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Michael E. Scharf
University of Nebraska-Lincoln

Blair Siegfried
University of Nebraska-Lincoln, bsiegfried1@ufl.edu

Lance J. Meinke
University of Nebraska-Lincoln, lmeinke1@unl.edu

Robert J. Wright
University of Nebraska-Lincoln, rwright2@unl.edu

Laurence D. Chandler
Northern Crop Science Lab, Fargo, ND

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Michael E. Scharf, Blair D. Siegfried, Lance J. Meinke,
Robert J. Wright,* and Laurence D. Chandler[†]

Department of Entomology, University of Nebraska–Lincoln, Lincoln, Nebraska 68583-0816
* Current address: South Central Research and Extension Center, University of Nebraska, Box 66, Clay Center, NE 68933.
[†] Current address: Northern Crop Science Lab, 1307 N 18th Street, Fargo, ND 58105-5677.
*Corresponding author:* B. D. Siegfried

Abstract

Elevated cytochrome P450 monooxygenase-based metabolism is associated with carbaryl and methyl parathion resistance in western corn rootworm (*Diabrotica virgifera virgifera* LeConte) populations in the state of Nebraska. The objectives of this study were to identify potential differences in cytochrome P450 in resistant and susceptible rootworm populations using the model substrate 4-chloro-*N*-methyl aniline (4-CNMA) and heme staining of microsomal proteins separated by SDS–PAGE. Baseline N-demethylation activity corresponded well with resistance to the *N*-methyl-substituted insecticide carbaryl among study populations. N-demethylation of 4-CNMA is NADPH dependent and inhibited by the cytochrome P450 inhibitor piperonyl butoxide, which confirms that the reaction is catalyzed by cytochrome P450. Following ingestion of the cytochrome P450 inducer pentamethyl benzene, N-demethylation activity increased significantly in the more susceptible rootworm populations. Hemoprotein staining of microsomal proteins separated by SDS–PAGE identified two groups of potential P450 isoforms in the 45- to 53-kDa range which appear more highly expressed (a) in resistant populations and (b) following PMB induction.

Keywords: cytochrome P450, induction, carbaryl, insecticide resistance, N-demethylation.

Cytochrome P450 monooxygenases (P450s) are an enzyme superfamily which occurs in all cellular organisms, and they are important to xenobiotic metabolism and insecticide resistance when they are overproduced (1). P450s are microsomal hemoproteins, inducible by numerous apolar substances, require NADPH as a cofactor for catalytic activity, and vary in molecular mass from 45 to 60 kDa (2). Relatively less is known of the molecular aspects of P450 induction and overexpression in insects. However, researchers have recently reported commonalities, such as a lack of induction in resistant individuals (3, 4), that are suggestive of mutations to trans-regulating genetic elements (3, 5–7) which may be involved in both induction and constitutive overexpression (3).

In Nebraska, populations of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte, which are resistant to organophosphate and carbamate insecticides, have recently been identified (8). However, a 1997 survey of United States populations indicated that carbaryl resistance is limited to Nebraska, with the highest levels focusing around Phelps and York Counties (9). Studies of insecticide synergism initially indicated that both oxidative and hydrolytic metabolism are involved in methyl parathion (10) and carbaryl resistance (9) in populations from isolated areas of Nebraska. In studies of [^{14}C]carbaryl metabolism, increased NADPH-dependent N-demethylation to 1-naphthyl acetamide, followed by hydrolysis to 1-naphthol, were observed in resistant pop-
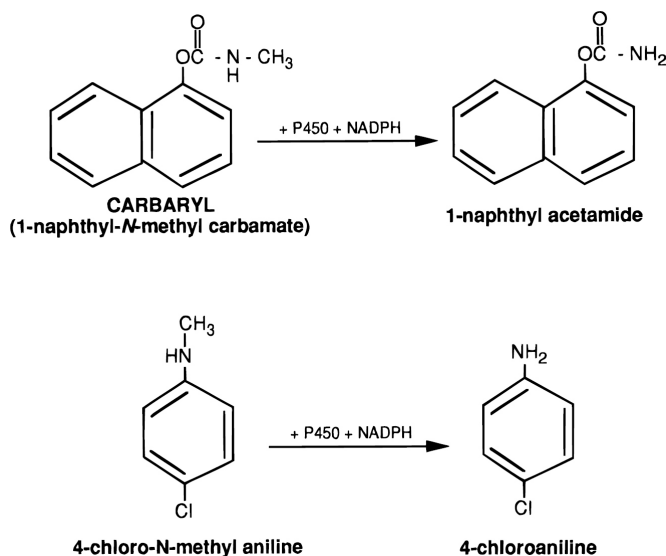
Figure 1. Chemical structures for carbaryl and 4-chloro-*N*-methyl aniline and their respective N-demethylated metabolites 1-naphthyl acetamide and 4-chloroaniline.

ulations (11). Increased formation of 1-naphthyl acetamide via N-demethylation (Figure 1), however, appears to be of greater importance to overall resistance levels than either esterase-based hydrolysis or P450-based ring hydroxylations of the parent insecticide carbaryl (11).

The present status of western corn rootworm insecticide resistance to organophosphate and carbamate insecticides in Nebraska and ongoing corn rootworm areawide management pilot programs in other areas of the United States (which are employing carbaryl; see Reference 12) have placed increased priority on developing insecticide resistance management programs for this species. Development of biochemical techniques to document changes in detoxification enzymes following insecticide selection would be useful for monitoring the effects of ongoing and future pest management programs. To be most effective, biochemical population assessment depends on a sound understanding of metabolic characteristics of insecticide-resistant populations (13). In other insects, studies of this nature have been important for identifying biochemical characteristics associated with P450-based xenobiotic resistance and enabled future purification,

immunological, and molecular work (14, 15). The research reported here focused specifically on biochemical characteristics of cytochrome P450 in insecticide-resistant versus insecticide-susceptible rootworm populations, and its objectives were to (1) examine P450 activity toward a model substrate and its inhibition by the P450 inhibitor piperonyl butoxide, (2) investigate the effects of induction on P450 activity, and (3) identify potential electrophoretic P450 isoforms which are associated with insecticide resistance and/or are responsive to induction.

Materials and Methods

Chemicals

Pentamethyl benzene, 4-chloro-*N*-methylaniline (4-CNMA), and 4-chloroaniline were obtained from Aldrich Chemical Co. (Milwaukee, WI). Piperonyl butoxide (PBO) was purchased from Crescent Chemical (Hauppauge, NY). Electrophoresis reagents and Tween-20 (EIA grade) were purchased from Bio-Rad Laboratories (Hercules, CA). Unless noted, all other chemicals were purchased from Sigma (St. Louis, MO) and were of reagent grade or better.

Rootworm Populations

Three populations of western corn rootworm beetles were obtained during late July and August of 1996. The susceptible Saunders County (Nebraska) population was collected from an area with little exposure to adult management practices and no reports of control failures. The York and Phelps County (Nebraska) populations came from areas where adult management has been practiced in excess of 10 years and where control failures have been reported. Field-collected beetles (at least 500) were shipped to the USDA-ARS Northern Grain Insects Research Laboratory in Brookings, South Dakota, and maintained there for oviposition. Approximately 50,000 eggs were obtained from each population and standard procedures were used to collect and maintain eggs until termination of diapause and to rear F_1 individuals to adults (16). One additional generation was cultured, and F_2 adults were shipped to the University of Nebraska–Lincoln in January–March 1998. Levels of carbaryl susceptibility (based on percentage mortality on a diagnostic concentration of 5.0 μg/ml carbaryl; see Reference 11) for these laboratory-reared populations were previously reported: Saunders (93.8%), York (28.8%), and Phelps (62.5%). For experiments described below, F_2 adults were maintained in plastic cages at room temperature on a 14:10 h(L:D) photoperiod and provided an artificial diet containing ground corn and bee pollen, with agar as a water source.

Induction of P450

Pentamethylbenzene (PMB) was used as an inducer of P450. Groups of beetles were isolated from colonies and provided with an agar water source containing 0.2% w/v PMB for 96 h and food as described above. This concentration of PMB was used as it has been previously shown to effectively induce P450 in a number of insect species (4, 17–19). Agar was prepared by boiling a solution containing 1.05 g bacto–agar (Difco, Detroit, MI) and 44 mg sorbic acid in 175 ml distilled water. As the solution cooled, 354 mg of PMB was added while stirring, and the solution was allowed to solidify for ca. 16 h at 4 °C.

Protein Preparations and Assay

Abdomens were dissected from frozen adult rootworms and homogenized with a Teflon glass Potter–Elvehjem-type homogenizer in 150 mM KPO_4 (pH 7.4) containing 10% v/v glycerol, 1 mM phenylmethylsulfonyl fluoride, 1mM phenylthiourea, 1 mM ethylenediamine tetraacetate, and 0.1 mM dithiothreitol (15). The homogenates were centrifuged at 10,000g for 15 min at 4 °C in a microcentrifuge, and the supernatant was filtered through glass wool and either used directly in model substrate activity assays or ultracentrifuged for use in SDS–PAGE. Ultra centrifugation was performed at 350,000g for 60 min in a Optima TLX ultracentrifuge (Beckman, Palo Alto, CA) and the resulting microsomal pellet resuspended in homogenization buffer containing 20% glycerol. Preliminary investigations indicated that >95% of N-demethylation activity is confined to the microsomal pellet when using this centrifugation protocol (M.E.S., unpublished). Protein concentration for all prep arations was determined with a commercially available bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) according to the manufacturer's instructions, using bovine serum albumin as the standard.

Model Substrate Metabolism

P450-dependent demethylation of the model substrate 4-chloro-N-methylaniline (Figure 1) was quantified by the method of Kupfer and Bruggerman (20). The assay was initiated by adding 250 μl of reaction mixture to a volume of 10,000g supernatant containing 0.5 mg protein as determined by the BCA protein assay. The reaction mixture was composed of Tris–HCl (0.4 M, pH 7.5), NADPH (1.8 mM), 4-CNMA (1.5 mM; stock prepared in ethanol at 20% v/v), and Tween-20 at a volume equivalent to that of substrate in ethanol. Blank reactions contained all components except that an equivalent volume of Tris–HCl was substituted

for NADPH. The reaction proceeded for 16 min with shaking at 30 °C and was terminated by adding 375 μl of stop solution [*p*-dimethyl-aminobenzaldehyde (15 mg/ml) in 3.0 N sulfuric acid]. Microcentrifuge tubes containing the stopped reaction were centrifuged for 15 min at 10,000*g* and 4 °C. The product, 4-chloroaniline, was quantified by comparing absorbance of supernatants at 445 nm to simultaneously determined standard curves (0–50 μmol) in a Beckman DU-65 spectrophotometer. Assays were replicated three times using three different protein preparations.

Inhibition of N-demethylation activity was examined using the cytochrome P450 inhibitor piperonyl butoxide using protein extracts prepared from the York (R) population, prepared as described above. Fifty microliters of protein extract containing ca. 0.4 mg protein was preincubated for 0.5 h at 4 °C with 5 μl PBO in ethanol (final concentration = 0.3125, 0.625, 1.25, 2.5, and 500 μM). Uninhibited and no-NADPH controls received an equivalent volume of eth-

anol (5 μl), and assays were replicated three times under the conditions described above using two different protein preparations.

SDS–PAGE and Heme Staining

Heme staining of PAGE-separated microsomal proteins was accomplished as described by Thomas *et al.* (21) and Waters *et al.* (14), with the exception of the use of tetramethyl phenylenediamine (TMPD, 22) as a reagent for hemoprotein visualization. Ten percent polyacrylamide gels were prepared and prerun for 20 min at 180 V and 4 °C to electrophorese ammonium persulfate into gels before protein was loaded (21). A volume of microsomal resuspension containing 75 μg protein (as determined by the BCA protein assay) was diluted 1:1 in SDS–PAGE sample buffer which contained no β-mercaptoethanol (0.5 M Tris, 10% w/v SDS, 53% v/v glycerol, and 0.05% w/v bromophenol blue). Protein in sample buffer was denatured by heating to 70 °C for 5 min and loaded onto
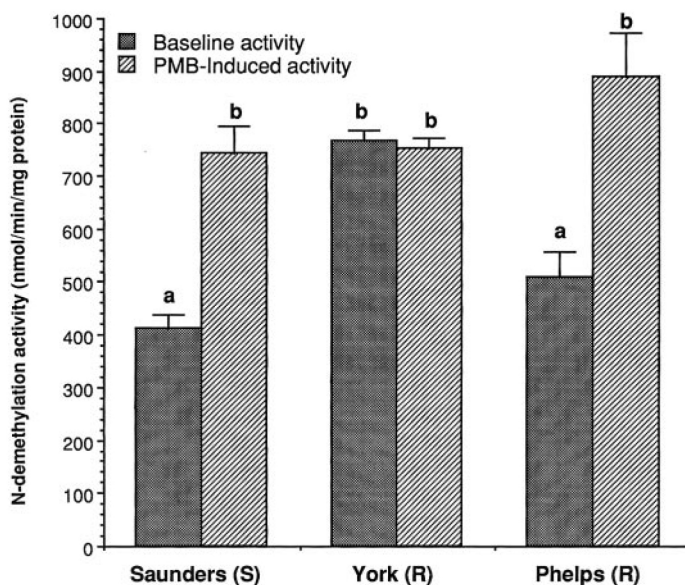
Figure 2. *In vitro*, NADPH-dependent N-demethylation activity toward the model substrate 4-chloro-*N*-methylaniline by protein preparations of insecticide-susceptible (S) and -resistant (R) western corn rootworm populations. Baseline and PMB-induced activity were determined for individuals held under normal laboratory conditions and with exposure to dietary pentamethyl benzene (PMB) for 96 h before assays, respectively. Bars with the same letter are not significantly different by the Fisher LSD (*t*) test ($P \leq 0.05$).

prerun gels. Electrophoresis was conducted at 110 V and 4 °C for 2.0 h. Gels were then placed into 50 ml of Tris–HCl (50 mM, pH 7.0) containing 15 mgTMPDand 75 μl of 30% hydrogen peroxide to visualize hemoprotein bands.

Results and Discussion

P450 Activity, Inhibition, and Induction

P450 activity was assayed *in vitro* using the model substrate 4-chloro-*N*-methylaniline (Figure 1), which shares an *N*-methyl group similar to the insecticide carbaryl. N-demethylation of 4-CNMA was significantly greater for the resistant York population than for the resistant Phelps (intermediate activity) and susceptible Saunders (lowest activity) populations, which were not significantly different ($P \leq 0.05$; Figure 2). These patterns of N-demethylation activity correspond with previously reported levels of carbaryl resistance and metabolism in the same laboratory-reared populations (11). Ingestion of PMB was associated with signifi-

cantly induced P450 activity for the Saunders and Phelps populations (Figure 2). These levels of induced N-demethylation activity (1.7- and 1.9-fold, respectively) are less than earlier results reported for *B. germanica* (2.5- to 8-fold; Reference 4) and *S. eridanea* (3.2-fold; Reference 19). In the York population, induction of N-demethylation activity did not occur. Although this occurrence cannot be resolved at the present time, this lack of induction may be associated with the York population having the highest constitutive (baseline) activity.

In the resistant population with the highest baseline N-demethylation levels (York), N-demethylation activity was significantly inhibited by PBO in a concentration-dependent manner (Figure 3). Carbaryl toxicity was previously shown to be synergized by PBO in field-collected beetles of the York population (9), and inhibition of N-demethylation activity by PBO suggests that the reaction is catalyzed by a cytochrome P450 involved in carbaryl metabolism. Because of the relative importance of N-demethylation to carbaryl resistance (see Refer-
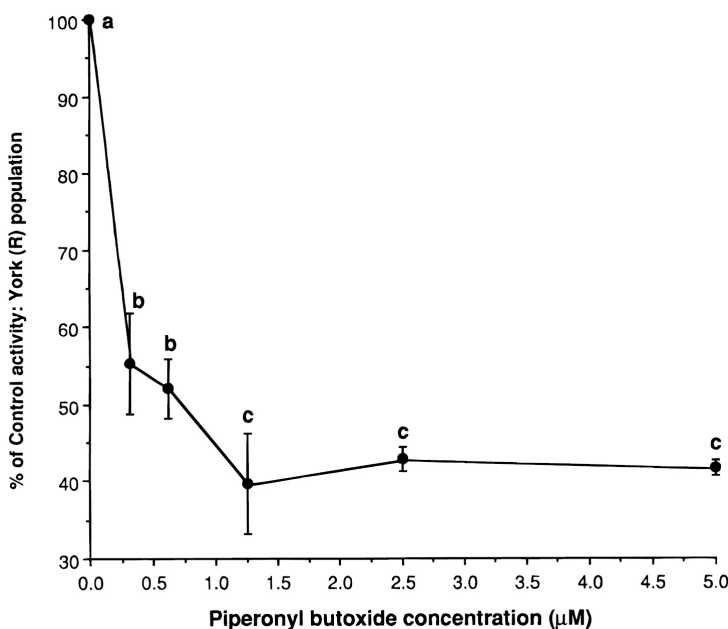
Figure 3. Inhibition of N-demethylation activity by the cytochrome P450 inhibitor piperonyl butoxide in protein preparations from the insecticide-resistant York population of western corn rootworm. Points with the same letter are not significantly different by the Fisher LSD (*t*) test ($P \leq 0.05$).

ence 11 and Figure 1), elevated N-demethyl-ation of the model substrate 4-CNMA (relative to susceptible baselines) may have potential as a marker for assessing evolving carbaryl resistance in rootworm populations.
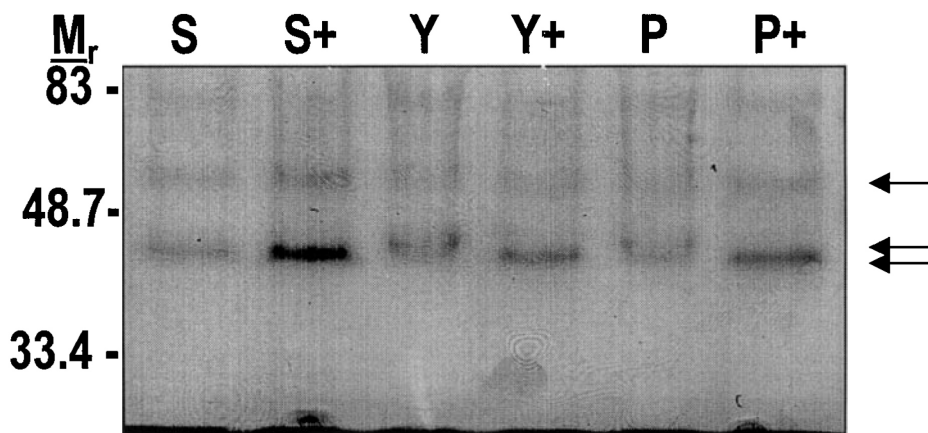
SDS–PAGE of Microsomes

Microsomes (75 μg/lane) from control and PMB-fed rootworm populations were separated on 10% polyacrylamide gels and stained to visualize hemoproteins (Figure 4). Following heme staining, proteins in the vicinity of molecular mass 46–53 kDa were stained in each uninduced population. In the resistant York and Phelps populations, two diffuse bands occurred at molecular mass 45–46 kDa in uninduced individuals and each was more intense than those in the susceptible Saunders population. Following induction, the molecular mass 45 kDa protein increased in intensity in all populations, but was most pronounced in the Saunders, followed by the Phelps populations. It also appears that a 46-kDa protein is less intense following induction in both resistant populations. SDS–PAGE results are in agreement with results of N-demethylation assays discussed above, although band intensities do not appear to directly cor-respond with N-demethylation activity results. Together, these findings suggest that potential P450 isoforms are constitutively overexpressed in insecticide resistant populations, while induction of various forms is greater for more susceptible populations. Because multiple P450 forms are clearly present, possibly several in a given heme-stained band, the isolation of distinct electrophoretic forms will be necessary to assist future studies of P450 in this species.

Conclusions

This study has identified patterns of P450-based activity toward the model substrate 4-chloro-*N*-methyl aniline, inhibition of this activity by PBO, potential electrophoretic P450 isoforms, and changes in both activity and isoform composition following PMB induction. It is apparent from results of earlier studies that cytochrome P450 is involved in carbaryl resistance (9, 11), and results of this study suggest that *in vitro* assays of N-demethylation activity may be used to assist in identifying potential carbaryl resistance in rootworm populations. Of the several potential P450 isoforms identified in the

Figure 4. Hemoprotein staining of SDS–PAGE gels for control and PMB-induced microsomes from insecticide-susceptible and -resistant western corn rootworm populations. Protein loadings were standardized (75 μg/lane) based on results of BCA protein assays (see "Materials and Methods"). Hemoproteins of molecular weights 45–53 kDa are indicated by arrowheads. S = Saunders population (susceptible); Y = York population (resistant); P = Phelps population (resistant); + = PMB-induced; M_r = molecular weight standards (83, 48.7, and 33.4 kDa).

present study, individual forms are most likely involved in resistance to, or bioactivation of, specific carbamate or organophosphate insecticides. Whether or not specific P450 isoforms responsible for N-demethylation of both carbaryl and 4-CNMA have been selected by insecticide exposure remains unclear.

The present insecticide resistance situation in Nebraska and the involvement of cytochrome P450-based metabolism are the primary reasons for undertaking this study. Current areawide management pilot programs are using carbaryl to manage rootworm populations (12) and, because of the potential for resistance development associated with these programs (23), further emphasize the need for a more detailed biochemical understanding of corn rootworm cytochrome P450. Techniques designed to monitor changes in cytochrome P450 isoform composition following insecticide selection are important to these formal pest management programs. Development of such techniques will be assisted by the findings presented here.

Acknowledgments

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