro* (37). Likewise, fat body lipase activity increases by 50% *in vitro* 10 min after additions of cAMP and cGMP. These results suggest that Lom-AKH-I and its related bioanalogs stimulate fat body lipolytic activity through mediation by the cAMP second messenger system.

Diacylglycerols produced in response to AKH are hydrophobic and are transported from the fat body to the flight muscles by carrier proteins in the hemolymph. A hemolymph lipoprotein (A_{yellow}) serves to transport diacylglycerol in resting locusts (39). However, in flying locusts or locusts treated with CC extract, the A_{yellow} protein combines with a second, nonlipid-bearing protein (C_L) to form a higher molecular weight lipoprotein complex (A^+) which transports the newly synthesized diacylglycerol (39-42). A third lipoprotein carries lipid both at rest and in response to CC extracts (43). Immature adult locusts (< 3 days old) show little lipid mobilization in response to injections of CC extracts (36,39) because little C_L protein is present and A^+ does not form (39,41). In addition, A_{yellow} of immature locusts fails to load with diacylglycerol (39).

AKH stimulates carrier protein formation and loading. A^+ forms within minutes after injection of CC extracts in *L. migratoria* (39). AKH secretion by flying locusts is prevented by injections of concentrated trehalose or sucrose (44), and diacylglycerol and A^+ lipoprotein formation does not occur in locusts loaded with these disaccharides (43). However, CC extracts still stimulate the production of both diacylglycerol and A^+ in disaccharide-loaded locusts. Since there is no evident change in protein levels during the experiment, A^+ must form from existing proteins

in response to the presence of AKH. Furthermore, both Lom-AKH-I and octopamine stimulate fat body lipid mobilization (45). Octopamine causes lipid release at any time, but AKH mobilizes lipids only in the presence of A_{yellow} and C_L when the A^+ lipoprotein can be formed. Lom-AKH-I appears to promote the loading of the lipoproteins for formation of the A^+ carrier protein (46).

Not only does Lom-AKH-I promote mobilization and release of fat body lipid stores, but it also increases the efficiency of lipid degradation by the flight muscles. Injection of a CC glandular lobe extract drastically reduces carbohydrate use during flight of *S. gregaria* (47). Presumably, the CC extract contains Lom-AKH-I which enhances diglyceride oxidation in preference to carbohydrate. This is supported further by a competition study with *in vitro* flight muscles that shows trehalose consumption is depressed by 75% when in the presence of a combination of lipid from A^+ lipoprotein and CC glandular lobe extracts (48). Extracts of CC glandular lobes stimulate the oxidation of palmitate and palmitoyl carnitine in preparations of flight muscle mitochondria (48), and the addition of 2-bromostearic acid, an inhibitor of mitochondrial carnityl acyl transferase, relieves the inhibition of carbohydrate utilization caused by lipid and CC extract (48). These results suggest that Lom-AKH-I can suppress flight muscle carbohydrate metabolism in favor of lipid as the principal energy substrate.

These results demonstrate that Lom-AKH-I modulates lipid metabolism at three levels in locusts: synthesis, transport and utilization. First, AKH activates lipolytic activity in the fat body to convert stored triacylglycerides to mobile diacylglycerides. Second, AKH promotes the production of the A^+ lipoprotein from the A lipoprotein and C_L protein to act as a hemolymph carrier for the mobilized diacylglycerides. Finally, AKH stimulates acyl carnitine transferase in flight muscle mitochondria to promote the use of lipid as the oxidative substrate of preference for energy production during migratory flight.

Considerations on the Actions of AKH/HGH Peptides

It is unfortunate that so many of the early physiological investigations were limited to the use of crude extracts of CC glandular lobes which have an uncertain composition. For example, an additional AKH exists in the CC glandular lobes of both *S. gregaria* and *L. migratoria* (49). Lom-AKH-II reportedly promotes hyperglycemia in locusts (50,51). Consequently, it is difficult to know from the early experiments exactly what effects on the fat body can be attributed to which AKH. This can be a source of confusion, as is evident from the previously described example that synthetic Pea-CAH-I and -II fail to stimulate cAMP elevation in preparations of *P. americana* fat body *in vitro*, whereas CC extracts elevate cAMP levels (25). Obviously, the elevation of fat body cAMP levels in cockroaches must result from the presence of factors in the CC other than the CAHs. A similar situation probably exists for locusts, as well. These alternative agents could be unidentified peptides or, even, neurotransmitter chemicals, such as octopamine (52).

The developmental stage of the insect may also affect its physiological response to the AKH/HGHs. For example, young (< 3 days) adult locusts show little lipid mobilzation in response to CC extracts (36,39). In *Manduca sexta*, CC extracts activate glycogen phosphorylase in larvae but elevate hemolymph lipids in adults. It was not possible to separate phosphorylase-stimulating activity from the adipokinetic activity during hormone isolation (54), and Mas-AKH isolated from the CC of adults stimulates fat body phosphorylase in larvae (55). It is now recognized that Mas-AKH regulates the mobilization of both larval trehalose and adult lipids (R. Ziegler, personal communication).

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Biosynthetic Effects of the *Blaberus* Hypertrehalosemic Hormone

Hyperglycemic peptides may have several physiological effects. A major action by all these peptides is to elevate hemolymph carbohydrate (trehalose) levels. In addition, the Pea-CAHs were isolated based upon their actions on muscle contraction in *P. americana* (10-12). Bld-HrTH was isolated based upon its hypertrehalosemic action (56), and cardioacceleratory effects have been confirmed for synthetic Bld-HrTH.

We have discovered that Bld-HrTH has two additional physiological actions in *B. discoidalis* that have not been reported previously for members of the AKH/RPCH family. Bld-HrTH stimulates both the synthesis of cytochrome hemes during fat body mitochondriogenesis and the rate of synthesis of export proteins by the female fat body.

Mitochondrial integrity influences the energy-generating capacity within a cell. *B. discoidalis* has proven a valuable animal for investigating the regulation of processes related to mitochondrial formation in higher animals (57). During the first week of adult life, the fat body of male *B. discoidalis* undergoes a maturational development that involves reorganization of the cytoplasmic organelles and metabolite stores as the tissue converts from a storage function to a biosynthetic function (57,58). An increasing biosynthetic capacity is indicated for the fat body based upon 2- to 3-fold increases in both total RNA content of the tissue (58) and respiratory enzyme activities of the mitochondria (59). Fat body synthesis of export proteins increases coincident with the increases in total RNA and respiratory activity (Figure 1).

The maturation of fat body mitochondria is affected by the neuroendocrine balance in adult male *B. discoidalis*. Removal of the CC arrests the maturation of the respiratory enzymes at their partly developed, 5-day level (58, Figure 1). Injections of CC extracts on days 0 to 5 result in precocious, 10-day levels of respiratory activity by 5 days of age. Administration of the uncoupling agent 2,4-dinitrophenol indicates that electron transport, rather than phosphorylation, is the rate-limiting step for respiration in fat body mitochondria (59). A situation that differs from vertebrate mitochondria where phosphorylation is limiting. This indicates that the levels of cytochrome enzymes available for electron transport in fat body mitochondria determine the respiratory and ATP synthesis capacities of the tissue and influence its biosynthetic potential.

Cytochrome synthesis was examined in the fat body of adult male *B. discoidalis* by measuring the synthesis of cytochrome hemes. Heme is synthesized from the condensation of succinate and glycine by aminolevulinic acid synthase to produce aminolevulinic acid (ALA), a specific heme precursor. A developmental pattern exists for the incorporation of [^{14}C]ALA into cytohemes of fat body mitochondria with a peak of synthesis between days 4 and 6 of adult age (60). CC ablation eliminates this peak of synthesis for cytohemes *a* and *b*; CC extract injections return the synthesis of cytohemes *a+b* to normal levels in CC-ablated cockroaches but have no effects on the synthesis of the *c*-type hemes for cytochromes *c* and c_1. The synthesis of cytohemes *a+b* in response to CC extracts requires a latent period of 24-48 hr to obtain a maximum response and is dose-dependent over a range of 0.01 to 0.08 CC pair (61). The active factor in CC extracts is sensitive to chymotrypsin but not to trypsin. This "cytochromogenic hormone" (CGH) is secreted on days 2-3 of adult age in males (62). Since maximal synthesis of cytohemes *a+b* occurs on day 4, CGH secretion on days 2-3 agrees with the earlier observation that CGH requires about 48 hr to produce its response (61).

Quite by coincidence, the isolation of both the cytochromogenic and hypertrehalosemic factors was undertaken at the same time in our laboratory using independent bioassays (56,61). Both cytochromogenic and hypertrehalosemic factors proved soluble in acidic acetone, and both activities migrated nearly identically on reversed phase HPLC. Final isolation and characterization of the hypertrehalosemic

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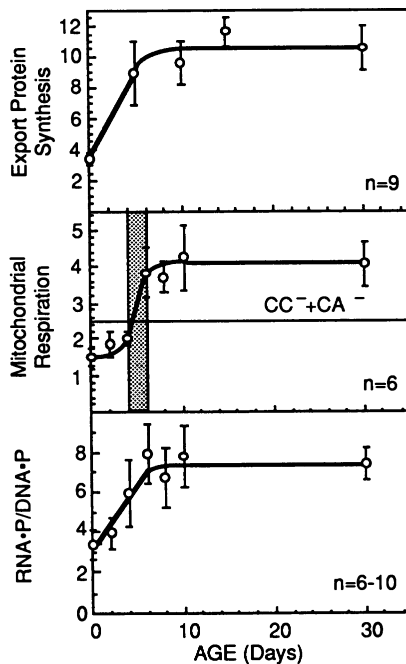

Figure 1. Age effects on the rate of synthesis of export proteins (upper), the rate of oxygen consumption by isolated mitochondria (middle) and the total RNA content (lower) for the fat body of adult male *B. discoidalis*.
Protein synthesis = dpm ($x10^3$) per mg soluble protein per hr; oxygen consumption = nmoles ($x10^3$) of O_2 consumed per mg mitochondrial protein per hr; RNA•P/DNA•P = µg RNA•P per µg DNA•P. Values are mean±SEM. In the figure for mitochondrial respiration, the horizontal line labeled $CC^{-}+CA^{-}$ denotes the respiratory level attained by animals cardiacectomized-allatectomized at day 0; the darkened area at days 4-6 denotes the period of maximal heme synthesis.

factor (Bld-HrTH) was accomplished and the synthetic peptide prepared (13). We have been unable to resolve Bld-HrTH from CGH chromatographically. Synthetic Bld-HrTH is active in the heme synthesis bioassay over a dose range that is an order of magnitude higher than its stimulation of trehalose synthesis *in vivo* (Figure 2). Therefore, we conclude that Bld-HrTH and the putative CGH are either identical or have a closely similar structure. Until we can demonstrate compelling evidence for a separate heme-synthesis stimulating peptide, we are considering the stimulation to cytoheme *a+b* synthesis to be an additional physiological action by Bld-HrTH. There are frequently two AKH/HGH-like peptides within a species, and regulation of heme synthesis may be a function of a second HGH-like peptide in *B. discoidalis*, although there is no evidence to support this speculation.

Other neurohormones of the AKH/RPCH family also stimulate the synthesis of cytohemes *a+b* in the fat body of *B. discoidalis*. At equivalent doses, Pea-CAH-I has as much activity as Bld-HrTH, but Pea-CAH-II and Lom-AKH-I are inactive (Figure 3). Therefore, the ability to stimulate developmentally-related heme synthesis in the fat body appears to be a property of many of the AKH/HGH peptides. The inability of Pea-CAH-II and Lom-AKH-I to stimulate heme synthesis in *B. discoidalis* does not indicate that these peptides do not have this effect in their host insect. Rather, these two peptides may differ so much in structure from Bld-HrTH that they fail to interact adequately with the fat body receptors of *B. discoidalis* to promote a response, but could be effective at higher doses.

The subcellular action by Bld-HrTH for induction of heme synthesis is uncertain, although present evidence suggests involvement of gene expression. Bld-HrTH-mediated heme synthesis is suppressed by alpha-amanitin, an inhibitor of mRNA synthesis (Figure 4); whereas, Bld-HrTH-mediated trehalose synthesis is unaffected by alpha-amanitin. Amanitin also inhibits the natural increase in heme synthesis that occurs at day 4. These results suggest that Bld-HrTH may promote gene expression related to heme synthesis. Gene expression as a response to Bld-HrTH agrees with the extended latent period necessary to attain maximal heme synthesis after Bld-HrTH treatment. In recent molecular cloning experiments, we identified a fat body mRNA that has a low constitutive level but increases by 50-fold in fat body of animals treated with Bld-HrTH. We have isolated the cDNA for this message and are in the process of determining its sequence. Comparisons with gene sequence databanks indicate the Bld-HrTH-responsive gene is probably a member of the cytochrome P-450 superfamily which agrees with the stimulation of heme synthesis by Bld-HrTH.

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Protein Synthesis

Lom-AKH-I suppresses fat body protein synthesis in locusts. Administration of glandular lobe extracts or synthetic Lom-AKH-I inhibits protein synthesis by 60% in immature and adult locusts (63). Lom-AKH-I does not mobilize lipids in larval locusts (36), and it is speculated that the major action of Lom-AKH-I in immatures is to regulate protein synthesis. The inhibitory effect of Lom-AKH-I on protein synthesis is observed for adult locusts *in vivo* at doses that are lower than those needed for lipid mobilization (64), and both arylphorin and vitellogenin synthesis are suppressed. The inhibitory action of Lom-AKH-I is also confirmed for *in vitro* protein synthesis by the muscle and gut, as well as the fat body. In preparations of dispersed fat body cells, synthetic Lom-AKH-I stimulated cAMP synthesis and lipid release but inhibited protein synthesis by 70% (38).

Since Bld-HrTH affects the synthesis of cytohemes *a* and *b* in the fat body, presumably as precursors for mitochondrial cytochromes aa_3 and *b*, we speculated that Bld-HrTH would influence the basal metabolic capacity of the fat body through effects on the rate of ATP synthesis. Hence, Bld-HrTH regulation of energy

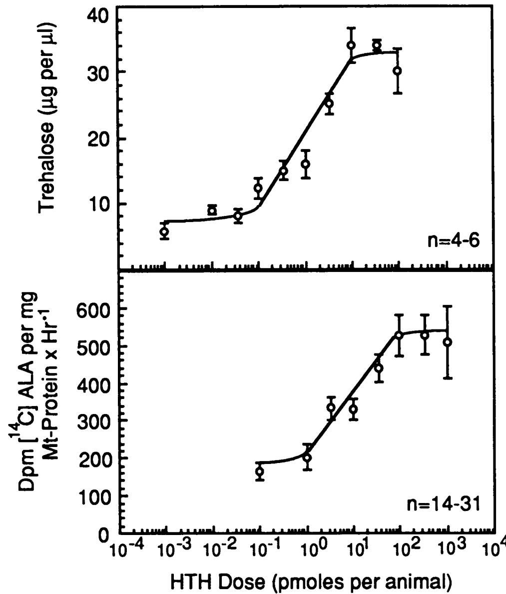

Figure 2. Dose-response effects by *Blaberus* hypertrehalosemic hormone on *in vivo* synthesis of trehalose (upper) and cytoheme *ab* (lower) in decapitated, adult male *B. discoidalis*.

Values are mean ±SEM. For cytoheme *ab* synthesis, animals are decapitated on day 0 and injected with the designated dose of Bld-HrTH daily on days 2, 3 and 4 and cytoheme *ab* synthesis measured on day 4 (60, 61). For trehalose synthesis, animals are decapitated on day 5 and the designated dose of Bld-HrTH injected on day 6 at time zero and the hemolymph carbohydrate measured 2 hr later.

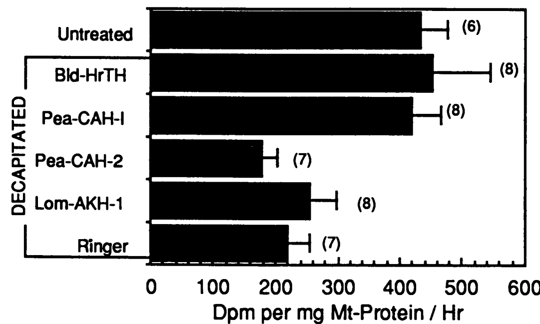

Figure 3. Effects of bioanalogs of *Blaberus* hypertrehalosemic hormone on cytoheme *ab* synthesis in the fat body of 4-day old, adult male *B. discoidalis*. Values are mean+SEM. All animals were decapitated on day 0 and received 20 pmoles of peptide on days 2, 3 and 4 followed by measurement of cytoheme *ab* synthesis on day 4.

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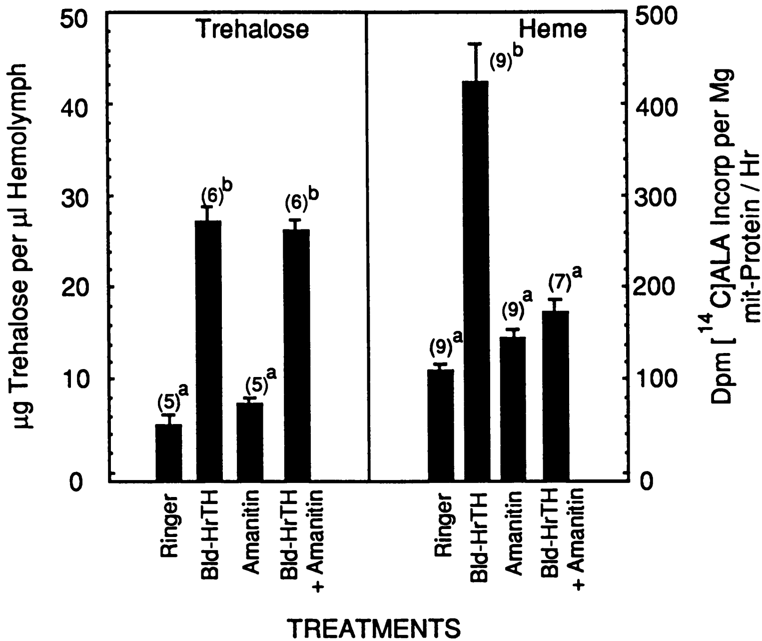

Figure 4. Effects of α-amanitin on *Blaberus* hypertrehalosemic hormone-dependent, *in vivo* synthesis of trehalose and cytoheme *ab* in decapitated, adult male *B. discoidalis.*

Values are mean+SEM for the number of individual animals shown in parenthesis. Similar small letters indicate no significant differences between means ($P \leq 0.05$) based on ANOVA and Fisher's PLSD test of individual means. Animals were decapitated on day 0 and injected with either Ringer, 100 pmoles of Bld-HrTH, 1.8 μg α-amanitin or both Bld-HrTH and amanitin on days 1, 2, 3 and 4 followed by determination of cytoheme *ab* synthesis on day 4 (60, 61). For trehalose synthesis capacity, animals were decapitated on day 0 and treated with either Ringer or 1.8 μg of α-amanitin on days 1, 2, 3 and 4 and the Bld-HrTH response determined on day 5 by injection of 20 pmoles of Bld-HrTH followed by measurement of hemolymph carbohydrate levels 2 hr later.

synthesis should be reflected by rates of energy-consuming processes such as protein synthesis.

The ability of Bld-HrTH to influence fat body endergonic metabolism was assessed by measuring its effects on protein synthesis during vitellogenesis in female *B. discoidalis*. Vitellogenesis is initiated in this insect without feeding or mating and increases in ovarian protein content are measurable by day 5. Both allatectomy and decapitation suspend ovarian protein formation and injections of JH-III or topical applications of methoprene, a JH analog (JHA), promote ovarian protein formation (65,66); however, ovarian protein contents in JHA-treated, decapitated females never attain those of normal females (66).

The rate of synthesis of soluble proteins (SP) was measured for fat body from decapitated females treated with JHA, CC extracts or both (66). CC extracts do not increase the capacity of the fat body for SP synthesis; whereas, JHA has a dose-response effect on SP synthesis. For tissue from females treated with a half-maximal dose of JHA, we found that additional treatment with either a CC extract or Bld-HrTH elevated the rate of SP synthesis to the maximal level observed in normal, vitellogenic females (66) (Figure 5). SDS-PAGE of the SPs demonstrates that the pattern of polypeptides synthesized depends on JHA and not the presence of CC extracts (66). The latter is also confirmed for synthetic Bld-HrTH.

From these results, the following explanation is proposed. JH controls the synthesis of specific proteins, presumably vitellogenins, by the adult female fat body. In the absence of JH activity, only constitutive, basal SP occurs. Under such conditions, neither the presence nor absence of Bld-HrTH affect SP synthesis since energy demands are minimal. However, when specific SP synthesis is stimulated by JH or JHA in the absence of Bld-HrTH, then SP synthesis is less than maximal amount since the energy synthetic capacity of the tissue is limited. In the presence of Bld-HrTH, the biosynthetic capacity of the fat body is enhanced by greater ATP production because both cytochrome heme synthesis and the amounts of available mitochondrial cytochromes are increased. Finally, Bld-HrTH promotes a 3-fold increase in the rate of total fat body RNA synthesis in adult male *B. discoidalis* (Figure 6). These findings suggest that Bld-HrTH may have a dual action on the translational capacity of the fat body. First, it may increase the numbers of ribosomal sites on which translation can occur, and second, an increased capacity for ATP production should permit higher rates of translation.

Direct *in vitro* effects on fat body protein synthesis were measured for Lom-AKH-I and Bld-HrTH in *B. discoidalis*. Unlike the direct inhibition of protein synthesis by Lom-AKH-I in *L. migratoria* (64); no inhibitory actions were noted when either 10 pmoles (= 5 nM) of Bld-HrTH or Lom-AKH-I were added directly to isolated fat bodies from *B. discoidalis*. The activity of both hormones was confirmed by their ability to stimulate trehalose elevation in the hemolymph of *B. discoidalis*. It is apparent that the inhibitory effects of Lom-AKH-I on locust fat body protein synthesis do not extend to the fat body of *B. discoidalis*. The ability of Bld-HrTH to stimulate fat body protein synthesis in *B. discoidalis* requires long-term exposure *in vivo*; no direct effects were noted.

Genetics and Synthesis of the AKH/RPCH Family

The adipokinetic hormones are becoming important models for research into the regulation of neurohormone synthesis in insects. Recently, we isolated the AKH structural gene from *M. sexta*. The gene for Mas-AKH was isolated from a genomic library of *M. sexta* using a 29mer probe that was designed based upon the nonapeptide hormone sequence and substituted with deoxyinosine to account for ambiguous codon positions (67). This probe selected the Mas-AKH clone from 2.5 x 10^5 recombinant plaques. The resulting Mas-AKH gene is intronless and codes for a ~0.6 kb message. From the longest open reading frame within the cloned sequence,

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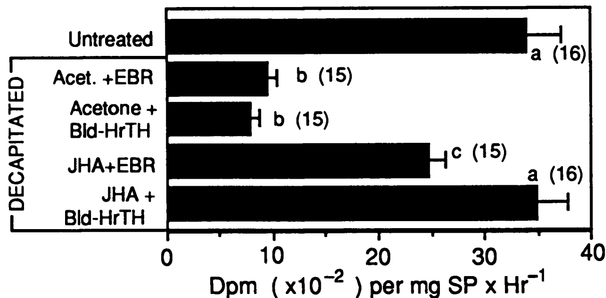

Figure 5. Effects of JHA (methoprene) and Bld-HrTH on the capacity for soluble protein synthesis by fat body from decapitated, female adult *B. discoidalis*.

Values are mean+SEM for the number of replicate individuals shown in parenthesis. Similar small letters indicate no significant differences between means (P≤ 0.05) based on ANOVA and Fisher's PLSD test of individual means. Animals were decapitated on day 0 and treated on days 3-14 with either 15 μg of methoprene applied topically to the abdomen on days 3, 6, 9 and 12 or by injection of 100 pmoles of Bld-HrTH daily, or both. Fat body was isolated on day 15 and the capacity for synthesis of export proteins measured (66). Acetone and Ringer were administered as control treatments for JHA and Bld-HrTH, respectively.

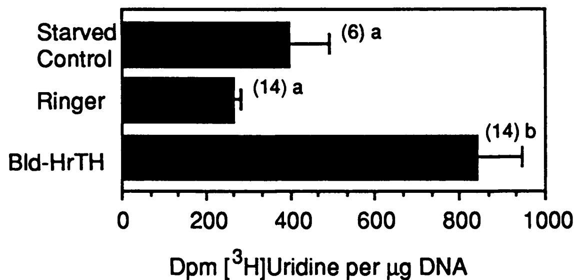

Figure 6. Effects of Bld-HrTH on the synthesis of total RNA in the fat body of decapitated, 4-day-old, adult male *B. discoidalis*.

Values are mean+SEM for the numbers of animals shown in parenthesis. Similar small letters indicate no significant differences between means (P≤ 0.05) based on ANOVA and Fisher's PLSD test of individual means. Animals were decapitated on day 0, treated on days 1, 2 and 3 with 100 pmoles of Bld-HrTH and incorporation of [^{3}H]uridine measured for total fat body RNA following 8 hrs of incubation *in vivo*.

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we deduced that the preproAKH consists of a 19 amino acid signal sequence, a single AKH peptide region followed by a Gly-Lys-Arg cleavage and amidation processing site and a 34 amino acid tail that contains no homology to AKH/RPCH family peptides (Table II). Northern analysis of RNA from the brain, the CC+CA and the ventral nerve cord of *Manduca* demonstrated that only the CC+CA contained RNA that hybridized to the Mas-AKH gene probe (67).

Table II. Comparison of amino acid sequences and organization for the preprohormones of Mas-AKH, Lom-AKH-I from *Schistocerca gregaria* (68), and Scn-AKH-I and -II from *Schistocerca nitans* (69)

	Signal	AKH	PS	C-terminal peptide
Mas-AKH:	MYKLTVFLMFIAFVIIAEA	QLTFTSSWG	GKR	AMTNSISCRNDEAIAAIYKAIQNEAERFIMCQKN
Lom-AKH-I:	MVQRCLVVALLVVVVAAALCSA	QLNFTPNWGT	GKR	DAADFGDPYSFLYRLIQAEARKMSGCSN
Scn-AKH-I:	MVQRCLVVALLVVVVAAALCSA	QLNFTPNWGT	GKR	DAGDYGDPYSFLYRLIQAEARKMSGCSN
Scn-AKH-II:	MRQGCALTLMLLVVVCAALSAA	QLNFSTGW	GRR	YADPNADPMAFLYKLIQIEARKLAGCSN

PS=processing site.

PreproAKH structures for Mas-AKH, Lom-AKH-I of *S. gregaria* (68) and Scn-AKH-I and -II of *S. nitans* (69) are compared in Table II. Although the amino acid compositions differ for the preproAKHs from the locusts and *M. sexta,* the organization of all the preprohormones is the same: each precursor consists of a signal sequence, a single copy of the hormone sequence, a cleavage-amidation processing site and a C-terminal peptide of about thirty amino acids. The three locust C-terminal peptides are clearly related, and upon alignment with one gap, the C-terminal peptide of *M. sexta* is 21-32% homologous with the locust sequences.

After cleavage of the signal sequence, the pro-AKH of *S. gregaria* exists as a homodimer consisting of two AKH-peptide tails linked by a disulfide bridge between the Cys residues in each tail (68). It is likely that the Scn-AKH-I and -II C-terminal peptides form homodimers, as in *S. gregaria,* or heterodimers if both proAKH-I and proAKH-II reside within the same neurosecretory vesicles. The proAKH structure of *M. sexta* is uncertain. Because the tail of *M. sexta* proAKH contains two Cys residues, it can form either a homodimer (either parallel or antiparallel) as in *S. gregaria,* an internal disulfide bridge to itself or a mixture of all structures.

The above findings on precursor structures suggest a common mechanism of AKH/RPCH peptide synthesis among insects as well as, perhaps, other arthropods and support the idea that these peptides constitute a true family in the evolutionary sense (14,70). However, care must be taken in addressing this question. For example, we have used the gene for Mas-AKH as a probe for the AKH gene in preparations of total DNA from *Heliothis virescens* and *Heliothis zea* (the latter species contains the identical Mas-AKH peptide), and no distinct hybridizing band was detected in either species, even at low stringency. Based upon this surprising finding, we can only conclude that the AKH genes have undergone extensive divergence between the Sphingidae and the Noctuidae, despite conservation of the identical amino acid sequence in the active hormone. Further research on family precursors from diverse species may provide a clearer portrait of the molecular evolution of this peptide family.

Structure-Activity Relationships of Bld-HrTH

Peptide hormones contain information within their structures that can be classified into

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definable regions (i.e. an "address" region and a "message" region). The address aligns the peptide with the receptor active site and is reflected by the 50% level of response (*potency*) to the peptide in a dose-response assay. The message is the portion of the peptide that activates the receptor to transduce information to the cell and is reflected by the maximum activity (*efficacy*) observed in the bioassay. Comparisons of dose-response data for Bld-HrTH and defined structural analogs using our *in vivo*, *B. discoidalis* hypertrehalosemic bioassay suggest several structure-activity relationships for Bld-HrTH.

In the *B. discoidalis* hypertrehalosemic assay, no other peptide was more potent than Bld-HrTH (71). This suggested that Bld-HrTH and its receptor(s) have co-evolved to maintain an optimal fit in *B. discoidalis*. Critical portions of the address and message are contained within the N-terminal octapeptide of the hormone because most of the octapeptide bioanalogs had reduced potencies but full maximum activities. The C-terminal dipeptide has some influence on the address since added potency is observed with decapeptides. Some bioanalogs such as Mas-AKH and Pea-CAH-II are devoid of activity in the *B. discoidalis* bioassay. Mas-AKH and Pea-CAH-II are the most divergent sequences from Bld-HrTH in the AKH/RPCH family, and their lack of activity suggests that large differences in receptor specificity have evolved between the insect species.

A series of single amino acid replacement analogs has helped to define the importance of each side chain and each terminus for hypertrehalosemic activity in *B. discoidalis* (72). The major amino acid replacements were Ala and Gly which result in an analog with a single deleted side chain. This approach suggests that positions 1, 4 and 8 are critical to the Bld-HrTH "address" (Fig. 7). Positions 3, 6, and 9 are important but less critical to the "address". Removal of the side chain at position 5 or the addition of a negative charge at the C-terminus result in analogs with reduced efficacy. This suggests that these groups are important to the "message". Residues in positions 1, 4 and 9 may also be part of the message, but since they are critical for the address, their role in the message cannot be demonstrated. Positions 2, 7, and 10 can tolerate changes in their amino acids composition without the analog showing significant changes in bioassay results relative to Bld-HrTH.

Other information has suggested that a folded conformation for Bld-HrTH may be important for the "address" of this peptide and its action in *B. discoidalis*. First, Chou-Fasman calculations (73) predict that residues 5-8 form a ß-turn. Also, Wheeler and coworkers have observed through CD analysis, evidence that Bld-HrTH forms a ß-turn in SDS-micelles (personal communication). We have compared Bld-HrTH to single amino acid replacement analogs at positions 5-7 and have found correlations between the amino acid frequency coefficients of Chou and Fasman, and relative analog potency. For example, Gly is found in the f_i position (Bld-HrTH position 5) of ß-turns slightly less often than Ser. [Gly^5]-Bld-HrTH is only slightly less potent than Bld-HrTH. On the other hand, Gly is found much less often than Pro at the f_{i+1} ß-turn position, and [Gly^6]-Bld-HrTH is much less potent than Bld-HrTH. Gly and Asn are found in almost identical frequency at the f_{i+2} position. Likewise, [Asn^7]-Bld-HrTH has identical potency with Bld-HrTH. Finally, in other studies we have found that position 7 of Bld-HrTH tolerates replacement by D-amino acids much better than the rest of the molecule. Tolerance of D-amino acids has been an important clue to the presence and position of a ß-turn in other small neuropeptides like Bld-HrTH, for example LHRH (74).

Conclusions

Our data suggest that Bld-HrTH in *B. discoidalis*, and likely the AKH/RPCH family in insects in general, has several regulatory effects on fat body metabolism in addition to the mobilization of stored metabolites. For example, Bld-HrTH appears to increase both the energy generation capacity of the tissue and its RNA complement. Bld-

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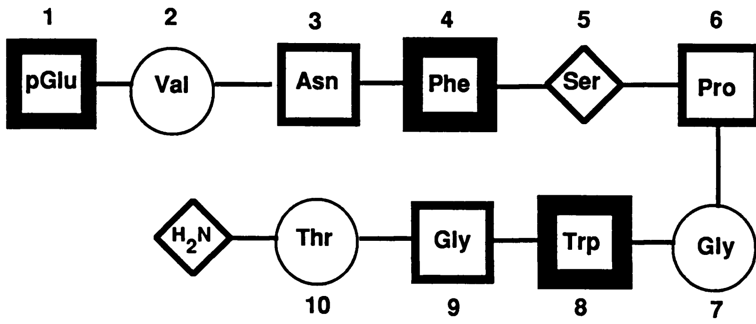

Figure 7. Proposed active structure for Bld-HrTH for hypertrehalosemic activity in *Blaberus discoidalis* cockroaches.
Residues have indication symbols for the effects of substitution on activity: (Bold squares) critical for potency; (squares) important for strong potency; (diamonds) strong effects on efficacy; and (circles) little or no direct influence on potency or efficacy.

HrTH-related increases in RNA enhance the capacity of the fat body for protein biosynthesis, an energy consuming event, at the same time that Bld-HrTH increases the exergonic potential of the tissue by elevating the electron transport capacity and the rate of ATP synthesis. In this way, Bld-HrTH regulates both the rates of of energy production and consumption within the fat body.
From the view that AKH/HGH hormones may determine energy production by the major biosynthetic tissue of the insect, Bld-HrTH compares with the action of the thyroid hormones in vertebrate animals. Just as Bld-HrTH synergizes the action of JH on the synthesis of putative vitellogenic proteins by the fat body; thyroid hormones have permissive actions and synergize the effects of gonadotropins on gonadal maturation in fish (75). The thyroid hormones may elevate the basal metabolic "tone" within a tissue to insure competency for meeting the demands of energy consuming processes (76). In the absence of adequate thyroid stimulation, biosynthetic processes may be diminished. The thyroid hormones may maintain the balance between anabolic and catabolic cellular processes in vertebrate animals. We propose that Bld-HrTH and the other AKH/RPCH family peptides may serve as the insect complement of the vertebrate thyroid hormones to regulate general fat body energy metabolism, in addition to promoting stored metabolite mobilization.

Acknowledgments

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Chapter 8

Chemical Identification of Insect Diuretic Peptides

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Diuretic peptides promote diuresis in insects in different ways. The chapter reviews the isolation and chemical identification of diuretic peptides from three insects: *Locusta migratoria, Manduca sexta*, and *Acheta domesticus*. The biological activities of the characterized peptides are discussed.

Physiological investigations on diuresis in insects have been very difficult but extensive. A voluminous literature exists on diuresis and its regulation; this has been reviewed comprehensively and recently (1,2) so no attempt will be made to do so here. However, it should be emphasized that diuresis is a complex process in insects. It is not clear whether a diuretic hormone should be defined as a material that increases urine secretion by the Malpighian tubules, one that decreases fluid resorption by the rectum, or one that increases fluid loss by the whole insect (1). In addition, there is growing evidence that multiple factors may act on even just one of these possible sites for a diuretic hormone. A particularly well studied example is the existence of three peptide factors affecting in different ways the Malpighian tubules of the mosquito *Aedes aegypti* (3). This review will focus on the status of isolation and chemical identification of diuretic hormones from insects, eschewing the considerable literature on biologically active factors for which no sequence information exists.

DIURETIC PEPTIDES IN LOCUSTA MIGRATORIA

Mordue's group isolated and partially characterized a diuretic peptide from the corpora cardiaca (CC) of *Locusta migratoria* (4). Batches of up to 150 CC were collected and extracted with 20% aqueous methanol. After centrifugation,

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the supernatant was concentrated to dryness, and the residue stored at -70° C until needed. The residue was solubilized in 0.1 % trifluoroacetic acid (TFA) and chromatographed first with a TSK 2000 SW size exclusion chromatography column. On this column the diuretic peptide DP-1 coeluted with a major constituent termed LCCP. This fraction from size exclusion chromatography was then purified by reversed-phase LC using Aquapore RP-300 (a wide-pore C_8 packing for peptides) with a gradient starting at 27% acetonitrile/0.1% aqueous TFA increasing by 0.88% min^{-1} of organic modifier.

Fractions were monitored for diuretic activity using a rapid assay based on the elevation of levels of cAMP in cultured Malpighian tubules treated with peptide (5). The diuretic activity in the active peak was confirmed using the much more laborious Ramsay-type (6) assay with isolated tubules. The amount of this peak recovered after 2 steps of purification was estimated from UV absorbance (based on glucagon as a standard) at 1.7 pmol/CC. Edman degradation gave the sequence ? -Gly- ? -Gly-Ile-Gln-Ala- ? -Val/Met-Tyr-Lys-, which would represent only about 1/5 of the full sequence expected for the reported M_r of 6,000-7,000 Da. These authors do not seem to have reported further details of this factor. In earlier work, Morgan and Mordue had reported (7) the existence of DP-2, a smaller peptide of ~1,000 Da which stimulated fluid secretion by Malpighian tubules but apparently not *via* cAMP. This peptide could therefore not be detected by the quick assay used for detecting DP-1.

Rafaeli *et al.* have recently reported the existence of an adrenocorticotropin (ACTH)-like material in locust CC (8) detectable by immunoassay. They also reported that ACTH at 10 mM stimulated liquid excretion from, and elevated levels of cAMP in, locust Malpighian tubules. Extracts of CC produced similar effects in the traditional in vitro assay (8). They believe that a diuretic peptide in locusts has at least immunological similarity to ACTH. While the partial sequence reported (4) by Morgan *et al.* has no apparent similarity to ACTH, it is conceivable that these groups could be investigating the same factor.

For some time Proux and others in Bordeaux had investigated (9) another factor from *Locusta migratoria* which was also reported to be a diuretic peptide with a quite different tissue localization, being mainly in the subesophageal and thoracic ganglia. This material was found to be cross-reactive with antibodies raised against the antidiuretic hormone of many higher vertebrates, arginine vasopressin (AVP). This material, the AVP-like factor, was shown to have diuretic activity in an assay based on the excretion of dye from the hemolymph of locusts injected with amaranth. Homogenates of 51,000 ganglia were extracted and fractionated by semi-preparative RPLC, with progress of the isolation being monitored using an AVP immunoassay. The column was eluted with a gradient of acetonitrile-0.1% aqueous TFA. In the first step, two immunoreactive zones were found and called F1 and F2 (10). Additional tests using the

amaranth excretion assay showed that only the slower eluting, less abundant factor (F2) stimulated dye excretion.

The second reversed-phase LC purification of both factors used a Vydac C_4 column eluted with a gradient of acetonitrile-0.1% aqueous heptafluorobutyric acid (HFBA). This gave a markedly different selectivity than the acetonitrile-0.1% TFA system for F1, with an extremely efficient removal of non-immunoreactive UV-absorbing materials. A somewhat different CH_3CN/0.1% HFBA program provided a substantial enhancement of the purity of F2, although less dramatically than for F1.

The third purification of both factors by reversed-phase LC was performed with gradients of 1-propanol-0.1% aqueous TFA. The immunoreactive F1 was separated from a number of impurities and coincided with a peak having low absorbance at 220 nm, and almost no absorbance at 280 nm, suggesting that F1 was essentially homogenous and that it did not contain Trp or Tyr. However, on this third chromatography of F2 in 1-propanol-0.1% aqueous TFA, the main immunoreactive fractions essentially coincided with major components that absorbed strongly at 220 and 280 nm. Rather than regarding the factor as substantially pure, the immunoreactive F2 was again chromatographed on a Vydac C_4 column, but with 1-propanol-0.1% aqueous HFBA. This fourth RPLC separation provided a very selective separation of the immunoreactive fractions from several strongly UV-absorbing impurities; absorbance in the zone corresponding to F2 was low at 220 nm and nil at 280 nm.

The immunoreactive zones from this purification were again separated on an isocratic 1-propanol-0.1% TFA program. An immunoreactive zone was isolated corresponding to a peak with low UV absorbance at 220 nm and undetectable absorbance at 280 nm. This highly purified F2 caused a strong enhancement of the rate of amaranth excretion in the *in vivo* *L. migratoria* assay.

In subsequent studies (11), these two factors were separately reduced and carboxymethylated (RCM) prior to sequence analysis. The results of thorough analysis of these peptides gave the surprising result that the two had identical amino acid compositions; moreover, both RCM-peptides were found to have the identical primary sequence Cys(CH_2CO_2H)-Leu-Ile-Thr-Asn-Cys(CH_2CO_2H)-Arg-Pro-Gly. These results were surprising considering the substantially different retention times of the native factors on RPLC.

Nonapeptides with the sequence determined for F1/F2 were synthesized using manual solid-phase methodologies as both the carboxyl-terminal amidated and free acid forms. On reversed-phase LC analysis, RCM-F1 and RCM-F2 were shown to coelute with the synthetic RCM-amide rather than the RCM-acid. The intact, synthetic nonapeptide Cys-Leu-Ile-Thr-Asn-Cys-Pro-Arg-Gly-NH_2 was shown by LC to have the same retention time as native F1 rather than F2. The factors were next analyzed using size-exclusion LC with RIA monitoring. The retention times of native F1 and F2 corresponded to M_r

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~700 and ~1,470, immediately suggesting that F2 might be a dimer of F1.

Synthetic F1 was treated using conditions known to convert oxytocin to a mixture of parallel and antiparallel dimers; RPLC separation gave F1 (20.1 min) and two more retained peaks at 42.0 and 45.3 min. The latter were analyzed by size-exclusion LC; both had M_r ~1,570. Comparative analyses by reversed-phase LC showed that the faster eluting dimer had retention (UV detection) virtually coincident with that of native F2 (separate analysis, fractions monitored by RIA).

To ascertain the relative configurations of the dimers, each was synthesized specifically, using protecting groups that allow directed formation of disulfide bonds. Comparison of retention behavior of the specifically synthesized dimers with those synthesized non-specifically showed that F2 corresponds to the antiparallel dimer (Fig. 1) and the slower eluting component to the parallel isomer.

The biological activities of synthetic and native peptides were assayed in vitro using isolated Malpighian tubules attached to the midgut. In this assay, a lack of stimulation in controls leads to gradually decreasing flow. In contrast, the flow remained roughly constant when the medium was complemented with physiological concentrations (ca. 1.7 nM) of synthetic or native F2, or with very high doses of synthetic F1. Physiological doses of the parallel dimer and native F1 had no effect.

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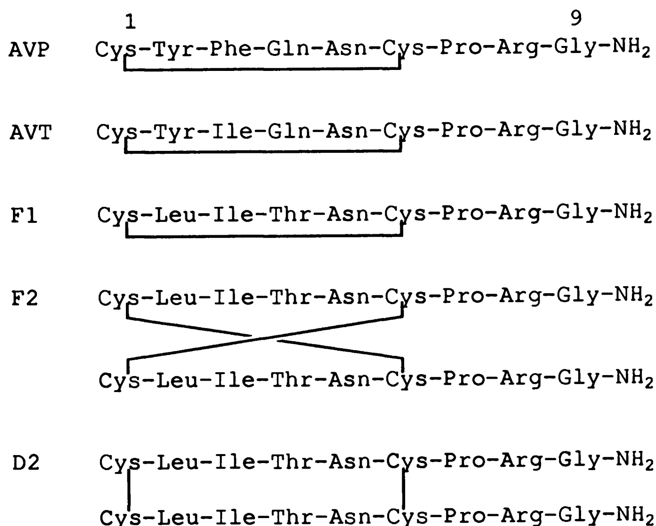

Fig. 1. The sequences of AVP, arginine vasotocin (AVT), F1, F2 (the AVP-like DH), and the parallel dimer D2.
(Adapted from ref. 11.)

To check whether the diuretic hormone acts on Malpighian tubules via cAMP, intact tubules were incubated with synthetic F2 for various periods of time, then extracted and assayed for cAMP using an RIA. Addition of the synthetic antiparallel dimer elevated tissue cAMP concentration at all times tested.

These data show the presence in subesophageal and thoracic ganglia of *Locusta* of an antiparallel dimeric peptide (F2), which was called the AVP-like insect diuretic hormone (AVP-like DH) (11). The sequence homology between the AVP-like DH and the vertebrate neurohypophyseal peptides is strong: Cys-(2)-(3)-(4)-Asn-Cys-Pro-(8)-Gly-NH_2. The AVP-like DH and AVP differ only at positions 2, 3, and 4, whereas the homology to the ancestral molecule arginine vasotocin is even stronger, with differences restricted to positions 2 and 4. Moreover, the existence of a neuropeptide as a dimer had only two precedents. Transforming growth factor-ß, a dimer (12), is encoded by a gene containing one copy of the 112-amino acid monomer. ß-Human atrial natriuretic polypeptide is an antiparallel dimer of the 28-amino acid α-human atrial polypeptide (13). The latter dimer also coexists in tissue with its corresponding monomer, a situation analogous to F1 and F2.

The considerable differences in tissue distribution of the AVP-like DH, as compared with the factors investigated by Morgan *et al.* and Rafaeli *et al.* from the corpora cardiaca is perplexing. It is possible that there may be a multiplicity of diuretic factors in the locust as has been reported for *Aedes aegypti* (3).

A DIURETIC PEPTIDE IN MANDUCA SEXTA

Many species of lepidopterans undergo a profound diuresis immediately after adult eclosion, such as with Danaus plexippus (14). However, wandering fifth stadium larvae of *M. sexta* lose ~30% of their body weight during the 48 hr period which precedes pupation, after they cease feeding and just before "wandering" behavior (in which they seek a pupation site). With *Manduca sexta*, the post-eclosion diuresis is less significant. A putative diuretic hormone has been identified (15) in *Manduca*, in which DH activity in fractions from purification steps was detected using an assay with newly emerged adult *Pieris rapae*. This *in vivo* assay was developed based on one previously reported for *Danaus plexippus* (14). Newly emerged adult butterflies were neck-ligated and beheaded, depriving the abdomen of a pulse of DH which ordinarily stimulates substantial loss of a clear fluid (~20% of the body weight) from the rectum. Neck-ligated animals excrete only a dark meconium (waste products of pupal metabolism), representing ≤9% of the body weight. Injection of DH-active fractions into ligated butterflies caused a typical normal urine excretion. Caution has to be taken to avoid evaporation of the excreted fluid in this gravimetric assay. Typically about ten fractions can be assayed per day.

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M. sexta DH was isolated in parallel with eclosion hormone (EH), whose purification and sequencing were described earlier (16). Both hormones were extracted together, but are separated by SP-Sephadex ion exchange chromatography. The strategy of subsequent purification steps is nearly identical for EH and DH, although the chromatographic conditions differ. Despite starting with a much cruder tissue source than the other isolations of diuretic hormones reported to date, only five LC isolation steps were used. The scheme is shown in Fig. 2.

The starting material for isolating the hormones was 10,000 heads of pharate adult *M. sexta*, cut off directly into dry ice. A 5 mm cork borer was used to "trim" a frozen tissue portion containing the brain and corpus allatum/corpus cardiacum complex away from the eyes, proboscis, antennae, and other parts of the head. The trimmed heads (0.42 kg) were homogenized in acetone to remove lipids. The extract was filtered, the acetone discarded, and the residue re-extracted with an acidic solvent containing protease inhibitors: 1 M acetic acid, 20 mM HCl, 0.1 mM PMSF, and 0.01 mM pepstatin A.

The extract was centrifuged and the supernatant loaded onto an SP-Sephadex C-25 column, which was eluted sequentially with 1 M acetic acid and 50 mM NH_4OAc (pH 4), followed by 50 mM, 100 mM, 200 mM, 400 mM, and 800 mM NH_4OAc (each at pH 7).

1) Homogenize 10,000 trimmed heads from pharate adults (420 g) with acetone, filter (delipidation)
2) Extract with strongly acidic buffer containing protease inhibitors
3) SP Sephadex chromatography; DH in 0.4 & 0.8 M NH_4OAc, pH 7.0
4) Vydac C_4 cartridge; DH in 35% CH_3CN/0.1% TFA fraction
5) Vydac C_4 semipreparative LC, 20-40% CH_3CN/0.1% TFA gradient
6) Vydac C4 semipreparative LC, 10-30% 1-propanol/0.1% TFA gradient
7) TSK SP-5PW LC, 0-0.4 M NaCl/0.02 M $H_2PO_4^-$, pH 6.25 gradient
8) Vydac C_4 analytical LC, 30-45% CH_3CN/0.1% HFBA gradient
9) Vydac C_4 microbore LC, 20-40% CH_3CN/0.1% TFA gradient

Yield: ~5 nmol pure DH (~24 µg). Purification factor: 18×10^6 from heads. Quantity of hormone: 0.5 pmol/head.

Fig. 2. Scheme for isolating *Manduca sexta* diuretic hormone.

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Aliquots from each fraction were desalted and assayed; eclosion hormone eluted in the 50 mM NH_4OAc fraction, allatotropin in the 100 and 200 mM NH_4OAc fractions (17), and diuretic hormone in the 400 and 800 mM NH_4OAc.

The large volumes of buffer were concentrated and desalted by applying them to 10 g of Vydac C_4 bulk packing contained in a polypropylene syringe barrel. The cartridge was washed with 0.1% TFA, and the desalted peptides were then eluted using step gradients of 20%, 35%, and 60% acetonitrile/0.1% TFA. Both EH and DH were recovered in the 35% acetonitrile/0.1% TFA fraction, freeing them of both very hydrophilic and hydrophobic constituents.

The active organic fraction was diluted with twice its volume of water and pumped onto a 25 x 1.0 cm Vydac C_4 semipreparative column equilibrated with 0.1% TFA. This procedure obviates the necessity of evaporating the organic solvent, which almost invariably causes loss of trace amounts of peptide. An acetonitrile/0.1% TFA gradient was used for the first semi-preparative LC step.

Active fractions from the first semipreparative RPLC purification were pooled, again diluted with water, and pumped onto the same semi-preparative column. A gradient of 10-30% 1-propanol/0.1% TFA was utilized for the second semi-preparative isolation.

In order to obtain different selectivity from reversed-phase, the subsequent step was an ion exchange LC separation using a TSK SP-5PW analytical column, equilibrated with 0.02 M sodium phosphate buffer, pH 6.25, eluted with a gradient of sodium chloride from 0-0.5 M. The active fractions from ion exchange LC purification were loaded directly onto a Vydac C_4 analytical column (15 x 0.46 cm) and eluted with an acetonitrile/0.1% HFBA gradient. However, the DH still appeared slightly impure and was purified on a 25 x 0.21 cm Vydac C_4 microbore column with acetonitrile/0.1% TFA gradient.

DIURETIC HORMONE SEQUENCE AND BIOLOGICAL PROPERTIES

Automated Edman degradation of intact DH (0.5 nmol, or 10% of the sample) yielded a single amino acid sequence, and residues 1-40 were assigned. DH was digested with trypsin and the fragments separated by RPLC. Sequence analysis of six fragments showed that one of these clearly represented the carboxyl terminus of DH, and demonstrated that DH is a 41 residue peptide terminating in Ile. The carboxyl terminus was shown to be amidated by comparison of RPLC retention times of this tryptic fragment and two synthetic hexapeptides (Asn-Phe-Leu-Asn-Asp-Ile) prepared in the amidated and free acid forms. Hence, the complete structure of DH was established as shown in Fig. 3; a structure also supported by amino acid analyses of intact DH and its tryptic fragments.

M. sexta DH, as well as an analogue with an acid at the carboxyl terminus, was synthesized using conventional automated solid phase methodologies (18). The synthetic

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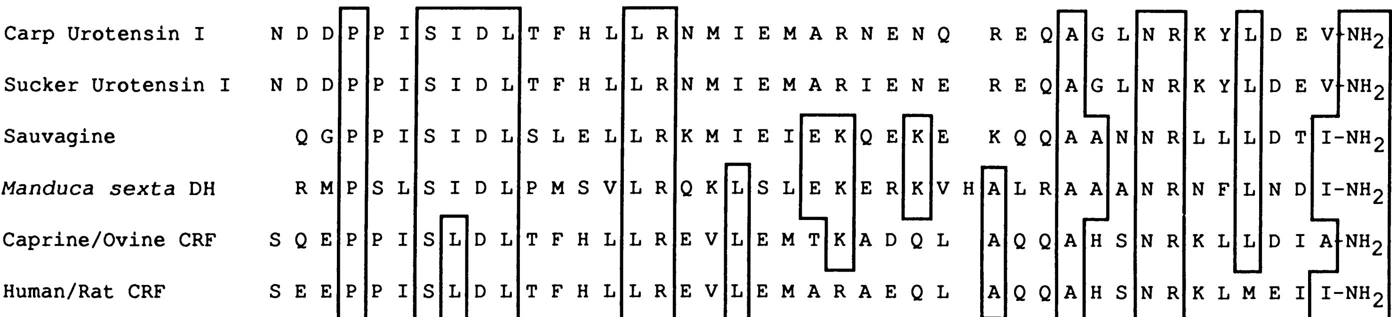

Fig. 3. Complete amino acid sequence of *Manduca sexta* DH and its similarity to the sequences of urotensin I (carp and sucker), sauvagine, and corticotropin-releasing factor (CRF) from four species of mammals.

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amidated and acidic forms were compared to native DH by reversed-phase LC analysis, which again established the identity of native DH with the synthetic amidated form. In the *Pieris* assay, synthetic DH and its acid analogue were found to have approximate ED_{50} values of 0.1 ng and 0.1 µg per animal, respectively, showing the native amidated form to be about 1000 times as potent as the acid analogue. Similarly, the effects of synthetic DH were investigated in *M. sexta* larvae *in vivo*. Late fifth stadium larvae, weighing ~9 g, excrete approximately one third of their body weight in water over a 48 hr period. When 0.1 µg of synthetic DH was injected into post-feeding, pre-wandering larvae, the rate of fluid loss was 2.3 times that of controls for those injected with synthetic DH, a highly significant difference at $P < 0.001$. Assuming a 50% fluid volume in these animals and a uniform distribution of DH throughout this fluid, the amount injected represents an *in vivo* concentration of only ~4 nanoMolar.

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A computer search for sequence similarity of *M. sexta* DH with known peptides and proteins located a substantial similarity with sauvagine (19). Sauvagine was isolated from skins of the frog *Phyllomedusa sauvagei* using a bioassay based on its potent antidiuretic effect in rats (20). If allowance is made for a one-amino acid insertion of histidine in *M. sexta* DH (position 27), 40% of the residues are identical. An additional 16 amino acids (39% of the sequence) in *M. sexta* DH could result from single-base mutations in the sauvagine gene. The location of these amino acids strengthens the supposition that histidine has been inserted in a well conserved sequence. Sauvagine is but one member of an important peptide family including corticotropin releasing factor (CRF) and urotensin I (21). CRF, isolated and identified first from sheep hypothalami (21), has been sequenced subsequently from the rat (rCRF, (21)), goat (cCRF, (22)), and cow (bCRF, (23)). The structure of human CRF was deduced from the sequence of its gene (hCRF, (24)). The sequence identity of *M. sexta* DH with the various forms of CRF and the urotensins is somewhat lower than for sauvagine.

The most studied physiological/pharmacological effects of the sauvagine/CRF/urotensin I family appear to be the stimulation of ACTH release and hypotensive/vasodilatory properties; the antidiuretic effect of sauvagine has been attributed to its potent hypotensive action (19). The potency and nature of the effects elicited by each peptide varies with the species in which it is tested. For example, urotensin I and sauvagine elicit a much more potent and selective hypotensive/vasodilatory effect in mammals than that caused by the mammalian form oCRF (25). In contrast, injection of 500 pmol of urotensin I, sauvagine, and oCRF did not stimulate fluid excretion in the *P. rapae* assay (15). For comparison, the ED_{50} of *M. sexta* DH is ~0.02 pmol. There is not at this time any data on biological activity of truncated analogues of *Manduca sexta* DH, although this would clearly be of interest.

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DIURETIC PEPTIDES IN THE CRICKET

Coast *et al.* recently reported (26) the isolation and identification of one of several peptides from *Acheta domesticus* which stimulates fluid secretion by isolated Malpighian tubules of this species. They isolated this peptide from surgically excised CC, which were extracted, heated at 100° C, centrifuged, and then fractionated sequentially on LiChrosorb RP8 and Aquapore RP300 columns, both eluted with acetonitrile gradients in aqueous TFA. A single major UV-absorbing peak was obtained, which had diuretic activity. The purity of the material, and its co-migration with diuretic activity, was confirmed on phenyl and octadecyl reversed-phase columns, and by size exclusion and ion exchange. The peptide was found to be blocked, but incubation with pyroglutamyl aminopeptidase liberated two peaks which were sequenced. Further analysis of the native peptide by fast atom bombardment mass spectrometry gave a molecular weight which, together with data from automated Edman degradation, confirmed the sequence as pGlu-Arg-Asp-Ile-Phe-His-Ala-Gln-Thr-Asp-Ile-Phe-Gln-Val-Pro-Lys-OH. Curiously, synthesis of this peptide gave a material which was indistinguishable by all chromatographic and mass spectral analyses, but which was without any biological activity. Few experimental details are provided in this brief report, but these authors are forced to conclude that one possibility is that the native peptide identified may have contained an extremely potent minor constituent. As discussed below, this possibility seems unfortunately rather likely.

CONCLUSIONS:

While several insect diuretic hormones have been chemically characterized to various degrees to date, only two of these show biological activity when the deduced sequence is chemically synthesized and tested. In each case four or five isolation steps using reversed-phase and/or ion exchange LC, each using a mobile phase providing rather disparate selectivity, have been required to isolate these factors from an enriched tissue source (dissected nervous tissue or "trimmed" insect heads). In those isolation procedures where incomplete sequences, or biologically inactive sequences have been obtained, less than three reversed-phase LC isolation steps have been used, so that the materials may not have been homogenous. The incredible abundance of AKH compared to other insect neuropeptides has probably mislead insect physiologists into underestimating the difficulty of obtaining a pure preparation of peptide hormone for characterization (27).

Only one of the four reports on characterization of diuretic peptides relied on a direct assay of the effects of extracts on Malpighian tubules (26). A more common strategy has been the use of a less laborious bioassay.

The two diuretic hormones with unambiguous biological activity are both strongly homologous to known peptide hormone families represented in vertebrates, fish, and birds, yet they are of different peptide families. Members of both peptide families have antidiuretic effects in mammals! The complexity of diuresis in insects suggests that a diversity of structures remain to be discovered.

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Chapter 9

Eclosion Hormone

From Genes to Behavior

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Eclosion hormone (EH) is a novel insect neurohormone that acts on the nervous system to trigger a stereotyped set of behaviors. The gene that encodes EH was isolated from the moth Manduca sexta and has provided a molecular means to determine the structure of EHs from a variety of insects. Only a few (2-4) neurons in the CNS produce EH and these cells appear to release the peptide both locally within the CNS and systemically into the blood. The former is responsible for causing the behavioral responses while the latter acts on various peripheral targets.

For behavior to be adaptive, it must be adjusted to fit the changing physiological and developmental conditions of the animal. Neuropeptides are a class of molecules that play an important role in these behavioral adjustments. The ecdysis system of insects has provided a useful model for examining the manner by which neuropeptides alter the functional state of the nervous system.

Insects show highly specialized behaviors that they use for ecdysis, the process of shedding the old cuticle at the end of a molt (1). These behaviors often involve the use of temporary muscles and joints that are not functional at other times, and at least some of the neural elements involved in ecdysis appear to be dedicated to these behaviors since they degenerate after the terminal ecdysis to the adult stage. Ecdysis behaviors are triggered by a unique chemical signal, the peptide hormone eclosion hormone (EH). This neuropeptide acts directly on a steroid-primed CNS to cause the coordinated release of the motor programs used for the escape from the old cuticle (2). Studies on the tobacco hornworm, Manduca sexta, indicate that the peptide is used for every ecdysis during the life-history of the insect (3). Also, EH bioactivity is found in the nervous systems of a wide variety of insects (3). Consequently, it appears that eclosion hormone is present throughout the insects, and its association with ecdysis is likely a very ancient one.

During the past few years, two major advances have been made in the ecdysis system that have provided new insights into the role of EH in the control of ecdysis behavior. The following sections discuss these advances and their implications for understanding the role of peptides in behavioral control.

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A Molecular Analysis of Eclosion Hormone.

Studies on the characterization of EH culminated in 1987 with the reports of the primary amino acid sequence of the hormones from Manduca sexta (4,5) and from Bombyx mori (6). EH is a 62 amino acid peptide with 3 internal disulphide bridges and no significant similarity to any other known peptide. Knowledge of its primary structure then provided an avenue for cloning the gene that codes for it (7). Based on the N-terminal sequence of Manduca EH, a 72 nucleotide oligomer was designed and used as a probe to isolate cross-hybridizing clones from a Manduca genomic library. A restriction fragment from one of the clones was sequenced and found to encode the information for residues 5-62 of EH. This restriction fragment was then used to isolate the corresponding cDNA clone from a library prepared from brain RNA. Sequence analysis of the genomic and cDNA clones have provided us with the following picture of the EH gene. It is found as a single copy per haploid genome and is comprised of 3 exons that are distributed over about 7.8 kb of DNA. The first exon is untranslated, while the second contains the hydrophobic signal sequence followed by the first 4 residues of EH. The remainder of the peptide is encoded by the last exon. The mature mRNA is about 0.8 kb in length and produces a single translation product, preEH, that is 88 amino acids in length. This small precursor contains a 26 residue signal peptide followed by a single copy of EH. Thus, after the signal peptide is cleaved off in the endoplasmic reticulum (8), the remainder has the structure of the mature EH molecule. Therefore, unlike many other biologically active peptides that are processed from large precursors that contain copies of other active peptides (9), the situation for EH is very simple -- the EH gene gives rise to only a single secretory product, EH.

The isolation of the EH gene from Manduca allowed a molecular approach to be used for the isolation of the corresponding genes from other insects (Horodyski, Riddiford and Truman, unpublished). One strategy was to use a DNA fragment that contains the Manduca EH coding region to identify cross-hybridizing clones from a Drosophila genomic library. Hybridization at low stringency resulted in the isolation of a clone that contained the major portion of the Drosophila EH gene. This gene codes for an 0.8 kb mRNA and conceptual translation of the coding region revealed a peptide that shows 69% identity to Manduca EH. In situ hybridization to the polytene chromosomes from larval salivary glands showed that the Drosophila EH gene is located on the right arm of chromosome 3 at location 90B. This region does not correspond to any known mutations, but stocks are available with deletions in this area to facilitate a screen for mutants at the EH locus.

A second strategy for the isolation of novel EH genes has involved the use of the polymerase chain reaction (PCR; 10). Comparison of the structure of Drosophila EH with those from the moths showed a marked conservation of the sequence in the regions of residues 14-21 and 49-57. Mixed synthetic oligonucleotides were made to these two regions and used as primers for PCR on the DNA isolated from various insects. The amplification products were then separated on gels, transferred to nitrocellulose and hybridized to an internal probe derived from a region of the Manduca EH gene corresponding to residues 21-48. Amplification products of the appropriate size that were recognized by the internal probe were produced from the DNA of a number of insects including Acheta domestica, Aedes aegypti, Tenebrio molitor, and Bombyx mori. The products from Aedes and Tenebrio have been sequenced and shown to be homologous to Manduca EH. Thus, PCR provides an efficient way to isolate homologous peptides from a diverse array of species. The information from these species will show how EH has diversified through insect evolution. Moreover, comparison of the structures of the

peptides from these diverse species should identify regions of the peptide that are most important for its biological activity.

EH Neurons and the Route of Activation of Ecdysis Behaviors.

EH was initially identified as a circulating neuropeptide that was thought to act back on the CNS to trigger ecdysis behaviors. In pharate adult Manduca sexta, large amounts of EH accumulate in the CC-CA complex prior to ecdysis and are released into the hemolymph late in the molt. A set of lateral brain neurosecretory cells (NSCs) that projected to the CC-CA were found to contain EH-like activity (11). It was clear, however, that these cells were not responsible for EH release in larvae or pupae since in these stages release of the hormone comes from the ventral nervous system (3). The recent discovery of the cells responsible for this release has prompted a re-evaluation of the route by which EH triggers ecdysis.

In Manduca larvae and pupae, stores of EH are detected in only one peripheral site, the proctodeal nerves, and depletion of hormone stores from this site occurs in conjunction with ecdysis. An unexpected finding was that the EH found in the proctodeal nerve actually arises in the brain, from 2 cells in each hemisphere whose axons project the length of the CNS to the neurohemal sites in the proctodeal nerves (12). These ventromedial cells (VM cells) show strong EH immunoreactivity, and in situ hybridization experiments show that they contain the EH mRNA (7). This brain-proctodeal system represents a novel neurosecretory pathway distinct from the classic medial and lateral NSC cell groups. Interestingly, the VM cells are then modified during metamorphosis in that they grow axon collaterals that extend to the CC-CA complex. Thus, for adult ecdysis these cells secrete from an anterior site as well as from the proctodeal nerves in the posterior end of the animal. These cells make a major contribution to the EH that is released at adult ecdysis but their relative contribution versus the lateral cells has yet to be determined.

Cells that have the same morphology as the VM cells and that are immunoreactive against the anti-EH antiserum are found in other insects such as Drosophila and the cricket Acheta domestica (Truman and Hewes, unpublished). In dragonflies, electrocautery of cells that correspond in location to the VM cells blocks ecdysis (13). These cells, therefore, seem to be associated with EH even in disparate insect groups. The unusual morphology of the VM cells deserves comment. In descending the length of the CNS enroute from the brain to the proctodeal nerves, these cells have access to all of the central ganglia. Indeed, in the cricket (and less so in Manduca), each ganglion receives a small spray of immunoreactive processes both as the axon enters and as it leaves the ganglion. This anatomical arrangement is consistent with a local release into the CNS as well as secretion into the systemic circulation.

The relative roles of systemically versus locally released hormone in the triggering of ecdysis has been explored for pupal ecdysis in Manduca (Hewes and Truman, unpublished). Prepupae that are partially dissected to expose the hindgut and its proctodeal nerve innervation nevertheless subsequently undergo ecdysis behavior. In such preparations, the hindgut can then be severed at its anterior and posterior borders and reflected back out of the body cavity into an adjacent well but with the proctodeal nerves still attached to the rest of the CNS. At the appropriate time, relative to developmental markers, EH then appears in the well followed about 40 min later by the onset of ecdysis behavior in the carcass. The significance of these results is that the animals are ecdysing at the appropriate time relative to the secretory activity of the VM cells but without the benefit of circulating EH. A

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similar conclusion comes from experiments in which a blood-tight ligature is placed anterior to segment A7, thereby isolating the only known source of circulating EH posterior to the ligature. Bioassays of the blood from these animals fail to show any EH activity, yet their ecdysis behavior is robust and occurs at the predicted time. Lastly, the surgical removal of the proctodeal nerve followed by the "back-firing" of the VM cells by electrical stimulation of the proximal stumps results in precocious activation of ecdysis behavior (about 40 min after the onset of stimulation). All of these results argue that circulating EH is not necessary to trigger ecdysis behavior and that peptide released from the axons that pass through the CNS will suffice.

What, then, is the role of the EH that is released into the circulation? For adult ecdysis, both the intersegmental muscles (14) and the epidermis (15) are peripheral targets that respond to circulating EH. In the case of pupal ecdysis in Manduca, the segmentally arranged Verson's glands release their contents at the time of ecdysis to establish the cement layer (16). These glands are also targets for EH (Hewes and Truman, unpublished). The ligated prepupae, discussed above, show normal ecdysis behavior but fail to secrete material from the Verson's glands. Injection of EH to supply circulating peptide then rescues this secretion. These results argue for the following conclusions. The VM cells apparently release EH not only systemically from the proctodeal nerves but also locally in the CNS from sites associated with the axons as they pass through each ganglion. The anatomical arrangement of the cells thus insures that their firing will distribute the neuropeptide to the entire CNS. The locally released material is sufficient to evoke the ecdysis response on an essentially normal time-course. Hormone that is in the circulation presumably acts back on the CNS to augment the effects of the locally released material, but it is not necessary. Blood-borne EH, however, is essential for the response of peripheral targets such as the dermal glands.

The conclusion that the VM cells release in both a local and a systemic mode leads to the question of which is the older function of these neurons? The VM cells do not belong to the "classic" neurosecretory cell groups in the brain. Rather, they more resemble a class of brain interneurons that descend from the brain through most or all of the CNS. This resemblance is even more striking in more primitive insects such as crickets in which the cells show segmentally arranged processes in each ganglion through which they pass. We suggest that initially the VM neurons may have been descending interneurons whose function was to distribute a unique peptide, eclosion hormone, throughout the CNS at the appropriate time to coordinate the behaviors of ecdysis. Peptide likely leaked from the CNS during such release episodes and, as ecdyses became more complex, peripheral tissues may have begun to use it as a cue to time their ecdysis-related activities. With evolutionary time, this peripheral signal may have then been reinforced by the axons extending out into the periphery (either at the CC-CA and/or the proctodeal nerve) to provide a more reliable circulating signal. In some cases, additional brain cells such as the lateral group Ia cells (10) may have been added to supplement the circulating levels. At present, this evolutionary scenario is largely speculation, but continuing comparative work should be enlightening.

Implications

Eclosion hormone is a unique neuropeptide whose actions are tied to a specific set of behaviors shown at ecdysis. The hormone, in turn, is produced by a gene whose sole product is EH. This gene occurs throughout the insects, and the association of EH with ecdysis behaviors may be an evolutionarily ancient relationship. An intriguing aspect of EH is the small number of neurons that actually express the

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peptide -- 2 pairs in the larval stages of Manduca and only one pair in adults or larvae of Drosophila. This extreme restriction suggests a unique role for this peptide in the behavior of insects.

An obvious question, then, is whether EH is unique in this respect or whether there are other peptides associated similarly with other behavioral changes. EH may be unique in the sense that it coordinates a large number of peripheral events that are associated with ecdysis behavior. Circulating hormone is needed to accommodate these peripheral targets and, accordingly, the few neurons must make sufficient peptide to be distributed throughout the whole body of the animal rather than through the local confines of the CNS. This requirement for greater amounts of peptide resulted in levels of material that made chemical isolation and sequencing feasible. Immunocytochemical studies continue to show an increasing richness of peptides in the insect CNS, a number of which are restricted to 1 or 2 pairs of descending brain interneurons that extend throughout the segmental CNS (unpublished). We think it is highly likely that many of these cells with their unique peptides are key switches (like the VM cells and EH) that chemically alter the CNS to bring about characteristic sets of behavioral responses. A major challenge for the future is determining the nature of the peptides that these cells contain and in deciphering the chemical code used for the regulation of insect behavior.

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Chapter 10

Pheromone Biosynthesis-Activating Neuropeptide

Regulation of Pheromone Production in Moths

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Pheromone production in females of many species of moths is controlled by a hormone, the pheromone biosynthesis activating neuropeptide (PBAN). PBAN was isolated from *Helicoverpa* (*Heliothis*) *zea* and identified as a 33 amino acid peptide. Amidation of the C-terminus is important for the biological activity of this hormone. Oxidation of two Met residues at postitions 5 and 14 enhanced pheromonotropic activity of PBAN. Stability of PBAN in saline can be increased by the substitution of Nle for Met at positions 5 and 14. Biological activity is mostly associated with brain-suboesophageal ganglion and corpora cardiaca of both female and male moths. Terminal abdominal ganglion does not appear to be directly involved in the control of pheromone production. PBAN is slowly degraded in hemolymph. Future possibilities of research on PBAN are discussed.

Mating in most species of moths is mediated through the production and release of species-specific sex pheromones by the females and, in a majority of the moths, reproductive activity is limited to the hours of darkness. To achieve this diurnal periodicity of mating, production of sex pheromone must also be synchronized (1). In the corn earworm, *Helicoverpa* (*Heliothis*) *zea*, and in many other species of moths, this synchronization is affected by a factor produced in the head of the female (2). The factor was identified as a peptide hormone, produced in the suboesophageal ganglion (SOG) and most likely

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released from the corpora cardiaca into the hemolymph at the onset of scotophase which induces pheromone production (3). The hormone has been designated pheromone biosynthesis activating neuropeptide (PBAN) and its pheromonotropic activity was found to be associated with crude extracts of brain-SOG from both females and males. Since males of several species of moths are known to produce complimentary pheromones (4), it is entirely possible that PBAN is also involved in controlling production of these male pheromones.

A key factor which facilitated the isolation and identification of PBAN and the follow-up research was the development of a simple and very sensitive bioassay (2). Briefly, the assay involves ligation of a female between head and thorax and injection of test material into the abdomen of the ligated female. The ovipositor bearing the pheromone gland is excised after 3 hours and extracted in an organic solvent containing an internal standard. The pheromone is quantified by capillary gas chromatography, using the internal standard method of quantitative analysis. PBAN was isolated using a three step chromatographic purification process (5), beginning with several thousand brain-SOG complexes dissected from both male and female *H. zea* moths. The intact peptide was sequenced by automated Edman degradation using a pulsed liquid phase-sequencer (6). The C-terminus was sequenced after carboxypeptidease P digest. Hez-PBAN has 33 amino acids (Figure 1 top), and a mol wt of 3900. Since the first report of PBAN in 1984 several reports have appeared showing similar activities in different species of moths (7-11). Recently, PBAN was isolated and identified from the silkworm, *Bombyx mori* (12). The Bom-PBAN is also made up of 33 amino acid residues of which 27 residues are identical to Hez-PBAN (Figure 1 bottom).

C-terminus of PBAN

Since we could not determine with certainty if PBAN exists as the free acid or amide, both forms of the peptide were synthesized. There was also an error in the assignment of position 23; instead of Asp the peptides were synthesized with Asn. However, it was subsequently shown that this substitution does not significantly affect biological activity. PBAN-OH and PBAN-NH2 were assayed at 1, 10, and 100 pmol doses. Significant pheromonotropic activity was obtained only from PBAN-NH2 at both 10 and 100 pmol doses (Figure 2). Subsequently PBAN-OH was also tested at 1000 pmol. At this dose it caused pheromone production that was not significantly different from pheromone produced in response to PBAN-NH_2 at 10 pmol. Since a single female has <10 pmol of PBAN, the C-terminus amidation appears to be very important for biological activity.

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Hez PBAN

5 10
Leu-Ser-Asp-Asp-Met-Pro-Ala-Thr-Pro-Ala-Asp-Gln-Glu-Met-

15 20 25
Tyr-Arg-Gln-Asp-Pro-Glu-Gln-Ile-Asp-Ser-Arg-Thr-Lys-Tyr-

30
Phe-Ser-Pro-Arg-Leu-NH_2

Bom PBAN

5 10
Leu-Ser-**Glu**-Asp-Met-Pro-Ala-Thr-Pro-Ala-Asp-Gln-Glu-

15 20 25
Met-Tyr-**Gln**-**Pro**-Asp-Pro-Glu-**Glu**-**Met**-**Glu**-Ser-Arg-Thr-

30
Arg-Tyr-Phe-Ser-Pro-Arg-Leu-NH_2

Figure 1. Amino acid sequences of PBAN from *Helicoverpa zea* (Hez-PBAN) top, and *Bombyx mori* (Bom-PBAN) bottom. Amino acids in Bom-PBAN sequence shown in bold letters indicate differences in primary structure compared to Hez-PBAN.

Figure 2. Dose responses of PBAN-Asn_{23}-OH and PBAN-Asn_{23}-NH_2 in *Helicoverpa zea* females. Error bars represent SEM, N=5.

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Oxidation of PBAN

The HPLC retention time of synthetic amidated PBAN was different from that of the natural PBAN. Since Hez-PBAN has two Met residues at positions 5 and 14 it was likely that these residues were oxidized during the purification of the natural peptide. It was reported (13) that oxidation of Met to methionine sulfoxide occurs during the isolation of peptides and proteins from natural sources. Oxidation of the synthetic peptide resulted in a product which was identical to the isolated natural peptide. Dose responses were determined for both reduced and oxidized synthetic PBAN (Figure 3). Reduced PBAN caused a significant pheromonotropic response at 2 pmol which more than doubled at the 4 pmol dose. Significant inhibition of pheromone production was observed at 8 and 16 pmol doses but not at the higher doses of 32 and 64 pmol (Figure 3A). The oxidized PBAN showed significant activity even at 0.5 pmol dose that was similar to the response obtained from 2 pmol of reduced PBAN (Figure 2B). Maximal response from the oxidized PBAN was obtained at the 2 pmol dose, with significant inhibition of biological activity at the 4 and 8 pmol doses (Figure 3B). The reason for higher activity in the oxidized PBAN is not clearly understood, but it is speculated that oxidized PBAN may very well be the active form of the hormone.

We have also noticed a significant to complete loss of activity when PBAN is dissolved in saline and stored in a freezer and thawed before use in the bioassay. In order to determine the effect of oxidation on prolonged biological activity, PBAN-$Nle_{5,14}$ was synthesized. The PBAN-Nle $_{5,14}$ analog was bioassayed at 10 and 100 pmol together with reduced PBAN. At 100 pmol dose the Nle $_{5,14}$ analog was as active as PBAN (Figure 4). However, at 10 pmol the analog had negligible activity. Subsequently Nle $_{5,14}$ analog and PBAN were dissolved separately at 100 and 10 pmol/20 µl of sucrose-phosphate buffer respecitvely. Each solution was divided into 2 parts, one portion was kept at 4°C and the second portion at 30°C. After 24 hr each solution was assayed. At 4°C, there was no loss of activity in PBAN or the Nle $_{5,14}$ analog. However, at 30°C PBAN suffered almost 80% loss in activity compared to only 20% in the Nle $_{5,14}$ analog. Thus PBAN-Nle $_{5,14}$ is more stable in solution and can be used in situations where storage may be necessary, even though it initially has about 10 fold less activity than PBAN.

Distribution of PBAN Activity in Various Tissues

It was reported earlier that PBAN is produced in the SOG (3). Because of significant pheromonotropic activity associated with the corpora cardiaca-corpora allata (CC-CA) complex, it was speculated that PBAN may be released

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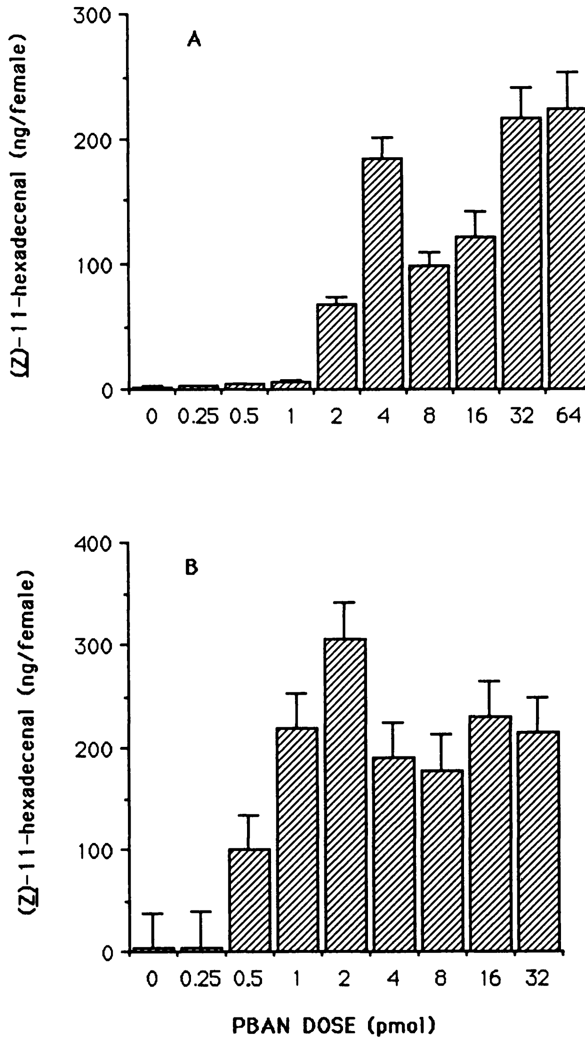

Figure 3. Dose responses of PBAN (A) and oxidized PBAN (B). Error bars represent SEM, N=10. (Figure 3A reproduced with permission from Ref. 6. Copyright 1989 American Association for the Advancement of Science.)

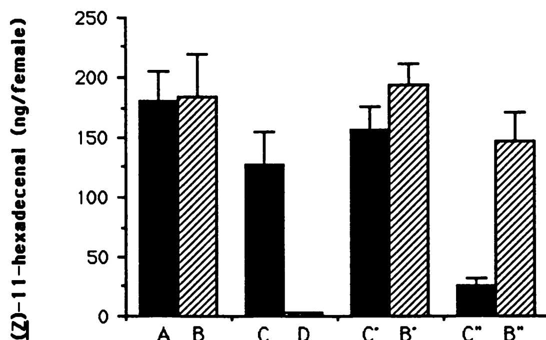

Figure 4. Pheromonotropic activity of PBAN at 100 pmol (A), PBAN-Nle $_{5,14}$ at 100 pmol (B), PBAN at 10 pmol (C), PBAN-Nle $_{5,14}$ at 10 pmol (D), PBAN at 10 pmol after 24 hr storage at 4°C and 30°C respectively (C′and C"), PBAN-Nle $_{5,14}$ at 100 pmol after 24 hr storage at 4°C and 30°C, respectively (B′ and B"). Error bars represent SEM, N=5.

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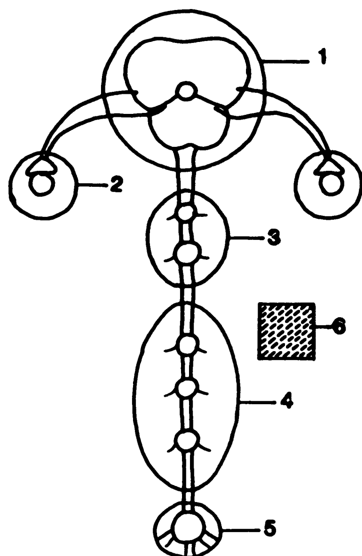
from this neurohemal site into the hemolymph (14). It has been suggested that PBAN may actually be transported through the ventral nerve cord to the teminal abdominal ganglion (TAG) (15), and that a second messenger may be involved between the TAG and site of pheromone biosynthesis in the pheromone gland. To check this possibility, various parts of the nervous systen from a single female *H. zea* female were removed in scotophase, divided as shown in Figure 5, and homogenized in insect saline. In adddition, 5 µl hemolymph from the same female was drawn and mixed with 15 µl of cold saline. The samples were then assayed in ligated females. Results are shown in Figure 5. It is evident that most of the activity is associated with brain-SOG followed by activity in CC-CA. Thoracic ganglia and various ganglia of the ventral nerve cord and hemolymph had very low activity. The low activity in hemolymph may be attributed presumably to the small amount of PBAN released to maintain pheromone production as well as to the ratio of hemolymph removed to that of total circulating hemolymph.

In another experiment, TAG was removed from virgin ligated females 1 hour before the 3rd scotophase. Three hours after surgery, females were injected with 10 pmol of PBAN in 20 µl sucrose-phosphate buffer. Sham operated females were used as controls. Pheromone was extracted after 3 hours. There was no significant difference in



Tissue	(Z)-11-hexadecenal ng/female ± SEM, N=8
1.	112.0 ± 14.7
2.	17.2 ± 2.5
3.	2.3 ± 0.6
4.	1.7 ± 0.4
5.	1.2 ± 0.2
6.	3.7 ± 1.2

Figure 5. Pheromonotropic activity associated with (1) brain-suboesophageal ganglion, (2) corpora cardiaca-corpora allata (3) thoracic ganglia (4) three anterior abdominal ganglia (5) terminal abdominal ganglion and (6) hemolymph

pheromone titer between the two groups (Table I), indicating that TAG is not important for pheromone production. However, the existence of a second messenger is not ruled out because the nerves leading from TAG to the pheromone gland were still intact and could have evoked the necessary response. In fact, in a recent study (Soroker, V.; Kamensky, B.; Raina, A. K.; Rafaeli, A. J. Insect Physiol., in press), it has been shown that intracellular c-AMP levels in the pheromone glands of *H. armigera* without the TAG, when incubated *in vitro* with Hez-PBAN, increased by 230% over control glands.

Table I. Role of terminal abdominal ganglion in pheromone production in ligated *Helicoverpa zea* females

Treatment	(Z)-11-hexadecenal ng/female ± SEM (N)
TAG Removed + 10 pmol PBAN	164.8 ± 12.9 (8)
Sham Operated + 10 pmol PBAN	172.3 ± 17.6 (5)

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To further demonstrate the possible release of PBAN from CC, the CC-CA complexes were dissected from scotophase and photophase females, homogenized in saline and assayed at 1 CC-CA eq/female for pheromonotropic activity. The experiment was also carried out with CC-CA from both scotophase and photophase males. Results are presented in Table II. It is evident that there is higher activity in females during the scotophase indicating that CC do not act as a storage site but as a release site. Presence of similar activities in CC of scotophase and photophase males may be due to the absence of a well defined diel periodicity of pheromone production and sexual receptivity in males as compared to that in females. For example *H. zea* females do not produce or release pheromone in the photophase, but if a calling scotophase female is brought in the vicinity of photophase males, such males do evert their hair pencils (a probable mechanism for dispensing pheromone) and attempt to mate with the female.

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Table II. Pheromonotropic activity asociated with the corpora cardiaca of female and male *Helicoverpa zea* adults

Donor	Phase	(Z)-11-hexadecenal ng/female ± SEM (N)
Female	Photophase	8.7 ± 1.3
	Scotophase	23.6 ± 6.3
Male	Photophase	27.1 ± 2.1
	Scotophase	30.2 ± 6.9

Stability of PBAN in Hemolymph

To determine as to how long PBAN would persist in the hemolymph, a ligated female was injected with 100 pmol of PBAN in 40 µl saline. Immediately after injection, and every 30 min thereafter, 5 µl hemolymph was drawn from this female, mixed with 15 µl cold saline and assayed inligated females. The results are shown in Table III. Significant activity was recovered in hemolymph immediately following injection of PBAN, which indicates rapid dispersal of the peptide. After 30 min, the pheromonotropic activity was highest. It decreased significantly after 1 hour but persisted at about the same level through the next hour. The results indicated a low rate of degradation of PBAN in hemolymph.

Table III. Stability of synthetic PBAN *in vivo*

Time (min) after injection of PBAN into the donor	pheromonotropic response of the recipient (Z)-11-hexadecenal ng/female ± SEM (N=5)
0	37.7 ± 3.5
30	136.6 ± 10.3
60	70.0 ± 7.0
90	30.8 ± 6.2
120	53.0 ± 5.5
Donor	271.7 ± 40.3

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Future research on PBAN

Elucidation of the primary structure of PBAN opened up a new field of research with immense possibilities. The exact site and the specific neurosecretory cells that produce PBAN need to be identified. We also need to determine where, when, and how PBAN is released. Development of antibodies for their use in immunohistochemistry should prove useful. A radioimmunoassay for PBAN will be useful to more precisely detect the hormone in various tissues such as hemolymph, and follow the time-course of release and determine PBAN titer at various times of the day. Identification of the receptors for PBAN is crucial to the study of its mechanism of action (how does PBAN induce pheromone production?).

There is strong evidence to suggest that the 33 amino acid Hez-PBAN may actually be a prohormone. A smaller portion of the molecule can induce pheromone production in ligated females (Raina, A. K.; Kempe, T. G. Insect Biochem., in press). Further studies on structure activity relationship can lead to the development of analogs that may act as superagonists or antagonists of PBAN. Based on that information one can then design PBAN mimics for practical use in disruption of normal reproductive behavior of various pest species.

We also need to identify the gene for PBAN. The gene can provide additional information about the nature of pro-PBAN (if it is different from PBAN), and also allow us to clone it in a baculovirus. A melanization and reddish coloration hormone (MRCH) was isolated from the head extracts of *B. mori* (16). Partial structure determination of the MRCH revealed that the amino acid sequence of this hormone and that of PBAN from *B. mori* are identical (12). It has also been observed that Hez-PBAN causes intense melanization in *H. zea* larvae (Raina, unpublished). Such larvae either die or produce deformed pupae. Infestation of *H. zea* and possibly larvae of other species of moths with the recombinant virus containing the PBAN gene may be

used to enhance mortality normally caused by the virus. The symptom can also be used to detect expression of the peptide gene. Research on several aspects mentioned in this section is already in progress in our laboratories and the preliminary results are very encouraging.

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Chapter 11

Insect Neuropeptides

Influence on Color Change in Insects and Chromatophoral Pigment Movements in Crustaceans

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This report focuses on the identification and sequence relations of the insect neuropeptides that can trigger color changes in crustaceans, with emphasis on the newly identified family of pigment-dispersing peptides. The latter show no relation to the insect melanization and reddish coloration hormone (MRCH) and are distinct from the adipokinetic hormone (AKH) family peptides, related to the crustacean red pigment concentrating hormone (RPCH). The pigment-dispersing factors (PDFs), identified from the insects *Romalea microptera* and *Acheta domesticus*, as well as the pigment-dispersing hormones (PDHs) of Crustacea, are octadecapeptides sharing several features. The function of PDFs in insects is unknown, although immunocytochemical localization points to a neuromodulatory role. In orthopterans PDH-immunoreactive neurons are located in the optic lobes, with fiber projections into the brain as well as arborizations in the optic lobes. Enzyme-linked immunosorbant assays (ELISA) confirm the optic lobes as a rich source of immunoreactive PDF in *Romalea*.

Among arthropods, crustaceans are well known for their ability to display reversible integumental color changes and eye pigment movements. The former are brought about by dispersion or concentration (aggregation) of pigment granules within epithelial chromatophores. Eye pigment movements, occurring during the course of light or dark adaptation, involve the migration of screening pigment granules within photoreceptor cells (retinular cells) and in certain extraretinular cells associated with the ommatidia. Whereas the pigment movements within the retinular cells appear to be directly influenced by conditions of illumination, the pigmentary responses of extraretinular

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eye pigment cells and epithelial chromatophores in Crustacea are controlled by neurosecretory hormones (*1-3*). These hormones are readily separable into two sets: the hormones responsible for chromatophoral pigment concentration and ommatidial dark adaptation belong to one set, and they are distinct from those causing chromatophoral pigment dispersion and ommatidial light adaptation (*2*, *4*).

Many insects show light/dark-adaptive eye pigment movements (*5*), but only a few species can undergo rapid integumental color changes (*6*). Screening pigment movements in insect compound eyes were once thought to be under nervous control, but recent evidence points to a direct light-induced pigment migration in retinular cells (*7*) and in extraretinular eye pigment cells (*8*). In contrast to Crustacea, there is no evidence for hormonal control of eye pigment movements in insects.

Neurohormones are, however, implicated in the control of color changes in insects. The somewhat slow, morphological color changes resulting from qualitative or quantitative alterations in pigmentation are regulated by multiple hormones--juvenile hormone, ecdysone, and neurohormones--the interplay of which seems to vary with the insect species (*6*).

This paper summarizes current knowledge of the chemistry of neurohormones involved in the regulation of color changes in insects, as well as the nature of other insect neuropeptides with chromatophorotropic actions in Crustacea--with emphasis on pigment-dispersing peptides.

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Melanization and Reddish Coloration Hormone (MRCH)

Among the neurohormones regulating insect pigmentation, primary structure is known for only MRCH. This hormone promotes the formation of melanin in the cuticle and ommochromes in the epidermis of armyworm species (*9*, *10*). Multiple forms of MRCH have been isolated from the heads of adult *Bombyx mori* (*11-13*) and one of these, MRCH-I, was found to be structurally identical to PBAN-I (pheromone biosynthesis activating neurohormone) isolated from the same source (*14*). Although this hormone acts only as PBAN in *Bombyx*, the same molecule serves dual functions in the cutworm *Spodoptera litura* by promoting cuticular melanization in the larval stage and sex pheromone production in the adult stage (Matsumoto, S.; Kitamura, A.; Nagasawa, H.; Kataoka, H.; Orikasa, C.; Mitsui, T.; Suzuki, A. *Insect Biochem.*, in press).

The PBAN-I (MRCH-I) isolated from *Bombyx* is structurally related to the PBAN characterized from *Heliothis zea* (*15*; see also article by Raina *et al.*, this volume). As indicated below, these peptides have common termini (N-terminal Leu, C-terminal Leu-amide), conserved chain length (33 residues), and show residue differences at only 7 positions.

Heliothis: LSDDMPATPADQEMYRQDPEQIDSRTKYFSPRLamide
Bombyx: LS*E*DMPATPADQEMY*QP*DPE*EME*SRT*R*YFSPRLamide

The N-terminal sequence of MRCH/PBAN shows some similarity to a portion of insulin-like growth factor-II, whereas the C-terminal sequence of MRCH/PBAN is nearly identical to the C-terminal pentapeptide sequence of leukopyrokinin (pGlu-Thr-Ser-Phe-Thr-Pro-Arg-

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Leu-amide) isolated from cockroach heads (*16*). MRCH/PBAN peptides do not resemble any of the crustacean chromatophorotropins, and the former peptides may not be active on crustacean chromatophores and eye pigment cells. It remains unknown whether MRCH influences rapid color changes brought about by intracellular pigment migration in insects.

Control of Rapid Color Changes in Insects

Rapid color changes, caused by pigment migrations within chromatophores or within epidermal cells, are displayed by only a few species of insects, e.g., *Corethra* and the stick insect *Carausius*. Ameboid chromatophores associated with anterior and posterior tracheal bladders contribute to color change in *Corethra*. Migration of the dark ommochromes from the base of the epidermal cell to its surface causes a rapid color change, body darkening, in *Carausius*. A darkening neurohormone produced in the brain is implicated in the control of these rapid color changes (*17-19*). This chromactivating hormone is cross-reactive: the extracts of *Corethra* heads cause darkening in *Carausius*, and *Carausius* organ extracts are active on *Corethra* as well as on *Carausius*. These reactions can also be triggered by brain extracts from a variety of insects lacking color change, pointing to a wide distribution of the darkening hormone among insects (*18*). Attempts have been made to characterize this hormone (*20*, *21*), but its structure remains unknown.

Differentiation of Chromactive Factors in Insect Nervous Tissues

Besides the insect darkening hormone discussed above, the insect nervous tissues contain factors which can trigger color changes in crustaceans. Initial studies examined the ability of insect tissue extracts to cause body blanching (due to chromatophoral pigment concentration) in prawns and shrimp (*22*). This blanching substance was found in the corpora cardiaca of many insect species (*23-25*), whereas the substance causing darkening in insects (*Corethra*, *Carausius*) was present in the central nervous system. Initial separation by paper electrophoresis (*26*, *27*) indicated that the observed activities are due to two distinct substances: Factor A, causing concentration of chromatophoral pigments in crustaceans and showing only a slight effect on color change in *Carausius*; Factor C, which lacks pigment-concentrating effect in crustaceans but causes darkening in *Carausius*. Factor A is present in crustacean sinus glands and insect corpora cardiaca, whereas Factor C is found in the brain of *Carausius* and absent in the corpora cardiaca (*26*, *27*).

Peptides of the RPCH/AKH Family

The similarity of the crustacean blanching substance (Factor A) found in crustacean sinus glands and insect corpora cardiaca has been confirmed by the structural identification of a family of neuropeptides common to arthropods. The first identified member of this family is RPCH (red pigment concentrating hormone), an octapeptide (pGlu-Leu-Asn-Phe-Ser-Pro-Gly-Trp-amide) isolated from eyestalks of the shrimp *Pandalus borealis* (*28*). Shortly thereafter, a related peptide--designated as adipokinetic hormone (AKH)--was purified from locust

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corpora cardiaca and shown to be a decapeptide: pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-amide (*29*). Besides their sequence similarity, the two peptides were found to be active in cross-tests. Initial tests indicated that RPCH and AKH are able to mimic each other and elicit hyperlipemia in locusts and butterflies, hyperglycemia in cockroaches, and concentration of chromatophore pigments in Crustacea (*29-32*). The relative potencies of the peptides varied with the test system, each hormone being generally more reactive in its own system.

The sequence of RPCH is identical among the crustacean species examined, the shrimp *Pandalus borealis* (*28*) and the crabs *Cancer magister* and *Carcinus maenas* (*33*). In contrast, as reviewed by Keeley *et al.* in this volume, the AKH-related peptides contain 8 to 10 residues and show considerable sequence heterogeneity, with as many as 13 different peptides identified so far from various insect species (*34*, *35*). Despite this heterogeneity, the RPCH/AKH peptides are members of a family with the following common features: pGlu as the blocked amino-terminus, Phe at position 4, Trp at position 8, and an amidated carboxyl terminus. The AKH-related peptides show varied functions in insects--hyperglycemia, hypertrehalosemia, hyperlipemia, cardioacceleration, myotropic action, or a combination of these roles--depending on the species. Thus, while maintaining some critical degree of sequence similarity, these peptides evolved appropriate structural modifications to serve unique functions in different species. It remains unclear whether the AKH-related peptides aid in the control of pigmentation or pigmentary migrations in insects.

Pigment-Dispersing Actions of Insect Nervous Tissue Extracts on Crustacean Chromatophores

Early workers noted that insect head extracts cause not only chromatophoral pigment concentration in shrimp and prawns, but also chromatophoral pigment dispersion in crabs (*22*). The insect factors responsible for the latter action did not receive much attention until recently. The discovery of structural similarity of RPCH and AKH (*28*, *29*) has triggered renewed interest in determining the relationship of insect pigment-dispersing factors and crustacean pigment-dispersing hormones.

In tests for melanophore pigment dispersion in eyestalkless individuals of the fiddler crab *Uca pugilator*, head extracts from the cockroach *Periplaneta americana* and the honey bee *Apis mellifera* showed strong activity, as did the crustacean eyestalk extracts (*36*). Head extracts of the butterfly *Danaus plexippus* and the mealworm *Tenebrio molitor* were, however, inactive in the chromatophoral dispersion assay.

In gel filtration chromatography, the pigment dispersing hormone (PDH) activity of *Uca* eyestalk extracts emerged in a single peak, whereas the activity of *Periplaneta* head extracts eluted in two peaks with apparent molecular masses of 3500 and 1000 daltons. The *Uca* PDH and the larger factor from *Periplaneta* showed nearly identical elution profiles. Despite this apparent similarity, the corresponding factor from *Periplaneta* did not seem to be identical to *Uca* PDH; the former was oxidation-resistant (*36*), whereas the latter was oxidation-sensitive (*37*). The factor from *Apis* appeared to be an oxidation-sensitive larger polypeptide, about 20,000 daltons (*36*). These initial

evaluations suggested that the pigment-dispersing factors are an assemblage of heterogeneous polypeptides.

Characterization of Pigment-Dispersing Factors in Insects

Large-scale isolation and purification of pigment-dispersing factors (PDFs) was undertaken in our laboratory, utilizing lyophilized heads of the cricket *Acheta domesticus* (*38*, *39*) and the lubber grasshopper *Romalea microptera* (*40*) as starting material, and identifying the active compound by assays for melanophore pigment dispersion in eyestalkless *Uca*. The protocols employed were a combination of methods proven successful for the isolation of PDHs from crustacean eyestalks (*41*, *42*).

The insect PDFs were isolated as follows. Lyophilized heads (100-150 g lots) were powdered and extracted in boiling water. The extract was cooled, acidified (by addition of acetic acid up to 5%), centrifuged, and the supernatant subjected to batch chromatography with Bio-Rex 70, a weakly acidic cation-exchange resin (Bio-Rad). The resin was added to the extract, the mixture stirred for 4 to 6 hours, and then poured into a column. The PDF adsorbed to the resin was recovered by elution with 50% acetic acid. About 90-95% of the PDF activity in the original extract could be recovered by this batch chromatography. This material was taken up in water and extracted with the organic phase of 1-butanol/ 0.1 M NH_3 (1:1, v/v). Repetitive extraction with the organic phase (up to five times) brought most of the PDF into this phase, which was then dried and subjected to an additional liquid-partitioning step. The dried material was taken up in 0.2 M HCl and partitioned with cyclohexane/butanol (4:1, v/v). Most of the PDF activity was retained in the aqueous phase, and this was lyophilized and further purified by a series of chromatographic steps: a) gel filtration, Sephadex G-25-50, eluant 1 M acetic acid; b) ion-exchange chromatography, CM-Sephadex C-25-120, eluant 0.075 M ammonium acetate pH 4.9; c) partition chromatography, Sephadex G-25-50, gel bed in an aqueous phase of butanol/acetic acid/water (4:1:5, v/v), eluant organic phase of the solvent system; d) reverse-phase HPLC, Whatman ODS-2, 30-45% acetonitrile with 0.1% TFA over a 30-minute gradient.

With head extracts from *Acheta* (*37*), as well as *Romalea* (*39*), the gel filtration step yielded two peaks of pigment-dispersing activity, emerging at about 0.5 and 0.8 column volumes, respectively. The first peak coincided with the elution profile of PDH from *Uca*; the PDF in this zone, like the *Uca* PDH, caused dispersion of chromatophoral pigments in *Uca* and did not cause pigment concentration. The second active peak, emerging at about 0.8 column volume in gel filtration, triggered not only melanophore pigment dispersion but also leucophore (white) pigment concentration in *Uca*. The two biological activities were inseparable from each other, even after ion-exchange chromatography, partition chromatography, and reverse-phase HPLC. This factor can be called pigment-dispersing/concentrating factor (PDCF).

Initial sequencing attempts with HPLC-purified *Acheta* PDCF indicated a blocked amino-terminus. It was deblocked by pyroglutamyl amino peptidase and subjected to gas-phase sequencing. The resulting data, along with spectral analysis, indicated that the purified peptide has the sequence: pGlu-Val-Asn-Phe-Ser-Thr-Gly-Trp-amide. This was also the deduced sequence for *Gryllus* AKH (*43*). The synthetic peptide,

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however, failed to induce melanophore pigment dispersion or leucophore pigment concentration. This indicates that the melanophore-dispersing/leucophore-concentrating chromatophorotropin is only a minor component of the purified material, and alternative strategies have to be developed for separating it from the *Gryllus/Acheta* AKH octapeptide. Our evaluations indicate that the corpora cardiaca are a richer source of PDCF in *Acheta*, and we hope to reinvestigate this peptide.

Fortunately, the PDFs from *Romalea* (*40*) and *Acheta* (*38*, *39*) were amenable to purification and characterization. The final yield of PDF was higher from *Romalea* (11 nmol from 3,000 heads) than *Acheta* (<0.3 nmol from 10,000 heads). We encountered difficulties in sequencing the latter peptide. Initial sequencing enabled the assignment of residues 1 to 12 for *Acheta* PDF (Asn-Ser-Glu-Ile-Ile-Asn-Ser-Leu-Leu-Gly-Leu-Pro), and a subsequent analysis permitted the identification of the C-terminal pentapeptide sequence for residues 14 to 18 as Val-Leu-Asn-Asp-Ala (*39*). Residue 13 could not be directly identified, but was assumed to be Lys or Arg in view of the peptide's susceptibility to trypsin. HPLC comparison of synthetic peptides showed that the Lys-13 analog displayed an identical retention time with the native peptide, whereas the Arg-13 analog eluted 0.6 min later. This indicated the presence of Lys at position 13, and thus enabled us to establish the octadecapeptide sequence of *Acheta* PDF.

Sequencing of *Romalea* PDF was straight forward, and it also proved to be an octadecapeptide (*40*). The *Romalea* peptide differs from *Acheta* PDF at a single position, the former having Leu-14 in place of Val-14. In these peptides the presence of Asp-Ala bond, positions 17-18, permitted the cleavage of the C-terminal residue by heating in dilute acid. HPLC analysis of the derivatized cleaved product enabled the identification of the C-terminus as Ala-amide.

Sequence Similarities of Insect Pigment-Dispersing Factors and Crustacean Pigment-Dispersing Hormones

Utilizing eye pigment migraton in the shrimp *Palaemon* as an assay, the major form of a light-adapting distal retinal pigment hormone was isolated from eyestalks of the shrimp *Pandalus borealis* (*41*) and identified as an octadecapeptide: Asn-Ser-Gly-Met-Ile-Asn-Ser-Ile-Leu-Gly-Ile-Pro-Arg-Val-Met-Thr-Glu-Ala-amide (*44*). This peptide can induce not only ommatidial light adaptation, but also chromatophoral pigment dispersion (*45-47*), and it is referred to as α-PDH in the recent literature. Another form of PDH, designated ß-PDH, which differs from α-PDH at six positions, was isolated from the fiddler crab *Uca pugilator* (*42*) and the shore crab *Cancer magister* (*48*). Since then peptides closely similar to ß-PDH have been identified from the brown shrimp *Penaeus aztecus* (*49*) and the crayfish *Procambarus clarkii* (*50*). Three forms of PDH--a variant of ß-PDH, a variant of α-PDH, and α-PDH--have been characterized from the shrimp *Pandalus jordani* (*51*).

As summarized in Table I, the identified crustacean PDHs are separable into two groups based on their net charge: α-PDH and its analogs, which have Gly at position 3; ß-PDH and its analogs, which are more acidic than the former because of Glu at position 3. The latter peptides are more widely distributed, whereas α-PDH has been found so far in species of *Pandalus*.

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Table I. Primary Structures of Pigment-Dispersing Peptides

Species	*Reference*	*Sequence*
Variants of ß-PDH		
Uca pugilator	42	NSELINSILGLPKVMNDA amide*
Cancer magister	48	NSELINSILGLPKVMNDA amide*
Procambarus clarkii	50	NSELINSILGLPKVMN*E*A amide
Penaeus aztecus	49	NSELINS*L*LG*I*PKVMNDA amide
Pandalus jordani	51	NSELINS*L*LGLPKVM*T*DA amide
Acheta domesticus	39	NSE*I*INS*L*LGLPKV*L*NDA amide
Romalea microptera	40	NSE*I*INS*L*LGLPK*LL*NDA amide
Variants of α-PDH		
Pandalus borealis	44	NSGMINSILGIPRVMTEA amide**
Pandalus jordani	51	NSGMINSILGIPRVMTEA amide**
Pandalus jordani	51	NSGMINSILGIP*K*VM*AD*A amide

* ß-PDH
**α-PDH

Based on net charge and sequence similarity, insect PDFs are more closely related to ß-PDH than to α-PDH. The *Acheta* PDF and *Romalea* PDF show 83% and 78% sequence similarity, respectively, to ß-PDH.

The insect PDFs and crustacean PDHs constitute a peptide family common to arthropods. This peptide family shows the following common features: amino-terminal Asn, carboxyl-terminal Ala-amide, conserved chain length (18 residues), and at least 50% sequence similarity. The PDH family peptides have no sequence relationship to any other hormonal peptides or proteins.

Relative Potencies of Insect PDFs and Crustacean PDHs

Since the function of PDFs in insects is unknown, they have been tested for melanophore pigment dispersion in the fiddler crab. In this assay *Acheta* PDF and *Romalea* PDF were equipotent, and both were about 50% as potent as fiddler crab's PDH (ß-PDH) and 10-fold more potent than α-PDH. The *Acheta* PDF and *Romalea* PDF differ from each other at position 14, with Val in the former and Leu in the latter. This substitution did not alter the melanophorotropic potency of the peptide. When compared to ß-PDH, *Acheta* PDF has three residue substitutions: position 4 (Ile for Leu), position 8 (Leu for Ile), and position 15 (Leu for Met). The substitution at position 8 does not seem to affect potency, because *Penaeus* PDH which has Leu-8 is as potent as ß-PDH.

The presence of Met at positions 4 and 15 in α-PDH and at position 15 in ß-PDH renders these peptides oxidation-sensitive. Replacement of Met-4 by Leu-4, as seen in ß-PDH, affords partial protection and leads to increased potency (*52*). Substitution of norleucine for Met-15 in ß-PDH (*53*) and for Met-4 and Met-15 in α-PDH (*54*) imparts oxidation-resistance and superpotency to these peptides. In view of this, the insect PDFs which are oxidation-resistant (due to

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Ile-4 and Leu-15) would be expected to be superpotent. Their 2-fold lower potency relative to ß-PDH is intriguing. The critical substitution counteracting superpotency and causing reduced potency is most likely the Ile-4, but this needs to be evaluated by tests with synthetic analogs.

Cytochemical Localization of PDH Immunoreactivity

An antiserum raised against synthetic ß-PDH (*55*) permitted immunocytochemical localization of reactive soma and tracts in crustacean (*55-57*) and insect nervous systems (*58*, *59*). In the grasshopper *Romalea microptera* (*58*), PDH-immunoreactive (PDH-IR) cells were found in the optic lobes but not in the brain. The optic lobe PDH-IR cells were in three groups: two groups near the posterior ventral and dorsal margins of the lamina, and the third group between the lobula and medulla. Fiber extensions from the former group were localized in the lamina and medulla, and projections could be seen in between optic cartridges. PDH-IR fibers and arborizations were also evident in the brain. The PDH-IR cellular distribution, as noted in *Romalea*, has recently been confirmed in several other orthopterans: locusts, *Schistocerca gregaria*, *Locusta migratoria*; crickets, *Acheta domestica*, *Gryllus bimaculatus*, *Teleogryllus commodus*; phasmid, *Extatosoma tiaratum*; and the cockroach, *Periplaneta americana* (Würden, S.; Homberg, U.; Dircksen, H.; Rao, K.R., in press). These investigators also found that the position and branching pattern of the PDH-IR cell group located between the lobula and medulla are the same as those proposed for circadian pacemaker neurons in crickets and cockroaches (*60*).

In contrast to the orthopterans, PDH-IR cells are distributed widely in the optic lobes, brain, and subesophageal ganglion of *Manduca sexta* (*59*). This differential distribution may reflect a diversity of functions which remains to be explored.

Immunoassay of PDF in Grasshopper Nervous Tissues

Immunocytochemical localization of PDF in insect tissues, as described in the previous subsection, was accomplished with an antiserum for crustacean ß-PDH. Evaluations by enzyme-linked immunosorbant assays (ELISA) revealed that the affinity of ß-PDH antiserum towards insect PDFs was 13-21%, relative to ß-PDH (*61*). An antiserum raised against crustacean α-PDH (*62*) showed much lower affinity towards insect PDFs, 0.04-0.12%.

More recently we prepared an antiserum against synthetic PDF of *Romalea*, and evaluated it by ELISA (Figure 1). This antiserum showed greater affinity for insect PDFs than towards crustacean α-PDH or ß-PDH. The IC-50 for *Romalea* PDF was about 50 fmol/well in ELISA. We have utilized this ELISA for estimating the quantitative distribution of PDF in the nervous tissues of *Romalea*.

Various parts of the nervous system were dissected, collected on dry-ice, lyophilized, and stored desiccated in a freezer until use. The tissues were extracted in acidified methanol (50% methanol with 0.1% acetic acid) and the extracts were dried by Speed-Vac, taken up in phosphate-buffered saline, and evaluated by an ELISA procedure described earlier (*63*). The results, shown in Table II, indicate that

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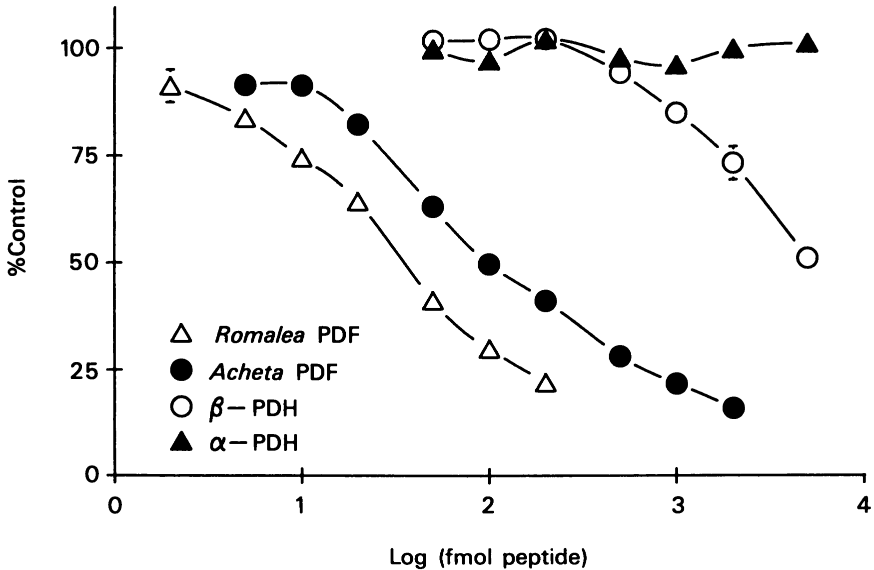

Figure 1. Dose-response antigen inhibition curves derived from ELISA (enzyme-linked immunosorbant assay), utilizing an antiserum raised against synthetic pigment-dispersing factor (PDF) of *Romalea*. Assay conditions: antigen coat, 200 fmol *Romalea* PDF/well; primary antibody dilution 1:140,000; secondary antibody (peroxidase-labelled antirabbit IgG), 1:1000; other details as described earlier (*63*). When no *Romalea* PDF is present in the assay, the obtained value is defined as 100%.

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PDF could not be detected in corpora cardiaca, subesophageal ganglia, thoracic nerve cord, and abdominal nerve cord. The optic ganglia are the richest source of PDF, the content in the brain being about 10-fold lower. This quantitative distribution is in concert with the finding of PDH immunoreactive cells in the optic lobes of *Romalea* (*58*). There was no significant sex-related difference in PDF content in the tissues examined (Table II).

Table II. Immunoreactive PDF in the Central Nervous System of the Lubber Grasshopper

	Mean ± S.D.	
Structure (N)[*]	*pmol/mg dry weight*	*pmol/structure*
Optic Lobes		
Male (5)	27.6 ± 6.3	48.2 ± 14.3[**]
Female (3)	18.1 ± 9.7	30.5 ± 13.4
Brain (without optic lobes)		
Male (5)	3.7 ± 1.6	3.3 ± 1.6
Female (3)	3.0 ± 1.0	3.1 ± 0.6
Corpora cardiaca	ND	ND[f]
Subesophageal ganglia	ND	ND
Thoracic nerve cord	ND	ND
Abdominal nerve cord	ND	ND

[*] N = Number of samples (5 animals/sample)
[**] Amount per pair of optic lobes
[f] ND: not detected; detection limit was 25 fmol/structure

The distribution of PDF is different from that of AKHs. The latter are present in high quantities in the corpora cardiaca--e.g., 450-1200 pmol per locust corpora cardiacum (*64*, *65*)--and at a much lower level in the brain (*64*).

When compared to the PDF content in brain plus optic lobes of grasshopper, 30-50 pmol/individual, most of the other neuropeptides occur in relatively low amounts: e.g., proctolin, 0.47-1.5 pmol/brain in *Schistocerca* and *Acheta* (*66*); 0.06-0.48 pmol leucokinins/head, 1.36 pmol leucopyrokinin/head, in *Leucophaea* (*34*).

Perspectives

Like the AKH/RPCH peptides, insect PDFs and crustacean PDHs are now an established family of neuropeptides common to arthropods. Whereas the functions of AKHs are varied and well defined in insects, the function of PDFs in insects remains unknown. The immunocytochemical distribution of PDF in the cephalic nervous tissues of orthopteran insects points to a neuromodulatory role, with potential functions in circadian rhythms; this would be an exciting area for further research.

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The relationship of insect PDFs to the hitherto uncharacterized insect-darkening factors, responsible for rapid color changes in *Corethra* and *Carausius*, merits examination. Critical tests have to be made to determine whether AKHs serve any role in the control of insect pigmentation. It is equally important to explore the extrapigmentary functions of PDH and RPCH in crustaceans, and progress is being made in this area. A neuromodulatory role has been demonstrated for RPCH (*67*), and suggested for PDH-like peptides (*68*), in the control of the motor system of the crustacean stomatogastric ganglion. RPCH seems to have a modulatory role in the control of crayfish swimmeret rhythm (*69*). RPCH and PDH are reported to have stimulatory and inhibitory roles, respectively, in the secretion of methyl farnesoate from crustacean mandibular glands (*70*).

Notwithstanding the mystery about their function in insects, PDH-related peptides should be identified from a wide variety of insects as well as other arthropods, and the genetic basis of their sequences will also have to be determined. The distribution and evolution of this peptide family are fruitful areas of investigation.

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Chapter 12

Egg Development Neurosecretory Hormone Activity in the Mosquito *Aedes aegypti*

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Egg development neurosecretory hormone (EDNH) is a bioactive neuropeptide responsible for the stimulation of ovarian ecdysteroid production in Aedine mosquitos. A consequence of this steroid production is the development of mature ovarian follicles. A brief history of the identification of EDNH and its relationship with juvenile hormone (JH) and ecdysone is presented. Current methods of isolation of the hormone involving high performance liquid chromatography (HPLC) and *in vivo* and *in vitro* bioassays are described. The existence of multiple molecular forms of EDNH defined by molecular weight (6.5 kD - 24 kD), retention by reverse phase-HPLC (RP-HPLC) and elution by ion exchange chromatography are discussed. EDNH activity in non-cerebral tissue is reported and prospects for the resolution of questions of multiple forms and their physiological significance are presented.

Egg development in Anophiline mosquitos begins at the time of adult emergence. Ovarian follicles double in diameter within the next 24 hours at which point two routes to maturation are available. In autogenous species (*e.g., Aedes atropalpus*), ovarian development proceeds uninterrupted so that by 72 hours after emergence mature vitellogenic eggs are present. In anautogenous species (*e.g., Aedes aegypti*), ovarian development proceeds for 24 hours after eclosion and is then arrested. The follicles remain at the resting stage until they are further stimulated by a peptide factor (2), the egg development neurosecretory hormone (EDNH), produced in the medial neurosecretory cells of the brain and released from the corpus cardiacum following a blood meal. Presumably, the same or a very similar factor stimulates ovarian development in autogenous species as well. Preceeding and following the mobilization of EDNH are, respectively, the action of juvenile hormone (JH), which is necessary for the preparation of ovaries to respond to EDNH, and ecdysone, which is involved in the post-EDNH production of yolk proteins.

The requirement of internal secretions for the successful development of mature follicles was recognized by Detinova (1) in studies on Anophiline mosquitos. These studies implicated the corpora allata as sites of secretion of a necessary hormonal factor (*i.e.*, JH). Clements (2) expanded upon Detinova's

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observations, and examined the effects of decapitation and ligation on ovarian development in both autogenous and anautogenous species. He concluded that an internal secretion was indeed necessary for ovarian development, and that the timing of the secretory release showed interspecific variation. Gillett (3) examined 4 anautogenous *Aedes aegypti* strains and found that in each a critical period of up to 7 hours after blood feeding existed during which decapitation prevented ovarian maturation. Since the corpora allata were presumably undisturbed, these observations suggested that a factor from the head, assumed to arise from brain neurosecretory cells (3), was required for ovarian maturation. Thus, in addition to the corpora allata factor released following adult emergence, a second factor, released after bloodfeeding, was equally important. Transfusion experiments (4) demonstrated that both factors were indeed hormonal. The temporal differences in their effects were illustrated by Lea (5) and the corpus cardiacum was identified as the storage/release site of the brain factor.

The secretions of the corpus allatum and the corpus cardiacum are, respectively, JH (6,7) and EDNH (8,9). Significantly, these hormones are released in sequence (For reviews, see references 10,11), with juvenile hormone stimulating both the development of follicles to the resting stage (6,7) , and rendering the fat body competent to respond to ecdysone (37). In response to EDNH (15,16), ecdysone is secreted by, and presumably synthesized in, the ovary (12-14). The ecdysone is then involved in vitellogenesis and ovarian maturation (10,11,17,18). The demonstration that mosquito ovaries were capable of secreting ecdysone (12) and that 20-hydroxyecdysone could stimulate ovarian maturation in decapitated mosquitos (14,18) formed the basis for development of two important bioassays (15,19) for monitoring EDNH activity during extraction and purification procedures. In our laboratory, we use both the *in vivo*, follicle maturation bioassay (19,20) and the *in vitro*, ovarian ecdysteroid synthesis bioassay (15,16), in modified forms, to detect EDNH activity.

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Bioassays and Tissue Extraction

The *in vitro* bioassay (15,16) has been modified in order to increase the rate of sample analysis. The minimum number of resting-stage ovaries required to give a reliable response to stimulation by brain extract was determined by incubating different numbers of ovaries in a fixed concentration of head extract. Total detectable ecdysteroid in post-incubation medium increased up through 40 ovaries per incubation (Table 1). Production actually decreased when 80 ovaries were used. Whether this reflects a feedback inhibition of ecdysteroid upon the ovary remains an interesting question. Table 1 suggests that the most efficient organ density, as expressed by the number of pg of 20-OH-ecdysone equivalents produced per ovary per 6 hr. incubation, was 40 ovaries per 100 μl incubation. To reduce demand upon resources we scaled down the assay by 75 percent and now obtain good results with 10 ovaries in 25 μl incubation volume, while maintaining the 1 ovary per 2.5 μl organ density (see Table 1).

As decapitation following bloodfeeding prevented ovarian maturation in anautogenous species (3) so did decapitation of autogenous females following emergence (14). *A. atropalpus* ovarian follicles increase in length 9-fold from emergence through 72 hours post-emergence (50 μm to 450 μm; 14). Decapitation within 30 minutes of emergence halts follicle growth at 75 μm and, when females are decapitated 4 hours after emergence, follicle length at 72 hours is 80 μm. No vitellin is detected in follicles of females decapitated within 4 hours of emergence (14). After 4 hours post-emergence, the effect of decapitation is dramatically reduced. These findings resulted in the development of an *in vivo* bioassay utilizing *A. atropalpus* (19,20). In its present form, our *in vivo* bioassay consists of

Table 1. Pg 20-OH-Ecdysone Equivalents At Various Organ Densities

Number of Ovaries	Avg. PG Produced	PG/Ovary
1	7.3 ± 3.6	7.3
2	29.5 ± 12.6	14.8
5	137.0 ± 43	27.2
10	220.0 ± 20	22.0
20	412.0 ± 38	20.6
40	1,298.0 ± 203	32.2
80	692.0 ± 98	8.6
10[a]	329.0 ± 44	32.9

Incubation volume = 100 µl except "a" which was 25 µl
Heads from resting stage females and males were extracted in *Aedes* saline on ice, centrifuged at 8,000xg for 5 minutes and the supernatant used in incubations. Concentration of head equivalent/µl was 0.2. Average ecdysteroid produced is expressed as the mean of 4 determinations ± SEM.

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decapitating *A. atropalpus* females within 2 hours of emergence, holding overnight (typically 18-24 hours), injecting with *ca.* 1 µl of test solution and visually scoring ovarian maturation 20-24 hours later. Details of scoring can be found in reference 20. Performance of the bioassay is illustrated in Figure 1 (see figure legend for details). A nearly linear response is obtained when from 0.125 through 1.0 head equivalent is injected into decapitated *A. atropalpus* females. Saline injected, decapitated females and intact, non-injected females show 0 percent and 100 percent vitellogenic follicles, respectively, when scored 24 hours after emergence.

Tissue extraction is done by either of two protocols (Figure 2) depending upon the experiment. Protocol I was initially used (16,21) in preparation for reverse phase liquid chromatography of EDNH. We developed protocol II as a modification to decrease tissue processing time and facilitate loading of large numbers of tissue equivalents onto the preparative C_{18} column system. Protocol I is still used in, for example, the preparation of extracts for high performance size exclusion chromatography (HP-SEC) and the processing of particularly fatty tissues prior to HPLC (*e.g.* abdomens and thoraces). Details of each protocol are given in the figure legend. Although we do not segregate male from female tissues, we do separate heads, thoraces and abdomens and process them separately. With regard to the head, EDNH activity is essentially the same in both male and female *A. aegypti* (15). Extracts of tissue by either protocol are stored at -15°C until bioassay or further processing. As purification proceeds, precautions are taken to reduce sample loss. For example, whenever possible, drying of fractions between chromatographic steps is avoided. Fractions of interest are pooled, concentrated under vacuum and applied to the next chromatographic step. In practice, we attempt to develop methods reproducible to a level such that fractions containing the biological activity of interest can be collected on the basis of retention time, eliminating the need for bioassays until the end fraction, the one to be used for

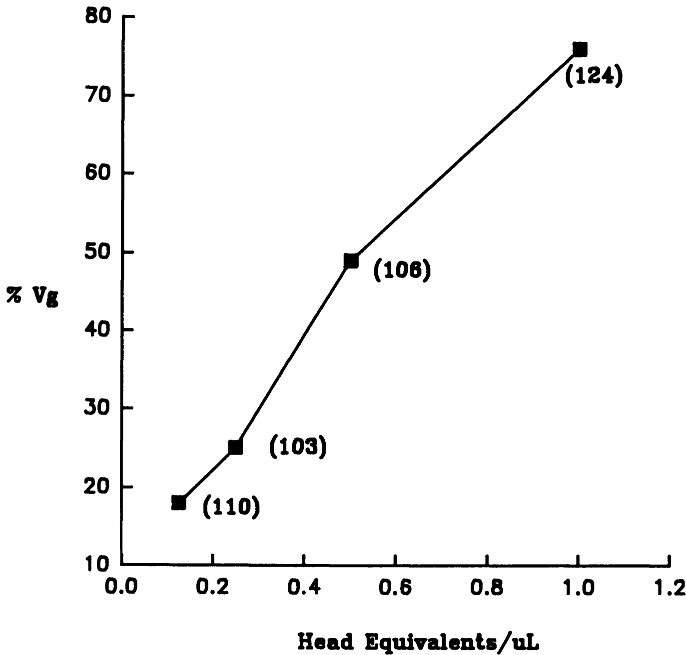

Figure 1. Dose response of ovarian development in decapitated *A. atropalpus* females injected with *A. aegypti* head extract. Crude extract was prepared by homogenizing lypohilized *A. aegypti* heads, on ice, in *Aedes* saline (154 mM NaCl, 1.36 mM $CaCl_2 \cdot 2H_2O$, 2.68 mM KCl, 1.19 mM $NaHCO_3$; pH 7.2) using an all glass homogenizer. Extract was prepared at 1 head per µl and dilutions of the 8000 xg supernatant were made with saline. Doses tested were 0.125, 0.25, 0.5 and 1.0 head equivalent per µl per female. Development was scored as follows: SC=2 for 24 hour level development; SC=0 for no development;SC=1 for intermediate development, and expressed as a percent of maximum possible development using the formula:

$$\% \, Vg = \frac{(N_F \times SC) + (N_I \times SC) + (N_O \times SC)}{(N_F + N_I + N_O) \times 2} \times 100$$

where N_F is the number of females showing full 24 hour development; N_I is the number with intermediate development; N_O is the number with no development. Morphology of development and scoring are described in reference 20. Numbers in parentheses represent total number of females injected and scored.

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Step	Protocol I	Protocol II
1	Dry, Sieve Tissue	Dry, Sieve Tissue
2	Homogenize Saline, Ice	Homogenize CH3OH/TFA, Ice
3	50,000xg 20 minutes, 4 C	50,000xg 20 minutes, 4 C
4	C-18 SepPak	SpeedVac
5	SpeedVac	Assay, LC
6	Assay, LC	

Figure 2. Protocols used for preparation of *A. aegypti* crude head extracts. Tissues were obtained from non-bloodfed females and males as described (16) except that sieving was done after fracturing and freeze-drying. This allowed handling of tissue at room temperature for the extended periods necessary for mass collection of heads, thoraces and abdomens. Homogenization is done using a Polytron high speed homogenizer (Brinkman Instruments) and PT-10 probe. For composition of *Aedes* saline, see Fig. 1. CH_3OH was 75 % in 0.1% TFA. C_{18} SepPak (Millipore Corp.) was prepared and used as described (16) except that the initial void volume was saved and processed through the re-equilibrated SepPak. Both the initial and void-processed 60% CH_3CN cuts were combined prior to drying in the Speed Vac (Savant Instruments).

critical bioassay, sequencing, etc., is obtained. Not only are time and material saved, but drying is eliminated until the final step. Inclusion of a "carrier" protein (22) (*e.g.* high purity bovine serum albumin or mouse ovalbumin) in fraction collection tubes aids sample recovery. We routinely include 10 µg of the carrier in each fraction. In addition, for those fractions which will be dried, each 1.5 ml Eppendorf polypropylene fraction tube is coated with ovalbumin by adding 1 ml of a solution of mouse ovalbumin (0.5 mg/ml in 60% CH_3CN, with 0.1% trifluoroacetic acid, TFA) and incubating overnight at 5°C. The tubes are emptied and air dried. After drying, tubes are used immediately or stored at -15°C. In our experience, treated tubes seem to yield somewhat higher recoveries of EDNH activity when compared with untreated tubes. The albumin does not interfere with either bioassay and elutes well past EDNH during RP-HPLC. Clearly, addition of exogenous protein is to be avoided when the fraction is destined for amino acid analysis or sequencing at the next step.

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Status of Cerebral EDNH

We have extracted and prepared EDNH from approximately 1 x 10^6 *A. aegypti* resting stage heads using both protocols. Following drying, (Step 5, Protocol I; Step 4, Protocol II), active extract is fractionated by RP-HPLC on a preparative scale C_{18} column (Waters µBondapak, 7.8 x 300 mm) using a CH_3CN/0.1% TFA versus 0.1% TFA gradient. Active fractions, eluted between 35 and 45 percent CH_3CN, are pooled, concentrated and loaded onto a cation exchange column (TSK-SP-5PW) with a mobile phase of 0.01 M ammonium acetate, pH 5.2, in 25% CH_3CN. A linear acetate gradient is run from 0.01M through 0.5 M. Three separate areas of activity are detected (at 0.27M, 0.35M and 0.42M acetate). The most active fractions, at 0.42M acetate, are loaded onto a Vydac C_4 column (4 x 150 mm) with a CH_3CN/0.1% TFA vs. 0.1% TFA system for further purification. The ion exchange results suggest the presence of at least three ionic forms of cerebral EDNH. This is similar to results recently reported by Matsumoto *et al* (25). We are now in the process of isolating post-ion exchange, reverse phase fractions for sequence analysis. Our first attempt revealed a 16 amino acid sequence of approximately 2000 MW, which we used in preparing synthetic sequences for both bioassay and antibody production. Peptides were synthesized on a Milligen/Biosearch 9600 instrument using tBoc amino protection, diisopropylcarbodiimide activation, and mixtures of dimethylformamide and dichloromethane as solvents. Coupling protocols were as supplied by the manufacturer, with only minor modifications.

The free amide peptide was synthesized on 4-methylbenzhydrylamine-substituted polystyrene resin, and the resin-bound peptide for antibody production was prepared on aminomethyl polystyrene. Cleavage/deprotection was done with hydrogen fluoride/anisole at 0°C. The free amide was not active in either bioassay. However, polyclonal antibody prepared against the resin-bound (23,24) sequence was immunologically reactive to EDNH reverse phase column fractions. We are now attempting to determine if the sequence represents an inactive fragment of authentic EDNH.

Multiple Forms of Cerebral EDNH

In processing head extracts prepared by either protocol with either preparative or semi-preparative C_{18} (µBondapak) columns, EDNH activity was detected in a number of non-contiguous fractions over a 10 percent CH_3CN gradient range. At least two different forms were detected, following RP-HPLC, as indicated by their activities in *in vitro* and *in vivo* bioassays (26). Multiple forms, whether artifacts or

true representatives of a peptide family, were also observed following ion exchange chromatography (25; and see previous section). It was suggested that one of the forms might have been a precursor to EDNH (26). At least three biologically active forms were identified following polyacrylamide gel electrophoresis (29), although extracts were of whole mosquitos.

Molecular weight estimates for cerebral EDNH vary from 6.5 kD (13) to 11 kd (25,27,28), 18.7 kD (29) and 24 kD (28). The 18.7 kD activity associated with egg development was extracted from whole *A. aegypti* (29). Standard open column methods were used in estimating the 6.5 kD, 11 kD, and 24 kD fractions. We have adapted high performance size exclusion chromatography (HP-SEC) methods, originally used for the preparation of *Lymantria dispar* prothoracicotropic hormone (PTTH; 30,31), to the fractionation of tissue extracts containing EDNH activity. With these methods, which include preparation of tissue via Protocol I, we find two molecular weight species of EDNH, estimated to be approximately 3.2 kD and 10 kD. Although widely varying methods were used to obtain the various molecular weight estimates, it is of interest to note that, in observing all molecular weights reported (13,25,27-29, this report), three catagories of sizes may be assigned; low (6.4 kD, 13; 3.2 kD, this report), medium (10 kD, 11 kD; 25,27,28 and this report) and high (18.7 kD, 24 kD; 28,29). These observations are reminiscent of the situation with PTTH, where multiple molecular weight forms have been regularly reported (30,38,39). In addition, ion exchange chromatography of *Bombyx mori* PTTH revealed at least three ionic sub-forms (40) a situation with some similarity to EDNH (25, this report). Such heterogenicity is suggestive of a peptide family. However, final resolution of these observations will only come about when amino acid sequences become available (25) and the techniques of molecular biology can be exploited. It is, however, tempting to speculate that some of these different forms of ecdysiotropins, separated by size, ionic character or relative solubilities, are authentic physiological variants. They may, as suggested (26), represent metabolic intermediates or a family of mature peptides. When the EDNH gene(s) is located, and the prohormone and mature peptide amino acid sequences are available, we can begin to address these questions.

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Non-cerebral EDNH

We have detected EDNH activity in isolated *A. aegypti* thoraces and abdomens, both *in vitro* and *in vivo*. Analysis of thoracic EDNH activity by HP-SEC revealed a molecular weight distribution identical to that found in cerebral extracts. When adjustments are made for the weight differences between lyophilized heads and thoraces, the amount of activity per tissue unit is nearly the same. Since all tissues are obtained from non-blood fed female, or male, *A. aegypti*, one presumes that release of EDNH, or EDNH-like material, from the corpus cardiacum has not yet occurred (9,32-34). It may be that some release of EDNH occurs prior to bloodfeeding, with the majority of the material remaining stored in the corpus cardiacum, and that the extraction and fractionation procedures used facilitate detection, of this released EDNH, in our bioassays. It is also possible, however, that some storage of the hormone occurs in neurohemal sites in the thorax. Endocrine-like cells, with structural aspects necessary for peptide production and secretion, have been found in *A. aegypti* abdomens (35). Thoracic endocrine complexes have been described in *A. aegypti* (36) and may serve as harbors of EDNH activity. The availability of specific antibody, dependent upon a well defined (*i.e.*, sequenced) antigen, will allow immunohistochemical examination of tissues and should aid in determining the relationship between cerebral and non-cerebral EDNH-like activities.

We have no evidence at present to indicate that thoracic EDNH is released.

Activity is only detected with tissue homogenates. Sensitive immunoassays (*e.g.*, enzyme linked immunosorbent assay, 43) will allow titering of activity in the hemolymph. The development of genetic probes will permit further analysis via the measurement of tissue specific mRNA levels. Discovery of non-cerebral, EDNH-like activity came through the use of the *in vitro* bioassay. No preparations of tissue extract, whether crude saline extracts or protocol I or protocol II extracts, elicited a response *in vivo*. Only after extracts prepared by protocol I were fractionated by HP-SEC was activity detected *in vivo*. This suggests that an inhibitor(s), different in molecular weight from either EDNH species, is present in the thorax. The question of whether the inhibition is specific (*i.e.*, an oostatic hormone) or non-specific (*i.e.*, generally toxic) remains open, although the thoracic inhibitor may be related to inhibitors of cerebral EDNH activity (16,20,41,42).

Summary

Egg development neurosecretory hormone, named for the effect upon the maturation of ovarian follicles (9) now presents, biochemically at least, a more complex picture than was originally anticipated. Fractionation and bioassays reveal more than one molecular form. Questions regarding the authenticity of the various forms and their physiological significance will only be answered when specific identities (*i.e.*, amino acid sequences and prohormone structures) are revealed. The imminent sequencing of at least one EDNH form (25) will no doubt lead to a rapid increase in the analysis of EDNH-active molecule(s). Once the path is open to the exploitation of molecular genetics, the study of mosquito, and in general all dipteran, reproductive physiology will accelerate. The EDNH/dipteran system should provide an ideal format for the analysis of synthesis, processing, secretion, receptor binding and turnover of neuropeptides just as it has provided an ideal setting for the study of reproductive physiology.

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Chapter 13

Mosquito Oostatic Hormone

A Trypsin-Modulating Oostatic Factor

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Mosquito oostatic hormone, a trypsin modulating oostatic factor (TMOF) has been purified from the ovaries of female *Aedes aegypti*. The hormone is a decapeptide of molecular weight 1047.6. The primary sequence has been determined using mass spectra recorded on a quadrupole Fourier transform instrument. Injection of synthetic analogs into mosquitoes, biting midges, flies and fleas inhibited proteolytic enzyme biosynthesis in the midgut. The target tissue of the hormone is midgut epithelial cells, and binding of [^{3}H]TMOF to midgut receptor was demonstrated *in vivo*. TMOF exhibits homology to mammalian, plant and several viral proteins. This is the first insect peptide hormone to share homology simultaneously with viral, plant and mammalian proteins. These results indicate that trypsin synthesis in mosquitoes and other insects may be regulated by sequence-related TMOFs that bind to midgut specific receptors.

Knowledge and understanding of how hormones regulate cellular processes in mosquitoes may be of great importance for controlling their reproduction. Conceivably, new types of insecticides may be developed that interfere with hormonal regulation, as for example, does the juvenile hormone analog (methoprene).

Although considerable research has been devoted in elucidating how biosynthesis of midgut proteases is initiated in mosquitoes, very little has been done to determine how this process is terminated. Thus, it is important to investigate the role of hormones in the control of digestion.

Antigonadotropins or factors that inhibit egg development, such as oostatic hormones and trypsin modulating oostatic factor (TMOF), have been demonstrated in the cockroaches, *Blattela germanica* and *Blatta orientalis*, (1) the eye gnat, *Hippelates collusor* (2), decapod crustaceans (3), and the house fly, *Musca domestica*, (4,5). In mosquitoes, Meola and Lea (6) and Else and

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Judson (7) similarly demonstrated an ovary-produced humoral factor secreted during vitellogenesis that inhibited yolk deposition in less developed follicles. However, the work of Meola and Lea (6) and Else and Judson (7) was done before adult previtellogenic follicular growth was recognized as a separate, hormonally-regulated process preceding vitellogenesis (8,9). Therefore, we repeated some of their work by implanting ovaries from blood-fed donors into females that had reached the resting stage (i.e., completed previtellogenic growth 3-5 days after emergence) and then fed them blood. Conversely, we implanted ovaries that had reached the previtellogenic resting stage into recipients that were blood fed 24 h earlier. In both cases, the larger ovaries inhibited the growth of the less mature ovaries (Table I). These results confirm earlier observations, indicating that the ovary secretes a humoral oostatic factor that inhibits egg development in less mature ovaries.

Table I. Inhibition of Egg Development in Previtellogenic Ovaries by Vitellogenic Ovaries

Female *Ae. aegypti* fed sugar for 3 days	Number of females (n)	Donor ovary yolk size (μm ± SEM)	Recipient yolk size (μm ± SEM)
a. Blood fed 24 h and implanted with an ovary (50 μm) removed from sugar-fed donor 3-5 days old and analyzed 48 h later	25	50 ± 10	380 ± 80
b. Implanted with ovaries (150 μm) removed from donors fed on blood for 24 h. Females were fed blood and analyzed 48 h later	30	400 ± 50	50 ± 20

Ovaries from sugar-fed females (3 days old) had reached the previtellogenic stage. Ovaries with yolk size < 70 μm did not move past the previtellogenic stage.

Mechanisms of Inhibition

General. Several mechanisms of inhibition have been suggested. In *Rhodnius prolixus*, oostatic hormone produced by the abdominal neurosecretory organs inhibits the action of JH on vitellogenic follicle cells, preventing the ovary from accumulating vitellogenin from the hemolymph (10,11). In the house fly

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M. domestica oostatic hormone seems to inhibit the release or synthesis of egg development neurosecretory hormone (EDNH) (12), but in mosquitoes the hormone seems to act directly on the ovary (6). *R. prolixus* oostatic hormone, which has been partially purified (13), is a small peptide of M_r 1,411 as determined on Sephadex G-50. Kelly et al. (5) prepared a crude extract of oostatic hormone from *M. domestica*, injected it into the autogenous mosquito *Aedes atropalpus*, and demonstrated inhibition of both egg development and ecdysteroid biosynthesis. They suggested that oostatic hormone functions at a point subsequent to release of EDNH. They were unable, however, to exclude the possibility that release of EDNH, itself, was inhibited.

Mosquito specific. Since 1985 my laboratory has been involved in the purification and sequencing of *Aedes aegypti* "oostatic hormone". The earliest results from my laboratory (14) indicated that the mosquito ovary was a rich source for "oostatic hormone". Starting with 4,800 pairs of ovaries I have purified the hormone 7,000-fold using low pressure chromatography. The hormone was found to be a small peptide-like molecule of M_r 2,200 at pH 4.5. Injection of the purified peptide into female mosquitoes inhibited yolk deposition and vitellogenin synthesis (14; Table II).

Table II. Inhibition of Egg Development with Purified Oostatic Hormone

Oostatic hormone (nmol)	Number of females	Yolk length (μm ± SE)
2.86	17	53 ± 7
1.45	18	82 ± 8
0.24	17	101 ± 5
Control	10	186 ± 4

Female *A. aegypti* were fed blood on a chicken, immediately injected with oostatic hormone, and 24 h later analyzed for egg development. M_r of oostatic hormone was used as 1047.6 based on mass spectra analysis of the purified hormone for nmol calculations.

Activity of the hormone in the ovary increased rapidly following blood feeding and reached a maximum at 48 h (14). The hormone did not block the release of EDNH from the mosquito brain and thus, we assumed that the hormone acts directly on the ovary either by preventing pinocytosis or by inhibiting ecdysteroid biosynthesis (14). It took several years of experimentation to find out that the primary target of the factor that we called "oostatic hormone" was not the ovary. Injection of partially purified "oostatic hormone" into female *Ae. aegypti* inhibited egg development, proteolytic enzyme activity (trypsin and chymotrypsin-like enzymes) and blood digestion in the midgut (15). Controls injected with insulin chain A did not affect these processes. A single injection of "oostatic hormone"

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preparation caused 1.7-1.5 fold reduction in trypsin-like enzymes activity as measured with α-N-benzoyl-DL-arginine-p-nitroanilide (BApNA), with a 10 h delay in peak activity (15). In collaboration with Dr. Y. Schlein of the Hebrew University of Jerusalem, Department of Parasitology, a quantitative and sensitive method to determine concentrations of trypsin and chymotrypsin-like enzymes in insects was developed (16). The assay is based on the specific binding of [1,3-^{3}H]diisopropylfluorophosphate (DFP) to trypsin and chymotrypsin-like enzymes. Trypsin-like enzymes can be determined specifically in the presence of 10 mM TPCK (tosylamide-2-phenylethyl chloromethyl ketone; chymotrypsin inhibitor) and chymotrypsin-like enzymes can be determined in the presence of 10 mM TLCK (tosyl-L-lysine chloromethyl ketone HCl; trypsin inhibitor). The assay sensitivity is limited only by the specific activity of DFP: available commercial preparations have sp. act. of 35 Ci/mmol and thus the lower limit of detection is 65 ng of either trypsin or chymotrypsin. Using this method I followed the biosynthesis of trypsin-like enzymes (chymotrypsin-like enzymes are responsible for only 7% of the proteolytic activity) in the midgut of female *Ae. aegypti*. A 4-fold reduction in [1,3-^{3}H]diisopropylphosphoryl-trypsin-like derivatives was noted after "oostatic hormone" treatment (15). Injection of "oostatic hormone" into decapitated and ovariectomized females, animals that do not synthesize ecdysteroids but synthesize protease in their midgut, inhibited trypsin-like enzyme biosynthesis and blood digestion in their midgut, indicating that "oostatic hormone" probably inhibits midgut cells that synthesize trypsin, and not the ovary or the brain's endocrine system Table III (15). *Ae. aegypti* "oostatic hormone" is not species specific and injections of the hormone into *Culex quinquefasciatus*, *Culex nigripalpus* and *Anopheles albimanus* cause inhibition of egg development, blood digestion and synthesis of trypsin-like enzymes (15). Since three of these mosquito species are important vector carriers of disease (yellow fever, malaria and encephalitis), it is important to find out the exact mechanism that terminates vitellogenesis and blood digestion.

Purification and Sequencing

To answer the question whether our hormone acts on the oocytes and is a true "oostatic hormone", or whether its function is to modulate midgut serine proteases and, thus should be called Trypsin Modulating Oostatic Factor (TMOF), we purified the "hormone" to apparent homogeneity, sequenced it and synthesized synthetic analogs. The "hormone" was isolated from 30,000 pairs of ovaries, 4 days after female *Ae. aegypti* were fed a blood meal. Ovaries were extracted in distilled water containing 1 mM phenylmethyl sulfonyl fluoride (PMSF), acidified to pH 4.5 and heat treated. The extract was centrifuged and the supernatant purified using DEAE, C_{18} reversed phase high pressure liquid chromatography (HPLC) and gel filtration on TSK G2000 SW column. About 549 nmol of purified "hormone" were obtained from 30,000 pairs of *Ae. aegypti* ovaries (i.e., 6 g of soluble protein) after

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Table III. Effect of Oostatic Hormone on Trypsin Synthesis in Decapitated and Ovariectomized Female *Ae. aegypti*

Female *Ae. aegypti* were fed blood on a chicken and then	*No. of* females	Trypsinlike activity[a] (BApNA) (nmol/min/midgut ± SE)
a. Immediately decapitated	25	3.74 ± 0.2
b. Immediately decapitated and injected with oostatic hormone (1.45 nmol)	25	1.63 ± 0.1
c. Immediately decapitated and injected with insulin chain A (1.45 nmol)	25	3.94 ± 0.3
d. Assayed 24 h later	25	5.96 ± 0.4
e. Immediately ovariectomized and injected with oostatic hormone (1.45 nmol)	25	0.22 ± 0.1
f. Immediately ovariectomized and injected with insulin chain A (1.45 nmol)	25	5.23 ± 0.2

[a]Ten female *Ae. aegypti* midguts were removed and analyzed for trypsin activity 24 h later. Results taken from (15).

the five-step purification procedure. For sequence analysis "oostatic hormone" was rechromatographed on reversed phase HPLC and a pure peptide (2.6 nmol) was collected at 18.8 min and sent to Dr. Donald F. Hunt, Department of Chemistry, University of Virginia, Charlottesville, VA who ran the sample using mass spectrometry with quadrupole Fourier transform (17). Bombardment of the sample (20-50 pmol) in thioglycerol with 6 kV cesium ions detected an abundant $(M + H)^+$ ion at m/z 1047.6. Data obtained from collision activated dissociation spectra recorded on a triple quadrupole mass spectrometer supported the structure of "oostatic hormone" as NH_2-Tyr-Asp- Pro-Ala-Pro-Pro-Pro-Pro-Pro-Pro-COOH. The earlier data of M_r 2000 was based on gel filtration, and was

not accurate due to the poly proline that interacted with the column matrix and gave a large apparent M_r (14).

A peptide having the identical sequence was synthesized using standard automated solid phase peptide techniques (18). The synthetic peptide was then chromatographed using C_{18} reversed phase HPLC. Native "oostatic factor" coeluted with the synthetic peptide indicating that "oostatic hormone" structure is NH_2-Tyr-Asp-Pro-Ala-$(Pro)_6$-COOH. A Corey-Pauling-Koltun (CPK) atomic model of the factor indicates that the carboxyl end of the molecule probably exhibits a left-handed helical conformation in solution due to the six proline residues (23).

Properties of Synthetic Hormone

Mode of action. The possibility that mosquito oostatic factor acts as an inhibitor of midgut trypsin and chymotrypsin enzymes using the same mechanisms as other inhibitors (TLCK, TPCK, soybean trypsin inhibitor) (16) was also investigated . These inhibitors bind to the active site and prevent hydrolysis of substrate. Female *Ae. aegypti* were fed blood on a chicken and 24 h later, when trypsin and chymotrypsin activities are at a maximum, midguts (5 per group) were removed, homogenized in 50 mM Tris-HCl buffer, pH 7.9, centrifuged at 12,000 g for 20 min at 4°C, and the supernatants collected. One group was incubated with 20 μg (19.5 nmol) of synthetic oostatic factor in 100 μl incubation mixture (a 7-fold higher quantity than is used *in vivo*) for 30 min and then [^{3}H]DFP added, whereas controls were incubated only with [^{3}H]DFP. At the end of incubation both groups were analyzed for [^{3}H]DIP-trypsin and chymotrypsin-like derivatives (16). No difference was found between the two groups (Table IV) which indicates that oostatic factor does not bind to the active site and does not inhibit enzyme activity, but probably the biosynthesis of trypsin and chymotrypsin.

Table IV. Does Synthetic Oostatic Factor Inhibit Trypsinlike and Chymotrypsinlike Activities?

Female *Ae. aegypti* midguts were analyzed for serine protease activity 24 h after a blood meal with	No. of Groups	[^{3}H]DIP-trypsin and chymotrypsin (cpm/midgut ± SEM)
a. Oostatic factor (20 μg)	3	47,330 ± 2,959
b. Without oostatic factor	3	49,891 ± 3,341

Six groups of female *Ae. aegypti* (5 per group) were fed blood, the midguts were dissected out, and analyzed 24 h later in the presence and absence of synthetic oostatic factor for [^{3}H]DIP-trypsin and chymotrypsin-like enzymes

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Species Specificity. Oostatic hormone is not species specific (14), when *Ae. aegypti* synthetic oostatic hormone was injected into *C. variipennis*, *Ae. aegypti*, *An. quadrimaculatus*, *Stomoxys alcitrans*, *Ctenocephalides felis* (Bouche) and *Lutzomyia anthophora* (Addis) synthesis of [1,3-^{3}H]DIP-trypsin-like isozymes was reduced (Table V).

Target Tissue. To study whether midgut cells have "oostatic hormone" specific receptors the hormone was labeled with [^{3}H]acetic anhydride to a specific activity of 0.53 Ci/mmol, and aliquots of 780 pmol in 0.5 μl were injected into sugar-fed female *Ae. aegypti*. At intervals following the injection, midguts were removed, washed 3 times in tissue culture medium TC 199 (GIBCO Laboratories), homogenized in 1 N NaOH (100 μl), centrifuged at 12,000g and the supernatant analyzed in a liquid scintillation counter. Fifteen-minutes after injecting "oostatic hormone" the rate of binding to gut receptor was 8.7 pmol/h/gut, the binding rate increased to 13 pmol/h/gut at 1 h and declined thereafter to 4 pmol/h/gut between 2-4 h, to 0.9 pmol/h/gut at 8 h, 0.13 pmol/h/gut at 30 h and 0.0089 pmol/h/gut at 96 h. At peak binding 1.7%-2.1% of injected "oostatic hormone" bound to gut receptor. These results indicate that the gut has hormone specific receptor and that the binding is a dynamic, receptor specific process. No binding to other tissues (brain, thorax, fat body, ovary and Malpighian tubules) was detected 24 h after injecting the "hormone". Thus, a more appropriate name for "oostatic hormone" is Trypsin Modulating Oostatic Factor (TMOF). The rapid decline in binding 1 h after injecting TMOF indicates that the factor is rapidly removed from the hemolymph or metabolized into an inactive molecule. Thus, a binding assay of TMOF to midgut receptor is a better way to express its activity.

Homology. An on-line computer search (MATCH and PIR accesses the National Biomedical, National Bioresearch, Medical Research Foundation) found that TMOF has significant homology to mammalian, plant and several viral proteins that are either synthesized by double stranded DNA viruses (Epstein Barr virus and Herpes simplex virus) or single stranded RNA viruses (Abelson murine leukemia virus, avian spleen necrosis virus, rubella virus, simian T-lymphotropic virus and HIV-2). This close homology (7 to 8 amino acids out of 10) may indicate that TMOF differs from other insect hormones that exhibit homology only to vertebrate proteins. Prothoracicotropic hormone (PTTH), and leucosulfakinin exhibit homology with human insulin, gastrin and cholecystokinin, respectively (19,20).

Model. Blood digestion plays a key role in the survival of vector arthropods and in the transmission of many important diseases such as malaria, dengue and Lyme disease (21,22). Collating these observations, a model for the control of trypsin biosynthesis in the mosquito is proposed. After the blood meal the midgut epithelial cells start to synthesize trypsin in concert with the neuroendocrine system that synthesizes oostatic hormone, as was proposed for *Rhodnius* by Davey and Kunster (11), which accumulates in the developing ovary as a prohormone. The

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Table V. Effect of Synthetic Oostatic Hormone on Trypsin Biosynthesis in Mosquitoes, Fleas, Flies and Sandflies

Females were fed blood and then	No. of females	[1,3-^{3}H]DIP trypsin per midgut (cpm)[a]
C. felis (Bouche)		
a. Immediately injected with oostatic hormone (477 pmol)	11	1,876
b. Control (not injected)	10	3,192
S. calcitrans		
c. Immediately injected with oostatic hormone (4.77 nmol)	14	5,644
d. Immediately injected with saline	9	10,385
L. anthophora		
e. Immediately injected with oostatic hormone (477 pmol)	4	1,802
f. Control (not injected)	8	2,492
A. quadrimaculatus		
g. Immediately injected with oostatic hormone (10 nmol)	16	41,042
h. Immediately injected with saline	10	60,868
A. aegypti		
i. Immediately injected with oostatic hormone (2.87 nmol)	17	13,625
j. Immediately injected with saline	7	99,167
C. variipennis		
k. Immediately injected with oostatic hormone (286 pmol)	6	10,186
l. Immediately injected with saline	10	25,999

Females were analyzed 24 h after the blood meal for [1,3-3H]DIP-trypsinlike derivatives. Oostatic hormone concentrations are based on M_r 1047.6 derived from mass spectra analysis.
[a]Results are average of 2 determinations.

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active hormone is released into the hemolymph 24 h later, and signals the midgut epithelial cells to stop the synthesis of trypsin-like enzymes which ceases 55 h later.

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Chapter 14

Housefly Oostatic Hormone

Methods for Isolation and Characterization

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House fly oostatic hormone (OH) was first described over 20 years ago. Only recently has appreciable progress been made toward its isolation and characterization. Due to significant technological advancements in HPLC over the last decade, a variety of highly specialized chromatographic tools are now available to assist in the purification of insect hormones, characteristically present in ng or pg quantities. Techniques which include size-exclusion, reverse-phase and normal-phase HPLC resulted in >1000-fold purification of house fly OH derived from ovaries of mature adults. Our data suggest that more than one oostatic factor may be present in the ovaries. Moreover, the most potent factors are hydrophilic and are of low molecular weight.

Since the report of the existence of an oostatic hormone in the corpora lutea of _Blatella germanica_ and _Blatta orientalis_ (1), research geared to the elucidation of mechanisms regulating oogenesis has uncovered oostatic hormones or factors in a variety of insects (Table 1). It is noteworthy that all of the species tabulated produce eggs in a cyclic manner and, except for _R. prolixus_, these factors have their origin in the ovaries.

House fly oostatic hormone (OH) was reported over two decades ago (2) and is a humoral factor produced in females possessing eggs in the vitellogenic phase (stages 4-10) of development (2, 3). Initial studies showed that stage 4-10 eggs in the first gonadotropic cycle retained in gravid females, completely inhibited previtellogenic eggs (stages 2-4) to develop beyond stage 4. Extracts, prepared from mature vitellogenic ovaries (stages 4-10) or from whole bodies or abdomens of mature females, injected into immature 24-hr-old females inhibited ovarian development. Moreover, whole body extracts prepared from immature females had no effect on ovarian development when injected into immature females.

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These results provided a basis for the hypothesis that house fly oostatic hormone "maintained the cyclicity of maturation in subsequent gonotropic cycles" (3).

Table 1. Insect Oostatic Factors

Year	Species	Source	References
1935	Blattella germanica	corpora lutea	Iwanoff & Metscherskaja (1)
"	Blatta orientalis	"	"
1958	Iphita limbata	ovaries	Nayar (18)
1967	Hippelates collusor	"	Adams & Mulla (19)
1968	Musca domestica	"	Adams et al. (2)
1972	Aedes aegypti	"	Meola & Lea (20)
"	Aedes solicitans	"	"
"	Aedes taeniorhynchus	"	"
1973	Rhodnius prolixus	endocr. organs	Huebner & Davey (21)
1980	Diploptera punctata	ovaries	Tobe (5)
1986	Aedes atropalpus	"	Kelly et al. (9)
"	Drosophila melanogaster	"	" "

Oostatic mechanisms have been studied in Rhodnius prolixus (4), Diploptera punctata (5), Aedes aegypti (6, 7), Aedes atropalpus (8, 9) and Musca domestica (3, 8) and the biochemical and physiological roles of the oostatic factors involved vary. In R. prolixus (4), the oostatic factor acts directly on the ovary by inhibiting juvenile hormone (JH) action on the follicle cells while in D. punctata (5) the factor suppresses JH synthesis by the corpus allatum. Unlike the JH-mediated mechanisms seen in R. prolixus and D. punctata, mosquito OH appears to affect ovarian development by inhibiting proteolytic enzyme synthesis and blood digestion in the midgut (6).

Although early work led to an erroneous conclusion that house fly OH affects ovaries by preventing JH synthesis and/or its secretion by the corpus allatum (10), subsequent studies (11) provided the basis for a different hypothesis, namely that OH affects ovarian development by inhibiting egg development neurosecretory hormone (EDNH) release and/or synthesis (3). However, more recent work (8), in which normal vitellogenic and steroidogenic responses to EDNH were inhibited in newly emerged decapitated female A. atropalpus that were injected with both EDNH and house fly OH, suggests that house fly OH may act at a level subsequent to EDNH release. However, because of experimental design, the possibility that EDNH release itself was affected could not be ruled out.

Despite intensive efforts to define oostatic mechanisms, surprisingly little is known regarding the chemical nature of these factors. In Rhodnius, the antigonadotropin was partially purified from ovarian homogenates by using low-pressure gel-filtration

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chromatography. Results from chromatography of acidified and non-acidified ovarian extracts on Sephadex columns and from treatments of active column fractions with heat, trypsin, tyronase, and sodium thioglycolate, suggested the factor was a protein-bound low molecular weight peptide (ca. 1.4 KD) lacking tyrosine and disulfide bridges. An insufficient supply of ovaries prevented further characterization of the peptide.

Compared to Rhodnius, much more is known in A. aegypti about the oostatic hormone. A high purification of mosquito OH from an ovarian extract was achieved by low pressure ion-exchange and gel-filtration chromatography and the hormone was characterized as a peptide-like molecule with an apparent molecular weight of 2.2 KD (7). Moreover, the hormone appears to exist as a trimer and octamer when it elutes from a Bio-Gel P-10 column with a pH 7.0 phosphate buffer. However, when a pH 4.5 sodium acetate buffer was used, the monomeric form prevailed.

In subsequent studies, reverse-phase high-performance liquid chromatography (RP-HPLC) was used to fully purify mosquito oostatic hormone and the ovarian-derived peptide was identified as H-Tyr-Asp-Pro-Ala-Pro-Pro-Pro-Pro-Pro-Pro-OH (12). The amino acid sequence of the decapeptide was determined by tandem quadrupole mass spectrometry and compared to sequences of other insect neuropeptides (13). It is a highly unusual peptide because it contains six consecutively-linked prolines in the carboxy terminus. Interestingly, the structure possesses 70% homology to HIV II (12).

Although considerable effort has gone into studying the physiological action of house fly OH, little has been reported with regard to the development of a systematic approach for its purification. A semi-purified extract was first prepared by grinding mature female house flies in 95% ethanol, filtering, dialyzing the filtrate, and precipitating the dialysate with acetone (14). This method produced a dry powder (acetone insolubles) that remained active for up to as long as 10 months when stored at -10°C under an inert atmosphere.

In a subsequent study, solvent partitioning and solid-phase extraction techniques were used to produce a partially-purified extract (15). The first step in the purification scheme involved partitioning of a highly concentrated ethanolic extract of house fly ovaries between chloroform and water. Further processing of the chloroform layer (i.e., evaporation, solvent exchange, and filtration through a bed of Porapac Q) eventually produced a methanolic extract with a 23-fold increase in OH activity over the crude extract.

High performance liquid chromatography is emerging as a powerful technique applicable to the purification of house fly oostatic hormone. Reverse-phase HPLC was first applied to purifying an extract of house fly ovaries prepurified through a C-18 Sep Pak cartridge (16). Chromatography on a Shandon C-18 column with a typical trifluoroacetic acid (TFA)-buffered acetonitrile-water gradient (45 min. total run) produced a cluster of active fractions near the solvent front (0-2.5 min). A fraction with low and inconsistent activity was also observed in the hydrophobic heart of the chromatogram (38-40 min). The possibility

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that a biogenic amine was responsible for activity in the early chromatographic fractions was investigated (16), but none of the amines studied co-chromatographed on the C-18 column with the inhibitory material derived from house flies, despite the fact that three of the amines were moderately inhibitory in the A. atropalpus in-vivo ovarian maturation assay.

Although RP-HPLC separated OH from the crude extract, the elution time of active fractions from the C-18 column and poor peak resolution in those fractions were troublesome. Nonetheless, these results were useful to characterize OH as a highly polar molecule (probably highly functionalized) and provided a compelling reason to employ other chromatographic techniques for its purification.

Further progress in isolation was made when RP-HPLC was used in combination with size-exclusion (SE) and normal-phase (NP) HPLC (17). A flow diagram of the purification scheme is shown in Figure 1. Initially, a TFA-buffered aqueous acetonitrile extract of ovaries was processed by SE-HPLC. This separation provided a series of active fractions eluting in the low molecular weight (≤2.5 KD) region of the chromatogram. Fractions corresponding to molecular weights greater than 2.5 KD lacked activity. Active fractions were combined and reprocessed on a Shandon Hypersil C-18 column using a linear gradient elution (1.0 ml/min) with acetonitrile/0.1 %TFA vs. water/0.1% TFA as solvents. The elution profile of UV detectable (214 nm) components in the ovarian isolate and areas in the chromatogram depicting activity are shown in Figure 2. Results from the RP-HPLC were similar to those described earlier (16): high oostatic activity associated with early eluting fractions (2-5 min) and high activity in a single fraction eluting later in the chromatogram (17-18 min). However, because fractions eluting between 40-45% acetonitrile on C-18 columns (16, 17) were inconsistently active, no further analysis was done on the active fraction collected at 17-18 min. A group of three unresolved UV-detectable peaks, correlating with oostatic activity, was observed in the highly polar region of the chromatogram (2-5 min). Improvements in the resolution of these peaks of the chromatogram were made by varying the type of C-18 column used, and by lowering the solvent flow rate. These improvements, however, were marginal at best and baseline separation of peaks in this area was never fully achieved. Nonetheless, variations in the potency of individual fractions obtained early in the chromatogram suggested that the oostatic factor either eluted as a broad peak over a series of adjacent fractions or each of the fractions contained a different oostatic factor.

Clearly, results from RP-HPLC of ovarian extracts in two separate studies (16, 17) were consistent; both showed the highly polar nature of OH. Also, the extreme retentiveness of this inhibitor on reverse-phase columns suggested that further purification of OH could be achieved on columns designed to separate by adsorption rather than by hydrophobic-absorption phenomena, i.e., silica gel columns.

Figure 3 illustrates a typical NP-HPLC analysis of the fraction eluting between 3-4 min from the RP-HPLC separation shown in Figure 2. Exceptional resolution of the fraction (containing 3.4 ovary

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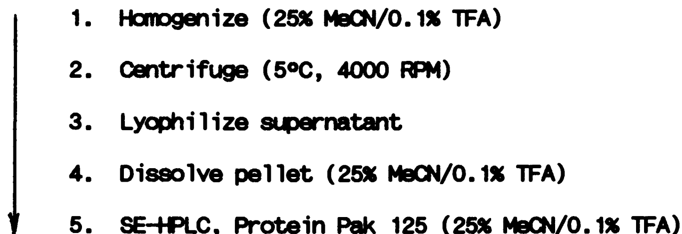
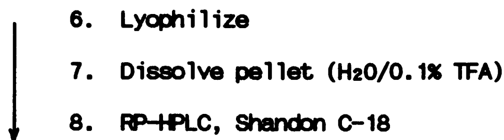
equivalents) was obtained on a 5µ-particle size Shandon Hypersil silica gel column eluted with 90% acetonitrile in 0.1% TFA for 16 min (1 ml/min) then 50% acetonitrile in 0.1% TFA (1.5 ml/min). Four fractions, designated 1-4, were collected and bioassayed. Fractions 2 and 4 were active but fraction 3 was the most inhibitory. Further analysis of fraction 3 by NP-HPLC showed it

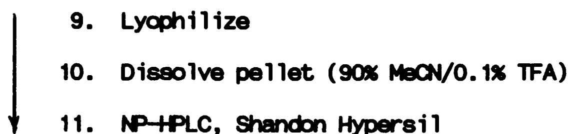
OVARIES



POOLED FRACTIONS (≤2.5 KD)



FRACTION 4 (3-4 min.)



FRACTIONS 1-4

Figure 1. Scheme to extract and purify house fly oostatic hormone.

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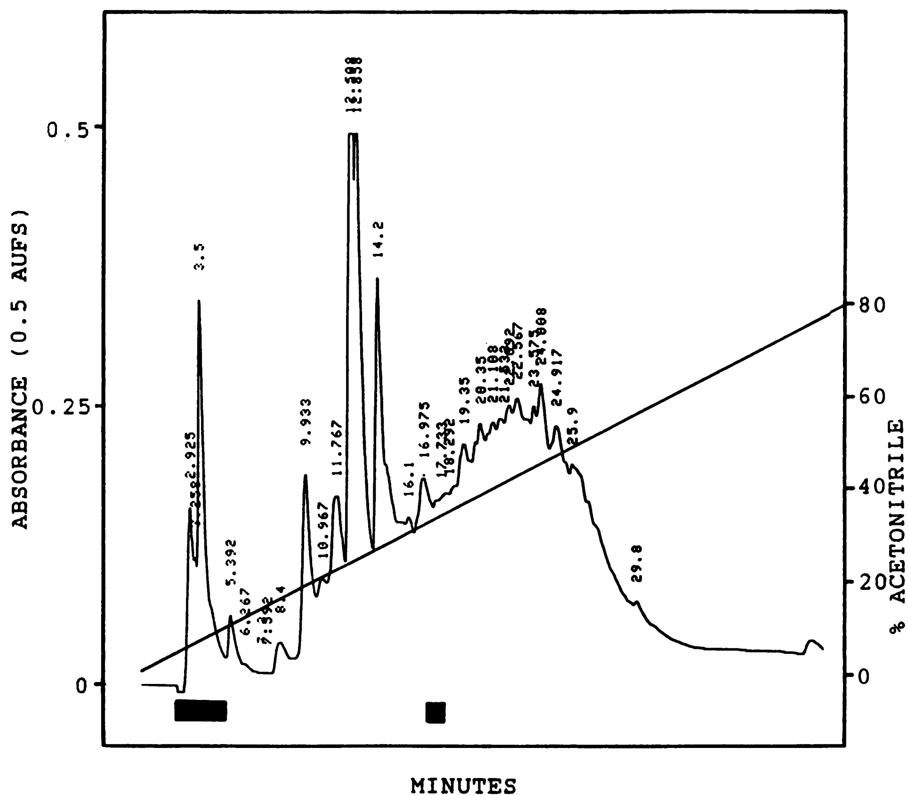

Figure 2. UV profile from reverse-phase HPLC of pooled active fractions obtained from size-exclusion HPLC. Bars under curve represent regions of the chromatogram with oostatic activity. Trace represents 5.8 ovary equivalents.

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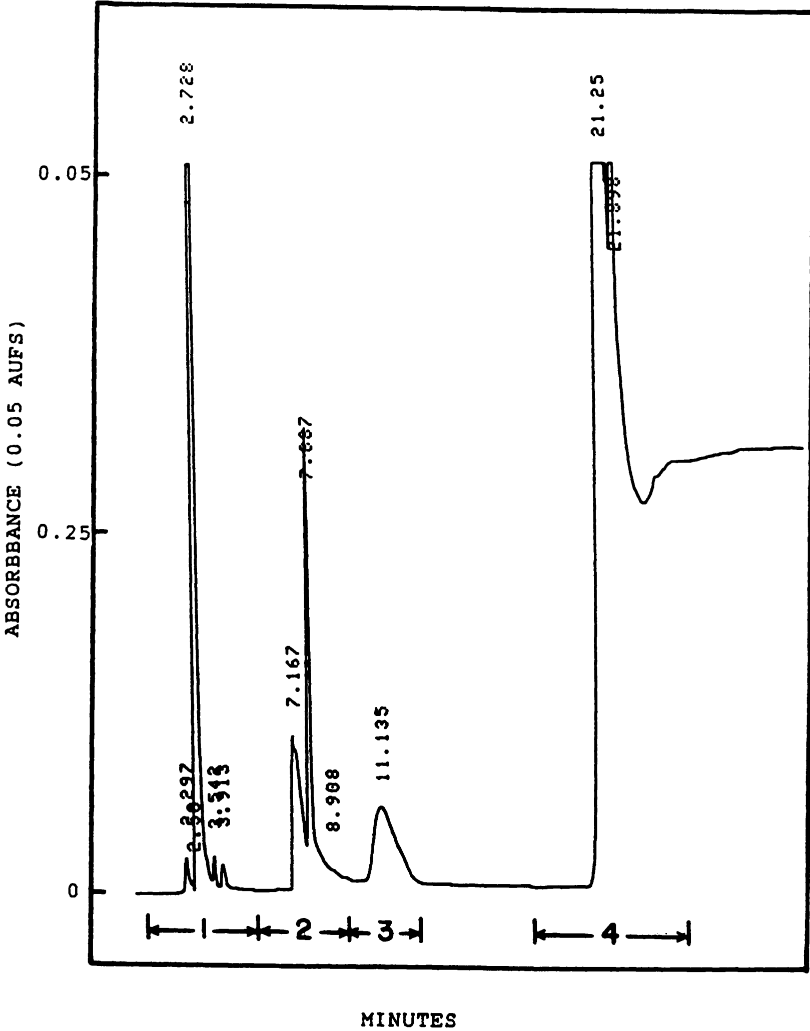

Figure 3. UV profile of a normal-phase HPLC of an early eluting fraction obtained by RP-HPLC. Detector response attenuated to 0.1 AUFS after 18 min.

to contain two UV-detectable peaks, one of which completely volatilized during lyophilization of the HPLC solvents. HPLC and bioassay data suggested that inhibitory activity elicited by fraction 3 was associated with the volatile component.

The addition of NP-HPLC into the scheme to purify OH was crucial since it provided adequate resolution of the highly polar components in the inhibitory fractions obtained by RP-HPLC. As a result, three regions in the NP chromatogram were discovered to possess oostatic activity. Since the NP-HPLC was conducted on only one of the three active fractions obtained by RP-HPLC, it is entirely possible that the remaining fractions may uncover other oostatic factors other than those described here. Although estimated molecular weights of these inhibitors ($\leq$ 2.5 KD) are reasonably close to that reported for mosquito OH (1047 amu), differences in chromatographic behavior between house fly OH and mosquito OH (12, 17) on reverse-phase columns suggest that structures of these two important hormones are different.

In summary, a combination of HPLC techniques were used to separate the oostatic factors endogenous to house fly ovaries. Further research is necessary for final purification and chemical characterization of these inhibitors. The mechanism for feedback regulation of ovarian maturation in house flies is complex and efforts to elucidate this mechanism will require knowledge of the number and chemistry of the oostatic factors involved. High performance liquid chromatography as a separative technique has already contributed greatly to this objective and the results presented here will be an integral part of the final purification of these factors.

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Chapter 15

Allatal Stimulation and Inhibition in Locusts

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The ability of the adult female locust to synthesize juvenile hormone (JH) is dependent on allatal maturation after adult emergence and by long-term (slow) and short-term (rapid) modulation of the competent corpora allata (CA). Allatal maturation is described in terms of increasing levels of terminal epoxidase and methyl transferase and to an increasing capacity of the CA to respond to allatotropin. Mature CA respond equally to farnesoic acid, the penultimate precursor of JH. Evidence exists that lateral neurosecretory cells of the brain are involved in long-term modulation of the mature CA. Short-term modulation is attributed to cerebral allatotropin and to ovarian allatostatin, which overrides allatotropic stimulation at the end of vitellogenesis. The rate-limiting step in JH biosynthesis, under short-term control, is concerned with mevalonate synthesis. This is concluded from *in vitro* factorial experiments with two 3-hydroxy-3-methyl-glutaryl CoA reductase inhibitors: hydroxymethylglutarate and mevinolin, with and without allatotropin and mevalonate.

The synthesis of juvenile hormone (JH) in the insect corpora allata (CA) is controlled by an array of inhibitory and stimulatory factors originating in different parts of the retrocerebral complex and in the ovary. The nature of the factors involved and their interactions seem dissimilar in different insect groups, and it is premature, based on what is known to date (1,2), to formulate a unified model of allatal control for all insects. Differences may truly exist, however they may be a consequence of information inadequate to generalize from. Rather, we present herein a model of allatal control in the adult female locust during reproductive maturation, which may serve

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as a reference point for evaluating other insect groups. Most of the work cited from our laboratory has been performed with the African migratory locust (Locusta migratoria migratorioides).

The basic methodologies of measuring JH titer and rates of biosynthesis will be mentioned here only briefly. JH titer is determined by either radioimmunoassay (3) or preferably by gas chromatography coupled with mass-spectrometry (4-6). Alternatively, JH biosynthesis can be determined with excised CA in vitro by the radiochemical (RCA) method (7). JH values obtained by titering reflect the equilibrium of rate of JH synthesis, its degradation and clearance from the hemolymph, its tissue uptake and excretion. The rate of de novo synthesis, as measured by the RCA method, is argued to correlate well with JH hemolymph titer (8,9). As we shall later show, the correlation of in vitro synthesis and in vivo titers is not always maintained in L. migratoria under all experimental situations.

Three-Component Model of Control of JH Biosynthesis

Developmental Maturation is a progressive acquisition of competence of the CA to synthesize JH, which normally proceeds in conjunction with developmental changes associated with increase in gland volume (10,11). Biosynthetic capacity increases markedly during the first 2-3 days after adult emergence (12,13). Total capacity of the CA is expressed and can be quantified when all rate-limiting steps in the pathway of JH synthesis are either fully activated or bypassed, and no inhibitors present. In practice this is done by incubating CA in vitro in the presence of farnesoic acid (FA), the penultimate precursor of JH. Exogenous FA supplements the endogenous pool within the CA and elicits a significant increase in synthesis, consistent with the suggestion that the last two steps in JH biosynthesis (epoxidation and methylation) are substrate limited, rather than being rate-limited by deficiency of the enzymes involved (8).

Long-Term Modulation of JH synthesis is ascertained by measuring the basal rates in vitro. Basal rates of excised CA are linear for at least 8 hr (14). Basal rates increase gradually during the first gonadotrophic cycle as vitellogenesis is attained and progresses. They continue to increase even after the CA have matured developmentally and attained maximal competence. A decrease in basal rates is observed after deposition of the first batch of eggs and in old non-vitellogenic females, while the level of allatal competence still remains high (13,14).

Short-Term Modulation - allatotropic and allatostatic - is superimposed on the basal activities of developmentally mature CA. Locust cerebral allatotropin was first demonstrated in vitro in methanolic brain and corpora cardiaca (CC) extracts (14,15) and allatotropin I was later partially purified and characterized (16). Allatotropin I is found in high concentrations in the brain, where it is presumably synthesized, and in corpora cardiaca (CC) to where it is presumably transported and released as required. The exact location of allatotropin synthesis in the brain is still unknown. Ovarian allatostatin rapidly depresses allatotropin-stimulated JH synthesis (17). This allatostatic factor is methanol soluble and

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resistant to boiling (16). Allatotropic/allatostatic modulations decay rapidly in vitro in the absence of the effector.

Sites of Synthesis and Release

The origin and transport of the "maturation factors" in the adult locust has been examined by evaluating the in vivo and in vitro effects of surgical interventions (Figure 1). Electrocoagulation of the median neurosecretory cells (MNC) of the pars intercerebralis of 2-day old adult female L. migratoria prevented the normal increase in both CA volume and epoxidase activity associated with JH synthesis (18). Electrostimulation of the MNC of 1-day old females enhanced JH biosynthesis in vitro within 2-3 days of the stimulation, whilst cautery of the cerebral axonal tracts of these cells (the internal cardiaca tract TCC-I), prevented both normal development and enhancement by electrostimulation (19). Electrocoagulation of the TCC-I in 8-day old females, in which the CA are functionally mature, did not affect their in vitro activity (20). These findings suggest that the MNC regulate the development of the CA during the first days after adult emergence, as would be expected for the hypothetical maturation factors. Their possible routes of transmission are not obvious. No direct nervous connection has been described between the MNC and the CA in L. migratoria (21,22). Furthermore, transection of the NCA-I in newly-emerged females impaired their basal activity, but did not effect subsequent JH synthesis in vivo (23,24). These observations are consistent with the possibility that the maturation factors are humorally transmitted to the disconnected CA. On the other hand, some evidence indicates that axonal transmission does have an effect on CA maturation. Thus, the assymetry in CA volume and activity between the paired CA (11,25) is more consistent with neural control of CA development.

The factors of long-term modulation most probably originate in the lateral neurosecretory cells (LNC) of the protocerebrum and are transported via the NCC-II to the CC and from there via the NCA-I to the CA. JH synthesis is first reduced as an immediate response to electrocogulation of the LNC in vitellogenic L. migratoria females, but thereafter recovers. Only after 30 hr does a final decline commence. NCA-I transection is a more mild treatment, and the initial transient reduction is not evoked. Here too the in vitro basal activity of the CA declines after 30 hr of nerve transection (20) suggesting that a stimulatory control factor is neurally transmitted. In Schistocerca gregaria, the decrease in CA activity after nerve transection takes about 6 hr (26), which is still within the range of long-term modulation. Destruction of the NCC-I has no effect on the basal in vitro activity of the CA in vitellogenic females, indicating that the MNC do not participate in long-term modulation. The denervation of mature CA specifically reduce their basal level of activity, but not their competence, as these glands can be stimulated by FA to the same degree as control intact CA (9), indicating the independence of long-term modulation and the maturation process.

The basal activity of CA from diapausing females of the Savio strain of L. migratoria, denervated in vivo (NCA-I transection), decreased over a four days period, after which they were found to be inactive when assayed in vitro (27). This indicates an axonal activating factor whose effect on basal activity decays over several days after nerve transection.

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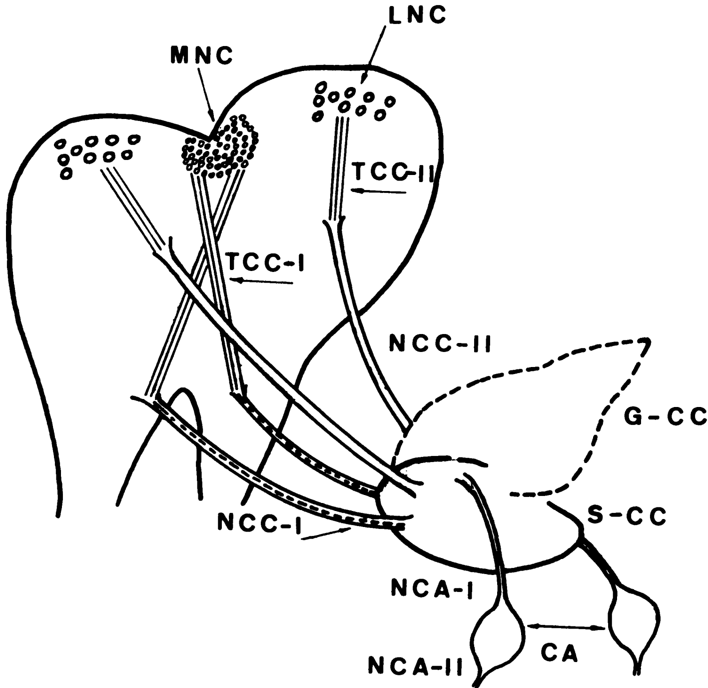

Figure 1. Schematic representation of the main features of the locust brain and retrocerebral complex. MNC = median neurosecretory cells; LNC = lateral neurosecretory cells; TCC-I = axonal intercerebral tracts from the MNC leading to the NCC-I, a ventral pair of nerves which innervate the CC. TCC-II = the axonal intercerebral tracts from the LNC leading to the NCC-II, a dorsal pair of nerves which innervate the CC. G-CC = the glandular lobe of the CC; S-CC = the secretory neurohemal lobe of the CC. NCA-I are a pair of nerves which lead to the CA. The NCA-II lead to the subesophageal ganglion.

Short-term modulation of locust CA activity was based on indirect evidence until the recent isolation and partial purification of allatotropin I (16). This is most probably the same factor described from the brain and CC of adult L. migratoria females (15). Allatotropin causes a transient stimulation of the rate of JH synthesis in the mature CA in vitro. In contrast to long-term activating factors, the effect of allatotropin I decays rapidly, within an hour of its absence (14). High levels of in vivo allatotropin-stimulated JH synthesis would therefore not be detected by the in vitro system. Allatotropic stimulation might also explain the sustained high levels of activity occasionally obtained with transplanted CA, where no nervous connections remain (28). A transient in vivo stimulation of JH synthesis that is not retained during the in vitro assay may explain the inconsistencies found between in vivo and in vitro JH synthesis in solitarious and gregarious L. migratoria (6). Titer determinations of allatotropin I are not yet possible, but semi- quantitative determinations, based on dose-response curves, suggest that high levels of cerebral allatotropin I are found at all stages of mature locusts (14), though less is found in the brains of newly fledged locusts (Abd El-Hadi, F.; Applebaum, S.W., unpublished data).

In diapausing females of the Savio strain of L. migratoria, the basal activity of the CA is totally inhibited in vivo, as established by the arrest of oocyte development and absence of JH in the hemolymph (27). However, these same glands are immediately activated when they are incubated in vitro (i.e., denervated and removed from their natural milieu), indicating that their maturation has not been retarded. In vivo denervation of the glands (NCA-I transection) in diapausing females elicits elevated JH titer and induces oocyte development, indicating that this short-term inhibition is axonally imposed.

Mode of Action

The development and deposition of eggs is cyclic in locusts, with the first cycle accompanied by parallel maturation of the CA (11,14). The latter has usually been evaluated by determining JH production in vitro in the presence of FA (8,13,29), but allatal response to allatotropin would seem to be more appropriate for portraying the physiological state of CA maturation. The rates of JH synthesis elicited by FA and allatotropin are equivalent only in mature CA. Immature CA of newly emerged adult females exhibit both low basal activity and low competence. Some small stimulation of JH synthesis is obtained in response to exogenous FA, indicating that the endogenous FA supply is limited relative to the capacity of the terminal enzymes. However, immature CA are no stimulated by allatotropin, possibly because they are deficient in rate-limiting enzymes, or alternatively, that they have not yet developed responsiveness to the hormone (Figure 2). Responsiveness is usually meant to describe the abundance of hormone receptors, but no studies on allatotropin receptors have been conducted in insect CA.

Considerable differences are found in the rates of JH synthesized by mature CA of different locust females and between the

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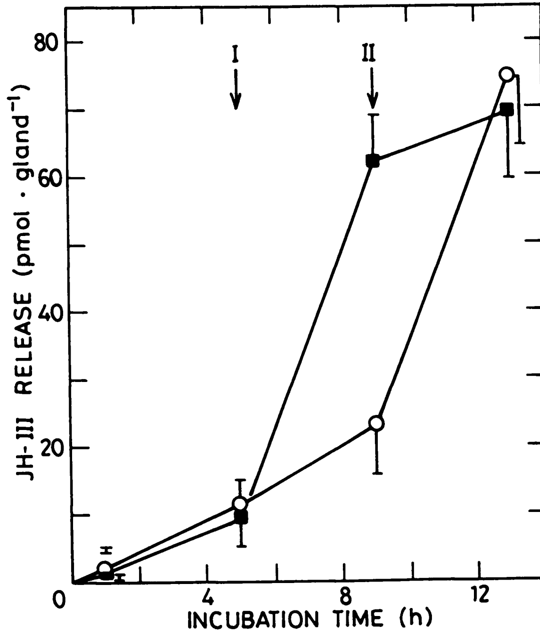

Figure 2. Cumulative JH synthesis in vitro by CA of immature (2-3 days) adult female locusts. The first 5 hr demonstrate basal synthesis. I = addition of either 50ul farnesoic acid (FA) (■) or allatotropin (O) for an additional 4 hr incubation. II = transfer of the allatotropin-treated CA to FA-supplemented media, and FA-treated CA to allatotropin-supplemented media. Values are the mean ± SE of 11 replicates. The error bars of the 1 hr incubation have been placed above and beside the values in order not to obscure them.

two glands of each locust (11,14,18). About the same degree of variation also exists in the competence of these glands and a good correlation was found between the basal and FA-induced rates of synthesis (13). Such a correlation was however not found in similar experiments with 8-day old females (9).

The biosynthetic maturation of the CA involves the elaboration of key enzymes at different levels of the biosynthetic pathway of JH. The first of these are rate-limiting enzymes, early in the biosynthetic pathway, amenable to activation or inhibition. The second of these, terminal epoxidase and methyl-transferase, are usually regarded as non-rate-limiting. Their increase in activity is directly correlated to the increase in CA volume (18,30). When the amount of these enzymes is marginal, as presumably occurs in immature CA, this also restricts the total capacity of the CA to synthesize JH. When mature CA are activated, the levels of rate-limiting enzymes supply sufficient precursor FA for maximal activity of the terminal epoxidase and methyl-transferase. This can be demonstrated by the rapid *in vitro* effect of allatotropin on *de novo* synthesis of JH from acetate (31).

Reserpine treatment retards the development of allatal competence of locust females for several weeks, but basal rates appear to be normal (32). This indicates that development of the two key enzyme systems are not dependent one on the other. Reserpine acts in vertebrate systems by depleting monoamine transmitter substances in the central nervous system (33) and has been shown to activate peptidergic neurosecretory pathways in insects (34,35). The differential response to reserpine in the locust CA suggests that part of the process of developmental maturation may be dependent on neurosecretion.

Other than the description of neural and humoral connections and interactions, which has been detailed above, little is known of the mode of action of **long-term modulating factors** on basal activity of the CA. Basal activity is marginal in mature nonvitellogenic older females and no correlation can be found in such cases between basal and FA-induced activity. The latter is often high, indicating that competence is retained (i.e., that the non-rate limiting terminal enzymes are abundant and active). We interpret such long-term control of the basal activity of the glands in terms of an equilibrium between active and inactive forms of the rate-limiting enzyme (or enzymes). We regard this equilibrium as subject to gradual change during the cycles of productivity.

Short-term modulation is superimposed by allatotropic and allatostatic factors on the basal, long-term modulated JH synthesis. The basal activity of locust CA is usually below the maximal potential for JH synthesis and is amenable to allatotropic stimulation. The structure of locust allatotropin has not yet been determined, but it is known to be a peptide, presumably of low molecular weight. Various observations indirectly support the probability that it is humorally transmitted. CA excised from mature virgin female locusts and from sibling mated females (Table 1) do not differ in their basal activity and responsiveness to allatotropin, but oocyte development is retarded in the virgin females (36). This implies that although maturation and long-term activation of the CA

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are unaffected by mating, allatotropin stimulates JH synthesis by the CA in vivo, hastening the maturation of oocytes. Allatotropic stimulation has been shown to decay rapidly, so that when the CA are excised and incubated in its absence, they revert to basal rates of JH production, which do not accurately represent the in vivo state of activity. The variation in basal activity of individual glands has been attributed to random changes in the physiological level of activation of the glands, due to a pulsatile on/off mechanism (18), but here too it is more probable that these differences reflect the inherent variability in the maturity of the glands.

Table 1. Effect of Mating on Oocyte Size and JH Synthesis In Vitro [1]

Treatment	Oocyte Size (mm)	Basal JH Synthesis (pmole/CA.hr)	Allatotropin-stimulated JH synthesis (pmole/CA.hr)
A	5.8±0.4	29.8±10.8	75.6±24.0
B	1.7±1.2	20.0± 8.1	65.8±20.6
C	1.1±0.7	40.1±16.8	81.6±32.5

[1] Values are the mean of 5 replicates ± SE.
A= Females and males caged together; 20 days after adult emergence.
B= Females and males separated by screen mesh; 20 days after adult emergence.
C= Females isolated from males in separate culture room; 20 days after adult emergence.

Cerebral levels of allatotropin are high after maturation and hemolymph levels are presumably subject to control by a releasing factor. Neuroparsins A and B are cerebral peptides produced in the MNC (37) which are transported to the CC, from where they have been isolated (38) and their sequences determined (39). Semi-isolated neuroparsin A retarded oocyte growth when injected and antiserum to neroparsin A induced green pigmentation and precocious sexual maturation, but neither had any effect on in vitro (40) or in vivo JH levels (Girardie, unpublished data, cited in 40). It is therefore unlikely that neuroparsin A reduces hemolymph levels of JH. It may perhaps be involved in inhibiting the release of allatotropin in appropriate physiological situations. All the data at hand suggest that the mature CA in fact synthesize JH at a basal rate which is only a part of their potential, and that they are then activated by allatotropin selectively.

The direct effect of an allatostatic ovary-derived factor was first reported in a survey of various tissue extracts of vitellogenic locust females on JH synthesis in vitro (16). We have recently found

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(17, Hirsch, J.; Applebaum, S.W., unpublished) that this allatostatic factor is degraded by trypsin and proteinase K, but not by chymotrypsin. Ovarian allatostatin is absent in the previtellogenic ovary. Its content is highest in the ovary of a female bearing oocytes of 3 mm length and later decreases. Thereafter, ovarian allatostatin accumulates in the hemolymph. In vitro, it overrides allatotropic stimulation, and we suggest that this may also be the situation in vivo with ovarian-derived allatostatin participating in the negative control of CA activity at the end of the gonadotrophic cycle (Figure 3).

As was previously stated, the amount of JH produced by mature CA is dependent on the activation state of the presumptive rate-limiting enzymes, but nothing is known of how allatotropin activates these enzymes or how allatostatin inactivates them. Experiments utilizing precursors were designed in order to identify the regulatory enzymes responsible for rate-limitation of JH synthesis. FA, the penultimate precursor of JH, enters after the rate-limiting stage and activates competent CA of vitellogenic females. We have recently shown that mevalonate (17), one of the first products within the biosynthetic pathway, also activates mature CA and by definition is therefore situated after the rate-limiting stage. Comparative dose-response curves with these two precursors show that the more removed the exogenous precursor is from JH, the higher the concentrations required for stimulation: FA is active in the micromolar range, whilst mevalonate acts in the millimolar range.

Mevalonate is produced by the action of 3-hydroxy-3-methyl-glutaryl (HMG) coenzyme A reductase. Two specific inhibitors of HMG-CoA reductase - mevinolin and hydroxymethylglutarate - were therefore included in factorial in vitro experiments, with and without mevalonate and/or allatotropin. Hydroyxmethylglutarate is a structural analog of HMG-CoA reductase, as is the fungal metabolite mevinolin. Both are competitive inhibitors of the vertebrate enzyme, and reduce cholesterol biosynthesis (41). We found that concentrations of 10^{-4}M mevinolin and 100mM HMG depressed the basal level of JH synthesis significantly, and allatotropin was unable in both cases to circumvent this inhibition. In contrast, mevalonate increased the JH synthesis to levels significantly higher than the basal levels, approaching those elicited by allatotropin (Abd El-Hadi, F.; Applebaum, S.W., unpublished data) (Figure 4). These results strongly suggest that mevalonate synthesis is rate-limiting JH synthesis. We are still left with the necessity of directly identifying the regulatory enzymes and how their activity is controlled at the molecular level. HMG-CoA reductase is an obvious candidate for this role. In vertebrates, it's activity is modulated by reversible phosphorylation (42). However, in experiments carried out in vitro with CA of the cockroach *Diploptera punctata*, no correlation was evident between activity of the enzyme and JH synthesis (43). The locust CA seems to be a preferred system for examining allatotropic regulation of JH synthesis, but pure synthetic allatotropin is essential for definitively establishing the presumptive role of reductase in the regulation of allatal activity. Efforts to purify and sequence allatotropin are in progress.

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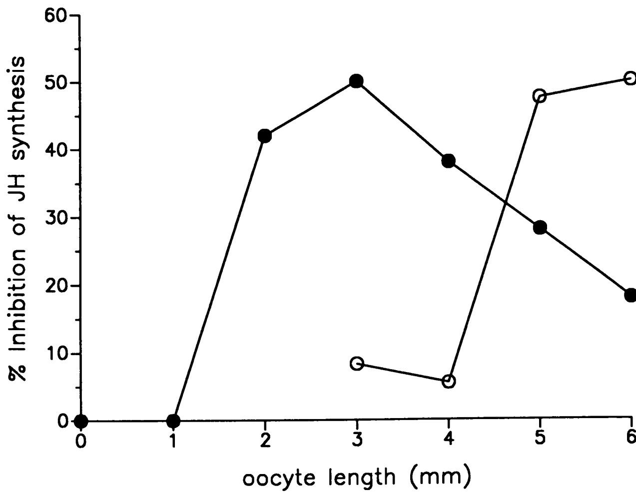

Figure 3. Inhibition of JH synthesis *in vitro* by allatotropin-stimulated CA of vitellogenic locusts. ●—● = ovarian extract; O—O = hemolymph extract. Values are the mean of 7-12 replicates.

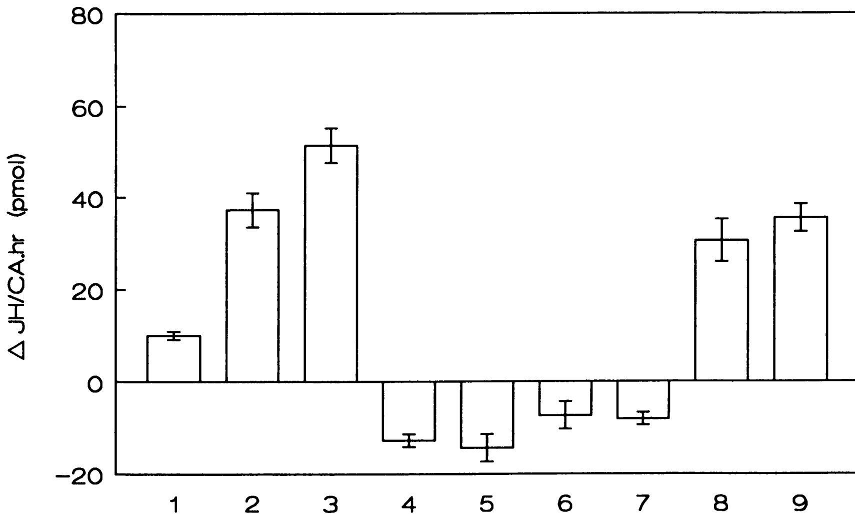

Figure 4. Differential JH synthesis *in vitro* (stimulated/inhibited minus basal rates) in the presence of mevinolin or HMG ± allatotropin or mevalonate. 1 = control (no additions); 2 = allatotropin; 3 = mevanolate; 4 = mevinolin; 5 = HMG; 6 = HMG + allatotropin; 7 = mevinolin + allatotropin; 8 = HMG + mevalonate; 9 = mevinolin + mevalonate. Values are the mean ± SE of 10-14 replicates.

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Chapter 16

Allatostatins

Neuropeptide Inhibitors of Juvenile Hormone Synthesis in Brain and Corpora Allata of the Cockroach *Diploptera punctata*

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The hypothesis that allatostatins in the brain are transported to the corpora allata (CA) by axons was substantiated by the binding of allatostatin antibodies to corpora cardiaca-CA complexes and by the demonstration of allatostatic activity in fractions from extract of 6000 pairs of CA that cochromatograph with synthetic allatostatins 1-4 on reverse phase HPLC. These fractions inhibited juvenile hormone (JH) synthesis in test CA in an *in vitro* assay. Two truncated peptides containing only the 6 and 5 C-terminal amino acids of allatostatin 4 (8 amino acids) were 10 and 100 times, respectively, less inhibitory than the complete peptide.

It is important to understand the regulation of the corpora allata (CA) because the modulation of the titer of juvenile hormone (JH), which parallels CA activity (1), is required for the maintenance of larval characteristics, the absence of JH is necessary for metamorphosis of larva to adult, and in the adult female, JH is required, in most species, for egg development.

Neurosecretory material in the brain was recognized as a likely source of inhibitory regulation of the CA in the cockroach *Leucophaea maderae* by Scharrer (2, 3). Final stage larvae molted to supernumerary larvae following severance of the nerves projecting from the brain to the CA (2). Also, unilateral transection of such nerves resulted in accumulation of neurosecretory material proximal to the cut, depletion in the corpora cardiaca (CC), and enlargement of the CA on the operated side. These observations suggested enhanced JH synthesis after denervation of the CA (3). In addition, Scharrer described nerve terminals containing neurosecretory granules within the CA (4).

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Scharrer hypothesized that neurosecretory cells of the brain that innervate the CC and CA transport inhibitory peptidergic factors to the CA.

Scharrer's hypothesis is also applicable to other species of cockroaches, such as Diploptera punctata. This viviparous cockroach has been a favorable experimental animal in which to test Scharrer's hypothesis further. The female mates immediately after adult emergence, and this mating is required to initiate an increase in JH synthesis and the resulting vitellogenic growth of oocytes. The CA of virgin females do not appear to produce enough JH to initiate normal vitellogenesis (5). However, following severance of the nerves between the brain and the CA, a cycle of JH synthesis and vitellogenesis occurs even in virgins (5). Thus JH synthesis in virgins is inhibited by innervation from the brain. A deficient nutritional condition can also inhibit the CA. A female deprived of protein as a larva develops eggs slowly due to limited synthesis of JH (6). Denervation of the CA restores normal JH synthesis and egg development in such females. These are examples of inhibition of the CA through intact nerves from the brain. CA also appear to be inhibited by brain factors traveling through the hemolymph in both larvae and adults of D. punctata (7-10).

Factors that inhibit the CA, called allatostatins (11), have been extracted from the brain as well as the CC and CA of adults (12) and from the brains of larvae (13, 14). Since one adult brain equivalent elicited about the same inhibition of JH synthesis in vitro as 10-20 pairs of CA (12, 15), brain tissue was used as a source of material for the identification of allatostatins (16). Following initial purification of active material on a reverse phase (RP) C_{18} cartridge, high pressure liquid chromatography (HPLC) was used to separate the active material into several fractions. Two of these fractions were further purified and four similar amidated peptides, allatostatins 1-4, have been isolated and sequenced (Fig. 1). Synthetic peptides have the same retention times as the native materials on HPLC. The order of activity of the allatostatins is 1 > 2 = 4 > 3. The action of these peptides on the CA in vitro is reversible. Allatostatin 1 also inhibits CA of adult Periplaneta americana (16).

Many questions remain to be answered with respect to these and other allatostatins that have not yet been identified. In the present paper we have asked whether the material extracted from the CA contains the same allatostatins that have been isolated from brain tissue. We have also determined what part(s) of the allatostatin molecule is important for its activity.

Materials and Methods

Immunohistochemistry. Antisera to synthetic allatostatins coupled to bovine serum albumin with glutaraldehyde (17) were generated in mice (18). Brain-CC-CA complexes, fixed in 4% glutaraldehyde, were incubated in diluted antiserum and the binding sites were visualized as described by Denburg et al. (19) except that horseradish peroxidase-diaminobenzidene was used instead of fluorescein. CC-CA were photographed in buffer with transmitted light.

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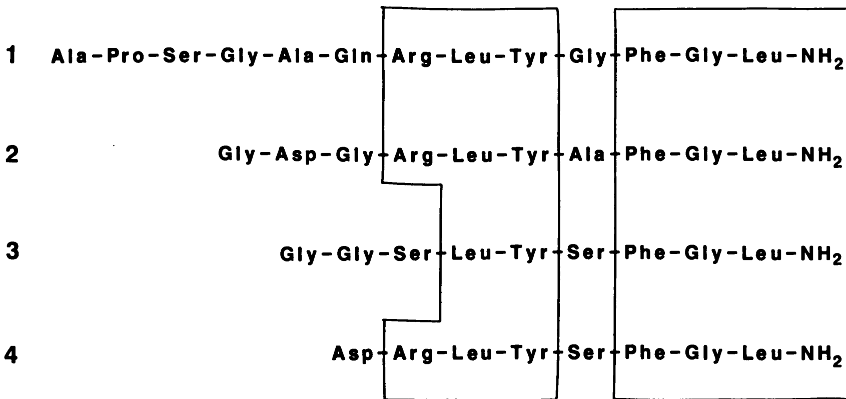

Figure 1. Sequences of four allatostatins isolated from brains of adult virgin female _Diploptera punctata_. The peptides are similar at the C termini and are amidated. Amino acids in identical positions are indicated by the boxes (modified from _16_).

Isolation of Allatostatins from CA. For the isolation of allatostatins from CA, 6000 pairs of glands were dissected from virgin and mated females. The extraction and isolation procedures were the same as those described in Woodhead et al. (16) except that the first HPLC separation employed a C_{18} RP column with a shallow gradient (10-35% over 50 min., 1 ml/min flow rate) of acetonitrile with 0.1% trifluoroacetic acid. Approximately 500 CA equivalents were applied for each of 12 runs. Synthetic allatostatins 1-4 were used as markers for collection of fractions. Two fractions were collected on the basis of the elution time of synthetic allatostatins: one expected to contain allatostatin 4 and the other to contain allatostatins 1, 2 and 3. These were separated by about 0.7 min. The second HPLC separation, on a C_8 RP column, was as described in Woodhead et al. (16), and peaks of UV-absorbing material were collected by hand. A third separation of the C_8 fraction which showed biological activity corresponding to the elution time of allatostatins 1 and 2 was performed on a C_{18} RP column as for the first separation. For this third separation 0.2 ml fractions were collected for the duration of time required to elute allatostatins 1 and 2.

Assay. *In vitro* assays for inhibition of JH III synthesis by material eluting from the columns near and at the same time as the synthetic allatostatins were performed using single CA from 2 day virgin females in 50 µl of medium 199 (GIBCO) (16). Control rates of synthesis of the glands were established in a 3 h incubation in untreated medium, then glands were transferred to medium containing eluted material. Results are expressed as percent inhibition of JH synthesis [1-(treated rate/untreated rate) x 100]. Four glands were tested for each fraction and the results expressed as the mean percent inhibition ± standard error of the mean (SEM). The quantity of material, expressed as CA equivalents, used in assays for each putative allatostatin (see figure legends and Results) was based on the size of the UV-absorbing peak compared to one of known activity from brain extract, on relative activity of the synthetic allatostatins (16) and on the amount of eluate collected.

Assay of Truncated Peptides. The truncated forms of allatostatin 4 were synthesized on a Pharmacia-LKB Biolynx model 4170 automated solid-phase peptide synthesizer by The Biotechnology Service Centre, Department of Clinical Biochemistry, Banting and Best Institute, Toronto, Canada. The assays comparing the activity of allatostatin 4 with the two analogues were carried out as for the HPLC eluates.

Results

Evidence for Allatostatins in CA. The amino acid sequences for the allatostatins that have been isolated from brain extract are shown in Figure 1. Our goal was to determine whether these allatostatins could be isolated from CA. Evidence for the presence of allatostatins in the CA is shown by the binding of allatostatin antibodies to CC-CA tissue (Fig. 2). Polyclonal mouse antibodies produced in response to peritoneal injections of synthetic

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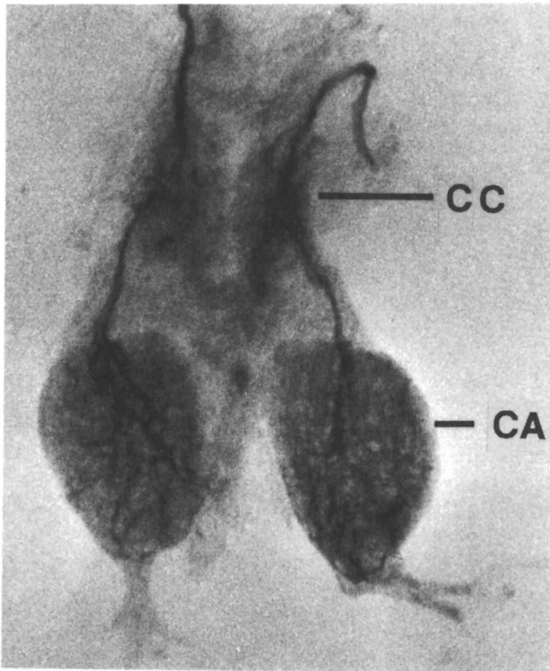

Figure 2. A pair of corpora allata (CA) and corpora cardiaca (CC) shows immunoreactivity to allatostatin 1 antibodies in a nerve descending from the brain through the CC to the CA. Glands from a day 1 last instar female larva; X 215.

allatostatin 1 coupled to bovine serum albumin bound to axons that traverse the CC and branch extensively in the CA (Fig. 2). This has been observed in CC-CA of adults as well as last instar females. However, since allatostatin 1 antibodies also bound to allatostatins 2, 3 and 4 in enzyme-linked immunosorbent assays (Stay, unpublished), polyclonal antibodies could not be used to identify the specific allatostatins within the CA. Hence the isolation of allatostatins from CA by extraction and HPLC separation was undertaken.

Isolation of Allatostatins from CA. A preliminary separation of extract of 200 CA on a C_{18} column revealed the presence of material that inhibited JH synthesis by CA *in vitro*. These factors eluted at the same times as the allatostatic material of brain extract. This included the areas of allatostatins 1-4 and several adjacent areas (16).

The elution times of synthetic allatostatins from the C_{18} column served as markers for subsequent collection of fractions from CA extract. Extract from 6000 pairs of female CA was separated on the C_{18} column, 500 pairs at a time. The material eluting at the time corresponding to that of synthetic allatostatin 4 was pooled and applied to a C_8 column immediately following an HPLC separation of synthetic allatostatin 4. Relevant parts of these chromatograms are shown in Figure 3 with the assay for the inhibition of JH synthesis by these eluates. A peak of UV-absorbing material occurred with the same retention time as that of synthetic allatostatin 4. The peak was about half the size as that from an extract of 1200 brains (16). The allatostatin extracted from CA inhibited JH synthesis 70 $\pm$ 8% when tested at 100 CA equivalents/CA.

Material eluting from the first C_{18} column at the same time as allatostatins 1-3 (~ 43.9 to 45.3 min) was collected together and reapplied to the C_8 column immediately after an HPLC separation of synthetic allatostatins 1-3. The relevant parts of these chromatograms are shown in Figure 4 with the assay for inhibition of JH synthesis by these eluates. A peak of material eluted with the same retention time as synthetic allatostatin 3. This peak was about half the size as that from an extract of 1200 brains (16). Because allatostatin 3 is less active than 4, the material was tested at 200 CA equivalents/CA; it inhibited JH synthesis 48 $\pm$ 6%. Although other material also eluted in the regions of allatostatins 1 and 2, collections were made according to the retention times of these allatostatins. The presence of allatostatins 1 and 2 is indicated by the inhibition of JH synthesis (84 $\pm$ 2% and 76 $\pm$ 0.3%) when tested at 100 CA equivalents/CA.

Allatostatins 1 and 2 which eluted between 41.95-44.1 min on the C_8 column were then separated on a C_{18} column immediately after separation of synthetic allatostatins 1 and 2. Figure 5 shows two peaks of UV absorbance in CA extract corresponding to the elution times of synthetic allatostatins 1 and 2. This material, collected in 0.2 ml aliquots, was tested at 300 CA equivalents/CA and showed 70-75% inhibition of JH synthesis.

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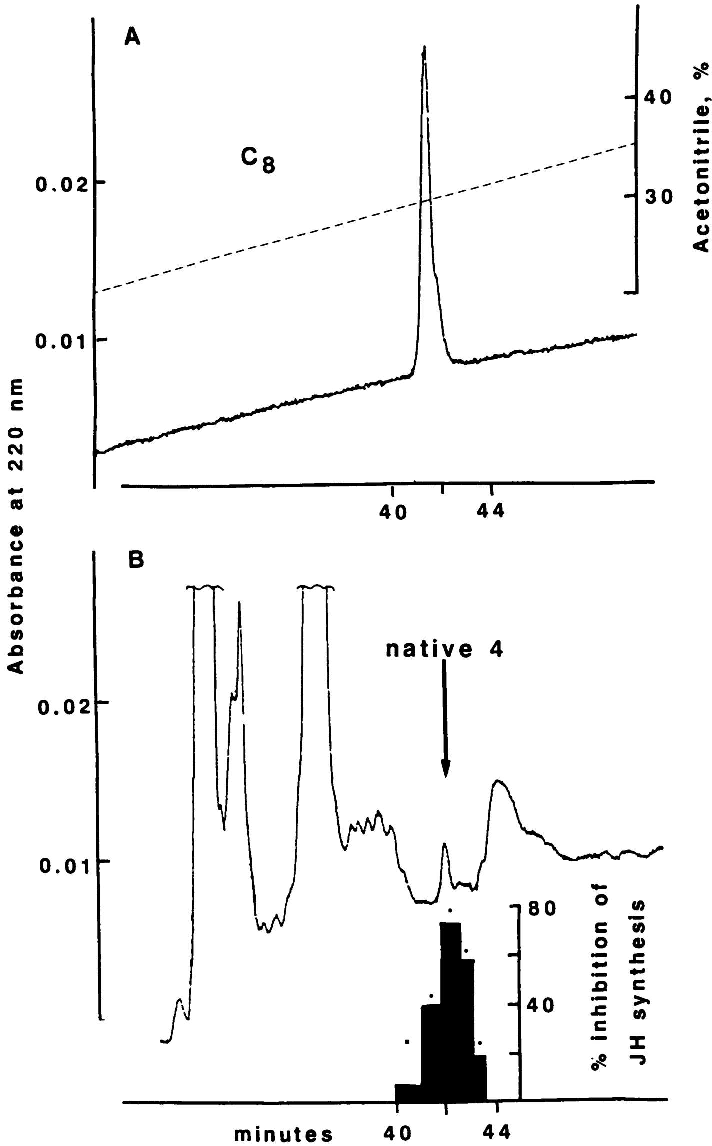

Figure 3. Isolation of allatostatin 4 from CA by HPLC on a C_8 column. A) Synthetic allatostatin 4 eluted with an acetonitrile gradient (dashed line). B) Extract of 6000 pairs of CA (collected from a C_{18} column in the region of allatostatin 4) run immediately following and with the same gradient as that in A. The histogram shows the inhibition of juvenile hormone (JH) synthesis of fractions eluting at the same time and adjacent to allatostatin 4. Fractions were tested on 3-4 CA at 100 CA equiv./CA. Dots indicate SEM.

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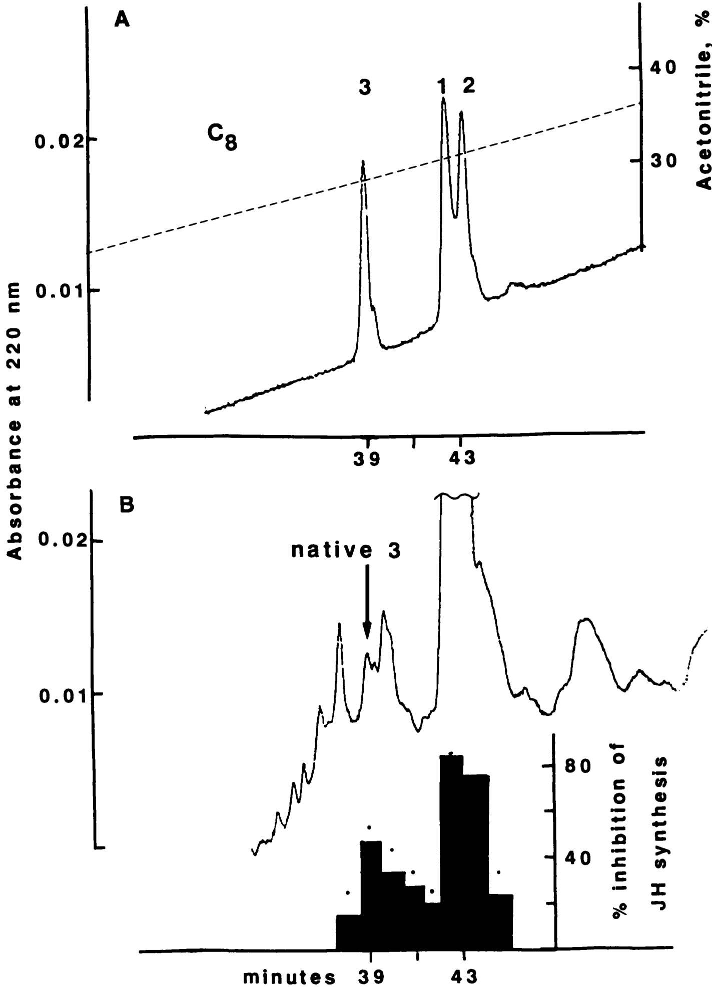

Figure 4. Isolation of allatostatin 3 from CA by HPLC on a C_8 column. A) Synthetic allatostatins 1, 2 and 3 eluted with an acetonitrile gradient (dashed line). B) Extract of 6000 pairs of CA (collected from a C_{18} column in the regions of allatostatins 1, 2 and 3) run immediately following and with same gradient as that in A. The histogram shows the inhibition of JH synthesis of fractions eluting before, at the same time as, and after allatostatin 3. These fractions were tested on 3-4 CA at 200 CA equiv./CA. Material eluting at the same time as allatostatins 1 and 2 was assayed at 100 CA equiv./CA. Dots indicate SEM.

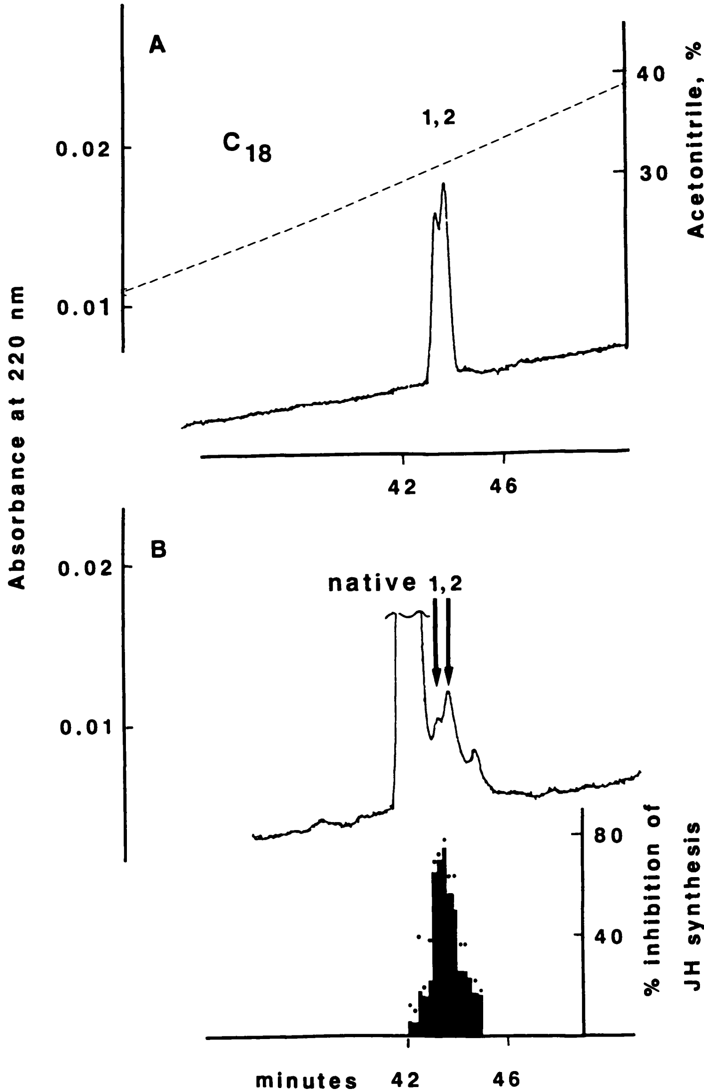

Figure 5. Isolation of allatostatins 1 and 2 from CA by HPLC on a C_{18} column. A) Synthetic allatostatins 1 and 2 eluted with an acetonitrile gradient (dashed line). B) Material eluting at the same time as allatostatins 1 and 2 collected from the C_8 separation shown in Figure 4B was run immediately following and with the same gradient as that in A. The histogram shows the inhibition of JH synthesis of 0.2 ml fractions collected in the regions of allatostatins 1 and 2. Three to four CA were assayed at 300 CA equiv./CA. Dots indicate SEM.

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Assays of Truncated Allatostatin 4. The amidated octapeptide, allatostatin 4, was synthesized in two forms shortened by 2 and 3 amino acids, respectively, at the N terminus (Fig. 6). The response of CA from 2 day virgin females to increasing concentrations of allatostatin 4 and its two analogues showed approximately a ten-fold decrease in activity as the peptide was shortened from 8 to 6 amino acids and a further ten-fold decrease as it was shortened from 5 to 4 amino acids.

Discussion

Extract of CA from *D. punctata* contains at least four allatostatins. These have the same retention times on HPLC as the allatostatins isolated from the brain and have similar abilities to inhibit the synthesis of JH by CA *in vitro*. Thus it may be concluded that the members of this family of related allatostatins produced by neurosecretory cells of the brain are transported to the CA where they act to depress the rate of JH synthesis. This neural method of delivery of peptidergic neurosecretion from brain to target organ is analogous to that of vertebrates in which hypothalamic factors are released into a portal system and delivered to the anterior pituitary, as Scharrer noted (4).

The distinct peaks of UV absorbance resulting from chromatography of 6000 pairs of CA occurred at the same retention times as synthetic allatostatins. These peaks were about half the size (Figs. 3-5) as those from an extract of 1200 brains (16) which would indicate that half as much allatostatin was recovered from 6000 CA as from 1200 brains. Thus this CA preparation contained 10 x less of each allatostatin as the brain preparation. In addition, the inhibition of JH synthesis resulting from 100 CA equivalents of eluates at the retention times of allatostatins 1, 2 and 4 and from 200 CA equivalents of eluate at the retention time of allatostatin 3 showed greater inhibition than 5 brain equivalents of eluates from the extraction of 1200 brains. This would be expected from the relatively greater concentration of CA material tested. The four allatostatins occurred in about equal amounts in the brain (16). Assay of the four allatostatins derived from CA showed 70-80% inhibition with putative allatostatins 1, 2 and 4 and ~ 50% inhibition with putative allatostatin 3. This also suggests that the four allatostatins occur in about equal amounts in CA.

Pratt et al. (20) have isolated and sequenced allatostatin 1 from the brain of adult female *D. punctata* using isolation procedures different from ours and also indicated that other allatostatins remain to be identified. With yet other methods Khan, Guan and Tobe (unpublished) also isolated and sequenced allatostatin 4 from extract of *D. punctata* adult brain. It will now be important to determine whether these peptides occur and act as allatostatins in other groups of insects. The corpora allata of *P. americana* are also inhibited by allatostatin 1 (16). Since *P. americana* is not closely related to *D. punctata* (21), it is likely that these allatostatins will be functional in other species of cockroaches. A cerebral allatostatic factor from last instar larval *Manduca sexta* inhibits JH I synthesis by CA *in vitro* (22). This factor is characterized as a protein of much higher

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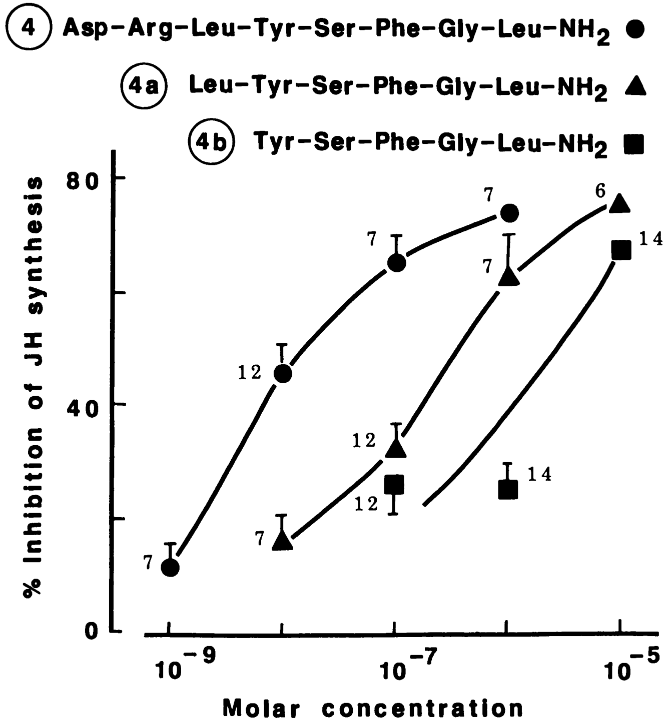

Figure 6. Responses of CA to synthetic allatostatin 4 (●) and two truncated peptides, 4a (▲) and 4b (■). Single CA were incubated for 3 h in untreated medium then transferred to medium with allatostatin 4 or one of the analogues. Numbers of CA assayed are shown beside the data points. Bars indicate SEM.

(6.8-13 kDa) molecular weight than that of the allatostatins of D. punctata that inhibit JH III synthesis.

The existence of multiple forms of the same peptide is not an unexpected finding. In Drosophila melanogaster a single gene codes for a family of Phe-Met-Arg-Phe-NH_2 (FMRFamide) related peptides which are similar at the C terminus (23), as are the allatostatins in D. punctata. Whether or not the different forms of allatostatins have different functions remains to be determined. It is clear that the different forms of allatostatins have different activity when tested on CA of 2 day virgin females (16), with allatostatin 3 showing the lowest activity and allatostatin 1 the highest activity. The most obvious structural difference in allatostatin 3 is the lack of arginine at the 7 C-terminal position. The truncated forms of allatostatin 4, which lack arginine in this position, also showed reduced activity compared to allatostatin 4, suggesting that arginine is important for the activity of the molecule. The deletion of leucine in position 6 from the C terminus reduced the activity of allatostatin 4 still further. Structure-function studies should be extended because such information may be helpful in the design of analogues with practical applications.

Several major questions with respect to these allatostatins remain to be addressed. One is how the allatostatins act at the cellular level to regulate JH production. Crude extract of brain was not effective with the penultimate precursor of JH, farnesoic acid, in the incubation medium, suggesting that allatostatins act prior to this step in the JH synthetic pathway (15). Indeed allatostatin 1 is also ineffective in inhibiting JH synthesis in the presence of farnesol, the penultimate precursor of farnesoic acid (20). The actual step(s) in the biosynthetic pathway affected by allatostatin can now be determined. It should also be possible to isolate the receptor for allatostatin and to identify the second messenger system involved. It will also be important to localize and define the cells which produce these allatostatins, whether they change during development, and how the release of allatostatins is modulated by stimuli such as mating, feeding and the state of ovarian development. Antibodies to the peptides will be useful in these studies. Finally, of major importance is the identification of the gene(s) for these peptides and the regulation of their expression. This information will contribute to our understanding of the evolution of the mechanisms involved in the regulation of juvenile hormone synthesis.

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Chapter 17

Two Types of Allatostatic Peptides from Brains of the Cockroach *Diploptera punctata*

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We isolated two members of a family of basic allatostatins: a tridecapeptide (ASAL) and a tyrosine-rich octadecapeptide (ASB2). Both have the familial C-terminal sequence (L/V)YXFGL-amide and rapidly inhibit juvenile hormone biosynthesis by corpora allata from adult females *in vitro*. Peptide ASB2 has an unprocessed potential dibasic cleavage site. Isolation and identification was aided by gel filtration sizing (TSK) and UV diode array detection. The structure-activity relationships of eleven substitution and deletion analogs of ASAL indicate that the consensus C-terminal segment is essential for signal transduction, whereas the variable N-terminal address segments promote high affinity binding.

The allatostatins are a family of brain peptides which rapidly and reversibly inhibit juvenile hormone biosynthesis in isolated insect corpora allata. Five allatostatic peptides from brains of the cockroach *Diploptera punctata* have now been structurally identified (1,2, our unpublished results), their size ranging from octapeptide to octadecapeptide. The currently identified allatostatins all possess the consensus C-terminal sequence (L/V)YXFGL-amide, which has no significant homology with other known peptides. It is likely that they are released locally from nerve terminals within the glands (3).

It is now well established, mainly from studies on immunological cross-reactivity, that many peptidic vertebrate neurohormones have structural counterparts in invertebrates or even in bacteria (4) and vice-versa (5). Thus, metazoan neuromodulators

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can have ancient evolutionary origins; this may be especially true of peptides such as somatostatin or insulin, whose distribution is not exclusively neuronal and which have wide-ranging effects on cell metabolism (6).

On the other hand, several invertebrate neuropeptide families have no known counterparts in the more widely studied vertebrates [allatostatic hormones, allatotropic hormone (7), pheromone biosynthesis-activating neuropeptide (8,9) etc.], and so may represent more recently evolved hormone/receptor systems associated only with the regulation and integration of phenomena which are sub-phylum specific. At present, we cannot exclude the possibility that the relatively late evolution of e.g. juvenile hormones and certain pheromones, which are crucial to the specialized developmental and reproductive biology of arthropods, was associated with an independent origin of the neuropeptides which now regulate their synthesis. However, it seems unlikely that this hypothesis can progress beyond mere supposition without analysis of the molecular evolution of the genes from which such neuropeptides are derived (in insects and other invertebrates). Accordingly, we believe that the isolation and sequencing of the progenitor gene constitutes an important part of an investigation of putative novel insect neurohormones, such as the allatostatins. The sequences of incompletely processed pro-hormones are of particular interest in the case of small hormones, such as the allatostatins, since they afford more information for the construction of selective nucleic acid probes. Furthermore, there is clear evidence in vertebrates, e.g. metorphamide (10) amidorphin (11) and cholecystokinin (12), that tissue-specificities in prohormone processing enzymes often generate functional hormones containing potential cleavage sites. Thus, the primary tissue extract must be protected from indiscriminate attack by mixed proteolytic enzymes if the integrity of the more elaborate peptide hormones and prohormones is to be preserved.

We describe an isolation procedure which yielded complete sequences for two different allatostatic hormones, one containing a tyrosine-rich 12-residue N-terminal extension containing a potential dibasic cleavage site. The high degree of structural homology in their C-terminal hexapeptide amide sequence justifies inclusion in a single family. However, the dissimilarity of their N-terminal sequence and significant difference in their biological effect at certain times suggest that they belong to different sub-families and probably interact with different classes of receptors.

Sample Manipulation and Bioassay

Control of Sample Loss. Throughout the isolations we aimed to minimize loss of bioactivity by avoiding procedures which would promote adsorption to surfaces or exposure to active degradative enzymes. In practical terms this meant (a) using recently siliconized pipette tips and collection tubes, (b) employing large-volume (up to 15 ml) injection loops for chromatography, thereby avoiding the need for prolonged room temperature vacuum evaporation, (c) using highly purified carrier polypeptides in 1-5 μg amounts in all except the final high performance liquid chromatography (HPLC)

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collection tubes, (d) regularly passivating all stainless steel components in the chromatography systems (except HPLC columns themselves) with 20% nitric acid (metal-free systems were not available to us). Initially, we used bovine serum albumin (BSA) as a protective carrier, after the first C_{18} Sep-Pak. Batches of 0.25 mg crystalline lyophylized BSA (Sigma) were washed on a C_{18} Sep-Pak with 35% CH_3CN in aqueous 0.1% trifluoroacetic acid (TFA), to ensure removal of any impurities which might contaminate allatostatin fractions, then eluted with 70% CH_3CN. TFA was substituted with 0.2% formic acid when providing carrier for DIOL Sep-Pak samples. However, we found BSA to be unsatisfactory in several HPLC systems, especially the TSK solvent (see below), because of progressive denaturation and occasional precipitation. Therefore, we subsequently employed oxidized insulin B chain as carrier for all steps between DIOL Sep-Pak and final RP_{18} HPLC. Batches of 0.25 mg of insulin B chain (Sigma) were passed twice through a 250 x 4.6 mm Zorbax RP_{18} Protein Plus column in a linear gradient of CH_3CN / aqueous 0.1% TFA, taking the center cut at each pass. This carrier eluted after the most hydrophobic allatostatin components in all RPLC systems tested, except those having gradients steeper than 1.5 % CH_3CN / min (which were not used in our final purification procedure).

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Preparation for Bioassay. The standard *in vitro* assay for allatostatins (1) is a two hour organ culture of corpora allata during which the inhibition of release of radiolabeled JH III from [^{3}H-methyl] methionine is measured; accordingly, the test sample must be free from unphysiological contaminants before incorporation into the modified tissue culture medium 199 (1). Our preparative-scale procedures all involved volatile modifiers (hydrochloric acid, formic acid, ammonium formate, TFA) which could be removed along with the solvents (ethanol, water, acetonitrile): this analytical workup of aliquots for bioassay was the only occasion when we used vacuum evaporation.

Volatile solvents and solutes, up to 0.3 ml, were removed by room temperature centrifugal vacuum (SpeedVac, Savant) in siliconized tubes pre-loaded with 200 μg BSA and 30 μg bacitracin. We found that trace amounts of mercaptoethanol seriously elevate the blanks in the standard radiolabeled isooctane partition assay (13), and residues of TFA require careful readjustment of the pH of the tissue culture medium. Therefore, evaporation was routinely extended for approximately 30 minutes beyond the time of apparent dryness to ensure good removal of solvent modifiers. Dried residues were dissolved in 0.85 ml of modified culture medium 199 (1) by one minute of ultrasonication through the walls of the plastic tube, then left to stand for ca. 30 minutes and finally vortex mixed.

Samples from TSK column chromatography and some chemical procedures contained non-volatile contaminants which we usually removed by dilution to below 5% CH_3CN with aqueous 0.1% TFA followed by trapping on C_{18} Sep-Pak as previously indicated (1); each de-salting yields the test material in 4 x 0.3 ml of volatile solvents, which are suitable aliquots for vacuum evaporation. We were interested to obtain evidence for or against the existence of cysteine or cystine in the allatostatins, and devised a small-scale

desalting procedure to investigate the influence of separate and consecutive treatments with dithiothreitol and iodoacetamide on biological activity. Preliminary experiments showed that allatostatic activity could be efficiently separated from reagents by passage over P-6 gel filtration beads (Calbiochem) swollen in 50% aqueous CH_3CN. We used unfractionated brain extracts after the first C_{18} Sep-Pak pre-purification (see below) corresponding to 50 brains. Samples were first reduced to dryness in the presence of 5 µg purified BSA, and then reacted with 1 mM dithiothreitol or 5 mM iodoacetamide in 50 µl of either 0.05M phosphate buffer (pH 7.4) or medium 199 at 30°C for 30 minutes in siliconized plastic stoppered tubes. Reagents and salts were removed by adding an equal volume of 0.2% TFA in CH_3CN and centrifuging through calibrated (internal volume = 0.5 ml) Biogel P-6 columns (MF-1 microfilter units, 0.2 um Nylon 66, Alltech Associates) which had been pre-equilibrated in the centrifuge in CH_3CN/ aqueous 0.1% TFA (1:1) and pre-treated with 25 ug purified BSA in the same solvent. Incubation and centrifuge tubes were washed through twice with 50 µl of equilibration solvent, and the combined centrifugates plus carrier peptide evaporated under centrifugal vacuum. The sample was then ready for bioassay or further reaction. Control incubations yielded average recoveries in the range 75-110% bioactivity, and the absence of any effect of the reagents led us to conclude that Cys was absent from the principal allatostatins.

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Extraction and Initial Purification

Our procedure began with extraction of brain-retrocerebral gland complexes from 10-12 day-old mated females in 0.2M HCl/75% ethanol (1). This was chosen to minimize proteolysis of hormones and partially processed pro-hormones, through denaturation/precipitation of contaminating enzymes. However, and notwithstanding our ultimate success, size analysis by TSK GW2000-SWXL column (see below) revealed that the bioactive supernatant after centrifugation at -20°C contained important quantities of material in excess of 10,000D, indicating incomplete precipitation of polypeptides. Much of this material co-eluted with the total bioactivity in the 18-35% CH_3CN fraction (0.1% TFA) during initial purification on C_{18} Sep-Pak. We routinely treated the sample with 1 mM dithiothreitol after the first C_{18} Sep-Pak, since this afforded repeatably high recoveries of bioactivity. A preliminary analysis of the bioactive fraction on RP-18 HPLC (35-70% CH_3CN in 0.1% TFA, at 1.75% per min) resulted in a broad spread of activity up to 40% CH_3CN (not shown), indicating multiple hydrophobic peptides.

We devised a second pre-purification step using Diol Sep-Pak and two consecutive gradients of increasing water content carried out first with 0.2% formic acid, then 0.1 % TFA as modifiers. Sep-Paks were attached with low pressure Teflon or Tefzel fittings to a Beckman 110A single channel pump fitted with manual sample injector and flow divertor valves to allow fast change-over of pre-mixed solvents from a 30 ml reservoir. Samples of up to 20 ml were loaded at 4 ml/min from large sample loops made from low pressure (300 psi rating) Teflon tubing; larger samples were loaded off the pump by gravity feed. Components were eluted from the Paks at 1.2 ml/min and

step-wise changes in the strength of the eluant were made every 1.2 ml (single Pak) or 1.8 ml (two Paks in tandem) with system dead volumes of 0.6 and 1.2 ml, respectively. Figure 1 shows how this procedure fractionated the bioactivity (expressed in allatostatic units, ASU, 1) into two principal components, eluting at roughly similar water concentrations. These elution profiles were used to define a batch procedure employing steps of 10-40% water (formic acid) and 20-38% water (TFA) for preparative fractionation (1). TSK gel analysis revealed that fraction A activity eluting in 0.2% formic acid was essentially free from contaminants larger than 5,000D, but that approximately 25 % of the UV absorbing material in the B fraction comprised components in the size range $(5\text{-}20)\times10^3$D, having no detectable allatostatic activity. In hindsight, a low pressure gel filtration of allatostatins B may have been helpful at this stage, although our attempts to achieve this by TSK 2000 HPLC were hampered by poor biological recoveries from large scale (> 2,000 brain equivalent) separations.

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Monitoring Bioactive Components by Size Exclusion

It became clear during methods development that brain-complex extracts could contain at least 7 different bioactive components, and we wanted to focus our purifications on examples of large and small allatostatins. Accordingly we carried out gel permeation analyses on a Toyosoda 300 x 7.8 mm G2000-SWXL column pumped at 0.2 ml/min with a premixed water based solvent (pH 6.0 - 6.5) containing 0.15% ammonium formate, 0.1% sodium sulfate, 2.5 M urea, 0.2% propane 1,2 diol, 33% CH_3CN. As with all other solvents used in this work, the solute modifiers were first dissolved in the aqueous component and pumped through a C18 Sep-Pak at 4 ml/min before mixing with CH_3CN and filtration to 0.2 µM (Nylon 66). Samples were evaporated to dryness with carrier peptide, redissolved in 110 µl of running solvent, filtered to 0.2 micron and 85 - 90 µl injected at the start of the run. The effluent was monitored at 225 or 276 nm and 12 second fractions collected manually into tubes preloaded with 0.5 ml aqueous 0.1% TFA containing 5 µg of carrier peptide. The solvent was chosen because of its ability to minimize physico-chemical interactions in our set of reference peptides, but proved to be rather vigorous and several synthetic peptides suffered hydrolysis during prolonged exposure.

Isolation of Pure Peptides A and B by HPLC

RP-4, Triethylamine/Formic acid. Methods development trials on 100-500 brain-equivalents gave good recoveries of both A and B bioactivities on a 75x4.6 mm RP-4 cartridge column (Pierce), using triethylamine/formic acid as modifier, and this column was used to separately prepare samples of the principal A and B active fractions (Figures 2 and 3). The less hydrophobic components ASB1 were poorly reproducible in their activity and retention time from batch to batch, possibly indicating variable processing of prohormone(s), and the low bioactivity became unworkable after subsequent RP-18 chromatography. Accordingly, we focused on the ASB2 activity which was repeatable, and had an apparent MW of 2,250 D (n=3) by TSK

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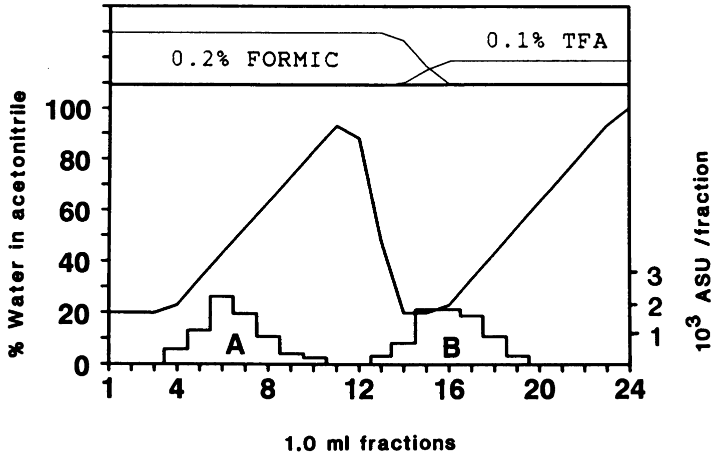

Figure 1. DIOL-Sep-Pak separation of allatostatins A and B. A typical analytical separation of extract from 430 brains, after pre-purification on RP-18 Sep-Pak. A single Pak was loaded and eluted at 0.5 ml/min with two consecutive gradients of 20-100% water in CH_3CN (5% /min) containing first 0.2% formic acid then 0.1% TFA as acid modifier. Fractions of 0.5 ml were assayed at appropriate dilution for biological activity.

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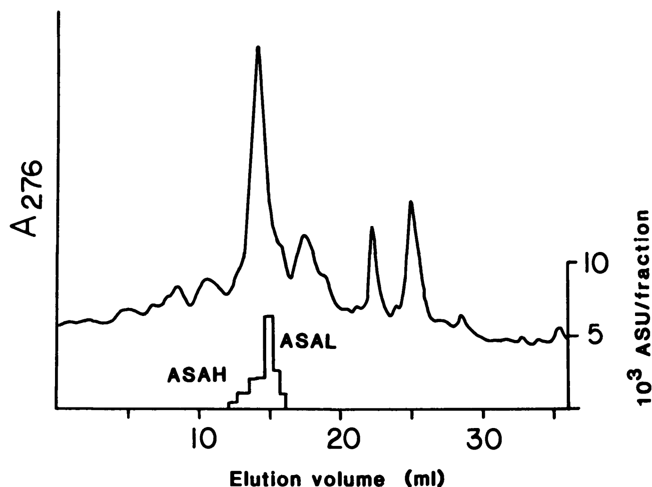

Figure 2. RP-4 reversed-phase HPLC profile for the initial separation of allatostatin A components. The A fraction from Diol Sep-Pak prepurification of extract from 12,500 brains, in 7.5 ml of initial solvent, was eluted at 0.5 ml/min by a linear gradient of 10-30% CH_3CN (0.5% /min) in aqueous 1% formic acid, 0.3% triethylamine (pH = 6.5). Suitable dilutions of aliquots of 0.5 ml fractions were bioassayed. The highest concentration of ASAL eluted at 16% CH_3CN (corrected for gradient elapsed volume).

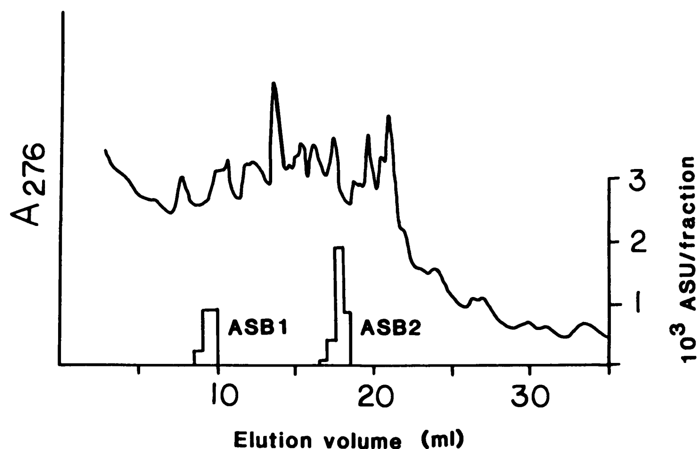

Figure 3. RP-4 reversed-phase HPLC profile for the initial separation of allatostatin B components. The B fraction from Diol Sep-Pak pre-purification of extract from 4,300 brains was eluted at 1 ml/min by a linear gradient of 10-50% CH_3CN (1% /min) in aqueous 1% formic acid, 0.3% triethylamine (pH = 6.5). Effluent was monitored at 276 nm (continuous line) and suitably diluted aliquots of 1 ml fractions were bioassayed. Elution of ASB2 was consistent at 24±0.5% CH_3CN (corrected for gradient elapsed volume), whereas ASB1 varied between runs.

analysis. The A activity routinely yielded a major peak of greatest hydrophobicity (ASAL) following a rather variable lesser spread of activity (ASAH); TSK analysis suggested approximate MWs of 980 and 1,900 D for ASAL and ASAH, respectively. We focused on ASAL as an example of the smallest natural peptide.

RP-18, TFA. We used an 115 x 2.1 mm (Pierce) semimicrobore column at 0.3 ml/min for the final chromatography, in order to increase the sensitivity of the flow-cell UV-absorption detector and to reduce final sample volumes. The ASAL and ASB2 fractions from RP-4 purification both independently gave only one bioactive peak on the higher resolution chromatography, but we failed to achieve spectral purity of either in a single pass. Figure 4 gives an example of a first RP-18 chromatogram of ASB2, developed with a 1% CH_3CN per min gradient. The sample is rich in non-aromatic-containing peptides, having relatively small extinction coefficients at low wavelengths, and pilot scale (1,000 brain-equivalent) total amino acid composition analysis confirmed the important mass contribution by small, low-wavelength shoulders on the peaks. Accordingly, we chose second, shallower, gradients which eliminated virtually all of the low-wavelength contamination of the ASAL and ASB2 components.

Both ASAL and ASB2 peptides exhibited tailing on this system, presumably due to their basicity and incomplete silanol-capping of the stationary phase. In addition to allowing cross-identification of components between different trial chromatograms, UV-spectral monitoring by diode array helped us distinguish between peak asymmetry and peak contamination. In order to deconvolute the spectral profiles we first established semi-empirical values for the partial extinction coefficients of key amino acids (Figure 5) in a set of reference peptides (see above) which eluted at similar CH_3CN concentrations to our unknowns in this system. The assignment of absolute coefficients for residues in different peptides eluting in gradients is prevented by the dependence on the context of the sequence and on the organic solvent concentration. However, the values given in this study had sufficient predictive power (Figure 5) to dictate the need for rechromatography. Ultimately, we obtained both ASAL and ASB2 at approx. 90% mass purity, suitable for gas-phase sequencing and tandem MS sequencing, in yields of 90 fmole/brain and 35 fmole/brain, respectively.

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Primary Sequence of the Peptides

The sequences are based upon automated Edman degradation (ASAL) or tandem MS (ASB2), and were confirmed by total amino acid composition analysis (ASAL), FAB^+ MW determination before and after methylation, and co-chromatography of synthetic and natural products yielding indistinguishable UV-spectra by diode array detection. Methylation experiments confirmed the amidation of the potential C-terminal carboxyl groups and partial N-terminal analysis of ASB2 established the free Glu carboxyl at position 7. The found MWs of 1,335.5 (ASAL) and 2,168.7 (ASB2) were in close agreement with theoretical values and revealed predictable errors of up to 25% in the size determinations by TSK chromatography (Figure 6). The Leu/Ile ambiguity at position 18 (C-terminus) in ASB2, resulting from the C-

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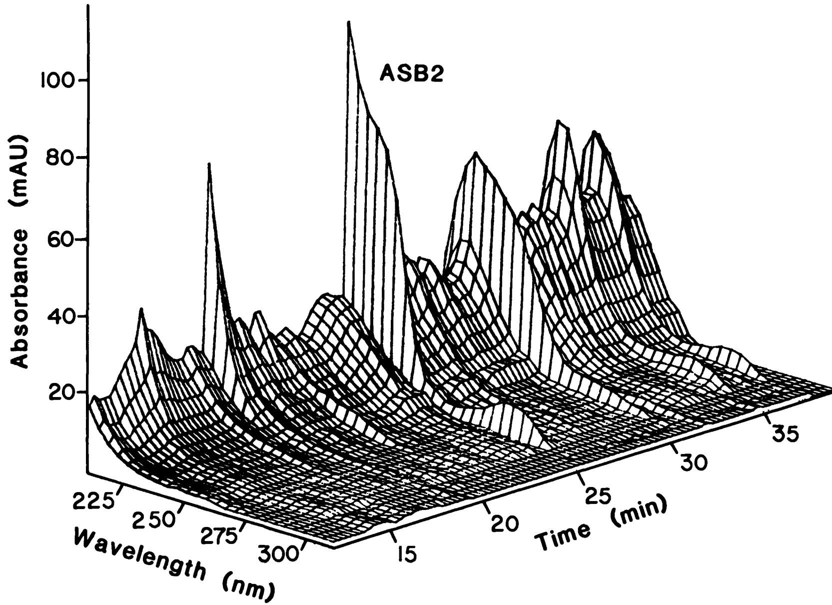

Figure 4. Initial RP-18 reversed-phase HPLC profile for the purification of ASB2 monitored by diode array UV detection. Combined fractions ASB2 from RP4-HPLC, representing extracts of 6,000 brains in 5.2 mls initial solvent, were eluted at 0.2 ml/min by a gradient of 10-50% CH_3CN (2% /min) in aqueous TFA (1-0.8%), monitoring at 210-320 nm. The strongly aromatic signal at 26 min, representing over 200 pmol ASB2, shows major impurities absorbing below 235 nm.

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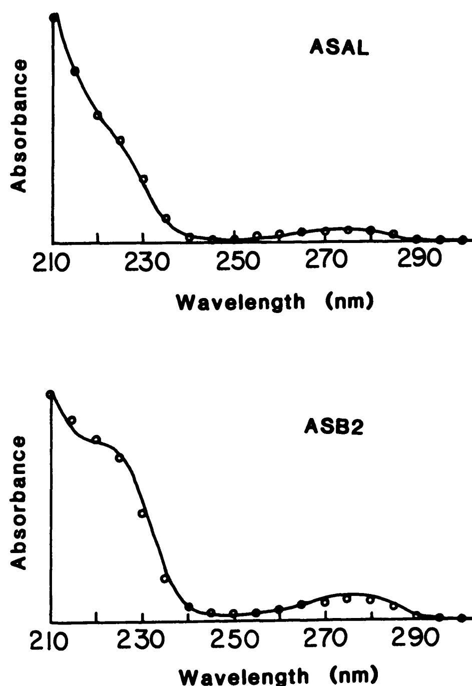

Figure 5A. UV spectra of allatostatins ASAL and ASB2 and the corresponding predicted spectra for peptides. Authentic spectra (continuous lines) were obtained by HPLC flow-cell diode array detection of final purification of ASAL and ASB2 from more than 10,000 and 5,000 brains, respectively. The spectra of the corresponding pure synthetic peptides from the same solvent gradients (not shown) were identical (ASAL) or showed only minor differences below 240 nm (ASB2).

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EXTINCTION COEFFICIENTS

nM	Tyr	Trp	Phe	His	Arg	Peptide
210	6000	24568	14140	5800	84	1085
215	8000	29384	8854	6206	53	669
220	9700	35400	4200	5220	30	350
225	10000	25311	1496	2900	15	224
230	7000	10620	456	1160	8	108
235	2766	4892	185	458	0	56
240	868	2424	145	80	0	21
245	439	1749	224	40	0	9
250	351	1948	342	0	0	5
255	390	2628	400	0	0	4
260	735	3308	416	0	0	2
265	981	4185	300	0	0	2
270	1226	5062	132	0	0	1
275	1400	5598	45	0	0	1
280	1303	5600	28	0	0	1
285	950	4630	24	0	0	1
290	300	4250	20	0	0	0
295	100	1488	18	0	0	0
300	43	0	16	0	0	0

Figure 5B. Partial extinction coefficients were approximated by best-fit deconvolution of spectra from a series of authentic 4 to 30-meric peptides eluting at between 12-48% CH_3CN.

proximal assignments by MS analysis, was resolved in favor of Leu by high-resolution HPLC separation of ASB2 and its Ile^{18} isomer. The sequence of ASAL is given in Table I, and the sequence Ala-Tyr-Ser-Tyr-Val-Ser-Glu-Tyr-Lys-Arg-Leu-Pro-Val-Tyr-Asn-Phe-Gly-Leu-amide has been assigned to ASB2 (our unpublished data).

Biological Activity

Slope of the Dose/Response Curve. The dose response curves obtained throughout the isolation of A and B allatostatins, using serial dilutions of bioactive fractions tested on corpora allata from our standard Day 10 pregnant females, resembled those for unfractionated extracts of brain-complexes and pure synthetic peptides. Specifically, the responses increased over a wide range of concentrations, and logit/log (Hill) plots yielded slopes of less than 0.8 (not shown). We adopted the minimal model of 1:1 stoichiometry for ligand/receptor interaction, and foresaw three possible contributory causes of shallow response curves (1) multiple receptors with graded affinities but additive effects (2) metabolic inactivation of the peptides during the course of two or three hour assays, and (3) desensitization (down-regulation) during the course of the assays.

Low Dose and High Dose Responses. A developmental comparison of the response curves towards ASAL clearly demonstrated the duality of the responses to nM and sub-μM concentrations in corpora allata from vitellogenic and late-pregnant females (14). Although the biological variation in our corpora allata makes the inhibition data too imprecise to constitute a valid test of specific models, Figure 7 illustrates how numerical simulation using the Hill equation (15) resolves the total response into high and low-dose components with identifiable estimates of binding affinities and maximum effect. No molecular paradigm has yet been identified to explain the apparent additivity of the low and high level responses. Analysis of the developmental data (14) reveals a response of relatively constant magnitude at micromolar levels of allatostatin, but major changes in the response to low levels with apparent Ki in the range 0.04-0.3 nM. The corpora allata from mated females show maximum sensitivity on Day 6, immediately after the termination of vitellogenesis, consistent with 85-87% of the biosynthetic flux being inhibited by an allatostatic interaction with an apparent Ki of 0.06 nM. Time-course studies using 0.69 nM ASAL on CA from Day 6 mated females (14) showed that the response was stable for at least three hours *in vitro*, thereby eliminating mechanisms (2) & (3)(listed above) in that particular case. It remains to be seen whether or not the same is true for both allatostatins over a range of concentrations and physiological gland donors.

Structure/Activity Studies on ASAL

All presently known allatostatins have strong structural homology in their amidated C-terminal hexapeptide moiety, aside from a neutral Val^{13} for Leu substitution in ASB2 and the various neutral hydrophilic variants of the type A allatostatins at position 10

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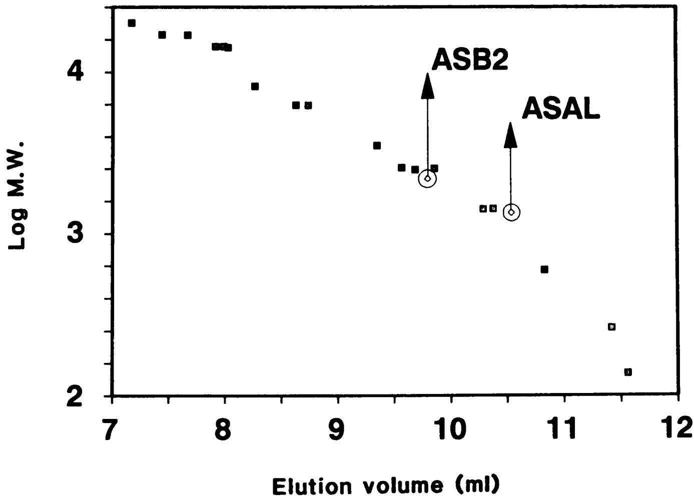

Figure 6. Estimation of MW of natural ASAL (tridecapeptide) and ASB2 (octadecapeptide) allatostatins by G2000-SWXL gel permeation chromatography. Each elution volume is the mean of two separate analyses of appropriate bioactivity after purification by RP4-HPLC. MWs were determined by FAB-MS. Calibration standards (squares) ranged from 137D (p-aminobenzoic acid) to 20.1kD (trypsin inhibitor).

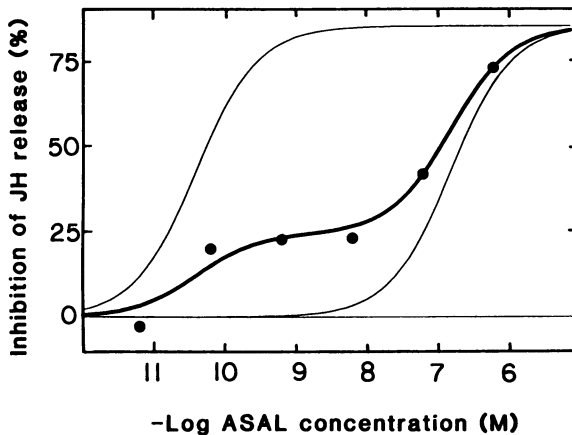

Figure 7. High/Low components in ASAL response. The dose/response curve towards ASAL given by corpora allata from Day 2 mated females in a standard two hour assay *in vitro* (solid circles) reveals two quasi-additive responses. Estimates of individual IC50 (Low, 0.04 nM; High, 180 nM) and maximum response (Low, 23%; High, 62%) values were obtained by minimizing the sum-of-squares deviation from computed idealized Hill plots (Log $(V_0/V_i - 1)$ vs Log(ASAL)). Each datum point represents measurement on 12-20 pairs of corpora allata. Chi-square = 0.8297, $P_{critical}$ = 0.975 .

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Table I. Type A Allatostatins - Structure/Activity

Peptide	Structure	pIC50
ASAL	Ala-Pro-Ser-Gly-Ala-Gln-Arg-Leu-Tyr-Gly-Phe-Gly-Leu-NH_2	9.5
[Lys^7]-ASAL	Ala-Pro-Ser-Gly-Ala-Gln-Lys-Leu-Tyr-Gly-Phe-Gly-Leu-NH_2	8.8
[D-Arg^7]-ASAL	Ala-Pro-Ser-Gly-Ala-Gln-<u>Arg</u>-Leu-Tyr-Gly-Phe-Gly-Leu-NH_2	8.1
[Asp^6,Ser^{10}]-ASAL-(6-13) -octapeptide amide (W4)	Asp-Arg-Leu-Tyr-Ser-Phe-Gly-Leu-NH_2	7.9
ASAL-(4-13)-decapeptide amide	Gly-Ala-Gln-Arg-Leu-Tyr-Gly-Phe-Gly-Leu-NH_2	7.6
ASAL-(6-13)-octapeptide amide	Gln-Arg-Leu-Tyr-Gly-Phe-Gly-Leu-NH_2	7.5
[Gly^{11}]-ASAL	Ala-Pro-Ser-Gly-Ala-Gln-Arg-Leu-Tyr-Gly-Gly-Gly-Leu-NH_2	NA
[Ala^{13}]-ASAL	Ala-Pro-Ser-Gly-Ala-Gln-Arg-Leu-Tyr-Gly-Phe-Gly-Ala-NH_2	NA
ASAL-oic acid	Ala-Pro-Ser-Gly-Ala-Gln-Arg-Leu-Tyr-Gly-Phe-Gly-Leu	NA
Des-Gly^{12},Leu^{13}-ASAL	Ala-Pro-Ser-Gly-Ala-Gln-Arg-Leu-Tyr-Gly-Phe-NH_2	NA
ASAL-oyl-Ala	Ala-Pro-Ser-Gly-Ala-Gln-Arg-Leu-Tyr-Gly-Phe-Gly-Leu-Ala	NA
ASAL-oyl-Ala amide	Ala-Pro-Ser-Gly-Ala-Gln-Arg-Leu-Tyr-Gly-Phe-Gly-Leu-Ala-NH_2	NA

Inhibition of JH release by CA from Day 10 pregnant females in a 2 hour *in vitro* assay is expressed as the -Log of the IC50 (M). NA : no activity at 10^{-5} M

(=15/ASB2). ASAL and ASB2 have indistinguishable dose/response curves in our standard bioassay on corpora allata from Day 10 pregnant females (1, our unpublished data). This similarity may result from different combinations of mechanisms 1-3 (see above) in the two cases. Nonetheless, the result indicates that both peptides contain fully effective messages, capable of suppressing ca. 90% of hormone synthesis in glands of appropriate physiological state, despite major structural differences in their N-terminal segments. This prompted us to begin exploration of the N-terminus as a source of sub-type address information. We report here the results of modifications of the ASAL sequence and their influence on the strength and sensitivity of the corpora allata response.

Single Residue Substitutions. Table I shows sequence comparisons between ASAL and eleven synthetic analogs. All peptides were synthesized, purified, verified and submitted for dose/response evaluation *in vitro* using corpora allata from Day 10 pregnant females, as previously described (1). The pIC50 values were computed from linear regressions of data as logit/log (Hill) plots. The major dissimilarities between the N-terminal segments of ASB2 and ASAL and other related allatostatins (2), suggested to us that this segment is not involved in signal transmission, but is an address segment which promotes binding to different receptor sub-types recognizing very similar message (C-terminal) segments. We have already reported that desamidation of the C-terminus of ASAL destroys all observable bioactivity (1) and here report the total inactivity at levels up to 10 μM of the Gly^{11} and Ala^{13} analogs and of the des-Gly^{12},Leu^{13}-ASAL. Replacement of the terminal amide by Ala or Ala-NH_2 also abolished all biological activity. This strongly implicates the C-terminal part of the peptide in crucial signal transmission. On the other hand, the substitution of Lys^7 or D-Arg^7 in the middle of the molecule had only quantitative effects on apparent binding strength (Table I) and did not reduce the magnitude of the response at concentrations above 100 nM (not shown). Thus, the correct Arg^7 in ASAL is required for strong binding, but it is already outside the message segment; this is consistent with it being within the A/B variable N-terminus.

N-terminal Truncation. Three N-terminally shorter sequence analogues of the tridecapeptide ASAL were recently identified from saline extracts of cockroach brains (2), being octa, nona- and decapeptides (W4, W3, W2) with two or three substitutions at positions 5-7 & 10, relative to ASAL. We can independently confirm the existence of one of these (W4, Table I) from partial sequence data from a larger peptide isolated from our acid/ethanol extracts (not shown). These N-terminally truncated variants appear to have higher interaction Ki values, but it was not clear whether that effect was due to the shorter address segment or to substitutions in what remained of it. Accordingly, we synthesized two N-terminally truncated analogues of ASAL, with no substitutions, and determined the responses. As shown in Table I, both the (4-13)-decapeptide amide and the (6-13)-octapeptide amide show substantial lowering of affinity due to loss of the first 3 or 5 residues respectively. The octapeptide amide W4 has a slightly lower IC50 than the ASAL-(6-13)-octapeptide amide

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from which it differs by Asp^6, Ser^{10} substitutions. All these compounds gave inhibitions of 85% or greater at concentrations of 1 μM or lower, indicating the presence of a effective message segment. This evidence directly establishes the importance of the N-terminal segment in promoting high affinity binding to the allatostatin receptors, and the major difference between ASB2 and the ASAL types in this segment makes it likely that they target different receptor sub-types.

Acknowledgments

We are grateful to L. Setyobudi, B.B. Smith, P.C. Toren, J.F. Zobel, D.F. Hunt, B.K. Stierwalt and E.G. Jaworski, for collaboration, facilitation and encouragement. Supported by NIH grant DK34549. Oregon Agricultural Experiment Station Technical Paper number 9263.

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Chapter 18

Myotropic Insect Neuropeptide Families from the Cockroach *Leucophaea maderae*

Structure–Activity Relationships

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Structure-activity relationship studies for three myotropic families of insect neuropeptides first isolated from Leucophaea maderae are discussed. Active core regions, representing the minimum number of residues required to elicit myotropic activity, are identified along with key structural attributes within the core regions. A select few of the fragments and/or analogs have proved more active than their parent peptides. The synthesis of active, conformationally-restricted analogs for two of the families has provided evidence for the presence of turn conformations within their proline-containing active core regions.

Nearly six decades passed between the first investigations into the role of neuropeptide hormones in insect physiology by Polish researcher Kopec (1) and the first report of the isolation and characterization of an insect neuropeptide, proctolin (2). A further period of almost eight years elapsed before an additional three insect neuropeptide structures were reported in the scientific literature. Since that time, the number of characterized neuropeptide structures isolated from insects has increased over ten-fold (3). The emergence of this plethora of primary structures has left researchers in the insect neuropeptide field with the daunting task of determining the relationship between peptide structure and biological activity. In this report, we review structure-activity relationship studies and present new data for three myotropic neuropeptide families: the sulfakinins, leucokinins and pyrokinins, all isolated originally from the cockroach Leucophaea maderae during this recent period of explosive growth. In addition, conformationally-restricted analogs have been synthesized for two of the families and found to retain significant activity, providing evidence for the presence of turn conformations.

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Sulfakinin Family

The sulfakinins constitute a family of real and putative peptide sequences characterized from the cockroach *Leucophaea maderae* (leucosulfakinin subfamily) and fly *Drosophila melanogaster* (drosulfakinin subfamily) with homology to the mammalian hormones human gastrin II and cholecystokinin (CCK) (Figure 1). The sulfated leucosulfakinins (LSKs) were identified in extracts of *L. maderae* corpora cardiaca, the major neurohumoral organs of insects which are analogous to the vertebrate hypothalamo-hypophyseal system (4,5). Utilizing an oligonucleotide probe mixture based on the amino acid sequences of LSK and the gastrin/CCK family, a gene has been cloned from *Drosophila* encoding for two putative LSK-like peptides (Figure 1), designated drosulfakinins (6). The DSKs and LSK-II share a common C-terminal octapeptide sequence and similarities in the remaining N-terminal regions. Subsequently, the two natural homologs perisulfakinin (PSK) (7) from *Periplaneta americana* and *Locusta migratoria*-sulfakinin (LMSK or LomSK) (Schoofs et al. *Physiol. Entomol.*, in press.) have been isolated and characterized (Figure 1). Perisulfakinin is identical to LSK with the exception of the conservative substitution of Asp for Glu at position 4, and demonstrates the same myotropic potency on a *Periplaneta* hindgut as does LSK on a *Leucophaea* hindgut preparation. The LMSK sequence represents an insertion of a Leu-Ala dipeptide block between the first and second residues of LSK-II. Unsulfated LSK-II was also identified in *Periplaneta* corpora cardiaca extracts (7). However, as the corpora cardiaca were extracted with Bennett's solution (7,8), containing 1% TFA and 1M HCl, the possibility that this material arose as a result of a loss of sulfate group from sulfated LSK-II cannot be ruled out.

Another structural aspect of the sulfakinins is their homology with FMRFamide, a molluscan peptide with cardioacceleratory and rectum contractile activity. Three of the four amino acids of FMRFamide match the C-terminal residues of all sulfakinin sequences (Figure 2) (9). Brownstein (10), and Greenberg and co-workers have noted a homologous relationship between FMRFamide and the mammalian Met-enkephalin-Arg^6-Phe^7. This led Greenberg *et al.* to suggest that the enkephalin and FMRFamide-like peptides may have co-evolved from an ancestral peptide YGG-FMRFamide (11,12,13), which has since been isolated from *Octopus vulgaris* (14). As depicted in Figure 2, the common C-terminal heptapeptide sequence of the sulfakinins and Met-enkephalin-Arg^6-Phe^7 (YGG-FMRFamide) share homology as well. In addition to the three C-terminal amino acids Met-Arg-Phe, the sulfakinins share a Gly and a lateral match of Tyr-Gly amino acid pairs. Thus structural similarities exist between gastrin/CCK and the sulfakinins, the sulfakinins and the FMRFamide family, the sulfakinins and Met-enkephalin-Arg^6-Phe^7, and FMRFamide and Met-enkephalin-Arg^6-Phe^7. These sequence homology relationships are diagrammed in Figure 3 (9). Through the intermediacy of the insect sulfakinins and the cephalopod YGG-FMRFamide, the figure relates the structures of the vertebrate gastrin/CCK family and mammalian Met-enkephalin-Arg^6-Phe^7. The similarity in sequences exhibited in Figure 3 suggests that they were derived from a common ancestral sequence (9).

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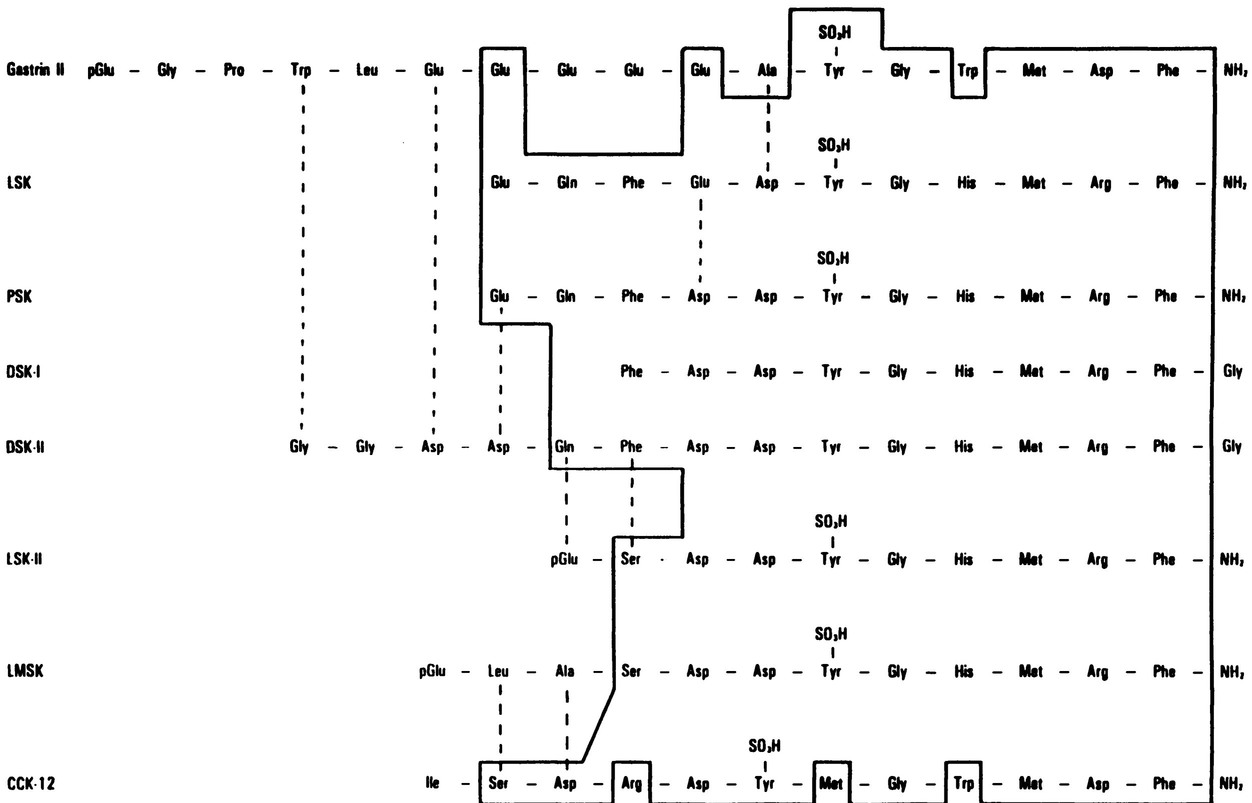

Figure 1. Sequence homologies among members of the insect sulfakinin family and human gastrin II and cholecystokinin-12 (CCK-12). Dashed lines denote residues in which the nucleotide codons could differ by a single nucleotide. Abbreviations: LSK = leucosulfakinin, PSK = perisulfakinin, DSK = drosulfakinin, LMSK = *Locusta migratoria*-sulfakinin.

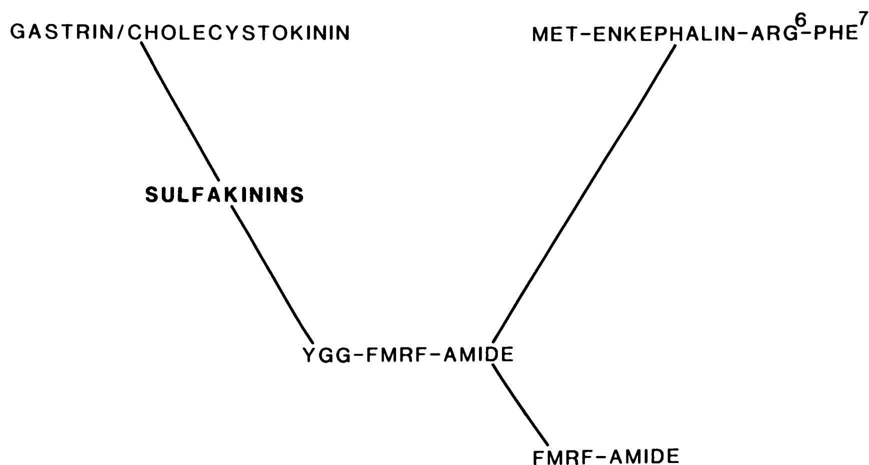

Figure 2. Sequence homologies among the common sulfakinin (SK) C-terminal heptapeptide, FMRFamide and mammalian Met-enkephalin-Arg^6-Phe^7. Solid lines denote identical amino acids and the dashed line connects two residues in which the nucleotide codons could differ by a single nucleotide.

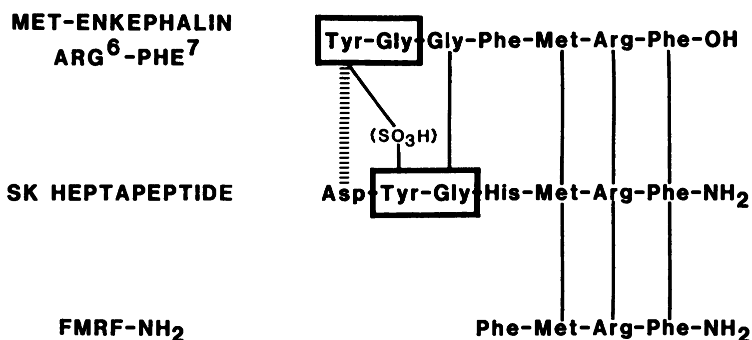

Figure 3. Schematic diagram of structural relationships between vertebrate and invertebrate neuropeptides discussed in the text. The similarity in sequences suggests that they may have been derived from a common ancestral source.

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Synthetic gastrin I (unsulfated), gastrin II, CCK-8, and the natural CCK analog caerulein were all found to be inactive on the isolated cockroach hindgut, demonstrating the importance of the C-terminal sequence differences between positions 8 and 10 of the sulfakinin family (His and Arg) and the corresponding positions in the gastrin/CCK family (Trp and Asp). In an effort to determine the relative importance to contractile activity of these C-terminal differences between the sulfakinins and the gastrin/CCK families, the Trp and Asp residues in the C-terminal regions of both human gastrin II and CCK-8 were sequentially replaced with the corresponding SK amino acids (Table I). While the synthesis of [His^{14}, Arg^{16}]gastrin II proved feasible, synthesis of the other gastrin/CCK analogs required the replacement of oxidation-susceptible Met with isosteric norleucine (Nle). Evaluation of the gastrin II and CCK-8 analogs on the cockroach hindgut preparation demonstrated that the introduction of a single residue (Arg) in the C-terminal positions of both gastrin II and CCK-8 transformed these inactive mammalian hormones into active SK analogs on an insect bioassay (9). The Arg-containing analog of gastrin II retained a significant level (20%) of the activity of LSK. The introduction of both His and Arg imparted little to the myotropic activity of the gastrin II analog, but conferred an approximately 4-fold increase in myotropic activity for the CCK-8 analog. The CCK analog displayed approximately 900-fold and 20-fold reductions, respectively, compared with the activity of the corresponding gastrin analogs (9,15). This lower activity appears largely due to the fact that the Tyr(SO_3H) moiety in the CCK analogs is shifted by one position towards the N-terminus relative to the sulfakinins and gastrin analogs (15). A more detailed investigation of the effect of sulfate position on sulfakinin myotropic activity is discussed later in this section.

An evaluation of a series of LSK fragments (Table II) demonstrated that a significant portion of the N-terminal region is not critical for myotropic activity on the cockroach hindgut. Five N-terminal residues can be removed without precipitating a total loss of activity. Removal of a sixth N-terminal residue (Tyr[SO_3H]) or the C-terminal amino acid led to inactive fragments. Thus, the C-terminal hexapeptide fragment constitutes the myotropic "active core" of the insect sulfakinins, retaining nearly 10% of the activity of the parent peptide. All of the sulfakinins share a common C-terminal heptapeptide sequence. Nonetheless, full activity requires the C-terminal octapeptide, which demonstrated a 7-fold increase in activity even over the parent peptide ([Nle^9] LSK) (Table I) (16).

In order to gain information about the relative importance of amino acid residues within the LSK active core region to myotropic activity, a series of octapeptide analogs with Ala replacements was synthesized (16). The Ala residue is of intermediate polarity and hydrophobicity in comparison with other natural amino acids. While LSK positions 7 (Gly) and 9 (Met), within the active core, demonstrate flexibility to Ala substitution without precipitous loss of hindgut contractile activity, all activity is lost upon similar substitution at positions 8, 10, and 11. The Gly at position 7 appeared most flexible to Ala replacement, as [4-11, Ala^7]LSK

displayed a potent threshold concentration value of 3.8 ± 0.66 x 10^{-10}M, representing 34% and 58%, respectively, of the myotropic activity of the parent fragment ([4-11, Nle^9]LSK) and of LSK itself. The inability to tolerate substitution of Ala for Phe is consistent with the importance of aromatic character in position 11 for SK myotropic activity.

In an effort to determine the importance of the basic character of the 8 and 10 positions to LSK myotropic activity, these residues were replaced by another basic amino acid, Lys. Replacement of the Arg^{10} with Lys led to a very active compound, [4-11, Nle^9, Lys^{10}]LSK, with a threshold concentration value of 2.0 ± 0.4 x 10^{-10} M, representing a retention of fully 65% of parent fragment activity and 430% of the activity of the full-length peptide ([Nle^9]LSK). Thus, basicity at the 10 position is a critical factor for LSK-receptor site interaction. Conversely, [4-11, Lys^8, Nle^9]LSK proved to be inactive on the isolated cockroach hindgut. However, the previously reported active gastrin and CCK analogs, containing key LSK amino acid substitutions (9,15), demonstrated that nonbasic Trp could replace His at LSK position 8 without complete loss of activity. Synthesis of [4-11, Trp^8, Nle^9]LSK showed that a retention of 5.2% of parent fragment activity (threshold concentration value: 4.26 ± 1.47 X 10^{-9} M) could be achieved by replacing His with Trp. This suggested that aromatic character and not basicity is a critical factor at the 8 position for LSK-receptor site interaction. Further confirmation of this assertion could be found with the insertion of an aromatic Phe residue in the 8 position, which led to a FMRFamide-like analog ([4-11, Phe^8, Nle^9]LSK) with a threshold concentration value of 5.16 ± 1.38 x 10^{-9} M, representing 4.3% of the parent fragment's activity. Aromatic residues are found at this relative position in the C-terminal regions of the invertebrate FMRFamide peptide family (Phe) (9,17), the mammalian hormone Met-enkephalin-Arg^6,Phe^7 (Phe) (9), the insect SK's (His), and the vertebrate gastrin/CCK peptide family (Trp) (9), suggesting conservation of aromatic character at this position throughout the molecular evolution of these homologous myotropic peptides.

When the His of LSK position 8 was replaced with 3-methylHis, the resulting analog ([3-$MeHis^8$]LSK) was found to have a threshold concentration value of 1.09 ± 0.22 x 10^{-10} M (Table II), a significant two-fold greater activity over synthetic, parent LSK. This proved to be the most potent LSK analog yet synthesized, suggesting it displayed a stronger ligand-receptor interaction than that of the natural product. Given the importance of aromatic character in the 8 position, the greater activity of [$3MeHis^8$]LSK may be attributable to an increase in electron density of the aromatic cloud provided by the electron-donating properties of the exocyclic methyl group.

Like the C-terminal amide moiety, the presence of a sulfate group is required for sulfakinin myotropic activity. Nevertheless, as the active CCK analogs attest, the position of the sulfate is less critical (15). With the apparent flexibility of sulfakinin activity in relation to movement of sulfate position in the sequence, it was of interest to determine the limits of sulfate mobility without precipitating complete loss of activity. A series

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Table I. Gastrin/Cholecystokinin Analogs with Sulfakinin Residue Substitutions

Peptide[a]	Sequence	Threshold Concentration ($\times 10^{-10}$M)
[Nle^{15},Arg^{16}]Gastrin II	pE-G-P-W-L-E-E-E-E-Glu-Ala-Tyr(SO_3H)-Gly-Trp-Nle-Arg-Phe-NH_2	11 ± 35
[His^{14},Arg^{16}]Gastrin II	pE-G-P-W-L-E-E-E-E-Glu-Ala-Tyr(SO_3H)-Gly-His-Met-Arg-Phe-NH_2	14 ± 7
[$Nle^{3,6}$,Arg^{7}]CCK-8	Asp-Tyr(SO_3H)-Nle-Gly-Trp-Nle-Arg-Phe-NH_2	9860 ± 740
[$Nle^{3,6}$,His^{5},Arg^{7}]CCK-8	Asp-Tyr(SO_3H)-Nle-Gly-His-Nle-Arg-Phe-NH_2	260 ± 65

[a]Abbreviations: Nle = norleucine; CCK = cholecystokinin.

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Table II. Myotropic Activity of Leucosulfakinin Analogs on Isolated Cockroach Hindgut (9,16)

Peptide Analog Subgroups	Peptide[a]	Peptide Sequence[b]	Threshold Concentration ($\times 10^{-10}$M)[b]	Activity as Percentage of Respective Parent Peptide
A	[1-10,Nle^9]LSK	Glu-Gln-Phe-Glu-Asp-Tyr(SO_3H)-Gly-His-Nle-Arg-NH_2	inactive[c]	(-)
	[Nle^9]LSK	Glu-Gln-Phe-Glu-Asp-Tyr(SO_3H)-Gly-His-Nle-Arg-Phe-NH_2	8.6 ± 2.8	(100%)[d]
	[3-11,Nle^9]LSK	Phe-Glu-Asp-Tyr(SO_3H)-Gly-His-Nle-Arg-Phe-NH_2	21 ± 7.5	(41%)[d]
	[4-11,Nle^9]LSK	Glu-Asp-Tyr(SO_3H)-Gly-His-Nle-Arg-Phe-NH_2	1.3 ± 0.55	(660%)[d]
	[5-11,Nle^9]LSK	Asp-Tyr(SO_3H)-Gly-His-Nle-Arg-Phe-NH_2	310 ± 90	(3%)[d]
	[6-11,Nle^9]LSK	Tyr(SO_3H)-Gly-His-Nle-Arg-Phe-NH_2	100 ± 37	(9%)[d]
	[7-11]LSK	Gly-His-Met-Arg-Phe-NH_2	inactive[c]	(-)
	[8-11]LSK	His-Met-Arg-Phe-NH_2	inactive[c]	(-)
B	[4-11,Ala^7,Nle^9]LSK	Glu-Asp-Tyr(SO_3H)-Ala-His-Nle-Arg-Phe-NH_2	3.8 ± 0.7	(34%)[e]
	[4-11,Ala^8,Nle^9]LSK	Glu-Asp-Tyr(SO_3H)-Gly-Ala-Nle-Arg-Phe-NH_2	inactive[c]	(-)
	[4-11,Ala^9]LSK	Glu-Asp-Tyr(SO_3H)-Gly-His-Ala-Arg-Phe-NH_2	32 ± 10	(4%)[e]
	[4-11,Nle^9,Ala^{10}]LSK	Glu-Asp-Tyr(SO_3H)-Gly-His-Nle-Ala-Phe-NH_2	inactive[c]	(-)
	[4-11,Nle^9,Ala^{11}]LSK	Glu-Asp-Tyr(SO_3H)-Gly-His-Nle-Arg-Ala-NH_2	inactive[c]	(-)
C	[4-11,Nle^9,Lys^{10}]LSK	Glu-Asp-Tyr(SO_3H)-Gly-His-Nle-Lys-Phe-NH_2	2.0 ± 0.4	(65%)[e]
	[4-11,Lys^8,Nle^9]LSK	Glu-Asp-Tyr(SO_3H)-Gly-Lys-Nle-Arg-Phe-NH_2	inactive[c]	(-)
	[4-11,Trp^8,Nle^9]LSK	Glu-Asp-Tyr(SO_3H)-Gly-Trp-Nle-Arg-Phe-NH_2	43 ± 15	(3%)[e]
	[4-11,Phe^8,Nle^9]LSK	Glu-Asp-Tyr(SO_3H)-Phe-Nle-Arg-Phe-NH_2	52 ± 14	(2.5%)[e]
	[3MeHis^8]LSK	pE-Q-F-E-Asp-Tyr(SO_3H)-Gly-3MeHis-Met-Arg-Phe-NH_2	1.1 ± 0.2	(200%)[f]

[a]Abbreviations: LSK = leucosulfakinin; Nle = norleucine; 3MeHis = 3 methyl histidine. [b]Average of five replicates. Threshold determination procedure described previously (9). [c]Inactive up to at least 1×10^{-5} M. [d]Activity expressed as percentage of threshold activity of [Nle^9]LSK (parent peptide) (9). [e]Activity expressed as percentage of threshold activity of [4-11,Nle^9]LSK (parent peptide). [f]Activity expressed as percentage of synthetic LSK (parent peptide) (4).

of Tyr(SO_3H) position-analogs (Table III) was synthesized to gain information about this question.

Movement of the sulfate moiety by one position towards the N-terminus ([Tyr(SO_3H)^5, Phe^6, Nle^9]LSK) led to a very significant 38% retention of parent peptide ([Nle^9]LSK) activity. Movement of the sulfate group by two to five positions towards the N-terminus (represented by analogs [Tyr(SO_3H)^4, Phe^6, Nle^9]LSK through [Tyr(SO_3H)^1, Phe^6, Nle^9]LSK) was accompanied by a drop of several orders of magnitude to a plateau of approximately 0.1-0.2% of parent activity. Thus, LSK myotropic activity displays some tolerance to movement of the sulfate towards the N-terminus, particularly at the 5 position. Movement of the Tyr (SO_3H) by one position towards the C-terminus ([Phe^6, Tyr(SO_3)^7, Nle^9]LSK) led to retention of a low but measurable 0.3% of parent peptide activity. However, biological evaluation of [Phe^6, Tyr(SO_3H)^8, Nle^9]LSK indicated that complete loss of activity occurs upon movement of the sulfate group by more than one position towards the C-terminus.

The structurally-related gastrin and CCK hormones share an overlapping spectrum of activities in mammals, including the stimulation of smooth muscle contraction in the intestines (5). Dockray (18) and Larrson et al. (19) have theorized that the two related mammalian hormones evolved from a common ancestral molecule. The great flexibility displayed by the sulfakinin myotropic receptor site to a shift of the sulfate group from position 6 ("gastrin position") to 5 ("CCK position") (LSK numbering) is consistent with this theory, as the receptor could accommodate any subsequent molecular evolution of an ancestral CCK hormone from LSK-II or another sulfakinin-like peptide (15).

As shown in Table III, one of the active Tyr(SO_3H) position analogs, [Phe^6, Tyr(SO_3H)^7, Nle^9]LSK, contains a sulfate moiety shifted by one position towards the C-terminus relative to the natural product. Contrary to expectations, the C-terminal pentapeptide fragment of this analog (Tyr(SO_3H)-His-Nle-Arg-Phe-NH_2) demonstrated reversible inhibition rather than stimulation of the spontaneous contractions of the isolated cockroach hindgut. In this respect, it behaved like the inhibitory leucomyosuppressin (pGlu-Asp-Val-Asp-His-Val-Phe-Leu-Arg-Phe-NH_2) (20,21), a natural FLRFamide peptide isolated from L. maderae. The shared property is probably a result of sequence similarities in the C-terminal tetrapeptide regions of the two peptides. This data underscores the importance of a C-terminal hexapeptide sequence for the stimulatory myotropic activity of the sulfakinins.

The Leucokinins

The leucokinins constitute a family of eight myotropic octapeptides also isolated from L. maderae cockroach head extracts (Table IV) (22). Three homologous myotropic neuropeptides termed achetakinins, were recently characterized from head extracts of the cricket, Acheta domesticus (Holman, G. M., et al. in Chromatogr. & Isolation of Insect Hormones and Pheromones, in press.) (Table IV). Both the leucokinins (Phe-X-Ser-Trp-Gly-NH_2, X=Tyr, His, Asn, Ser) and the achetakinins (Phe-X-Pro-Trp-Gly- Met-NH_2, X=Tyr, Ser, Asn) share

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a common C-terminal pentapeptide sequence, analogous to the common C-terminal pentapeptide sequence contained in the tachykinins (Phe-X-Gly-Leu-Met-NH_2, X = Tyr, Phe, Ile, Val).

The related achetakinins double the rate of fluid secretion by isolated Acheta Malpighian tubules (Coast, G. M., et al. J. Insect Physiol., in press.). In addition, some leucokinins stimulate fluid secretion and/or depolarize transepithelial membrane potentials in the malpighian tubules of the yellow fever mosquito, Aedes aegypti, in a fashion similar to several uncharacterized peptides isolated from mosquito head extracts (23). Thus, the neuropeptide family of leucokinins, achetakinins, and homologs may function in the control of water and ion balance, as well as myotropic activity, in a number of insects. To determine the relationships of the C-terminal sequence of the leucokinins to myotropic activity, analogs were synthesized and tested on the isolated cockroach hindgut.

Truncation of the most active of the leucokinins, L-VIII (Table IV), by one amino acid at the C-terminus or by more than three residues at the N-terminus, led to myotropically inactive fragments (Table V, Group A). Conversely, the synthetic C-terminal pentapeptide fragments (Table V, Group B) demonstrate significant hindgut contractile activity relative to the parent peptides (Table IV). Indeed, the core leucokinin pentapeptide fragment (LPF: Phe-X-Ser-Trp-Gly-NH_2) with Tyr in position 2 (LPF[Tyr^2]) retained all of the activity of its parent, L-VIII, while the related achetakinin pentapeptide core, APF[Tyr^2] (Table V, Group F), proved more active than its parent, A-I (Table IV) (25). The requirement for a C-terminal amide is shown by the inactivity of the C-terminal acid form of LPF[Tyr^2] (Table V, Group A).

The effects of replacing each amino acid in the active core were examined. Position 2 of the LPFs tolerates substantial variability, not only to those amino acids in the analogous position of leucokinins (X = Tyr, His, Asn, Ser), but also to Phe, Ala, Thr, Asp, Lys, and Leu (Table V, Group C). Even a peptide substituted with the modified amino acid N-methyl Tyr retains activity (Table V, Group C). The lowest threshold concentrations are attained with the uncharged, aromatic residues Tyr or Phe in position 2 (Table V, Groups B and C). Position 2 does show some selectivity, however, as LPF[$DAla^2$] (Table V, Group C) failed to show activity. While Ala substitution of positions 2, 3, or 5 of the active core LPF[Tyr^2] resulted in significant retention of myotropic activity, the analogous substitution of non-aromatic Ala for Phe^1 or Trp^4 leads to inactive peptides. In contrast, when other aromatic residues are placed in positions 1 and 4, significant retention of activity is observed (LPF[Tyr^1, Tyr^2], LPF[Tyr^2, Phe^4], LPF[Trp^1, Tyr^2, Phe^4]) (Table V, Group E), and APF[Phe^2, Tyr^4] (Table V, Group F). The high activity of achetakinin APF[Tyr^2] demonstrates that the Ser^3 of LPF can be readily replaced by Pro, raising the possibility of a turn in the active core. Indeed, by Chou-Fasman secondary structure prediction rules for proteins (29), a β-turn conformation would be favored for the first 4 residues of all the LPF sequences derived from the known leucokinins (LPF[Tyr^2], LPF[Ser^2], LPF[His^2], LPF[Asn^2]) and for APF[Tyr^2] (25).

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Table III. Myotropic Activity of a Series of Tyr(SO_3H) Position-Analogs of Leucosulfakinin (LSK)

Peptide[a]	Sequence	Threshold Concentration Value (x 10^{-10}M)[b]
[Phe^6,Tyr(SO_3H)^8,Nle^9]LSK	Glu-Gln-Phe-Glu-Asp-Phe-Gly-Tyr(SO_3H)-Nle-Arg-Phe-NH_2	Inactive [0%][c]
[Phe^6,Tyr(SO_3H)^7,Nle^9]LSK	Glu-Gln-Phe-Glu-Asp-Phe-Tyr(SO_3H)-His-Nle-Arg-Phe-NH_2	2880 ± 530 x 10^{-10}M [0.30%]
[Nle^9]LSK	Glu-Gln-Phe-Glu-Asp-Tyr(SO_3H)-Gly-His-Nle-Arg-Phe-NH_2	8.60 ± 2.80 x 10^{-10}M [100%]
[Tyr(SO_3H)^5,Phe^6,Nle^9]LSK	Glu-Gln-Phe-Glu-Tyr(SO_3H)-Phe-Gly-His-Nle-Arg-Phe-NH_2	23 ± 4 x 10^{-10}M [37.72%]
[Tyr(SO_3H)^4,Phe^6,Nle^9]LSK	Glu-Gln-Phe-Tyr(SO_3H)-Asp-Phe-Gly-His-Nle-Arg-Phe-NH_2	4420 ± 93 x 10^{-10}M [0.19%]
[Tyr(SO_3H)^3,Phe^6,Nle^9]LSK	Glu-Gln-Tyr(SO_3H)-Glu-Asp-Phe-Gly-His-Nle-Arg-Phe-NH_2	5420 ± 690 x 10^{-10}M [0.16%]
[Tyr(SO_3H)^2,Phe^6,Nle^9]LSK	Glu-Tyr(SO_3H)-Phe-Glu-Asp-Phe-Gly-His-Nle-Arg-Phe-NH_2	6660 ± 1230 x 10^{-10}M [0.13%]
[Tyr(SO_3H)^1,Phe^6,Nle^9]LSK	Tyr(SO_3H)-Gln-Phe-Glu-Asp-Phe-Gly-His-Nle-Arg-Phe-NH_2	4000 ± 1040 x 10^{-10}M [0.21%]

[a]Abbreviations: LSK = leucosulfakinin; Nle = norleucine. [b]Values in brackets represent activity expressed as a percentage of parent [Nle^9]LSK. [c]Inactive up to at least 1 x 10^{-6}M.

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Table IV. Myotropic Activities of the Leucokinins and Related Achetakinins on the Isolated Cockroach Hindgut (20,25)

Peptide[a]	Peptide Sequence	Threshold Concentration[b] ($\times 10^{-10}$M)
L-VIII	Gly-Ala-Asp-Phe-Tyr-Ser-Trp-Gly-NH_2	0.3 ± 0.05
L-VI	pGlu-Ser-Ser-Phe-His-Ser-Trp-Gly-NH_2	0.5 ± 0.1
L-IV	Asp-Ala-Ser-Phe-His-Ser-Trp-Gly-NH_2	1.0 ± 0.1
L-III	Asp-Gln-Gly-Phe-Asn-Ser-Trp-Gly-NH_2	0.9 ± 0.2
L-VII	Asp-Pro-Ala-Phe-Asn-Ser-Trp-Gly-NH_2	1.0 ± 0.5
L-V	Gly-Ser-Gly-Phe-Ser-Ser-Trp-Gly-NH_2	0.4 ± 0.1
L-I	Asp-Pro-Ala-Phe-Ser-Ser-Trp-Gly-NH_2	2.0 ± 0.5
L-II	Asp-Pro-Gly-Phe-Ser-Ser-Trp-Gly-NH_2	2.0 ± 0.4
A-I[c]	Ser-Gly-Ala-Asp-Phe-Tyr-Pro-Trp-Gly-NH_2	0.9 ± 0.5
A-II[c]	Ala-Tyr-Phe-Ser-Pro-Trp-Gly-NH_2	0.5 ± 0.3
A-IV[c]	Asn-Phe-Lys-Phe-Asn-Pro-Trp-Gly-NH_2	2.0 ± 1.6

[a]L = leucokinin; A = achetakinin. [b]Average of five replicates. Threshold concentration determination procedure has been previously described (16). [c]Holman, M., Nachman, R., and Wright, M. in Chromatogr. & Isolation of Insect Hormones and Pheromones, in press.

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Table V. Myotropic Activities of the Leucokinin Fragments/Analogs on Isolated Cockroach Hindgut

Group	Peptide[a]	Peptide Sequence	Threshold Concentration[b] (X 10^{-10})
A	[1-7]L-VIII	Gly-Ala-Asp-Phe-Tyr-Ser-Trp-NH_2	inactive[c]
	[5-8]L-VIII	Tyr-Ser-Trp-Gly-NH_2	inactive[c]
	[6-8]L-VIII	Ser-Trp-Gly-NH_2	inactive[c]
	LPF[Tyr^2]-OH	Phe-Tyr-Ser-Trp-Gly-OH	inactive[c]
B	LPF[Tyr^2]	Phe-Tyr-Ser-Trp-Gly-NH_2	0.2 ± 0.06
	LPF[Ser^2]	Phe-Ser-Ser-Trp-Gly-NH_2	2 ± 0.5
	LPF[His^2]	Phe-His-Ser-Trp-Gly-NH_2	3 ± 1.2
	LPF[Asn^2]	Phe-Asn-Ser-Trp-Gly-NH_2	4 ± 1.3
C	LPF[Phe^2]	Phe-Phe-Ser-Trp-Gly-NH_2	0.2 ± 0.08
	LPF[Ala^2]	Phe-Ala-Ser-Trp-Gly-NH_2	8 ± 1.9
	LPF[Thr^2]	Phe-Thr-Ser-Trp-Gly-NH_2	8 ± 2.5
	LPF[Asp^2]	Phe-Asp-Ser-Trp-Gly-NH_2	12 ± 3.9
	LPF[Lys^2]	Phe-Lys-Ser-Trp-Gly-NH_2	23 ± 4.2
	LPF[Leu^2]	Phe-Leu-Ser-Trp-Gly-NH_2	260 ± 113
	LPF[NCH_3Tyr^2]	Phe-MTyr-Ser-Trp-Gly-NH_2[e]	99 ± 19.5
	LPF[$DAla^2$]	Phe-DAla-Ser-Trp-Gly-NH_2	inactive[d]

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D	LPF[Ala^1,Tyr^2]	Ala-Tyr-Ser-Trp-Gly-NH_2	inactive[c]
	LPF[Ala^2]	Phe-Ala-Ser-Trp-Gly-NH_2	8 ± 1.9
	LPF[Tyr^2,Ala^3]	Phe-Tyr-Ala-Trp-Gly-NH_2	0.4 ± 0.1
	LPF[Tyr^2,Ala^4]	Phe-Tyr-Ser-Ala-Gly-NH_2	inactive[c]
	LPF[Tyr^2,Ala^5]	Phe-Tyr-Ser-Trp-Ala-NH_2	6 ± 0.9
E	LPF[Tyr^1,Tyr^2]	Tyr-Tyr-Ser-Trp-Gly-NH_2	12 ± 2.8
	LPF[Tyr^2,Phe^4]	Phe-Tyr-Ser-Phe-Gly-NH_2	470 ± 109
	LPF[Trp^1,Tyr^2,Phe^4]	Trp-Tyr-Ser-Phe-Gly-NH_2	6,640 ± 970
F	APF[Tyr^2]	Phe-Tyr-Pro-Trp-Gly-NH_2	0.4 ± 0.060
	APF[Phe^2,Tyr^4]	Phe-Phe-Pro-Tyr-Gly-NH_2	550 ± 135
	APF[Leu^2]	Phe-Leu-Pro-Trp-Gly-NH_2	14 ± 2.7
G	LPF[Cys^0,Tyr^2,Cys^5]	Cys-Phe-Tyr-Ser-Trp-Cys-NH_2	3,100 ± 700
	cyclo(LPK[Cys^0,Tyr^2,Cys^5])	cyclo(Cys-Phe-Tyr-Ser-Trp-Cys-NH_2)	9,100 ± 640

[a]Abbreviations: L = leucokinin, LPF = leucokinin pentapeptide fragment (Phe-X-Ser-Trp-Gly-NH_2), APF = achetakinin pentapeptide fragment (Phe-X-Pro-Trp-Gly-NH_2). [b]Average of five replicates. Threshold concentration determinations procedure described previously (16). [c]Inactive up to at least 1 x 10^{-6}M. [d]Inactive up to at least 1 x 10^{-5}M. [e]MTyr = NCH_2Tyr.
SOURCE: Reprinted from ref. 25

Consistent with the presence of a turn conformation in the C-terminal region is the activity retained by a conformationally constrained analog, cyclo-[Cys-Phe-Tyr-Ser-Trp-Cys-NH_2] (Figure 4). The reduced potency of this cyclic analog compared with LPF[Tyr^2] may be predominantly attributable to replacement of Gly^5 by Cys^5 since the linear precursor, Cys-Phe-Tyr-Ser-Trp-Cys-NH_2, demonstrates a similar threshold concentration (Table V, Group G) (25). Synthesis of other constrained analogs, including one linking the C- and N-termini with an amide bond, are currently underway.

The Pyrokinins

At present, the pyrokinins make up a small family of myotropic insect peptides which share the common C-terminal pentapeptide C-terminal sequence Phe-X-Pro-Arg-Leu-NH_2 (X = Thr, Val, Ser). The first such neuropeptide was isolated from L. maderae cockroach head extracts and was termed leucopyrokinin (LPK or Lem-PK) and reported in 1986 (24). Leucopyrokinin (pGlu-Thr-Ser-Phe-Thr-Pro-Arg-Leu-NH_2) shares a 50% sequence homology with the cardioacceleratory P. americana neuropeptide CC-2 (M-2) (pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-NH_2), a member of the hyperglycemic/red pigment-coloration hormone (RPCH) family (5). Nevertheless, the sequence differences between the two peptides have profound consequences for their physiological activity profiles. Neither CC-2 nor the related CC-1 proved active on the isolated cockroach hindgut bioassay. Furthermore, LPK lacks the Trp residue that is critical for both hypertrehalosemic and RPCH activities (26). Indeed, LPK does not demonstrate RPCH activity in the dwarf crayfish Camberellus shufeldti up to a 5 nmol concentration (Ranga Rao and Nachman, unpublished data.). Thus, from physiological and structural perspectives, LPK and the hyperglycemic neuropeptides are distantly related. The recent isolation of both locustapyrokinin (Lom-PK) (Schoofs L., et al. Gen. Comp. Endocrinol., in press.), locustamyotropin II (Lom-MT-II) (Glu-Gly-Asp-Phe-Thr-Pro-Arg-Leu-NH_2) (Schoofs, L., et al. Insect Biochem., in press.), and locustamyotropin I (Lom-MT-I) (Gly-Ala-Val-Pro-Ala-Ala-Gln-Phe-Ser-Pro-Arg-Leu-NH_2) (27) from the locust Locusta migratoria demonstrate that the presence of pyrokinins is not limited to the cockroach. Lom-PK demonstrates 10% of the myotropic activity of LPK on the heterologous cockroach hindgut isolate. The locust pyrokinins elicit a stimulatory myotropic response on the isolated locust oviduct (Schoofs, L., et al. Insect Biochem., in press.).

When the full structure of the pheromone biosynthesis activating neuropeptide (PBAN) from the corn earworm Heliothis zea was recently reported (28), we noted that the sequence contained the pyrokinin C-terminal pentapeptide fragment Phe-X-Pro-Arg-Leu-NH_2 (X = Ser), identical to that contained in locustamyotropin I (Figure 5) (27). Indeed [Nle^5, Nle^{14}]PBAN demonstrates LPK-like contractile activity in the heterologous cockroach hindgut and oviduct assays, retaining a full 25% of LPK activity on the isolated Leucophaea oviduct (Holman, G. M. and Nachman, R. J., unpublished data.). Thus, PBAN can be considered a member of the pyrokinin family of insect neuropeptides.

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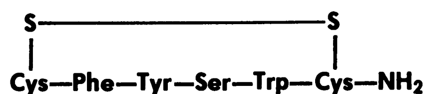

Figure 4. Cyclic analog of the leucokinin active core (X = Tyr) with a disulfide bond linkage.

Heliothis Pheromone Biosynthesis Activating Neurohormone	Leu · Ser · Asp · Asp · Met · Pro · Ala · Thr · Pro · Ala · Asp · Glu · Glu · Met · Tyr · Arg · Glu · Asp · Pro · Glu · Glu · Ile · Asp · Ser · Arg · Thr · Lys · Tyr	· Phe · Ser · Pro · Arg · Leu · NH_2
Locustamyotropin–I	Gly · Ala · Val · Pro · Ala · Ala · Gln	· Phe · Ser · Pro · Arg · Leu · NH_2
Leucopyrokinin	pGlu · Thr · Ser	· Phe · Thr · Pro · Arg · Leu · NH_2
Locustamyotropin–II	Glu · Gly · Asp	· Phe · Thr · Pro · Arg · Leu · NH_2
Locustapyrokinin	pGlu · Asp · Ser · Gly · Asp · Gly · Trp · Pro · Gln · Gln · Pro	· Phe · Val · Pro · Arg · Leu · NH_2

Figure 5. Members of the pyrokinin family that share the common C-terminal pentapeptide Phe-X-Pro-Arg-Leu-NH_2. The Pheromone Biosynthesis Activating Neurohormone (PBAN) is from Heliothis zea.

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Leucopyrokinin also stimulates contraction of the oviduct of L. maderae (Holman, G. M. and Nachman, R. J., unpublished data.). Whether PBAN is capable of stimulating contractions of the hindgut, oviduct or other tissue has yet to be determined in an insect from which it has been isolated. It is possible that the C-terminal pyrokinin pentapeptide sequence represents a myotropic component of the physiological profile of PBAN.

The initial choice of analogs for structure-activity studies was based on the homologous relationship between LPK and inactive CC-2 (Table VI) (29). The four amino acid positions uncommon to both LPK and CC-2 were interchanged in LPK to determine the residues most critical for activity. The replacement of Thr and Ser in the N-terminal region of LPK for the corresponding CC-2 amino acids Leu and Thr led to analogs which retained 28% and 77% of the activity of the natural product. Conversely, substitution of Asn for Arg and Trp for Leu in the C-terminal region led to a drop to an activity plateau of 0.1%. These data are consistent with the high myotropic activity observed for natural pyrokinins which diverge only at the N-terminal region of the sequences (Table VI). It was apparent from these results that the C-terminal differences between LPK and CC-2 account for their disparate activities.

The relative insensitivity of LPK to N-terminal amino acid replacement suggested an evaluation of truncated LPK analogs would prove fruitful. Surprisingly, removal of the first amino acid at the N-terminus (pGlu) effected an increase in activity over the natural product. The resulting fragment, [2-8]LPK, demonstrated 144% of the activity of the parent peptide. Fragment analogs resulting from the removal of a second and third N-terminal amino acid ([3-8]LPK and [4-8]LPK) retained a significant 59% and 30% of LPK myotropic activity, respectively. The fragment [4-8]LPK proved to be the active core of LPK, as the removal of more than three N-terminal amino acids resulted in the inactive compound, [5-8]LPK (29).

As alluded to earlier, amino acid substitution within the active core risked substantial loss of myotropic activity. Replacement of Pro at position 6 with highly flexible Gly retained only 0.1% of LPK activity. Even analogs [Ala^8]LPK and [Lys^7]LPK, with conservative amino acid changes at the C-terminus of the active core, retained only 0.04 and 0.4% parental core sequence activity, respectively. However, replacement of Phe at position 4 with Tyr (also aromatic in character) demonstrated a larger 5% retention of LPK activity. The greatest flexibility within the active core was found at position 5, as conservative replacement of Thr with Ser allowed for a 25% retention of LPK activity. This is consistent with the variability observed in the natural pyrokinins at this position. The C-terminal acid analog proved 1000-fold less active than the natural product, demonstrating the importance of the amide group at this site.

While the replacement of the Pro at position 6 with D-Ala resulted in only 0.02% of LPK activity, unusual contractile activity was observed. Unlike LPK and its analogs, the myotropic activity of [D-Ala^6]LPK remained for up to 5 minutes after the peptide-containing saline in the bioassay chamber was replaced with fresh saline. This demonstrated that at least a great portion of the

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Table VI. Myotropic Activity of Leucopyrokinin (LPK) Analogs on the Cockroach Proctodeum

Peptide	Sequence	Threshold Concentration[a] x 10^{-10}M (% of LPK)
LPK:	pGlu-Thr-Ser-Phe-Thr-Pro-Arg-Leu-NH_2	6.5 ± 1.5[c] (100)
CC-2:	pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-NH_2	---- (0)[b]
[Thr^{3}]LPK:	pGlu-Thr-Thr-Phe-Thr-Pro-Arg-Leu-NH_2	8.4 ± 1.0 (77)
[Leu^{2}]LPK:	pGlu-Leu-Ser-Phe-Thr-Pro-Arg-Leu-NH_2	23 ± 7 (28)
[Asn^{7}]LPK:	pGlu-Thr-Ser-Phe-Thr-Pro-Asn-Leu-NH_2	6800 ± 900 (0.1)
[Trp^{8}]LPK:	pGlu-Thr-Ser-Phe-Thr-Pro-Arg-Trp-NH_2	5400 ± 700 (0.1)
[Asn^{1}]LPK:	Asn-Thr-Ser-Phe-Thr-Pro-Arg-Leu-NH_2	14 ± 8 (46)
[Ser^{5}]LPK:	pGlu-Thr-Ser-Phe-Ser-Pro-Arg-Leu-NH_2	26 ± 16 (25)
[Tyr^{4}]LPK:	pGlu-Thr-Ser-Tyr-Ser-Pro-Arg-Leu-NH_2	130 ± 40 (5)
[Ala^{8}]LPK:	pGlu-Thr-Ser-Phe-Thr-Pro-Arg-Ala-NH_2	1500 ± 900 (0.4)
[Asn^{1}]LPK-OH:	Asn-Thr-Ser-Phe-Thr-Pro-Arg-Leu-OH	1900 ± 400 (0.3)
LPK-OH:	pGlu-Thr-Ser-Phe-Thr-Pro-Arg-Leu-OH	7000 ± 1700 (0.1)
[Lys^{7}]LPK:	pGlu-Thr-Ser-Phe-Thr-Pro-Lys-Leu-NH_2	16000 ± 3000 (0.04)
[D-Ala^{6}]LPK:	pGlu-Thr-Ser-Phe-Thr-D-Ala-Arg-Leu-NH_2	28000 ± 6000 (0.02)
[2-8]LPK:	Thr-Ser-Phe-Thr-Pro-Arg-Leu-NH_2	4.5 ± 0.7[c] (144)
[3-8]LPK:	Ser-Phe-Thr-Pro-Arg-Leu-NH_2	11 ± 20 (59)
[4-8]LPK:	Phe-Thr-Pro-Arg-Leu-NH_2	22 ± 10 (30)
[5-8]LPK:	Thr-Pro-Arg-Leu-NH_2	---- (0)[b]

[a]Average of five replicates. Determination of threshold concentrations is described in Methods. [b]Inactive up to a concentration of at least 5 x 10^{-6}M. [c]Statistically significant difference ($p < 0.05$).

Reprinted from ref. 29

activity was due to the D-Ala containing peptide rather than the presence of any [Ala^6]LPK as an inadvertent contaminant. The unusual persistence of the contractile stimulation suggests a strong ligand-receptor interaction, perhaps because the kink imparted to the structure by the D-Ala residue approximates a proline bend. The possibility that [D-Ala^6]LPK can act as an LPK antagonist is under investigation.

The existence of a Pro residue in the pyrokinins raises the possibility of the presence of a turn structure in the active core region. Indeed, a β-turn is predicted by Chou-Fasman (30) rules for the sequence Thr-Pro-Arg-Leu within the active core. The significant activity of the cyclic LPK analog *cyclo*-[Asn-Thr-Ser-Phe-Thr-Pro-Arg-Leu] (Figure 6), in which the N- and C-termini are linked by an amide bond, is consistent with the presence of a β-turn. This cyclic analog demonstrated a threshold concentration value of 3.5 x 10^{-8}M or a significant 4% of its linear counterpart.

Conclusions

While these three insect neuropeptide families (sulfakinins, pyrokinins, and leucokinins) were originally discovered in the Madeira cockroach, members have since been found in other species, suggesting that they are widely dispersed throughout Insecta. All of the families inclusively share a C-terminal amidated active core, a region that represents the minimum number of residues required to elicit myotropic activity on the isolated cockroach hindgut. For the leucokinins and pyrokinins, this active core is the C-terminal pentapeptide sequence. For the sulfakinins, it is the C-terminal hexapeptide sequence containing a sulfate group on the Tyr residue. These core regions have demonstrated a high degree of conservation over the evolutionary history of the families. Considerable variability is allowed in the N-terminal region. Whether these variable regions serve to direct the message encoded by the core to specific receptor targets or potentiate activity on the same receptors has yet to be determined, and awaits an accounting of the full spectrum of activities for each of the families.

Replacement of the C-terminal amide with an acid moiety reduces the myotropic potency of the pyrokinins and eliminates activity of the leucokinins and sulfakinins. During the course of these structure-activity studies, several fragments and/or analogs were found to exhibit greater activity than the parent natural products. The fragments [2-8]LPK (Table VI), [5-9]A-I (APF[Tyr^2]; Table IV), and [4-11,Nle^9]LSK demonstrated 1.5-, 2- and 7-fold increases in myotropic activity over their parent peptides. In addition, the amino acid substitution of His with 3-MeHis in position 8 of LSK (Table II) led to a 2-fold improvement in activity over the parent neuropeptide.

Armed with the knowledge of the structural requirements for activity gleaned from these primary structure-activity studies, we have now begun to examine some of the 3-dimensional conformation requirements via the preparation of conformationally-constrained analogs of the active core regions. In this paper, we have presented biological data on cyclic analogs of both the pyrokinin

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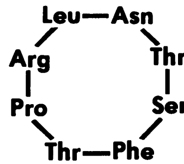

Figure 6. Cyclic analog of leucopyrokinin.

and leucokinin families that retain significant biological activity. The application of nuclear magnetic resonance and circular dichroism spectroscopy in conjunction with computer modeling/graphics methodologies on these and other conformationally-constrained analogs will enable us to gain a better understanding of messenger-receptor conformational interactions displayed by these insect neuropeptides. It may then be possible to unlock the key to their activity.

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Addendum

During preparation of this manuscript, a report noting the homology between PBAN from Bombyx and LPK appeared (Kitamura, A., et al. Biochem. Biophys. Res. Commun. 1989, 163, 520-526). In addition, the authors reported that while removal of the C-terminal residue of the 33 amino acid PBAN eliminates activity, only the C-terminal decapeptide is required to elicit a measure of pheromone biosynthesis. The pyrokinin pentapeptide core comprises half of that PBAN decapeptide sequence.

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Chapter 19

Pheromone Biosynthesis-Activating Neuropeptide Hormone of *Heliothis zea*

Isolation and Characterization

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A pheromone biosynthesis activating neuropeptide hormone (Hez-PBAN) controlling sex pheromone production in moths was isolated from the brain-subesophageal ganglion complexes of the adult corn earworm, Heliothis zea. Hez-PBAN, a 33-residue C-terminally amidated peptide with a molecular weight of 3900, was purified by several high performance liquid and size- exclusion chromatographic steps. Its primary structure was determined by a combination of (1) amino acid analysis, (2) automated Edman degradation, (3) Californium-252 time-of- flight plasma desorption mass spectrometry, and (4) C-terminal sequencing with carboxypeptidase P. Natural Hez-PBAN was found to have undergone oxidation of both its methionines to the corresponding sulfoxides during isolation and purification. Synthetic Hez-PBAN, when oxidized, was shown to exhibit identical chromatographic and spectroscopic properties as the natural material. Synthetic Hez-PBAN at a dose of 2-4 picomoles induced production of a normal quantity of sex pheromone in ligated H. zea females.

We recently reported the primary sequence of the pheromone biosynthesis activating neuropeptide hormone (Hez-PBAN) that controls sex pheromone production in adult female moths of the corn earworm, Heliothis zea *(1)*. Hez-PBAN is a 33 residue C-terminally amidated peptide with a molecular weight of 3900 daltons. This paper describes the details of the isolation and characterization of this first member of a new family of insect neuropeptides.

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Methods and Materials

Tissue Extraction and Sample Preparation. Brain-subesophageal (BR-SOG) complexes were dissected from adult male and female H. zea and quick-frozen on dry ice. Batches of BR-SOG from 50-75 insects were stored frozen in 2-ml polypropylene centrifuge tubes (Sarstedt) in 250 μl of a solvent consisting of 5% formic acid (v/v) (Fisher), 15% trifluoroacetic acid (v/v) (TFA, Baker), and 1% NaCl (w/v) (Fisher) in 5N HCl (Baker). Batches of tubes equivalent to 1000-2000 BR-SOG were processed together. The contents of the tubes were allowed to thaw and quickly homogenized with a Polytron homogenizer (Brinkmann) equipped with a microtip at maximum speed. The homogenate was centrifuged at 4000 rpm at 5° C. The pellet was re-extracted with 250 μl of solvent and the combined supernatants from ca. 250 BR-SOG were adsorbed onto a C-18 Sep-Pak preparative cartridge (Waters), prepared by washing sequentially with 10 ml each of acetonitrile and 0.1% TFA. A Model RP-SY (FMI) lab pump was used in the application of liquids to the Sep-Pak. After the supernatant was recycled through the Sep-Pak 2 or 3 times, the Sep-Pak was washed with 3 ml of 0.1% TFA to remove buffer, and the adsorbed peptide fraction was eluted with 2 ml of 80% aqueous acetonitrile containing 0.1% TFA. The resulting Sep-Pak eluates were each concentrated on a Model SVC200H Speed-Vac (Savant) to remove acetonitrile, adjusted in volume to 500 μl with 0.1% TFA, and extracted three times with ethyl acetate. The combined ethyl acetate extracts were back-extracted with 0.1% TFA, and the combined aqueous phases concentrated on the Speed-Vac. The concentrated aqueous extract was filtered through a Millex HV (Millipore) filter prior to analysis by HPLC Steps A, B, C, and D or A, E, and D, as described in the following section.

HPLC Purification Steps. Reverse phase (RP) HPLC Step A: Filtered samples were fractionated on a Supelcosil LC-18DB column with a Pelliguard guard column (Supelco) at ambient temperature on a Model 840 liquid chromatograph with autosampler (Waters). The sample was injected onto the column and eluted with a concave gradient (Waters curve 7) over one hour at 1.0 ml/min beginning with 10% acetonitrile (0.1% v/v TFA) and 90% aqueous 0.1% TFA, and ending with 60% acetonitrile (0.1% v/v TFA) and 40% aqueous 0.1% TFA. The eluant was monitored spectrophotometrically at 214 nm and by fluorescence (excitation = 230 nm, emission >300 nm). Fractions were collected over 1 min intervals, with both the autosampler and fraction collector cooled to 0-5° C. Fractions with the same retention times from multiple runs were pooled in the fraction collector and dried in the Speed-Vac concentrator (ca. 500 BR-SOG/run).

RP-HPLC Step B: Dried, pooled 1-min fractions were further purified by reversed phase HPLC on a Zorbax C-8 150 SP column (DuPont) on a 1090 liquid chromatograph equipped with a photodiode array detector and Chemstation (Hewlett Packard). The samples were eluted with a linear gradient from 5 to 60% acetonitrile in 0.25 N triethylammonium phosphate (TEAP), pH 2.20, over one hr at 28° C and 0.4 ml/min. Fractions were collected over 1 min intervals and concentrated in the Speed-Vac to remove the acetonitrile.

RP-HPLC Step C: Concentrated fractions were further purified using the same 1090 chromatograph and Zorbax column as in Step B, but with a linear gradient of 10 to 50% acetonitrile (0.1% v/v TFA) over 1 hr at 28° C and 0.4 ml/min. Fractions were collected over 0.5 or 1.0 min intervals and dried in the Speed-Vac concentrator.

RP-HPLC Step D: Dried fractions were further purified using the same 1090 chromatograph and a Vydac 218 TP54 C-18 column (Separations Group) with a linear gradient from 10 to 50% acetonitrile (0.1% v/v TFA) over 1 hr at

28°C and 0.4 ml/min. Fractions were collected over 0.5 min intervals and dried in the Speed-Vac.

High performance size-exclusion chromatography (HP-SEC) Step E: Dried, pooled 1-min fractions from RP-HPLC Step A were further purified by high performance size-exclusion chromatography on a series of four Protein-Pak 125 columns (Waters). The columns were eluted isocratically with 40% aqueous acetonitrile containing 0.1% TFA at ambient temperature and 1.0 ml/min, using the same 840 chromatograph described in RP-HPLC Step A. The columns were calibrated with a series of molecular weight standards as described previously *(2)*. Fractions collected over 1 min intervals were dried in the Speed-Vac concentrator.

Amino Acid Analysis. Vapor phase hydrolysis with HCl containing 0.1% sodium sulfite and amino acid analysis were performed as previously described *(3)*.

Sequence Analysis. Amino acid sequence analysis was performed on a Model 470A Vapor Phase Sequencer (Applied Biosystems) or on a Model 477A Pulsed-Liquid Sequencer (Applied Biosystems) using reagents and method cycles supplied by the manufacturer. The resulting phenylthiohydantoin (PTH) residues were analyzed on a Model 120A On-Line Analyzer (Applied Biosystems).

C-Terminal sequence analysis with carboxypeptidase P (Boehringer-Mannheim) was performed as described previously *(1)*.

Mass Spectrometry. Samples were introduced into a Californium-252 time-of-flight plasma desorption mass spectrometer in a 1% TFA solution applied to a nitrocellulose-covered aluminized Mylar foil. The samples were run at 15 kV accelerating volts with a 30-cm flight path, and the signal was accumulated for 19 hr *(1)*.

Synthesis. Solid-phase synthesis of Hez-PBAN was performed as described previously *(1)*. The crude peptide was initially purified by RP-HPLC on an Ultrasphere column (4.6 cm X 25 cm, Beckman) with a gradient of 0-60% acetonitrile (0.1% v/v TFA) at ambient temperature and 1.5 ml/min. Final purification of the major peak was achieved by semi-preparative RP-HPLC on a Supelcosil LC-18DB column (Supelco, 1 cm x 25 cm) with a Pelliguard guard column (Supelco) at 28 C on a Model 820 liquid chromatograph with Model 712 refrigerated automated injector and Model 490E detector (Waters). Samples were eluted over one hour at 2.8 ml/min with a linear gradient of 10-60% acetonitrile (0.1% v/v TFA). Fractions were collected over 0.1 min and dried in a Speed-Vac concentrator. Each 0.1 min fraction was analyzed for purity under the conditions of RP-HPLC Step D. The purified synthetic peptide was subsequently sequenced on the Model 477A sequencer.

Reactions of Hez-PBAN. A dried aliquot of purified Hez-PBAN was allowed to react with pyroglutamate aminopeptidase as described previously *(4)*. The quenched reaction mixture was analyzed under the conditions of RP-HPLC Step D. A dried aliquot of purified synthetic Hez-PBAN was oxidized by reaction with DMSO/HCl/acetic acid, as described previously *(1)*. The major product was purified by the conditions of RP-HPLC Step D. The purified Hez-PBAN disulfoxide was subsequently sequenced on the Model 477A sequencer.

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Results

Hez-PBAN was isolated batchwise from BR-SOG complexes of H. zea by a sequence of four (RP-HPLC Step A ⇒ RP-HPLC Step B ⇒ RP-HPLC Step C ⇒ RP-HPLC Step D) HPLC steps. As previously described *(2)*, fractionation of extracts prepared essentially by the procedures of Bennett et al. *(5)* by RP-HPLC Step A demonstrated a continuum of biological activity in fractions eluting between 44 and 52 min, with three distinct zones of activity. These zones were designated PBAN I, II, and III.

As most of the biological activity was associated with the PBAN I zone eluting between 44 and 46 min, our initial efforts were directed towards the purification of this zone, which was further purified by RP-HPLC Step B (Figure 1). The fraction eluting between 33 and 34 min, later shown to contain most of the biological activity, was further purified by RP-HPLC Step C (Figure 2). Final purification of the Hez-PBAN I peak eluting at 35.1 min was achieved by RP-HPLC Step D (Figure 3).

The Hez-PBAN I peak eluting at 28.8 min appeared to be homogeneous as evidenced by superimposition of its normalized upslope, apex, and downslope UV spectra (Figure 3, right insert). The presence of a tyrosine residue(s) in the sequence was suggested by strong minima at 283 and 276 nm in the second derivative UV spectra *(6, 7)* between 250-310 nm (Figure 3, left insert). In addition, there was spectral evidence for phenylalanine by the presence of a weak minimum at 255 nm.

Alternatively, Hez-PBAN I was purified by a sequence of three reverse phase HPLC steps (RP-HPLC Step A ⇒ HP-SEC Step E ⇒ RP-HPLC Step D). Biologically active fractions from RP-HPLC Step A, as described above, were further purified by HP-SEC Step E, and as described previously *(1)*, the major zone of biological activity was found in fractions eluting between 33 and 35 min, and corresponding to a MW of ca. 4200 Daltons. These fractions were finally purified by RP-HPLC Step D to give a single, pure product.

The second zone of biological activity (PBAN II) eluting between 46 and 49 min was similarly purified by the three-step HPLC purification scheme

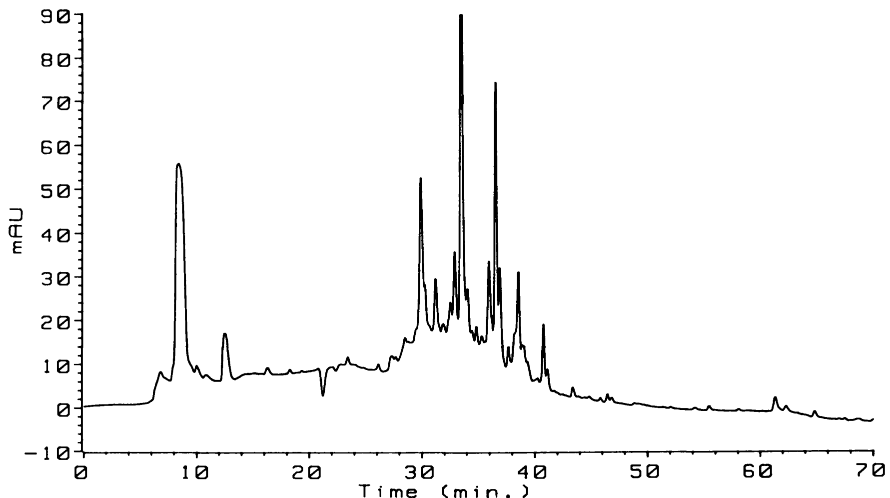

Figure 1. Elution profile using RP-HPLC Step B ($0.100A_{210}$ full scale) of fraction from RP-HPLC Step A eluting between 45-46 min derived from 1757 BR-SOG of H. zea.

utilized for PBAN I. The resulting purified product appeared to be identical to that from the PBAN I zone, displaying identical retention time and UV spectra in RP-HPLC Step D.

Amino acid analysis performed on ca. 25 pmol of purified Hez-PBAN indicated the following composition: Asp (5.2), Glx (4.5), Ser (2.2), Gly (2.6), Arg (2.7), Thr (1.6), Ala (2.2), Pro (3.5), Tyr (1.7), Met (1.0), Ile (0.8), Leu (1.6), Phe (0.8), and Lys (0.9).

Because of the number of pyroglutamyl-blocked insect neuropeptides reported to date *(8)*, and the high Glx content found in the peptide, a purified sample was treated with pyroglutamate aminopeptidase prior to automated Edman degradation. However, the retention time of the resulting putatively deblocked peptide was unchanged when re-analyzed under the conditions of RP-HPLC Step D, suggesting that no reaction with the enzyme had occurred and that the amino-terminus did not contain pyroglutamic acid. Automated Edman degradation by the vapor-phase sequencer of both the enzyme-treated (Table I, run 1) and untreated (Table I, run 2) Hez-PBAN yielded information on the first 14 amino acid residues and confirmed the presence of a free N-terminus in Hez-PBAN.

Automated Edman degradation was performed on ca. 200 pmol of purified Hez-PBAN using the pulsed-liquid sequencer. Data were obtained from 33 cycles (Table I, run 3). However, the residues at positions 23 and 32 could not be ambiguously assigned. A second attempt on the same instrument using sample purified from the PBAN II zone confirmed the earlier sequence and established the residues at positions 23 and 32 (Table I, run 4).

In order to determine the carboxy-terminus of Hez-PBAN, ca. 200 pmol of purified peptide was digested with carboxypeptidase P. Released amino acids were periodically analyzed as their PTC derivatives. Leu was found to be the C-terminal residue followed by Arg, Pro, Ser, Phe, Tyr, and Lys, respectively, thus confirming the automated Edman degradation data (Table I, run 5). However, none of the data could distinguish between a C-terminal amide or free acid.

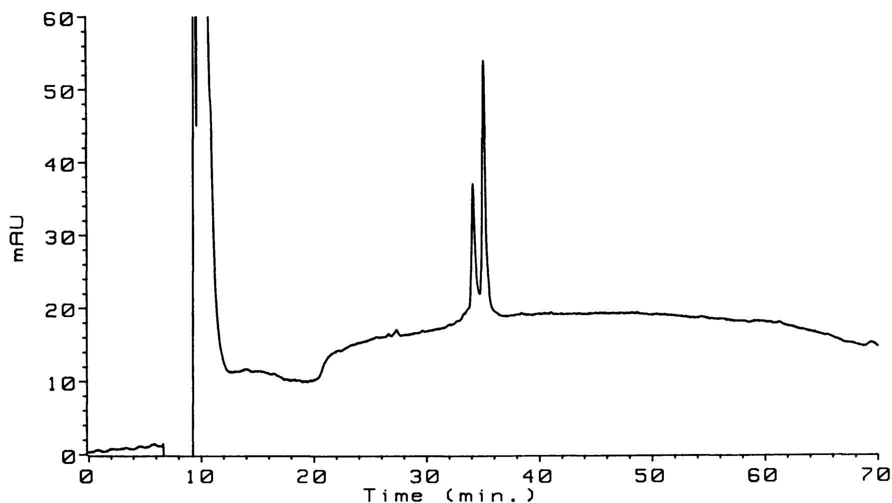

Figure 2. Elution profile using RP-HPLC Step C ($0.060A_{210}$ full scale) of fraction from RP-HPLC Step B eluting between 33-34 min.

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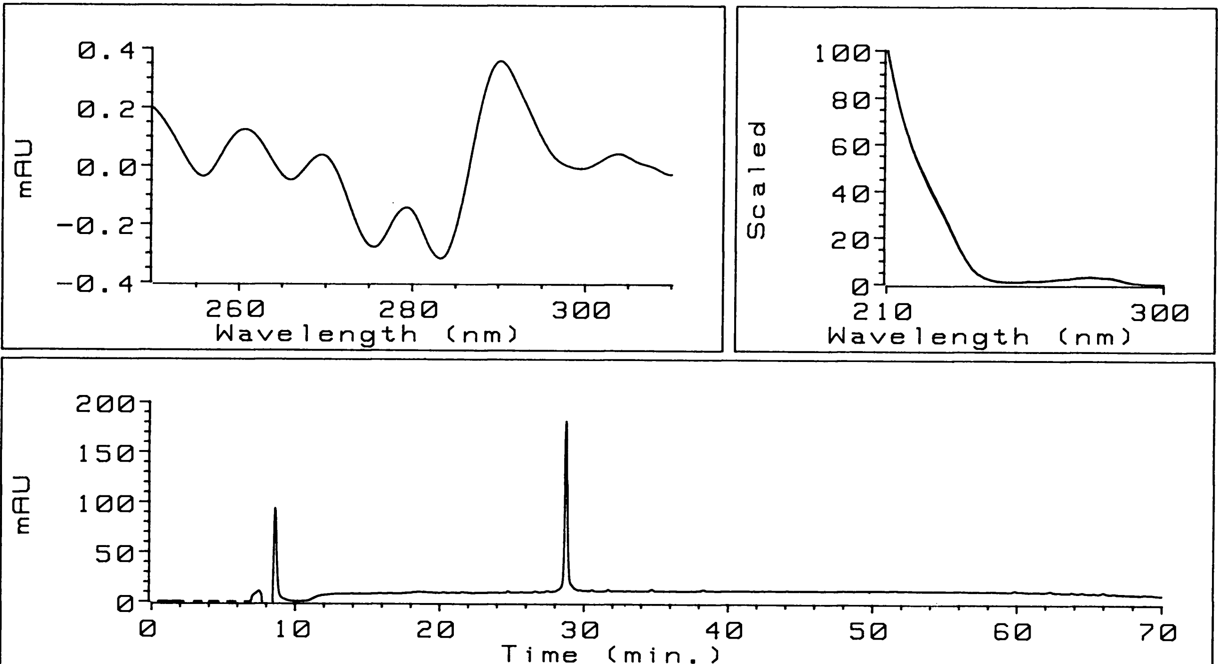

Figure 3. Elution profile using RP-HPLC Step D (0.200A_{210} full scale) of pooled Hez-PBAN fractions eluting between 35-36 min by RP-HPLC Step C and derived from 5922 BR-SOG of H. zea. Overlay (right insert), of normalized UV spectra of the upslope, open and downslope of the peak eluting at 28.8 min (insert right). Second derivative spectrum (spliced) of Hez-PBAN between 250-310 nm.

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Table I. Sequence Analysis of Hez-PBAN

Run No.[a]	10 20 30
1	LSDDMPATXXD...
2	LSDXMPATPADXXM...
3	LSDDMPATPADQEMYRQDPEQIXSRTKYFSPXL
4	LSDDMPATPADQEMYRQDPEQIDSRTKYFSPRL
5	...KYFSPRL

[a]Run 1, ca. 5 pmol, Model 470A sequencer; Run 2, ca. 50 pmol, Model 470A sequencer; Run 3, ca. 200 pmol, Model 477A sequencer; Run 4, ca. 75 pmol, Model 477A sequencer; Run 5, C-terminal analysis with carboxypeptidase P, ca. 200 pmol; pmol estimates for Runs 1-4 are based on yields of Leu on the first cycle.

Californium-252 time-of-flight plasma desorption mass spectrometry performed on ca. 30 pmol of Hez-PBAN indicated two molecular ion peaks (Figure 4). The first peak at m/z 3934 $[(M+H)^+]$, presumed to be the singly charged molecular ion, and a second broader peak centered at m/z 1966 $[(M+2H)^{2+}]$, assumed to be the doubly-charged molecular ion, corresponded to molecular weights of 3933 and 3930, respectively.

Because C-terminal amides have often been reported in insect neuropeptides *(8)*, a 33 residue C-terminal amide consistent with the sequence data was synthesized by solid-phase methods. The peptide was purified by HPLC and its structure confirmed by automated Edman degradation. Californium-252 time-of-flight plasma desorption mass spectrometry provided additional evidence for the structure via a very broad peak for the singly-charged molecular ion at m/z 3902-3906. Because the calculated MW of Hez-PBAN (3899.6, based on the most abundant ion in the isotope cluster) was seen to differ from that observed in the mass spectrum of the isolated native peptide by ca. 32, it was presumed that the native peptide had undergone oxidation of both its methionines to their respective sulfoxides during the course of its isolation and purification.

Final structural proof of Hez-PBAN came from demonstration of identical properties of both the isolated native and synthetic Hez-PBAN disulfoxides. Synthetic Hez-PBAN was oxidized to its disulfoxide by the method of Wagner and Fraser *(9)* Both native Hez-PBAN and synthetic Hez-PBAN disulfoxide demonstrated identical retention times. In addition, the elution profile of a mixture of equal amounts of native and synthetic Hez-PBAN disulfoxides displayed one peak under the conditions of RP-HPLC Step D. Superimposition of its normalized upslope, apex, and downslope UV spectra indicated peak homogeneity.

Based on all of the data presented, it is proposed that Hez-PBAN is a 33-residue C-amidated peptide having a MW of 3900 Daltons and the following primary structure:

Leu-Ser-Asp-Asp-Met-Pro-Ala-Thr-Pro-Ala-Asp-Gln-Glu-Met-Tyr-Arg-Gln-
-Asp-Pro-Glu-Gln-Ile-Asp-Ser-Arg-Thr-Lys-Tyr-Phe-Ser-Pro-Arg-Leu-NH_2.

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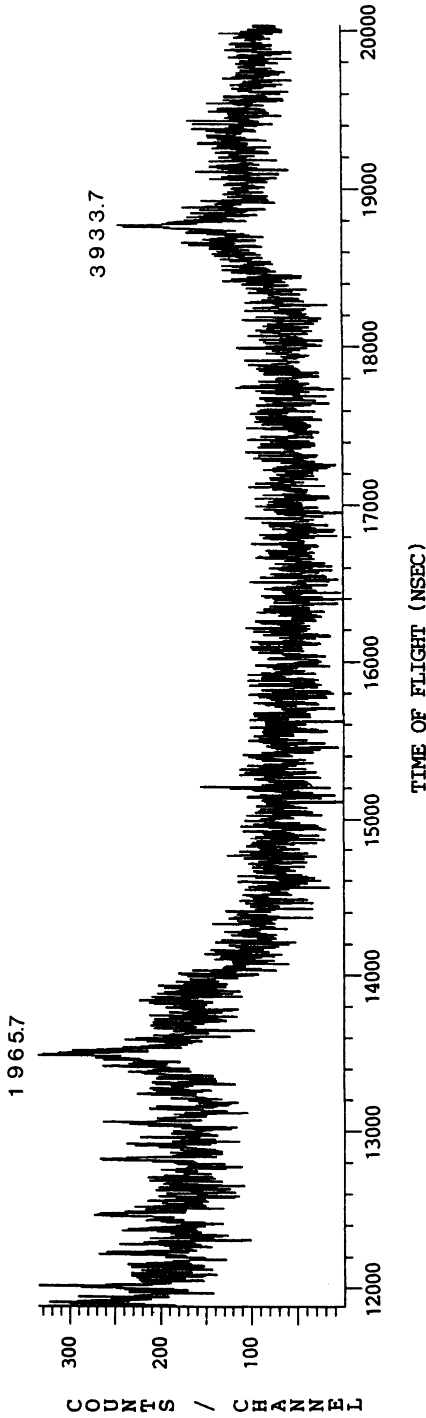

Figure 4. Partial Californium-252 time-of-flight plasma desorption mass spectrum of Hez-PBAN.

Synthetic Hez-PBAN demonstrated biological activity in a dose-responsive manner when injected into ligated female adult H. zea. All of the usual components of the pheromone were produced by the synthetic material, and in the same proportions as those elicited by the native peptide *(1)*.

Discussion

Hez-PBAN was isolated on the basis of an in vivo bioassay in ligated female adult H. zea. Peptides were initially extracted from BR-SOG tissue into a solvent system consisting of 5% formic acid, 15% TFA, 1% NaCl, and 1N HCl, as first described by Bennett et. al *(5)* and designed to precipitate proteins, prevent the binding of peptides to structural proteins, and inhibit enzymatic degradation of peptides. Care was exercised throughout all of the subsequent steps to prevent thermal, chemical, or enzymatic degradation of the native peptide.

Purification of Hez-PBAN was achieved via a four-step RP-HPLC procedure. One chromatographic mode was used throughout, but a change in buffer and/or column at each step was used to effect purification. Alternatively, Hez-PBAN was purified by a sequence of three HPLC (RP-HPLC ⇒ HP-SEC ⇒ RP-HPLC) steps. In this case, changes in chromatographic mode, in addition to changes in buffer and/or column, were used to achieve purification in only three steps. The purity of the product isolated by either procedure was comparable. Similar multi-step procedures have been employed in the isolation of insect neuropeptides in the AKH/RPCH-family *(3, 10, 11)* and in the isolation of mammalian neuropeptides from brain and hippocampus *(12)*.

The primary structure of Hez-PBAN was determined by a combination of automated Edman degradation, C-terminal sequence analysis and plasma desorption mass spectrometry. The amino acid analysis was in good agreement with the proposed sequence except for extraneous glycine and low methionine. Glycine is known to be an ubiquitous contaminant commonly resulting in high background glycine values, particularly in high sensitivity amino acid analysis *(13)*. Low methionine results from its destruction during hydrolysis *(13)*. The MW of Hez-PBAN (3900) is in good agreement with the estimate (ca. 4200) from HP-SEC, attesting to the utility of HP-SEC in molecular weight estimation *(14)*.

Native Hez-PBAN underwent oxidation of its two methionines during the course of its isolation and purification. Oxidation of methionine to methionine sulfoxide is known to occur during the isolation of peptides and proteins from natural sources *(15, 16)*. It was observed that purified samples of synthetic Hez-PBAN were easily oxidized to a mixture of oxidation products resulting from the oxidation of Met^5 and/or Met^{14} to their respective sulfoxides. These observations are consistent with the isolation of Hez-PBAN from the PBAN II zone. We speculate that the continuum of biologically-active HPLC fractions observed between 44 and 52 min after RP-HPLC Step A, represents Hez-PBAN (PBAN III), a mixture of Hez-PBAN monosulfoxides (PBAN II), and Hez-PBAN disulfoxide (PBAN I), all of which were biologically active.

Hez-PBAN represents the first member of a new family of insect neuropeptides. Recently *(17)*, the primary structure of PBAN from the silkworm, Bombyx mori (Bom-PBAN) was reported (Table II). Like Hez-PBAN, Bom-PBAN is a C-terminally amidated 33-residue peptide with 27 residues identical to those of Hez-PBAN. Interestingly, its methionines also oxidized during isolation.

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Sequence homology has been noted previously *(17)* between the N-terminus of Hez-PBAN and insulin-like growth factor II, and the C-terminus with the insect peptide leucopyrokinin (Table II) isolated from cockroach heads. The striking similarity of the first 16 residues of the melanization and reddish colorization hormone (MRCH) of B. mori *(18)* and Bom-PBAN has led to the suggestion by Kitamura et al. *(17)* that they are identical (Table II), thus implying a dual role for this hormone in the larvae (melanization and reddish colorization) and adult moth (pheromone biosynthesis).

Table II. Amino Acid Sequence of Hez-PBAN and Related Peptides

	10 20 30
Hez-PBAN...	LSDDMPATPADQEMYRQDPEQIDSRTKYFSPRLNH2
Bom-PBAN...	LSEDMPATPADQEMYQPDPEEMESRTRYFSPRLNH2
MRCH-1...	LSEDMPATPADQEMYQ...
Leucopyrokinin	pETSFTPRLNH2
Insulin-like Growth Factor II...	LLETYCATPAKSEOH
	55 60 65

The discovery of these pheromone biosynthesis activating neuropeptides could form the basis for development of new biorational control agents for the disruption of the essential processes of development and reproduction in moths *(19)*.

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Chapter 20

Adipokinetic Hormone Neuropeptide Family

Applying Recombinant DNA Techniques

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The application of recombinant DNA techniques to the study of neuropeptide biosynthesis is essential because these techniques facilitate structural analyses and evolutionary studies, clarify biosynthetic pathways, provide the necessary background and probes for analysis of synthesis regulation at the levels of mRNA and gene transcription and assist in classical genetic experimentation. Examples of these benefits are cited and problems encountered in insect systems are discussed.

The analysis of neuropeptides in insects may lead to the development of both specific insect control strategies and model systems that clarify the role of neuropeptides in all animals. Therefore it is important to vigorously pursue neuropeptide studies and to exploit the available technologies to the fullest. This paper will review the importance of current recombinant DNA techniques to the study of neuropeptide biology. Studies of the adipokinetic hormone (AKH) family will be emphasized as examples, but other peptides will be mentioned to present a broader picture of the advantages and problems associated with the techniques.

There are several general reasons for studying neuropeptide related mRNAs and genes including:

I. Efficient determination of primary sequences
II. Determination of biosynthetic pathways
III. Analysis of the regulation of synthesis
IV. Facilitation of genetic strategies to study function
V. Clarification of evolutionary relationships

and these will be considered in turn.

<u>Primary Sequences</u>

One of the most obvious benefits of modern DNA technology is the explosion in available structural data as a result of the relative

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ease of DNA sequencing. This technology is particularly useful in certain special cases. For example the determination of long amino acid sequences is usually simpler at the DNA than the protein level. While this is typically not a problem with neuropeptides it will become important as investigators begin to study the receptor molecules to which neuropeptides (and potential control agents) are targeted. There will also be instances in which a neuropeptide is too large to be sequenced completely from its amino terminus, and so scarce that extensive structural studies such as complete tryptic mapping are unfeasible. In this instance, amino terminal data can provide a basis for complete structural determination by cDNA cloning.

Recombinant DNA techniques have been particularly useful in providing the primary amino acid sequences of neuropeptide precursor proteins. These proteins vary in size, but are sometimes quite large. Since precursors function as metabolic intermediates they invariably are found in very low abundance if they are detectable at all. Thus it is usually necessary to use recombinant DNA techniques to determine the amino acid sequence of a neuropeptide precursor, and fortunately a search of recombinant libraries can usually be based on the structural information provided by the neuropeptide itself. The importance of nucleic acid approaches is illustrated by the case of **Drosophila** FMRFamide-related peptides (1,2). The precursor is large and there is no recognized rich source of the peptides (much less their precursor) so direct structural determination at the protein level would have been very difficult indeed. The grasshopper AKH peptides, on the other hand, were exceptional in that the precursor proteins (less their signal sequences) were identified and sequenced at the protein level thanks to intense synthesis in the corpora cardiaca (C.C.) and the diligence of Hekimi et al. (3). Even in this nearly ideal case, as we shall see, the structures of the mRNAs prove to be very useful (4,5).

The precursors for the grasshopper AKHs, **Manduca** AKH, the **Drosophila** peptides related to FMRFamide, and drosulfakinin all contain additional peptides. Thus the determination of neuropeptide precursor structure often pays the dividend of leading to the identification of other interesting peptides.

If one grants that recombinant DNA techniques have utility in the determination of new sequences, when can they be exploited? The answer is whenever two fundamental technical problems, how to obtain a library containing the recombinant of interest and how to obtain a probe suitable for screening the library, can be solved. The most obvious place to begin a cloning project is often with the construction of an appropriate cDNA library. This is usually much more challenging than the construction of genomic libraries, and a number of difficulties may arise. Scarcity of starting material is a common problem in insect biochemistry, and it certainly was a problem in the case of cloning of the **Schistocerca nitans** AKH cDNAs. Although the C.C. is an abundant source of the peptides it is also a tiny structure, and it was necessary to collect nearly 2000 organs in order to obtain a manageable level of poly(A)$^{+}$ RNA at the end of the preparation. Even given adequate amounts of starting material, the preparation of high quality RNA is not

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necessarily assured. Techniques which work in one tissue in one organism may be spectacularly unsuccessful in another setting. For example the guanidine thiocyanate extraction procedure which worked well for us in previous work (6,7) gave low yields when applied to **Schistocerca nitans** C.C., while modifications of another technique proved entirely satisfactory (8). Researchers studying insects should be mindful of the fact that they are likely to be in the position of extracting RNA from a novel tissue or in a species not previously studied.

There is a another set of problems which may occur during the construction of a cDNA library. For example, some RNAs are difficult to convert into cDNAs. This was to some extent true of AKH I, which is predicted by computer modeling to have a substantial amount of secondary structure (5,9). Rat cholecystokinin mRNA also proved to be a temperamental template and the cDNA clones derived from it were short by over 100 nucleotides (10). Such problems can form a significant barrier to beginning a new project, since it is possible that the short segment of the mRNA coding for a known neuropeptide is inaccessible to reverse transcription. However, once a library is constructed, it is available as a resource for many investigators with many interests, and screening an existing library requires only a modest expenditure of time and resources. It is also likely that as experience in the field accumulates current problems will be addressed more easily. For example, in some cases it may be possible to avoid the construction of libraries from a number of closely related species by using known nucleotide sequence data from one species and the technique of polymerase chain reaction (11) to generate the related recombinants from another species.

The problem of obtaining a cDNA library can sometimes be avoided, at least initially, by beginning at the genomic level. Thus in the cases of the drosulfakinin and the FMRFamide related peptides of **Drosophila** precursors, investigators exploited the small **Drosophila** genome, and began their screens at this level (1,2,12). In these cases the absence of a rich source of mRNA meant that cDNA libraries would be less predictable and likely more difficult to screen. It is important to realize that this situation does not apply to all insects. Acridid grasshoppers, for example, have genomes several times the size of mammals and thus nearly 100 times the size of **Drosophila** (13). This means that the screening of a grasshopper genomic library requires a very accurate probe capable of avoiding the large number of chance matches that arise in such a large collection of sequences, and that a large number of recombinants must be screened. For example, at the beginning of our search for the **Schistocerca nitans** AKH genes we estimated that we would have to screen over 1 million clones to have a 90% chance of encountering a true positive. And it appears that this was a reasonably accurate estimate.

The other tool necessary for a successful cloning project is a probe with specificity adequate for the library of interest. The specificity required will vary greatly depending on the complexity of the library. Most often, cloning projects begin with the use of synthetic oligonucleotides whose sequences are based on available protein sequence data. In fact this technique has been employed in

the cases of AKH I and II, Manduca AKH, drosulfakinin, and the **Drosophila** FMRFamide precursor (1,2,4,5,12,14) often, however, with special modifications. For example, in the case of AKH I and II, specific mRNA priming was used to improve the specificity of the probes (5,15). Probe specificity is often a problem in projects based on short neuropeptide sequences. Bradfield and Keeley (14) dealt with the similar difficulties in the **Manduca** AKH sequence through the use of oligonucleotides containing deoxyinosine. While they were successful, screening required very carefully adjusted conditions, and it was their conclusion that the modestly sized **Manduca sexta** genome stretched the power of the technique to its limit.

On the other hand two groups have obtained precursor clones without the benefit of any protein data from the species under investigation. Schneider and Taghert (2) combined the use of oligonucleotides directed at the molluscan peptide FMRFamide together with a cDNA clone of the **Aplysia** precursor to obtain a related gene in **Drosophila.** Nichols et al. (12) based a search for a **Drosophila** peptide related to gastrin and cholecystokinin primarily on the protein sequence of the cockroach peptide leucosulfakinin (16). In both cases the small size of the **Drosophila** genome expedited the search. Unfortunately such strategies will not always be successful. For example comparison of the **Schistocerca nitans** AKH I and II precursors' nucleotide sequences with that of **Manduca** AKH shows no significant homology. Thus a cDNA of a precursor for one of these AKHs would not be useful in screening a library from the other species. It also should be kept in mind that success of such a strategy does not obviate the need for structural studies at the protein level. Nonetheless, when successful, this kind of a strategy can rapidly advance the area under study.

Antibodies can also be used as probes to screen expression libraries, and this strategy has been used successfully in the cloning of vertebrate neuropeptides (17). It is likely that this technique will be helpful in cases in which structural data is particularly difficult to obtain and a variety of highly specific antibodies is available. While these and other techniques will prove valuable in special circumstances, screening with synthetic oligonucleotide probes based on protein data is likely to be the principal method of identifying other peptide precursors in the near future.

Determination of Synthetic Pathways

Determining the structure of a cDNA not only provides structures of final products, but also reveals much about how those products are formed. For example, it was possible to sequence the proAKH I and II peptides, but it would not have been obvious whether there were larger very short lived precursors to these rather small peptides without comparison to the structures derived from the mRNAs. Thus it was important to first accurately establish the completeness of the cDNA clones and then inspect the inferred precursor protein structures. Both precursors were found to contain additional amino terminal sequences which were very hydrophobic and likely to be

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signal sequences. The von Heinje algorithm (18) predicts cleavage just prior to the Gln which begins both mature AKH I and the isolated proAKH I dimer (in the form of pGlu). This algorithm predicts cleavage one amino acid closer to the amino terminus in the case of AKH II, but the algorithm makes this error fairly often (20% of cases). Such "prepro" sequences are generally very useful in predicting the general scheme of synthesis, but it is not possible to decide which potential processing sites are actually used. For example, both proAKH I and II contain the sequence arg-lys in their carboxyl terminal peptides which apparently are not processed in the C.C. at least. Similarly it would not be possible to predict **a priori** that the single cysteines in these propeptides would oxidize to form peptide dimers (3,19). Nonetheless the inferred protein sequence sets the boundaries for what is possible, and often predicts the existence of unrecognized peptides which should be sought in the animal, such as the many interesting peptides in the **Drosophila** FMRFamide related peptide precursor (1,2).

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Regulation of Synthesis

A striking feature of neurotransmitter chemistry is the precise regulation of synthesis, that, for example, results in a few neurons synthesizing a particular neuropeptide while all the rest do not. It appears from the vertebrate literature that mature neurons also precisely regulate the rate of neuropeptide synthesis (20), though the functional significance of this regulation has not yet been demonstrated, and it seems probable that insects will show this phenomenon as well. It is likely that much of this regulation will be at the level of transcription, and so an understanding of the phenomena of interest will require analysis of both mRNA and gene structures as well as the identification of regulatory sites and factors. While such analyses are just beginning in several insect neuropeptide systems, the results will be of general interest because of the power of simpler neural systems to clarify functional consequences of neural cell biology.

Genetic Studies

Biologists and biochemists interested in insects benefit from the facts that **Drosophila** is such a favorable organism for genetic research and has such a rich history of previous studies. Particularly because genetic studies have such power in the analysis of complex biological phenomena, it seems important to use genetic techniques to clarify the role of neuropeptides and the regulation of their synthesis to the functioning of the insect nervous system. One might also imagine using mutants to clarify the potential of control agents designed to either block or over - stimulate neuropeptide receptors. It is thus not surprising that several groups have begun the study of neuropeptide precursors in **Drosophila** and certainly more will follow. We have joined in by recently isolating an AKH peptide family member from **Drosophila** (Schaffer, M.H.; Noyes, B.E.; Slaughter, C.A.; Thorne, G.C.; Gaskell, S.J. Biochem. J., in press.), and using this sequence to

identify a genomic clone. However, the complexity of genetic studies should not be underestimated. Despite the long history of **Drosophila** genetic research, it is likely that informative mutants and deficiencies will need to be newly constructed, as in the case of **Drosophila** FMRFamide (1,2). The work may well be further complicated by the possibility of the mutants having very subtle phenotypes. Even so an impressive array of tools is available to address these problems, and the advantages of adding genetic approaches to the other ways of studying neuropeptide function justify the considerable effort it is likely to require.

While **Drosophila** is certainly the insect which has been most intensively studied, it is worth mentioning that genetic studies may become important in other genera as we struggle with problems of insecticide resistance, for example. Fortunately, other insects do have some of the features which make **Drosophila** favorable for study, such as polytene chromosomes, and it may be that recombinant DNA research combined with such features will encourage much more work in other species.

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Evolutionary Studies

It seems only common sense that the study of genes and their structure should be invaluable to the study of evolution, and indeed that is the case. One can already observe some of these benefits in the work done on neuropeptides. The determination of precursor structure is useful in identifying or confirming evolutionary relations. Although the relationship between AKH I and II and **Manduca** AKH was already quite striking, it is the case that the **Manduca** AKH is one of the least typical family members (being 9 amino acids in length instead of 8 or 10) (21), and it is possible that such a structure might arise due to convergent evolution. This possibility is argued against by the observation that the precursor peptides show the same general organization and that each of these precursors bears a significantly higher degree of homology toward the other two than to any other sequence in the protein sequence data banks (4,5,14). The **Drosophila** FMRFamide related precursor provides a more striking example. Although only one of the peptides predicted by the infered precursor structure has been isolated from the animal, others will doubtlessly follow, and it is easy to imagine that the relationship between some of the more divergent phe-amide peptides would be cause for speculation and debate in the absence of the precursor structure. In general, it is difficult to be confident about the evolutionary significance of homologies in short neuropeptides and the analysis of precursor structures can help clarify relationships.

While more distant relationships are best detected by comparison of protein structures, interesting relationships may be seen by analysis of untranslated regions of mRNA sequences. Comparison of the **Schistocerca nitans** AKH I and II cDNA sequences reveals a high degree of homology over the translated region (63.8% identity over 218 nucleotides with 5 gaps), but no significant homology in the untranslated regions. This is in contrast to the relationship between AKH I cDNA sequences of **S. nitans** and **S. gregaria** which show a high degree of homology throughout the

available sequences, both translated and untranslated regions (95.2% identity over 330 nucleotides with two gaps). These results strongly suggest that AKH I and II diverged prior to the separation of **S. nitans** and **S. gregaria.** Comparison of the **Locusta** and **Schistocerca** AKH I precursor carboxyl terminal amino acid sequences reveals an odd result. It happens that there is only one difference between **L. migratoria** and **S. gregaria** while there are two differences between **S. gregaria** and **S. nitans.** This might call into question the categorization of these species, but comparison at the cDNA nucleotide level supports the very close relationship between **nitans** and **gregaria.**

Taghert et al. (22) have illustrated another use of structural comparison by studying the gene structure of the FMRFamide related precursor in both **Drosophila melanogaster** and **Drosophila virilis.** They observe interesting short regions of sequence homology 5'to the transcribed region which may indicate conserved regulatory sequences.

Thus the application of recombinant DNA technology to the study of neuropeptide biosynthesis has enhanced, and will continue to enhance, our understanding of the structure, function, and evolution of these interesting modulatory agents. Some of the most exciting advances, such as the insights provided by genetic studies, are yet to come.

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Chapter 21

Molecular Genetics of the *Drosophila* FMRFamide Neuropeptide Gene

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We are studying insect neuropeptides with the intention of utilizing the advanced genetic and molecular techniques of *Drosophila* to address questions regarding neuropeptide gene function and regulation. We isolated a *Drosophila* gene that encodes a series of FMRFamide-like neuropeptides. Molluscan FMRFamide was the first representative isolated from what has turned out to be a very large family of biologically active molecules (Price, D.A.; Greenberg, M.J. Science 1977, 197, 670-671); *Drosophila* preproFMRFamide resembles many other neuropeptide precursors in that it contains many related neuropeptides, and also has protein regions not obviously coding for neuropeptides. It is expressed by a small subset of neurons within the CNS, but these neurons are not obviously related by their ancestries, their positions or their apparent functions. This chapter summarizes our recent results detailing the structure, expression, evolution and genetics of *Drosophila FMRFamide*.

The gene (1,2) is present in single copy in the haploid genome and is transcribed as a single RNA class of ~1700 nucleotides (nt) (3). The 347 amino acid preproFMRFamide precursor is encoded entirely within the second of two exons. The first exon comprises just 106 bp of 5' untranslated sequence. The precursor contains 13 separate neuropeptides that contain FMRFamide-like sequences, but each is extended 3-5 amino acids in the N-terminal direction. Some of the extension sequences are tandemly repeated but most are unique. This complex structure raises the question of whether or not such variations in peptide sequence indicate a diversity of functions or simply variations in structure that are permitted through natural selection. The gene is first expressed in late embryonic stages and then actively transcribed throughout all subsequent post-embryonic stages. We analyzed the cellular pattern of expression

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using specific DNA and antibody probes. In situ hybridization revealed a stereotyped and discrete set of signals in positions that were highly correspondent with those of FMRFamide-immunoreactive neurons. Interestingly, signal intensities were reproducibly cell-specific: this suggests that distinct neurons possess different steady-state amounts of neuropeptide RNA. We were able to map the time at which different neurons first started to express the gene and found that it varied for specific cell groups and spanned both embryonic and larval stages (Schneider, O'Brien and Taghert, submitted). Finally, we found that most of the adult pattern of neuropeptide gene expression could be accounted for by that of persistent larval neurons. Only a small component appeared to be "adult-specific" (O'Brien, Schneider and Taghert, submitted). An antiserum to a small peptide epitope (not FMRFamide) present on the precursor protein was raised in rabbit and affinity purified. Immunocytochemistry with this antiserum displays a pattern highly correspondent to that generated by the DNA probes (Schneider and Taghert, unpublished results).

Cloning and sequencing the same gene in *D. virilis* (4) provided useful comparative data with which to formulate testable hypotheses concerning *FMRFamide* gene regulation and function. First we found that the deduced *virilis* proFMRFamide precursor contains only ten copies of FMRFamide-like peptides, three fewer than that of *D. melanogaster*. Secondly, inspection of the individual peptide sequences gave insight into the degree to which insect neuropeptide structures have drifted since speciation (estimated to approach 50 Myr, 5). In addition, we compared DNA sequences 5′ upstream of the gene in the two species and identified a large number of conserved sequence regions that represent candidate cis-acting elements for the regulation of the gene. Such a conserved mechanism of transcriptional regulation is credible due to the fact that the pattern of neurons that express FMRFamide-like peptides was indistinguishable between the two species. We are continuing our analysis of the neuropeptide gene promotor using DNAse footprinting and P element transformation techniques.

Finally, initial progress has been made in the genetic analysis of the *FMRFamide* locus. The gene is located in polytene interval 46C1-2 on the right arm of the 2nd chromosome. To date, the only known deficiency of the region uncovered the segmentation gene, *even-skipped*, but did not delete the *FMRFamide* gene (J. Nambu and R. Scheller, personal communication). Because such a deficiency would be of value in our analysis, we have recently created 5 stocks with x-ray irradiation to revert a closely-positioned P element that contained alcohol dehydrogenase (*Adh*). These animals are currently being analyzed to see if the nearby *FMRFamide* gene was also deleted (O'Brien and Taghert, experiments in progress). Success in this initial aspect of mutagenesis will permit future attempts to create point mutations in the gene for a more precise genetic dissection. Our goal in these experiments is an in vivo examination of the physiological and developmental consequences of manipulating neuropeptide gene structure and dosage.

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In summary, our studies have been designed to formulate a basis on which to create and test specific hypotheses concerning the structure, function and regulation of a neuropeptide gene. A molecular genetic analysis of *Drosophila* neuropeptides holds much promise for investigations of neuropeptide physiology in that it suggests ways to address neuropeptide functions comprehensively. It also offers techniques to identify and characterize other gene products with which neuropeptides may interact.

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Chapter 22

Baculovirus-Directed Foreign Gene Expression

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The baculovirus expression vector is the only eucaryotic DNA viral vector for the cloning and expression of genes in lepidopteran insect cells and insects. It is now a major tool and model for the studies of the molecular biology of insect systems. The baculovirus vector is a helper-independent recombinant baculovirus which during infection can express abundant quantities of recombinant mRNA and proteins of foreign genes. Chimeric genes coding potentially any peptide or protein can now be expressed under the transcriptional regulation of baculovirus promoters representing genes of immediate early, delayed early, late and very late classes. The majority of recombinant proteins produced in the baculovirus system are functionally authentic and although differences in glycosylation appear to exist, this has not been shown to be detrimental to the "authenticity" or function.

The genetic engineering of a baculovirus polyhedrin gene promoter (1,2) pioneered the use of recombinant techniques for the cloning and expression of foreign genes using a recombinant baculovirus infection of cultured lepidopteran insect cells (2) or insects (3). A major area of interest in the development and use of heterologous expression systems is the ability to produce authentic recombinant proteins. The expression of recombinant proteins in baculovirus infected cells has been remarkably successful: the foreign gene is often highly expressed and the majority of recombinant proteins expressed to date (more than 125) are functionally authentic. The major area for application of baculovirus expression vectors (BEV) has been the production of human, vertebrate and viral proteins for studies of structure and function or for development as therapeutics, diagnostics or vaccines (4,5). The study of the processing, cellular localization and secretion of a variety of recombinant proteins during baculovirus infection has rapidly given significant details on the nature of lepidopteran cellular processing pathways for a comparison with the more comprehensively studied pathways in procaryotic and eucaryotic systems. This has rapidly advanced our knowledge of the molecular biology of insect cells (6,7). The use of the BEV for cloning and study of genes of agricultural value is now becoming of major focus as another important application for genetically engineered baculoviruses (8).

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Baculovirus Biology

There are more than 500 species of insect baculoviruses named according to the genus and species of host in which discovered (9). The major cytopathic and diagnostic character of the subgroup A occluded baculoviruses is the accumulation of numerous viral occlusions (often called polyhedra) in the nucleus of the infected cell. The nucleus increases significantly in volume extending nearly to the cell plasma membrane and under phase scope or dark field objectives will exhibit a highly refractile image. Subgroup A baculoviruses are also referred to as the nuclear polyhedrosis viruses (NPV). At the ultrastructural level the nucleocapsids are either enveloped singly (*S*NPV) or as multiples (*M*NPV).

In susceptible insects the viral occlusion, assembled primarily of the viral-encoded polyhedrin protein, dissolves in the high pH environment of the gut lumen to release the virions which then enter the columnar epithelial cells by fusion of cell microvillar membrane and viral envelope. Keddie *et al.* (10) show that viral replication may occur in both the regenerative and columnar cells, but the nature of virus entry and replication in the regenerative cells is not comprehensively studied. In the columnar cell viral occlusions, and presumably polyhedrin protein synthesis, are not produced in significant quantity in lepidopteran insects. Instead, the virus buds from the columnar cell into the hemacoel and is a source of inoculum for secondary infection. This form is called the extracellular virus (ECV). At one time the concensus was that the ECV directly invaded all of the other cells and tissues to effect secondary infection resulting in death of the insect. Keddie *et al.*, however, cast some doubt on this generalization by showing that there is a distinct temporal pattern of tissue infection which correlates with a tissue contact-mediated infection process. In the majority of secondary infected tissues the virus acquires an envelope in the infected cell nucleus, and concomitant with the synthesis and assembly of the polyhedrin protein, numerous particles become embedded in the viral occlusion. The virus can be purified from the occlusion by a dilute alkaline saline treatment and banding on sucrose or cesium chloride gradients. Purified as such this viral form is referred to as the polyhedral derived virus (PDV). Packaged in the viral occlusion the virus is highly stable in the extracellular state and if protected from ultraviolet light or extreme variation in temperature will be highly stable for long periods of time in the laboratory or environment.

Both ECV and PDV are infectious for susceptible cultured lepidopteran cells. However, the ECV is much more highly infectious when compared on a physical particle: infectious particle ratio. For natural routes of infection in the insect, the viral occlusion and the PDV is far more highly infectious than ECV(11). The occluded baculoviruses, therefore, have a biphasic life cycle: the PDV and viral occlusion are the most efficient viral forms for natural infection of the insect, and the ECV form is the most efficient for secondary infection of tissues in the insect and cells in culture. The polyhedrin gene is not essential for cell culture infection.

Autographa californica MNPV (AcMNPV)

The genetically complex Ac*M*NPV prototype baculovirus genome is a double-stranded, covalently closed circular molecule with superhelical conformation and a size of approximately 130 kbp (12). Regulation of viral gene expression is temporally organized relative to the time of viral DNA replication which occurs at approximately 6 hours post infection in cultured insect cells. By our functional criteria, some of the early genes (Table I) are described as immediate early because they are expressed immediately in a viral infection or transient expression assay, or do not require

transactivating viral factors in a transient expression assay. These are IE0 (13), IE1 (14) and IEN (15). An example of a delayed early gene is 39K, of unknown function, which is optimally transactivated by IE1 through a transcriptional enhancer (16,17). The late genes expressed after viral DNA replication are presumed to represent mostly virus structural proteins and the very late regulated but abundantly expressed polyhedrin and p10 genes.

Table I. Ac*M*NPV Genes

GENE	RNA	PROTEIN	CLASS	REFERENCE
IE-0	2.1 kb (spliced)	636 aa	Immed. early	Chisholm & Henner 1988(13)
IE-1	1.9 kb (unspliced)	582 aa	Immed. early	Guarino & Summers 1986(14)
IE-N	1.3	NA	Immed. early	Carson *et al*., 1988(15)
39K	0.9	NA	Delayed early	Guarino & Summers 1987(18)
DNA pol	3.0	114 kDa	Early	Tomalski *et al*., 1988(19
35K	1.07	35 kDa	Early	Friesen & Miller, 1987(20)
94K	2.63	94 kDa	Early	Friesen & Miller, 1987(20)
ETL	1.7	28 kDa	Early	Crawford & Miller, 1988(21)
p26	1.1	26 kDa	Early	Liu *et al*., 1986(22)
gp67	2.1	67 kDa	Late	Whitford et al., 1989(23)
25K	1.6	25 kDa	Late	Beames & Summers,1988(24)
6.9K	0.5	6.9 kDa	Late	Wilson *et al*., 1987(25
39K Capsid	2.2	39K	Late	Thiem & Miller, 1989(26)
10K	0.8	10 kDa	Very late	Kuzio *et al*., 1984(27
Ph	1.4	33 kDa	Very late	Iddekinge *et al*.,1983(1)

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Baculovirus Expression Vector (BEV)

The BEV is a helper independent DNA virus expression vector which replicates in insects or insect cells (28-30). Insect baculoviruses are reported mostly from insect arthropods, but a few are also known from non-insect arthropods (31,9).

Other than cloning and expression of a foreign gene in an invertebrate cell environment, the unique character of the BEV is due to the abundant expression achieved by correct insertion of a foreign gene under the transcriptional regulation of the baculovirus polyhedrin gene promoter. The particularly strong polyhedrin promoter is one major advantage in comparison with other higher eucaryotic expression systems.

Construction and Selection of a Recombinant Baculovirus (28,32,33)

The polyhedrin gene maps to the EcoR-I fragment (7.0 kbp) of the Ac*M*NPV genome. The gene was cloned and sequenced (1)(Figure 1). By standard mutagenesis techniques some of the N-terminal coding sequences for the gene were removed and artificial cloning linkers inserted (2). Constructs were made which allowed the fusion of N-terminal polyhedrin protein sequences to the foreign protein or for the production of non-fused recombinant proteins (3,29,32). Most of the foreign genes inserted are

either genomic or cDNA clones with highly variable numbers of non-translated 5' and 3' DNA sequences flanking the coding sequences and which contain their own translation initiation and termination codons (Table II). The BEV vectors also contain the non-translated 3' and polyadenylation sequences. A transfer vector containing the polyhedrin gene promoter and the foreign DNA sequence of choice are then mixed in the appropriate ratio with carefully purified Ac*M*NPV DNA and introduced into *Spodoptera frugiperda* Sf9 cells by standard transfection (28) or electroporation procedures (34). Standard plaque assays are then used to visualize the occlusion negative (⁻occ) plaques which should represent the recombinant baculovirus in which the hybrid gene has been substituted for the polyhedrin gene by homologous

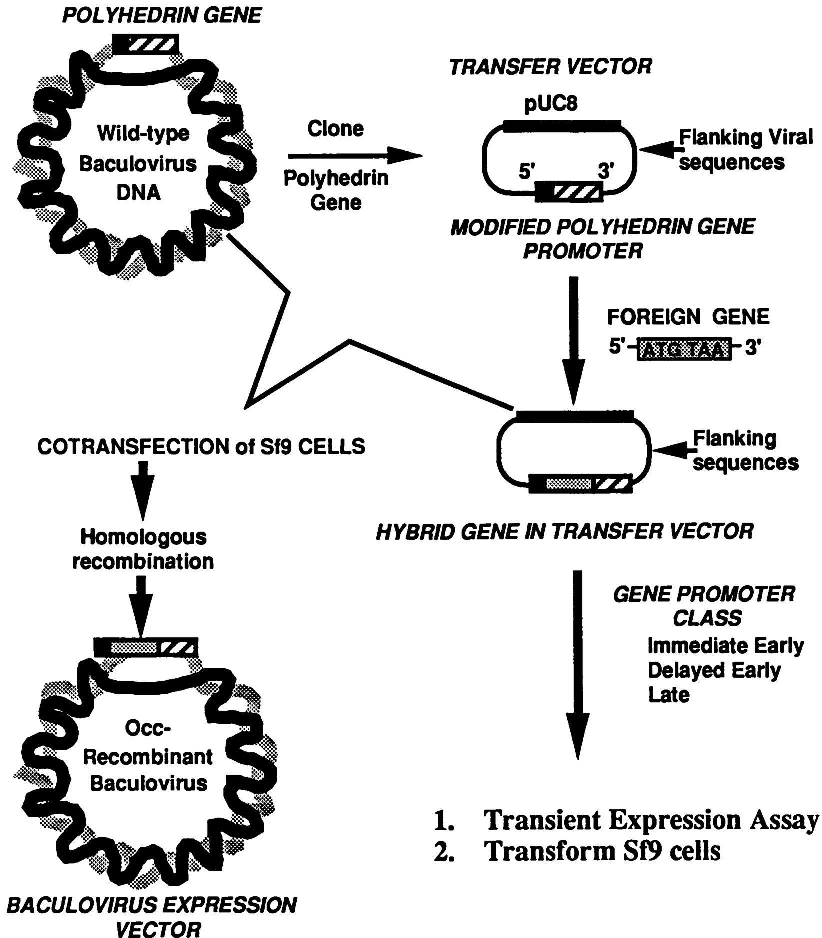

Figure 1. Helper-independent baculovirus vector (*Autographa californica* nuclear polyhedrosis virus).

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recombination of the approximately 2.5 kbp each of 5' and 3' DNA sequences flanking the hybrid gene construct. The recombination frequency should be between 0.1% and 5%. The recombinant virus is repeatedly plaque purified until no wild-type virus is present and adjusted to a high titer which, under optimal conditions, should be 8.5-9.0x10^6 pfu/ml. The position and orientation of the foreign gene should be verified both in the transfer vector and recombinant virus. Dependent on the properties of the recombinant protein a variety of screening or detection strategies are possible: plaque hybridization with RNA or DNA probes, detection with monoclonal or polyclonal antibodies, enzymatic assay or some other functional assay, or the use of new colormetric screening vectors (35-37).

The production and screening for a recombinant baculovirus should be accomplished in 4-6 weeks. However, the expression of a gene construct can be evaluated more quickly by transfection of Sf9 cells with the transfer vector and subsequent infection with wild-type virus. It has been our experience that sufficient expression can be obtained in order to evaluate recombinant protein size and processing. Alternatively, inserting a foreign gene under the transcriptional regulation of the IE-1 promoter and evaluation of expression in a transient expression assay will also allow rapid determination of the expression and properties of the recombinant protein or modified form thereof (38). Foreign gene expression can also be evaluated by either of these assays and should take no longer than 2-5 days (Figure 1).

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Limitations of Polyhedrin Directed Foreign Gene Expression

Polyhedrin expression can be variable dependent upon the cell line or insect tissue in which the virus replicates. Optimal replication of most lepidopteran baculoviruses usually occurs in fat body tissue, but fat body cell lines are not presently available. Optimal expression in cultured Sf9 cells is always attained in T-flasks. Care must be taken to avoid shear stress through routine handling or pipetting; the cells must be "healthy" and >97% viable. To avoid the effects of shear stress, Pluronic F68® is essential (39,40). Expression is significantly reduced under suspension culture conditions.

Constructs of the Polyhedrin Gene Promoter and Constructs Thereof Used for Foreign Gene Expression

The non-translated polyhedrin gene leader sequence is predominantly AT in composition and transcription initiation occurs in a TAAG motif highly conserved in late Ac*M*NPV genes (41-43). Vectors optimal for steady state recombinant mRNA synthesis retain the non-translated polyhedrin leader as intact to the ATG translation initiation codon. Some vectors extend into the ATG (YM1) or into the N-terminal DNA sequence of the polyhedrin gene with site-directed mutations to alter the ATG codon to an ATT (pVL941)(32). These vectors are optimal for foreign gene transcription and should produce steady state quantities similar to that of the polyhedrin mRNA in wild-type virus infected cells (44).

The ideal construct of the 5' end would insert the foreign gene ATG (+1) adjacent to the last nucleotide of the polyhedrin gene non-translated leader:

POLY- ACCTATAAAT–ATG FOREIGN GENE.
(-10 at the first A of ACCTATAAAT; -1 at the final T; +1 at the A of ATG)

Polyhedrin is abundantly expressed at levels of 500-1000 micrograms per milliliter of 1-3x10^6 infected cells and accumulates in the cell nucleus. Foreign genes

are considered poorly expressed at 1-5 ug/ml, some are expressed at 10-50 ug/ml and there are reports of levels of recombinant protein expression similar to that of polyhedrin. The largest insert of foreign DNA expressed was a 6.6kb cDNA for the poliovirus genome (45). The polyprotein for poliovirus was processed and assembled into non-infectious capsids. The largest recombinant protein expressed was the chronic myelongenous leukemia - associated P210 BCR-ABL oncogene protein (46). A partial list of all recombinant proteins expressed is given in Table II and includes genes from humans, other vertebrates, several RNA and DNA viruses, plants, invertebrates, and procaryotes.

Processing of Recombinant Proteins

Apparently the majority of protein processing pathways known to exist in higher eucaryotic cells also function in baculovirus infected cells. Some of these are listed in Table II. Caution is recommended in making such a recommendation since the predominance of data has been generated in only one cell line (Sf9, 2) in continuous culture for many years.The majority of the recombinant proteins exhibit biological or functional properties very similar to the authentic proteins.

Table II. Processing of Recombinant Proteins

		AMIDATION	
human pre-pro gastrin releasing peptide		no	Lebacq-Verheyden *et al.* 1988(47)
		GENE SPLICING	
silk moth chorion chromosomal genes		yes	Iatrou *et al.* 1989(48)
simian virus 40 small t antigen		yes	Jeang *et al.* 1987(49)
		yes	Lanford 1988(50)
simian virus 40 large t antigen		partial*	Lanford 1988(50)
		MYRISTYLATION	
hepatitis B virus surface antigen		yes	Lanford *et al.* 1989(51)
HIV p17		yes	Overton *et al.* 1989(52)
HIV p55gag		yes	Gheysen *et al.* 1989(53)
polyoma virus middle t antigen and pp60 (c-src)		yes	Piwnica-Worms *et al.* 1990(54)
		N GLYCOSYLATION	
Campoletis sonorensis polydnavirus	WHV2	yes	Blissard *et al.* 1989(55)
	WHV1	no	Blissard *et al.* 1989(55)
Drosophila Kr protein		no	Ollo & Maniatis 1987(56)
fowl plague influenza virus hemagglutinin		yes	Kuroda *et al.* 1989(57)
fowl plague virus hemagglutinin gene		yes	Kuroda *et al.* 1989(57)
" " " " "		yes	Kuroda *et al.* 1990(58)
hamster prion protein		partial*	Scott *et al.* 1988(59)
hepatitis B virus surface antigen		yes	Lanford *et al.* 1989(51)
herpes simplex virus type 1 glycoprotein D		yes	Krishna *et al.* 1989(60)
HIV envelope protein gp160		yes	Rusche *et al.* 1987(61)
" " " "		yes	Wells & Compans 1990(62)
human beta galactosidase		yes	Itoh *et al.* 1990(63)
human CD4		partial*	Webb *et al.* 1989(64)
human complement C1r proenzyme		yes	Gal *et al.* 1989(65)

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Table II. (Con't)

	N GLYCOSYLATION (Con't)	
human EGF receptor	yes	Greenfield *et al.* 1988(66)
" " "		Greenfield et al. 1987(67)
human erythropoietin	yes	Wojchowski *et al.* 1987(68)
" "		Quelle *et al.* 1989(69)
human glucocerebrosidase	yes	Martin *et al.* 1988(70)
human interleukin 2	no	Smith *et al.* 1985(71)
human hIR extracellular ligand binding protein	yes	Sissom & Ellis 1989(72)
human nerve growth factor receptor	no	Vissavajjhala & Ross 1990(73)
human respiratory syncytial virus F and G glycoproteins	yes	Wathen *et al.* 1989(74)
human T-lymphocyte surface antigen	yes	Richardson *et al.* 1988(75)
human tissue plasminogen activator	yes	Jarvis *et al.* 1989(6)
human tissue-type plasminogen activator	yes	Steiner *et al.* 1988(76)
infectious hematopoietic necrosis virus glycoprotein	yes	Koener & Leong 1990(77)
fowl plague influenza virus hemagglutinin	yes	Kuroda *et al.* 1989(57)
" " " " "		Kuroda *et al.* 1990(58)
Japanese encephalitis virus envelop protein	yes	Matsuura *et al.* 1989(78)
measles virus H and F protein	yes	Vialard *et al.* 1990(79)
mouse entactin	no	Tsao *et al.* 1990(80)
mouse interleukin 3	yes	Miyajima *et al.* 1987(81)
murine coronavirus JHM E2 peplomer glycoprotein	yes	Yoden *et al.* 1989(82)
murine immunoglobulin heterodimer	yes	Hasemann *et al.* 1990(83)
newcastle disease virus hemagglutinin-neuroaminidase	yes	Nagy *et al.* 1990(84)
parainfluenza Type 3 virus hemagglutinin-neuroaminidase glycoprotein	yes	Van Wyke Coelingh *et al.* 1987(85)
phaseolus bulgaris B-phaseolin	yes	Bustos *et al.* 1988(86)
pseudorabies virus gp50	yes	Thomsen *et al.* 1990(87)
rabies virus glycoprotein G	yes	Prehaud *et al.* 1989(88)
ricin ß-chain	yes	Piatak *et al.* 1988(89)
simian virus 40 large and small T antigen	yes	Lanford 1988(50)
sindbis virus E1	yes	Oker-Blom & Summers 1989(90)
vesicular stomatitis virus glycoprotein	yes	Bailey *et al.* 1989(91)

*Partial = both glycosylated and nonglycosylated forms were produced.

	O GLYCOSYLATION	
Drosophila Kr protein	no	Ollo & Maniatis 1987(56)
human hIR extracellular ligand-binding protein	no	Sissom & Ellis 1989(72)
human nerve growth factor receptor	no	Vissavajjhala & Ross 1990(73)
human respiratory syncytial virus F and G glycoproteins	no?*	Wathen *et al.* 1989(74)
pseudorabies virus gp50	yes	Thomsen *et al.* 1990(87)

* It is unclear to what extent O-linked glycosylation occurred.

Continued on next page.

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Table II. (Con't)

	PALMITOYLATION	
ha-ras p21 protein(soluble)	no	Page *et al.* 1989(92)
(membrane associated)	yes	Page *et al.* 1989(92)
simian virus 40 T antigen	yes	Lanford 1988(50)
	PHORBOL ESTER BINDING	
protein kinase C-y	yes	Patel & Stabel 1989(93)
	PHOSPHORYLATION	
chronic myelongenous leukemia-associated P210 BCR-ABL	yes	Pendergast *et al.* 1989(94)
Drosophila Kr protein	yes	Ollo & Maniatis 1987(56)
hepatitis B virus core and precore antigens	yes	Lanford & Notvall 1990(95)
human c-fos protein	yes	Tratner *et al.* 1990(96)
human c-myc protein	yes	Miyamoto *et al.* 1985(97)
human EGF receptor	yes	Greenfield *et al.* 1988(66)
human epidermal growth factor receptor (tyrosine kinase domain)	yes	Wedegaertner & Gill 1989(98)
human immunodeficiency virus p24	yes	Overton *et al.* 1989(52)
human immunodeficiency virus tat protein	no?*	Jeang *et al.* 1988(99)
human insulin receptor ß-subunit	yes	Herrera *et al.* 1988(100)
human insulin receptor - tyrosine kinase domain	yes	Ellis *et al.* 1988(101)
human multidrug resistance 1	yes	Germann *et al.* 1990(102)
human papillomavirus Type 18 E6 protein	no?*	Grossman *et al.* 1989(103)
human T cell leukemia virus type 1 trans-activator protein p40	yes	Nyunoya *et al.* 1988(104)
" " " " "	yes	Jeang *et al.* 1987(49)
human terminal transferase	yes	Chang *et al.* 1988(105)
maize transposable element Ac protein	yes	Hauser *et al.* 1988(106)
mouse p53	yes	O'Reilly & Miller 1988(107)
murine platelet derived growth factor receptor	yes	Morrison *et al.* 1989(108)
polyoma virus middle T antigen	yes	Forstova *et al.* 1989(109)
polyoma virus middle t antigen and pp60 (c-src)	yes	Piwnica-Worms *et al.* 1990(54)
protein kinase C-y	yes	Patel & Stabel 1989(93)
simian virus 40 large T antigen	yes	Lanford 1988(50)
" " " " " "	yes	O'Reilly & Miller 1988(107)
simian rotavirus NS26	yes	Welch *et al.* 1989(110)
Xenopus serine-specific protein kinase pp90-rsk	yes	Vik *et al.* 1990(111)

* It is unclear whether the authentic protein is phosphorylated.

	PROTEIN SECRETION	
bovine coronavirus hemagglutinin-esterase (truncated form)	yes	Parker *et al.* 1990(112)

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Table II. (Con't)

PROTEIN SECRETION (Con't)		
Campoletis sonorensis polydnavirus		
WHV2	yes	Blissard *et al.* 1989(55)
WHV1	yes	Blissard *et al.* 1989(55)
hematopoietic necrosis virus glycoprotein	no	Koener & Leong 1990(77)
hepatitis B virus envelope protein		
M protein	yes	Price *et al.* 1988(113)
S protein	limited	Price *et al.* 1988(113)
hepatitis B virus surface antigen	yes	Kang *et al.* 1987(114)
hepatitis B virus surface antigen	no	Lanford *et al.* 1989(51)
hepatitis V virus core and precore antigens	yes	Lanford & Notvall 1990(95)
HIV-1 gp160	yes	Wells & Compans 1990(62)
HIV-1 Pr55gag	yes	Gheysen *et al.* 1989(53)
human B-galactosidase	yes	Itoh *et al.* 1990(63)
human B-interferon	yes	Smith *et al.* 1983(2)
human complement component C1r	yes	Gal *et al.* 1989(65)
human erythropoietin	yes	Wojchowski *et al.* 1987(68)
human gastrin releasing peptide precursor	yes	Lebacq-Verheyden *et al.* 1988(47)
human glucocerebrosidase	partial	Martin *et al.* 1988(70)
human hIR extracellular ligand-binding protein	yes	Sissom & Ellis 1989(72)
human hst-1 transforming protein	yes	Miyagawa *et al.* 1988(115)
human interleukin 2	yes	Smith *et al.* 1985(71)
human nerve growth factor receptor	yes	Vissavajjhala & Ross 1990(73)
human plasminogen	yes	Whitefleet-Smith *et al.* 1989(116)
human respiratory syncytial virus F and G glycoproteins	yes	Wathen *et al.* 1989(74)
human T11 T-lymphocyte surface glycoprotein	yes	Richardson *et al.* 1988(75)
human tissue plasminogen activator	partial	Jarvis *et al.* 1989(6)
Hyalophora cecropia attacin	yes	Gunne *et al.* 1990(117)
mouse interleukin-3	yes	Miyajima *et al.* 1987(81)
newcastle disease virus hemagglutinin-neuroaminidase	yes	Nagy *et al.* 1990(84)
potato tuber protein-patatin	yes	Andrews *et al.* 1988(118)
pseudorabies virus gp50	yes	Thomsen *et al.* 1990(87)
ricin ß-chain	yes	Piatak *et al.* 1988(89)
simian sarcoma virus PDG7-ß protein	partial	Giese *et al.* 1989(119)
urokinase type plasminogen activator	yes	Devlin *et al.* 1989(120)

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Multiple Promoter Constructs

Because the transcriptional promoters for polyhedrin and p10(121-124) have been functionally mapped, it is possible to engineer these promoters to express a coding DNA sequence and position the constructs in non-essential locations of the viral genome. With multiple promoters positioned in non-essential regions it is possible to express several copies of the same, or different, gene(s) either to enhance production of a selected recombinant protein or to produce homo-oligomeric or hetero-oligomeric assemblies of proteins (35,125,126)(Table II). Theoretically, this can be done with baculovirus gene promoters representing any class of genes (Table I).

If there are potential limitations for expressing a foreign gene with the very late and stringently regulated promoters of polyhedrin and/or p10, the IE1 gene promoter or the 39K gene promoter can be inserted into non-essential locations to express a gene product immediately upon viral entry to the susceptible cell (IE1) or after 2 hours infection (39K). Both genes express good levels of steady state mRNAs continuously through 24-36 hours post infection (15-17,38).

Summary and Discussion

Kirschbaum (127) summarizes a well-known theme in the areas of applied insect pathology relative to the historical progress of this discipline: "Although the pathogens or microbial products currently used to control insects (i.e. biological insecticides) offer certain advantages over chemical insecticides, they also have certain disadvantages that have limited their application." The disadvantages are primarily a limited host range and the fact that microbial agents are biological agents subject to many biological and physical factors which can alter virulence or infectivity therefore resulting in variable insecticidal effects when applied for such purposes. However, the growing social and scientific awareness of health hazards posed by the use of artificial chemicals for crop protection and public health will result in the limited and restricted use of such chemicals in the near future. As a result, naturally occurring microbial agents which are genetically engineered provide new opportunities for crop protection by providing environmentally safe pest control approaches which may now be more desirable than chemicals. Also, the several insect pathogenic viruses, bacteria and fungi that have been tested by Environmental Protection Agency safety testing protocols now provide another significant advantage: the majority of these insect pathogens which occur naturally in nature have a highly restricted host range and are not pathogenic for non-target organisms (7,128). If the factors which control host range are not altered through the genetic engineering of an insect pathogenic agent, then the insecticidal agent will be targeted and expressed only in the target host(s) which is susceptible to the pathogen. If this major advantage can be coupled with environmentally safe genetic engineering strategies, it will be possible to improve upon some of the major disadvantages which have limited effective use of insect pathogenic viruses for crop protection or public health.

Another consideration in genetic engineering strategies would be to express insect specific factors or to identify metabolic or developmental targets that are specific to pest insect biochemistry, metabolism and development systems. Insects and other invertebrates provide a large diversity of species (129) representing the greatest degree of evolutionary relationships with plants, other insects and animals, and pathogens. There should be a plethora of insect-specific mechanisms to target for insecticidal effects: we are only limited by our lack of knowledge of the molecular biology, biochemistry and physiology of invertebrate systems. Recent reports do emphasize the use of synthetic insect specific toxin genes (30,130), cloned juvenile esterase (131) or insect neuropeptides (132,133) as preliminary excursions into testing such approaches.

The development and use of a viral cloning and expression vector which functions in insects and the discovery of baculovirus gene promoters (IE1) which will express genes in non-infected insect cells and immediately upon entry into the insect (Jarvis *et al.*, 1990 submitted) provides at least two rapid and efficient entry level tools for the cloning and study of genes of agricultural and medical value. As new genes from invertebrates are identified and cloned, they can be amplified and studied with these vectors. Recombinant DNA techniques can be used to rapidly study the properties or functions of these gene products and, therefore, assess any practical

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characteristic of medical or agricultural application. Alternatively, peptides or proteins may be designed and delivered by such systems for temporal, developmental or tissue specific expression or targeted to a specific cellular location.

The study of the molecular biology of invertebrate systems is quite rich in opportunity. Insects and other invertebrates serve in a central capacity or role as vectors or reservoirs of plant and animal disease agents and are themselves susceptible to numerous pathogens. The diversity of species that have evolved in a comparable diversity of environmental situations has generated this vast array of relationships, many of which are essential linkages in a disease cycle or fundamental to crop protection.

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