Cytochrome P450 Monooxygenase Activity in the Dark Southern Subterranean Termite (Isoptera: Rhinotermitidae)

STEVEN M. VALLES, WESTE L. A. OSBRINK, FAITH M. OI,[1] RICHARD J. BRENNER, AND JANINE E. POWELL[2]

Center for Medical, Agricultural and Veterinary Entomology, USDA-ARS, 1600 SW 23rd Drive, Gainesville, FL 32608

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ABSTRACT Microsomal oxidases were characterized using surrogate substrates in the economically important dark southern subterranean termite, *Reticulitermes virginicus* (Banks). Aldrin epoxidase activity required NADPH and was inhibited by carbon monoxide and piperonyl butoxide (I_{50} = 4.72 ± 0.31 X 10^{-6} *M*), indicating that the enzyme(s) involved was a cytochrome P450 monooxygenase. Alclrin epoxidase activity was highest at 22°C and pH 7.2. Also, the activity was linear up to 0.5 mg of protein per incubate and increased with reaction time up to 15 min. Although neither substrate nor cofactor was found to be a limiting factor, aldrin epoxidase activity failed to produce a linear response with respect to time at temperatures >22°C, indicating enzyme inhibition. Although increased incubation temperature >22°C resulted in decreased aldrin epoxidase activity, similar heat treatments did not result in a concomitant increase in cytochrome P420. Significant variation (2.7-fold) in aldrin epoxidase activity was observed among 4 *R.virginicus* colonies collected from different locations in Florida. Adclitionally, cytochrome P450 and b_5 content, cytochrome c reductase, and methoxyresorufin O-demethylase activities were mensurecl in *R.virginicus.*

KEY WORDS *Reticulitermes virginicus,* termite, cytochrome P450, tletoxication

SUBTERRANEAN TERMITE DAMAGE, treatment of current infestations, and prophylactic treatments to prevent initial and subsequent infestations cost Americans an estimated $2 billion annually (Potter 1997). Historically, subterranean termite control has been accomplished by placing an insecticide barrier at the soil-structure interface. Organochlorine insecticides served in this capacity through 1988 when the Environmental Protection Agency banned their use in the United States (Cold et al. 1996). The insecticide barrier method of termite control still currently predominates in subterranean termite control; however, pyrethroid and organophosphate termiticides have usurped the organochlorines in this role. Unfortunately, pyrethroid and organophosphate termiticides lack the persistence of the organochlorines raising concern about their long-term effectiveness. Despite the recent introduction of the new barrier termiticide imidncloprid (Moffat 1993) and bait toxicants such as hexaflumuron (Su et al. 1997), termite control remains a difficult prospect and urgently needs alternative treatment or preventive methods.

Although insecticides have been used for decades against subterranean termites, very little is known about their detoxicntion capacity. In fact, the 1st report characterizing detoxication euzymes in subterranean termites was published in 1994 by Haritos et al. (1994) who found an active and competent cytochrome P450 monooxygenase system composed of multiple isoforms in the Australian termites *Mastotermes darwiniensis* Froggatt and *Coptotermes acinaciformis* (Froggatt). Aside from this report, little information is available concerning detoxication enzymes in the Isoptera. This scarcity of information prompted us to characterize cytochrome P450 monooxygenases in the economically important dark southern subterranean termite, *Reticulitermes virginicus* (Banks).

Materials and Methods

Insects. Four colonies of *R. virginicus* were collected from field sites in Florida. Colony 02 was collected 25 September 1997 from a wooden porch at Jacksonville Naval Air Station, Jacksonville, FL. Colonies 21 and 26 were collected 20 August 1997 from pine logs within a stand of pine trees at the Alachua County fair grounds, Gainesville, FL. Colony 71 was collected 29 October 1997 from pine logs within a stand of pine trees at the University of Florida's natural area teaching laboratory, Gainesville, FL. Termites were brought back to the laboratory in infested wood that was subsequently placed into a plastic trash can (43 cm diameter by 65 cm high) with moist vermiculite at the bottom. Moistened, corrugated cardboard was rolled into cylinders (10 cm diameter by 15 cm high) and placed on top of infested wood. Termites that entered the cardboard were gently knocked from

[1] Department of Entomology, Auburn University, 208 Extension Hall, Auburn University, AL, 36849.

[2] USDA Forest Service, P. 0. Box 928,201 Lincoln Green, Starkville, MS, 39759.

it into a tray and isolated from debris by allowing them to cling to moistened paper towels. Termites were then tapped off into a clean tray, Termites were identified using soldier keys from Miller (1949), Scheffrahn and Su (1994), and Hostettler et al. (1995).

Preparation of Microsomes. A mixture of soldier and worker termites (1 g) was homogenized in 40 ml of a protective buffer (0.1 M sodium phosphate buffer, pH 7.5, containing 10% glycerol, 0.1 mM dithiothreitol, 1 mM ethylenediaminetetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, and 1 mM phenylthiourea) by using a motor-driven Teflon pestle and glass mortar (Thomas Scientific, Swedesboro, NJ). The homogenate was filtered through 2 layers of cheesecloth and centrifuged at 10,000 X ,q for 15 min. The supernatant was filtered through glass wool and further centrifuged at 105,000 X g for 1 h in a Beckman 1.8-70 M ultracentrifuge (Beckman, Palo Alto, CA). The resulting pellet (microsonies) was washed twice and suspended in 0.1 M sodium phosphate buffer, pH 7.5, at a concentration of 1 mg protein per milliliter. These procedures were performed at 0-4°C. When pH studies were conducted, termites were homogenized in sodium phosphate buffer as described above at pH 6.6, 6.9, 7.2, 7.5, 7.8, and 8.1.

Enzyme Assays. Microsomal epoxidase activity was measured by the epoxidation ofaldrin to dieldrin (Ray 1967). The 5-ml reaction mixture contained microsomal protein; 0.1 M sodium phosphate buffer, pH 7.5; an NADPH generating system composed of 1.8 μmol NADP, 18 μmol glucose-6-phosphate, 1 U of glucose-G-phosphate dehydrogenase; and 250 nmol aldrin in 0.1 ml of ethylene glycol monomethyl ether. Incubations were carried out at 22°C in a shaking water bath for 15 min. However, when conducting the temperature experiments, incubations occurred at 18, 22, 26, 30, and 34°C. When time course experiments were conducted, all components were incubated in a common vessel (250-ml flask) at 22°C for 6, 9, 12, 15, and 18 min with 0.25 mg of microsomal protein per incubate. Aliquots (5 ml) were removed by pipette at the appropriate times. Reactions were terminated by the addition of 11 ml of *n*-hexane and the product. dieldrin, was extracted by shaking for 1 h followed by centrifugation for 3 min. An aliquot of the supernatant was removed and dried over anhydrous Na_2SO_4. The sample was 'analyzed by gas chromatography on a Hewlett-Packard model 5890 gas chromatograph (San Fernando, CA) quipped with a split/splitless injector (operated in splitless mode) and an electron capture detector. A 15-m DBGO8 capillary column (J & W Scientific, Folsom, CA) was used for all analyses. Injector, detector, and column temperatures were set at 250, 300, and 210°C, respectively.

Microsomal *O*-dealkylase activity was measured using the substrates methoxyresorufin, ethoxyresorufin, and pentoxyresorufin (Mayer et al. 1977). The 2.015-ml reaction mixture contained 0.1 M sodium phosphate buffer, pH 7.5; microsomal protein, 5 μl of 0.5 mM alkylresorufin substrate in ethylene glycol monomethyl ether; and 10 μl of 0.2 mM NADPH. The buffer and protein were incubated at 25°C in a water bath for 3 min. The reaction was initiated by the addition of substrate followed immediately by NADPH. Specific activity was determined on a Shimadzu model RF-5301PC spectrofluorophotometer (Columbia, MD) by the formation of the product resorufin and was based on the initial linear reaction rate. The excitation and emission wavelength settings and their respective slit settings were 560 nm (1.5 nm) and 580 nm (3.0 nm).

Total cytochrome P450 and cytochrome b_5 were determined by the method of Omura and Sato (1964). Microsomes were suspended in 0.1 M sodium phosphate buffer, pH 7.5. For cytochrome P450, the sample cuvette was saturated with carbon monoxide (carbon monoxide bubbled gently into the cuvette for 1 min), followed by the addition of ≈3-4 mg of $Na_2S_2O_4$ to both reference and sample cuvettes. For cytochrome b_5 the sample microsomes were treated with $Na_2S_2O_4$ only. The mixtures were stirred with a glass rod for 10 s, and immediately scanned from 500 to 400 nm. Spectra were recorded until no further increase in cytochrome P450 content occurred on a Varian model Bio 3 uv:vis spectrophotometer (Sugar Land, TX) equipped with a diffuse reflectance accessory. Change in absorbance was measured between 490 and 450 nm, and 424 and 409 nm, for cytochrome P450 and cytochrome b_5, respectively. The molar extinction coefficient of91 and 185 mM^{-1} cm^{-1} was used to calculate the specific content of cytochrome P450 and b_5, respectively.

Cytoclirome c reductase activity was determined by the reduction of cytochrome *c* (Masters et al. 1967). The G-ml reaction mixture contained 0.1 mg of microsomal protein, 5 μM sodium cyanide, and 2.5 μM cytochrome c in sodium phosphate buffer, pH 7.5. The mixture was shaken for 20 s, then 2.5 ml was pipetted into sample and reference cuvettes. Fifty microliters of sodium phosphate buffer, pH 7.5, was added to the reference ouvette and SO μl of NADPH generating system (as described in the aldrin epoxidase assay) was added to the sample cuvrtte to start the reaction.

Protein determinations were made by the method of Bradford (1976) by using bovine serum albumin as the standard. Analysis of variance (ANOVA) was used to analyze the aldrin epoxidase activity among the 4 colonies. Means were separated by the Scheffé multiple comparison procedure. Each experiment was replicated at least 3 times with duplicate determinations.

Results

Table 1 shows that aldrin epoxidase activity of microsomal preparations from soldiers and workers of the dark southern subterranean termite required NADPH and was inhibited by carbon monoxide. Piperonyl butoxide, a well-known inhibitor of cytochrome 1'450 monooxygenases, also inhibited the reaction with an I_{50} value of 4.52 ± 0.31 X 10^{-6} *M*. The reduced carbon monoxide difference spectrum of microsomes prepared from the termites showed a maximum at 450 nm with negligible cytochrome P-120

Table 1. Effect of cofactor and inhibitors on aldrin epoxidase activity of colony 21 of the dark southern subterranean termite, *R. virginicus*

Incubation mixture	Aldrin epoxidase activity (% of control ± SEM)
Complete (control)	100
− NADPH	2.45 ± 1.14
+ Carbon monoxide[a]	42.42 ± 8.21
+ Piperonyl butoxide (M)	
10^{-4}	6.84 ± 0.89
10^{-5}	23.37 ± 2.09
10^{-6}	70.63 ± 1.22

Mean ± SE of 3 experiments, each with duplicate determinations. Control epoxidase activity, 241.6 ± 17.5 pmol/min/mg of protein.
[a] Bubbled gently for 1 min before incubation.

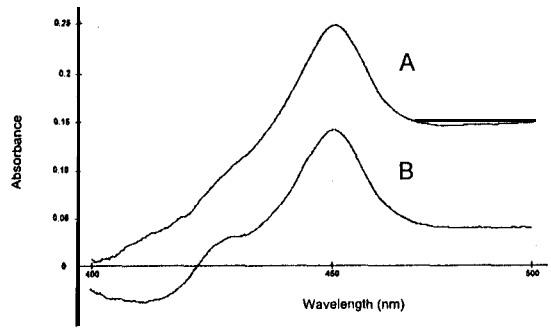

Fig. 1. Reduced carbon monoxide of microsomes prepared from *Reticulitermes virginicus* soldiers and workers (colony 21). (A) Measured immediately and,(B) after a 10 min incubation at 34°C.

(degraded cytochrome P450) formation (Fig. 1A). The spectrum produced from microsomes incubated at 34°C for IO min before carbon monoxide treatment, reduction with $Na_2S_2O_4$, and subsequent scanning also exhibited a negligible amount of cytochrome P420 (Fig. 1B).

Effects of time, tissue quantity, pH, and temperature on aldrin epoxidase activity are shown in Fig. 2. Activity was highest at 22°C, dropping steadily with increasing temperature beyond 22°C (Fig. 2A). At 30 and 34°C, activity was 76 and 51% that at 22°C, respectively. Fig. 2B shows that epoxidase activity was highest at pH 7.2. Aldrin epoxidase activity was linear up to 0.5 mg of protein per incubate (Fig. 1C) and increased with reaction time up to 15 min (Fig. ID). We were only able to achieve linearity with respect to time when the incubation temperature was set at 23°C.

Cytochrome P450 and cytochrome b_5 content, cytochrome c reductase, aldrin epoxidase, methoxy-

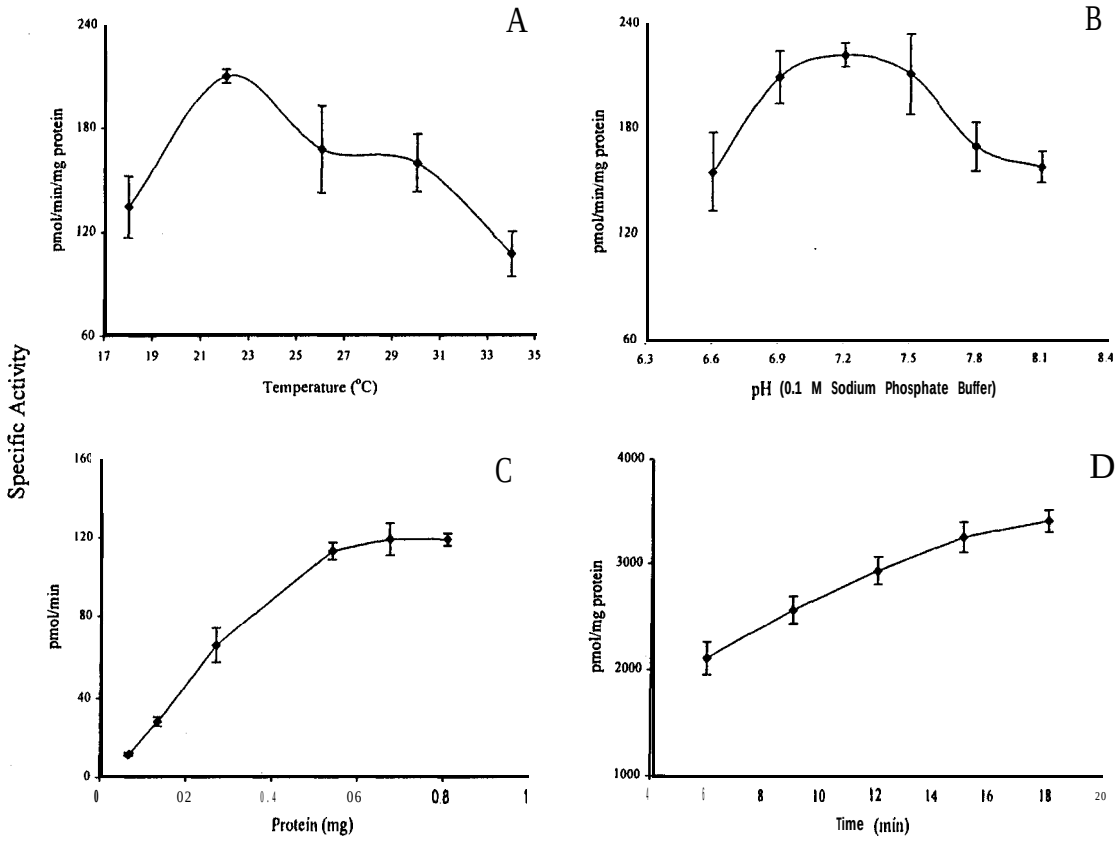

Fig. 2. Effectsof (A) temperature,(B) pH, (C) protein quantity, and (D) time course of aldrin epoxidase activity in colony 21 of *R. virginicus* soldiers and workers. Mean ± SE of 3 experiments, each with duplicate determinations.

Table 2. Detoxication enzyme levels in colony 21 of the dark southern subterranean termite, R. *virginicus*

Detoxication enzyme	Specific activity (nmol/min/mg of protein)
Cytochrome P450[a]	0.17 ± 0.01
Cytochrome b_5[a]	0.14 ± 0.01
Cytochrome *c* reductase	11.42 ± 0.34
Aldrin epoxidase	0.21 ± 0.004
Methoxyresorufin *O*-demethylase	0.0045 ± 0.0002
Ethoxyresorufin *O*-deethylase	N D
Pentoxyresorufin *O*-dealkylase	ND

Mean ± SE of 3 experiments, each with duplicate determinations. ND, not detectable.
[a] Measured in nmol/mg of protein.

resorufin, ethoxyresorufin and pentoxyresorufin *O*-dealkylase activities are summarized in Table 2. ANOVA indicated that aldrin epoxidase activity was significantly different among the 4 *R. virginicus* colonies collected from different locations and ecological settings (Table 3). Activity ranged from 172.5–464.7 pmol of dieldrin formed per minute per milligram protein with colony 26 exhibiting the highest activity.

Discussion

Results of this study indicated clearly that *R. virginicus* workers and soldiers contained an active microsomal oxidase system. However, because we used whole body homogenates as the enzyme source, the contribution of wood-digesting or other microorganisms in the termite gut toward the detoxication enzyme activities cannot be ruled out. The aldrin epoxidase activity required NADPH and was inhibited by carbon monoxide and piperonyl butoxide indicating that the enzyme(s) involved was a cytochrome P450 monooxygenase. Cytochrome P450 content was 0.17 nmol/mg of microsomal protein, which was very similar to the values reported by Haritos et al. (1994) for the Australian termites, *M. darwiniensis* (0.142 nmol/mg of protein) and *C. acinaciformis* (0.128 nmol/mg of protein). Additionally, aldrin epoxidase activity was similar between *R. virginicus* and the Australian termite species.

In preparing microsomes for use in cytochrome P450-catalyzed assays, care must be taken to prevent degradation or inhibition of the enzymes from endogenous inhibitors (Hodgson 1985). The most common inhibitors of microsomal oxidases are eye pigments such as xanthomatin (Schonbrod and Terriere 1971) and proteolytic enzymes released from the alimentary canal during homogenization (Valles and Yu 1996). Termite soldier and worker castes do not have eyes but the liberation of other inhibitors upon homogenization and subsequent microsome preparation must be considered. The microsome preparation scheme for *R. virginicus* had no apparent detrimental effects on cytochrome P450 as evidenced by negligible cytochrome P420 formation in the reduced carbon monoxide difference spectrum (Fig. 1A) and active aldrin epoxidase and methoxyresorufin 0-demethylase activities. However, we observed rapid loss of aldrin epoxidase activity when microsomes *were* incubated at temperatures >22°C. Although increased incubation temperature (>22°C) resulted in decreased aldrin epoxidase activity, similar heat treatments did not significantly increase cytochrome P420 content (Fig. 1B). At temperatures >22°C, aldrin epoxidase activity was linear for several minutes only, indicating limitations of substrate or cofactor(s), enzyme inhibition or some other enzyme-related factor, such as degradation. However, experiments conducted with increased quantities of substrate (aldrin), NADPH, or both did not provide a linear response with respect to time above 22°C (data not shown). Time-dependent linearity was achieved only when the temperature was reduced to 22°C indicating that the microsomal epoxidase was apparently heat sensitive. Subterranean termites have been reported to prefer lower temperatures typical of the soil environment in which they live (Holdaway and Gay 1948). For example, Smith and Rust (1994) reported that when western subterranean termites, *Reticulitermes hesperus* Banks, were exposed to a temperature gradient of 5.5–43.6°C, they preferred temperatures between 14 and 19°C.

Although no apparent degradation of cytochrome P450 was indicated by the reduced carbon monoxide difference spectrum in response to heat treatment, this was no assurance that the enzyme was catalytically active. Alternatively, phospholipid alterations, or inhibition of electron flow to cytochrome P450 could occur at higher incubation temperatures causing the observed inhibition of aldrin epoxidation.

Cytochrome P450 content and aldrin epoxidase activity were comparable to *M. darwiniensis* and *C. acinaciformis*. Aldrin epoxidase activity varied 2.6-fold among the 4 colonies of *R. virginicus* collected from different sites in Florida. It is not known whether these colony differences represent inherent variation among this species, stage-dependent or other developmental effects, or a direct response to environmental factors (i.e., enzyme induction).

Table 3. Aldrin epoxidase activity of R. *virginicus* colonies collected from different locations

Colony	Collection location	Ecological niche	Aldrin epoxidase activity (pmol/min/mg protein)
02	Jacksonville, FL	Structural lumber	152.5 ± 8.6a
21	Gainesville, FL	Pine log	206.4 ± 4.9a
71	Gainesville, FL	Pine log	26.9.7 ±13.0b
26	Gainesville, FL	Pine log	464.7 ±11.0c

Mean ± SE of 3 experiments, each with duplicate determinations. Means with a different letter are considered significantly different at the $P = 0.05$ level by the Scheffé multiple comparison procedure.

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