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Biorational Suppression of Lepidopteran Pests of Field Crops and Stored Products

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Title

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ABSTRACT

The mode of action of the chitin synthesis inhibitors (CSIs), diflubenzuron, chlorfluazuron, and teflubenzuron, was investigated and the role of detoxification of the CSIs on their comparative potency and resistance was studied. Results with tissue culture experiments demonstrated that the CSIs do not inhibit chitin synthesis by blocking uptake of the amino sugar precursors by epidermal cells. Ultrastructural analysis of the subcellular effects of the CSIs indicated that these compounds were highly selective with respect to chitin synthesis; a phenomenon that could be clearly demonstrated because as little as a 15 minute application was all that was needed in the tissue culture assays. The long exposures involved with treating intact insects could not distinguish secondary effects of the CSIs from its primary action on inhibiting chitin synthesis. Moreover, in contrast to earlier in vivo studies all three CSIs were similar in their effectiveness in vitro. Thus, the comparative toxicity results that demonstrated markedly different activity for these CSIs must result from differential metabolism and/or transport rather than differential activities at the target cell level. This interpretation was substantiated by direct biochemical studies that showed that oxidase and esterase inhibitors were synergistic with respect to the toxicity of diflubenzuron, but antagonized the toxicity of chlorfluazuron. Teflubenzuron was synergistic with the esterase inhibitor but not by the oxidase inhibitor. Thus, the differential effects of the CSIs in vivo may reflect their differential metabolism.

OBJECTIVES

The overall objective of the research was to promote the effective utilization of CSIs for the protection of field crops and stored products from losses caused by pest insects. We had the following specific goals: (1) To determine the effects of CSIs and an IGRs on the life cycle of the Indian meal moth and Spodoptera species; (2) To test the "precursor transport" hypothesis of mode of action of the CSIs in target cells in vitro; and (3) To assess the role of enzymatic detoxification and penetration of CSIs in their comparative potency and resistance. These objectives were adhered to in the cooperative research program with particular success resulting in several publications on both the Indian meal moth and Spodoptera.

TECHNICAL REPORT

Part I: MODE OF ACTION

Introduction:

Benzoylphenyl ureas are selective insecticides that inhibit chitin synthesis in insects, although the manner by which they do this is not known. Extensive studies have been carried out to determine the toxicity and effectiveness in controlling insect pests, both in a field environment and under laboratory conditions. For example, DFB inhibited DNA synthesis in Tenebrio molitor. This action may have resulted from an inhibition of uptake of nucleosides, as was demonstrated in a Manduca sexta cell line. Despite numerous studies, the mode of action of chitin synthesis inhibitors has not been determined. It has been postulated that benzoylphenyl ureas may inhibit chitin synthesis by affecting transport and/or utilization of precursor amino sugars in target cells. For example, Retnakaran et al. suggested from ultrastructural and autoradiographic evidence that transport of chitin precursors by epidermal cells in the spruce budworm was blocked by benzoylphenyl ureas. Similarly, Mayer et al. postulated that chitin synthesis inhibitors act by affecting cell membrane transport. Finally, Mitsui et al. demonstrated that DFB blocked directional transport of UDP-N-acetylglucosamine across gut epithelium in the cabbage armyworm.

We examined the hypothesis that chitin synthesis inhibitors acted by limiting precursor uptake in wing imaginal discs of the Indian meal moth, Plodia interpunctella. We showed that DFB and TFB inhibited ecdysteroid-dependent incorporation into chitin of [^{14}C]-GlcNAc, without inhibiting precursor uptake. Therefore, inhibition of chitin synthesis by benzoylphenyl ureas could not be explained by their effect on the uptake of amino sugar by this target tissue. We tested this hypothesis further by studying the effects of CFA, a potent benzoylphenyl urea compound, on chitin synthesis by wing imaginal discs from the fall armyworm, Spodoptera frugiperda, an agricultural pest of immense importance. Our studies on [^{14}C]-GlcNAc uptake and incorporation by S. frugiperda and P. interpunctella wing imaginal discs have yielded a highly responsive system for investigating ecdysteroid-dependent chitin synthesis in a target tissue in vitro.

Materials and Methods:

S. frugiperda and P. interpunctella were reared on artificial diet according to standard conditions. Mesothoracic imaginal wing discs were dissected from last instar larvae at the end of the feeding period and cultured in modified Grace's medium (GIBCO, Grand Island, NY). 20-hydroxy ecdysone (20-OHE, (Simes, Milan, Italy), was dissolved in modified Grace's medium and added to the cultures 24 hours after dissection of the wing discs. The

wing discs were incubated with hormone for periods ranging from 2 - 96 h. Hormone exposure was terminated by removing the wing discs from the culture dish, rinsing them twice in fresh medium and transferring them to new dishes with 1 ml of hormone-free culture medium.

The wing disc cultures were treated with the following chitin synthesis inhibitors: CFA, wettable powder, purity 80% (-IKI-7899 or CGA-112,913, DFB, 97% technical grade and TFB, 97% technical grade (CME 134). The inhibitors were added at concentrations that ranged from 0.1 nM to 100 μ M, and the duration of exposure of the cultures was for periods ranging from 15 minutes to 72 hours. The inhibitors were dissolved in DMSO and added in 2.5 μ l of stock solution to make the desired final concentration in the culture dish. Exposure of the wing discs to the inhibitors was terminated in the same manner as in the case of 20-OHE treatment. Control wing discs were subjected to the same timing protocol, but instead of inhibitor they were treated with 2.5 μ l of DMSO (with or without 20-OHE).

Uptake of [14 C]GlcNAc (NEC-723) and its incorporation into chitin was tested at the end of the 20-OHE exposure or 48 hours after hormone treatment, unless otherwise indicated. Following incubation for 4 hours with 0.5 μ Ci of [14 C]GlcNAc at 26°C, 95% r.h. as described previously the wing discs were rinsed twice in isotope-free medium. The wing discs were assayed for [14 C]GlcNAc uptake by dissolving them in 150 μ l of Soluene 350 (Packard, Downers Grove, IL) for 24 hr at 4°C, followed by the addition of CytoScint (ICN, Irvine, CA) for measurement of radioactivity. The Packard Tricarb 4000 scintillation counter had an efficiency of 92%, and quenching was checked with an external standard. The results were obtained in dpm.

To test for incorporation of [14 C]GlcNAc into chitin the wing discs were exposed to isotope and placed in a microcentrifuge tube containing 300 μ l of 50% KOH, which was then heated in a boiling water bath for 2 hours. The residue was collected from the digest by vacuum-filtering. When the residue was placed in a chitinase solution (Sigma, 10 mg/ml at pH 5.2) 96.3 % of the radioactivity was removed. For the experiments, the filters were placed in scintillation vials with CytoScint and the radioactivity was measured as above.

RESULTS

Effects of time of application of CFA, DFB and TFB on uptake and incorporation of [14 C]GlcNAc by wing discs:

The purpose of these experiments was to determine the effects of time and duration of application of CFA, DFB and TFB on [14 C]-GlcNAc uptake and chitin synthesis in the cultured wing discs. In our work with P. interpunctella, wing discs were

exposed to the chitin synthesis inhibitors continuously beginning with the onset of a 24 h 20-OHE treatment. While effective, this continuous treatment with inhibitors allowed for the accumulation of side effects of these agents. Therefore, in a series of experiments we found that CFA would inhibit chitin synthesis in the S. frugiperda wing discs when applied for a 24 h period before, during or after a 24 h 20-OHE treatment. Then, we examined shorter time intervals of treatment with the chitin synthesis inhibitors and found a 4 h treatment to be sufficient.

In a typical series of experiments the wing discs were treated with inhibitors for 4 hours at the start of the culture period, with 20-OHE added at 24 h of culture, and the experiment terminated at 72 h of culture (see the attached publications for full details). CFA had no effect on uptake of [^{14}C]GlcNAc, whereas DFB and TFB reduced uptake by about 40% and 52%, respectively, as compared with the controls treated with 20-OHE and DMSO. CFA, DFB and TFB almost completely inhibited incorporation of [^{14}C]GlcNAc by the wing discs. Clearly, all three inhibitors blocked chitin synthesis in the wing discs even when added for only 4 h at the start of culture. Moreover, there was limited inhibition of uptake of GlcNAc by wing discs treated with DFB or TFB, but CFA inhibited chitin synthesis without any apparent diminution of precursor uptake.

To further discover how short a treatment with inhibitor would be effective in blocking chitin synthesis, we tested very brief periods of exposure of the wing discs to CFA at the start of culture. Thus, CFA (100 μM) was added at the start of the culture period for times ranging from 15 minutes to 2 hours. 20-OHE was added at 24 h of culture as before, and the imaginal discs were harvested at 72 h of culture following the 4 h incubation with isotope. Even a 15-minute pulse of CFA at the start of the culture period resulted in an 89% reduction in chitin synthesis. Thus, even a brief treatment with CFA at the start of culture prevented the wing discs from synthesizing chitin when tested 72 h later, and demonstrated the irreversible nature of inhibition of chitin synthesis by CFA in this system.

Dose-dependent inhibition of [^{14}C]GlcNAc incorporation by CFA, DFB and TFB:

Reports on the in vivo activity of benzoylphenyl urea compounds indicate that TFB and CFA are considerable more potent than CFA. Consequently we wished to test whether these differences in activity were apparent from direct effects on the target tissue. Therefore, the purpose of this experiment was to compare the effectiveness of different concentrations of CFA, DFB and TFB on inhibiting chitin synthesis in S. frugiperda wing discs in vitro. 20-OHE (2 μM) was added after 24 h of culture and continued until 72 h of culture as before. The cultured wing discs were exposed to various concentrations of CFA, DFB and TFB

for 4 hours at the end of the hormone treatment period, simultaneously with [^{14}C]GlcNAc incubation. This inhibitor treatment period was selected because our results described above indicated that under these conditions chitin synthesis was inhibited without any corresponding effects on GlcNAc uptake. We found that all three inhibitors blocked chitin synthesis in a similar, dose-dependent manner. Incorporation of [^{14}C]GlcNAc was inhibited almost totally by all three inhibitors at 100 μM and 10 μM . Lower doses of the inhibitors resulted in decreased effectiveness, with DFB being significantly more potent at 0.1 μM . At the lowest concentration tested (0.0001 μM), CFA and DFB were not effective, whereas TFB showed slight inhibition of chitin synthesis. Thus, under conditions in which long term side effects of inhibitor treatment were minimized by treating the wing discs at the end of the culture period, there was little difference in the effectiveness of CFA, DFB and TFB in vitro.

FIGURE 1

INHIBITION OF CHITIN SYNTHESIS IN *PLODIA INTERPUNCTELLA*

FIGURE 2

INHIBITION OF CHITIN SYNTHESIS IN SPODOPTERA FRUGIPERDA

DOSE-RESPONSE DATA FOR CHITIN INHIBITORS

DISCUSSION:

Insect wing imaginal discs respond readily to ecdysteroids by synthesizing chitin when cultured in a controlled in vitro environment. Our work with P. interpunctella wing discs resulted in the establishment of an experimental protocol for chitin synthesis stimulation by 20-OHE, which provided the basis for investigating the action of chitin synthesis inhibitors. However, we have found that cultured wing discs of S. frugiperda are much more favorable for these in vitro experiments.

The potency of CFA in blocking chitin synthesis was not affected by the time of its application to the cultures. Thus, CFA exposures before, during and after 20-OHE treatment were equally effective. This was true for all three inhibitors when they were applied for 4 hours at the very beginning or at the very end of the 3 day culture period. It was striking to find that a CFA pulse as short as 15 min at the beginning of the culture period blocked ecdysteroid-stimulated [^{14}C]GlcNAc incorporation into chitin when tested 3 days later. Such results suggest immediate and irreversible binding of the inhibitor molecules to some unknown target sites in the imaginal disc cells.

There is little correlation between reduction in uptake of [^{14}C]GlcNAc and the inhibition of chitin synthesis. Thus, the reduced uptake of [^{14}C]GlcNAc cannot be the primary explanation for the mode of action of these chitin synthesis inhibitors. Although we observed in our current work that DFB and TFB, but not CFA, reduced uptake of the precursor when applied at the beginning of the culture period, this reduction in uptake was not always correlated with inhibition of chitin synthesis even for DFB and TFB, while CFA never inhibited GlcNAc uptake. The immediate and irreversible response of wing imaginal discs cultured in vitro to the benzoylphenyl urea compounds casts a new light on their mode of action.

The action of the chitin synthesis inhibitor, chlorfluazuron, also was investigated in Spodoptera frugiperda wing imaginal discs cultured in vitro using electron microscopy and cytochemical labeling with a lectin, wheat germ agglutinin (WGA), to monitor morphogenesis and the presence and localization of chitin and non-polymerized N-acetyl-D-glucosamine (GlcNAc) (see attached preprint for details). Our primary objective was to determine the selectivity of action of CFA on development of wing imaginal discs, i.e. which of the 20E-stimulated developmental processes are disrupted or affected by CFA. At the same time we sought to gain a better understanding of the mode of action of CFA on its target tissue. To achieve these goals, we conducted ultrastructural and cytochemical analyses of the cultured wing discs treated with CFA and the lectin, WGA, which

is specific for GlcNAc and its polymers. We found that chlorfluazuron selectively inhibited 20-hydroxyecdysone-stimulated chitin synthesis and procuticle deposition in imaginal discs, without otherwise affecting their morphogenesis.

Part II: TOXICOLOGY AND RESISTANCE

INTRODUCTION:

Benzoylphenylureas are selective insecticides which act on insects of various orders by inhibiting chitin formation, thereby affecting the elasticity and firmness of the endocuticle and disrupting normal molting. Diflubenzuron (DFB), the most investigated compound of this group, acts on the larval stage. The search for effective benzoylphenyl ureas has led to the development of new compounds which are considerably more potent than DFB against various agricultural pests. Resistance is the result of increased metabolism and excretion as well as of reduced cuticular penetration. The potency of insect control agents is known to be limited by oxidative, hydrolytic and glutathione transferase reactions which vary in their relative significance, depending on the species and the compound involved. Hydrolysis has been identified as the major route of DFB detoxification. The major hydrolytic metabolites of DFB in Spodoptera species are 4-chloroaniline and 4-chlorophenylurea. Labeled DFB and p-nitroacetanilide were used as substrates in the determination and partial characterization of carboxylamidase activity in vitro, in the prepupae of the stored product pest, P. interpunctella. The aim of this work was to determine possible synergism of DFB by hydrolase, oxidase and glutathione transferase inhibitors, aiming at inhibiting detoxification of this BPU in two larval stages of P. interpunctella.

A primary consideration in use of any novel pesticide is development of resistant strains, particularly in species that have developed resistance to insecticides that are commonly used. Strains of the housefly, Musca domestica L. that are resistant to conventional insecticides are variously cross-resistant to diflubenzuron. However, chlorfluazuron and teflubenzuron are equally toxic to strains of Tribolium castaneum that are resistant and susceptible to malathion. In a previous study a field strain of S. littoralis resistant over 100 times to pyrethroids and organophosphorus insecticides as compared to a laboratory susceptible strain, showed a mild tolerance (~5 times) to teflubenzuron.

The present study evaluated possible cross-resistance between pyrethroids and two benzoylphenyl ureas - diflubenzuron and chlorfluazuron in S. littoralis larvae. In addition DFB hydrolase activity was characterized in Spodoptera littoralis and detected in Spodoptera exigua. Hydrolytic detoxification processes and enzyme activity have been determined using labeled diflubenzuron. Inhibition of detoxifying enzymes weakens the defense system of the insects and this in turn increases the toxicity of the insecticide. This strategy can be utilized only if a precise evaluation is made of the kinetic characteristics of DFB hydrolases from various insect sources. Our study was

undertaken to determine the hydrolytic detoxification mechanism of DFB in the stored product lepidopteran pest Plodia interpunctella. Evaluation of this mechanism in vitro together with the use of inhibitors, may help in developing methods to reduce detoxification mechanisms of this compound as well as other BPUs, which may lead to enhanced potency.

MATERIALS AND METHODS:

Teflubenzuron (TFB) was obtained from Shell Agar GmbH (Ingelheim, Germany), hexaflumuron (HFM) from Dow Chemicals, and chlorfluazuron (CFA) from Ishihara (Japan). Radiolabeled [^{14}C]-diflubenzuron ([^{14}C]DFB, 15 mCi/g, uniformly labeled on the aniline ring) and technical DFB (99.5%), along with its metabolites 4-chloroaniline and 4-chlorophenylurea, were gifts from Duphar B.V. An organophosphorus insecticide, tested as a carboxylamidase inhibitor, was an emulsifiable concentrate of 72% S,S,S-tributylphosphoro-trithioate (DEF, Shell Agar GmbH). DFB oxidase inhibitor pipronylbutoxide (PB), technical grade (Pazchem, Tel Aviv) and the inhibitor of the conjugation pathway, diethylmaleate (DEM) and p-nitroacetanilide (pNAA) were obtained from Merck.

Plodia interpunctella was reared under standard laboratory conditions. First instar and 4th instar larvae, 0-24 h after ecdysis, were fed with culture medium treated with various concentrations of DFB, HFM, TFB and CFA. Each test group contained 60-75 larvae. First and 4th instar larvae were reared on growth media containing DFB, DEF or a mixture of DEF and DFB (the final concentration of each compound was 100 ppm). Homogenates of the prepupae that developed from these insects, were prepared in 0.1M phosphate buffer, pH 8.0, using an IKA, Ultra-Turax homogenizer. The homogenates were centrifuged for 10 min at 10,000 x g and the supernatant was used as the enzyme solution.

The Egyptian cotton leafworm S. littoralis, a well defined susceptible strain, yellow-eyed was reared on castor bean leaves under standard laboratory conditions of $25 \pm 1^\circ\text{C}$, 65% RH and a 16 h photoperiod. The field resistant strain was collected in 1990 from a cotton field in the Bet She'an area and since then was reared on castor bean leaves under standard laboratory conditions. Preliminary bioassays indicated that the field strain was resistant to most of the conventional insecticides and especially pyrethroids. This resistance probably developed from the high selection pressure of pyrethroids and organophosphates (10-12 treatments per season) that were applied to cotton field prior to 1990. All bioassays were done with 3rd instar ($20 \pm 3\text{mg}$) 24 h after ecdysis. Larvae were offered castor bean leaves treated with the test compound. The treatment was done by dipping the leaves in the appropriate solution. After 2 h of air drying at room temperature, the treated leaves were offered to the

larvae for 2 d and then replaced by untreated leaves. Mortality was determined after 6 days when all the untreated larvae (control) had molted to the fourth instars. Full details of the biochemical procedures are provided in the attached preprints.

RESULTS:

Cotton fields in Israel, prior to 1990, were heavily sprayed with pyrethroids and organophosphates (10-12 treatments per season) against aphids, lepidopterous and whiteflies. We expected that insects collected from such fields would be resistant to conventional insecticides. Preliminary assays indicated that the field strain is very resistant to pyrethroids and to a lesser extent to organophosphates. According to LC_{50} values, the field strain was ~120 times more resistant to cypermethrin than the susceptible laboratory strain. This strain showed a very mild (not appreciable) tolerance to diflubenzuron (1.5 times) and chlorfluazuron (1.2 times). These results indicated that no appreciable cross-resistance exists between pyrethroids and the two tested benzoylphenyl ureas - diflubenzuron and chlorfluazuron. According to LC_{50} values chlorfluazuron (0.13-0.15 ppm) is 120-150 times more potent than diflubenzuron on both the susceptible and the pyrethroid resistant strains. In a similar study, a multiresistant strain of S. littoralis (over 100 times resistant to pyrethroids and organophosphates), collected from cotton fields in 1987 showed a mild cross resistance (5 times) to teflubenzuron. Some cross resistance (2-3 times) between conventional insecticides and teflubenzuron was reported for the diamondback moth, Plutella xylostella. However teflubenzuron and chlorfluazuron showed similar potency on strains of Tribolium castaneum that are resistant and susceptible to malathion.

Determination of the toxicity of four BPUs - DFB, HFM, CFA and TFB - on 1st - instar larvae of P. interpunctella, revealed the highest toxicity with CFA, followed by HFM and TFB. DFB was the least toxic; it did not induce 100% mortality even at a concentration as high as 100 mg a.i./kg. Assays with 4th instars revealed a similar pattern of activity to that of 1st instars. The LC_{50} value of DFB on 4th instars was achieved at a dose as high as 300 mg a.i./kg and the relative toxicity of TFB, HFM and CFA was 315, 865 and 6056 times greater than DFB, respectively. The potency of DFB on 4th instars is much lower than that on 1st instars. On the other hand, TFB, HFM and CFA showed similar toxicity on 1st and 4th instars. These findings may result from higher DFB detoxification in 4th-instar larvae as compared with 1st-instar larvae. Similar results were obtained with Tribolium castaneum.

The importance of oxidases in the process of detoxification of DFB in the phenomena described above was examined by addition of 100 ppm PB to the medium. PB acted as an antagonist to DFB,

resulting in a decrease in larval mortality in all treatments. These results indicate that oxidation is not a predominant pathway in DFB detoxification in 1st instar larvae of P. interpunctella. Addition of 100 ppm DEM, the glutathione transferase inhibitor, to growth media of both instar larvae, alone and together with DFB, resulted in a considerable antagonism in 4th instar larvae; the effect on 1st instar larvae was additive. These findings suggest that glutathione transferase may play some role in DFB detoxification in 1st - instar but not in 4th instar larvae.

The highest DFB hydrolase activity was obtained with homogenates of prepupa. When activity was measured in 4th-instar larvae using an enzyme solution obtained from homogenate of the whole larvae or of the gut, hydrolysis was 13% and 12%, respectively. The products include 4-chloroaniline (4-CA) and 4-chlorophenylurea (4-CPU), the former being the major metabolite. A considerable amount of radiolabeled polar metabolites was observed at the origin of the TLC plate. These metabolites are extractable with methanol but not with acetonitrile and are considered to be conjugated polar metabolites. The amount of these metabolites increased with the time of reaction.

Diffubenzuron hydrolase activity was inhibited in vitro by profenofos and DEF at a concentration of 10^{-5} M, with profenofos being more effective. In other assays enzyme activity was inhibited in vivo by 40% following the addition of 100 ppm DEF to the diet of 4th-instar P. interpunctella larvae. The enzyme activity was inhibited by 38% upon addition of 10^{-4} M pNAA to the reaction mixture; 66% of 4-CA release was reduced, whereas the release of 4-CPU was not affected.

The activity of pNAA hydrolase was examined at the optimal conditions in various stages of P. interpunctella and with a single substrate concentration of 3×10^{-4} M. Specific activities of 0.24 ± 0.01 , 0.20 ± 0.01 , 0.23 ± 0.02 and 0.15 ± 0.02 μ mol/min/mg protein were obtained with homogenates of prepupa, wandering stage, 4th midinstar and 3rd midinstar, respectively; hence, prepupae were used in all assays.

The inhibition of hydrolysis by DFB was substrate concentration-dependent; the inhibitory effect decreased as the substrate concentration increased. A plot of the data by the method of Ackermann and Potter indicated that the inhibition of hydrolysis was dependent upon enzyme concentration. The inhibitory effect decreased as the enzyme level was elevated.

Enzyme activity was inhibited by the hydrolase inhibitors, DEF and profenofos, the latter showing a greater effect at relatively low inhibitor concentrations. The IC_{50} for profenofos was 2×10^{-7} M, while that for DEF was approximately 10^{-1} M. A

similar pattern of enzyme inhibition was obtained with enzymes extracted from S. litoralis.

INHIBITION OF pNAA HYDROLYSIS WITH *PLODIA INTERPUNCTELLA* BY THE
HYDROLASE INHIBITORS PROFENOFOS (●) AND DEF (○).

MORTALITY CURVES FOR 4TH INSTAR PLODIA INTERPUNCTELLA

FIGURE 6

DOSE-RESPONSE DATA FOR CHLORFLUAZURON AND DFLUBENZURON EFFECTS ON
SUSCEPTIBLE AND RESISTANT STRAINS OF SPODOPTERA LITTORALIS

DISCUSSION:

Benzoylphenylureas (BPU) are insect growth regulators, which act on various orders of insects and are considered important components in integrated pest management (IPM) programs. The toxicity of these compounds depends on their retention in the larval body and their transport to the biochemical sites. An understanding of the mechanism of detoxification of BPUs in general, and of DFB in particular, is of importance in elucidating mechanisms of resistance to these important insecticides. According to LC values, larvae of 1st instar P. interpunctella showed 11-210 times greater natural tolerance to DFB than to other BPUs, while 4th instar larvae showed 315-6056 times greater tolerance to this compound.

The high toxicity of CFA, HFM and TFB to this insect pest is probably due to the greater retention of these BPUs in the larval body. Greater retention is often the result of a slower detoxification procedure in the insect body. This in turn depends on the presence of degradative enzymatic activities. We showed that a hydrolytic activity exists in the stored-product insect P. interpunctella. Our results indicate that hydrolysis is an important route of DFB detoxification in 4th instar larvae of P. interpunctella, as expressed by a considerable synergism upon addition of DEF to a diet containing DFB). This synergism probably results from inhibition of DFB hydrolase activity. These findings concur with those obtained with Spodoptera littoralis.

At the optimal conditions of the in vitro assay, two metabolites were observed: 4-CA and 4-CPU. The first was the major one, with a level of about 5-8%, and the second was the minor metabolite with a level of about 2-3%. These results resembled those reported for S. littoralis. Resistance to various insecticides and DFB tends to increase with the developmental larval stages. The level of chitinolytic enzymes was reported to increase during larval-pupal development in Ephestia cautella. DFB hydrolase activity also accelerates in the prepupal stage of P. interpunctella, which explains the fact that DFB had lower toxicity in the later larval stages.

Both hydrolytic and oxidative processes have been reported to be involved in the metabolism of various insecticides such as pyrethroids or DFB in insects. The hydrolase inhibitors profenofos and DEF synergized the toxicity of pyrethroid compound. These compounds inhibited carboxyhydrolases in various insect species such as P. interpunctella (this study), S. littoralis, S. exigua, T. castaneum among others. Inhibition of detoxifying enzymes may help to overcome resistance due to hydrolysis or oxidation and is important in elucidating mechanisms of resistance to BPUs. The fact that pNAA degradation was inhibited by these two classical hydrolase inhibitors

indicates the presence of hydrolytic enzymes in P. interpunctella which enable metabolism of carbonylamide site pNAA.

In conclusion, our findings indicate that pNAA may serve in some cases as a surrogate substrate for DFB hydrolyzing enzymes. The assay is easy to perform, accurate, and can be used for evaluating mechanisms of detoxification and resistance to DFB and other BPU's. The findings reported herein may serve as an additional tool in evaluating detoxification processes in insects.

The results presented in this study also indicate that chlorfluazuron which is considered to be an efficient insecticide is equally potent on pyrethroid resistant and susceptible strain of S. littoralis larvae. These findings may have important practical implication for controlling this lepidopterous pest in cotton and other field crops.

COOPERATION

The research projects at Bet Dagan and Gainesville have proceeded according to an overall plan that was jointly conceived by the principal investigators on this project. Both Dr. Shaaya and Dr. Ishaaya visited Gainesville, Florida during the course of the project to discuss research results, plan experiments and confer on manuscripts. In addition the principal investigators have been in frequent communication through the mails.

While the two Spodoptera species studied in Gainesville and Bet Dagan were necessarily different, both laboratories used the same Plodia interpunctella strain, which originated with the Gainesville colony. To accommodate the investigation of different species of Spodoptera in Israel and the United States the Gainesville laboratory engaged two scientists with previous experience with the same Spodoptera species studied in Israel. This enabled us to apply their knowledge to Spodoptera frugiperda, a species that is available in Gainesville. USDA funds were used to supplement the BARD grant for these postdoctoral scientists, and their tenure in Gainesville was extended through the use of local funds.

Thus, as detailed in the original proposal, the special expertise and focus of the two laboratories was complimentary so that the combined efforts of the principal investigators were successfully applied to the objectives of this BARD grant. The first two publications under this grant have been published, and several more are in preparation, submitted or accepted.

EVALUATION

Biorational pesticides, such as insect growth regulators (IGRs) and chitin synthesis inhibitors (CSIs), which do not have adverse impact on the environment, are of critical importance in controlling pest insects that have become resistant to traditional insecticides. The objective of the project was to enhance the effective use of these compounds by obtaining new insights on their mode of action, comparative toxicity and resistance. The research resulted in new knowledge of the actions of the potent CSIs, chlorfluazuron, diflubenzuron and teflubenzuron. We determined the mode of action of the CSIs on transport and incorporation of precursors of chitin and glycoproteins in imaginal discs and cell lines in tissue culture. At the same time we investigated the CSI detoxifying enzymes to elucidate the basis of increased stability of CSIs in larvae. Inhibition of these enzyme systems, in some cases antagonized or synergized the activity of the CSIs.

Specifically, we tested the hypothesis that the inhibition of chitin synthesis by benzoylphenyl ureas could be explained by their effect on the uptake of GlcNAc into chitin. The test system consisted of organ cultures of wing imaginal discs from Plodia interpunctella and Spodoptera frugiperda. These wing discs synthesize chitin in response to 20-hydroxyecdysone, a non-steroidal ecdysteroid mimic. Chlorfluazuron, diflubenzuron and teflubenzuron inhibited ecdysteroid-dependent chitin synthesis in these cultured wing discs. Although chitin synthesis was inhibited, there was no corresponding diminution of amino sugar uptake by the imaginal discs. From these observations we conclude that the inhibition of uptake of amino sugars does not account for the ability of these chitin synthesis inhibitors to prevent chitin synthesis in pest insects.

Recent results with S. frugiperda showed that benzoylphenyl ureas can inhibit chitin synthesis in imaginal discs in vitro, with as little as a 15 minute exposure 3 days before assay for synthesis provides a unique system for distinguishing primary from secondary effects of the inhibitors. Previous work in vivo was unable to determine which effects of the inhibitors were "side effects", and which represented the primary mode of action of the inhibitor. The new ultrastructural analysis at the Gainesville laboratory builds on this significant foundation and emphasized the selectivity of the CSIs on chitin synthesis when optimal conditions in tissue culture are used to study the CSIs.

Comparative toxicity studies showed that for Plodia interpunctella that at the fourth larval instar teflubenzuron was more than 300 times more active than diflubenzuron, while chlorfluazuron was 6000 times more active than diflubenzuron. An esterase inhibitor, which inhibited carboxylamidase, synergized diflubenzuron activity. These results and others of a related

nature provide critical information on the detoxification of the CSIs. Thus, hydrolases seem to play an important role in diflubenzuron detoxification, while oxidation and glutathione transferases seem to be of minor importance. Both hydrolase and oxidase inhibitors were synergists of the toxicity of diflubenzuron, indicating the importance of these enzyme systems in the detoxification process. By contrast these metabolic inhibitors did not enhance the activity of more stable benzoylphenyl ureas, such as chlorfluazuron. Most notably, the metabolic inhibitors blocked the activity of the stable compound, chlorfluazuron, as measured by larval mortality and body weight. Taken together these findings provide key information for understanding and managing resistance to the CSIs by target pest insects.

The toxicological and biochemical research of the chitin synthesis inhibitors demonstrated the importance of resistance and synergism studies with these compounds. Clearly, there are important differences in the toxicology of the various benzoylphenyl ureas, and the stability of these compounds in the target insect at vulnerable stages of development will be key to effective utilization of CSIs in insect control.

PUBLICATIONS

Published or accepted:

Benzoylphenyl ureas inhibit chitin synthesis without interfering with amino sugar uptake in imaginal wing discs of *Plodia interpunctella*. H. Oberlander, D. L. Silhacek, E. Leach, I. Ishaaya, and E. Shaaya. Arch. Insect Bioch. and Physiol. 18:219-227 (1991)

Chitin synthesis in *Spodoptera frugiperda* Wing imaginal discs: I. Chlorfluazuron, diflubenzuron, and teflubenzuron inhibit incorporation but not uptake of [14-C]N-acetyl-D-glucosamine. P. Mikolajczyk, H. Oberlander, D. L. Silhacek, I. Ishaaya, and E. Shaaya. Arch. Insect Bioch. and Physiol. 25:245-258 (1994).

Chitin synthesis in *Spodoptera frugiperda* wing imaginal discs. II. Selective action of chlorfluazuron on wheat germ agglutinin binding and cuticle ultrastructure. G. Zimowska, P. Mikolajczyk, D. L. Silhacek and H. Oberlander. Arch. Insect Bioch. and Physiol. (Accepted, 1994).

In preparation:

Hydrolase activity in *Plodia interpunctella*: Use of diflubenzuron and p-Nitroacetanilide as substrates. S. Greenberg-Levy, I. Ishaaya, E. Shaaya, D. L. Silhacek and H. Oberlander.

Toxicity and synergism of DFB in two larval stages of the stored product pest *Plodia interpunctella*. S. Greenberg-Levy, E. Shaaya, M. Kostjukovsky, I. Ishaaya, D. L. Silhacek and H. Oberlander.

Response of susceptible laboratory and resistant field strains of *Spodoptera littoralis* to diflubenzuron and chlorfluazuron. I. Ishaaya, S. Yablonski, E. Shaaya, H. Oberlander and D. L. Silhacek.