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Bruce D. HAMMOCK
Coauthors:
Yehia A. I. ABDEL-AAL, Mohamed ASHOUR, Adrian BUHLER,
Terry N. HANZLIK, Richard NEWITT, and Thomas C. SPARKS*
Departments of Entomology and Environmental Toxicology
University of California
Davis, California 95616 U.S.A.
and
*Department of Entomology
Louisiana State University
Baton Rouge, Louisiana 70803 U.S.A.

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SUMMARY

Inspite of rapid developments in a number of alternative approaches for the control of agricultural pests, the need for chemical pesticides in both developed and developing countries has never been greater. Not only are higher standards of environmental and human safety required in many countries, but much higher efficacy also is needed. However, we see a decrease in the number of companies involved in insecticide development and discovery and a reduction in the number of new compounds reaching the market. It is critical for economic and humanitarian reasons that we continue to develop methods to control agricultural pests.

This chapter will address the problem of pesticide development using recent work from this laboratory as an example. It will be argued that the rate of discovery of new compounds can be accelerated by exploiting fundamental knowledge and by integrating several successful approaches to pesticide discovery.

Some approaches being followed in this laboratory will be explored. Our work concentrates on exploiting a fundamental knowledge of the endocrine regulation of larval-pupal transformation in pest Lepidoptera. An overview of the system will first be presented followed by a more detailed discussion of the role of juvenile hormone esterase in insect metamorphosis. One can disrupt insect development by the inhibition of this enzyme using either organophosphates or a series of beta-thioltrifluoropropanones as "transition state" mimics. Alternatively one could stimulate production of the juvenile hormone esterase as is done naturally by a *Chelonus* parasite of some pest species. This stimulation could be accomplished by mimicking the natural processes in pest insects or by infection with a baculovirus carrying the esterase gene. Thus, the work discussed will cover both chemical and biochemical methods of pest control. It will be shown that the two approaches are, in fact complementary. Such multidisciplinary approaches to the discovery of new insect control agents seem critical to the development of future innovative materials for pest control.

In Lepidoptera it is widely accepted that a reduction in juvenile hormone titer followed by two bursts of the steroid hormone ecdysone leads to pupation. Evidence from this laboratory indicates that in pest noctuiids that this dogma holds except that a second prepupal burst of juvenile hormone is needed before the actual ecdysis occurs to the pupa. Evidence also will be presented that the reduction in juvenile hormone titer is effected not only by a reduction in biosynthesis, but also by an increase in metabolism of the hormone. This

degradative metabolism is mediated largely by a single, highly specific enzyme here after known as JH esterase. A variety of circumstantial experiments indicate that this enzyme is needed for the reduction in JH titer. The most direct experiments come from treating larvae with inhibitors of the enzyme. Several phosphoramidothiolates have been shown to be potent, selective inhibitors of the enzyme *in vitro* and *in vivo*. These compounds will delay or block normal metamorphosis by slowing the reduction of the normal JH titer and delaying the release of ecdysone. A series of polarized ketones also have been made based on transition state theory which are highly potent inhibitors of JH esterase. By varying the structure of these compounds, one can make potent inhibitors of a variety of toxicologically significant esterases.

Alternatively, one could artificially induce the JH esterase leading to cessation of feeding and precocious development. Work in this laboratory indicates that the enzyme is under different regulatory mechanisms depending upon the developmental stage of the insect. While investigating the mechanism by which *Chelonus* parasites cause precocious development, it was found that JH esterase appears in the hemolymph one stadium early. With this encouragement it became important to develop molecular probes to investigate further the regulation of this key enzyme and possibly to isolate the gene. To this end, trifluoroketone ligands were attached to Sepharose and an affinity purification procedure developed for the enzyme. The procedure may give purification factors of over 1000 as well as almost quantitative yield of an apparently homogeneous enzyme. Thus, a combination of chemical and biochemical approaches has led to rapid progress on the exploitation of this system for insect control.

INTRODUCTION

Numerous problems face the agricultural chemicals industry, but none are so profound as the reduction in the discovery and development of new compounds. In this manuscript we will address this problem using examples from the insect control area, but many of the problems and possible solutions apply to agricultural chemicals in general. Dr. Robert Metcalf (1980) documented and presented reasons for the decline in the discovery of new insecticides, and this subject has been treated recently in the light of problems with pest resistance to insecticides (Hammock & Soderlund, 1985). There are a variety of factors contributing to the dearth of new materials, and these factors probably have acted synergistically resulting in a dramatic and precipitous decline in new compounds. As outlined by previous workers, these problems exist with increased costs of discovery, registration, production, and marketing. On the optimistic side, if improvements can be made in several of these areas, the recovery observed, may be similarly dramatic.

It is absolutely essential that such recovery occur. It is widely agreed that some form of pest control is essential to maintain the high level of agricultural productivity and profitability expected in developed countries. Recent tragedies in the third world illustrate the urgent need for the control of agricultural and stored product pests in these areas. The ominous rise of malaria and other tropical diseases serves as a continual reminder that man needs new and better weapons to combat vectors as well.

Mature compounds vanish from the market for a variety of reasons, with resistance in target species being a prime problem. Thus, the arsenal of materials available for pest management becomes more restrictive at a time when advances in integrated pest management illustrate a need for a variety of compounds with varying levels of persistence and selectivity. If we are to exploit our knowledge of pest ecology, it is essential that new materials enter the market.

It was previously argued that the high risk and uncertainty of discovery was a major factor in the decline of new compounds (Hammock & Soderlund, 1985). Although a variety of factors are combining to make pesticide development more attractive in the future, of these factors it is discovery that we as scientists can influence most directly. Thus, discovery will be emphasized in this manuscript. It will be argued that we finally are understanding enough about some aspects of target and nontarget biochemistry that we can begin biorational design of pesticides in earnest. Discovery of promising biological activities will be accelerated by advances in biological and chemical technologies and by the development of new paradigms for the development of compounds. The above areas are often viewed as mutually exclusive approaches. Work from our laboratory will be used to illustrate that chemical, biochemical, and molecular approaches are, in fact, complementary.

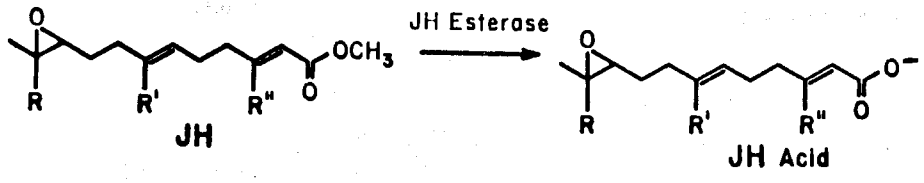

Fig. 1. The structures of the major juvenile hormones. JH I: R=R'=ethyl, R"=methyl. JH II: R=ethyl, R'=R"=methyl. JH III R=R'=R"=methyl. JH II is the major homolog detected so far in *Trichoplusia ni*. Hydrolysis of the conjugated methyl ester is the major route of metabolism in this species and is mediated by juvenile hormone esterase.

BACKGROUND

Approach

In the above paragraphs a major, international problem was outlined. In the following paragraphs, this article will become increasingly myopic as first a rationalization of our study of metamorphosis is presented with a brief outline of the biology of larval pupal transformation in the Lepidoptera in general and the Noctuiidae in particular. Although there are several hormones known and probably many more involved in regulating metamorphosis, this article emphasizes juvenile hormone. In fact it will emphasize work largely from this laboratory and those of our collaborators on a single esterase involved in the degradation of juvenile hormone (Fig. 1). This rapid limitation of the scope of the article will allow some aspects of the work to be presented in detail. However, these details will be used to illustrate at least one approach to solving our global problem of a failure to control pests of agriculture and human health.

Target for Study and Exploitation

A variety of factors have gone into our selection of the larval pupal transformation in the Lepidoptera as a system to investigate. A major reason for this selection is that it illustrates some of the fundamental puzzles in developmental biology which we have yet to explain in any species. However, there are practical reasons as well for our selection of this target. Most of our research is carried out with the family Noctuiidae. This family includes some of the most destructive pests known such as the genera *Heliothis*, *Spodoptera* and *Trichoplusia*. If a highly selective control agent could be developed for this family, it could be profitably marketed even in the current economic situation. Use of pesticides to control this family has resulted in environmental contamination, pest resurgence and pest resistance. Thus there is a great need for environmentally compatible compounds active on this group of insects.

As will be discussed below, metamorphosis is a critical and vulnerable time in the insect's life. The metamorphic events are regulated by a variety of nervous, endocrine and neuroendocrine signals each subject to disruption. We are working on the hypothesis that disruption of these regulatory systems may be amplified many times in the survival capacity of the insect. As we gain a deeper understanding of metamorphosis, we will discover still more targets for disruption. Hopefully, a variety of these targets can be exploited for insect control in a selective and environmentally compatible fashion.

Overview of Metamorphosis

Our understanding of metamorphosis has evolved from the research of a great number of scientists. Kopec', Wigglesworth, Williams, and certainly Fukuda (1944) are pioneers in this field. Two epithelial hormones are involved in metamorphosis. Ecdysone, a water soluble steroid hormone, promotes molting. Ecdysone is produced in response to a neurohormone known as prothoracicotropic hormone or PTTH just before each molt. The second epithelial hormone is known as juvenile hormone. This hormone has a terpenoid backbone and contains two hydrolytically unstable functionalities, a 10,11-epoxide and a terminal methyl ester conjugated with the 2,3 double bond (Fig. 1). Simplistically, when the juvenile hormone titer is high, secretion of ecdysone results in a larval to larval molt. However, when the juvenile hormone titer is low in the presence of 20-

hydroxyecdysone, each subsequent molt leads to a more advanced stage culminating in the adult form in holometabolous insects. During metamorphosis, many of the larval tissues are histolyzed and replaced with rapidly proliferating tissues arising from tiny embryonic centers known as imaginal disks which are carried by the larva since early embryonic development. Thus it becomes clear that the level of juvenile hormone is critical in regulating metamorphosis (Fig. 2). If JH levels remain high, the insect fails to undergo metamorphosis correctly and may become a giant larva. This effect has been exploited commercially with the development of juvenoids. Since the larval or feeding stage is destructive in the Lepidoptera, a more attractive approach to control this insect group would be to artificially reduce the JH titer in an early instar. This approach has led to the discovery of several diverse chemical structures which give anti-juvenile hormone effects. Numerous reviews of the endocrine control of metamorphosis are available (de Kort & Granger, 1981). For reviews dealing specifically with the metabolism of JH see Hammock & Quistad 1976, 1981, Richards, 1981; and Hammock, 1985.

ROLE OF JH ESTERASE IN METAMORPHOSIS

JH Esterase Activity Correlates with Declines in JH Titers

It is widely assumed among endocrinologists that hormone titers are regulated by variations in the rates of biosynthesis against a rather constant level of degradation. Biosynthesis is certainly a critical mechanism of regulation in the Lepidoptera. Many years ago Williams (1961) demonstrated that the corpora allata which produce JH reduce (yet do not halt) their activity in the early last larval instar. This reduction in JH titer is thought to allow the release of PTTH and thus a small burst of ecdysone which reprograms critical tissues for the forthcoming metamorphosis. It also results in a behavior known as wandering in which the insect searches for an appropriate site to pupate. Why JH production by the corpora allata was reduced yet not eliminated at this time of precipitous JH decline was not undestood. However, a subsequent prepupal burst of JH is seen in many species (Fig. 2).

A partial answer to this question was provided by Weirich and his coworkers (1973) at Zoecon when it was noted that the rate of JH degradation in the hemolymph of *Manduca sexta* increased dramatically in the early last larval instar. However, to go further it is important to examine the metabolism of JH. There are many likely sites for oxidative metabolism in JH, yet in most insects one of two hydrolytic pathways predominate.

Simplistically, one would expect both the epoxide and the ester of JH to be very unstable in water. However, the epoxide appears to be stabilized by the lipophilic environment offered by the JH backbone. This stability is further increased by the ethyl substituent present on the epoxide in most Lepidoptera examined. As will be further discussed later, the conjugation of the ester with the 2,3-olefin greatly stabilizes this functionality. Thus, in a biological system, the hormone appears rather stable unless there is an esterase or epoxide hydrolase responsible for its degradation. In the Lepidoptera so far studied, ester hydrolysis is the predominate pathway of metabolism (Hammock, 1985).

There are Two Major Peaks of JH Esterase in the Ultimate Instar

At this point, work from this laboratory will be emphasized. By monitoring JH hydrolysis in a variety of tissues, it was found that the hemolymph and fat body contributed the majority of the hydrolysis on a total body basis. In these two tissues there were two peaks of JH esterase activity. One peak occurred early in the last larval instar (a prewandering peak) and the other late in the instar (a post wandering or prepupation peak) (Vince & Gilbert, 1977; Sparks *et al.*, 1979). Using isoelectric focusing and a variety of other biochemical tests, it appeared that the esterase activity in these two peaks as well as artificially "induced" peaks probably was due to a single enzyme identical in each case (Sparks & Hammock, 1979a,b; Wing *et al.*, 1981). In *M. sexta* the enzyme from both peaks was affinity purified to homogeneity and the enzyme from each peak showed identical specific activity and electrophoretic mobility on SDS PAGE (Abdel-Aal & Hammock, unpublised information). Further work on purification supports this early hypothesis, but it needs to be retested using the more sophisticated tools now available.

Regulation of the prewandering peak. Since both of

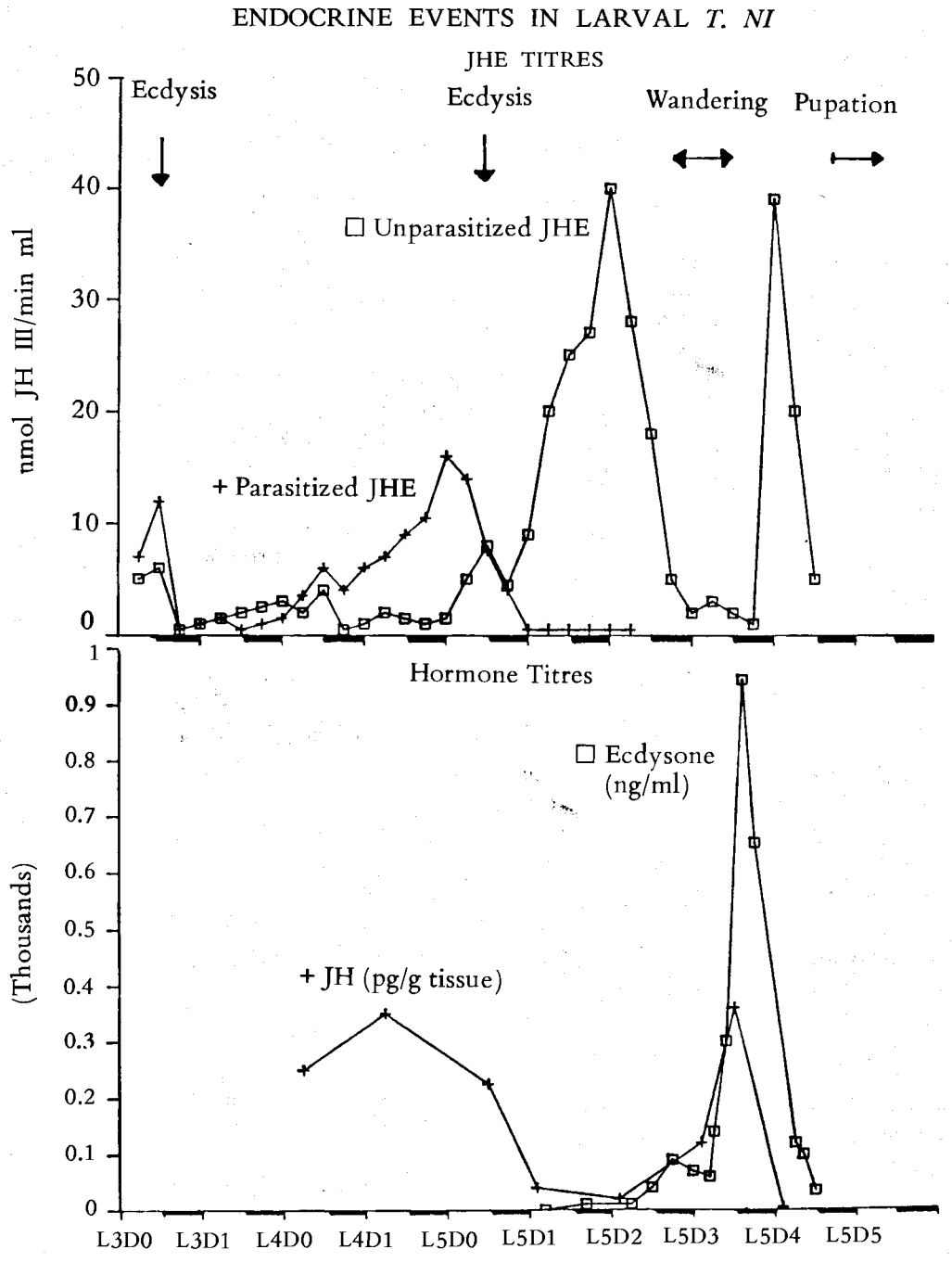

Fig. 2. Endocrine events in the last larval stadium of normal and parasitized *T. ni*. Of special note are the peaks of JH esterase occuring just prior to and just after wandering in the last larval instar. These are termed the pre and post wandering peaks, respectively.

these peaks correlate with a decline in the JH levels in the insect, it is tempting to assume a cause-effect relationship. It also becomes interesting to question how this enzyme is regulated. In the case of the prewandering peak of activity, ligation posterior to either the thorax or head blocked the production of the enzyme. It then was tempting to conclude that JH production by the corpora allata in the head might induce production or activation of the enzyme. It now appears that JH may modulate the level of the enzyme, yet application of JH or juvenoids to headless larvae failed to increase the activity of JH esterase in the hemolymph (Sparks & Hammock, 1979b).

However, in isolated abdomens, Grace Jones from this laboratory, demonstrated that implantation of the brain, subesophageal ganglion or a combination of these tissues increased the amount of JH esterase activity in the hemolymph (Jones *et al.*, 1981). Sally Levinson further demonstrated that an inadequate food supply and specifically limited protein resulted in a decrease in activity in this first peak of activity. Interestingly, a low protein diet resulted in a faster decline in JH esterase activity than did ligation. These experiments indicate that regulation of the first peak of JH esterase activity may be under neuroendocrine control and that it certainly is tied closely to environmental cues (Hammock *et al.*, 1981). Such nervous input often is mediated through the neuroendocrine system. However, at this point several experiments support the regulation of the prewandering peak by a neuroendocrine mechanism, but this mechanism must be treated as hypothetical.

Regulation of the post wandering peak. Regulation of the second peak of activity appeared to be similar based on the initial observation that ligation posterior to the thorax or head caused either a total blockage of the second peak or a great reduction in the activity present at this time. However, the similarity ends at this point for JH and a variety of juvenoids can stimulate the appearance of the peak in the wandering stage where JH esterase normally is absent or in isolated abdomens from post wandering animals which contain little JH esterase. Implantation of brains or subesophageal ganglia appear to do little to increase the esterase levels. A working hypothesis is that in *Manduca sexta* and *Trichoplusia ni* JH has no direct effect on increasing production or activity of JH esterase until after a critical time in the insect's development which occurs after wandering. How JH actually increases the production or activity of JH esterase remains to be determined.

The involvement of JH in the production of the post wandering peak of JH esterase activity in *Manduca sexta* was shown by allatectomy (Sparks *et al.*, 1983). However, these experiments were carried out most completely by Hone in *T. ni.* Removal of the corpora allata which produce JH was shown to reduce the JH esterase titer in a time dependent manner which correlated with results from ligation experiments and with the appearance of a prepupal burst of JH discussed below. Reimplantation of corpora allata or application of JH to allatectomized animals resulted in a dose dependent increase in JH esterase activity (Jones & Hammock, 1983).

Biological significance of regulatory mechanism. It appears rare in biology that a single protein is first under the control of a neuroendocrine mechanism and then later under the control of an epithelial hormone. However, it is possible to rationalize this scenario of regulation in terms of what we know of the biology of these animals. If the first or prewandering peak of JH esterase is responsible for the decline in JH in the early last larval instar, this esterase must not be under positive feed back control from JH itself. If this were the case, production of the esterase would be "induced" at an early stage, and the insect would develop precociously. Furthermore, timing of the "decision" to pupate is critical in the survival of the insect. Some lepidopterous species have a variable number of larval instars and most can at least vary the amount of time spent in the feeding stage. It is essential that the larva acquire sufficient food reserves to support metamorphosis and subsequent reproduction. On the other hand it is important that it completes development as soon as possible to minimize chances of mortality due to predation and environmental factors. It is thus not surprising that reduction in JH titers and the appearance of JH esterase are tied closely to an assessment of the quantity and quality of the insect's food. Such rapid adjustments are usually under the control of nervous and neuroendocrine systems (Hammock *et al.*, 1984a; Hammock, 1985).

Why then should the postwandering peak be under a separate regulatory mechanism? First, we must question the hypothesis of why a prepupal burst of JH occurs. According to the classical model proposed by Wigglesworth (1970), the JH titer should simply decline leading to pupation. Thus, a variety of

hypotheses have been developed to explain the appearance of this prepupal burst of activity. One attractive hypothesis developed in *M. sexta* is that it blocks precocious development of certain tissues. In *T. ni* this peak of JH is critical for actual ecdysis to occur. Its absence caused by allatectomy or a variety of chemical agents leads to teratogenic events or to a larva apparently frozen in the prepupal stage (Sparks 1984, Sparks *et al.*, 1985; Jones & Hammock, 1985).

Not only does this prepupal burst of JH appear critical for ecdysis to the pupa, but many workers have shown that even a trace of JH in the early pupal stage will profoundly disrupt insect development. Thus, this burst of JH must be of a very short duration. The fact that the appearance of JH at this time in development causes the appearance of an esterase that rapidly degrades it, assures that the JH will not be present to disrupt further development (Hammock, 1985).

BIOCHEMISTRY OF JH ESTERASE

During the above discussion the term JH esterase was used repeatedly. Our working definition of this term indicates an enzyme which degrades JH, but does not imply a physiological role. However, its timely appearance in a variety of species certainly indicates that it may have a physiological role. Investigations into its biochemistry further indicate its specialized nature. JH is quite stable to base hydrolysis since the S_N2 mechanism envisioned for this process takes the trigonal carbonyl group out of conjugation with the 2,3 olefin and thus increases the activation energy of the reaction. Since a similar mechanism is envisioned for serine esterases and proteases, one would anticipate that the majority of esterases present in insects would not be able to hydrolyze this conjugated ester efficiently (Hammock, 1985). This is exactly what was found by Sparks who reported that general esterases (defined as those enzymes hydrolyzing alpha naphthyl acetate) contribute very little to the hydrolysis of JH relative to the so called JH esterase (Sparks & Hammock, 1979a).

The esterase appears to only hydrolyse methyl esters at an appreciable rate. A variety of experiments involving competitive inhibition by alternate substrates and by the action of inhibitors further indicate a preference for methyl esters on a lipophilic backbone with great steric similarity to JH (Sparks & Rose, 1983). Kinetic studies by Abdel-Aal on the esterase from *M. sexta* indicates a K_m of about $3 \cdot 10^{-8}$M and a V_{max} of 1000 nmoles/min/mg protein using JH I (Abdel-Aal & Hammock, 1985a). The enzyme also shows a slight preference for the natural 10R,11S optical isomer over the 10S,11R (Abdel-Aal, Sparks, Prestwich, unpublished). Thus, biochemically, this enzyme appears uniquely suited to turn over JH very rapidly.

INHIBITION OF JH ESTERASE

Inhibition by Organophosphates

A great deal of circumstantial evidence points to a regulatory role for JH esterase. However, direct proof is the most convincing. This has now been provided using selective inhibitors for the enzyme from several chemical classes. Sparks and Hammock (1980) found JH esterase from *T. ni* to be refractory to inhibition by a variety of compounds which commonly inhibit carboxyesterases. However, some phosphoramidothioates were much stronger inhibitors of JH esterase than other critical esterases such as acetylcholinesterase. Thus, these compounds could be used to test the hypothesis that JH esterase has a regulatory role. It was found that topical application of these compunds to either *M. sexta* or *T. ni* would extend the feeding stage just as would application of JH. Studies by Schooley, Baker and coworkers have shown by glc/ms analysis that application of these compounds results in failure of the insect to clear JH from its body. When the compounds are applied at the time of the second peak of JH esterase, a variety of teratogenic effects occur including the appearance of larval structures and cuticle in the pupa (Sparks & Hammock, 1980; Jones & Hammock, 1985).

Inhibition by Trifluoromethylketones

The toxicity of the above compounds limits the range in which they can be used effectively, and their inhibition of the majority of the carboxyesterase activity in insect hemolymph makes the *in vivo* experiments somewhat equivocal. The discovery of a second series of JH esterase inhibitors has provided further proof to the hypothesis of a regulatory role for JH esterase. These compunds contain a trifluoro-

methylketone moiety (Sparks & Hammock, 1980; Hammock *et al.*, 1982, 1984b; Prestwich *et al.*, 1984). It is thought that the electron withdrawing properties of the trifluoromethyl group shift the equilibrium of the adjacent carbonyl group from the trigonal to the hydrated tetrahedral form. Linus Pauling in 1946 and 1948 hypothesized that any catalyst functions by lowering the activation energy of a reaction and thus by stabilizing or binding to transition state(s) along the reaction. This theory has been advanced by a variety of workers to explain the catalytic mechanisms of enzymes (see reviews by Wolfenden, 1976, and Abdel-Aal & Hammock 1985).

$RC(O)CF_3$

Substituted Trifluoroketone

It is widely accepted that serine esterases have a true reaction intermediate as the acetylated enzyme as well as two transient intermediates which are tetrahedral hydrates. These transient intermediates probably approach in structure the transition states in which bonds are being made or broken between the trigonal and tetrahedral forms of the substrate. Since the trifluoroketones tend to exist in a tetrahedral state, they certainly have some resemblance to the transient intermediates and probably to the transition states as well. Thus it is not surprising that some members of this series are potent inhibitors of JH esterase.

The aliphatic trifluoromethyl ketones described above supported the hypothesis that JH esterase was specialized to hydrolyze JH since the most potent compounds had MR's very similar to JH II. However, the compounds were disappointing *in vivo* in that they gave little inhibition of the esterase. However, this might be anticipated since they are apparently reversible inhibitors of the enzyme. However, it was found that placement of a sulfur in a thioether linkage beta to the carbonyl greatly increased the potency of the resulting substituted 3-thio-1, 1, 1-trifluoro-2-propanones.

$RSCH_2C(O)CF_3$

3-Substituted Thio-1,1,1-trifluoro-2-propanones

It initially was rationalized that the pi electrons of the thioether were mimicking the 2,3-olefin. It subsequently has been found that the presence of the sulfur increases the potency of this series of compounds to a variety of serine esterases. Whether the pi electrons of the sulfur act to mimic the olefin of JH or are involved in hydrogen bonding with the active site of the JH esterase is not clear. However, the sulfur bestowes an average of 2.5 kcal/mole of additional bond energy with the JH esterase (Abdel-Aal & Hammock, 1985b). This bond strength is in the range of that of typical hydrogen bonds. It has been noted by Musker (unpublised observations) that there is a surprisingly strong hydrogen bond between the beta-sulfur and the hydrated carbonyl in crystal structure. Thus, the exact role of the sulfur remains hypothetical.

This series of compounds proved useful for a variety of reasons. One reason was that they were active *in vivo* and resulted in an extended feeding state in *T. ni* as would be expected. Since the compound used in this study proved very selective for JH esterase compared to other hemolymph esterases, it was strong evidence for the involvement of JH esterase in clearance of JH. It is hoped that both the selective organophosphate and trifluoroketone inhibitors of JH esterase will be useful in numerous of laboratories to investigate the role of JH hydrolysis in a variety of organisms and to inhibit JH metabolism when investigating the biological activity and binding of JH.

Practical Application of Trifluoroketones and Transition State Theory

It is unlikely that inhibition of JH esterase will be of applied significance for a variety of reasons. First, the enzyme is produced in large excess relative to JH, and one must inhibit the greater proportion of the enzyme in *T. ni* to obtain a biological effect. Kinetic support for this statement is provided below. The biological effect is similar to that obtained with juvenoids in that only certain stages are sensitive. Thus a great deal of damage would be done before the compounds can act. This has been an objection to the use of juvenoids on field and row crops. Although this objection does not rule out the use of juvenoids in agricultural situation, it certainly indicates that different pest management strategies and economic thresholds will have to be used, and thus use of juvenoids will meet resistance from designers of pest management systems based on quick acting compounds. The same cautions would apply to JH esterase inhibitors. Like juvenoids, the biological effect may be to extend the last larval stadium and thus increase damage. Finally, these

compounds are far less active than juvenoids while offering no major advantages.

As will be discussed below, the major advantage offered by these compounds is in advancing our understanding of metamorphosis and the biochemistry of JH esterase. However, this series of compounds may have application to insect control based on the endocrine system, to other areas of toxicology, and certainly as an illustration of a useful paradigm for the development of selective drugs and toxins.

Use in Endocrinology

It is possible that JH esterase will have a more critical role in some species or that the enzyme has as yet undiscovered roles in different stages of pest insects. If so the present compounds may have utility. Synthesis of compounds which had a structural backbone very similar to JH yet contain the trifluoroketone moiety failed to increase the inhibitory potency of the compounds to the JH esterase of *T. ni*. However, the compounds do bind to JH esterase with a high affinity. If this functionality were added to highly active juvenoids such as the poly aromatic compounds under development by several companies, the compounds' activity might increase due to inhibition of the esterase and the fact that the esterase could bind, protect and then release the juvenoid at the most critical time in the insects' development.

Use in Behavior

Ferkovich and coworkers (1973, 1982) as well as a variety of subsequent authors have emphasized the importance of antennal esterases in the catabolism of some pheromone acetates (see Morse & Meighen, 1984 for recent literature). Thus some years ago we prepared high specific activity radiolabels for the two major components of the sex pheromone of *T. ni*. The minor component was produced by tritiating lauryl alcohol by the Wilzbach process and then reacting it with acetic anhydride, while the major component was made by reacting Z_7-dodecenol with tritiated acetic anhydride. Based on these labels, rapid assays for the esterase activity in male antennae were developed based either on thin layer chromatography on prelayer plates or on partitioning of the radiolabeled acetic acid released by the pheromone. As expected, the trifluoroketones were potent inhibitors of this esterase preparation.

$CH_3(CH_2)_{11}OAc$

$Z_7\ CH_3(CH_2)_3CH=CH(CH_2)_6OAc$

Two Components of *T. ni* Pheromone

Thus, trifluoroketone mimics of the major and minor components of the *T. ni* and the *Pectinophora gossypiella* (pink bollworm) pheromones were made. The resulting compounds had slightly higher volatility than the natural compounds as determined by gas liquid chromatography and smelled identical to the natural materials. This later point has no behavioral significance, rather it illustrates how similar the trifluoromethyl ketones were to the natural compound using a critical, but poorly defined detector. Thus, 1, 1, 1-trifluoro-2-pentadecanone and Z-1, 1, 1-trifluoropentadec-10-ene-2-one should mimic the above two components of the *T. ni* pheromone while $Z_8\ Z_{12}$ 1, 1, 1-trifluorononadec-8,12,-diene-2-one mimics one of the two major components of the pheromone of *P. gossypiella*.

$CH_3(CH_2)_{12}C(O)CF_3$

$Z\ CH_3(CH_2)_3CH=CH(CH_2)_7C(O)CF_3$

Trifluoromethylketone Mimics of the *T. ni* Pheromone

The trifluoromethylketones demonstrated similar or greater response than the natural compounds on electroantennagrams. In each case the duration of the response was longer for the trifluoromethylketone than for the natural compound presumably because it could not be metabolized by the antennal esterases. Other trifluoromethylketones such as the corresponding benzyl compound gave only a background response on an electroantennagram as did related compounds lacking the trifluoromethylketone moiety. However, the benzyl trifluoromethylketone was able to prolong the response caused by the natural compounds, presumably by inhibiting the antennal esterase.

Based on laboratory and limited wind tunnel experiments it was hoped that these comounds might force the male moths to move out of the pheromone plume as they oriented up wind due to a prolonged response caused by esterase inhibition. However, in the field Gaston found that the trifluoromethylketone mimics of the two major components of the *T. ni* pheromone and the benzyl trifluoromethylketone

failed to enhance or reduce the attraction of male moths to pheromone baited traps. In addition, these compounds had little biological effect when placed alone in traps.

Thus, again these compounds failed to have a useful field application. However, their strong response on electroantennagrams indicates a similarity to the natural compound which might be exploited in other species. It is hoped that these compounds will be useful to insect behaviorists and the affinity columns discussed below could be very useful in isolating these biologically interesting enzymes.

Inhibition of Toxicologically Significant Esterases

As one varies the substituent on the trifuloroketone moiety, the selectivity for the inhibition of a variety of different esterases can be modified. For example Ashour has shown that the compounds can inhibit a variety of carboxyesterases from mammalian liver acting on substrates such as malathion, diethyl succinate, 4-nitrophenyl acetate, and clofibrate. As with JH, these compounds also can act as synergists *in vivo*. The compounds then can be used to test the role of esterases in xenobiotic metabolism *in vivo*, to distinguish among mammalian carboxylesterases by differential inhibition, and to study the catalytic mechanism and possibly to purify these enzymes.

In addition to the role of esterases in xenobiotic metabolism, some of these enzymes are certain to have physiological roles. Possibly one such enzyme is Johnson's neurotoxic esterase. Inhibition of this enzyme followed by aging is associated with a delayed neuropathy resulting in loss of peripheral nerves. The late Ron Talcott found that some of the trifluoromethylketones reported to be very active as inhibitors of JH esterase were potent but reversible inhibitors of the neurotoxic esterase from human brain. In addition, the compounds could block the inhibition of the enzyme by the potent delayed neurotoxin, mepafox. Thus, the trifluoromethylketones may have utility in purifying and studying this enzyme. It is possible that one could block the appearance of delayed neuropathy by rapid administration of the appropriate trifluoromethylketone to protect the neurotoxic esterase from irreversible binding. Since some of these compounds reversibly inhibit acetylcholinesterase, they could be used like phosphonates to protect this enzyme as well.

The above enzymes are important to study in nontarget species since the selectivity of many pesticides is based on the differential metabolism by carboxyesterases. Similarly, carboxyesterases are important to study in target species because they may be responsible for resistance. In addition to providing basic information on these enzymes, it is possible that polarized ketones can find use as synergists or that this functionality can be incorporated into other structures such as pyrethroids to enhance their biological activity.

In addition to the applications discussed above and affinity purification outlined below, the compounds may have utility in investigating the mechanism of esterase action. As NMR's are developed with better signal to noise ratios, larger probes and greater field strength, the use of F^{19} spectra will become more routine. These compounds offer the possibility of examining a very sensitive nucleus not present in most biological molecules as it interacts with enzymes. The chemical shifts of F^{19} as a trifluoromethylketone reacts with an enzyme or hydrates are very large and this nucleus also is useful for reflecting its hydrophobic environment.

Transition State theory as a **Paradigm**

In the foreseeable future, the fine tuning of biological activity to obtain useful drugs or agricultural chemicals will be done largely by activity directed synthesis. Selectivity between target and nontarget organisms probably will be detected in the greenhouse or field. However, total reliance on a random synthetic approach to discover initial biological activity is no longer economically acceptable. The frequency of discovery of lead structures and novel biological activities will increase in the future for many reasons. Certainly among these reasons will be sysnthesis directed at inhibiting key enzymes. In the past this synthetic effort often has been directed at mimicking the substrate or product and in some cases the attachment of a reactive functionality. Work on suicide substrates is helping to refine synthetic approaches leading to highly active molecules. Similarly, the idea of designing compounds to mimic transition states or transient intermediates rather than substrates or products may be useful. The above work on JH esterase illustrates one such approach, but two other examples will be cited from somewhat distant and

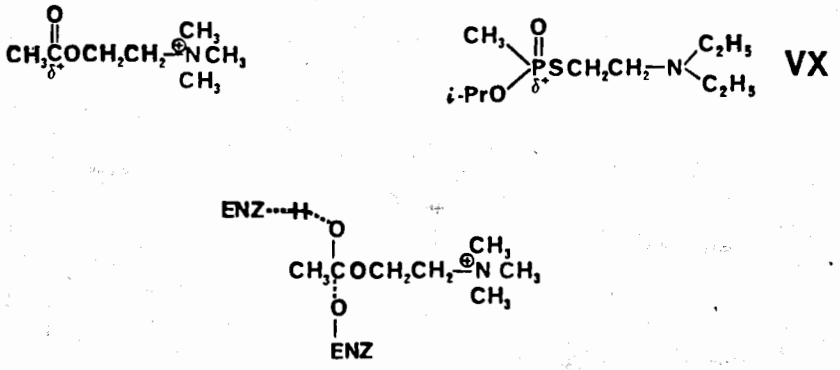

Fig. 3. Similarity of an organophosphate inhibitor of acetylcholinesterase to both the substrate acetylcholine and a tetrahedral transient intermediate probably similar to a transition state along the reaction coordinate. Note that the pentavalent phosphate center is tetrahedral.

recent history.

Optimization of a wide variety of compounds inhibiting acetylcholinesterase was done on the basis of mimicking the substrate, acetylcholine. It has been argued by Abdel-Aal and Hammock (1985b) based on theoretical grounds that N-methyl carbamates are actually transition state mimics. A similar argument can be made with organophosphates as illustrated in Figure 3. One can assume that initial binding of the organophosphate to acetylcholinesterase is due to the polarized P=O group mimicking the carbonyl group of the substrate. However, one can also assume than the affinity of organophosphates for acetylcholinesterase is due to pentavalent, tetrahedral phosphate mimicking both the trivalent carbonyl and tetravalent tetrahedral intermediate. Thus the phosphate group must have some similarity to several structures along the reaction coordinate. Probably the tight binding of the phosphate to the target enzyme before phosphorylation is due, in part, to its resemblance to a transition state which binds very tightly to the enzyme. Since so many compounds have been made in this series, it is unlikely that a new paradigm will aid in further discovery in this group of insecticides, but it may be useful in exploiting other enzyme systems.

One possible area of interest is the sulfonyl urea herbicides where the kinetics of enzyme inhibition by these compounds is suggestive of slow-tight binding inhibition. This type of inhibition often is seen with transition state mimics. As will be discussed below, the trifluoromethylketones have proven extraordinarily successful in affinity purification. In the future one might consider using biologically active compounds to isolate the receptor upon which they act. The receptor in turn could be used for further optimization of structure, the discovery of new lead structures, and possibly could be exploited directly through biotechnology as discussed in the CONCLUSION below. This approach could be very useful with the benzoylphenyl urea insecticides where a detailed knowledge of their mechanism of action is still lacking and where knowledge of such a mechanism could lead to the discovery of improved structures. In this example many of the same compounds used as haptens for the development of pesticide immunoassays also could be used for isolation of a biochemical target by affinity chromatography.

APPLICATION OF TRIFLUOROKETONES IN AFFINITY PURIFICATION

Since some trifluoroketones have been shown to bind selectively and tightly to JH esterase it seemed reasonable to use them in an attempt to affinity purify the enzyme. To this end butane dithiol was reacted with 3-bromo-1, 1, 1-trifluoroacetone to give the corresponding monosubstituted thiol. This compound in turn was attached to epoxyactivated Sepharose resulting in a trifluoroketone immobilized on a solid support. This affinity ligand proved to be extraordinarily successful in that a few microliters of gel

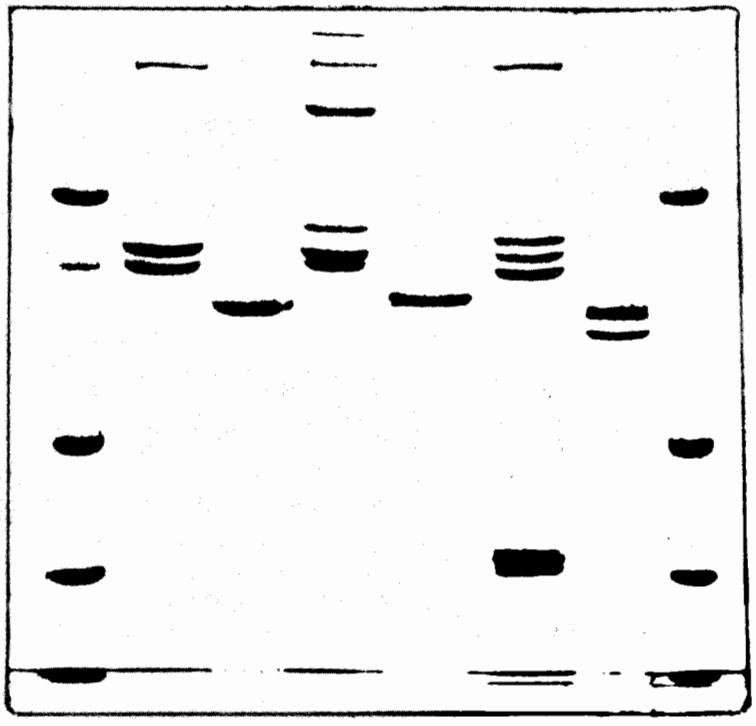

Fig. 4. SDS PAGE gel of crude hemolymph and affinity purified JH esterase from three species. Tracks are described from left to right. Track 1. Standards. Track 2. Hemolymph from *Manduca sexta*. Track 3. JH esterase from *Manduca sexta*. Track 4. Hemolymph from *Heliothis virescens*. Track 5. Hemolymph from *Heliothis virescens*. Track 6. Hemolymph from *Bombyx mori*. Track 7. JH esterase from *Bombyx mori*. Track 8. Standards.

could extract all of the JH esterase from many milliliters of hemolymph.

By optimizing conditions this affinity column proved successful in the isolation of JH esterase from 3 strains of *Bombyx mori, Heliothis virescens, Heliothis zea, Manduca sexta,* and *Trichoplusia ni* (Fig. 4). In most cases the recovery of enzyme activity was very high and the SDS gel in Fig. 4 indicates that purification was to apparent homogenity in species which had but one JH esterase. Fluorograms following the radiolabeling of the apparent catalytic site of the enzyme with ^{3}H paraoxon further indicated apparent homogenity in most cases and that multiple esterases rather than impurities were contained in the other preparations. The high yield of the enzyme obtained from this column and the almost 1000X purification often obtained has allowed the determination of a partial sequence from the *M. sexta* enzyme. In is likely that this and slightly modified columns will prove very useful in the purification of esterases from a variety of tissues and species.

EXPLOITATION OF KNOWLEDGE OF INSECT METAMORPHOSIS

Development of Hormone Agonists and Antagonists

The exceptionally high activity of juvenoids as well as their selectivity provides ample evidence for the utility of the endocrine system as a target for the development of insect control agents. Numerous sites of attack are available including disruption of biosynthesis, release and processing of both epithelial and neurohormones, further development of both mimics and antagonists of these hormones, and a variety of other approaches as yet unanticipated. However, it is possible that basic research has progressed far enough on several fronts that direct work on exploitation can commence. For example, the studies on PTTH by Ishizaki, Suzuki and associates (1984) will soon have progressed to the point where they can be exploited by a variety of techniques.

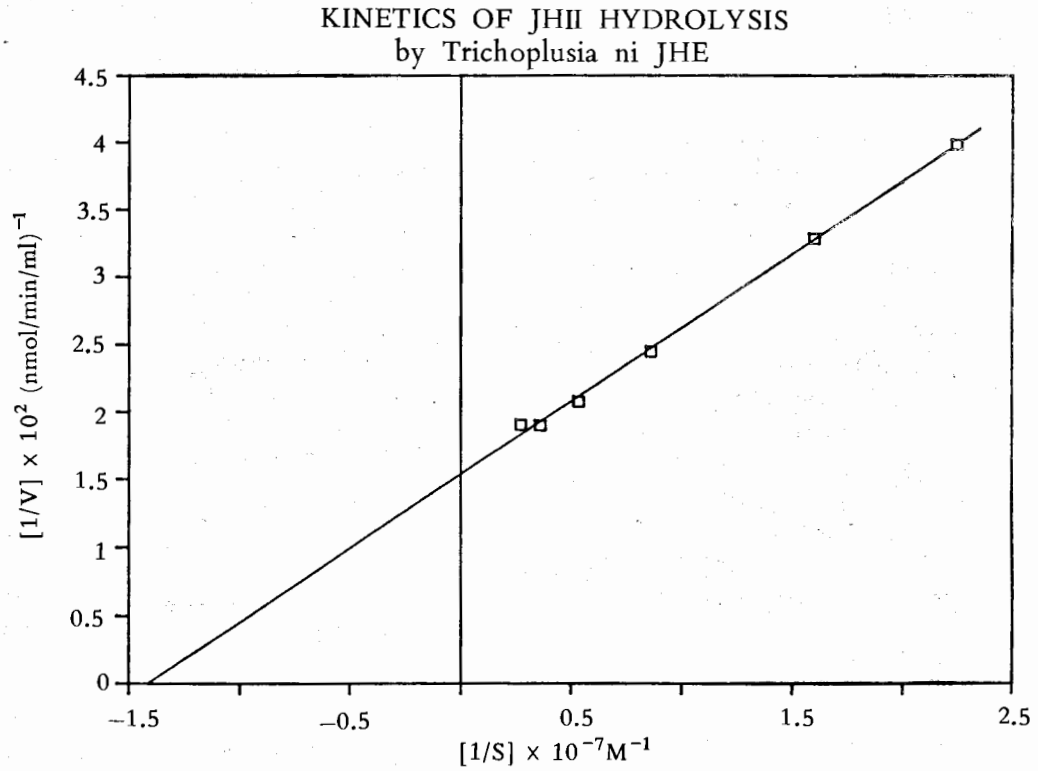

Fig. 5. Lineweaver Burk plot of the kinetics of hydrolysis of JH II by the JH esterase of *Trichoplusia ni*. For details see Table I.

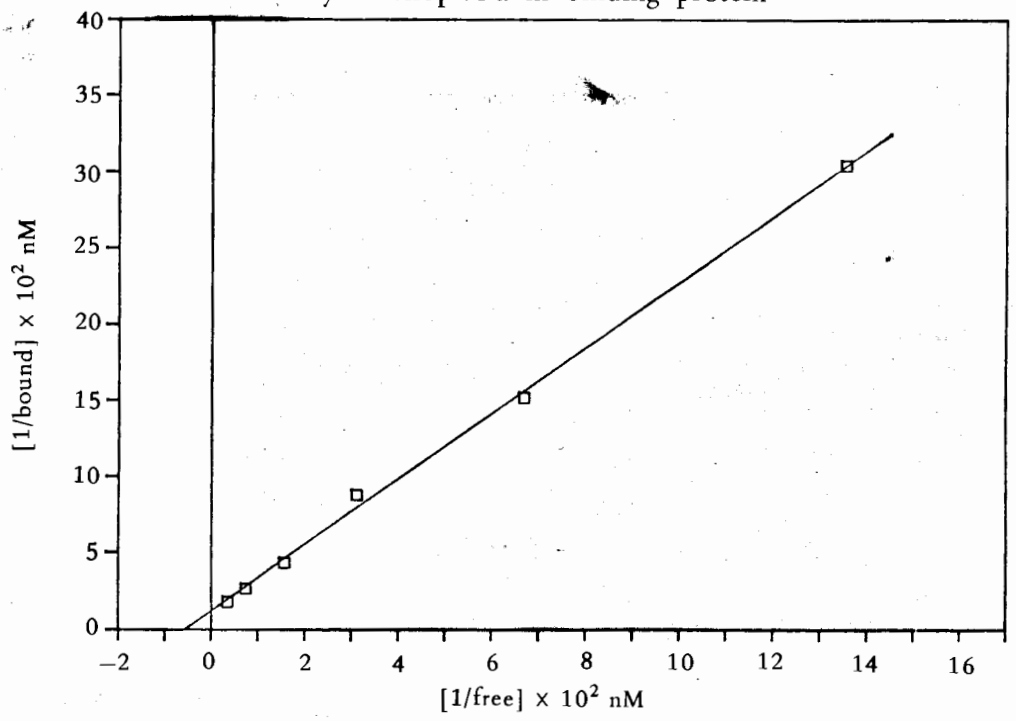

Fig. 6. A bound/free plot of the binding of JH II to the hemolymph binding protein of *Trichoplusia ni*. For details see Table I.

Our current effort is to exploit our knowledge of JH esterase for insect control. It was discussed above that inhibition of this enzyme does not appear to be a promising approach. However, inducing the artificial production of the enzyme could well be profitable. Kinetic studies indicate that when the enzyme is present in much higher quantities than the intrinsic hormone titer, substantial reductions in the titer of JH can be anticipated (Table 1).

For example, the trifluoromethylketones have been very useful in obtaining kinetic data on JH hydrolysis and binding in hemolymph. These and other inhibitors allowed us to study the binding protein without interference from JH esterase. They allowed the estimation of catalytic sites in the whole hemolymph due to the slow tight binding nature of their inhibition by JH esterase, and they ultimately allowed the isolation of the enzyme.

Kinetic studies on the hydrolysis of JH II by prewandering JH esterase from *T. ni* hemolymph (Fig. 5) and JH II binding to and dissociation from the binding protein of the same but inhibitor treated hemolymph (Fig. 6) were done to construct a limited model describing the capacity of the hemolymph to hydrolyze JH in the presence of the binding protein. These and other significant kinetic parameters are shown in Table 1.

The JH II titer in *T. ni* is far below the K_m of JH esterase. Therefore the k_{cat}/K_m ratio is the right kinetic parameter for projecting the capacity of JH II hydrolysis under *in vivo* conditions. A value of $4.5 \cdot 10^8$ M^{-1} min^{-1} for this parameter is near the diffusion controlled encounter of the enzyme and the substrate. This coupled with the often high molar equavalency of the esterase argues against using inhibitors of this enzyme for practical insect control. One can extend the argument since JH esterase can even hydrolyze the bound form of JH II on the binding protein since in the whole thermodynamic box of these components the $t_{0.5}$ for JH II hydrolysis is about 150 times shorter than the $t_{0.5}$ for the association of the JH to the binding protein. Therefore, a substantial reduction in JH titer is anticipated when even a small amount of the esterase is present.

Natural Anti-Juvenile Hormone Effects

Precocious reductions in JH titer have profound developmental effects as amply illustrated by the development of several classes of chemical anti-juvenile hormones. Such apparent anti-JH effects also occur in nature. One such example is shown in Fig. 7. In this case when a prasite in the genus *Chelonus* stings the egg of a noctuiid larva such as *T. ni* a parasite egg is laid within the host egg. The host

Table 1 Kinetic Parameters of JHE and JH Binding Protein(s) from *T. ni* using JH II as Substrate.

Kinetic parameter	JHE	JH Binding Protein
Molar concentration in the plasma	1.49×10^{-8}	8.1×10^{-6}
k_{cat}	31.8 min^{-1}	—
K_M(M)	7.06 1 10^{-8}	1.75×10^{-7}
V_{max} nmoles/min/ml	65.0	—
$k_d min^{-1}$	—	0.0932
$k_a M.^{-1} min^{-1}$	—	5.33×10^5
k_{cat}/M_M $M.^{-1} min^{-1}$	4.50×10^8	—
	$[k_{cat}/K_M] \times E_t$ (Min.$_{-1}$) 6.72×10^2	$k_a \times$B.P. concentration (Min.$_{-1}$) 4.32
$t_{0.5}$ Sec.	0.062	9.625
Relative $t_{0.5}$	1.00	155.24

undergoes apparently normal development, but will form a prepupa one instar early. The diminutive prepupa of the host spins a silk cocoon and then development of the host halts while the parasite rapidly gains weight, exits and consumes its host, and then spins its own cocoon inside that of its host. In some cases the parasite fails to develop, yet some event early in the host's development causes the host larva to pupate precociously (Jones *et al.*, 1981; Jones 1985; Buhler *et al.*, 1985).

The mechanism by which the parasite causes the precocious development has not been elucidated, however it does not appear to be a simple anti-JH effect. Rather a complex series of metamorphic events including the appearance of JH esterase occur one instar early (Fig. 2). These observations indicate that the appearance of JH esterase is one of the earliest developmental events signaling metamorphosis. Thus an understanding of its regulation seems likely to shed light on the events which trigger the onset of metamorphosis. The biological effects of parasitization by *Chelonus sp.* indicates that materials with anti-hormone effects are promising candidates for the control of Lepidopterous larvae since they eliminate the last and most destructive larval instar of the pest (Buhler *et al.*, 1985).

Use of JH Esterase Message

At some point we may be able to exploit the structure of the factors which initiate the series of metamorphic events or even those factors which cause the initial production of JH esterase. However, more fundamental information on the biology and biochemistry of the metamorphic event is required before one can move in the above direction. However, our present knowledge of the amino acid sequence of JH esterase can lead directly to the isolation of its message. As will be explained below such information can be used with existing biotechnology for insect control. In addition such molecular probes will assist greatly in searching for the factors which regulate other events in metamorphosis. For instance isolation

Fig. 7. Effect of the egg-larval parasite *Chelonus* sp on the development of *T. ni.* The mature male wasp is shown in addition to a normal wandering fifth stage larva (largest larva) and a normal penultimate larva (smallest larva). Successful parasitization and even unsuccessful parasitization result in small larvae which wander one or two instars early (two intermediate sized larvae). Each of these wandering larvae will spin a cocoon, but only the normal fifth stage larva will pupate.

of unique mRNA's that appear before the JHE message may yield these factors which will prove still more useful in insect control.

Key experiments run by Sparks indicate that JH esterase produced at inappropriate times may be lethal to the insect. In early instar *M. sexta,* injection of picomoles of affinity purified JH esterase caused the clear anti-JH symptom of darkening of the epidermis. In *T. ni* prepupae the enzyme delayed pupation as does administration of chemical anti-juvenile hormones (Sparks *et al.*, 1985). For years it has been assumed that knowledge of the structure of a peptide or protein could only be used for leads in directed synthesis programs. Such directed approaches to pesticide discovery in which fundamental knowledge of biology stimulates sysnthetic effort has been termed a **Third generation approach to pesticide chemistry** (Sparks & Hammock, 1983; Hammock, 1985). As crystal structures are used with computer aided design, we can anticipate that such third generation approaches will become even more fruitful. Yet there are indications that such knowledge also can be exploited directly for insect control using biological means.

Isolation of the message for a low abundance protein is complex yet apparently straight forward if an amino acid sequence adequate to develop an unambiguous probe can be obtained. The affinity purification system described above yields sufficient JH esterase for sequence analysis. Not only is this genetic information of value in the study of the regulation of the enzyme, but it can be exploited directly. Work from the laboratories of Summers and Miller have indicated that the baculovirus isolated from the noctuiid *Autographa californica* contains a powerful promoter which can be used to induce insect cell lines to produce foreign proteins such as beta-interferon and beta-galactosidase (Smith *et al.*, 1983; Pennock *et al.*, 1984). Recently, Maeda *et al.* (1985) have used a related nuclear polyhedrosis baculovirus as a vector to transfer the human alpha interferon gene to last instar larvae of *Bombyx mori*. The infected larvae were found to produce microgram amounts of interferon in a time dependent fashion.

Along this line a number of laboratories are working on the hypothesis that with improved vectors, the genetic sequences for key peptides or proteins could be used to kill pest insects. Neurohormones and receptors are likely targets for such work, yet sequence information is available for only a limited number of hormones and no receptors from insects. In addition, it is yet unclear what processing and secretion signals are required by nonendocrine cells expressing these peptides unnaturally. Selective toxins are another target for incorporation yet they may have two disadvantages. One disadvantage is that regulatory agencies may be slow to approve genetically engineered toxins for use even if they are highly selective. Also, if the resulting toxin kills the viral infected cells, the infection of the organism may be terminated before lethal concentrations of the toxin are produced.

In contrast, JH esterase offers some advantages as a target. As a naturally secreted enzyme with a high turnover number and low K_m, its effectiveness will be amplified. Since the major tissues of the insect larva are known to produce the enzyme, one can anticipate the processing machinery to be in place. How such production will be influenced by the regulatory mechanisms for JH esterase is uncertain. Since it is an endogenous enzyme of the pest insect, it may face fewer regulatory hurdles than toxins. Finally, JH esterase is anticipated to disrupt the development of the entire animal and rapidly halt feeding prior to the most damaging last larval instar. Yet is should not be toxic to any individual cell and its presence should not impede the proliferation of the virus. Thus, it is possible that our fundamental knowledge on JH esterase can be exploited directly using biological approaches. It is this direct exploitation of biological knowledge for insect control which has been termed a **Fourth generation approach to pesticide development** (Hammock, 1985).

CONCLUSIONS

The above discussion illustrates how chemistry and biology have been combined to enhance our understanding of an important biological system. In this case a partial understanding of a biological system has led to the synthesis of a series of very potent, biologically active compounds using a paradigm known as transition state theory. These compounds, in turn, have allowed us to determine some of the properties of juvenile hormone esterase, to demonstrate its biological role and finally to purify this low abundance enzyme in high yield. Sequence information obtained upon the pure enzyme may allow the isolation of the message and gene for JH esterase. The gene may be of direct utility in the development of bioengineered pesticides, however the resulting probes certainly will be of value in furthering our understanding of metamorphosis. A deeper understanding of this critical developmental event is certain to delineate further targets in pest insects which can be exploited for insect control by both classical chemistry and by biotechnology. Of the many problems in pesticide development, the uncertainty and high expense of discovery is among the most critical. However as argued below, the combination of rapidly advancing technologies is certain to increase our probability of discovering bioactive compounds. In Figure 8 an argument is presented that we must make the most of and integrate all approaches to pesticide development. Ordish (1967) and Williams (1967) defined first through third generation pesticides based

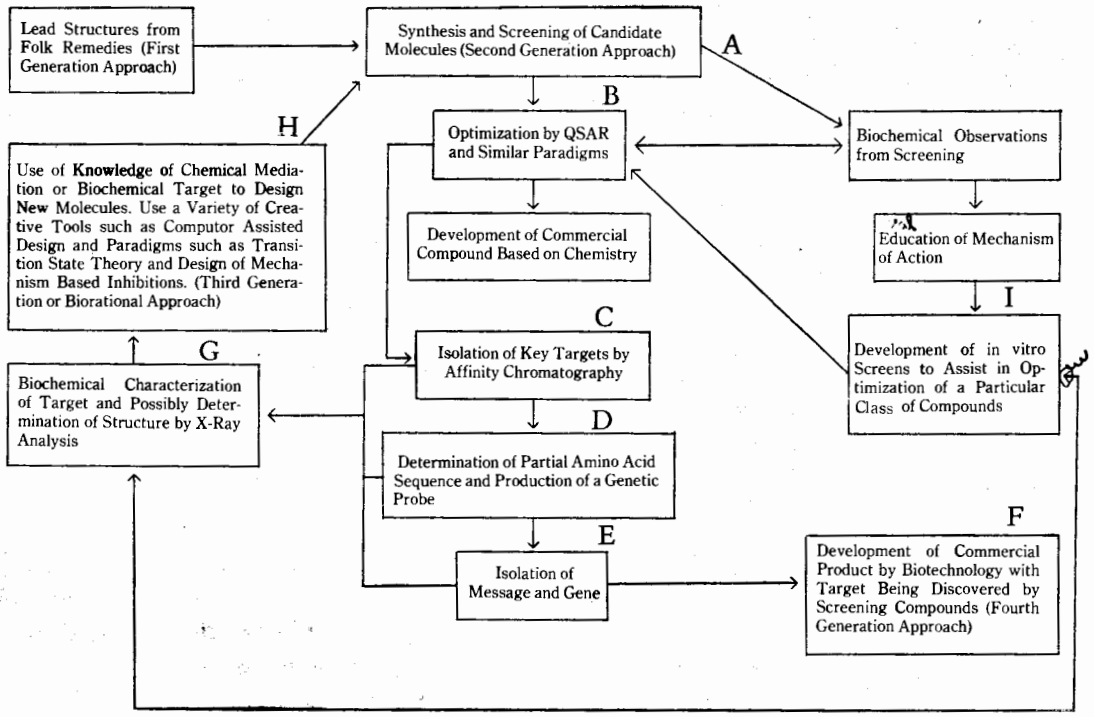

Fig. 8. Flow diagram of methods to integrate approaches in the development of bioactive molecules. This diagram indicates that first through fourth generation approaches to the discovery of biologically active molecules complement each other and should be used in combination. Novel suggestions are that second generation approaches (A and B) can lead to the isolation of essential biological molecules in pest species by affinity chromatography (C). Once the biological target is isolated straight forward biological techniques can in some cases lead to the development of a commercial product using biotechnology (F). This same work can lead to enhanced knowledge of a key target (G) which may be exploited using a combination of third (H) and second generation approaches (A).

on compound type. It seems more reasonable to categorize pesticides based upon the approach used to discover them (Sparks & Hammock, 1983; Hammock, 1985). Thus, first generation compounds are based on folk remedies, second generation compounds arise from screening and structure activity studies, third generation compounds come from biorational approaches and fourth generation compounds from direct exploitation using biotechnology.

The second generation approach has resulted in the discovery of the majority of compounds on the market. Although this approach is unattractive due to the large number of compounds that must be synthesized for each active material, other approaches used in isolation usually have failed. It is most reasonable to assume that the second generation approach will be the mainstay of the industry with lead structures arising from first and third generation approaches. Third generation approaches (H, Figure 8) are anticipated to play an increasing role in discovery of active molecules and in stimulating creative thought among chemists. This process is certain to occur as new paradigms for the discovery of active molecules evolve and as computor aided design becomes increasingly available to the bench chemist.

It is important to address some of the novel approaches outlined in Figure 8. In many cases, biological activity is found, but even after massive attempts at optimization, the activity is not good enough to be commercialize. It is no longer acceptable to waste this expensive research by storing and forgetting it in company files. Even if a series of active compounds does not appear promising to commercialized, the compounds possibly can be used to study and isolate the biochemical target, be it receptor or enzyme. Undoubtedly structures valuable for use in affinity chromatography were synthesized in approaches A and B during an attempt to optimize structure. Thus, isolation of a target now known to be essential for survival of a pest species can be accomplished rapidly. The biochemical techniques needed to characterize a macromolecule and to isolate its gene and/or message are becoming increasingly straight forward (Figure 8, D and E). In some cases this information can be exploited directly by biotechnology to produce new products for agriculture (F, Figure 8).

The partial reliance of biotechnology efforts on knowledge generated from synthesis and screening clearly is illustrated by recent work on the molecular biology of resistance to the herbicides glyphosate and the sulfonyl ureas. It is critical to realize that unsuccessful leads can be exploited by biotechnology as well as commercial products. Thus, there should be a close relationship between biotechnology efforts and classical synthetic efforts.

This additional knowledge about a critical target also can be exploited in terms of rational synthesis based on now proven paradigms for discovery. One of the more elegant ways would be to generate a crystal structure of an affinity purified or genetically engineered pest target (8, Figure 8). We should not anticipate that commercial products will be developed exclusively by computer in the near future, but the value of such creative tools as computer aided design in reducing on the average the number of compounds which must be synthesized and tested for a success is certain to be dramatic. Such knowledge will help in developing better *in vitro* assays which occasionally help in structure optimization. Thus, biochemical knowledge about a target is much less expensive to obtain now than even a few years ago. This knowledge not only has the marginal advantage of providing better *in vitro* bioassays, but it can be used directly to design active materials using both third and fourth generation approaches. Thus, we feel that progress in the pesticide field will occur at an accelerating rate. This scientific progress coupled with beneficial changes in regulatory policy, development strategies, and social acceptance will help us to again attack problems in pest control in both developed and developing countries.

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