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Effects of the synergist *S,S,S*-tributyl phosphorotrithioate on indoxacarb toxicity and metabolism in the European corn borer, *Ostrinia nubilalis* (Hübner)

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Abstract

The toxicity of the oxadiazine insecticide indoxacarb to the European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae), was evaluated in the presence and absence of *S,S,S*-tributyl phosphorotrithioate (DEF), an inhibitor of hydrolytic metabolism. Bioassays involving topical application of different concentrations of indoxacarb to third instars of a susceptible *O. nubilalis* laboratory strain were performed, and in vitro metabolism experiments involving [^{14}C] indoxacarb were examined to determine the role of hydrolytic metabolism in indoxacarb activation. Indoxacarb toxicity decreased in the presence of DEF indicating antagonism of toxicity. Results of in vivo and in vitro inhibition experiments indicated a reduction of indoxacarb activation and formation of the hydrolytic metabolite. These results are consistent with the proposed mechanism of hydrolytic activation for this compound.

Keywords: *Ostrinia nubilalis*; Indoxacarb; Oxadiazine; DEF; Synergist; Insecticide metabolism

1. Introduction

Indoxacarb is the first member of the oxadiazine insecticides and exhibits strong activity against lepidopteran pests of vegetables, tree fruits, cotton, corn, peanut, soybean, alfalfa and other crops [1]. Activity of this compound has also been shown against some homopteran and coleopteran species [2]. Indoxacarb is a highly active insecticide when ingested [2], although contact by direct spray or exposure to dried residue [3] has been shown to have activity in certain situations. Upon ingestion, larvae stop feeding after a short period of time and exhibit mild convulsions or a passive paralysis, which is an irreversible condition [4].

Indoxacarb is a pro-insecticide, and must undergo metabolic activation of the parent compound. This process involves hydrolysis to the *N*-decarbomethoxyllated metabolite [4], which is much more toxic than the parent compound. The activated metabolite of indoxacarb has a unique mode of action involving the blocking of sodium channels of nerve cells, resulting in paralysis and death of the target pest species [5]. Neurotoxic symptoms lead to a rapid and irreversible cessation of feeding [2]. Symptoms of exposure to indoxacarb as a contact insecticide are similar but slower to develop [2]. Experiments involving indoxacarb metabolism in lepidopteran larvae indicate that at least 90% of the parent compound is converted to the activated metabolite 4 h after ingestion [2]. Tissue localization studies with fifth instar *Manduca sexta* larvae have shown that the fat body and midgut are the most active tissues catalyzing the activation of indoxacarb [4].

S,S,S-Tributyl phosphorotrithioate (DEF) is a widely recognized insecticide synergist that functions by inhibiting hydrolytic metabolism, and is commonly used in experiments involving metabolism of insecticides with ester linkages [6–8]. Synergists are often used to inhibit detoxification in cases where resistance has evolved. However, as hydrolysis is a fundamental step in the activation

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of indoxacarb, it is anticipated that the use of DEF should antagonize toxicity by inhibiting activation of the parent compound. Metabolism studies in several lepidopteran larvae show that orally administered [^{14}C] indoxacarb is rapidly converted to the decarbomethoxyllated metabolite [4], and the metabolic conversion is correlated with appearance of neurotoxic symptoms. However, there is little information regarding metabolism of topically applied indoxacarb.

The objectives of this study were to test the effects of the synergist DEF on indoxacarb toxicity and metabolism in *Ostrinia nubilalis* (Hübner) when topically applied. Bioassays involving topical application of indoxacarb in the presence and absence of DEF and in vivo and in vitro inhibition by DEF of [^{14}C] indoxacarb metabolism were compared. Results of these experiments provide insight into the role of hydrolytic metabolism in the activation of indoxacarb in a representative lepidopteran.

2. Methods and materials

2.1. Insects and rearing

Fourth instars were randomly selected from a susceptible *O. nubilalis* laboratory colony maintained at the University of Nebraska and used in all experiments. The laboratory colony was initially derived from field collections in 1995 [9] and reared for 90 generations without exposure to insecticides. Rearing methods were based on those developed at the USDA-ARS Corn Insect Research Unit, Ames, IA [10,11].

2.2. Chemicals

[^{14}C] Indoxacarb (27.3 mCi mmol^{-1}) and non-radiolabeled indoxacarb (95% purity) were supplied by DuPont Agricultural Chemical (Newark, DE). [^{14}C] Indoxacarb was purified by thin layer chromatography (TLC) using a 250 μm thick silica plate (Sigma Chemical Company, St. Louis, MO). The plate was developed in toluene:acetone (70:30), and the labeled compound was identified by co-chromatography with a cold indoxacarb standard visualized under UV light. The radioactive band was scraped from the plate and eluted from the silica with repeated rinses in acetone. *S,S,S*-Tributyl phosphorotrithioate (DEF) was obtained from Chem Services (West Chester, PA).

2.3. Bioassays

Susceptibility of *O. nubilalis* to indoxacarb was determined by exposure of fourth instars to varying concentrations of indoxacarb prepared in acetone. Insecticide dilutions were topically applied to the dorsal abdomen in 1 μl acetone using a Hamilton syringe (Reno, NV) with a repeating dispenser. Control larvae were treated with acetone only. To evaluate the effect of DEF on indoxacarb toxicity, both control and indoxacarb treated fourth instar *O. nubilalis* were treated with 1 μl of 5 μg/μl DEF 4 h before treatment with indoxacarb. Larvae were placed in plastic Petri dishes after treatment and maintained at 27 °C, 24 h scotophase, and 80% RH. Treated larvae were provided with a wheat germ-based diet [10] throughout exposure. Mortality was evaluated at 24 and 48 h after treatment. Larvae that did not respond to probing were considered dead. A single bioassay consisted of seven doses of indoxacarb plus a control, with 16 larvae exposed at each concentration. Each bioassay was repeated three times. Dose-mortality data were analyzed by probit regression using POLO-PC [12] to calculate lethal doses and their 95% confidence intervals, slopes and their standard errors. Mortality was corrected for control mortality using the method of Abbott [13].

2.4. In vivo inhibition experiments

Fourth instars were topically treated with 0.5 μl DEF (5 μg/μl) or 0.5 μl acetone (control) using a Hamilton syringe. Midguts of 40–60 treated larvae were dissected 4 h after treatment as described by Siqueira et al. [9] with slight modifications. Dissected midguts were vortexed briefly in ice-cold 0.2 M sodium phosphate, pH 7.0 (buffer A), and centrifuged for 5 min at 5000*g* at 4 °C to remove gut contents. After centrifugation, the pelleted midgut tissue was weighed and homogenized in 1 ml buffer A using a Potter-Elvehjem homogenizer. Midgut homogenates were centrifuged for 10 min at 12,000*g*, and the supernatant collected and filtered through glass wool. Protein concentration was determined using BCA protein assay (Pierce, Rockford, IL) [14], and esterase activity was quantified through assays with the model substrate *p*-nitrophenyl acetate (pNPA) as described by Zhou et al. [15].

To measure indoxacarb metabolism by midgut preparations, the 12,000*g* supernatant from tissue homogenates was incubated with [^{14}C] indoxacarb for 2 h at 30 °C. The concentration of protein samples added to each reaction was approximately 0.7 mg/ml in a total volume of 500 μl. Reaction mixtures consisted of 50 μl of the 12,000*g* supernatant, 440 μl buffer A, and 4.4 μM [^{14}C] indoxacarb (total volume of 500 μl). Each treatment was replicated three times and the entire experiment was repeated with two different preparations. To determine the rate of recovery and to measure non-enzymatic conversion of indoxacarb, control incubations consisting of 490 μl buffer A and an equal concentration of [^{14}C] indoxacarb were conducted. Reactions were stopped by vortexing with 500 μl of ethyl acetate. A combined ethyl acetate extract (2 × 500 μl) of the incubation was evaporated to dryness under N_2, dissolved in 30 μl of acetone, and applied to a TLC plate which was developed in toluene:acetone (70:30) followed by dichloromethane:acetone:ethyl acetate (95:2:3). All samples, as well as the cold standards were applied to the TLC plate using disposable micropipettes. TLC plates were exposed to X-ray film (Kodak X-Omat AR) for 7 days at −20 °C. Radiolabeled indoxacarb and