Journal of the American Mosquito Control Association, 13(3):233–237, 1997

HEME PEROXIDASE ACTIVITY MEASURED IN SINGLE MOSQUITOES IDENTIFIES INDIVIDUALS EXPRESSING AN ELEVATED OXIDASE FOR INSECTICIDE RESISTANCE

WILLIAM G. BROGDON,[1] JANET C. McALLISTER[1] AND JOHN VULULE[2]

ABSTRACT. Optimum conditions are described for a simple, rapid, microplate-based assay that indirectly measures the differences in oxidase levels between individual susceptible, resistant, or induced mosquitoes. A small proportion (0.01–0.1) of a single mosquito is used, allowing multiple replicates of the oxidase assay. Cytochrome C is used as a positive control. The levels of oxidase found in sample populations of pyrethroid-susceptible, pyrethroid-resistant, and phenobarbital-induced *Anopheles albimanus* mosquitoes are characterized with the assay.

INTRODUCTION

Xenobiotic detoxification in organisms is accomplished by families of enzymes that hydrolyze, oxidize, and conjugate injurious compounds into less toxic, more water-soluble products that can be readily excreted. Among the most important detoxification enzymes are the mixed-function, or P450, oxidases (also termed oxygenases). These enzymes are present throughout the tissues of plants and animals and catalyze an enormous range of biochemical reactions essential to life (Agosin 1985, Hodgson 1985). Our particular interest in oxidase enzymes involves detoxification of insecticides by disease vectors. In biochemical detection of insecticide resistance (Brogdon 1989), it is necessary that enzyme levels be determined in single mosquitoes, because populations often consist of mixtures of susceptible and resistant individuals. Methods have been developed for the measurement of levels of esterases and glutathione s-transferases (Brogdon and Dickinson 1983, Brogdon and Barber 1990a) in single mosquitoes. A simple method for detecting elevated levels of oxidase in single insects has not been available.

There have been 2 approaches to measurement and study of oxidase activity in insects (Agosin 1976). The first approach has been to evaluate spectral and catalytic properties of oxidases associated with the membrane, using techniques such as the determination of carbon monoxide inhibition of enzyme spectral characteristics (Omura and Sato 1964). The second approach has been to attempt the solubilization, resolution, purification and reconstitution of the enzyme system. This has required measurement using specific substrates of known oxidase enzyme functions, such as: *O*-dealkylation using 7-methoxy-4-methylcoumarin (Feyereisen and Vincent 1984), 7-ethoxycoumarin (Patil et al. 1990), or methoxyresorufin (Mayer et al. 1977); aldrin epoxidation to dieldrin (Yu et al. 1971, Feyereisen 1983); or aryl hydrocarbon hydroxylase (Dehnen et al. 1973). All of these methods suffer from the drawback that pooled microsomal fractions from a number of insects must be used; none of these methods can be used to measure differences in oxidase levels in single mosquitoes.

An indirect alternative is to measure the level of heme-containing enzymes (including the cytochrome oxidase enzymes) in single insects. The levels of these enzymes should be correlated with the peroxidase activity of the heme group. Such a technique would provide a useful means for measuring large-scale differences in oxidase levels characteristic of insecticide resistance and oxidase induction, provided that the heme peroxidase levels are low but measurable in susceptible, noninduced mosquitoes.

The present study had 3 objectives. The first was to develop a method for measuring basal levels of heme peroxidase activity, thus, hemoprotein levels, in susceptible populations of anopheline mosquitoes. Second, sample populations were compared using the assay to determine if enzyme levels are enhanced by induction or by the presence of insecticide resistance. Third, reliability of the technique was evaluated through analysis of within-run and between-run variability of results. The means for running a standard curve of enzyme activity and an internal standard were also detailed. The standard curve was designed to show the linearity of the heme peroxidase assay with hemoprotein concentration. Cytochrome C was used as an internal standard for each microplate. Cytochrome C was chosen because of its wide availability and low cost. This method is intended to provide a simple means of quantifying oxidase activity in mixed populations of resistant and susceptible mosquitoes.

MATERIALS AND METHODS

Mosquitoes: Reference strains of *Anopheles albimanus* Wied. used in this study are maintained at the Centers for Disease Control and Prevention in Atlanta, GA. Resistant mosquitoes were from a

[1] Entomology Branch F-22, Division of Parasitic Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Public Health Service, U.S. Department of Health and Human Services, Chamblee, GA 30341.

[2] Kenya Medical Research Institute, Nairobi, Kenya.

strain of *An. albimanus* from Guatemala that contains oxidase resistance to permethrin, but not the high esterase resistance that also occurs in the country (Brogdon and Barber 1990b). This colony is periodically selected to preserve the mechanism. The association of the elevated oxidase measured in these sample populations with insecticide resistance has been confirmed via a single-family isolate from the parent strain through bioassay (resistance level >100× based on dose-response), complete synergism by piperonyl butoxide, and induction experiments. Details of these experiments will be reported elsewhere.

Sample preparation: Adult 3- to 4-day-old, nonbloodfed female mosquitoes were frozen and homogenized in 100 µl assay buffer in plastic microcentrifuge tubes using plastic pestles. Late 4th-instar larvae and pupae were transferred alive into assay buffer for homogenization. Homogenates were diluted to 1 ml with additional buffer. For bloodfed mosquitoes, the abdomen was removed before homogenization.

Enzyme assays: The method for enzyme assays was an adaptation of that for detecting heme peroxidase activity on electrophoresis gels (Thomas et al. 1976) using the substrate 3,3′5,5′-tetramethylbenzidine (TMBZ). Both the free base and dihydrochloride forms of TMBZ were obtained from Sigma Chemical Company, St. Louis, MO. The TMBZ working solution was prepared fresh daily by dissolving 50 mg TMBZ free base or dihydrochloride in 25 ml absolute methanol and adding 75 ml 0.25 M sodium acetate buffer (pH 5.0; pH adjusted with acetic acid).

To 100 µl of mosquito homogenate, 200 µl of TMBZ solution were added in assay wells, followed by 25 µl of 3% hydrogen peroxide (commercial grade). The plate was read in a microplate-reading spectrophotometer at test wavelengths of 630–690 nm at time $T = 0$ and at intervals thereafter, depending on the purpose of the experiment. Mosquitoes were conveniently assayed in sample sizes of 32, since 3 replicates of 32 mosquitoes fill a single microplate.

Standard curves (8 replicates) for heme peroxidase were prepared using dilutions of a commercial preparation of cytochrome C (Sigma Chemical Co., St. Louis, MO). Mosquito protein was measured on 50-µl aliquots of assay homogenates using the Bradford assay (Bradford 1976; Brogdon 1984a, 1984b). This was used as a general indicator of size and to test for differences in size among different populations.

Population comparisons: Individual mosquitoes from 4 populations were compared. A permethrin-susceptible control population was compared with a resistant strain (LD_{50} > 100× the control population) with elevated oxidase levels. A portion of a population sample of a mixed resistant colony (ranging from susceptible individuals to highly resistant) was then induced with 0.1% phenobarbital (PB), an oxidase inducer, that was provided overnight in a 10% sucrose solution provided to nourish the mosquito colony. Sucrose/PB was replaced with normal 10% sucrose the next morning to prevent mortality from PB. Mosquitoes were collected 24 h after initial exposure to PB along with mosquitoes from the same sample population that had not been induced. Another sample population from the mixed resistant colony was selected at the LD_{50} level with 0.025% permethrin-impregnated resistance test papers (World Health Organization, Geneva, Switzerland). A sample population of the F_1 generation from survivors was compared for oxidase activity with the original population values for the parent strain.

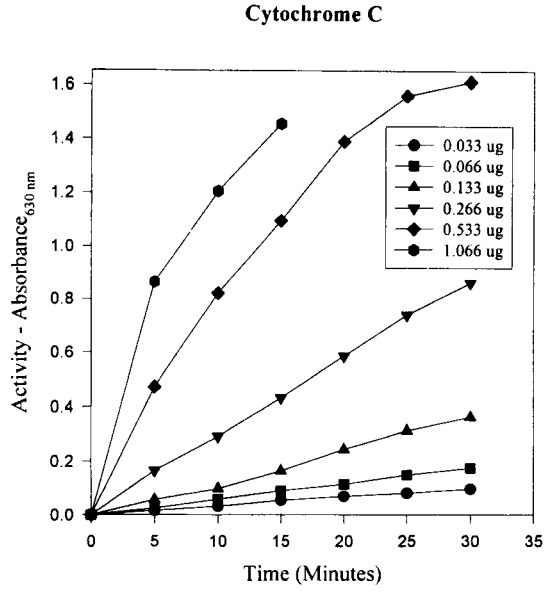


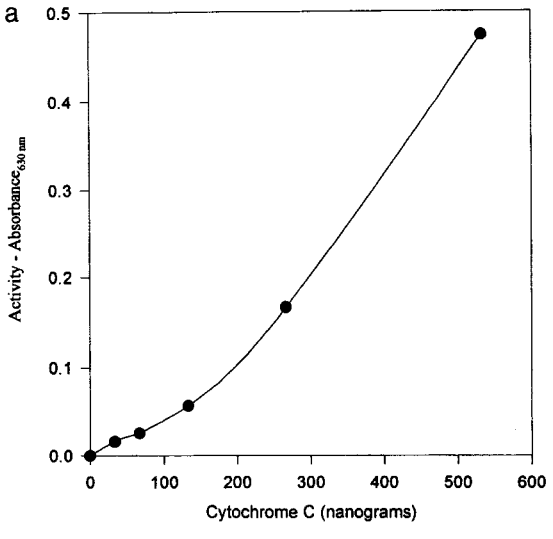
Fig. 1. Linearity of TMBZ substrate peroxidation for 0.03 to 1.06 µg of cytochrome C. $n = 8$ replicates.

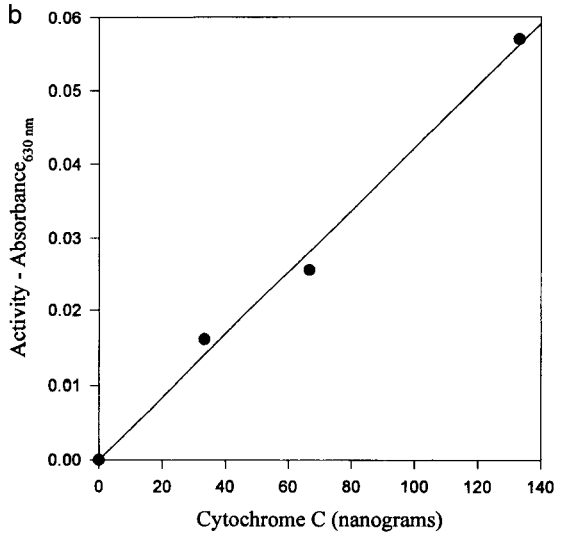
RESULTS

Standard curves: The cytochrome C standard showed linear peroxidation kinetics for at least 30 min at concentrations below 0.5 µg (Fig. 1). Above that concentration, linearity was lost after 5 min. Therefore, initial enzyme rates measured at $T = 5$ min were used for all microassays.

The complete standard curve for 0.03–1.066 ng of cytochrome C is accurately described by an exponential equation $y = ae^{bx}$ (Fig. 2a). However, for practical purposes, the data may be adequately described using 2 linear equations of the form $y = mx + b$ (Figs. 2b–c). The first describes the curve from 0 to 140 ng ($r^2 = 0.99$), and the second describes the curve from 0.150 to over 1 µg ($r^2 = 0.99$).

Comparison of sample populations: The control and resistant sample populations were significantly





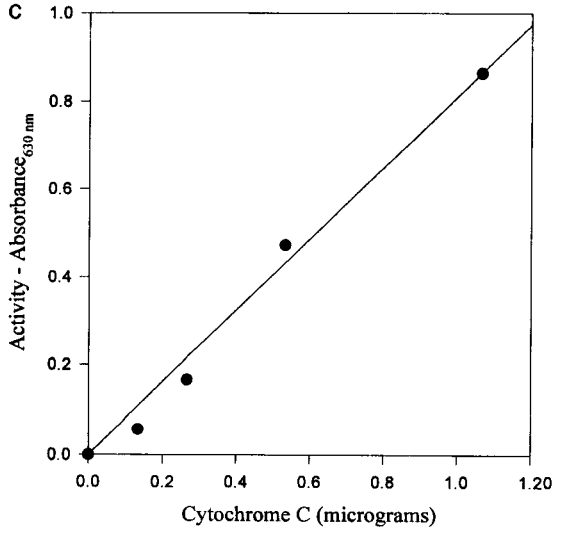


Fig. 2. Standard curves for cytochrome C. a. Cubic spline curve of range 0–660 ng. b. Linear regression of range 0–140 ng (r^2 = 0.994). c. Linear regression of range 0.150–1.066 μg (r^2 = 0.991). In each case, n = 8 replicates.

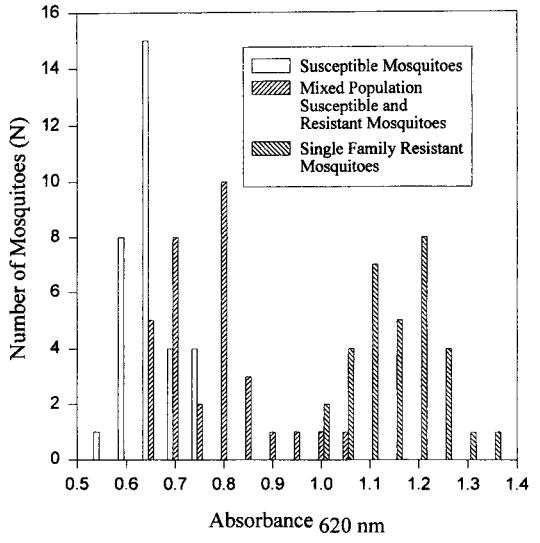


Fig. 3. Comparison of permethrin-susceptible and -resistant sample populations of *Anopheles albimanus*. For each population, n = 32.

different (Mann-Whitney rank-sum test, $P < 0.001$) in oxidase activity (Fig. 3). The single-family isolate from the resistant population showed a resistance level in bioassays of greater than 100× and was completely synergized by piperonyl butoxide. The resistant strain of *An. albimanus* used for selection and induction experiments contains both resistant and susceptible individuals, similar to a field population. The portion of the resistant population with absorbance of ≥0.3 (the resistance threshold) was equivalent to the portion of the population surviving beyond the upper range limit of a susceptible population in bioassays of the resistant strain (data not shown).

The induced and control resistant mosquitoes from the same population showed significantly different oxidase levels (t-test, $p < 0.001$), confirming induction by phenobarbital (Fig. 4). Exposure to permethrin selected for significantly higher oxidase levels (Mann-Whitney rank-sum test, $P < 0.001$) than did the unselected portion of the sample (Fig. 5). In these experiments, protein assays conducted in triplicate on every mosquito showed that none of these populations differed significantly in the sizes of individual mosquitoes (data not shown).

Within-run and between-run variation: In all experiments involving individual mosquitoes, the maximum coefficient of variation (SD/$\bar{x}$) was 7%

←

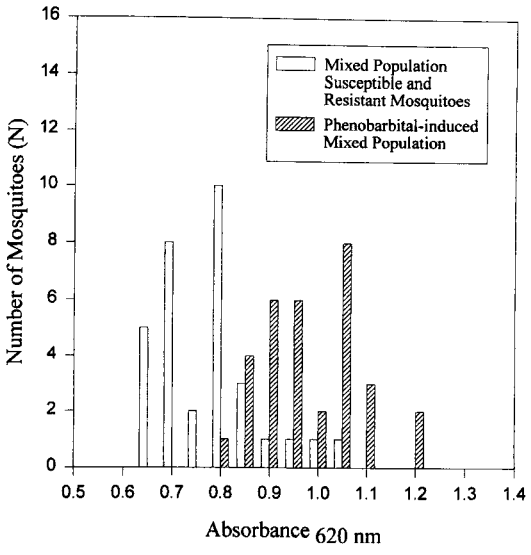


Fig. 4. Comparison of permethrin-resistant and phenobarbital-induced sample populations of *Anopheles albimanus*. For each population, $n = 32$.

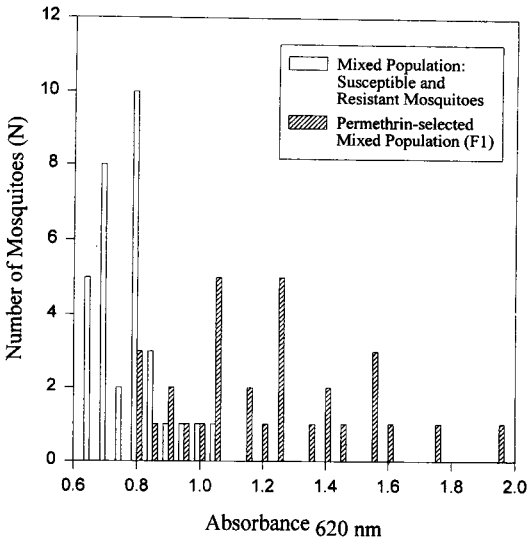


Fig. 5. Comparison of permethrin-resistant and permethrin-selected sample populations of *Anopheles albimanus*. For each population, $n = 32$.

and the average was 2.5%. The day-to-day variation in assays was 15%.

DISCUSSION

Use of heme peroxidation to analyze variation in oxidase levels does not provide information on specific oxidases. This is also true of Omura and Sato's (1964) carbon monoxide method which is broadly specific for hemoproteins (Agosin 1985). However, there are a number of advantages in using a more general oxidase assay. First, microsomal preparations have low reproducibility (Agosin 1985) and are not possible with single mosquitoes. Moreover, correlations of *in vitro* mono-oxygenases with their *in vivo* functions are difficult to obtain due to the multiplicity of closely related enzymes competing for the same substrate, and the broad, overlapping substrate specificities of enzymes in the family (Agosin 1985, Scott et al. 1994). In addition, it is rare to obtain linear reaction plots with the specific substrates of various oxidases due to the high lipophilicity of the substrates, multiple forms of the enzyme, and the complex nature of the membrane (Hodgson 1985). In fact, enzyme rates in these reactions depend not on the concentration of substrate, but on the absolute amount (Lewis et al. 1967). Finally, association of a specific oxidase with insecticide resistance or enzyme induction is extremely difficult, even with purified microsomal enzymes (Plapp and Casida 1969). For these reasons, we feel that a generic assay for inferring differences in oxidase level based on hemoprotein levels is a practical option that is broadly applicable to resistance detection or measurement of oxidase enzyme induction.

This biochemical microassay must be correlated to bioassay data or known experimental variables from the same sample population for the results to be comparable to those of other sample populations. This is an indirect assay that would be sensitive to misinterpretation in the presence of an unknown hemoprotein or interfering substance. Every effort must be made to minimize experimental variables or to quantify them, as in a protein microassay run on the same sample to correct for mosquito size.

3,3′5,5′-Tetramethylbenzidine is a nonmutagenic, noncarcinogenic substrate (Holland et al. 1974, Garner 1975), commonly used in kinetic ELISA (Hancock and Tsang 1986). The TMBZ has the additional advantage of a molar extinction coefficient that is 10^{-100} that of other hydrogen donor substrates, making it possible to detect enzyme activity in small portions of single mosquito preps. Reading the results at absorbances ranging from 630 to 690 nm is simple due to the broad absorbance peak at 655 nm (Bos et al. 1981).

Although it is an indirect assay, the method described here has a number of practical applications where a difference in oxidase levels is suspected. The technique allows for rapid determination of resistance frequency estimates, and the simplicity of the method makes it potentially available to even the most financially pressured mosquito control organizations.

ACKNOWLEDGMENTS

This work was supported, in part, by World Health Organization TDR grant 910637.

REFERENCES CITED

Agosin, M. 1976. Insect cytochrome P-450. Mol. Cell. Biochem. 12:33–44.

Agosin, M. 1985. Role of microsomal oxidations in insecticide degradation, pp. 647–730. *In:* G. A. Kerkut and L. I. Gilbert (eds.). Comprehensive insect physiology, biochemistry, and pharmacology, Volume 12. Pergamon Press, Oxford.

Bos, E. S., A. A. Van der Doelen, N. Van Rooy and A. H. W. M. Schuurs. 1981. 3,3′,5,5′-Tetramethylbenzidine as an Ames test negative chromogen for horseradish peroxidase in enzyme-immunoassay. J. Immunoassay 2:187–204.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248–254.

Brogdon, W. G. 1984a. Mosquito protein microassay—I. Protein determinations from small portions of single-mosquito homogenates. Comp. Biochem. Physiol. 79B: 457–459.

Brogdon, W. G. 1984b. Mosquito protein microassay—II. Modification for potential field use. Comp. Biochem. Physiol. 79B:461–464.

Brogdon, W. G. 1989. Biochemical resistance detection: an alternative to bioassay. Parasitol. Today 5:56–60.

Brogdon, W. G. and A. M. Barber. 1990a. Microplate assay of glutathione s-transferase activity for resistance detection in single-mosquito triturates. Comp. Biochem. Physiol. 96B:339–342.

Brogdon, W. G. and A. M. Barber. 1990b. Fenitrothion-deltamethrin cross-resistance conferred by esterases in Guatemalan *Anopheles albimanus.* Pestic. Biochem. Physiol. 37:130–139.

Brogdon, W. G. and C. M. Dickinson. 1983. A microassay system for measuring esterase activity and protein concentration in small samples and in high-pressure liquid chromatography eluate fractions. Anal. Biochem. 131:499–503.

Dehnen, W., R. Tomingas and J. Roos. 1973. A modified method for the assay of benzo(a)pyrene hydroxylase. Anal. Biochem. 53:373–383.

Feyereisen, R. 1983. Polysubstrate monooxygenase (cytochrome P-450) in larvae of susceptible and resistant strains in house flies. Pestic. Biochem. Physiol. 19:262–269.

Feyereisen, R. and D. R. Vincent. 1984. Characterization of antibodies to house fly NADPH-cytochrome P-450 reductase. Insect Biochem. 14:163–171.

Garner, R. C. 1975. Testing of some benzidine analogues for microsomal activation to bacterial mutagens. Cancer Lett. 1:39–42.

Hancock, K. and V. C. W. Tsang. 1986. Development and optimization of the FAST-ELISA for detecting antibodies to *Schistosoma mansoni.* J. Immunol. Methods 92: 167–176.

Hodgson, E. 1985. Microsomal mono-oxygenases, pp. 225–321. G. A. Kerkut and L. I. Gilbert (eds.). *In:* Comprehensive insect physiology, biochemistry, and pharmacology, Volume 11. Pergamon Press, Oxford.

Holland, V. R., F. L. Saunders, F. L. Rose and A. L. Walpole. 1974. Studies in peroxidase action. 18. The oxidation of 4-chloraniline—further studies. Tetrahedron 30:3299–3302.

Lewis, S. E., C. F. Wilkinson and J. W. Ray. 1967. The relationship between microsomal epoxidation and lipid peroxidation in houseflies and pig liver and the inhibitory effect of derivatives of 1,3-benzodioxole (methylenedioxybenzene). Biochem. Pharmacol. 16:1195–1210.

Mayer, R. T., J. W. Jermyn, M. D. Burke and R. A. Prough. 1977. Methoxyresorufin as a substrate for the fluorometric assay of insect microsomal o-dealkylases. Pestic. Biochem. Physiol. 7:349–354.

Omura, T. and R. Sato. 1964. The carbon dioxide binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J. Biol. Chem. 239:2370–2378.

Patil, T. N., R. A. Morton and R. S. Singh. 1990. Characterization of 7-etoxycoumarin-O-deetylase from malathion resistant and susceptible strains of *Drosophila melanogaster.* Insect Biochem. 20:91–98.

Plapp, F. W., Jr. and J. E. Casida. 1969. Genetic control of housefly NADPH-dependent oxidases: relation to insecticide chemical metabolism and resistance. J. Econ. Entomol. 62:1174–1179.

Scott, J. A., F. H. Collins and R. Feyereisen. 1994. Diversity of cytochrome P450 genes in the mosquito, *Anopheles albimanus.* Biochem. Biophys. Res. Commun. 205:1452–1459.

Thomas, P. E., D. Ryan and W. Levin. 1976. An improved staining procedure for the detection of the peroxidase activity of cytochrome P-450 on sodium dodecyl sulfate polyacrylamide gels. Anal. Biochem. 75: 168–176.

Yu, S. J., U. Kiigemagi and L. C. Terriere. 1971. Oxidative metabolism of aldrin and isodrin by bean root fraction. J. Agric. Food Chem. 19:5–9.