WESTERN REGIONAL PROJECT PROPOSAL

BIORATIONAL METHODS FOR INSECT PEST MANAGEMENT (IPM): BIOORGANIC AND MOLECULAR APPROACHES

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TITLE: BIORATIONAL METHODS FOR INSECT PEST MANAGEMENT (IPM): BIOORGANIC AND MOLECULAR APPROACHES

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STATEMENT OF THE PROBLEM:

As the millennium closes and the global economy becomes a reality, the U.S. faces compelling challenges in the production of food, fiber, and raw wood products. Rapid worldwide population growth coupled with urban expansion and societal concerns about agricultural and forested land use patterns have resulted in an increased demand for food, fiber, and timber from a shrinking landbase. Until very recently, many of our concerns for increased production were simply met by increasing synthetic inputs such as pesticides and fertilizers into agricultural and forest ecosystems. This led to cosmetically appealing, abundant, and high quality food and fiber sources. However, the myriad problems caused by emphasis on pest control based on programmatic use of toxic chemicals versus pest management based on monitoring, thresholds, and judicious use of treatments are all too familiar to humankind. In only a few decades, these practices have resulted in contamination of water supplies, biomagnification of toxins through the food chain, and a host of human health-related concerns. Primary among these is food safety for children, who are endangered by short-term and chronic exposure to the insecticidal agents or their metabolites. As a result, the availability of these management tools is continuously decreasing due to loss of efficacy, increasingly stringent regulatory requirements, and growing concern about real and perceived risks to societal well being. In many cases only one or a few chemical insecticides may be registered and efficacious for the control of a given insect pest. Yet, consumers retain very high expectations for both the quality and availability of food and fiber, making real world pest management a very different reality for current and future generations. Without these agents to rely on we must seek to use every benign tool available to manage these pests with increased attention to the stewardship of our environment. The goal of this project is to identify and utilize semiochemicals, bioregulatory molecules, and highly-specific plant and microbial metabolites as major components in the integrated management of insect pest species.

JUSTIFICATION:

The use of chemical pesticides in agriculture has generated an abundance and quality of food and fiber unequalled in the history of mankind. Despite strong current advocacy of low tillage sustainable agricultural practices, the universal expectation of cheap, plentiful agricultural products and freedom from disease eliminates the possibility of returning to more pastoral, noninterventional agricultural methods. Effective, economical control of pest insects for agricultural and public health protection is one of the cornerstones of

modern life in developed countries.

With our current pesticides, the efficacy, ease of use, and incompatibility with other pest control strategies discouraged the active development of alternative methods of insect control until recently. This reliance on a single crop protection strategy has rendered many crops critically vulnerable to both deregistration of the pesticides currently registered for use on a given crop, and to the development of resistance in populations of key pest species. The development of resistance has been exacerbated by the widespread misuse of pesticide; continuous pressure due to spraying on a calendar schedule whether it was needed or not, the spraying of "cocktails" of several pesticides at once, and the reluctance to rotate pesticides to preserve their field efficacy. These practices have accelerated the development of cross-resistance to a broad spectrum of pesticides with different modes of action. It is widely recognized that continued development of chemical pesticides with the same modes of action or the same receptor target as pesticides in current use is a case of diminishing returns; the effective lifetime of toxin compounds which are merely variations on current pesticides is increasingly brief. The development of juvenile hormone (JH) analogs, an ecdysone agonist or "molt accelerating chemical and plants bioengineered with *B. t.* toxins are prime examples of new pesticides with novel modes of action. However, the JH analogs are only effective against those insects where adults are the problem, the ecdysone agonists are still unproven, and insects are all but certain to develop resistance to *B. t.* toxins, thus requiring a continuing process to develop novel approaches to insect control.

The goal of this project is to develop new and effective pest management tactics based on the chemistry, biology, and biochemistry of insects and plants. As an added benefit, these tactics will generally be mutually compatible with and complementary to other environmentally sound methods of insect control, for example the use of biological control agents such as parasitic and predatory insects. This is of crucial importance because future pest control efforts will of necessity be characterized by the integration of several control strategies, rather than total reliance on a single strategy as is often the case with current programs based on insecticide use.

RELATED CURRENT AND PREVIOUS WORK

Objective 1. SEMIOCHEMICAL SYSTEMS

Chemical signals are used by insects to orient, survive and reproduce in their specific environments. This reliance on chemical cues is evolutionarily linked to the development of chemosensory organs and cells early in the history of arthropods, probably even before the development of light sensitive organs (Snodgrass, 1926), and offers a number of opportunities for insect control. Natural chemicals which transfer information between organisms are classified generally as semiochemicals.

When used for intraspecific communication, these chemicals are called pheromones. When used at the interspecific level, they are called allelochemicals, which can be partitioned by cost-benefit analysis as kairomones when the species responding to the chemical message benefits, allomones when the species producing the chemical message benefits, and synomones when both benefit. These classes often overlap and the same compounds can serve both intra- and interspecific functions. In order to understand and manipulate the various semiochemicals to maximize their usefulness in integrated pest management, research is needed on all aspects of these molecular communication systems. This includes the initiation and regulation of biosynthesis as well as transmission, perception, molecular transduction, and integration of the information imparted by these components to responding organisms.

Semiochemicals are used as increasingly important components of integrated pest management strategies for a growing number of insect species (Arn and Lewis, 1997; Borden, 1997; Minks, 1997; Sanders, 1997; Staten et al., 1997). For example, sex pheromones, an intensively studied class of semiochemicals, are used at several levels in insect pest management (Howse et al., 1998). Pheromones are used in traps to monitor changes in population levels. This monitoring allows pest managers to time insect control measures accurately and effectively to coincide with population increases near the economic threshold. Highly sensitive pheromone-based monitoring is also crucial for detection of incipient infestations of introduced or exotic insects, such as the Mediterranean and Mexican fruit flies (Heath et al., 1997) or for detection of incipient infestations of introduced pests with constantly expanding ranges, such as the gypsy moth (Sharov et al., 1997) or the pink bollworm (Baker et al., 1990).

Secondly, sex pheromones are used as treatments to reduce pest populations through mating disruption, or sexual confusion, by inundating a crop area with pheromone so that males cannot locate females, thus preventing reproduction. This strategy has proven to be highly effective with insects such as the oriental fruit moth (Rice and Kirsch, 1990), the pink bollworm (Staten et al., 1997), and the diamondback moth (McLaughlin et al., 1994), to name but a few. Thirdly, insect sex and aggregation pheromones and other attractants have been commercialized for mass trapping of several insect species, such as bark beetles (Borden 1990) and stored product pests (Burkholder, 1990). Recent work has also demonstrated that semiochemically based mass trapping will work effectively as a treatment against other pest species, such as nitidulid beetles in Australia (James et al., 1996). Interruptive or antagonistic pheromones to prevent colonization of crops or commodities are also being explored, and this is an active area of applied research with bark beetles (Borden, 1997).

The early enthusiasm that centered on semiochemicals as magic bullets for the management of all insect populations has been appropriately attenuated, but there is a strong consensus that basic

research into the chemistry, biosynthesis, regulation, binding and degradation of semiochemicals will play an increasingly important role in future insect management programs (Howse, 1998). Today, pheromone components and the semiochemically-induced behavioral responses for over 1,500 insect species have been described (Voerman, 1988; Arn et al., 1992), and there is a growing awareness of the complex interaction between host plants and pheromone production in insects (Landolt and Phillips, 1997). Many of the successful early studies in this field are the result of collaborations between biologists and chemists to elucidate the structures of the chemical cues. Only in the last two decades have biochemists, and more recently, molecular biologists, combined efforts with chemical ecologists to attempt to more clearly understand the fundamentals of insect pheromone biosynthesis, regulation and degradation (Prestwich and Blomquist, 1987; Cardé and Minks, 1997; Tillman et al.,1999).

Another rapidly expanding focus of semiochemical research is the identification and function of semiochemicals that attract beneficial insects to their hosts. Our knowledge of beneficial insect pheromones is still rudimentary, with only a few pheromones indicated for a plethora of parasitoids and predators. However, both kairomones (Mendel et al., 1995, Feener et al., 1996, Arakaki et al., 1997) and synomones (De Moraes et al., 1998, Rose et al., 1998) are now well known to be used by parasites and predators to locate hosts. These compounds show considerable promise in the mass rearing and entrainment, and manipulation of beneficial insects (Tumlinson, 1993).

From 1994 through 1999, the multistate research project format has expedited the development of knowledge regarding semiochemically based pest management tactics. One of the major focal points of research by project collaborators has been to develop an understanding of the mechanisms by which semiochemicals are produced, regulated, and perceived, and how the semiochemical message is translated into a behavioral response. One area of dramatic progress that has benefitted from project collaborative ties has been the elucidation of the molecular regulation and *de novo* synthesis of pheromones in bark beetles (Seybold et al., 1995; Tillman et al., 1998; Tittiger et al., 1999). Additional progress was made on bark beetle cuticular hydrocarbons, which may have close-range pheromonal activity (Page et al., 1997). This work involved collaboration between Page (USDA Forest Service), Seybold, and Blomquist. Furthermore, Paine et al. (1999) reported the identification of semiochemicals associated with the Jeffrey pine beetle, *Dendroctonus jeffreyi*, a bark beetle that kills pines in the Sierra Nevada. This work was carried out at Riverside by Millar (CA) with collaboration with Seybold and Blomquist, who are proceeding with the study of the regulation and biosynthesis of several of the pheromone components (1-heptanol and frontalin).

Semiochemical research often focuses on the identification or synthesis of a new semiochemical, or determination of the specificity of a semiochemical blend, but few research groups have had the expertise or

resources to carry a semiochemical project through all its various stages. We will proceed from initial identification and synthesis of semiochemicals through large-scale field tests. We will also research effective technology for dissemination of semiochemicals for commercialization, and will have a particular focus on the utilization of semiochemical interactions across trophic levels. The collaborating scientists in this project, with diverse backgrounds and expertise, and with a wide variety of resources (from analytical instruments to access to field plots and field stations, collaborations with state extension service specialists, etc.) will facilitate carrying projects through from inception to application.

In short, the work described in this section is designed to take advantage of collaborative work on the analytical chemistry, biology, chemical ecology, physiology, neurophysiology, biochemistry, and molecular biology of semiochemicals in selected insect pests in order to elucidate, develop, and implement new applications for insect management.

Objective 2: PHYTOCHEMICALS

Phytopagous insects account for roughly half of the total number of insect species (Brattsten and Ahmad, 1986). Plants are the primary producer of food for both livestock and humans. Therefore, phytophagous insects are often in direct competition with human food and fiber production. A historical overview of insecticide development opines that, at present, there is a “renaissance of integrating chemicals and biologicals for sustainable pest control with human safety” (Casida and Quistad, 1998). In contrast to the limited number of artificial pest control agents, plants have produced a wide variety of secondary metabolites to target the large number of insect species (Rosenthal and Janzen, 1979; Whittaker and Feeny, 1971). The mechanisms of many of these are poorly understood. Identification of such phytochemicals, and understanding their mechanisms at the molecular level, will provide the inspiration, and the knowledge, for engineering novel and more effective pest control agents. Better chemicals for integrated pest management are desirable for at least three important reasons: (1) A more effective response to growing pesticide resistance in insects. (2) better restriction of the toxicity of applied chemicals to the target species, and (3) chemicals to be synthesized by transgenic plants that will inhibit insect predation while maintaining flavor and nutrient values for humans.

Phytochemicals have been employed for insect control for hundreds of years (Jacobson & Crosby, 1977). These have been overtly toxic compounds. Such toxic effects include irritation, inhibition and disruption of sensory organs, muscle contractility, digestive processes, active transport, water balance, intermediary metabolism, and neurotransmission (Gershenzon & Croteau, 1992). However, it is now recognized that plants are quite sophisticated and utilize compounds with low intrinsic toxicity to defend against insect attack. (Roitberg

& Isman, 1992; Rosenthal & Berenbaum, 1991; Tallamy and Raup 1991). Such compounds are diverse in both chemistry and mode of action. The action is often subtle. Plants lessen the feeding damage by modification of insect behaviors that include, but are not limited to, host selection, feeding, oviposition, development, reproduction, and pheromonal and kairomonal communications (Lewis and Tumlinson, 1988; Maxwell & Jennings, 1980; Price *et al*., 1980). Phytochemicals affect multiple crucial aspects of insect fitness, for example, the probability of survival to reproductive age, development time, and fertility (Istock, 1981; Slansky and Scriber, 1985; Scriber and Ayers, 1988). Population dynamics are also modulated by phytochemicals (Raffa & Berryman, 1987). It is clear that any one plant may produce multiple compounds, each directed to different targets on the offending insect. It is our intention to develop the understandings to employ such a multivariant approach in a rational strategy for integrated pest management.

Plant phenolics play important roles in protection against environmental stresses (e.g., herbivory, infection, UV radiation). The role of phenolics in plant defense against herbivores has been a particularly intense area of study and has been the basis of several plant defense theories (Appel,1993). The toxicity of many phenolics from simple phenolic acids to complex polyphenols has been attributed to their ability to function as prooxidants (Appel, 1993; Summers and Felton, 1994).

The rate-limiting step in the synthesis of most phenolics is dependent upon the enzyme phenylalanine ammonia lyase (PAL, EC 4.3.1.5) which catalyzes the deamination of phenylalanine to form cinnamic acid. The ability to produce transgenic plants with selective modification of PAL activity has provided a powerful tool to assess the in planta function of phenolics. Recent findings indicate that overexpression of PAL activity may not provide effective antiherbivore defense and may actually suppress the plant's ability to mount induced responses to herbivory (Bi et al., 1997; Felton et al., 1999). Further research on the role of plant phenolics as defenses against herbivores is needed.

Although ryanodine was originally discovered in a screen for insecticides, most of the work on the biological effects of ryanodine have been conducted in vertebrates. The ryanoids are known to be powerful modulators of calcium ion metabolism, and thus represent an entre into a novel aspect of insect control. The pharmacology and physiology of the ryanodine receptor have been reviewed (Sutko *et al*., 1997; Sitsapesan and Williams, 1998) Ryanoids themselves have seen limited (but effective) use as pesticides, at least in part due to the limited distribution in plant species and the complexity of the structure. However, because they are environmentally friendly pesticides and because they can potentially open an independent mode of pest control, the biochemistry of the ryanoids deserves far more attention. The ryanodine receptor (RyR, also called the calcium-induced calcium release channel, CICR) is the historically accepted target of

ryanodine. Recent research indicates that the full implications of the ryanodine receptor for signaling in insect muscle cells remain to be determined (Hinton, 1998). However, the discovery of multiple ryanodine receptor isoforms and the documentation of the presence of ryanodine receptors in most cell types (all muscle types, neurons and non-excitable tissues) indicates a role for the ryanodine receptor that extends far beyond muscle contraction. To this point, it has been suggested that some ryanoids (*e.g.*., ryanodol) may act at a site different from the classic ryanodine receptor (Lemberg and Casida, 1994). For example, a member of the ryanodane diterpene family has antifeedant activity (Gonzalez-Coloma *et al*., 1996).

Computational techniques have had an enormous impact on both basic research and product development. The introduction of comparative molecular field analysis (CoMFA, Cramer *et al*., 1988) and similar three dimensional quantitative structure activity relationship (3D-QSAR) techniques have provided researchers with robust analytical tools with a high degree of predictive accuracy. The general area of QSAR has been the subject of many reviews and monographs (*e.g.*, Hansch and Leo, 1995). These techniques have been applied to ryanoids (*e.g.,* Welch, 1998). An understanding of the mechanism by which ryanoids modulate intracellular calcium metabolism can lead to development of a powerful new tool for integrated pest management. Using QSAR it is possible to identify the molecular determinants for receptor-ligand recognition and for modulation of calcium channel function. Such insights can be applied in at least to ways: (A) genetic manipulation of plants to produce ryanoids with desired biological properties and (B) engineering of ryanomimetics with a structure suitable for economic synthesis yet with specificity for the target.

Objective 3. <u>TRANSGENIC PRODUCTS</u>

A recent cover article in Chemical and Engineering News, titled “Transforming Agriculture”, described how transgenic crops are fundamentally altering agriculture and agrochemical industries (Thayer, 1999). Examples given in this article are that half of the 72 million acres of soybeans planted in the US this year will be sown with seeds genetically engineered to be resistant to herbicides. Last year about 19.5 million acres were planted with crops genetically engineered to be insect resistant, primarily due to the production of the toxin of *Bacillus thuringiensis* by the plants. Five years ago when we wrote our initial proposal only field trials of genetically engineered plants were under way!

There can be enormous advantages to insect control when the insecticidal material is produced by the plant. For example, corn borers are very hard to control with conventional insecticides because once the borer has gotten inside the plant, it cannot be contacted by sprayed insecticides. A similar situation exists with cotton bollworms. Other benefits to use of plants genetically modified to produce insect toxins is that use of wide-spectrum insecticides will be greatly reduced,

allowing build-up of natural predators of insect pests. Because of their size and properties, the research technologies needed to identify and implement gene products as plant protection agents are appreciably different from those used for synthetic or natural chemical pesticides (Bowers 1991).

New opportunities for intervening in pest-crop interactions result from our ability to manipulate macromolecules that play important roles in both the normal physiology of higher organisms and in the frequently 'aggressive' interactions occurring in host/predator, pest/parasite and host/disease associations. A number of intervention opportunities can be explored, such as incorporation of insect-selective proteinaceous toxins derived from other organisms into plants, or interference with the ability of pest insects to sustain physiological homeostasis, especially when the insects are subject to external stress. Genetic engineering methods now provide great impetus to the generalized agronomic use of gene products, because these methods allow bioactive gene products to be generated *in situ,* where they can act to increase crop resistance or decrease pest fitness. Several commercial crops engineered with specific *B.t.* genes directly toxic to Lepidoptera or Coleoptera are now in widespread commercial use. However, these innovations represent only a tiny fraction of the potentially useful plant gene products. Exposure of insect populations to a constant selection pressure of *B.t.* toxin is just as likely to cause emergence of resistant strains as daily sprays of insecticide, so development of crops engineered with different types of toxins or other physiologically potent gene products, is necessary. Some alternatives to *B.t.* are only recently discovered, and others await sequence determination, and/or explored for their effectiveness as crop protection agents. Accordingly, one thrust of this objective is to expand the scope of evaluation and exploitation of known and new peptide sequences obtained from a wide variety of biological sources. Another thrust of this objective is developing rapid receptor-ligand assays for hormones affecting fluid homeostasis in lepidopteran pests; these assays will allow high-throughput screening to identify low molecular weight compounds which mimic or antagonize the effects of the natural hormones.

Gene product studies will focus on several major areas. First, it has been appreciated for many years that natural developmental or metabolic hormones of insect pests can provide lead compounds for insect-specific control agents such as insect growth regulators (Kiussi 1992; Menn and Borkovec 1990), a number of which are now produced commercially. Other types of insect hormones such as the neuropeptide hormones regulating the lepidopteran corpora allata (source of the developmentally crucial juvenile hormone) (Kramer et al. 1993, Kataoka et al. 1989), and insect diuretic hormones, have also come under intensive study over the last decade (Schooley 1991). Diuretic hormones are particularly attractive as lead compounds for developing insect control tactics because they are also known to be important mediators of some classical insecticides (Maddrell and Casida 1971). Indeed, control of diuresis in insects is complex, as three different classes of peptide have been characterized that act through different second messengers:

The CRF-like DH act through cyclic AMP (Coast *et al.*, 1991), the myokinins act via elevation of intracellular Ca^{2+} (Coast, 1996; O'Donnell *et al.*, 1996) (presumably through the phosphoinositide pathway), and CAP2b acts through cyclic GMP (Davies *et al.*, 1995).

Investigations of insect viral toxicity enhancement strategies have also proceeded with several transgenic viruses having undergone field testing. These viruses are generally family or genus specific. Successful approaches include viral expression of insect hormones (Maeda 1993), insect-specific neurotoxins (McCutchen et al. 1991), or insect enzymes (Hammock et al. 1990). Recombinant viruses expressing toxins and JH esterase increase the speed of kill of the viruses and appear to be innocuous to beneficial insects (Hammock et al. 1993). This general area involving development of genetically modified pathogens for insect control, with enhanced toxicity to specific insects, a clearly defined activity spectrum, and no deleterious effects to any type of nontarget organisms, holds tremendous promise. However, studies to date with insect-specific neurotoxins have utilized only a couple of toxins described much earlier in the literature. What appears to be needed to improve the viability of this approach are other toxins from venoms with greater insecticidal activity and better selectivity in terms of vertebrate toxicity. Further study of such toxins would be expedited by collaborations within this project.

CRIS SEARCH

In summary, what makes this project unique is that it pulls together a diverse group of scientists to focus on the common goal of developing safe and sustainable methods of insect control from natural sources. A survey of the CRIS system using general keywords such as "natural products, phytochemical, transgenic, and semiochemical" and more specific keywords such as "phenolic, terpenoid, coumarin, and sex pheromone" came up with a number of Hatch projects and Regional Research projects or Coordinating Committees with potential overlaps of effort. A closer look at the titles, the projects and the scientists performing the work showed that essentially no overlap exists with the proposed work.

In summary, a detailed search of the CRIS system revealed no overlap of subject material or duplication of effort between any existing project or coordinating committee with the subjects and proposed work in this project proposal.

OBJECTIVES

Objective 1: To identify and understand the origin and perception of insect semiochemical systems with the goal of applying this knowledge to insect management.

Objective 2: To identify and understand the physiological mode of action of plant metabolites for development of novel biorational methods of insect management.

Objective 3: To develop insect regulatory peptides and proteins, including various enzymes and insect-specific toxins as novel agents for plant protection.

PROCEDURES

OBJECTIVE 1: To identify and understand the origin and perception of insect semiochemical systems with the goal of applying this knowledge to insect management.

This multistate project will be the focal point for collaborative studies aimed at gaining an integrated understanding of the role and nature of semiochemicals in target pest insects. One semiochemical system that has been intensively investigated during the previous performance period (Seybold et al., 1995; Ivarsson et al., 1997, 1998; Page et al., 1997; Tillman et al., 1998, 1999; Tittiger et al., 1999) is that of pine bark beetles, which kill trees that occur both in production-oriented forests and in the urban-wildland interface. Seybold (MN), Blomquist (NV) and Tittiger (NV) will continue their productive collaborative studies into the next performance period on *de novo* aggregation pheromone biosynthesis in the two economically important genera of bark beetles, Ips and Dendroctonus. The research will focus on the intermediates in the biosynthetic pathways, the enzymes that catalyze the biosynthetic reactions, the molecular-level regulation of these enzymes, and the site of synthesis. This work will involve analysis of the metabolic fate of radiolabeled pheromone precursors, isolation and identification of unique intermediates, northern blot analysis of transcripts for uniquely regulated genes in the pathways, enzyme assays of proteins expressed from isolated genes, identification of regulatory regions in the isolated genes, and *in situ* hybridization techniques with nucleic acids and antibodies to localize the site of pheromone synthesis. The formal addition of Tittiger (NV) and his experience and expertise in molecular techniques to this project will insure rapid progress in the molecular aspects of this work. He is leading a group that will identify the controlling elements of JH regulated genes in an attempt to better understand the mechanism of action of JH and to isolate, sequence and model the prenyl transferases involved in producing monoterpenoid pheromones in insects. Most of the studies will involve the classical, mevalonate-based isoprenoid pathway, and the interaction of juvenile hormone (JH) and perhaps as yet unidentified hormonal peptides as regulators of this pathway. The latter endocrinological studies will be pursued using decapitation, implantation, and hormonal rescue techniques. Cuticle wax hydrocarbons of bark beetles represent a relatively unstudied source of semiochemicals and Page (USDA Forest Service) and Seybold (MN) plan to initiate studies of proximal behavioral responses of *Ips* spp. to hydrocarbons.

A second collaborative project involving isoprenoid pheromone biosynthesis concerns the sesquiterpene sex pheromones of stink bugs (Hemiptera). Due to increasing use of highly specific insecticides, these insects are increasingly recognized as resurgent secondary pests of agricultural crops such as pome fruits and cotton. Millar (CA) has isolated a variety of sesquiterpenes (e.g. zingiberene, bisabolene epoxide) from the red-shouldered stink bug, *Thyanta pallidovirens*, and from another stink bug, *Acrosternum hilare*. Millar and Seybold (MN) plan labeling studies with ^{14}C-mevalonolactone followed by radio-HPLC analysis to determine whether synthesis of these sesquiterpenes may be *de novo* or from plant derived isoprenoid precursors.

A third project involving isoprenoid pheromones is designed to understand regulation of isoprenoid pheromone production at the molecular level in the cotton boll weevil and is being pursued by Tittiger (NV) and Blomquist (NV). The male boll weevil uses a monoterpenoid based pheromone system, and the understanding that has been gained from studying bark beetle pheromones will be used to make rapid progress with the boll weevil pheromone system. The boll weevil currently accounts for over $300 million dollars per year in damage and supression costs, and is arguably the most economically important agricultural pest in the United States. The identification of JH-regulated genes in male bark beetles and boll weevils represents a new tool with great potential for answering a long standing, fundamental question: how does JH regulate a gene?

Bartelt (USDA-ARS) has a long track record working on the analytical chemistry, biochemistry, and behavior associated with the pheromone systems of agriculturally important sap beetles in the family Nitidulidae (e.g. Bartelt and Weisleder 1996; Dowd and Bartelt 1993; Nardi et al. 1996; Petroski et al. 1994). These insects have also been identified as the primary vectors responsible for overland spread of oak wilt disease, *Ceratocystis fagacearum*, in the upper midwest. Seybold (MN) has begun working on the life histories and chemical ecology of several nitidulid species (*Colopterus truncatus* and *Carpophilus sayi*) that are known to be associated with oak wilt mats (sources of fungal innoculum) freshly wounded oak tissue. Bartelt and Seybold plan to collaborate on the identification of kairomonal and pheromonal attractants and interruptants for these species. Preliminary wind tunnel studies in Peoria suggest that coniferous monoterpenes will partially interruptant the response of the closely related *Carpophilus lugubris* to oak wilt volatiles. Identification of attractants would allow the development of mass trapping as a treatment to reduce populations of the oak wilt vectors, while identification of interruptants would provide a tool for preventing colonization of mats on infected trees or wounds on uninfected trees. Both control techniques may have utility in slowing the overland spread of oak wilt disease in the upper midwest. Finally, chemotaxonomic analyses of the cuticular hydrocarbons of *C. sayi* and *C. lugubris* are planned to begin to clarify their species status.

This regional project will provide a mechanism for the initiation and continuation of collaborative projects between project members working on all aspects of insect semiochemistry, and more broadly, the development of applications for natural products from any source for insect monitoring and control. Participants bring a broad range of expertise to the project, covering aspects from basic biology and life history of insects and insect-host associations, through to state-of-the-art identification and synthesis of novel compounds for modification of insect behavior (e.g., pheromones), or direct control. Networks of interlocking collaborative projects are under way, in advanced stages of planning, and/or awaiting approval of pending grant applications. Millar (CA) and Aldrich (MD) are cooperating on studies of pheromones of a number of hemipteran pests. Sex or aggregation pheromones have been identified for several stink bug and mirid bug species, and work on a number of other species is in progress. Field studies are focussing on trap designs and trapping protocols. Jackson (MT) and Millar (CA) are cooperating on the synthesis of new sex pheromones for several *Drosophila* spp., and Millar (CA) is assisting Bjostad (CO) with the identification of Geometrid moth pheromones. Blomquist (NV) and Millar (CA) have organized a collaboration on the identification and synthesis of pheromones of cerambycid beetles. This insect family includes notorious pests such as the Asian longhorned beetle which is causing havoc in hardwoods in the northeastern US. Weaver (MT) and Bartelt (USDA-ARS) and Coss (USDA-ARS) are working on isolating the pheromone communication system of the wheat stem sawfly, a severe regional pest of wheat production in the Northern Great Plains. The focus will be to develop monitoring and trapping systems for use for this pest, and to explore mating disruption. This species has been invulnerable to conventional chemical and physical controls for more than a century. In addition, Weaver (MT) and Jackson (MT) are working with Bartelt (USDA-ARS) to investigate kairomones and allomones in this multitrophic system involving crops and feral grasses as a reservoir for the sawfly and two important species of braconid parasitoids. Elucidation of these semiochemicals will provide valuable information for cereal crop breeders to use in enhancing novel forms of crop resistance to this pest. Multimethylene interrupted alkadienes are a group of unique cuticular hydrocarbons, some with semiochemical activity, observed on the Caribbean fruit fly (*Anastrepha suspensa*) and a number of Drosophila species. A collaborative effort between Jackson (MT) and Millar(CA) involves the organic synthesis of these compounds for testing. The genetics of biosynthesis of these compounds are understudy in a collaborative effort between Jackson(MT) and Etges (AK). Additional pest insects are being investigated to determine if they use multimethylene interrupted alkadienes as semiochemicals.

Objective 2: To identify and understand the physiological mode of action of plant metabolites for development of novel biorational methods of insect management.

The Welch (NV) laboratory has shown that the electrostatic and steric (Lennard-Jones) molecular determinants of ryanoid insect toxicity are centered in completely different loci than those that determine mammalian toxicity. This work will be expanded to include new compounds, including ryanomimetics, and to the biochemical characterization of the mechanism of insect ryanoid toxicity. The procedures are outlined in Appendix D (Welch).

The Felton (AK) laboratory has been active in the exploration of the role plant phenolics play in regulation of insect predation. This laboratory will continue to investigate the interrelationships between activity of enzymes of the phenolic biosynthetic pathways and the ability of plants to blunt insect feeding damage.

In summary, the engineering of novel chemicals for both chemical synthesis and transgenic plants is the long term objective of this research. Continued advances in genetics and molecular biology, including gene transfer among plant species, holds promise for the improvement of agricultural plant cultivars by building on the foundation of natural mechanisms for resistance to pests. However, the creation of desirable cultivars requires a profound and detailed knowledge of (A) the phytochemical resources available for manipulation and (B) the interactions of these phytochemicals with both human and insect consumers. The investigators in this section will be focused on specific classes of phytochemicals as lead compounds for superior chemicals for application and for transgenic plant cultivars.

OBJECTIVE 3: To develop insect regulatory peptides and proteins, including various enzymes and insect-specific toxins, as novel agents for plant protection.

The little mentioned disadvantage of crops genetically modified to produce *B. t*. or similar toxins is that the constant exposure of the insect pest to a toxin provides a persistent selection pressure comparable to daily spraying of conventional insecticides, practically guaranteeing the development of mutant pests with resistance mechanisms to *B. t*. Thus, there is a pressing need for continued research into alternative proteins with insecticidal activity so that once *B. t*. resistance evolves, second generation toxins will be available for genetic engineering into crops. The chief problem with this approach is that most proteins are probably degraded by digestive proteases in the midgut of lepidopteran larvae, the most important crop pests. Lepidopteran midgut is rather unique; it is highly alkaline (pH 10). The crystalline toxin of *B. t*. is insoluble at this high pH and binds to a specific protein in the midgut, and then causes septicemia. Thus it is desirable to find toxic gene products that are poorly digested, which can enter the circulatory system.

The Hammock lab at UC Davis is working on isolating and identifying insect-specific toxins from the venom of scorpions. Venoms will be

collected by electrically stimulated milking. The venom components will be separated by a number of successive purifications using ion exchange and reversed-phase HPLC. At each step of purification fractions will be monitored by injection bioassay for LD_{50} in several insect species and in mice. Fractions that are selectively active on pest insects, with low toxicity to mice, will be further purified, finally using microbore HPLC. They will then be analyzed by MALDI and electrospray mass spectrometry to determine both purity and molecular weight. Most scorpion toxins are small, highly disulfide-branched proteins. These will be reduced, the free Cys residues carboxamidomethylated, and the protein sequenced. The protein sequencing will be done by the Schooley group at UNR. Following sequencing, the corresponding gene will be synthesized and cloned into baculovirus vectors for expression. The recombinant baculoviruses will be evaluated as biological insecticides. In addition, the isolated toxins will be tested for oral toxicity on insects by incorporating into an artificial diet and feeding to insects. Because these toxins are exceptionally highly branched with disulfides, they may be resistant to proteolysis in the insect digestive tract so that oral activity is not out of the question. If oral activity is detected, then initially tobacco plants will be genetically modified to express a synthetic gene encoding the toxins. The synthetic gene will be designed using the tobacco codon bias. Recombinant plants will be grown and infested with *Manduca sexta*, an insect which thrives on tobacco plants, to assess the efficacy of the toxin against this pest. If the plant proves less susceptible to insect attack after this modification, then the stage will be set for engineering this gene into agronomically useful crops such as cotton and corn; these two crops account for a very large percentage of pesticide use.

The Schooley lab at UNR is actively investigating the structure and function of insect diuretic and antidiuretic hormones. It has long been thought that upsetting the water balance of insects is a very promising strategy for insect control. Currently three peptides have been completely sequenced which promote diuresis in *Manduca sexta*; these are *M. sexta* DH (Kataoka *et al.*, 1989), *M. sexta* DPII (Blackburn *et al.*, 1991), and *Manduca* kinin [M. Blackburn, unpublished, cited in (Kingan *et al.*, 1997)]. Nachman at USDA/ARS/FAPRL has already modified insect kinins to make them resistant to specific proteases, with maintenance of high diuretic activity (Nachman *et al.*, 1997). The biologically essential core of the kinins is only five amino acid residues, whereas *M. sexta* DH and *M. sexta* DPII are 41 and 30 amino acids long. Unfortunately, to date the kinins have not been shown to cause *in vivo* fluid loss from *Manduca sexta*; only *M. sexta* DH and *M. sexta* DPII have been shown to do so (Blackburn *et al.*, 1991; Kataoka *et al.*, 1989). We know from deletion experiments (P. Dey and D. Schooley, unpublished) that only a few residues can be deleted from the amino terminus of these hormones before activity is lost; any alteration of the carboxyl terminus causes a loss of activity. Thus, it is a far more challenging problem to try to modify such large peptides to be both resistant to proteases and still biologically active. We have determined the partial

protein sequence of an antidiuretic peptide in *M. sexta* which is larger still (8,710 Da); we have nearly completed determining the structure of a gene encoding this peptide from a cDNA library. Once we have the complete sequence, we hope to do a number of studies on its biological effects *in vivo* and *in vitro*. Then we hope to proceed with isolation of the receptor of this peptide. We plan to do this by expression cloning of a hindgut cDNA library. We shall screen for cells expressing the antidiuretic hormone receptor by making [^{125}I] labeled antidiuretic hormone, and selecting clones that specifically bind to this ligand. Once positive clones are located, they will be grown up and the receptor sequenced. The ultimate aim will be to express the receptor in a baculovirus system. This exact approach has been done successfully for the *M. sexta* DH receptor by Reagan (Reagan, 1994; Reagan, 1995). Accessibility of the antidiuretic hormone receptor will allow high-through put screening to locate low molecular weight antagonists of the antidiuretic hormone. Such an antagonist promises to be an effect way of disrupting fluid homeostasis in insects, because such an antagonist would block the ability of the larvae to resorb water from the rectum, causing dehydration of the larvae.

Dr. Shinji Nagata of the Schooley lab has already started to construct a cDNA library from Malpighian tubules of *Manduca sexta* for the purpose of isolating a receptor for *M. sexta* DPII. This peptide binds to the *M. sexta* DH receptor with only 1/10th the affinity of *M. sexta* DH (Reagan, 1994). It seems highly likely that separate receptors exist for this diuretic hormone; the homologous vertebrate hormones corticotropin releasing factor and urocortin have at least two receptors (Baram *et al.*, 1997; Perrin *et al.*, 1995) with differing affinities for the two ligands. The strategy to be used in isolating the DPII receptor, expression cloning and screening with [^{125}I]*M. sexta* DPII, is identical with that used by Reagan earlier. Again, widespread availability of this receptor (in contrast to the *M. sexta* DH receptor,which is patented by Novartis) should allow any number of institutions to screen fermentation broths or combinatorial libraries to locate low molecular weight materials that have the desired biological activity, but which lack the digestibility and penetration problems expected with the endogenous peptide hormones.

Schooley (NV) and Hammock (CA) will also isolate and characterize new insect enzymes and the corresponding genes involved in the control and expression of developmental hormones. A specific example is the insect juvenile hormone epoxide hydrolase, one of two important enzymes involved in the metabolic inactivation of juvenile hormone. Once identified, its gene will be incorporated into appropriate vectors for deployment as developmentally disrupting toxicity enhancers, specifically targeting lepidopteran pests, as has been successfully accomplished with JH esterase (Hammock et al. 1990, 1993).

EXPECTED OUTCOMES

The expected outcome of the proposed research is a better understanding of unique processes in insects that have the potential to be exploited for insect control. It is impossible to predict with any degree of accuracy to what extent this new understanding would lead to new and or improved insect control techniques, but, based on past history, it is likely that one or more of the dozens of processes insect scientists are studying will lead to new and or improved insect control techniques. The work accomplished during this project will be published in mainstream scientific journals, and patents will be sought where appropriate.

ORGANIZATION

Leadership of the project will be provided by an Executive Committee
consisting of the project chairman and secretary, to be elected from the project members, and the project section leaders. The chairman will be responsible for calling, organizing, and chairing the annual meeting, preparation and submission of the annual report, coordination with the project's Administrative Advisor, and any other matters relating to the project as a whole (official correspondence, etc.). The secretary will be responsible for recording and circulating minutes of the annual meeting and correspondence with project members about project business. The secretary will substitute for the chair in the latter's absence. The role of the section leaders is described below.

The Technical Committee will be comprised of all principal investigators in the project (all are currently faculty at land-grant institutions, or USDA-ARS personnel). The Technical Committee will be organized into sections corresponding to each of the three major foci of the project, with a member designated on a rotating basis to lead each section.

Collaborations between research groups and disciplines, shared use of specialized equipment or facilities, and free exchange of results and ideas will be encouraged to the greatest extent possible.

Project members will submit a concise, detailed annual report to the chairman, for collation and circulation to all project members. Each report will be reviewed by members of the appropriate section. The sections will evaluate and critique each report, and the section head will prepare and provide a summary to the report's author. At the annual project meeting, each project leader will orally present a brief outline of progress and of anticipated work for the coming year. The section head will then offer the critique for discussion by the entire committee. During the meeting, plans for collaborations between and within subcommittees will be finalized. An annual report will be prepared under the guidance of the project chairman.

Title: Biorational Methods for Insect Pest Management (IPM): Bioorganic and Molecular Approaches.

SIGNATURES

Administrative Advisor

5/20/99
Date

CHAIR, REGIONAL ASSOCIATION OF DIRECTORS

7/14/1999
Date

_____ _____

Administrator, Cooperative State Research, Education and Extension Service

Date

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ATTACHMENT 1: PROJECT LEADERS

REGIONAL PROJECT TITLE: Biorational methods for insect pest management (IPM): Bioorganic and molecular approaches.

LOCATION	INVESTIGATOR	SPECIALIZATION
A. EXPERIMENT STATIONS		
Arizona	W.S. Bowers	Entomology and Chemical Ecology
Arkansas	G.W. Felton	Plant Natural Products
California	B.D. Hammock	Transgenic Insects
	J.G. Millar	Semiochemistry and Chemical Ecology of Insects
	I. Kubo	Organic Chemistry
Minnesota	S.J. Seybold	Chemical Ecology
Montana	L.L. Jackson	Pheromone Biochemistry
	D. Weaver	Insect Behavior
Nevada	G.J. Blomquist	Pheromone Biochemistry
	D.A. Schooley	Insect Hormones
	Claus Tittiger	Molecular Biology of Pheromone Production
	W.W. Welch	Protein Chemistry
B. USDA		
Berkeley	M. Page	Chemical Communication and Taxonomy
Peoria	R. Bartlet	Pheromone Chemistry and Biochemistry

ADMINISTRATIVE ADVISOR	**R. S. Pardini**
CSREES REPRESENTATIVE WASHINGTON DC	**D. Jones**

RESOURCES

PARTICIPANT	STATE CONTRIBUTION TO OBJECTIVES 1	2	3	4	5	SY % Research	% Extension	% Teaching	RESOURCES SY	PY	TY
ARIZONA		X									
W. S. Bowers						.00	.90	.10	0.2	0.3	0.1
ARKANSAS		X									
G. W. Felton						0.1	.00	0.1	.01	.00	0.5
CALIFORNIA	X		X								
B. D. Hammock						.75	.00	.25	.15	0.5	1.0
									0.1	0.5	1.0
J. G. Millar						.01	.00	0.1	.15	0.5	1.0
I. Kubo						.80	.00	.20			
MINNESOTA	X										
S. J. Seybold						.70	.00	.30	.15	0.5	1.0
MONTANA	X										
L. L. Jackson						100	.00	.00	.35	.00	.00
									0.3	.00	0.3
D. K. Weaver						100	.00	.00			
NEVADA	X		X								
G. J. Blomquist						.75	.00	.25	0.2	.00	.25
									0.2	.00	.25
D. A. Schooley						.75	.00	.25	0.2	0.2	0.2
									0.3	.00	.00
C. Tittiger						100	.00	.00			
W. W. Welch						.65	.00	.35			
ARS-BERKELEY	X										
M. Page							N/A		.15	.15	.15
ARS-PEORIA	X										
							N/A				
R. Bartlet									0.1	.00	.00
TOTALS						.00	8.32	1.9	2.36	2.35	5.65

*Cooperator

CRITICAL REVIEW

OBJECTIVE 1 was to determine the chemistry, biology, biochemistry and neurophysiological mechanisms of perception of specific semiochemicals potentially useful for control of insect pests, and to study semiochemicals in model insect systems for later application to target pest insects.

Research in this area has been performed on bark beetles, fruit flies, several hemipteran pests (stink bugs), the wheat stem sawfly, and a number of other insects. Interesting new pheromones have been identified by Millar's group at the Riverside AES from the red-shouldered stink bug *Thyanta pallidovirens*, which produces a specific triunsaturated C_{10} acid methyl ester, together with three sesquiterpenes, as its pheromone blend. The male-produced pheromones in another species, *Acrosternum hilare*, is a specific mixture of a sesquiterpene and the epoxide of its geometrical isomer. This research is driven by the emergence of various true bugs as major crop pests as a result of decreased use of insecticides for control of lepidopteran species, and a need for monitoring their populations.

A collaboration between the Jackson group at Montana and Etges at Arkansas has shown pheromone differences between two populations of *Drosophila mojavensis*. These populations are isolated by the Sea of Cortez in Mexico; the mainland population has changed its preferred host plant, and also synthesize larger quantities of C_{33} and C_{35} alkadienes than the Baja population. Jackson believes these differences are the beginning of speciation of the two populations. Different dimethyl-branched C_{35} hydrocarbons have been identified from the cuticle of *D. navajoa*, but not in *D. arizonae*, although these species share an isomeric dimethyl-branched C_{35} hydrocarbon. Studies continue to determine whether these hydrocarbons are genuine pheromones. Pheromones are being identified from *D. mettleri*, guided by bioassay, and several possible pheromones are under investigation.

Weaver and others at Montana State are continuing investigation of the chemical ecology of the wheat stem sawfly, *Cephus cinctus* (Hymenoptera) and two of its parasitoids in cereal crops and grasses, in collaboration with Bartelt at USDA-ARS, Peoria. These studies on its overwintering and mating behavior on emergence have clarified how and when the female calls, including a sensitivity to temperature and proper illumination. A wind tunnel choice olfactometer has been investigated and a volatile collection system set up with the goal of isolation and identification of semiochemicals. Bioassay of these components will begin in January, when a new population of animals is available.

Bartelt from Peoria discussed fundamental investigations of the reliability and reproducibility of a micro solid phase extraction probe for collection and identification of volatiles from insects. This polymer-coated silica fiber was found to be robust, homogenous between different fibers, and very well-behaved for collection and characterization of semiochemicals.

A large body of work was accomplished by the Blomquist group at Nevada, by Seybold who is now at Minnesota and by Tittiger who we propose to add to the revised Regional Project W-189 on bark beetle pheromones. When this work started five years ago, the general conclusion regarding bark beetle pheromones was that most were obtained by the direct hydroxylation or minimal modification of tree derived precursors. Studies in the Blomquist laboratory used radiotracer techniques to directly demonstrate *de novo* aggregation pheromone production in *I. pini* and *I. paraconfusus* males from [1-^{14}C]acetate and (*RS*)-[5-^{3}H]mevalonolactone. In addition, they demonstrated both *de novo* synthesis and the isoprenoid nature of frontalin by showing the incorporation of ^{14}C-mevalonate into this pheromone component.

To evaluate the relative contribution from *de novo* and host precursor-related pheromone biosynthesis, the masses and enantiomeric compositions of ipsdienol in California populations of *I. pini* were examined from fed and unfed insects exposed to volatile myrcene. The observation that myrcene-treated beetles produce nearly racemic ipsdienol suggests that although myrcene is readily hydroxylated by male *I. pini*, it is not metabolized to the behaviorly active pheromone blend. Unfed, JH-treated males produce enantiomeric blends resembling the naturally occurring composition, suggesting that the majority if not all of pheromone produced arises from *de novo* synthesis and not from exposure to host myrcene.

The *in vivo* incorporation of radiolabeled acetate into ipsdienol by male *I. pini* increased with increasing topical JH III dose. The *in vivo* incorporation of radiolabeled mevalonolactone into ipsdienol by male *I. pini* was relatively high at all JH III doses, and did not significantly increase with increasing dose of JH III. Males fed on host phloem also significantly increased the incorporation of ^{14}C-acetate into ipsdienol. These data constitute direct evidence for the isoprenoid pathway in *de novo* ipsdienol biosynthesis, and indicate that JH III influences steps prior to mevalonate formation in this pathway.

HMG-CoA reductase (HMG-R) is a key regulatory enzyme in mammalian isoprenoid (cholesterol) biosynthesis, suggesting that HMG-R might also function as a key regulated enzyme in *de novo* monoterpenoid pheromone biosynthesis in *Ips* spp. Studies comparing HMG-R activity in fed, JH III-treated, and control male and female *I. pini* indicate that HMG-R activity is stimulated by feeding in females and by both feeding and JH III treatment in males. This indicates that in the natural setting, feeding on host phloem stimulates JH III biosynthesis by the CA of male *I. pini*, with JH III specifically increasing the activity of HMG-R in the biosynthetic pathway. The following preliminary results suggest that JH III acts by inducing transcription of the *HMG-R* gene.

Studies using polymerase chain reaction (PCR) and northern blot analyses showed that topical application of JH III to male *I.*

paraconfusus and *I. pini* increased the amount of HMG-R mRNA in a dose and time dependent manner.

Furthermore, this induction occurs in thoracic tissue of *I. paraconfusus*, and presumably *I. pini*. This finding is consistent with *in vitro* radiochemical data which shows that ipsdienone (a pheromone precursor) is produced in thoracic issue of male *I. paraconfusus*. Work is currently underway using *in situ* techniques to pinpoint the exact location of pheromone synthesis. The current picture of pheromone regulation in *I. pini* involves the feeding-induced production of JH III, with JH III then inducing *HMG-R* transcription or increasing HMG-R mRNA stability in male thoracic tissue.

Recent work has resulted in the cloning and sequencing of both HMG-R and HMG-CoA synthase (HMG-S) in another bark beetle, *Dendroctonus jeffreyi*. In male *D. jeffreyi* , topical treatment with JH III results in labeling of frontalin by ^{14}C-mevalonate, the induction of frontalin production, and increased transcript levels of both HMG-R and HMG-S, suggesting that both of these enzymes are regulated at the transcriptional level by JH.

The novel and exciting work demonstrating that JH induces pheromone production in Ips and Dendroctonus bark beetles at the transcription level by increasing the message for HMG-R and HMG-S opens new avenues for investigation. Work underway includes recovering and identifying regulatory elements involving promoters and conserved gene structures of the regulated enzymes in an attempt to better understand the mechanism of action of JH at the molecular level, the isolation, sequencing and modeling of the prenyl transferase involved in producing monoterpenoid pheromones in bark beetles, and work to understand the details of the biosynthetic pathways for the bark beetle isoprenoid derived pheromones ipsenol, ipsdienol and frontalin. In addition, work is underway in the cotton boll weevil, in which males also produce monoterpenoid pheromone components whereas females produce a sesquiterpenoid pheromone, to understand the regulation of pheromone production.

Objective 2. to discover, identify, and determine the physiological mode of action of plant metabolites toxic to insects, for development into biorational pesticides.

Research from the lab of Felton (Arkansas AES) and collaborators at Arkansas, Samuel Roberts Noble Foundation, and the Salk Institute explored genetic manipulation in plants of the systemic acquired resistance to pathogen infections, and the jasmonic acid signaling system for defense against insect attack. These studies show an antagonism in genetic manipulation of these pathways; that is, manipulation to enhance resistance to tobacco budworm attack increased susceptibility to viral pathogens. Conversely, enhanced resistance to viral pathogens resulted in decreased resistance to tobacco budworm. These result imply limitations in exploitation of genetic manipulation of plants to enhance resistance to both pathogens and insects.

Welch (Nevada AES) reported results on assay of a number of analogues of the natural insecticide ryanodine in grasshoppers. Comparison of their toxicity to grasshoppers using a comparative molecular modeling approach showed that alteration of two specific parts of the ryanodine molecule, the pyrrole ring and the isopropyl group, can drastically alter the relative toxicity of ryanodine to mammals *vs*. insects. It is thus clearly possible to synthesize analogues of ryanodine with enhanced toxicity to insects and decreased toxicity to mammals.

Objective 3. To develop peptides and proteins as novel agents for plant protection, and increase our understanding of the roles that peptides and proteins can play in pest control.

A collaborative study by Schooley and Welch (both Nevada AES) was reported on molecular modeling of the two diuretic hormones of the lepidopteran *Manduca sexta*. These diuretic hormones differ significantly in size (30 *vs*. 41 amino acids) and in primary sequence (only nine identical amino acid residues). The results of the modeling study showed that both hormones fold into a helix-loop-helix motif, with very similar shape except for a loop region, which is believed to be unimportant in determining the biological activity. To support the model, an analogue of the 41 amino acid *M. sexta* DH was designed and synthesized with a disulfide bridge locking the molecule into the predicted conformation. The EC_{50} value for this analogue was statistically the same as the EC_{50} values for the native hormone and the analogue containing two Cys residues, but lacking the disulfide bond.

Hammock (UC Davis AES) is isolating toxins from scorpion venoms monitored by bioassay on insects vs. mice. Those toxins which have a high toxicity to insects, but low toxicity to mice, are purified, then subjected to Edman degradation analysis and mass spectral analysis. Following sequencing a gene for the toxin is synthesized chemically and inserted into a pUC derived transfer vector for the baculovirus expression system. Transfection of this plasmid with a baculovirus results in the toxin gene being placed under a powerful insect selective promoter. The virus is then evaluated for an increased speed of kill when administered to pest larvae.

The Schooley group also studied the inactivation of the larger of the two *M. sexta* DH, Mas-DH, by incubating it *in vitro* with larval Malpighian tubules (Mt), the target organ of the hormone. The medium was analyzed, and degradation products were identified, using on-line microbore reversed-phase LC coupled to electrospray ionization mass spectrometry (RPLC-ESI-MS). This sensitive technique allowed identification of metabolites of Mas-DH, present at an initial level of ~1 µM. An accurate M_r value for a metabolite is usually sufficient for unambiguous identification. Mas-DH is cleaved by Mt proteases initially at Leu^{29}–Arg^{30} and Arg^{30}–Ala^{31} under the assay conditions; some Mas-DH is also oxidized apparently at Met^{2} and Met^{11}. The proteolysis can be inhibited by 5 mM EDTA, suggesting that divalent metals are needed for

peptide cleavage. The oxidation of the hormone is inhibited by catalase or 1 mM Met, indicating that H_2O_2 or related reactive oxygen species are responsible for the oxidative degradation. The Schooley group also identified two diuretic hormones from the beetle *Tenebrio molitor*, Tem-DH_{47} and Tem-DH_{37}, where the subscripted number denotes the number of amino acid residues. Neither of these hormones has appreciable activity on Mt of *M. sexta*, but are active in the nM range on *T. molitor* Mt. They have novel C-termini, with the C-terminus being in the free acid form rather than having the usual amidation. They also isolated two DH from the Pacific beetle roach, *Diploptera punctata*. One of these, Dip-DH_{46} is extremely homologous to the DH of *Periplaneta americana*, whereas Dip-DH_{31} has a novel sequence with greater similarity to calcitonin than the CRF-like DH. While Dip-DH_{31} stimulates cAMP production by Mt of *Schistocerca americana* and *D. punctata*, it has no effect on Mt of *M.sexta*. Curiously, these two DH act via the same second messenger, cAMP, but show strongly synergistic effects in *D. punctata*. As yet another example of two DH occurring in a single species of insect, the Schooley group isolated and sequenced two DH from the white lined Sphinx moth, *Hyles lineata*, Hyl-DH_{41} (= [Q^{27}]Mas-DH) and Hyl-DH_{30} (= [E^{9}]Mas-DH). Each differs from the known Mas-DH and Mas-DPII at only a single amino acid residue. Recently they also isolated a DH from a dampwood termite, *Zootermopsis nevadensis*. The 46 residue hormone Zon-DH_{46} has a higher degree of sequence similarity to the DH of *Periplaneta americana* than does the DH of the ovoviviparous cockroach *D. punctata*. A second DH in *Z. nevadensis* proved too unstable to acidic conditions to isolate using RPLC.