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[12] Biomechanisms Regulating Growth and Development

George L. Steffens and Theron S. Rumsey, Editors

Invited papers presented at a symposium held May 3–7, 1987, at the Beltsville Agricultural Research Center (BARC), Beltsville, Maryland

Organized by the BARC SYMPOSIUM XII COMMITTEE
Theron S. Rumsey and G. L. Steffens, Co-Chairpersons

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FOREWORD

The Annual Beltsville Symposium provides a forum for interactio among scientists involved in research that is vitally important to agr culture and to the agricultural sciences. The Twelfth Symposium in th series focused on the unifying biochemical and physiological mecha isms controlling growth and development of biological systems – an mals, plants insects. Unraveling the complex biochemical mechanism associated with the sequencing of organism growth and developme and identifying, locating, and manipulating key control mechanisms a essential in utilizing the full potential of biotechnology for improving t composition and quality of agricultural products and the profitability agriculture. Accordingly, speakers directed their remarks to basic aspec of biological mechanisms in their area of specialization with consider tion given to current status, future direction, potential impact, ar limitations to progress.

The Symposium addressed fundamental questions in:

- Tissue specific gene regulation: cell division and differentiation
- Mechanisms for regulating hormone concentration
- Hormonal regulation of growth and development
- Non-hormonal regulation of growth and development
- Nutritional regulation of growth and development

Because the backgrounds of the symposium attendees covered a wi spectrum in the basic biological and physical sciences, each topic w introduced by a brief overview, but general reviews were avoided in fav of findings from on-going research projects.

The symposium brought together a distinguished group of invit scientists from around the world who are leaders. Many attendees ma poster presentations which increased the exchange of ideas and s mulated informal discussion.

Emerging technologies for controlling growth and development w contribute markedly to improving the quality of the human diet and the well-being of agricultural industries.

WALDEMAR KLASSEN, Director

12] Strategies for the discovery of insect control agents: exploitation of biomechanisms regulating insect development

B.D. HAMMOCK, L.G. HARSHMAN, M.L. PHILPOTT, A. SZÉKÁCS, J.A. OTTEA, R.A. NEWITT, V.J. WROBLEWSKI, P.P. HALARNKAR and T.N. HANZLIK
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Abstract The development of environmentally acceptable methods of insect control reamins critical to agricultural productivity and profitability in developed and developing countries. In this laboratory we are attempting to exploit knowledge of endocrine regulation of larval-pupal transformation in the Lepidoptera to illustrate new paradigms for the development of insect control agents. One approach employed transition state theory to design powerful inhibitors of an esterase which specifically removes insect juvenile hormone and initiates pupation. Inhibition of this enzyme retards pupation. These inhibitors were used as affinity ligands to purify the enzyme. Injection of the enzyme disrupts normal insect development so methods to elicit precocious expression of the enzyme represents a second approach to developing control agents.

Introduction

The problems facing both US and world agriculture are changing as fast as the technologies which we can bring to bear on them. Only a few years ago we faced the problem of world hunger, and the solution of higher productivity seemed obvious. Agricultural science has been extraordinarily successful during the last few years in increasing food production in developed as well as in many underdeveloped countries. We have been so successful that many consumer countries have now become net exporters of food, and the agricultural community in this country is depressed in part due to overproduction.

Need for enchanced profitability in agriculture. We clearly cannot justify further applied research simply to increase productivity, yet starvation and malnutrition remain major problems in the world. Certainly some malnutrition stems from political instability, but it is trite to attribute agricultural problems simply to faults in distribution. Donations of food to depressed areas of the world help to relieve some immediate problems of starvation, but in both capitalistic and communistic societies, generosity is only a superficial solution. The long term solution comes from the ability of citizens of hungry nations to purchase food and the ability of producer nations to provide the food at a price that the world's hungry can afford. This goal can be accomplished by increasing the profitability of agriculture in both developed and develop-

ing nations. The goal encompasses the paradox that a sure way to increase our overseas market for high quality food is to improve the ability of developing nations to meet at least the minimum nutritional requirements of their populations.

Thus, our goal in research is turning from agricultural productivity to profitability. There will be many aspects to increasing profitability, but the control of pests will remain a key element in this goal. Hollingworth [1] and Hammock and Soderlund [2] cited a number of references outlining arguments that artificial vs. natural means of pest control are essential for high productivity and profitability in agriculture and that the use of such agents may in fact increase. The financial rewards are tremendous for the successful development of a pesticide. However, in a business community obsessed with the quarterly earnings statement and hostile takeover, the long term fundamental research needed for such success is unattractive.

Improving the rate of discovery. There are numerous approaches that can be taken to decrease the risk involved in the development of classical pesticides, and these are discussed elsewhere [1–3]. However, there are two solutions to this dilemma that can be addressed by fundamental research in regulatory biology. The first of these solutions involves increasing the rate of discovery of biologically active materials. Hammock *et al.* [4] and Geissbuhler *et al.* [5] discuss some of the paradigms that may lead to the discovery of new agents, and some of these approaches will be reiterated here. An alternative solution involves nonclassical approaches to the control of insect pests. A theme of this chapter to be further developed in the conclusion is that development of classical and nonclassical pesticides are not mutually exclusive endeavors.

Need for alternatives to classical pesticides. There are a number of reasons why alternatives to classical pesticides are attractive, three of which are listed. First, there is the hope that with nonconventional pesticides a large component of the registration and/or production costs can be eliminated. Maybe of greater importance is the hope that delays in registration can be avoided as well (this is critical if the time restraints of venture capitalists funding some of this work are to be met).

Second there has been the realization that industrial scale organic synthesis involves major risks, especially evident in the aftermath of disasters at the Sandoz plant along the Rhine and at Union Carbide's facility in Bopal, India.

A third reason to pursue alternative technologies is that pesticides may actually be banned in some areas. In the past, most of the compounds

removed from production had outlived their usefulness or presented significant risks to humans or to the environment. In the future, the heightened awareness of the public toward toxics in the environment may bring even greater restrictions on pesticide use. However, numerous organizations now exist to crusade for their version of a clean environment. Since perpetuation of the organization depends upon a succession of crusades, the banning of pesticides in general is a likely target. In states with a predominantly urban population, the banning of pesticides is a far more attractive goal for these organizations than more meaningful environmental crusades. They can depend upon a population that lacks the information and training to make an informed decision in this highly complex and controversial area, yet is convinced that pesticides offer far greater health risks than tobacco, diet or a host of other environmental chemicals. Since agriculture no longer is a dominant political force in many areas, some politicians may lack the courage to advocate a more rational stand. The complete banning of pesticides also is an attractive crusade because reduction of pesticide use in California, for instance, would not have the immediate impact on the daily lives of most people that more courageous steps to improve human and environmental health would entail.

Classical pesticides will continue to be the mainstay of agricultural production worldwide. However, a general ban on pesticides in even a very restricted area such as California, could represent a major market for innovative replacements. Complete banning of pesticides is unlikely to lead to a dramatic improvement in environmental health and could be disastrous to the agricultural economy in a single region. However, this worst-case scenario could foster a revolution in the development and implementation of alternative technologies. Such a large scale experiment would be very interesting in allowing us to evaluate the impact of the loss of pesticides on the economy, food quality and human health.

Exploitation of regulatory mechanisms. Regardless of whether one is searching for classical pesticides or alternative strategies, we still are faced with the problem of finding biological activity which can be exploited differently in pest and beneficial species. Thus the topic of this years' Beltsville Symposium in Agricultural Research on Biomechanisms Regulating Growth and Development is very appropriate. Regulatory systems are key to the survival of any species. In these systems we see both marked diversity and surprising levels of conservation. It also has been argued that disruption of a regulatory system in pest control offers distinct advantages; small changes in a regulatory system may result in a profound change in the ability of the organism to survive or reproduce.

At the University of California in Davis, we have been attempting to exploit the regulation of larval-pupal transformation in lepidopterous insects as a target for the development of both classical and nonclassical pest control agents. We have targeted one insect family, Noctuidae, as representing a group of pestiverous insects capable of supporting the development of an insecticide, should a family specific material be found. In our work we attempt to integrate both applied and fundamental research. Certainly, all of the advances in this field are based on a fundamental knowledge of the insect endocrine system. Possibly of greater long term importance, the agents which we have developed in an attempt to disrupt insect development for agricultural application also can be used as probes to extend our fundamental investigations of insect development biology. A second approach to the development of insect control agents is to integrate aspects of chemistry, biochemistry and molecular biology to develop bioactive materials. This approach will be discussed in greater detail in the conclusion to this chapter.

The insect endocrine system as a target for disruption

Rationale for target selection. The endocrine system has been targeted because it is hoped that the amplification of a response resulting from the disruption of a regulatory system will lead to highly active materials. The complexity of the endocrine system of insects is only now becoming appreciated as many new peptide mediators are being described. The system of peptidergic chemical mediators is a promising target for insect control, but in this chapter we discuss work on the epithelial hormones which control metamorphosis.

Metamorphosis is common to our most damaging insect pests yet appears to have evolved independently in insects. Thus, there is the hope of developing insect specific control agents. Some insect pests have been controlled by delaying metamorphosis, whereas many scientists hope to control insects as discussed later by accelerating metamorphosis. This acceleration should terminate the feeding phase of crop pests and lead to the death of the insect.

Overview of endocrine regulation in Lepidoptera. The regulation of metamorphosis is certainly much more complex than the "high-low-no" hypothesis formulated in the 1960's and quoted in most entomology textbooks. However this simple model is adequate background to understand most aspects of the approach presented in this chapter. According to this hypothesis, an increase in the level of the oxygenated sterol, 20-hydroxyecdysone, leads to a molt. In the presence of the terpenoid hormone, juvenile hormone (Fig. 1), the molt will be largely isometric

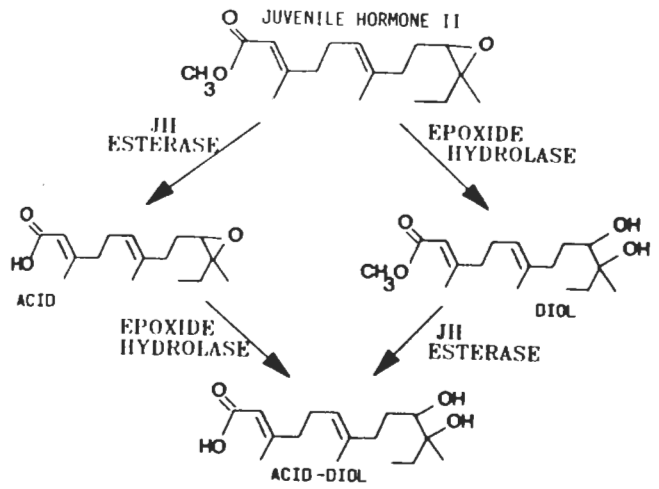


Figure 1. Structure and hydrolytic pathways of metabolism of juvenile hormone II. The juvenile hormones are terpenoid methyl esters containing a 10,11 epoxide moiety. The juvenile hormone homologs vary in the number and position of six rather than the usual five carbon isoprene units. Juvenile hormone II (shown) is the principal homolog in the Lepidoptera examined.

with an increase in size from one larval form to another. If there is a reduction in juvenile hormone at the time ecdysone is present, an anisometric molt will occur from a larva to a pupa [6]. This reduction in the titer of juvenile hormone near the start of the last larval instar is due to a variety of factors. Certainly there is a reduction in biosynthesis probably mediated by both innervation and neurosecretory innervation. However, there is a dramatic increase in the level of an esterase which is very efficient in hydrolyzing low titers of juvenile hormone and reducing it to very low levels. These low levels of juvenile hormone permit the release of the prothoracicotropic neurohormone (PTTH). This event, in turn, leads to a release of ecdysone which, in the absence of juvenile hormone, is leading to metamorphosis.

de Kort and Granger [7] provide an overview of the regulation of metamorphosis, whereas recent literature on the influence of PTTH on ecdysone production and the effects of ecdysone and juvenile hormone on protein expression are listed by Smith *et al.* [8] and Riddiford [9]. Since literature dealing specifically with juvenile hormone metabolism was reviewed recently [10, 11], the following overview will describe the current status of research and illustrate how an understanding of insect

metamorphosis can be used to develop leads for classical and nontraditional pest control agents. In this laboratory we have concentrated on disruption of juvenile hormone metabolism as a target.

Juvenile hormone metabolism

The presence of a number of sites for enzymatic degradation of the juvenile hormone molecule makes a comprehensive study of its metabolism formidable. Juvenile hormone contains ester and epoxide moieties that are subject to hydrolysis, as well as regions of unsaturation at carbons 2,3 and 6,7 that have the potential for oxidation and Michael addition. While exclusion of cofactors [12], addition of inhibitors [13] or selective extraction [14] have been used to exclude the effects of competing enzymes on the enzyme activity under study, the most widely used assays for the collective detection of all possible metabolites have employed thin-layer chromatography (TLC) [12, 15–17]. Whereas chromatographic assays tend to be labor intensive, the simultaneous assay of a number of different enzyme activities (in tissues where they are present together) probably more closely reflects the biological reality of juvenile hormone metabolism than do assays that detect only one type of activity at the exclusion of others [18].

Using such a TLC procedure, a scheme for juvenile hormone metabolism in Lepidoptera that emphasized ester hydrolysis and epoxide hydration was reported [15] (Fig. 1). Patterns of juvenile hormone metabolism vary with the species of insect studied [10], but two routes of metabolism are currently held as the predominant initial pathways for juvenile hormone metabolism in Lepidoptera. There is an indication of sulfate conjugation in some species [19]; however, pathways of conjugation have not been studied in detail. Conjugation with glutathione by glutathione *S*-transferase appears to have little role in juvenile hormone metabolism, at least in the insect species studied so far. These observations may not be surprising since both the epoxide and conjugated olefin are hindered and poorly reactive with glutathione.

The hydrolytic pathways of metabolism involving esterase and epoxide hydrolase appear to be more important in the species studied than oxidative pathways. Subsequent studies have concentrated mainly on ester hydrolysis possibly because of the availability of rapid assays [20]. In the Lepidoptera there is evidence that the enzymes which hydrolyze the highly stable conjugated ester of juvenile hormone are quite specific. For this reason the enzymes that metabolize juvenile hormone are termed juvenile hormone esterase.

Because the analytical methods are more difficult, research on juvenile hormone metabolism by epoxide hydrolases (EH) lags far behind work on esterases. This situation is unfortunate since a number of reports have demonstrated the occurrence of this enzyme activity in lepidopterans and other species [15, 16, 19] and have suggested that levels of EH may be a factor in the regulation of juvenile hormone titer [21]. However, this chapter will concentrate on the enzymes involved in ester hydrolysis of juvenile hormone in the Lepidoptera.

Regulation of juvenile hormone esterase

In most lepidopterans, two major peaks of hemolymph juvenile hormone esterase activity are found in the last larval stadium [22]. The first peak (associated with the prewandering phase of development) occurs early in the instar and supposedly correlates with a decline in circulating juvenile hormone titers. The second peak occurs late in the last stadium just prior to pupation. The enzyme(s) responsible for both peaks of activity appear to be similar electrophoretically and with respect to other biochemical characteristics [23, 24]. The fat body possesses the highest specific juvenile hormone esterase activity of major tissues aside from the hemolymph and commonly is believed to be the site of synthesis of both peaks of hemolymph activity [25]. This idea is supported by the observation that increases in fat body juvenile hormone esterase activity correlate well with corresponding increases in hemolymph activity and that the fat body protein(s) appear biochemically similar to the hemolymph protein(s) [25, 26]. This situation appears to be true for both the constitutive protein as well as the enzyme 'induced' in fat body by topically applied juvenile hormone or juvenile hormone mimics. The current hypothesis regarding the regulation of hemolymph juvenile hormone esterase activity suggests that juvenile hormone directly induces the fat body to synthesize the juvenile hormone esterase comprising the prepupal peak, while head factor(s) other than juvenile hormone induce the synthesis of juvenile hormone esterase in the prewandering peak [27, 28]. At this time in development, juvenile hormone appears to increase juvenile hormone esterase activity only in the presence of factors from the head. It should be cautioned as well that the majority of the work supporting this model has been carried out in one species, *Trichoplusia ni*.

The exact role of these putative head factors in inducing juvenile hormone esterase or modifying other developmental responses is uncertain as these factors have neither been isolated or characterized to any extent. Similarly, the increase in juvenile hormone esterase activity observed after *in vivo* application of juvenile hormone or other juvenoids

has not been unequivocally demonstrated to be a direct result of increased juvenile hormone esterase synthesis by the fat body. Therefore, it is still uncertain whether the regulation of hemolymph juvenile hormone esterase levels occurs solely at the level of transcription or involves post-translational processing events and/or active secretion from the fat body or other tissues. Fat body organ cultures have been used to address these questions and clarify the induction response observed after application of juvenile hormone [26, 29, 30]. Results from these studies, although suggestive of increased protein synthesis as a result of juvenile hormone application, are far from conclusive. Present studies in this laboratory are employing immunochemical methods to detect the specific incorporation of radiolabelled precursors into juvenile hormone esterase from the fat body in the absence and presence of the supposed inducing agents. However, to fully understand the regulation of the expression of the juvenile hormone esterase gene, it will be necessary to study the system at the level of the mRNA which can be accomplished by developing the appropriate cDNA or complementary RNA probes.

Inhibition of juvenile hormone esterase by organophosphates

In the ultimate larval instar, the rise in juvenile hormone esterase activity concurrent with the decline in juvenile hormone titer suggests that there is a cause and effect relationship between the two events. At best this is a correlation, and it was even reported in *Manduca sexta* [31] that the appearance of juvenile hormone esterase followed the fall of juvenile hormone in the hemolymph. Since endocrine regulation in vertebrates usually is assumed to occur via changes in biosynthesis against a background of constant degradation, such observations raised the question if juvenile hormone esterase is intimately involved in the regulation of juvenile hormone. Certainly it could have simply a scavenger role, pulling the hormone from lipid depots by mass action. Alternatively, the enzyme's name could be a misnomer, and its association with the reduction of juvenile hormone simply a coincidence.

Direct support for the hypothesis that juvenile hormone esterase is involved in hormone regulation comes from inhibitor studies. Juvenile hormone esterase can be distinguished from many other esterases by the observation in *M. sexta* that O,O-diisopropylphosphofluoridate (DFP) inhibits many nonspecific esterases [32], whereas juvenile hormone esterase activity remains relatively unaffected [33, 34]. Other serine esterase inhibitors have been screened *in vitro* for their ability to inhibit juvenile hormone esterase, not only in *M. sexta* [35] but also in *T. ni*, *Tenebrio molitor*, *Musca domestica* [36], *Blaberus giganteus* [37], and *Acheta*

domesticus [38]. It appears that there are major differences in the susceptibility of the esterases in different species which metabolize juvenile hormone to the corresponding acid. Carbamates, paraoxon and derivatives, among others were relatively poor inhibitors compared to the phosphoramidates O-ethyl-S-phenyl phosphoramidothiolate (EPPAT) and the S-benzyl-O-ethyl derivative (BEPAT). In *M. sexta* EPPAT and BEPAT showed I_{50} values of 4×10^{-9} M and 7×10^{-9} M, respectively. Among the other inhibitors, methyl paraoxon had the highest activity, with an I_{50} of 1×10^{-7} M [35]. In the other studies [36, 37], EPPAT and BEPAT also displayed the most potent inhibition of juvenile hormone esterase, although the obsolute I_{50} values varied.

EPPAT was the least acutely toxic of the effective inhibitors and was therefore chosen to assay for *in vivo* inhibition of juvenile hormone esterase in *T. ni* [36]. A single 22 μg dose of EPPAT given topically on day 1, one day before the prewandering peak of juvenile hormone esterase, reduced the level of juvenile hormone esterase activity to 10% of normal for a period of several hours without any visible behavioral changes. When larvae were treated three times a day 1 and 2, the average juvenile hormone esterase activity was reduced to 15% of normal, and the larvae remained in a prewandering state for 1 to 3 days longer than normal. This delay in the onset of prepupation events was also seen in *M. sexta* [35]. A similar delay in the time of pupation was achieved by treating larvae with a juvenile hormone mimic, the juvenoid epofenonane [36].

It was demonstrated [36] that EPPAT does indeed slow *in vivo* hydrolysis of juvenile hormone by topically applying juvenile hormone radiolabelled with tritium to larvae after treatment with EPPAT and assaying the relative proportions of juvenile hormone vs. juvenile hormone acid by thin layer chromatography. An even clearer demonstration of the role of juvenile hormone esterase came from direct analysis of juvenile hormone titers in larvae of additional ages with and without EPPAT treatment. In both cases juvenile hormone titers were very low, but they were significantly higher in EPPAT treated insects. A delay in the time of pupation induced by EPPAT is similar to that achieved when larvae are topically treated with the hormone mimic epofenonane [36] indicating that even low titers of juvenile hormone can block the release of PTTH and the events leading to pupation. The results are consistent with the classic understanding that juvenile hormone levels must drop for PTTH to be released and the events leading to pupation to occur [39]. The inhibition of juvenile hormone esterase produces the same behavioral response and presumably the same biochemical effect as maintaining

a high juvenile hormone titer suggesting that juvenile hormone esterase is quite important, if not essential, in regulating juvenile hormone levels in the hemolymph and subsequently the development of these larvae.

From a practical standpoint, it is clear that inhibition of juvenile hormone esterase, at least in the ultimate instar, is not the solution to controlling insect pests in that there is little economic benefit to extending the feeding stage of a phytophageous pest. However, selective inhibitors such as EPPAT have proven useful in testing the hypothesis that juvenile hormone esterase is involved in juvenile hormone regulation. One major criticism of this approach is that EPPAT inhibits most carboxylesterases which can be detected in the insects studied. Thus, its effect could be due to inhibition of these enzymes or even to an unanticipated biological effect of the compound itself. Thus, a series of inhibitors of radically different chemical structure and greater specificity for juvenile hormone esterase were needed.

Use of transition-state theory in the development of juvenile hormone esterase inhibitors

The observed specificity of the phosphoramidate inhibitors, such as EPPAT, toward juvenile hormone esterase raised the hope of designing still more specific inhibitors. The basic paradigm in this research was transition state theory as it applies to the mechanism of enzymatic reactions. This theory was first advanced by Pauling [40] in 1948. Enzymes are catalysts and thus promote a reaction without influencing its equilibrium constant. Thus, they must stabilize and therefore bind to the transient, unstable intermediate(s) of a reaction known as the transition-state, in order to lower the activation energy of that reaction. Based on this idea, extremely potent inhibitors can be developed for a given enzymatic reaction if one can synthesize "transition state mimics": stable chemical compounds resembling the transition state [41].

Since general ester hydrolysis proceeds through a tetrahedral, transient intermediate and a transition state resembling this intermediate, a polarized ketone was a likely candidate to mimic this state. These ketones are sensitive to nucleophilic attack, therefore in the presence of trace amounts of water, the keto-form will be hydrated and be in equilibrium with the corresponding geminal diol. The geminal diols are tetrahedral in geometry as has recently been shown by X-ray crystallography and in theory resemble the transition state of the hydrolysis of esters. Placing a perfluoroalkyl group next to a carbonyl is one way to develop a highly polarized ketone. These ketones should bind strongly to esterase enzymes by forming hemiketals with the serine present at the

active site of the enzyme. There are some problems with semantics as transition state theory is applied to these compounds, since they can represent both the substrate and a transient intermediate. They even can form a covalent adduct with the enzyme which is dependent on the enzyme's catalytic mechanism. Thus, polarized ketons could be considered as suicide substrates. However, it is useful in both the design of these compounds and in discussion to think of them as transition state mimics.

In this line of thinking, a series of juvenoid-like trifluoroketones have been synthesized. Among the compounds of structure *A*, 1,1,1-trifluoro-2-tetradecanone (TFT) was found to be a highly active and selective *in vitro* inhibitor of juvenile hormone esterase (I_{50}: 1×10^{-7}M) as compared to α-naphthyl acetate esterase (α-NaE) or trypsin [35, 36, 42]. However, they were unable to mimic the *in vivo* activity of juvenile hormone as EPPAT did. This lack of inhibition failed to support the theory that juvenile hormone esterase was involved in the regulation of juvenile hormone.

$$CH_3(CH_2)_n\text{–}C(O)\text{–}CF_3 \qquad (A)$$

$$CH_3(CH_2)_n\text{–}SCH_2\text{–}C(O)\text{–}CF_3. \qquad (B)$$

Introducing a sulfur atom β to the carbonyl significantly increased the activity of the resulting 3-substituted thio-1,1,1-trifluoro-2-propanones (*B*) against juvenile hormone esterase. Based on this finding, a series of aliphatic, aromatic [43, 44] and terpenoid [45] derivatives were synthesized. The most compound of these series was 3-octylthio-1,1,1-trifluoro-2-propanone (OTFP).

The most inhibitory compounds from both groups have molar refractivities (MR) very similar to the substrate juvenile hormones [46], which supports the picture of a transition-state mechanism for these compounds (Fig. 2). It was rationalized that the pi electrons of the thioether moiety in the second group would bioisosterically mimic the double bond conjugated to the ester carbonyl group of juvenile hormone [43], however, the compounds showed elevated inhibitory potency against some general carboxylesterases as well [43, 47]. In addition, while TFT and the aliphatic trifluoromethyl ketones appeared to be classical competitive inhibitors, the presence of the sulfur altered the kinetics of inhibition of many of the trifluoromethyl ketone sulfides (*B*) into a reversible but slow and tight binding inhibition mechanism against serine esterases [43, 48, 49]. This type of inhibition often has been observed with transition state inhibitors and other highly potent compounds [46, 50].

The presence of the sulfur moiety in OTFP increased its binding

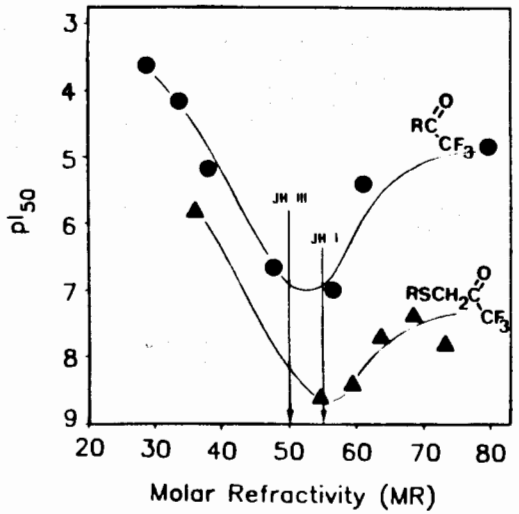


Figure 2. Inhibition of juvenile hormone esterase by trifluoromethyl ketones as a function of molar refractivity. The pI_{50} of inhibition of the enzyme by the structures shown is plotted as a function of molar refractivity which largely is a steric parameter paralleling carbon chain length for this series [46]. Both the less potent aliphatic series (●) and more potent thioether series (▲) show maximum potency at a MR of approximately 55 which is the MR of juvenile hormone I and II as shown by the arrow. Redrawn from [42, 43, 66].

energy to juvenile hormone esterae by approximately 10.5 kJ/mole [4] over the corresponding aliphatic compound. This increased potency and possibly the slow, tight binding mechanism of inhibition led to clear activity *in vivo*. As with EPPAT or juvenile hormone application, treated larvae remained in the prewandering phase for longer periods of time, consumed significantly more food and attained a greater ultimate weight than did untreated larvae. This delay in pupation caused by two structurally and mechanistically distinct inhibitors of juvenile hormone esterase provides strong evidence for the hypothesis that juvenile hormone titer is regulated in part by juvenile hormone esterase.

Affinity purification of juvenile hormone esterase

There has been interest in purifying juvenile hormone esterase for over a decade. However, the low abundance of the enzyme (approximately 2 micromolar catalytic site at maximal hemolymph titers in *M. sexta*) and

the difficulty in obtaining large amounts of hemolymph as starting material has made attempts at purification largely futile exercises [51–53]. The availability of trifluoromethyl ketones as potential affinity ligands was thus very attractive.

Figure 3 shows the simple route for the synthesis of the immobilized ligand. Bromotrifluoroacetone was reacted with equimolar butane dithiol to yield a monosubstituted thioether as the major product. This material was allowed to react with epoxy activated Sepharose leading to another thioether linking the trifluoromethyl ketone to the affinity matrix. The resulting affinity matrix proved to be exceptionally powerful in binding the juvenile hormone esterase activity from hemolymph while leaving most other proteins behind.

Elution proved to be very difficult because the esterase was bound to the ligand by a reversible covalent bond. The bond was readily reversible only under conditions when the enzyme was catalytically active, so denaturing conditions proved ineffective in removing the juvenile hormone esterase from the column. Reagents which should compete with the enzyme for the trifluoromethyl ketone also proved ineffective probably because of the uncharacteristic chemical properties of the highly polarized carbonyl. It was not even possible to get large amounts of

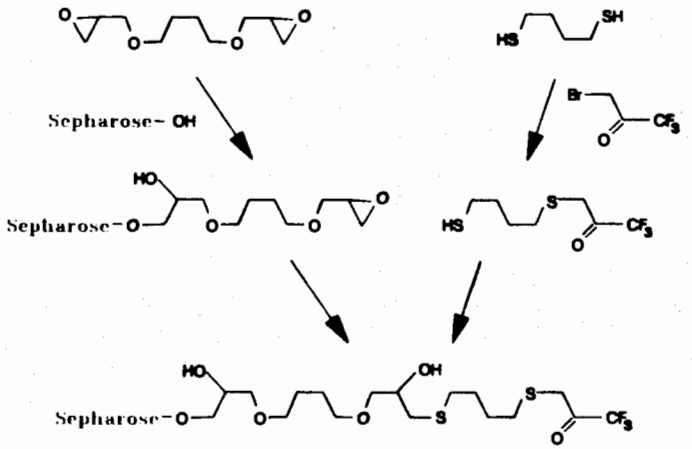


Figure 3. Synthetic pathway leading to an affinity gel for the purification of carboxylesterases. 1,4-Butane dithiol (right) is reacted with 3-bromo-1,1,1-trifluoropropan-2-one to yield the corresponding thio ether. This ether, in turn, is stirred with epoxy activated Sepharose prepared by the reaction of the gel with butane diol diglycidyl ether (left) to yield the affinity gel below.

enzyme off of the column using gradients of high concentrrations of very potent trifluoromethyl ketone inhibitors [54].

The slow tight binding nature of the covalent bond between the enzyme and the affinity ligand proved to be the source of difficulty in eluting the enzyme. Once the slow tight binding phenomenon was considered, it was obvious to incubate the enzyme loaded column with large amounts of free inhibitor to displace the enzyme from the column. The inhibited enzyme could be decanted off of the affinity gel and the activity recovered by dialysis (Fig. 4) [4, 11, 24, 50, 54, 55].

This method of purification led to high yields and even quantitative recovery of enzyme activity from a variety of species. There always is the danger that application of the affinity system will lead to overlooking important forms of the enzyme, however the high yields of catalytic activity recovered from most species suggest that the important forms of the enzyme are in fact being purified. Of great importance was the fact that this procedure provided enough pure enzyme to raise antibodies and carry out biochemical characterizatiion of the activity.

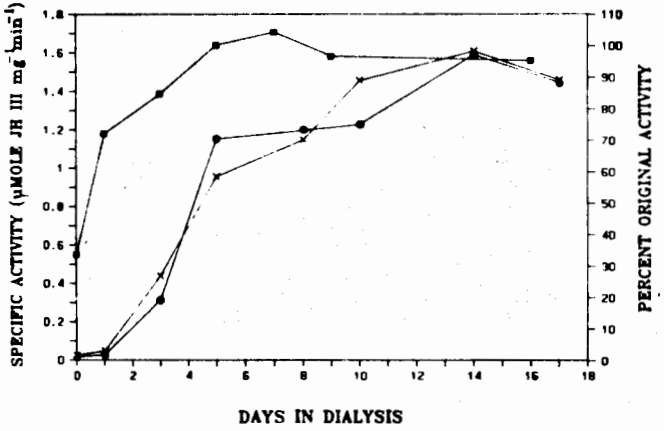


Figure 4. Recovery of juvenile hormone esterase activity during dialysis following elution from an affinity matrix by OTFP. Five ml solutions of 220 ng/ml and 260 ng/ml of purified protein from hemolymph (●) and a whole-body homogenate (×), respectively, were dialyzed in the presence of 200 μg/ml BSA in 1.5 L of buffer at room temperature and monitored during the period required to reach maximum activity. A control of previously purified esterase (210 ng/ml) from prepupal homogenate that had been reinhibited by a concentration of 10^{-7} M OTFP (■) was treated similarly to observe any loss of activity during the procedure. The protein concentration of each sample remained constant during the procedure.

Biochemical and immunochemical properties of juvenile hormone esterase

Physical properties. The juvenile hormone esterases from a variety of lepidopterous species appear very similar in regard to their pattern of appearance during development [22] and several of their biochemical properties, however there are clear differences among the species. These differences indicate that it may be dangerous to extrapolate information from one species to another. The esterases so far studied are approximately 60 000 in molecular weight based on SDS polyacrylamide electrophoresis and have acetic pI's. Gel permeation data indicate that the enzymes from *M. sexta* and *T. ni* are monomeric at least in dilute solution (Fig. 5).

Kinetic properties. The juvenile hormone esterases studied appear to have relatively low K_M's on the order of 10^{-7} M. The maximal velocities of hydrolysis vary for purified enzymes from about 800 for *Bombyx mori* to over 4000 nmol/min/mg protein for *Heliothis zea*. These data indicate that the enzyme should be able to extract juvenile hormone from lipid depots or from moderate affinity binding sites by mass action [4, 11]. Since the concentration of enzyme catalytic site far exceeds the concentration of substrate, the rate of hydrolysis of constant levels of juvenile hormone in an insect should be a direct function of enzyme concentration. Thus, as the level of juvenile hormone drops in the last stadium, the large increase in juvenile hormone esterase in hemolymph and tissue may be necessary to remove the last traces of hormone.

The actual rate of hydrolysis of juvenile hormone by juvenile hormone esterase is not high when compared to enzymes such as catalase or acetylcholinesterase [56]. Under *in vivo* conditions the rate limiting step in the degradation of juvenile hormone by the enzyme will be the binding of the enzyme to the substrate since it is very unlikely that an enzyme will simultaneously encounter two substrate molecules. If we assume the role of juvenile hormone esterase is to remove the last traces of the hormone from the tissues, it is quite reasonable to sacrifice a high turnover for a low K_M. For instance the juvenile hormone esterase purified from *T. ni* turns over the model substrate 4-nitrophenyl acetate at almost the same rate as juvenile hormone. However, the low K_M of the enzyme for juvenile hormone confirms that the enzyme is highly specific for juvenile hormone in terms of K_{CAT}/K_M.

Immunochemical properties. Antibodies raised to the juvenile hormone esterase of *T. ni* are capable of precipitating all of the hydrolytic activity on juvenile hormone in the hemolymph of pre and postwandering larvae

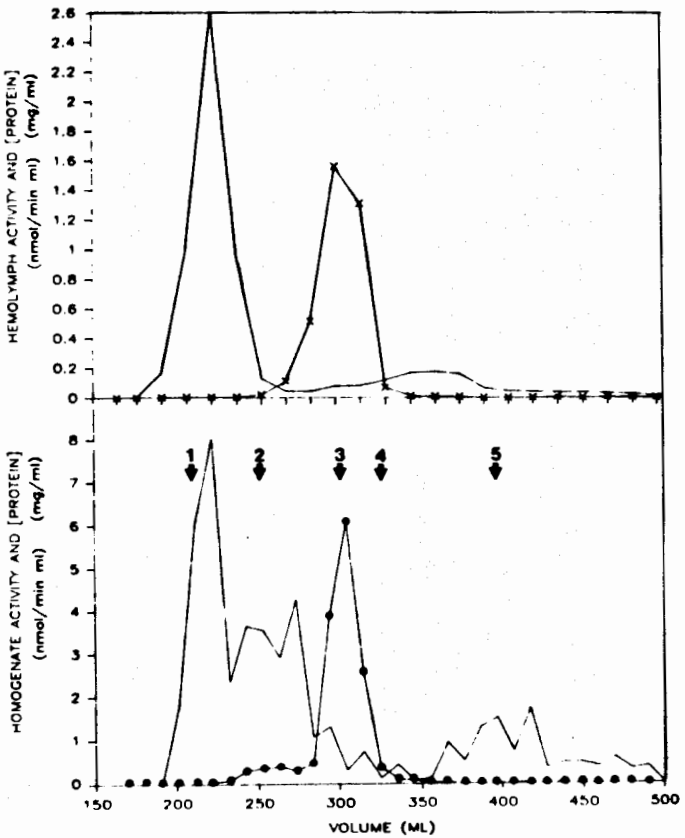


Figure 5. Gel permeation chromatography of juvenile hormone esterase activities from *T. ni*. Esterase activities and protein concentrations were measured from eluted fractions of a gel filtration experiment that used 10 ml each of diluted hemolymph (×) and a prepupal homogenate (●) prepared at pH 7.4. The major peaks of juvenile hormone esterase activity accounted for 90% and 88% of the activities present in the starting homolymph and homogenate, respectively. Filtration was conducted on a 2.5 × 95 cm column of Sephacryl S-200. Flow rate was 20 ml/hr and fractions of 15 and 10 ml were collected for the hemolymph and homogenate preparations, respectively. Protein concentrations of the fractions are indicated with (–) and the elution of the molecular weight standards are indicated with arrows, 1, Blue Dextran, 2,000 kD; 2, immunoglobulin G, 150 kD; BSA, 66 kD; 4, ovalbumin, 45 kD; 5, cytochrome c, 12.5 kD.

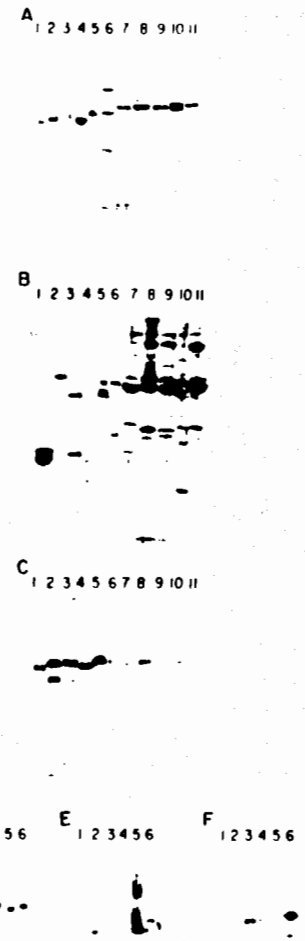
indicating that the enzymes are very similar [54]. These data provide further proof that most of the juvenile hormone esterase activity in the hemolymph at peak levels are due to the affinity purified enzyme. These antibodies also detect the esterase from the fat body in early instars and from the hemolymph at early molts [24], providing evidence that the same or a very similar enzyme is produced throughout larval development at lower levels.

As shown in Figure 6 antibodies raised to juvenile hormone esterase from *Heliothis virescens*, *H. zea*, *M. sexta*, and *T. ni* partially cross react on both ELISA's and Western blots. Thus, probably in some cases the antibodies were directed to antigenic determinants which were common among several of the species while other antigenic determinants were distinct.

An interesting situation arose when the antibody raised to apparently homogeneous juvenile hormone esterase from *T. ni* was used in a Western blot against crude serum. The antibody was found to detect many of the proteins in the serum. When this antibody was first treated with serum to remove nonspecific antibodies, only juvenile hormone esterase was detected on Western blots of crude serum [54]. Thus, it appeared that juvenile hormone esterase had an antigenic determinant in common with many of the proteins in the hemolymph of *T. ni*.

Glycosylation of juvenile hormone esterase. Pure juvenile hormone esterase was found to react with dansyl hydrazine following oxidation with periodate and to react with lectin (Fig. 6). Both of these observations indicate that the enzyme, like many hemolymph proteins, is glycosylated. Reaction of the *T. ni* esterase with the lectin concanavalin A suggests a mannose or N-acetylglucosamine content for the enzyme. Failure of the enzyme endo H to remove significant carbohydrate from the esterase suggests that the carbohydrate is not of the 'high mannose' type. When the esterase was exposed to peptide: N-acetylglycosidase F, there was a loss of lectin affinity, a small reduction in molecular weight and a shift of most of the bands to a more acidic pI. These data suggest that the enzyme has asparagine-linked oligosaccharides which probably are of a hybrid structure [54].

Similarities and differences among species. The similar kinetic properties discussed above and similar timing of appearance during development [22] suggest that the enzyme has a role which largely is parallel among species. The size and pI of the juvenile hormone esterases also are very similar. There are however numerous differences. Some are relatively subtle such as slight changes in the potency of inhibitors, while others may be significant in the biology of the enzyme.



The electrophoretic patterns are very interesting. The esterase from *M. sexta* appears as one sharp band on SDS-PAGE yet has two distinct catalytic sites and shows two approximately equal bands on isoelectric focusing, both of which are labeled with ^{3}H ethyl paraoxon. However, N-terminal amino acid analysis of this mixture suggests a single sequence (Fig. 7). Some of the other species show single and some multiple bands, but in each case the bands are labeled with paraoxon suggesting that they are esterases. Slicing of IEF gels followed by incubation of the eluted proteins with juvenile hormone confirms that they can be termed juvenile hormone esterases. *H. virescens* shows a single band on SDS-PAGE and IEF, yet shows two overlapping N-terminal sequences (Fig. 7). *H. zea* shows two clean bands on both SDS-PAGE and IEF the upper of which is glycosylated. *B. mori* shows at least three bands on both SDS-PAGE and IEF while *T. ni* shows one and possible more poorly resolved bands on SDS-PAGE but at least three bands on IEF [4, 11, 23, 50, 54, 55].

It is tempting to assume that the multiple forms of juvenile hormone esterase are due to differential glycosylation and that this glycosylation is a signal for processing or transport of the enzyme. The situation is, however, confusing. In *T. ni* at least the three major forms appear to be glycosylated. However, when the sugars are removed with endo F, at least three forms remain with two major ones at different isoelectric points. These data suggest that glycosylation alone does not account for the different bands on isoelectric focusing [54]. The juvenile hormone

Figure 6. Characterization of juvenile hormone esterase from five species of Lepidoptera. Hemolymph proteins (lanes 7–11) and affinity purified preparations of juvenile hormone esterase (lanes 1–5) containing some contaminating proteins were resolved by SDS-PAGE and stained with Coomassie Blue (A) or transferred to nitrocellulose filters for lectin blotting (B) or immunoblotting (C–F). Lectin blotting was conducted by first probing the filter with biotin conjugated to the lectin Concanavalin A which binds to proteins that have mannose or glucosylamine moieties. The bound lectin was then visualized by incubating the filter with avidin conjugated to horseradish peroxidase and a subsequent incubation with a chromophore substrate. Immunoblotting was done with standard procedures by first probing the filters with antisera raised against juvenile hormone esterase from three lepidopteran species. Visualization of the bound antibodies was done with incubations with alkaline phosphatase conjugated to goat IgG raised against IgG from rabbit and a chromophore substrate. A 1:750 dilution of antisera raised against juvenile hormone esterase from *H. virescens* was used for the immunoblot in (C) while a higher dilution of 1:1000 of the same antisera was used for the immunoblot in (D). Dilutions of 1:1000 of antisera against juvenile hormone esterase from *M. sexta* and *T. ni* were used for the blots in (E) and (F), respectively. For the gel and blots in A–C, the lanes were each loaded with 1.2 μg of juvenile hormone esterase from *Bombyx mori* (lane 1), *H. virescens* (lane 2), *H. zea* (lane 3), *M. sexta* (lane 4) and *T. ni* (lane 5). Hemolymph protein in the amount of 5 μg each were loaded into lines 7–11 in the same order as the purified proteins. Lane 6 contained 1 μg each of standards having molecular weights of 97 kDa, 67 kDa, 45 kDa, 31 kDa, 25 kDa and 14 kDa. The blots in D–F used protein loadings of 100 ng of juvenile hormone esterase from *B. mori* (lane 2), *H. virescenes*, (lane 3), *H. zea*, (lane 4), *M. sexta* (lane 5), and *T. ni* (lane 6). Lane 1 in blots D–F contained 500 ng of the molecular weight standards described above.

Heliothis virescens

(75%) WQETNSASVLAHLDSGIIAGVPASADAIKEASP-G

(25%) SAWQETN -----

Manduca sexta

RIPSTEEVVVRIESGWIAGLK--AEGNK-YA-E-

Trichoplusia ni

LP-LS-DAEAPS-LS-KADPI

Figure 7. N-terminal sequences of juvenile hormone esterases from three species. The amino acid sequences of the N-terminals of affinity purified juvenile hormone esterase from the larval hemolymph of three species of Lepidoptera are shown in one letter abbreviations. Each sequence was from apparently homogeneous, affinity purified enzyme which showed one band on SDS-PAGE. Two partially overlapping sequences were obtained from the same preparation of juvenile hormone esterase from *H. virescens*. One sequence represented 25% of the total signal and had a two amino acid extension upon an amino acid sequence identical to the five terminal amino acids of the other sequence. The data were obtained with a Beckman model 890M liquid phase sequencer. Amino acid derivatives were verified on two different chromatography systems (HPLC, TLC or GLC). Underlined amino acids denote where only one chromatography system derived unambiguous data while hyphens denote places in the sequences where the data was ambiguous on both systems. The proteins were not carboxymethylated prior to sequencing.

esterase from the majority of the species examined does not appear to contain sugars based on lectin blotting. However, a careful study of enzyme from different tissue compartments at different times during development has not been made. An exception is *H. zea* where one of the two forms appears heavily glycosylated (Fig. 6).

One of the most notable differences among the enzymes is their response to organic cosolvents [57]. Most soluble enzymes are denatured by exposure to high concentrations of organic solvents. In contrast, the rate of hydrolysis of juvenile hormone by both the hemolymph and purified juvenile hormone esterase from *M. sexta* were dramatically increased. It is possible that the solvents are mimicking some endogenous regulator, however it also is possible that the solvents simply are increasing the turnover number of the enzyme by speeding diffusion of the product from the enzyme's surface. Interestingly, the juvenile hormone esterases from other species seem to be either unaffected, denatured, or

capable of switching from ester hydrolysis to transesterification by employing an alternate nucleophile such as ethanol.

Thus, the biochemical properties of the juvenile hormone esterases from several different species are consistent with their hypothetical role. Overall juvenile hormone esterase activity appears to be due to very similar proteins. However, the marked differences observed are very interesting if only from a biochemical standpoint. As we examine the biochemical properties of these enzymes in more detail, possibly we will find biological reasons for the different properties. Detailed studies on the *in vivo* biological activity and turnover may help to explain the reasons for these apparently superficial differences.

Biological activity of exogenous juvenile hormone esterase

It appears that, at least in the ultimate instar of lepidopteran larvae, inhibition of juvenile hormone esterase is not a promising means for disrupting larval development and controlling insect pests. This strategy will not be as effective as mimicking juvenile hormone chemically. Even hormone mimics or juvenoids are of questionable utility with many field and row crop pests since they extend the destructive feeding stage. Thus, the alternative strategy becomes attractive. Previous attempts to develop anti-juvenile hormone agents have relied on chemical approaches. This work has concentrated on blocking biosynthesis of the hormone with the use of inhibitors or developing receptor antagonists [58]. The development of synthetic anti-juvenile hormones has been exciting but has not led to a commercial product. This laboratory has targeted increased degradation of juvenile hormone as a potential means to produce anti-juvenile hormone effects. It is believed that by turning-on juvenile hormone esterase activity at inopportune times in development, normal larval growth will be disrupted.

The aggressive nature of juvenile hormone esterase in terms of k_{CAT}/K_M ratio and the strict regulation of juvenile hormone esterase during development suggests that the inopportune appearance of juvenile hormone esterase may have profound effects on the physiology of insects. If the "hi-low-no" hypothesis is correct, then the premature appearance of large amounts of juvenile hormone esterase might lower the juvenile hormone titer significantly to induce precocious pupation. Since juvenile hormone is a feeding stimulant, even a slight reduction in titer might halt feeding in crop pests.

The affinity purification of juvenile hormone esterase has enabled us to concentrate sufficient quantities of this enzyme to investigate whether juvenile hormone esterase, when injected into larvae at times when it is

normally absent, can significantly affect the juvenile hormone titer to disrupt development. *M. sexta* was chosen for the initial experiments because its large size provided the greatest quantity of biomass for purification of juvenile hormone esterase. Second, third, and fourth instar larvae were injected on the last day of each instar with between 0 and 6 μg of purified juvenile hormone esterase in 2 μl of phosphate buffer (the fifth instar of this species was avoided because large amounts of juvenile hormone esterase were already present and the first instar was not used because of the size of the larvae). Control injections contained 6 μg of either hemolymph from which the juvenile hormone esterase activity had been removed or bovine serum albumen.

With the protein concentrations used, no visible response to the injections was seen in any control larvae or fourth instar larvae injected with juvenile hormone esterase. The most profound response was seen in second instar larvae and to a lesser degree in third instar larvae. In these second instar larvae, injections of juvenile hormone esterase resulted in cessation of feeding, a blackening of the larvae 16–48 h after injection followed by their eventual death. This biological activity of juvenile hormone esterase was dose-dependent over a range of protein concentrations from 0.75 μg to 5 μg (Fig. 8).

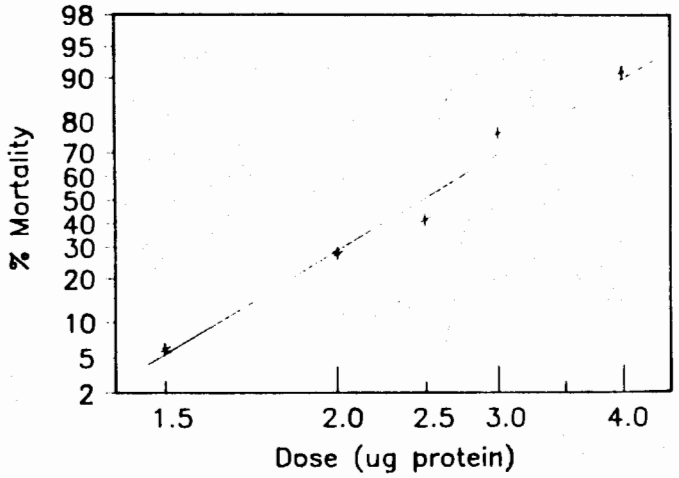


Figure 8. Probit analysis of mortality induced by injection of juvenile hormone esterase. Increasing dose of apparently homogeneous enzyme with a specific activity of 500 nmol/min/mg protein was injected laterally in 2 μl of buffer in the first abdominal segment of day 2 larvae of *M. sexta*. Mortality was recorded at the next molt.

It is interesting to note that the dose dependent mortality caused by juvenile hormone esterase occurred over such a narrow range of protein concentrations. One interpretation of the mortality is that the juvenile hormone esterase may be depressing the juvenile hormone titer below a critical level where the physiology of the developing larva is irreparably disturbed. These larvae may not be competent to initiate pupation immediately. Another possibility is that the juvenile hormone titer must be suppressed below a critical level for a particular duration of time or during a specific time in development. To date these criteria have not been met with the current injection technique. Certainly a third possibility is that the toxicity observed with juvenile hormone esterase is unrelated to it hydrolysis of juvenile hormone. These conclusions are being further investigated with determinations of the active life of the enzyme after injection, the use of multiple injections, and more extensive research in other instars and species.

The lack of clear cut anti-juvenile hormone effects are disappointing to an endocrinologist. However, they are exciting from the perspective of insect control. Picomole levels of the enzyme immediately halt feeding and rapidly lead to the death of the insect. Thus juvenile hormone esterase and the proteins regulating it have become obvious targets for development of insect control agents using recombinant DNA technology.

Approaches to the isolation of the message and gene for juvenile hormone esterase

The first hurdle in attempting to exploit juvenile hormone esterase is to isolate the gene and message coding for it. There are some difficulties in that such a low abundance protein is expected to have a low abundance message. The genomic library also presents a problem since the Lepidoptera have a surprisingly large genome and amplification of genomic libraries in this order has proven difficult.

Genomic approach. We have constructed a genomic library for *H. virescens* using a strain of bacteriophage λ, EMBL3, as the vector. Bacteriophage λ was suited for this work, in part, because it accepts a relatively large DNA insert. Plasmid vectors can only accept small DNA fragments, while cosmids will accept larger DNA fragments, but they are more difficult to use than bacteriophage [59]. EMBL3 is a λ construct that has a particularly useful host selection system for DNA inserts. The *E. coli* host strain P2392 is a P2 lysogen which will only grow λ with a DNA insert. Consequently, it is possible to determine what proportion of the available vector has incorporated foreign DNA.

To prepare a representative DNA library, it is necessary to have high

molecular weight DNA for insertion into the vector. There are numerous methods for obtaining pure DNA [60]. The isolated DNA should be in the range of 100 Kb or larger. This ensures that the DNA has been handled gently and has few non-cohesive ends after digestion by an appropriate restriction enzyme for insertion into the vector. After partial digestion, the genomic DNA was size-sorted on a sucrose gradient to collect a suitable range of inserts. The library was then completed by ligating the vector arms with inserts, and packaging this combination. The library can be amplified by rearing it on the host but this process has yielded ineffective libraries in Lepidoptera. Consequently, it is thought best to screen lepidopteran libraries without amplification.

The genomic library is currently being screened with single-strand DNA probes. We have sequenced the N-terminus of juvenile hormone esterase and used the least ambiguous portion to make oligonucleotide probes for all codon combinations. An alternative is to make a single large probe from an "optimized sequence" [61]. The oligonucleotides were constructed with a 3′ terminus so that they could be used to detect homologous DNA or cDNA, or hybridized to messenger RNA. The probe was end-labelled with ^{32}P and hybridized to nitrocellulose or nylon filters, which were used to lift plaques derived from the genomic library. The methods used are essentially those described by Woods [62] and Wahl *et al.* [63]. The capability of a probe to recognize a homologous sequence depends on the specific activity of the labeled probe [64]. Thus, it is a routine procedure to separate unlabeled [γ-^{32}P] ATP and unlabeled oligonucleotides from labeled oligonucleotides [64]. Positive plaques are purified, and their identity confirmed by subcloning into an expression vector and screening with antibody to juvenile hormone esterase.

cDNA approach. The alternative strategy was to prepare and screen a cDNA expression library [65]. mRNA was isolated by centrifuging homogenized fat body tissue from *H. virescens* through cesium chloride in a fixed angle rotor [66]. Poly A mRNA was then isolated by passing the isolated RNA through an oligo dT cellulose column. The integrity of the poly A mRNA was determined by *in vitro* translation.

Using the isolated message, cDNA was prepared using the Amersham cDNA system. Phosphorylated linkers were attached to the cDNA. The cDNA inserts were then ligated to bacterophage λ gt 11. This is an expression vector which produces a fusion protein corresponding to the cDNA insert and the N-terminal portion of the Lac Z gene. Thus, it is possible to determine what proportion of the phage have an insert by plaque color. With the expression library we were able to screen for cDNA inserts coding for part of the juvenile hormone esterase gene using

antibody to the enzyme from *H. virescens* (Fig. 9). Positive clones were verified by hybridizing a single stranded ^{32}P-labeled oligonucleotide probe made from sequenced esterase. The final verification will be to sequence the positive clones and match the nucleotide sequence with a corresponding sequence from the N-terminus of selected portions of juvenile hormone esterase.

Strategies to exploit the message for juvenile hormone esterase

In order to control insect populations, agents which disrupt their endocrine systems must be effectively applied to the organism. In the Lepidoptera it is very unlikely that a large molecular weight protein can penetrate the gut. Thus, it appears that insecticides based on recombinant DNA technology will be most effeciently delivered by a microbial agent. Baculoviruses which infect a specific group of insects are one

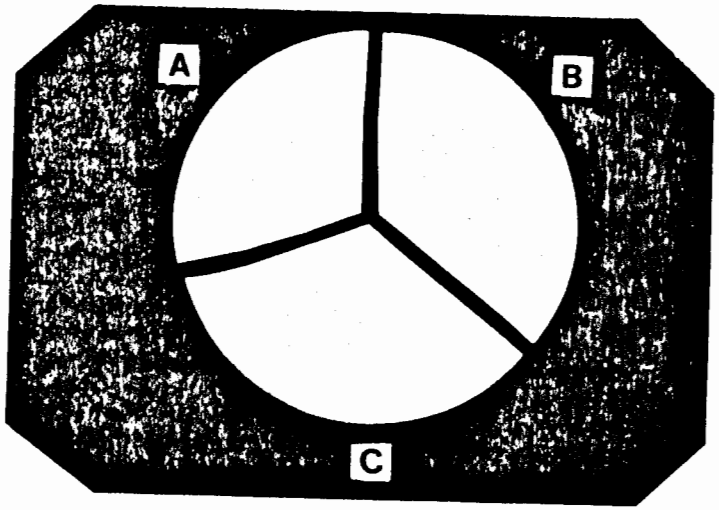


Figure 9. Cloning of the juvenile hormone esterase message from a cDNA expression library. A λgt11 expression library was constructed from cDNA of mRNA from *H. virescens* as noted in the text. The library was then screened with antisera against juvenile hormone esterase by standard procedures for a clone coding for a fusion protein of β-galactosidase and the esterase. A positively responding clone was isolated which reacted with antisera (A) but not against preimmune antisera (B) or antisera preincubated with purified juvenile hormone esterase (C). This result strongly suggested the clone contains DNA coding for an antigenic determinant from juvenile hormone esterase. Further characterization of the clone is presently being undertaken as described in the text.

possible delivery system. The baculoviruses are well-studied [67, 68] and have recently been used as expression vectors for interferon [69] and a variety of other proteins of pharmaceutical interest. Numerous laboratories are working to employ these and related expression systems to engineer insect specific control agents. We are particularly interested in *Autographa californica* which is a taxa-specific virus found in noctuid species [70, 71].

Successful development of such an engineered microbe would solve some of the problems introduced in the preface. For instance, the production of such a microbe would not involve the dangers associated with large scale chemical synthesis, and it could serve as a pest control agent in a situation where classical pesticides were banned. As a highly specific agent for our worst pest problems, it may reduce the use of other pesticides by avoiding destruction of natural enemies and resulting problems with pest resurgence and resistance. Since the virus could be produced in some less developed areas, it would help to deliver insect pest control to the regions of the world most in need of it. Whether this approach will result in the reduction of registration costs remains to be seen.

Conceptually, expression of juvenile hormone esterase offers a number of advantages as a control agent. First, we are attempting to enhance the production of an enzyme which is present at low levels continually during the life of the insect (unpublished data). Thus, we know that the machinery for the production and processing of the enzyme is present. This target also offers the possible advantage in the registration process of being a natural protein in the environment and even in the pest insect. A current problem with baculoviruses is that infected insects continue to feed for several days. The injection experiments described above indicate that expression of low levels of juvenile hormone esterase may lead to a cessation of feeding which is very desirable in an agricultural context. A number of laboratories are striving to insert a gene for a cytotoxin behind the polyhedron promoter of the baculovirus. In addition to the problems of registration to be faced with such an approach, it is possible that expression of a cytotoxin could kill infected cells and prevent the spread of infection. In contrast, juvenile hormone esterase is thought to be nontoxic to individual tissues but to kill the insect by disrupting the regulatory biology of the insect as a whole. Finally, production of baculoviruses has proven difficult. In this case, infected larvae can be treated with a juvenoid lacking an ester moiety to prolong their life and result in a greater production of virus.

Until this approach is proven to be successful in this or another laboratory, it is simply a pipe dream. The approach is intellectually very

attractive, but there are numerous problems to overcome. For instance, it may prove very difficult to include two polyhedron promoters in a single virus and obtain a virus that both will infect insects and express lethal levels of an inserted protein. Use of other promoters in the virus is attractive, but untested.

There are numerous other possible, but more distant approaches. For instance, nucleotide sequences coding for anti-sense mRNAs [71] could be inserted into the virus. In this scenario the goal would be to suppress the intra-cellular level of the enzymes which act to control juvenile hormone titers. Eventually, we anticipate that the most effective way to deliver these specific biological insecticides will be to incorporate them into economically valuable plants. This approach will be very difficult, but it may be possible to use chimera insect and plant viruses to have nucleotide insecticides cultured in plants and expressed in insects. Perhaps more reasonably, one could incorporate small viruses into plant genomes as vectors. Especially attractive are the microbes that are commensal in insects or can multiply in both insects and plants. In the rapidly developing field of biotechnology, the possibilities appear endless. However, the greatest practical value for the message and gene for the juvenile hormone esterase may be as a probe for the further investigation of insect regulatory biology. Such research is certain to yield other targets which can be expressed by both chemical and recombinant technologies.

Conclusion

Several points related to pesticide discovery are illustrated by the approaches outlined in this manuscript. It is widely accepted that the success rate from screening of random chemistry will continue to decrease [3, 4]. Thus it is important that chemists develop paradigms for the synthesis of active molecules which increase their likelihood of success. No single method will dominate the field, but as discussed here, a combination of modern approaches will increase the likelihood of discovery.

Paradigms for synthesis. It is very unlikely that synthetic inhibitors of juvenile hormone esterase will become commercial pesticides since they act to increase the destructive stage of many crop pests and they are less active than actual hormone mimics. Some of these inhibitors are promising as synergists for classical pesticides [47] and possibly as inhibitors of essential proteases and esterases such as those involved in neurohormone processing [72]. However, the relatively small amount of effort that was required to obtain very high *in vitro* as well *in vivo* activity attests to the

power of using a transition state model in the discovery of biological activity [4]. It has been pointed out that several classical pesticides such as the organophosphates, carbamates, and sulfonyl ureas act, in part, as transition state mimics [4, 46]. It is important that the approach of mimicking a transition state or transient intermediate is applied to synthetic efforts in addition to the mimicking of a substrate or product. With the increasing power of computer assisted molecular modeling [5, 73], the integration of these techniques into a comprehensive synthesis program becomes more viable.

Exploitation of comparative biochemistry. The most dramatic example of how biochemical differences between target versus nontarget organisms can be used is illustrated by the discovery of juvenile hormone mimics. In this case fundamental work in physiology and natural product chemistry lead to the discovery of the structures of the natural insect juvenile hormones. By a combination of approaches, commercial mimics of these materials were optimized at very low cost to society [74]. These approaches are being followed with the development of a wide variety of fungicides, herbicides, and insecticides.

To a lesser degree, the studies reported here are continuing to exploit the insect endocrine system. As we learn more of the developmental biology of insects, additional targets will become clear such as disruption of the regulation of juvenile hormone esterase or even disruption of the early events which initiate the entire molting process.

An approach to basic biology which is often overlooked by funding agencies as well as scientists, is that the chemicals which are developed in efforts to exploit fundamental knowledge of pest biochemistry can be used as probes to further the understanding of that system. Minsky [75] argued that, "to understand how something works, it helps to know how it can fail." Our knowledge of the nervous system, for instance, comes largely from studying why it fails when exposed to pharmaceuticals and toxins.

In this study a knowledge of insect endocrinology led to the discovery of a series of very potent, and selective enzyme inhibitors which were active *in vivo*. These agents then allowed us to test the hypothesis that juvenile hormone is regulated, in part, by degradation and that juvenile hormone esterase is a key element in this process. It is important to realize that successful chemistry not only extends from biology, but that successful research in biology also is catalyzed by innovative chemistry.

Use of ligands in receptor studies and affinity chromatography. The work in this chapter illustrates that remarkable purification of juvenile hormon esterase was obtained using a transition state ligand attached to a solid matrix. This affinity purification has accelerated greatly our work

on the characterization and molecular biology of the enzyme. One must then ask if this technique is more generally applicable to the field of pesticide chemistry.

As mentioned before, the chemicals which have resulted from attempts to develop commercial pharmaceuticals and toxins often have proven valuable as probes for the understanding of biological systems. Although enzyme inhibitors and receptor ligands have proven useful in characterizing catalytic processes and receptor interaction, industry in general has not found these approaches cost effective in developing new materials. We propose that developments in biological and chemical technologies have advanced to the point where exploitation of active ligands could be very practical.

In any research program the majority of exciting biological activities cannot be optimized to yield a commercial product. However, a large amount of very expensive research must be completed to determine that a lead is, in fact, dead. The use of linear free energy parameters to develop quantitative structure activity relationships and other similar approaches have been used with great success for optimizing structure [5]. However, these approaches will not support an intuitive jump to a radically different chemical structure attacking the same biological target. Thus, most attempts to optimize structure fail to lead to a commercial product. From the standpoint of both society and industry, this research effort has been wasted.

It is widely accepted in industry that mechanism of action studies rarely lead a synthetic effort. However, with affinity chromatography, industry may be in a position to exploit the expensive failures just described. A key to successful affinity chromatography is to have a variety of ligands of varying affinity and a good appreciation for the structure activity relationships among them. This situation is exactly what exists as a major attempt to optimize structure is finally deemed a failure. At this point it is relatively inexpensive to attach several of these ligands to a solid support and to attempt biospecific elution with a more potent ligand. One even could attempt binding the protein to the ligand based on hydrophobic interaction followed by biospecific elution. Based on a minimal investment, this approach is likely to yield a pure or highly enriched protein compared to the cost of the data already generated for a series of compounds. Such an approach could be applied in an attempt to isolate the elusive receptor of the acyl urea insect growth regulators or to provide a rapid isolation technique for the enzymes involved in the biosynthesis of aromatic amino acids in plants. The question then arises whether industry can exploit this information effectively to develop practical compounds.

Approaches to discovery of selective toxins. In 1967 Ordish [76] and Williams [77] defined first, second, and third generation approaches to the discovery of pesticides in terms of the type of compound found. We later modified this approach to define the generations in terms of the approach to discovery [10, 78]. Thus the first generation approach involves exploitation of folk remedies which arose, in theory, from a nonsystematic screening process. The second generation approach involves a systematic effort in which screening leads to the discovery of biological effects, and this activity is optimized based on bioassay. A third generation approach involves exploitation of knowledge of the comparative biology and biochemistry in target and nontarget organisms. Recent events in biology have lead to a fourth generation approach where bioactive agents arise from direct exploitation of biotechnology [10].

It is tempting to reject the classical approaches to discovery, especially in light of the increasing cost of finding new activities [5]. However, the second generation approach has, and will remain, the mainstay of our effort to develop selective toxins [2]. A more reasonable alternative is outlined in Fig. 5 where the approaches are integrated in an attempt to exploit all avenues to discovery. There are many levels at which these processes can be integrated and several will be illustrated. However, we will emphasize a central role for affinity purification in this process since it has not been widely applied.

It has been previously discussed that few compounds reach commercial use without an integration of at least second and third generation approaches. Several commercial compounds resulted from direct exploitation of first generation approaches. The pyrethriod insecticides clearly came from a second generation optimization of a first generation lead. Key in this development was a knowledge of the relative levels of esterase and oxidase activities in some target vs. nontarget species. Alternatively, the juvenile hormone mimics or juvenoids are the most obvious examples of a third generation approach; however, the commercial structures arose from classical structure optimization. In fact, some of the new compounds arose from a random screening program.

It is certain that serendipity will continue to play a major role in the discovery of novel biological activities. This process, in part, involves a numbers game of simply synthesizing and screening more compounds than the competition. Increasingly, the cost of this approach will force scientists to emphasize creative ways to improve their rate of success.

Foremost in increasing the likelihood of discovery is the realization that creativity flourishes in an exciting, stimulating environment. A synthesis program which emphasizes creative chemistry as well as active interaction with biologists is more likely to yield exciting discoveries. A

synthetic effort directed at a key regulatory or catalytic process may not lead to commercial exploitation of the targeted system, but due to conservation of biochemical mechanisms, it is more likely to yield useful biological activity than random synthesis. Rather than visualizing modern tools such as computer aided design or transition state theory as a direct route to commercial activity, it is better to visualize them as providing a creative stimulus for enhancing the likelihood of serendipitous discovery.

Integration of approaches. Figure 10 illustrates one mechanism by which such approaches could be integrated. Synthesis and screening (I) are of course central to the process. Ultimately it will be screening and structure optimization (II) that lead to new products (III). In addition to random synthesis, leads should enter the system from observations on folk medicines and plant-pest interactions (IV). This input represents a first generation approach. Of increasing importance will be leads from third generation approaches which arise from knowledge of the comparative biochemistry of target and nontarget species (V). In addition to input in the discovery process, third generation approaches (V) are having an increasing impact on structure optimization (II). In this case our knowledge of environmental chemistry allows desirable properties to be tailored into the molecule. An appreciation of chemical-biological interactions can assist in optimizing selectivity by taking advantage of the comparative biochemistry of target and nontarget species.

For years it has been debated in industry whether studies on the mechanism of action assist in the discovery and optimization of structures. It seems certain that the role of biologists and biochemists in this process will increase in significance. However, the approach can be justified simply on the basis of providing creative stimulation for synthetic efforts. It is essential that biologists with an appreciation for the target organisms be involved in the discovery process (VI). Biological observations do lead to insight regarding the mechanism of action of toxins (VIII), which in turn leads to the *in vitro* screens discussed by Geissbuhler *et al.* [5] (VIII).

The cost effectiveness of mechanistic studies on toxin action (VII) has often been questioned. It can be very expensive to get from a rough estimation of a possible mechanism (VII) to the detailed characterization of a biochemical target useful in directing synthesis (IX). This expense argues for better interaction among industrial, government and academic laboratories and especially for the need for the U.S.D.A. to investigate pesticide mechanism of action [2]. However, there are now tools to drive more quickly to isolation of a biochemical target and also more promising ways to exploit such knowledge directly rather than simply using it as a stimulus for creativity (V).

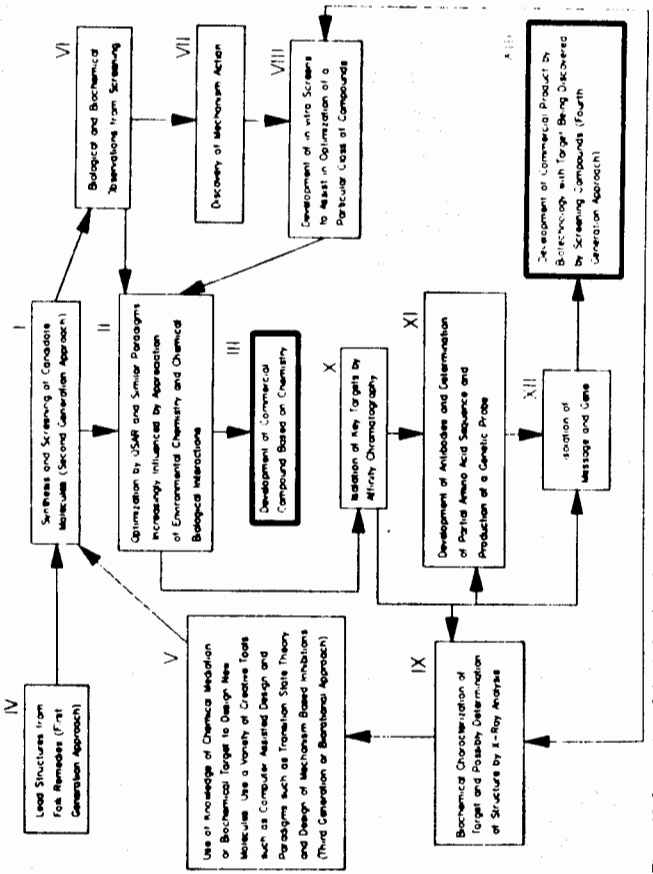


Figure 10. Integration of classical and modern approaches to the discovery of pesticides based on synthetic chemistry (III) and biotechnology (XIII). Affinity chromatography (X) is suggested as a novel way to link classical and molecular approaches (see text). Redrawn from [4].

One approach involves the use of affinity chromatography to isolate biochemical targets (X). Often this approach allows one to move directly toward the isolation of a molecular target. In this case synthesis and screening not only have led to the discovery of chemical activity, but they may have led to the discovery of a new site of action. The isolated target can be characterized biochemically which may allow the rational design of suicide substrates or transition state mimics (IX). It also may allow one to obtain a crystal structure which is a great asset in creative approaches to synthetic chemistry.

The tremendous advances which have occurred in the area of molecular biology may permit the direct exploitation of the affinity purified protein. A pure or even enriched protein allows one to develop antibodies and to determine peptide sequences which can be used to generate cDNA probes (XI). The immunochemical and cDNA probes can lead directly to the isolation of the gene and message (XII). Several of the numerous avenues of exploitation of this information are discussed here with regard to juvenile hormone esterase, and many more have been outlined in the numerous reviews of recombinant DNA technology. However, two points need to be raised.

First, it could be argued that the cost of isolating a target protein, even by affinity chromatography, is too expensive relative to its utility in directing further synthetic efforts or as a lead in biotechnology. The major point is that the lead can be used in both approaches.

Second, genetic engineers in different companies have been racing each other to exploit the messages and genes which have obvious application in agriculture. Soon we will run out of targets for which convenient probes already exist. Just as we must ultimately rely on serendipity for the discovery of most lead structures, screening will play a major role in finding new leads to exploit with biotechnology. For instance the acyl urea insect growth regulators are promising insecticides, but possibly more exciting, they have demonstrated the existence of a protein of unknown function which is probably unique to, and absolutely critical for, the survival of many insects. Such a protein is an obvious target for exploitation with recombinant DNA technology (XIII).

The study of the biological mechanisms which regulate the growth and development of living systems is certain to provide entertainment for many generations of scientists. It is exciting that our generation has witnessed the first direct application of this fundamental knowledge to the improvement of agricultural productivity and profitability. It seems certain that further exploitation of these regulatory systems will become increasingly important in agricultural research and development.

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