This is to certify that the

dissertation entitled

Glutathione S-transferase in Aedes aegypti

presented by

David F. Grant

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Entomology/Environ. Tox.

Major professor

Date 14 November 1986

GLUTATHIONE S-TRANSFERASE IN *Aedes aegypti*

By

David F. Grant

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Entomology

1986

ABSTRACT

GLUTATHIONE S-TRANSFERASE IN *Aedes aegypti*

By

David F. Grant

Glutathione S-transferase enzymes (GSH-trans) were characterized in permethrin and DDT resistant and susceptible strains of *Aedes aegypti*. The predominant GSH-trans, designated GSH-trans-1, was purified from susceptible larvae using S-hexyl-glutathione affinity chromatography and anion exchange chromatography. GSH-trans-1 has a molecular weight of 26.8 x 10^3 kD, and an isoelectric point of 5.0. Rabbit antiserum directed against susceptible larvae GSH-trans-1 was specific for a single band in both resistant and susceptible strains using Western blotting. Enzyme kinetic data support a sequential mechanism. There was no significant difference in enzyme kinetic constants (Km and Vmax) when comparing GSH-trans-1 from susceptible and resistant strains. GSH-trans-1 enzyme activity varied during larval development in both resistant and susceptible strains, but was approximately 1.7 times greater in resistant larvae and pupae throughout development. GSH-trans-1 amount (measured using a radioimmunoassay) also varied during development, and was generally higher in resistant larvae and pupae. GSH-trans-1 enzyme activity on day X was significantly correlated with GSH-trans-1 enzyme amount on day X-1 (where X = days 1 through 6), suggesting that the enzyme becomes catalytically active approximately 24 hours after it is

synthesized.

Another GSH-trans enzyme, GSH-trans-2, also was found in resistant and susceptible Aedes aegypti larvae. GSH-trans-2 was partially purified from resistant larvae using S-2,4-dinitrophenyl-glutathione affinity chromatography and anion exchange chromatography. GSH-trans-2 has a molecular weight of 28 x 10^3 kD, and an isoelectric point less than 5.0. GSH-trans-2 was not cross reactive with GSH-trans-1 antiserum. GSH-trans-2 enzyme activity varied during development of both susceptible and resistant larvae, but the pattern was different than that for GSH-trans-1. Throughout development, GSH-trans-2 was substantially higher (approximately 8 fold) in resistant strains of Aedes aegypti compared to susceptible strains.

ACKNOWLEDGEMENTS

I thank Dr. Fumio Matsumura and Dr. Rich Merritt for their generous intellectual and financial support of my doctoral research program. I appreciate the freedom, and especially the opportunity they gave me, to pursue my research and career goals.

I thank the other members of my guidance committee (Dr. G. Bush, Dr. D. Newson, and Dr. C. Sweeley) for their many helpful suggestions and editorial comments. I thank Dr. J. Miller for the untiring generosity with which he provided his time, lab space, and professional guidance.

Finally, I thank my wife Terry, for the many sacrifices she has willingly made during the last four years, and for understanding why this work is important to me.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

GENERAL INTRODUCTION

Since the first report of insect resistance in 1914 (Melander, 1914) the phenomenon of resistance has grown exponentially until today there are over 450 insect species that are resistant to insecticides (Georghiou and Saito, 1983). As new insecticides become available for commercial use, many insects display multiple or cross resistance to the new insecticides rendering them useless. Even insecticides that have previously been thought to be "resistance proof" such as the biological insecticide Bacillus thuringiensis, are now thought to be vulnerable (McGaughey, 1985). Metcalf (1980) has said; "The concerted effect of the exponentially increasing costs of insecticide development, the dwindling rate of commercialization of new material, and the demonstration of cross or multiple resistance to new classes of insecticides almost before they are fully commercialized makes insect pest resistance the greatest single problem facing applied entomology."

In addition to its direct importance, insecticide resistance provides a model for studying and testing hypotheses of selection and the evolutionary mechanisms of multicellular animals. The processes by which eucaryotes respond to stress are just beginning to be appreciated. In many ways, the problem of insecticide resistance is analogous to the problem of multidrug- resistance found in cell lines treated with chemotherapeutic drugs (Shen, et al., 1986), multidrug-resistance in bacteria (Davis and Rownd, 1972), and multidrug-resistance in yeast (Cohen and Eaton, 1979). Whether or not the mechanisms are similar remains to be seen. Insecticide resistance is

therefore of tremendous importance to human health, applied entomology, and as a model in understanding the mechanisms of evolution and genetic plasticity.

Aedes aegypti is a vector of both neurotropic (dengue) and viscerotropic (yellow fever) flaviviruses. These diseases still persist with an estimated 0.5 to 2 million human cases of dengue and 200 clinical cases of yellow fever annually (Harwood and James, 1979). Furthermore, since Aedes aegypti is widespread in tropical areas of the world, including the Southeastern United States, the potential exists for future epidemics of both dengue and yellow fever. The problem of mosquito resistance in underdeveloped countries is especially acute since new environmentally safe insecticides are prohibitively expensive.

Aedes aegypti has a long history of resistance problems (Chadwick et al., 1977). Recent studies have been directed toward understanding the mechanism of DDT resistance (McDonald and Wood, 1979a and 1979b; Rathor and Wood, 1981) and the genetic relationship between DDT and permethrin resistance (Malcolm, 1983a and 1983b; Chadwick et al., 1984; Brealey et al., 1984). The results suggest that DDT and permethrin resistance are due to increased metabolism and/or decreased target-site sensitivity, the relative importance of each differing in larvae and adults. Aedes aegypti selected with DDT are generally cross-resistant to permethrin and vice versa. The molecular basis for DDT and permethrin resistance, and cross-resistance is poorly understood since the metabolic enzymes and target-sites have not been purified and compared in resistant and susceptible individuals.

The work in this dissertation is an attempt to develop a model for understanding the molecular mechanisms involved in metabolic

insecticide resistance. As with all systems that are initially developed as models, the approach, rather than the results, should be considered more generally applicable to other systems. The approach taken in this work is to lay the foundation for understanding one system in considerable detail. From this information, testable hypotheses can be developed for other systems and for other insects.

CHAPTER 1

Glutathione S-transferase-1 in *Aedes aegypti* larvae: Purification and Properties

INTRODUCTION

Glutathione S-transferase (EC 2.5.1.18, GSH-trans) conjugates reduced glutathione (GSH) to a wide variety of electrophilic compounds (Mannervik, 1985). When conjugated to GSH, potentially toxic substrates are more water-soluble, and generally less toxic. Originally, GSH-trans enzymes were recognized and named based on their activities with various classes of electrophilic substrates such as aryl, alkyl, arene etc. (Boyland and Chasseaud, 1969). Recent work in vertebrates, however, has shown that these different isozymes are not substrate specific. The enzymes were found to be composed of binary combinations of at least 6 distinct subunits (Mannervik, 1985), and that subunit similarities correlated with substrate specificity similarities.

Insect GSH-trans enzymes are not nearly as well understood, since most of the data have been obtained using semi-purified enzymes. Multiple forms of GSH-trans have been found in the housefly (Clark et al., 1986), grass grub (Clark et al., 1985), and cockroach (Usui and Fukami, 1977), while only one form was found in wax moths (Chang et al., 1981) and Mediterranean fruit flies (Yawetz and Koren, 1984). These studies were designed to assess how GSH-trans enzymes are related to the development of insecticide resistance, especially resistance to organophosphates (Usui and Fukami, 1977; Oppenoorth et al., 1979). Very little information is known, however, about the biochemical mechanisms controlling the expression and regulation of GSH-trans enzymes (or other potential insecticide-metabolizing enzymes) at the molecular level. Much needs to be learned about how,

when, and where these enzymes are expressed in resistant and susceptible insects.

In this chapter I present data describing what apperars to be the predominant form of GSH-trans in susceptible strains of Aedes aegypti larvae and pupae. These data include: purification scheme, molecular weight, isoelectric point, kinetic constants, and expression during larval and pupal development.

Materials and Methods

Insects

The Rockefeller (Rock) strain of Aedes aegypti was obtained from Notre Dame University and is an unselected reference strain. Adults were reared at 25°C and 50-70% relative humidity in 25 cm square, screen cages, with a 16 hr light (1 hr dawn, 1 hr dusk), 8 hr dark photoperiod. Raisins were hung in the cages for food. Adult females were fed using guinea pigs. Larvae were reared in distilled water in covered enamel or plastic pans. The larvae were fed ground liver powder dissolved in distilled water (0.25 g/d). Under these conditions, larvae reach fourth instar in 5 days, pupate in 7 days and emerge in 10 days. Eggs were collected on filter paper in 400 ml beakers half filled with distilled water. The eggs were stored desiccated for up to two months and were hatched over a 2 hr period in degassed distilled water.

Enzyme Assays

Enzyme activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB), and glutathione (GSH) as substrates. CDNB was dissolved in substrate buffer (100 mM KH_2PO_4, 15% glycerol, pH 6.8) by stirring overnight at room temperature to give a final concentration of 1.07 mM (=substrate solution). GSH was made fresh daily as a 155 mM stock solution in ion exchange equilibration buffer (20 mM Tris, 2.0 mM dithiothreitol (DTT), 15% glycerol, pH 7.8) and was kept on ice during use. Change in absorbance was measured for 1 min at 340 nm in a waterbathed (24°C) Gilford 2600 spectrophotometer with an attached Hewlett Packard model 7225 A plotter. Absorbance values were converted using a molar extinction coefficient of 9.6 $mM^{-1}cm^{-1}$ (Habig et. al., 1974). Routine assays consisted of 2.1 ml substrate buffer, 0.8 ml of substrate solution, 50 ul of enzyme solution, and 100 ul of GSH solution, added last. Velocity plots were linear for approximately 2 min. Nonenzymatic conjugation (assays conducted without enzyme) was subtracted from all assays. Protein was measured by the method of Bradford (1976), as modified by Spector (1978). BSA was used as the protein standard.

Purification of GSH-trans-1.

All purification steps were done at 4°C. Approximately 5 g (wet weight) of 5 or 6 day old larvae were homogenized in 30 ml homogenization buffer (50 mM Tris, 10 mM cysteine, 10 mM DTT, 15%

glycerol, 0.2 mM PMSF added fresh, pH 7.8) in a glass/glass homogenizer. The homogenate was centrifuged (20 min, $75x10^3$x g, 4^oC), filtered through a 0.2um sterile filter, then loaded onto a 3 ml S-hexyl-glutathione affinity column (prepared as described in Mannervik and Guthenberg, 1981), pre-equilibrated with 20 ml of homogenization buffer. The column was washed with 20 ml of ion exchange elution buffer (20 mM Tris, 2 mM DTT, 300 mM NaCl, 15% glycerol, pH 7.8), then eluted with a linear 0-555uM gradient of S-hexyl-glutathione in ion exchange elution buffer. Active fractions were pooled and washed two times on a centricon-30 microconcentrator (Amicon) with ion exchange equilibration buffer. The retentate was then loaded onto a 0.75 x 20cm DEAE-Sepharose column equilibrated with 30 ml of ion exchange equilibration buffer. The DEAE column was eluted overnight with a linear 0-300 mM NaCl gradient in ion exchange equilibration buffer. Active fractions were pooled, washed two times on a centricon-30 and frozen at -80^oC. For enzyme kinetic studies, purified GSH-trans-1 was stored in ion exchange equilibration buffer with GSH added to give a final concentration of 300 uM.

Electrophoresis

Electrophoresis was used for determining purity, molecular weight, and isoelectric points. For routine experiments, discontinuous SDS polyacrylamide electrophoresis (Disc-SDS-PAGE) with 3.3% stacking and 11% resolving gels were used according to Laemmli (1970). Narrow range analytical isoelectric focusing gels (pH 4.5-6.0, 0.8mm, 5.5% acrylamide) were prepared and run according to manufacturer's recommendations (Pharmacia). Proteins were stained with

Coomassie brilliant blue. For determining the molecular weight of native GSH-trans activity, a 2 x 47 cm Sephadex G-100-50 column was used. Molecular weight and isoelectric point standards were obtained from Sigma.

Enzyme kinetics

Kinetic assays were conducted similarly to those described above for routine enzyme assays. A 5 x 5 matrix of CDNB concentrations (0.16, 0.08, 0.04, 0.02, 0.01 mM), and GSH concentrations (0.4, 0.2, 0.1, 0.05, 0.025 mM) was used. Substrates were diluted so that equal volumes were used in each assay. Assays consisted of 2.0 ml of substrate buffer, 0.4 ml of CDNB solution, 50 ul of a 50-fold dilution of purified stock GSH-trans-1 (0.22 ug), and 50 ul of GSH solution, added last. Each assay in the 5 x 5 matrix was done randomly. The 5 x 5 matrix assay was replicated three separate times for one enzyme preparation. All kinetic assays were conducted within 1 week after enzyme purification. The data were analyzed using a weighted (Wilkinson, 1961) least-squares linear regression model.

Preparation of anti-GSH-trans-1 antibody

Approximately 320 ug of purified GSH-trans-1 was mixed 1 : 1 with Freund's complete adjuvant (Sigma) and 160 ug injected according to standard methods (Weir, 1978) into each of two New Zealand White rabbits. Booster shots (160 ug/rabbit) were given one month later in incomplete adjuvant. Each rabbit's serum titer was tested 10, 14, and 21 days after boosting using standard ELISA techniques (Weir, 1978).

Preimmune serum was used as a control. Approximately 40 ml of positive serum was collected by cardiac puncture and frozen in 1 ml aliquots at -80°C.

Detection of GSH-trans-1 during larval development

Western blotting. Western blotting was used to determine anti-GSH-trans-1 serum specificity and to qualitatively assess the expression of GSH-trans-1 in 1 hr, 1d, 2d, 3d, 4d, 5d, 6d, and 8d old Aedes aegypti larvae. Rock larvae were hatched and distributed among 12 pans so that their density in the pans was inversly proportional to their age at sampling (ie. 6d old larvae samples were grown at a much lower density than were 2d old larvae samples). At the indicated times, either larvae or pupae were collected, washed on a cloth or wire screen, dried, and homogenized in 10 ml of homogenization buffer. The samples were centrifuged, filtered (0.2 um), and frozen in aliquots at -80°C. Samples were run on Disc-SDS-Page gels, and either stained with Coomassie blue, or transferred to nitrocellulose (0.2 um pore size, Schleicher and Schull) as described in Burnette (1981) using a Bio-Rad Trans-blot cell. The nitrocellulose sheet was incubated with two changes of blotting buffer (10 mM KH_2PO_4, 137 mM NaCl, 0.02% sodium azide, 0.25% Tween-20, 0.25% gelatin, pH 7.4) for 1 h with shaking at room temperature to block remaining protein binding sites. The sheet was then placed in 150 ml of 1:10000 anti-GSH-trans-1 serum in blotting buffer and incubated for 4 hr with shaking at room temperature Bound IgG was detected with alkaline-phosphatase conjugated goat-anti-rabbit IgG (Sigma, 1:1400) as described by Blake et al. (1984) using 1 M diethanolamine (pH 9.6) substrate buffer.

Radioimmunoassay. A radioimmunoassay was developed to measure the amount of GSH-trans-1 in developing Aedes aegypti larvae and pupae. Ten ug of purified GSH-trans-1 was iodinated with 1 mCi of ^{125}I using the Biorad Enzymobead radioiodination reagent. Iodinated protein (tracer) was purified on a 1 x 8 cm Sephadex G-25 column equilibrated with blotting buffer, then frozen at -80°C in aliquots. Anti-GSH-trans-1 serum titer was determined from an antibody dilution curve. Assays were incubated overnight at room temperature in a total volume of 500 ul. Five hundred ul of goat-anti-rabbit IgG bound to Staphylococcus aureus cells (Calbiochem) was added to separate bound from free tracer. After 1 hr at room temperature, tubes were centrifuged and the supernatant removed by aspiration. The pellets were then washed with 1 ml of blotting buffer, centrifuged, aspirated, and counted in a Hewlett-Packard gamma counter. No serum and preimmune serum tubes were used as controls. Purified GSH-trans-1 diluted in blotting buffer was used to prepare standard curves. Data were expressed as ug GSH-trans-1/mg total protein generated from a percent bound vs ln(GSH-trans-1) standard curve. A standard curve was included with each independent assay. Developing larvae and pupae samples (prepared as described above) were diluted (4 different dilutions for each of 2 independent assays) in blotting buffer to match the concentration range of the standard curves.

Enzyme assays. GSH-trans enzyme activity was measured in developing larvae and pupae as described above.

RESULTS

Purification. The S-hexyl GSH affinity column procedure provided

a very efficient first step in purifying GSH-trans-1 from Aedes aegypti (Table 1). Much lower efficiencies were obtained for this step when cysteine and DTT were not included in the homogenization buffer. After affinity chromatography however, cysteine was not necessary. Anion-exchange chromatography lead to an approximate doubling of the specific activity with only a 10% loss of recovery. The resulting enzyme preparation was apparently homogeneous as shown using SDS-Disc electrophoresis (Figure 1). A molecular weight of 50000 was obtained after gel filtration on a Sephadex-G-100 column, (data not shown) indicating that the native enzyme is composed of two identical subunits each with a molecular weight of 26.8 kD (Figure 1). Narrow range analytical isoelectric focusing also resolved only one major peak (Figure 2) having an isoelectric point of 5.0.

To assess the specificity of the anti-GSH-trans-1 serum, crude protein samples from developing larvae and pupae were separated on SDS-Disc gels (Figure 3), blotted onto nitrocullulose, and probed with a 1:1000 dilution of the immune serum. Only one band was detected for each age class of larvae or pupae (Figure 4), indicating a highly specific immune serum. All of these bands comigrated with purified GSH-trans-1. By knowing the amount of total protein and the amount of standard GSH-trans-1 used for the immunoblot, GSH-trans-1 represents approximately 0.5% of the total protein in Aedes aegypti. This amount is not enough to see in the total protein electrophoresis gel stained with coomassie blue (Figure 3).

Enzyme kinetics. Intersecting reciprocal plots (Figure 5) support a sequential mechanism for GSH-trans-1 using CDNB and GSH as substrates at pH 6.8 (Segel, 1975). Since the reciprocal plots for both graphs intersect below the 1/GSH and 1/CDNB axes, binding of

Table 1. Purification of GSH-trans-1 from 5 day old Rock *Aedes aegypti* larvae (n = 4 separate experiments).

Fraction	Enzyme activity (umol/min/mg ± SD)	Fold purification (± SD)	% Recovery (± SD)
Homogenate	0.59 ± 0.04	1	100
Affinity chromotography	118 ± 7.0	201 ± 12	73 ± 8
Anion exchange chromotography	208 ± 15	358 ± 37	61 ± 3

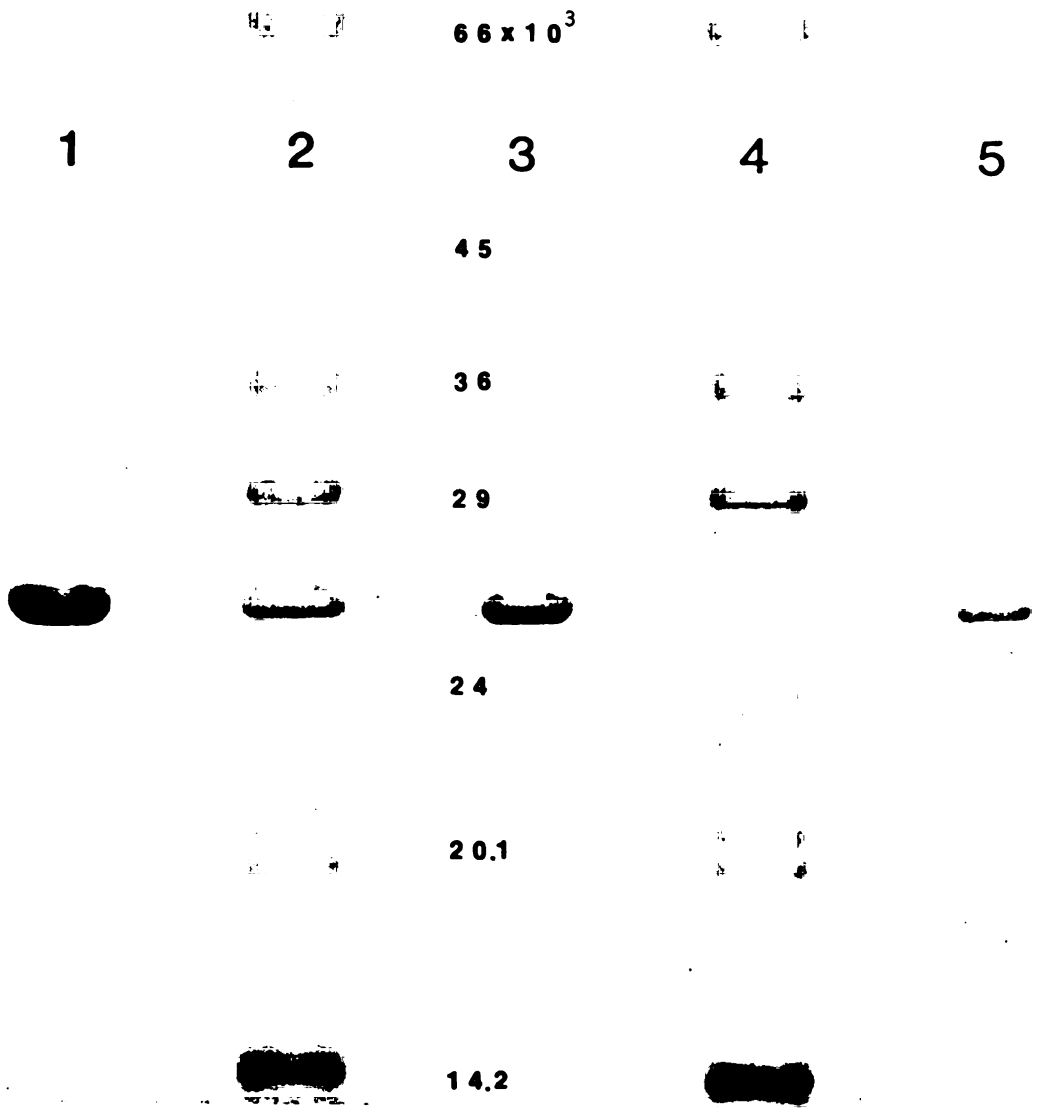

Figure 1. SDS-Disc electrophoresis (12% resolving gel, 3% stacking gel of purified GSH-trans-1 from fourth instar (6 day old) Aedes aegypti (Rock strain) along with molecular weight standards. 20 ug GSH-trans-1 (lane 1); 10 ug GSH-trans-1 + molecular weight markers (lane 2); 10 ug GSH-trans-1 (lane 3); molecular weight markers (lane 4); 5 ug GSH-trans-1 (lane 5). Molecular weight of GSH-trans-1 subunit = 26800 daltons (SD=200, n = 3 separate determinations).

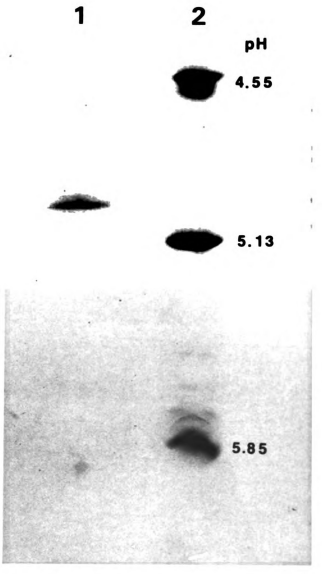

Figure 2. Narrow range (pH 4-6.5) analytical isoelectric focusing (5% gel) of GSH-trans-1 from fourth instar (6 d old) Aedes aegypti (Rock strain). 3.0 ug purified GSH-trans-1 (lane 1); pI standards (lane 2).

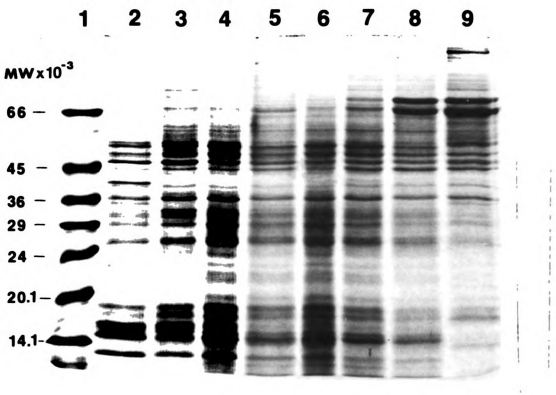

Figure 3. SDS-Disc electrophoresis (11% resolving gel, 3.3% stacking gel) of total protein from Aedes aegypti (Rock strain) during development. Molecular weight standards (lane 1). Lanes 2-9 each contain 40 ug total protein. 1 hr old larvae (lane 2); 1 day old larvae (lane 3); 2 day old larvae (lane 4); 3 day old larvae (lane 5); 4 day old larvae (lane 6); 5 day old larvae (lane 7); 6 day old larvae (lane 8); 8 day old pupae (lane 9).

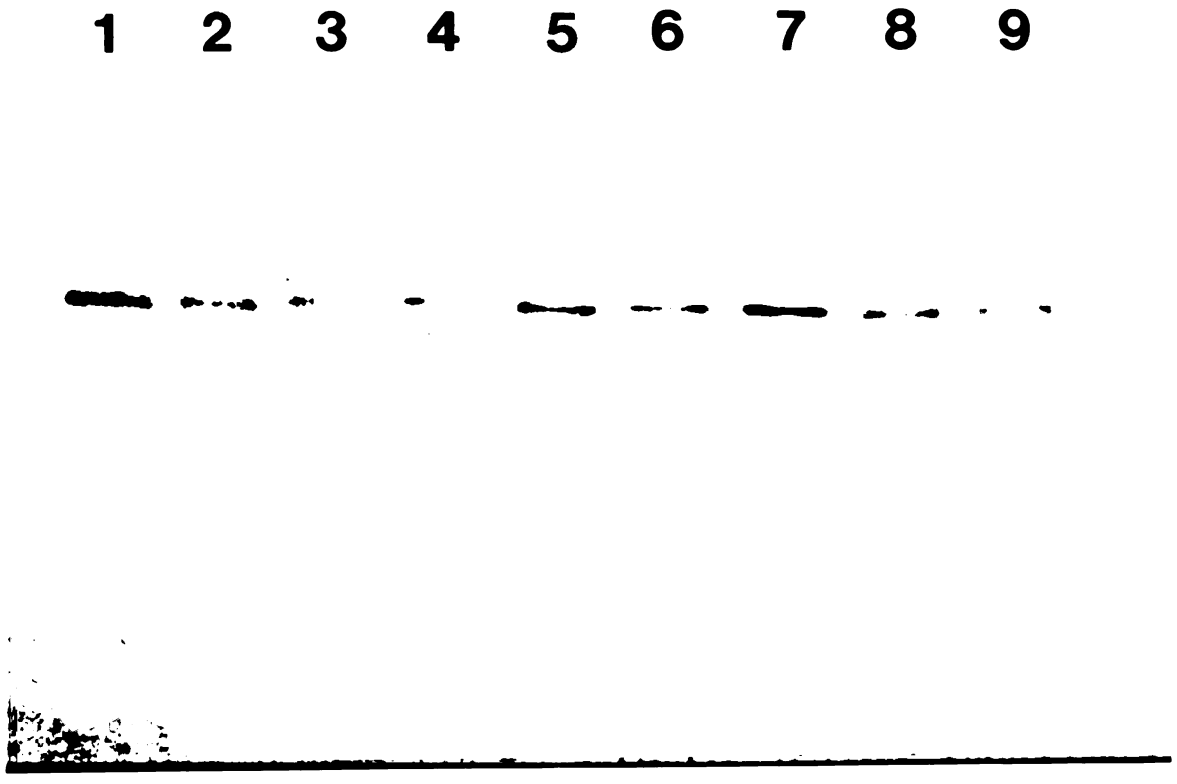

Figure 4. Anti-GSH-trans-1 serum immunoblot of total protein from Rock strain of Aedes aegypti during development. Proteins were run on SDS-Disc 11% resolving, 3.3% stacking gels then transferred to 0.2 um nitrocellulose membrane overnight. Antigens were detected as described in materials and methods. 25 ng GSH-Trans-1 standard (lane 1); Lanes 2-9 each contain 5.7 ug total protein. 1 hr old larvae (lane 2); 1 day old larvae (lane 3); 2 day old larvae (lane 4); 3 day old larvae (lane 5); 4 day old larvae (lane 6); 5 day old larvae (lane 7); 6 day old larvae (lane 8); 8 day old pupae (lane 9).

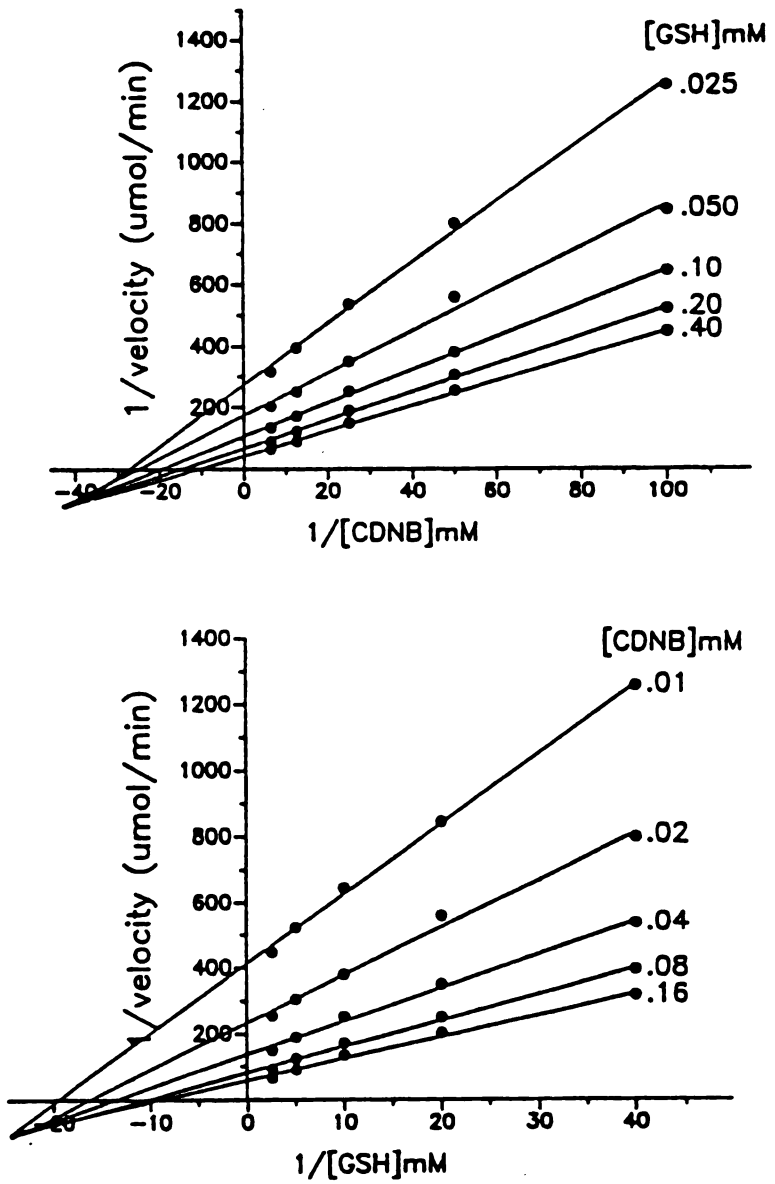

Figure 5. Lineweaver-Burk plots of GSH-trans-1 from Rock strain of Aedes aegypti. Each point is the mean of three separate assays.

either CDNB or GSH decreases the apparent affinity of the enzyme for the other substrate (Segel, 1975). The replots of the slopes and intercepts (Figure 6) give the kinetic constants for GSH-trans-1 (± SD): KmGSH = 0.05 mM ± .016; KmCDNB = 0.025 mM ± .009; Vmax = 218 umol/min/mg GSH-trans-1 ± 31. The Vmax value is consistent with the specific activity value of the purified enzyme after anion exchange chromatography (Table 1).

GSH-trans-1 during development. During development of Aedes aegypti larvae there is a definite pattern of change in GSH-trans enzyme activity (Figure 7). A minimum occurs at days 2 and 3 and a maximum at day 5. One hour after hatching, larvae have a considerable amount of enzyme activity, slightly more that pupae just before they emerge as adults (192 h old). These changes in activity during development suggest that GSH-trans activity is under some type of regulatory control.

Anti-GSH-trans-1 serum was shown by Western blotting to be specific for one band on SDS-Disc gels, however it is necessary to find appropriate serum and antigen concentrations in order to develop a quantitative radioimmunoassay. With no competing unlabeled GSH-Trans-1, a serum dilution of 1:14000 bound approximately 50% of the total ^{125}I-labeled GSH-trans-1 (relative to maximun binding, Figure 8). A 1:300 dilution of preimmune serum bound less radioactivity than did a 1:153600 dilution of immune serum. Percent bound was inversely related to log(ng GSH-trans-1) in the range of 3 to 50 ng GSH-trans-1 using a 1:8000 immune-serum dilution (Figure 8, inset). Radioimmunoassays were performed using a 1:10000 immune serum dilution, and standards in the range of 3-36 ng purified GSH-trans-1. The amount of GSH-trans-1 (Figure 9) generally follows the overall

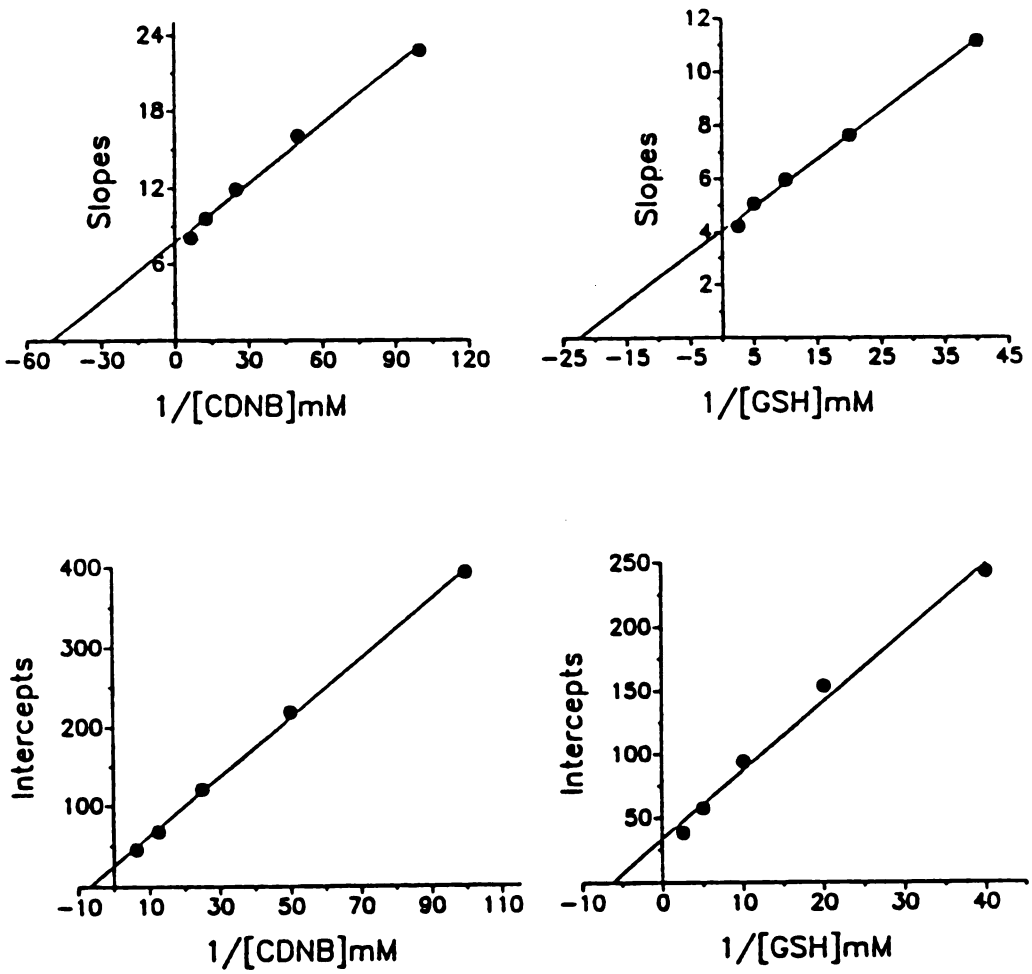

Figure 6. Replots of slopes and intercepts from primary plots shown in Figure 5.

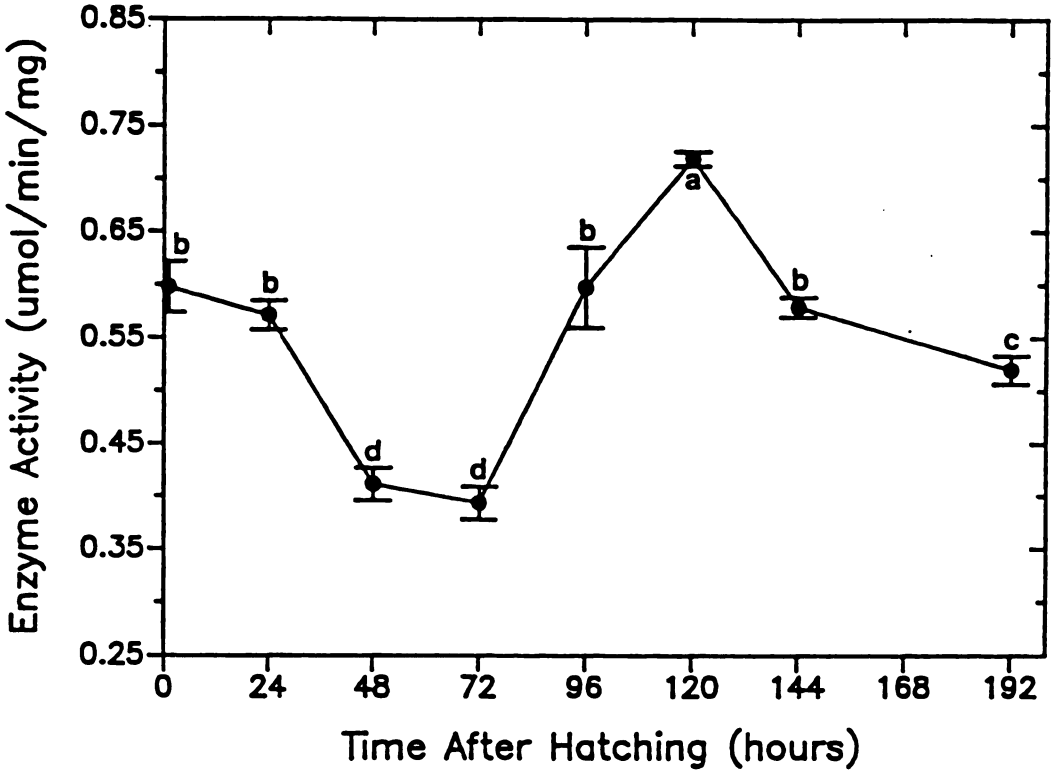

Figure 7. Total GSH-trans enzyme activity during development of Rock strain of Aedes aegypti. Larvae pupate at approximately 168 hours. Data are means ± SD ($n \geq 3$). Means with different letters are significantly different from each other ($P \leq .05$).

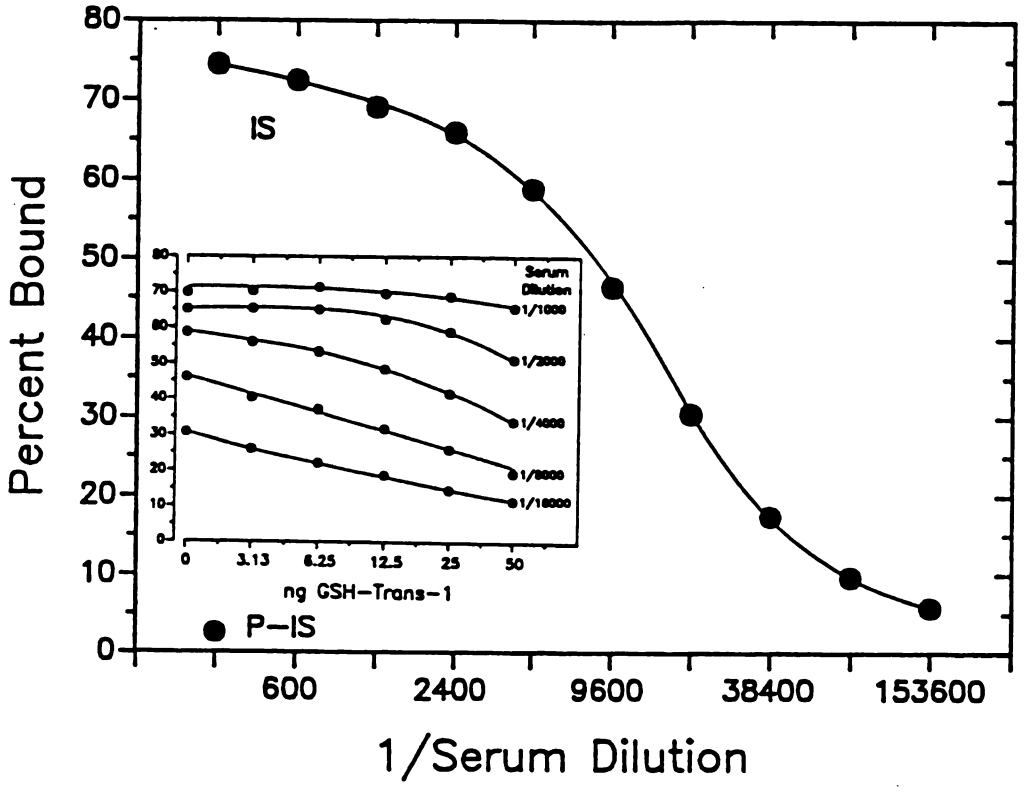

Figure 8. Anti-GSH-trans-1 serum dilution curve using iodinated GSH-trans-1 from Rock strain of Aedes aegypti. Inset: standard curve plots used to estimate serum dilution, and GSH-trans-1 concentration range for radioimmunoassays. IS = immune serum, P-IS = preimmune serum. SD $\leq$ size of symbols (n=2).

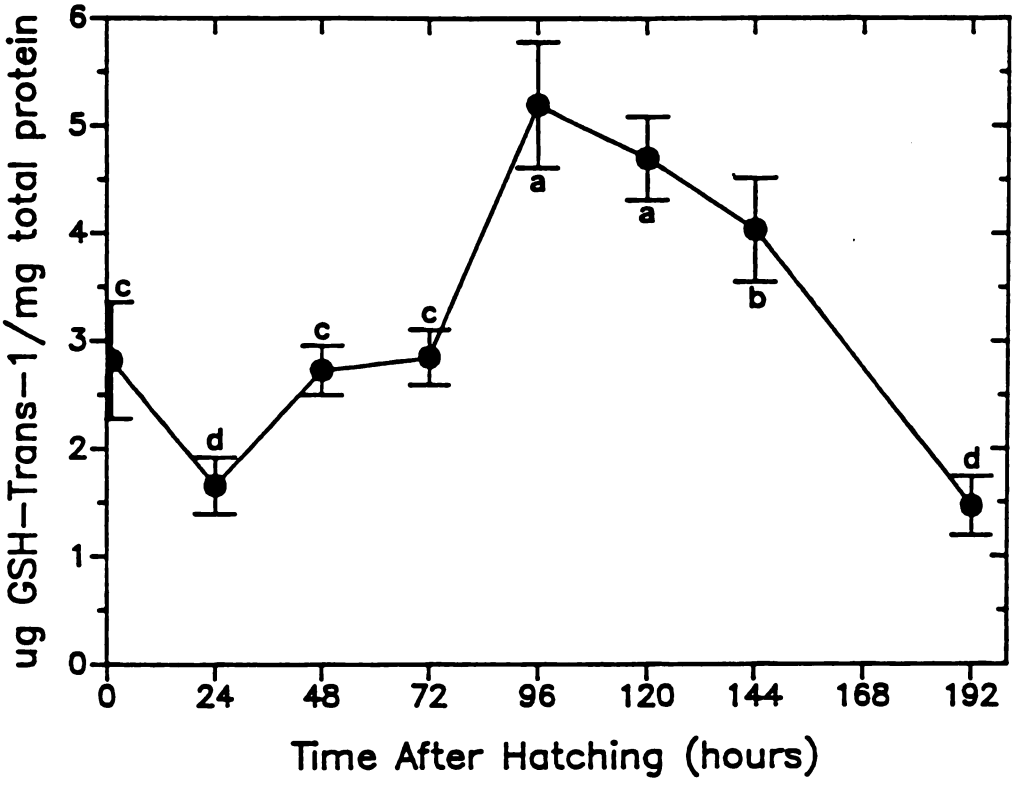

Figure 9. GSH-trans-1 enzyme amount during development of Rock strain of Aedes aegypti. Data are means ± SD (n=8). Means with different letters are significantly different from each other ($P \leq .05$).

pattern found for GSH-trans enzyme activity during development of Aedes aegypti. There is a peak of GSH-trans-1 amount at days 5 and 6 and a minimum in 1 day old larvae and in pupae. Further analysis of Figures 7 and 9 shows however, that the amount of GSH-trans-1 is not highly correlated with total GSH-trans activity when compared on a day to day basis ($r = 0.49$). For example, there is a significant increase in GSH-trans-1 protein between days 1 and 2, but a significant decrease in enzyme activity during the same period. In general, it appears that the amount of GSH-trans-1 protein more closely predicts the enzyme activity 24 h later ($r = 0.78$), but the relationship is still only marginally significant ($0.10 > P > 0.05$) suggesting that GSH-trans-1 does not account for the total GSH-trans activity found in Aedes aegypti.

DISCUSSION

Purification of GSH-trans-1 from Aedes aegypti was complicated initially by rapid loss of activity after homogenization. This was not a problem after cysteine and DTT were included in the homogenization buffer. Others have also found that factors (most likely quinones) present in insect crude homogenates rapidly denature GSH-trans (Clark et al., 1985; Chang et al., 1981; Motoyama et al., 1978). If 10 mM cysteine was included in buffers used following affinity chromatography however, GSH-trans-1 activity eluted from the anion-exchange column as a broad peak with a shoulder. When cysteine was not used following affinity chromatography, the GSH-trans-1 activity peak was sharper and had little or no shoulder. The shoulder could have either been an additional GSH-trans enzyme which remained

active only when cysteine was included in the anion-exchange steps, or that cysteine modified the enzyme , perhaps by forming a mixed disulfide. Ramage and Nimmo (1983) found that GSH-trans from rainbow trout displayed anomolous isoelectric behavior after a GSH-affinity column was used during initial purification. They concluded that GSH may form a mixed disulfide with the GSH-trans during elution from the GSH-affinity column. Cysteine may act similarly at high concentrations

When percent recoveries are less than 100% it is difficult to determine whether loss of enzyme activity is due to inactivation of the enzyme being purified, or due to loss of other isozymes or allozymes. Sixty-one percent recovery during purification is reasonably high however, and suggests that if other GSH-trans are present in 5 d old Aedes aegypti, they probably comprise $\leq$ 20% of the total activity measurable with CDNB. If more than one enzyme is present, other substrates such as 3,4-dichloronitrobenzene would probably give higher percent recoveries since they are generally considered more enzyme specific (Mannervik, 1985).

The Aedes aegypti GSH-trans-1 subunit molecular weight is very similar to isozyme subunits found in rat and man (Mannervik, 1985), and in other insects (Motoyama and Dauterman, 1978). This subunit size has apparently been well conserved during evolution. The GSH-Trans-1 Western blot analysis indicates that there are no post-translational modifications occurring that change the molecular weight of the antigenic sites on the subunit in Aedes aegypti. Once the antigenic sites are synthesized, the subunit primary structure is not altered, at least not altered enough to be seen using 11% SDS-gels.

Kinetic constants for GSH-trans-1 were obtained in order to

provide data to distinguish among possible isozymes in other strains of Aedes aegypti. The data do not provide information about whether the proposed sequential mechanism is random or ordered (Jakobson et al., 1979), but do discount the possibility of a ping-pong mechanism (Segel, 1975). Because the 1/velocity versus 1/substrate reciprocal plots intersect below the 1/substrate axis, the apparent affinity of the enzyme for the second substrate is decreased after binding to the first substrate. Apparent affinity is used here since a rapid equilibrium mechanism cannot be assumed. The 1/velocity versus 1/substrate plots are linear over the concentrations used, providing no strong evidence for deviations from Michaelis-Menton kinetics as found by both Chang et al. (1981) and Askelof et al. (1975). Stock enzyme preparations used in the present study however, contained 300 uM GSH. GSH is thought to be involved in forming kinetically stable activity states of GSH-trans enzymes (see discussion in Mannervik, 1985). The relationship between how GSH can stabilize enzyme activity and also alter enzyme isoelectric point is unclear. It seems evident that the results obtained from both kinetic experiments and purification procedures are heavily dependent on storage and buffer composition. The replots of the slopes and intercepts versus 1/substrate are not linear (Figure 6). This is most likely due to variability in the velocity measurements at low substrate concentrations.

There are two other reports of kinetic constants for GSH-trans in insect species using GSH and CDNB as substrates. The wax moth enzyme has a Vmax and KmGSH similar to Aedes aegypti, but an approximate 20-fold higher KmCDNB (Chang et al., 1981). The Mediterranean fruitfly enzyme has a 10-fold lower Vmax and a similar KmCDNB (Yawetz and

Koren, 1984).

The regulation and control of GSH-trans enzymes during development is a problem that has not been addressed in insects. The data presented here clearly show that changes occur for both GSH-trans enzyme activity and GSH-trans-1 enzyme amount during growth of Aedes aegypti larvae. Apparently after the larvae hatch there is either an increased rate of degradation, decreased rate of synthesis, or both. Synthesis of the protein then begins at days 2 and 3 and reaches a maximum at day 4. Enzyme activity, on the other hand, decreases until day 3, then begins to increase until day 5. There is little information available describing hormonal activity or timing of molting in Aedes aegypti larvae. With the rearing conditions I used, there are 4 molts between 0 and 120 h (Christophers, 1960) This would mean that the larvae molt every 30 h if all instars live equally long. Since it seems probable that younger instars develop faster than do older instars (Mills and Lang, 1976; Christophers, 1960), instar duration probably varies during development. Enzyme activity and amount measurements were made using homogenates consisting of many individuals, and done in 24 h intervals. The data therefore probably would not be sensitive enough to detect defined developmental stages.

Enzyme activity on day X is more highly correlated with enzyme amount on day X-1 (where X = days 1 through 6), than they are on equal days (Figures 7 and 9). This result suggests that Aedes aegypti has two forms of GSH-trans-1 that are recognized by the anti-GSH-trans-1 serum; a form that has no enzyme activity and a form that has enzyme activity. The inactive form is apparently synthesized, then converted to the active form 24 h later. Obviously this hypothesis will require further verification. The Western blot analysis shows that if there

are two GSH-trans-1 forms, the inactive form has a molecular weight (or subunit molecular weight) very similar to the subunit molecular weight of the active form. It would be very informative to do the same experiment using isoelectric focusing to separate the proteins before blotting. Post-translational modifications known to be involved in enzyme activation are phosphorylation, attachment of sugars, and disulfide bond formation (Wold, 1981). Disulfide bond formation between subunits is an obvious first choice for explaining enzyme activation in this system. Perhaps more than one modification is necessary.

Further study will be required to determine how environmental factors such as crowding, temperature, insecticide treatment, etc., affect expression of GSH-trans-1 during larval development. Such information will be very useful in understanding how these enzymes are regulated, and at what level insecticide selection operates on this system.

CHAPTER 2

Glutathione S-transferase 1 and 2 in Susceptible and Insecticide Resistant *Aedes aegypti* Larvae

INTRODUCTION

Insect toxicologists generally recognize four major enzymes or enzyme systems that are responsible for the initial metabolism of an insecticide (Matsumura, 1975). The first of these is the cytochrome-P-450 mediated mixed-function oxidase (MFO) system. This system is comprised of a family of isozymes (5-20 different isozymes) with overlapping catalytic efficiencies for a range of lipophilic substrates. These enzymes catalyze hydroxylation, heteroatom oxidation, epoxidation, group transfer, and other reactions (Guengerich and McDonald, 1984). The second major class of detoxification processes is the carboxylesterase (CE) system. This enzyme system catalyzes hydrolysis of ester bonds in compounds such as organophosphates and pyrethroids. The third type of enzyme important in insecticide metabolism is called DDT-dehydrochlorinase (DDTase). This enzyme converts DDT to the non-toxic DDE analog, and has been found mainly in diptera. The enzyme requires reduced glutathione (GSH) as a cofactor, but the mechanism of catalysis is unknown. The last major class of enzymes conferring matabolic resistance is the glutathione S-transferase system (GSH-trans). This enzyme catalyzes the conjugation of GSH to electrophilic sites on insecticides. In rat liver, there are 6 GSH-trans isozymes (Habig et al., 1974), 3 to 4 in houseflies (Clark and Dauterman, 1982), and apparently one in wax moths (Chang et al., 1981). GSH-trans isozymes demonstrate overlapping substrate specificity (Habig et al., 1974). Catalytic properties are

relatively easy to obtain for GSH-trans because of available spectrophotometric methods (Habig et al., 1974) and ease of purification using GSH-trans affinity columns (Simons and Jagt, 1977).

It is widely believed that insects don't develop metabolic resistance to insecticides, insecticides only select for those variant individuals that already possess metabolic resistance to the insecticide. Resistance in a population then develops as a result of selection based on Hardy-Weinberg principles of allele frequency. Initially, "resistant allele" frequency is very low. As susceptible alleles are eliminated during selection, resistant allele frequency approaches 1 and the population becomes resistant. Is this hypothesis really true, and if so what is the mechanism by which these "already metabolically resistant" variants arise? Is it merely a function of random gene mutation and recombination? Can the selection process itself affect the normal mechanisms producing heritable changes in metabolic capabilities? Is the development of one type of metabolic resistance related in any way to other metabolic resistance mechanisms? What is the relative importance of structural vs regulatory gene variation? Surprisingly few of these questions have been addressed at the molecular level in resistant insects. Metabolic resistance to insecticides occurs when the insecticide is metabolized at a rate that lowers its concentration at the target site below the toxic level. Metabolically resistant insects convert the insecticide to a non-toxic product faster than do susceptible insects. This implies that the enzyme or enzymes catalyzing this process have either different kinetic characteristics that allow then to detoxify the insecticide faster, or are more abundant. Since the production of either an

altered enzyme or more abundant enzyme must be heritable to provide resistance, alleles and/or allele frequencies have changed in resistant insects.

There are three known mechanisms that can lead to a more efficient detoxifying enzyme; point mutations, deletions, and insertions (Wagner et al., 1980). On the other hand, there are many known, and no doubt, many unknown mechanisms that could be involved in producing more of a particular enzyme. Three of these are mutation of a regulatory gene, recombination of regulatory and structural genes, and duplication of such genes. Mutation of regulatory genes may cause different DNA or protein binding characteristics leading to, for example, increased rates of messenger RNA (mRNA) synthesis or post-translational activation, or decreasing enzyme degradation. Recombination (either reciprocol or non-reciprocol) can lead to structural or regulatory gene amplification and gene conversion (Redei, 1982). There are several new theories of how gene expression is temporally and quantitatively regulated, including the possible role of repetitive sequences (Davidson and Britten, 1979), transposition (Campbell, 1983), and the firone hypothesis (Varshavsky, 1981). Davidson and Britten's (1979) model proposes that differences in the amount of a particular gene product (an enzyme for example) may be due to differences in nuclear RNA (nRNA) processing rather than due to differences in gene transcription or copy number. In terms of metabolic resistance, more enzyme could be produced by a change in the regulation of nRNA processing rather than a change in the regulation of transcription, enzyme activation or inactivation.

The theory of transposition (McClintock, 1952) has greatly modified concepts of genetic plasticity. No longer is a population considered merely a catalog from which natural selection chooses the most fit individuals. Transposable elements may code for promoters, genes for drug resistance, genes that regulate their own transposition, genes for protein kinase activity, and during transposition, gene amplification, deletion, inversion and cointegrate formation can occur (Campbell, 1983). In fact, transposable elements are the basis for what has been coined "the new Lamarckianism (Campbell, 1983) because of their before-mentioned ability to lead to inducible heritable changes in the genome. An example of such an adaptive change is provided by Tomich et al. (1980). They found that transposition of a transposable element conferring erythromycin resistance in bacteria was induced by low concentrations of erythromycin, leading to a stable, resistant population. The ability of environmental stress to lead to heritable changes in plants is just beginning to be appreciated. These changes may be due to both transposition, and gain or loss of repetitive sequences (Marx, 1984).

Another relatively new concept is the firone hypothesis of Varshavsky (1981). This theory states that DNA replication within any given chromosomal domain could be increased by substances (called firones) of either intra- or extracellular origin. The increased DNA replication leads to gene amplification, and depending on the amplified gene, to an increase in fitness. Two recent tests of this hypothesis have been made. Bojan et al. (1983) tested the hypothesis that a tumor promoter (the firone) could increase the frequency of mouse and hamster cells resistant to methotrexate by

causing amplification of dihydrofolate reductase, the enzyme which detoxifies methotrexate. The results were inconclusive and the authors recommended caution in accepting the model. Hayashi et al. (1983), on the other hand, found that potent tumor promoters of three classes greatly increased the appearance of cadmium-resistant cells due to amplification of proteins which detoxify cadmium. The resistance was independent of the presence of tumor promoter once it was established. It should be noted that selection with methotrexate or cadmium alone also leads to resistance due to amplification of the appropriate detoxifying protein. Resistance due to gene amplification has been inferred also in studies with aphids (Devenshire, 1977). More recent evidence shows that amplification of an esterase gene leads to organophosphate resistance in Culex quinquefasciatus (Mouches, 1986).

Another interesting aspect of metabolic detoxification systems is that of how (or whether) various classes of reactions (i.e. MFO vs. GTH-trans vs. CE) are coordinated by some type of common regulatory mechanism. This point is particularly important because of the common phenomenon that the development of one type of metabolic resistance system (e.g. MFO), is often associated with increases in other systems such as GSH-trans or CE. This phenomenon is called multiple metabolic resistance (Plapp and Wang, 1983). For example, in Enterobacteriaceae resistance to a wide variety of antibiotics appears simultaneously (Davies and Rownd, 1972). Similar multiple drug resistance occurs in the yeast Saccharomyces cerevisiae (Cohen and Eaton, 1979). The heat shock genes of Drosophila also provide an example where a single stimulus can coordinately induce synthesis of several different proteins (Nussinov and Lennon,

1984). Studies with mice have revealed that at least 20 different MFO enzymes, and a variety of drug-conjugating enzymes appear to be associated with the same genetic locus (Nebert et al., 1984). This locus has been termed Ah (for aryl hydrocarbon hydroxylase). The Ah locus produces a cytosolic receptor which binds a variety of inducers causing the coordinate induction of these detoxifying enzymes. Plapp (1984) has proposed a similar mechanism for multiple metabolic resistance based on enzyme induction studies and classical genetic analysis in the housefly. Plapp and Wang (1983) have shown also that inducibility of these enzymes may be a function of the level of resistance. More resistant houseflies had less inducible MFO and GSH-trans than did susceptible flies.

In summary, classical theories state that insecticides only select variants. How these variants arise is unknown but is assumed to be due to mutation and recombination. Newer theories suggest that the selection process itself may be important, especially in enzyme regulation and coordination. I do not wish to suggest that any of the above mechanisms is more or less likely. Until the above models are tested by comparing the molecular biology of metabolic enzyme gene expression in resistant and susceptible insects, all should be considered possible, and initial steps should be taken to experimentally test these alternatives.

In this chapter I present data that: 1) support the hypothesis developed in chapter 1 that there exists an active form and an enzymatically inactive form of GSH-trans-1 in Aedes aegypti larvae, 2) show that a new GSH-trans enzyme (GSH-trans-2) exists in Aedes aegypti larvae, and 3) show that insecticide resistant Aedes aegypti larvae express more GSH-trans-1 and GSH-trans-2 enzyme activity.

MATERIALS AND METHODS

Bioassays

Bioassays were performed on fourth instar (5 d old) larvae with technical permethrin, allethrin, deltamethrin, or p,p-DDT (99.9%). Six concentrations with two replicates of each concentration were used. Each bioassay was done randomly in 150 ml glass beakers containing 100 ml distilled water, 0.4 ml acetone carrier, and between 15 and 25 larvae. Twenty-four hr LC-50 values, and 95% fudicial limits were determined using probit analysis (Finney, 1952).

Strains

Two strains were used: the Rockefeller strain (Rock) is a susceptible strain (see Chapter 1) that has never been selected with an insecticide, and the Isla Verde strain (IV), which was originally resistant to DDT and dieldrin when collected. Both strains were from the Notre Dame Vector Biology Laboratory, and have been reared at M.S.U. since 1970. During this time the IV strain had lost resistance and was essentially identical to the Rock strain. Over a two year period the IV strain was selected with an approximate LC_{75} concentration of DDT, and, independently, an LC_{75} concentration of permethrin. The IV line selected with DDT was selected for nine

generations (IVF_{9-DDT}) while the IV line selected with permethrin was selected for 23 generations (IVF_{23}). Earlier generations of the permethrin selected line (IVF_5 and IVF_{13}) as well as the original two strains (Rock and IV) were also maintained. Buffers, enzyme assays, electrophoresis, enzyme kinetics, and radioimmunoassays were performed essentially as described in Chapter 1.

Anion exchange chromatography

During initial purification of GSH-trans-1 from IVF_{23}, percent recovery values were approximately half those normally obtained for the Rock strain. To determine if additional GSH-trans enzymes were present in the IVF_{23} strain, but were not being detected, a slightly different purification assay scheme was used. The IVF_{23} crude homogenate (in homogenization buffer) was loaded onto a 2 x 47 cm Sephadex-G-50-50 column equilibrated with ion exchange equilibration buffer. The column was eluted with ion exchange equilibration buffer. GSH-trans active fractions were pooled and loaded on to a 0.75 x 20 cm DEAE-Sepharose column also equilibrated with ion exchange equilibration buffer. The DEAE column was then eluted with a 0-600 mM NaCl gradient in ion exchange equilibration buffer. Enzyme activity was measured in 2 ml fractions off the column.

Partial purification of GSH-trans-2

GSH-trans-2 was partially purified using a combination of S-(2,4-dinitrophenyl)-GSH (S-DNP-GSH) affinity chromatography and DEAE-Sepharose chromatography. The S-DNP-GSH was synthesized as described

by Dykstra and Dauterman (1978). The ligand was attached to epoxy-activated Sepharose according to Mannervik and Guthenberg (1981). Three day old IVF_{23} larvae were homogenized in homogenization buffer and loaded onto an S-hexyl-GSH affinity column wich was connected to the S-DNP-GSH column. Both columns were preequilibrated with homogenization buffer. After running on the homogenate, the two columns were disconnected and the S-DNP-GSH column was eluted with 5 mM S-DNP-GSH in ion exchange equilibration buffer. Active fractions were pooled, loaded onto a 0.75 x 20 cm DEAE-Sepharose column and eluted with a 0-400 mM NaCl gradient. Active fractions were pooled, concentrated with a centricon-30 micro-concentrator and frozen at $-80^{o}C$.

Separation of GSH-trans-1 and GSH-trans-2 in crude homogenates

Anion exchange chromatography was used to separate GSH-trans-1 and GSH-trans-2 in crude homogenates so that the activity of each enzyme could be measured during development, and in the different strains. The method used to separate the enzymes was developed as follows: Five day old IVF_{23} larvae were homogenized in homogenization buffer and run through a Sephadex G-50-50 column equilibrated with ion exchange equilibration buffer. Active fractions were pooled and frozen in 10-2.5 ml aliquots at $-80^{o}C$. For each experiment one of the aliquots was thawed and diluted with 600 mM NaCl ion exchange equilibration buffer to give a final NaCl concentration of between 50 and 350 mM NaCl. Total GSH-trans activity (umol/min/ml) was then measured using 0.5 ml of this solution. The remainder of the solution (approximately 5 ml) was run through a 3 ml DEAE-Sepharose column

equilibrated with ion exchange equilibration buffer with the same NaCl concentration as that of the sample. After the material had run through the column, one ml of buffer was added to the top of the column, and the material eluted (eluate) was collected (1 ml). GSH-Trans enzyme activity (umol/min/ml) was then measured in the eluate and expressed as a percentage of the total GSH-trans enzyme activity present. This was repeated for 8 different NaCl concentrations. The results are expressed as percent enzyme activity in the eluate versus NaCl concentration.

RESULTS

Bioassays. There were no major differences in the bioassay results of the Rock and the parent IV strain using 4 different insecticides (Table 2). The two strains apparently are identical. Selection of the IV strain with permethrin led to a rapid development of resistance to DDT (Table 3). Seven-thousand ppb is beyond the level of water solubility for DDT. Permethrin selection also resulted in resistance to permethrin but not nearly as dramatic as that for DDT. Selection with DDT led to high DDT resistance and moderate permethrin resistance.

Physical and kinetic comparisons. Isoelectric point, molecular weight, enzyme kinetic constants, and antigenic specificity during development were compared in the Rock and IVF_{23} strains to determine if selection led to an altered GSH-trans-1 enzyme. GSH-trans-1 was purified as described in Chapter 1. There was no difference in the isoelectric point of GSH-trans-1 in the two strains (Figure 10). Western blotting shows that GSH-trans-1 from Rock and IVF_{23} have the

Table 2. Bioassay results of initial strains.

Parameter	Insecticide	Strain	
		Rock	Isla Verde
LC_{50} (ppb)	DDT	32	26
95% fud. lim.		25–40	13–46
Slope ± SE		2.5 ± .34	2.3 ± .46
LC_{50} (ppb)	Allethrin	88	106
95% fud. lim.		77–98	95–117
Slope ± SE		4.4 ± .67	4.9 ± .68
LC_{50} (ppb)	Permethrin	1.36	1.01
95% fud. lim.		1.2–1.6	0.5–1.3
Slope ± SE		5.7 ± 1.1	3.5 ± 1.1
LC_{50} (ppb)	Deltamethrin	0.09	0.12
95% fud. lim.		0.08–0.10	0.09–0.18
Slope ± SE		5.0 ± 0.72	4.3 ± 0.96

Table 3. LC_{50} values (ppb) for five strains of Aedes aegypti fourth instar larvae (95% fudicial limits).

Strain	Insecticide	
	DDT	Permethrin
Rock	32 (25-40)	1.4 (1.2-1.6)
IV	26 (13-46)	1.0 (0.5-1.3)
IVF_5 Permethrin selected	377 (89-1069)	4.2 (3.7-4.9)
IVF_{13} Permethrin selected	>7000	8.6 (7.5-10.1)
IVF_{23} Permethrin selected	>7000	14.2 (11.5-19.0)
IVF_{9-DDT} DDT selected	>7000	5.4 (4.9-5.9)

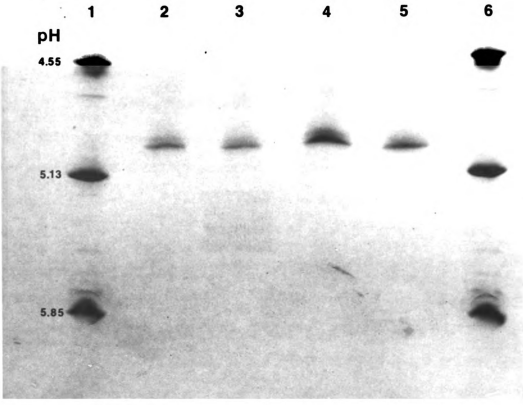

Figure 10. Narrow range (pH 4-6.5) analytical isoelectric focusing (5.5% gel) of GSH-trans-1 from fourth instar Rock and IVF_{23} strains of Aedes aegypti. pI standards (lane 1); 1.9 ug purified GSH-Trans-1 from IVF_{23} (lane 2); 1.6 ug purified Rock GSH-trans-1 (lane 3); 3.7 ug purified GSH-trans-1 from IVF_{23} (lane 4); 3.0 ug purified GSH-trans-1 from Rock (lane 5); pI standards (lane 6).

same molecular weight, and are both recognized by an antiserum developed against Rock GSH-trans-1. Like the Rock strain, IVF_{23} expresses GSH-trans-1 throughout larval development (Figure 11). Enzyme kinetic constants also were not significantly different between the two strains, $P \geq .20$ (IVF_{23} KmGSH = 0.095 $\pm$ .06, KmCDNB = 0.04 $\pm$ .02, Vmax = 254 $\pm$ 100).

Anion exchange chromatography. Crude homogenates of Rock and IVF_{23} 5 d old larvae were each run through a Sephadex G-50-50 column. The void volumes were loaded separately onto a DEAE-Sepharose column and the elution profiles compared (Figure 12). IVF_{23} has two distinct peaks of GSH-trans activity; GSH-trans-1 eluting at approximately 125 mM NaCl and a new enzyme (GSH-trans-2) eluting at approximately 300 mM NaCl. The Rock strain shows detectable, but very low amounts of GSH-Trans-2. When a Sephadex G-50-50 preparation of the IVF_{23} strain was adjusted to a given NaCl concentration, and run through a 3 ml DEAE-Sepharose column of the same NaCl concentration, the percent enzyme activity in the eluate shows a pattern indicating the presence of two distinct enzymes (Figure 13). Figure 13 is essentially the summation of the enzyme activity curve shown in Figure 12 with the inflection points of Figure 13 corresponding to the peaks in Figure 12. Notice however, that the NaCl concentrations required to elute activity do not closely correspond. Apparently it requires a higher salt concentration to elute proteins off a DEAE-column than it does to keep them from binding. The recovery of GSH-Trans enzyme activity is not significantly different from 100% at 300 mM NaCl or greater. This indicates that all GSH-trans activity in 5 d old IVF_{23} elutes off a DEAE-Sepharose anion exchange column between 0 and 400 mM NaCl. It is clear from Figure 13 that GSH-trans-1 can be separated from GSH-

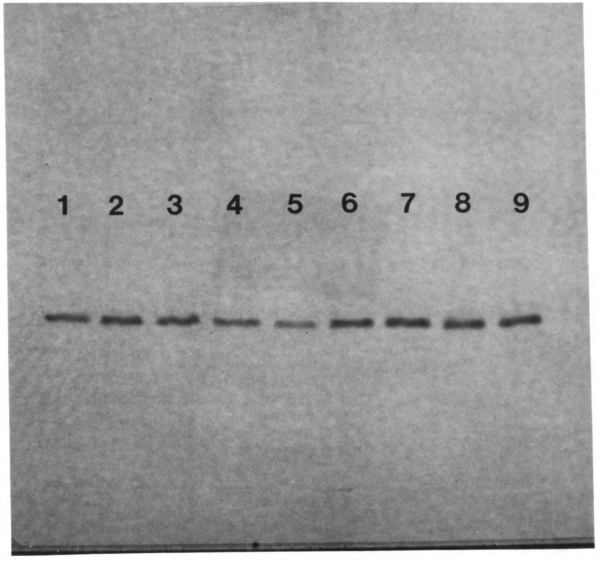

Figure 11. Anti-GSH-trans-1 serum immunoblot of total protein from IVF_{23} strain of Aedes aegypti during development. Proteins were run on SDS-Disc 11% resolving, 3.3% stacking gels then transferred to 0.2 um nitrocellulose membrane overnight. Antigens were detected as described in materials and methods. 25 ng GSH-Trans-1 standard (lane 1); Lanes 2-9 each contain 5.7 ug total protein. 1 hr old larvae (lane 2); 1 day old larvae (lane 3); 2 day old larvae (lane 4); 3 day old larvae (lane 5); 4 day old larvae (lane 6); 5 day old larvae (lane 7); 6 day old larvae (lane 8); 8 day old pupae (lane 9).

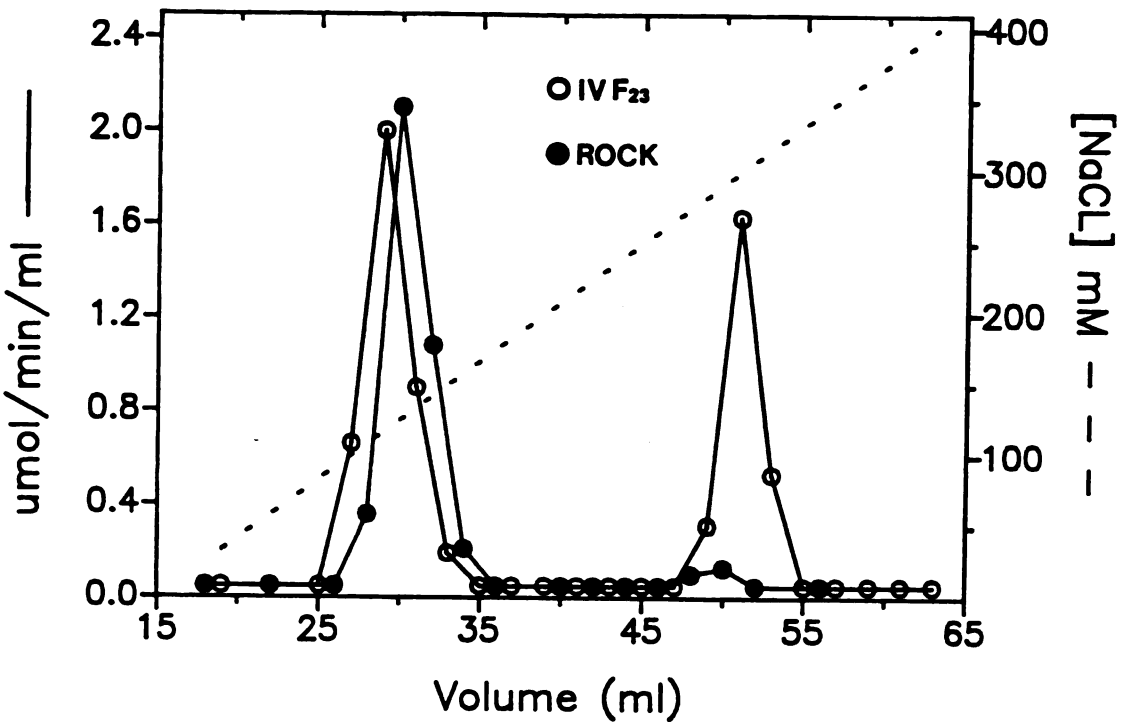

Figure 12. DEAE-Sepharose elution profile of GSH-trans enzyme activity. A crude homogenate of either the Rock or IVF_{23} strain was first fractionated on a Sephadex G-50 column. Active fractions were loaded onto a DEAE-Sepharose column and eluted with a linear NaCl gradient.

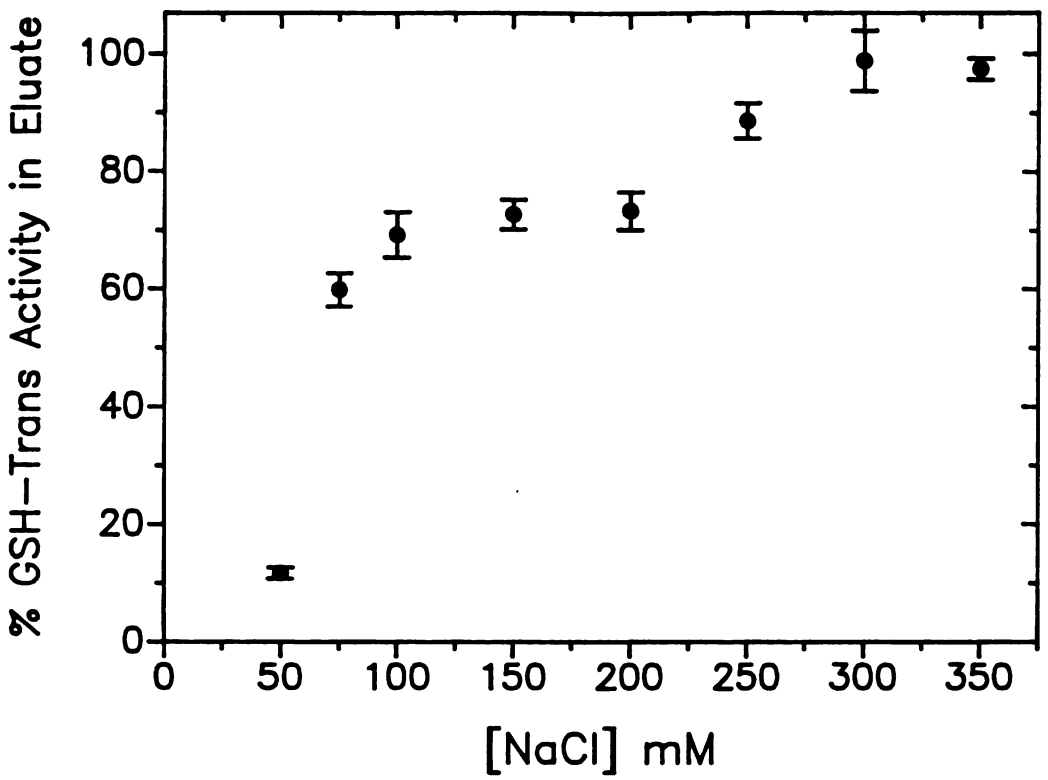

Figure 13. IVF_{23} GSH-trans crude homogenate enzyme activity (umol/min/ml) passing through a DEAE-Sepharose column expressed as a percent of the total GSH-trans enzyme activity (umol/min/ml) in the crude homogenate. The DEAE-Sepharose column was equilibrated with NaCl concentrations equal to that of the homogenate. Each point is the mean $\pm$ SD (n=3).

trans-2 by homogenizing the larvae in homogenization buffer containing 160 mM NaCl, and then running the homogenate through a small DEAE-Sepharose column equilibrated with the same buffer. The GSH-trans-1 activity will pass through the column, while the GSH-trans-2 activity will be bound. This forms the basis of the separation scheme used to measure GSH-trans-1 and, by difference, GSH-trans-2 activity in crude homogenates of Aedes aegypti.

Enzyme activity. During development of both the IVF_{23} and the IV strains of Aedes aegypti larvae there is a definite pattern of GSH-Trans-1 expression (Figure 14). A minimum for both strains occurs at day 2 with peaks at 1 h and 5-6 days. GSH-trans-2 on the other hand, shows a different pattern (Figure 15). For this enzyme the activity is maximal at day 2, then decreases gradually during larval development. GSH-trans-2 is undetectible at 192 hours in the IV strain. GSH-trans-1 enzyme activity in the IVF_{23} strain averages approximately 1.75 times the activity found in the IV strain during development. GSH-trans-2 activity is approximately 8 times higher in the IVF_{23} strain. Comparing GSH-trans-1 in 5 d old larvae of all six strains shows that insecticide-selected strains have statistically significant higher enzyme activity (Figure 16), although the differences are not large. GSH-trans-2 enzyme activity is also higher in all but one resistant strain (Figure 17). The interstrain differences for GSH-trans-2 are much larger, however. The Rock and IV strains are not significantly different for either enzyme comparison. GSH-trans-1, but not GSH-trans-2, is higher in the IVF_5 strain compared to the two susceptible strains.

The amount of GSH-trans-1 protein in the IVF_{23} and IV strains (using radioimmunoassay, Table 4) generally follows the pattern shown

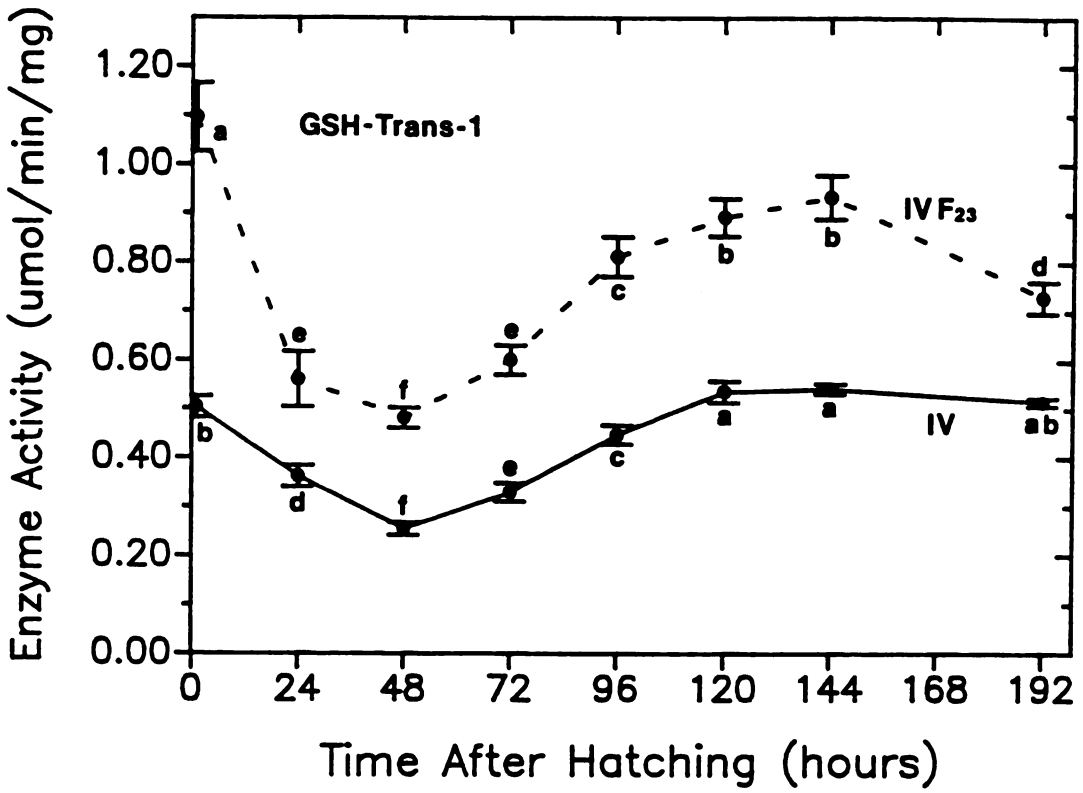

Figure 14. GSH-trans-1 enzyme activity during development of IVF_{23} and IV strain of Aedes aegypti. Data are means ± SD ($n \geq 3$). Within strain means with different letters are significantly different from each other ($P \leq .05$).

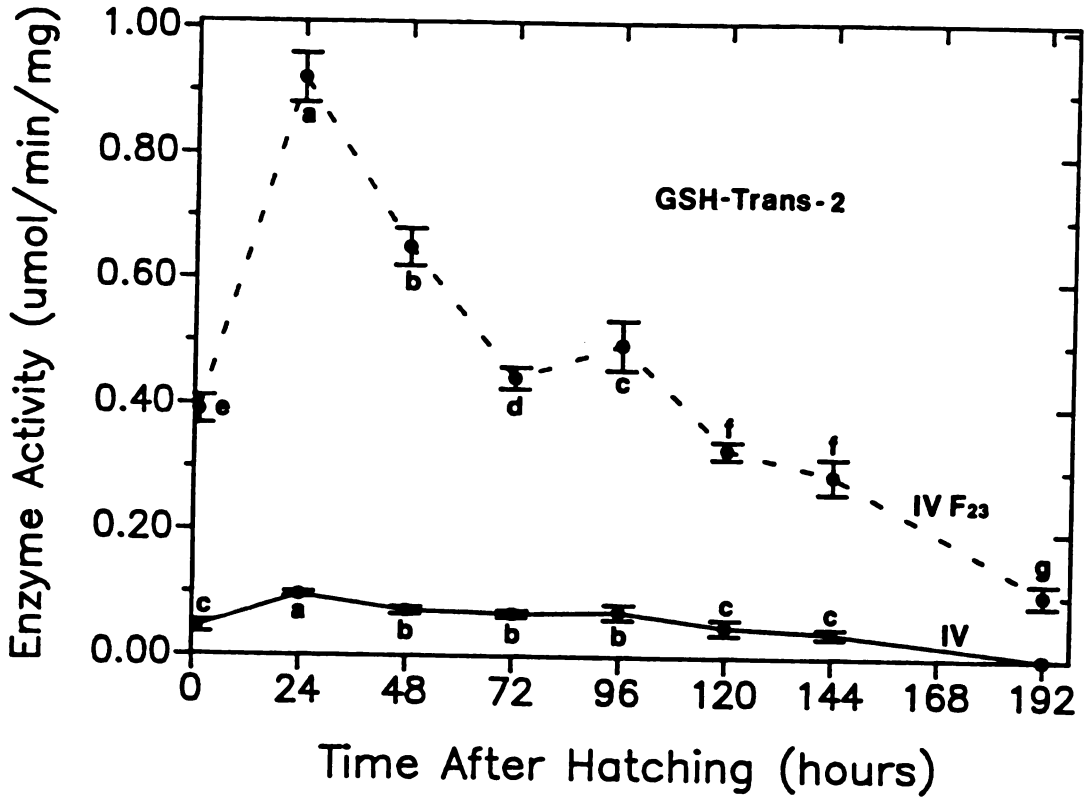

Figure 15. GSH-trans-2 enzyme activity during development of IVF_{23} and IV strain of Aedes aegypti. Data are means ± SD ($n \geq 3$). Within strain means with different letters are significantly different from each other ($P \leq .05$).

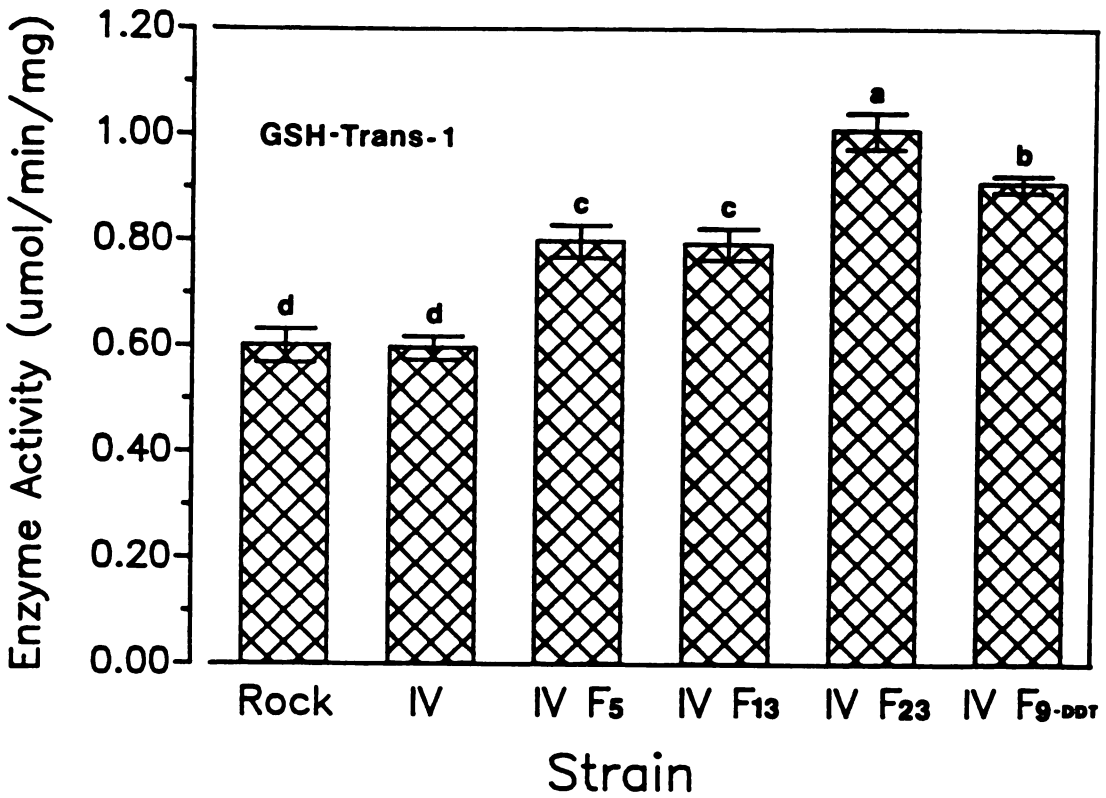

Figure 16. GSH-trans-1 enzyme activity in 6 strains of Aedes aegypti. Activity was measured in 5 d old larvae. Data are means $\pm$ SD ($n \geq 3$). Means with different letters are significantly different from each other ($P \leq .05$).

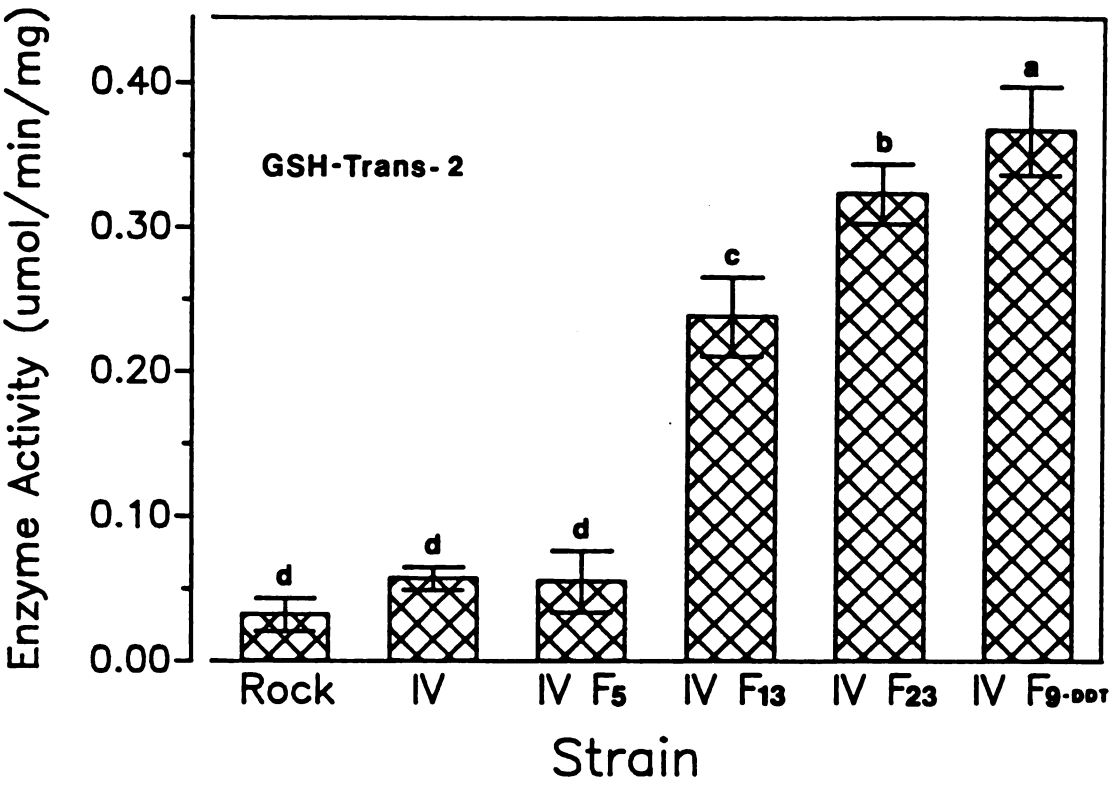

Figure 17. GSH-trans-2 enzyme activity in 6 strains of Aedes aegypti. Activity was measured in 5 d old larvae. Data are means ± SD ($n \geq 3$). Means with different letters are significantly different from each other ($P \leq .05$).

Table 4. Radioimmunoassay comparing amount of GSH-trans-1 during development of IV and IVF_{23} (n = 2 separate experiments, 4 replicates each).

Time After Hatching (hr)	Strain[a] (ug GSH-trans-1/mg protein ±SD) IV		Strain[a] (ug GSH-trans-1/mg protein ±SD) IVF_{23}		Comparison[b] Between Strains
1	3.6 ±0.6	b,c	3.2 ±0.2	c,d	ns
24	2.8 ±0.4	c,d	2.6 ±0.2	d	ns
48	3.4 ±0.5	b,c	3.6 ±0.1	b,c,d	ns
72	4.3 ±0.6	b	5.2 ±0.4	a,b,c	**
96	5.7 ±1.2	a	7.1 ±0.8	a	*
120	5.1 ±0.9	a	7.5 ±0.6	a	***
144	4.4 ±0.8	b	5.8 ±0.5	a,b	***
192	2.1 ±0.4	d	4.2 ±0.7	b,c,d	***

[a]Within strain means are significantly different ($P \leq 0.05$) from each other if they do not share a common letter. IV strain data analyzed using the Student-Newman-Kuels (Zar, 1974) test. IVF_{23} strain data analyzed using the nonparametric method of Dunn (1964). Bartlett's method was used to test for homogeneity of variences (Zar, 1974).

[b]Between strain means were tested using ANOVA (Zar, 1974), where alpha equals: 0.05 (*), 0.01 (**), or 0.001 (***). ns = not significantly different.

for GSH-trans-1 enzyme activity shown in Figure 14. There is a minimum in 1 d old larvae and a maximum in 4 and 5 day olds. After day 2 the IVF_{23} strain has significantly more GSH-trans-1 protein than does the IV strain. When GSH-trans-1 enzyme amount and GSH-trans-1 enzyme activity in IVF_{23} are graphically compared during development (Figure 18), the pattern found for the Rock strain (using total GSH-trans, see Chapter 1) is again evident: enzyme activity on day X seems correlated with enzyme amount on day X-1 (for X = 1-6 days), but not with enzyme amount on day X. The IV strain (data not shown) shows the same pattern. The relationship between GSH-trans-1 enzyme activity on day X, and GSH-trans-1 enzyme amount on day X-1, is highly significant for both the IV and IVF_{23} strains (Figure 19). Neither strain showed a significant relationship when the data (not shown) were compared on a same day basis.

GSH-trans-2 was partially purified from 3 d old IVF_{23} using a combination of S-DNP-GSH affinity chromatography and anion exchange chromatography (Figure 20). The protein has a molecular weight of $28x10^3$, slightly larger than GSH-trans-1.

DISCUSSION

The bioassay data show that the IV strain had lost all resistance to DDT before selection was begun. It also was not significantly different from the Rock strain using 3 other insecticide bioassays. After selection with either DDT or permethrin however, DDT resistance rapidly returned. The mechanism of DDT resistance in Aedes aegypti is unknown but could be due to any one or a combination of 3 mechanisms:

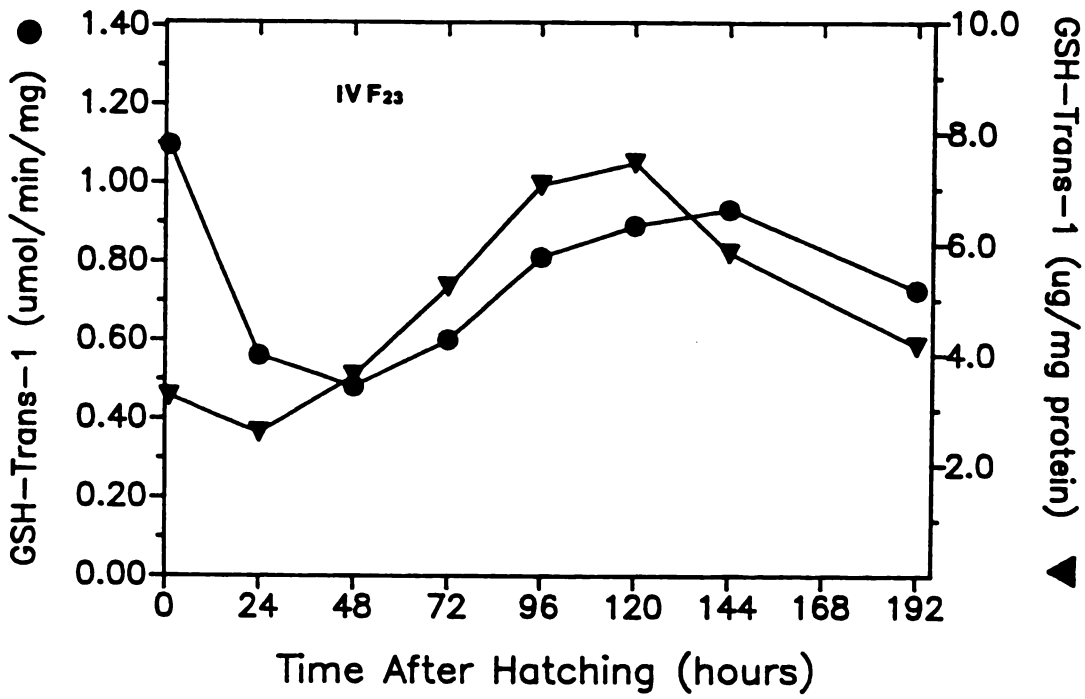

Figure 18. GSH-trans-1 enzyme activity and enzyme amount during development of IVF_{23} strain of Aedes aegypti. Data are means ($n \geq 3$).

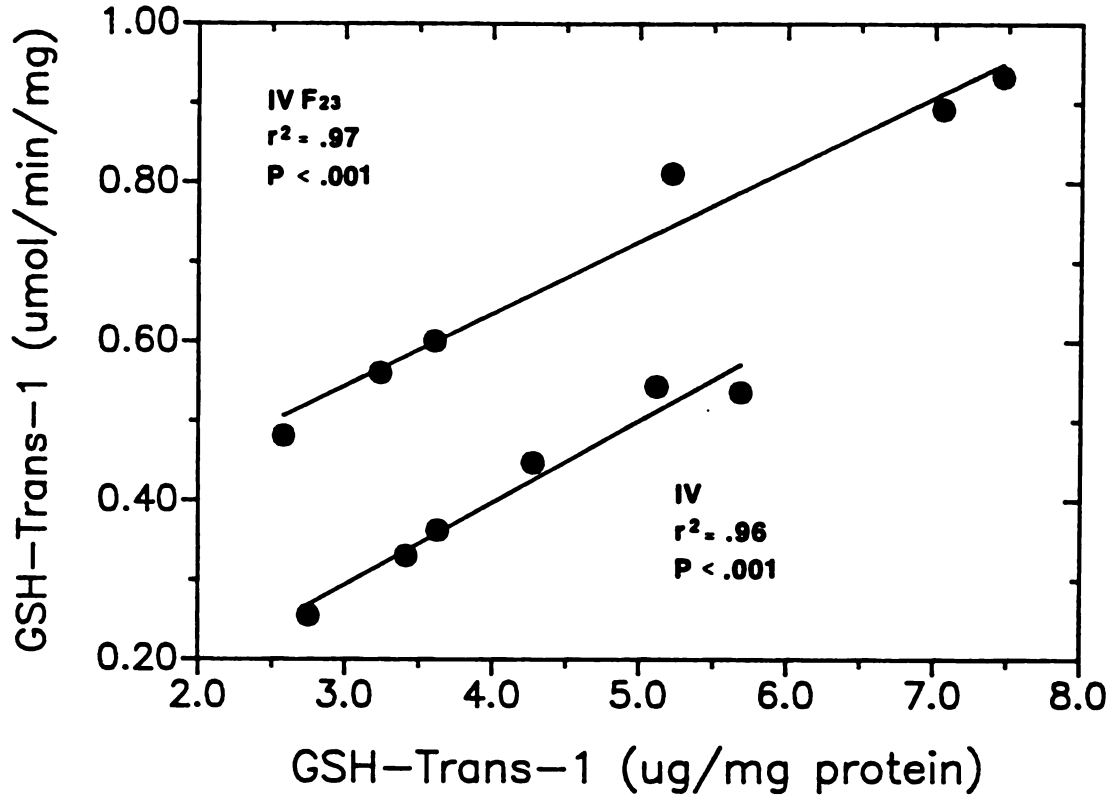

Figure 19. Relationship between GSH-trans-1 enzyme activity on day X, and GSH-trans-1 enzyme amount on day X-1 (where X = days 1-6) for IV and IVF_{23} strains of Aedes aegypti larvae.

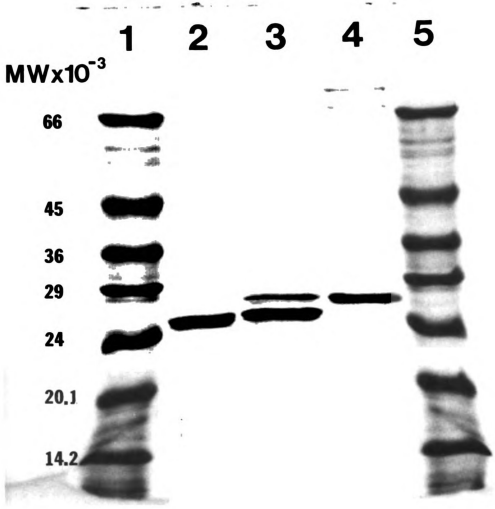

Figure 20. DISC-SDS-PAGE gel comparing pure GSH-trans-1 and partially purified GSH-trans-2 from IVF_{23} strain. Molecular weight standards (lanes 1 and 5); 10 ug GSH-trans-1 (lane 2); 10 ug GSH-trans-1 + 5 ug GSH-trans-2 prep. (lane 3); 10 ug GSH-Trans-2 prep. (lane 4).

1) increased metabolism to DDE, 2) reduced target site sensitivity, or 3) reduced internal content of DDT due to either reduced penetration or increased excretion (McDonald and Wood, 1979a, 1979b; Rathor and Wood, 1981). The relative imporance of these mechanisms apparently differs in adults and larvae. Increased metabolism is normally thought of as being due to DDTase (Kimura and Brown, 1964). There is some recent tentative evidence that DDTase is a GSH-trans isozyme (Clark and Shamaan, 1984; Clark et al., 1986), but this hypothesis will require further verification. Others have also found cross resistance between DDT and pyrethroids in Aedes aegypti (Chadwick et al., 1984; Prasittisuk and Busvine, 1977; Chadwick et al., 1977; Rongsriyam and Busvine, 1975). A genetic analysis by Malcolm (1983a,b) suggested that DDT and pyrethroid cross resistance mapped to allelic genes on chromosome III in adults. No mechanism for the cross resistance was suggested however. Permethrin and DDT are thought to have similar modes of action (Matsumura, 1975). If the mechanism of resistance to DDT and permethrin is due to a single allele, then an obvious candidate is the target site. The validity of conclusions concerning field development of resistance based on comparisons among inbred laboratory strains is, however, speculative at best (Uyenoyama, 1986). The options for response of a population are a function of the genetic diversity present.

From the electrophoretic, Western blotting, and kinetic assay results it seems reasonable to assume that GSH-trans-1 has not been altered by selection with permethrin. There are no significant differences in GSH-trans-1 from IVF_{23} or the Rock strains. If differences in enzyme characteristics existed between Rock and IVF_{23}, it would be logical to then compare IV with IVF_{23} and Rock. I will

assume, therefore, that the GSH-Trans-1 in IV is also the same.

The small amount of GSH-trans-2 in 5 d old Rock larvae (approximately 10% of the total enzyme activity) explains why it was not found during initial purification. GSH-trans-2 did not bind efficiently to the S-hexyl-GSH affinity column, and therefore, it was never seen after subsequent anion exchange chromatography. There have been other reports of isozymes of GSH-trans that do not bind to an S-hexyl-GSH affinity column (Mannervik, 1985). GSH-trans-1 was separated from GSH-trans-2 by running the IVF_{23} crude homogenate first through the S-hexyl-GSH affinity column, then through the S-DNP-GSH affinity column. This is the first report of using an S-DNP-GSH affinity column for purifying GSH-trans enzymes. This affinity matrix bound GSH-trans enzyme very effectively, but elution required S-DNP-GSH concentrations that were beyond its solubility at $4^{o}C$ in ion exchange equilibration buffer. It also did not seem to be as specific as the S-hexyl-GSH affinity coulmn as seen in the DISC-SDS gel of the GSH-trans-2 preparation. Further characterization of this ligand will be required before it should be considered for routine use.

Sephadex G-50-50 chromatography proved to be nearly as effective as cysteine in removing factors that denatured GSH-trans activity in crude homogenates. Without this step, anion exchange chromatography was very inefficient in eluting GSH-trans-1 and GSH-trans-2 activity peaks. This is probably because endogenous inhibitors and enzyme were both initially bound on the column within the first 0.5 cm, and this high concentration led to rapid inactivation of enzyme activity. Once these inhibitors were removed with the Sephadex G-50-50 column, this was no longer a problem. This procedure was later adopted for routine purification of GSH-trans-1 prior to S-hexyl-GSH affinity

chromatography. This prevented darkening of the affinity gel which occurred when crude homogenates were used.

GSH-trans-1 and GSH-trans-2 could be individually assayed during development of Aedes aegypti larvae by taking advantage of their differing isoelectric points. Figure 13 shows that any NaCl concentration between approximately 125 mM and 200 mM would give essentially the same results.

As found for the Rock strain, IVF_{23} and IV appear to regulate the expression of GSH-trans-1. Activity first decreases, then increases during development. The mechanism of regulation could be transcriptional, translational, or post-translational. The data do not exclude any of these possibilities. It is clear in both strains that GSH-Trans-2 is regulated differently than GSH-trans-1. Rather than decreasing after hatching, GSH-trans-2 increases. It then gradually decreases during the remainder of larval development.

The data presented strongly suggest that GSH-trans-1 and GSH-Trans-2 are isozymes rather than allozymes. They have a different molecular weight and isoelectric point, they do not share antigenic determinants, and their expression is not correlated. The only obvious characteristic they have in common, is the ability to conjugate CDNB with GSH. Once GSH-trans-2 is purified and an antibody is available, this hypothesis can be verified by testing individuals for the presence or absence of both enzymes using Western blotting.

GSH-trans-1 enzyme activity was compared in 5 day old larvae in six different strains. The differences between some of the strains are statistically significant, but not particularly impressive. These differences may or may not be biologically significant. GSH-trans-2,

on the other hand, is consideralby higher in 3 of the 4 selected strains. The mechanism(s) underlying these differences would be much easier to determine for GSH-trans-2. It remains to be seen whether the physical characteristics of GSH-trans-2 (kinetic constants, isoelectric point, etc.) are the same or different among the strains. It is worth pointing out that both GSH-trans-1 and GSH-trans-2 in the IVF_{23} strain are higher than those in the IV strain throughout larval development, not just in fourth instar larvae when the selection occurred.

The importance of the GSH-trans-1 enzyme analysis is to show the relationship between enzyme amount and enzyme activity during development. The results support the hypothesis proposed in Chapter 1. Instead of total GSH-trans enzyme activity however, the IV and IVF_{23} comparisons are between GSH-trans-1 enzyme activity and GSH-Trans-1 enzyme amount. This leads to a higher coefficient of determination and significance value. In comparing the IV and IVF_{23} regression lines, it is interesting to note that for a given amount of GSH-trans-1, there is more enzyme activity in the IVF_{23} strain. This will require direct verification by devising a technique for measuring both the inactive and active forms of GSH-trans-1 in the various strains. The data do suggest there is some variability in the mechanism that controls the relative distribution of GSH-trans-1 between active and inactive forms. This would explain, for example, why the amount of GSH-trans-1 in IV and IVF_{23} are not significantly different at 1, 24, and 48 hours old (Table 4), yet enzyme activity is higher during this time (Figure 14). It is also possible that another GSH-trans enzyme, not recognized by the anti-GSH-trans-1 serum, is present in early instars of IVF_{23}.

An obvious question raised by these results is why selection with permethrin leads to changes in GSH-trans enzyme activity. GSH-trans enzymes are not thought to be involved in the direct detoxification of these compounds (Matsumura, 1975). The most logical explanation is that there is some physical or functional linkage between permethrin resistance and GSH-trans expression. This linkage, whether physical, functional, or fortuitous, is a critical component in the resistance problem. Its solution will require a thorough understanding of the molecular genetics and biochemistry of the enzyme systems involved.

GENERAL DISCUSSION AND CONCLUSIONS

GSH-trans enzymes are involved in the detoxification of a variety of electrophilic compounds. They are important components of the metabolic defense mechanism of vertebrates (Mannervik, 1985) and invertebrates (Motoyama and Dauterman, 1980). They are studied in insects mainly because of their supposed involvement in insecticide resistance, especially organophosphate resistance (Motoyama and Dauterman, 1974). The emphasis of these insecticide resistance studies has been to determine if specific patterns of GSH-trans enzyme expression are associated with resistance. The results suggest that resistant and susceptible insects have basically the same GSH-trans isozymes. The differences lie only in the relative expression of the different forms (Clark and Dauterman, 1982). My results agree with this general conclusion, although not designed to test this hypothesis. Rather, what this research is directed towards is to understand the mechanisms that cause these different relative rates of GSH-trans expression.

To do this it is necessary to first understand the GSH-trans enzyme system. In this regard, insecticide resistant insects have not been used to their fullest potential. Resistant insects can be thought of as eukaryotic mutants, possessing altered traits that can be used as tools for studying the function of basic biological systems. I consider the permethrin and DDT resistant IV strains as mutants from which a wealth of information on the regulation and expression of GSH-trans enzymes can be obtained. From such studies not only will fundamental biochemical mechanisms be discovered, but the applied problem of insecticide resistance can be better understood.

My results lead to the following conclusions about GSH-trans enzymes in Aedes aegypti larvae: There are at least two distinct GSH-trans isozymes, GSH-trans-1 and GSH-trans-2. Both enzymes are expressed throughout larval development, but their patterns of expression are different. The two enzymes have different isoelectric points, subunit molecular weights, and antigenic determinants. The kinetic constants for GSH-trans-1 are the same in susceptible and insecticide resistant strains. GSH-trans-1 enzyme activity is slightly higher in permethrin and DDT selected strains. GSH-trans-1 amount is also slightly higher in insecticide-selected larvae older that 48 hours. GSH-trans-1 is catalytically active approximately 24 hours after it is synthesized. The mechanism of this activation is unknown. GSH-trans-2 enzyme activity is substantially higher in three out of four insecticide selected strains compared to the susceptible strains.

LITERATURE CITED

LITERATURE CITED

Askelof, P., C. Guthenberg, I. Jakobson, and B. Mannervik. 1975. Purification and characterization of two glutathione S-aryltransferase activities from rat liver. Biochem. J. 147:513-522.

Blake, M. S., K. H. Johnston, G. J. Russell-Jones, and E. C. Gotschlich. 1984. A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on western blots. Anal. Biochem. 136:175-179.

Bojan, F., A. R. Kinsella, and M. Fox. 1983. Effect of tumor promoter 12-O-tetradecanoylphorbol-13-acetate on recovery of methotrexate-, N-(phosphonacetyl)-L-aspartate-, and cadmium-resistant colony-forming mouse and hamster cells. Cancer Res. 43:5217-5221.

Boyland, E. and L. F. Chasseaud. 1969. The role of glutathione and glutathione S-transferases in mercapturic acid biosynthesis. Adv. Enzymol. 32:173-219.

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.

Brealey, C. J., P. L. Crampton, P. R. Chadwick, and F. E. Rickett. 1984. Resistance mechanisms to DDT and transpermethrin in Aedes aegypti. Pestic. Sci. 15:121-132.

Burnette, W. N. 1981. "Western blotting": Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. 112:195-203.

Campbell, A. 1983. Transposons and their evolutionary significance. In Evolution of genes and proteins. (Nei, M. and R. K. Koehn Eds.) Sinauer Associates Inc. Sunderland, Mass. pp. 258-279.

Chadwick, P. R., J. F. Invest, and M. J. Bowron. 1977. An example of cross-resistance to pyrethroids in DDT-resistant Aedes aegypti. Pestic. Sci. 8:618-624.

Chadwick, P. R., R. Slatter, and M. J. Bowron. 1984. Cross-resistance to pyrethroids and other insecticides in Aedes aegypti. Pest. Sci. 15:112-120.

Chang, C. K., A. G. Clark, A. Fieldes, and S. Pound. 1981. Some properties of a glutathione S-transferase from the larvae of Galleria mellonella. Insect Biochem. 11:179-186.

Christophers, S. R. 1960. Aedes aegypti (L.) the yellow fever mosquito, its life history, bionomics and structure. Cambridge University Press. London. 739 pp.

Clark, A. G. and W. C. Dauterman. 1982. The characterization by affinity chromatography of glutathione S-transferases from different strains of housefly. Pest. Biochem. and Physiol. 17:307-314.

Clark, A. G., G. L. Dick, S. M. Martindale, and J. N. Smith. 1985. Gluthathione S-transferases from the New Zealand grass grub, Costelytra zealandica, their isolation and characterization and the effect on their activity of endogenous factors. Insect Biochem. 15:35-44.

Clark, A. G. and N. A. Shamaan. 1984. Evidence that DDT-dehydrochlorinase from the housefly is a glutathione S-transferase. Pest. Biochem. Physiol. 22:249-261.

Clark, A. G., N. A. Shamaan, M. D. Sinclair, and W. C. Dauterman. 1986. Insecticide metabolism by multiple glutathione S-transferases in two strains of the housefly, Musca domestica(L). Pest. Biochem. Physiol. 25:169-175.

Cohen, J. D. and N. R. Eaton. 1979. Genetic analysis of multiple drug cross resistance in Saccharomyces cerevisiae: a nuclear mitochondrial gene interaction. Genetics 91:19-33.

Davidson, E. H. and R. J. Britten. 1979. Regulation of gene expression: possible role of repetitive sequences. Science 204: 1052-1059.

Davis, J. E. and R. Rownd. 1972. Transmissible multiple drug resistance in Enterobacteriaceae. Science 176:758-768.

Devonshire, A. 1977. The properties of a carboxylesterase from the peach potato aphid, Myzus persica (Sulz), and its role in conferring insecticide resistance. Biochem. J. 167:675-683.

Dunn, O. J. 1964. Multiple comparisons using rank sums. Technometrics 6:241-252.

Dykstra, W. G. and W. C. Dauterman. 1978. Excretion, distribution and metabolism of S-(2,4-dinitrophenyl) glutathione in the American cockroach. Insect Biochem. 8:263-265.

Finney, D. J., 1952. Probit Analysis. Second ed., University Press, Cambrige.

Georghiou, G. P. and T. Saito. eds. 1983. Pest resistance to pesticides. Plenum Press, New York. 809 pp.

Guengerich, F. P. and T. L. McDonald. 1984. Chemical mechanisms of catalysis by cytochromes P-450: A unified overview. Acc. Chem. Res. 17:9-16.

Habig, W. H., M. J. Pabst, and W. B Jakoby. 1974. Glutathione S-transferases, the first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249: 7130-7139.

Harwood, R. F. and M. T. James. 1979. Entomology in human and animal health. Macmillan Publishing Co., Inc. New York. 548 pp.

Hayashi, K., H. Fujiki, and T. Sugimura. 1983. Effects of tumor promoters on the frequency of metallothionein I gene amplification in cells exposed to cadmium. Cancer Res. 43:5433-5436.

Jakobson, I., M. Warholm, and B. Mannervik. 1979. Multiple inhibition of glutathione S-transferase a from rat liver by glutathione derivatives: kinetic analysis supporting a steady-state random sequential mechanism. Biochem. J. 177:861-868.

Kimura, T. and A. W. A. Brown. 1964. DDT-Dehydrochlorinase in Aedes aegypti. J. Econ. Entom. 57:710-716.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage t4. Nature 227:680-685.

Malcolm, C. A. 1983a. The genetic basis of pyrethroid and DDT resistance inter-relationships in Aedes aegypti I. Isolation of pyrethroid resistance factors. Genetica 60:213-219.

Malcolm, C. A. 1983b. The genetic basis of pyrethroid and DDT resistance inter-relationships in Aedes aegypti II. Allelism of R^{DDT2} and R^{py}. Genetica 60:221-229.

Mannervik, B. 1985. The isozymes of glutathione transferase. Adv. Enzymol. 57:357-417

Mannervik, B. and C. Guthenberg. 1981. Glutathione transferase (human placenta). Methods in Enzymol. 77:231-235.

Marx, J. L. 1984. Instability in plants and the ghost of Lamarck. Science 224: 1415.

Matsumura, F. 1975. Toxicology of Insecticides. Plenum Press, New York. 503 p.

McClintock, B. 1952. Chromosome organization and gene expression. Cold Spring Harbor Symp. Quant. Biol. 16:13-47.

McDonald, A. E. and R. J. Wood. 1979a. Mechanisms of DDT resistance in larvae of the mosquito Aedes aegypti L. Pestic. Sci. 10:375-382.

McDonald, A. E. and R. J. Wood. 1979b. Resistance in larvae of the mosquito Aedes aegypti L.; the effect of DDT selection. Pestic. Sci. 10:383-388.

McGaughey W. H. 1985. Insect resistance to the biological insecticide Bacillus thuringiensis. Science 229:193-195.

Melander, A. L. 1914. Can insects become resistant to sprays? J. Econ. Entomology. 7:167-173.

Metcalf, R. L. 1980. Changing fole of insecticides in crop protection. Ann. Rev. Entomol. 25:219-256.

Mills, B. J. and C. A. Lang. 1976. The biosynthesis of deoxyribonucleic acid during the life-span of the mosquito. Biochem. J. 154:481-490.

Motoyama, N. and W. C. Dauterman. 1974. The role of nonoxidative metabolism in organophosphorus resistance. J. Agric. Food Chem. 22:350-356.

Motoyama, N. and W. C. Dauterman. 1978. Molecular weight, subunits, and multiple forms of glutathione S-transferase from the housefly. Insect Biochem. 8:337-348.

Motoyama, N. and W. C. Dauterman. 1980. Glutathione S-transferases: Their role in the metabolism of organophosphorus insecticides. Rev. Biochem. Toxicol. 2:49-69.

Mouches, C., N. Pasteur, J. B. Berge, O. Hyrien, M. Raymond, B. R. de Saint Vincent, M. de Silvestri, and G. P. Georghiou. 1986. Amplification of an esterase gene is responsible for insecticide resistance in a California Culex mosquito. Science 233:778-780.

Nebert, D. W., H. J. Eisen, and O. Hankinson. 1984. The Ah receptor: binding specificity only for foreigh chemicals? Biochem. Pharmacol. 33: 917-924.

Nussinov, R. and G. G. Lennon. 1984. Structural features are as important as sequence homologies in Drosophila heat shock gene upstream regions. J. Mol. Evol. 20:106-110.

Oppenoorth, F. J., L. J. T. van der Pas, and N. W. H. Houx. 1979. Glutathione S-transferase and hydrolytic activity in a tetrachlorvinphos-resistant strain of housefly and their influence on resistance. Pest. Biochem. Physiol. 11:176-188.

Plapp, F. W. Jr. 1984. The genetic basis of insecticide resistance in the housefly: evidence that a single locus plays a major role in metabolic resistance to insecticides. Pest. Biochem. Physiol. 22:194-201.

Plapp, F. W. Jr. and T. C. Wang. 1983. Genetic origins of insecticide resistance. In Pest Resistance to Pesticides. (Georghiou G. P. and T. Saito, Eds.). Plenum Press, New York. pp. 47-70.

Prasittisuk, C. and J. R. Busvine. 1977. DDT-resistant mosquito strains with cross-resistance to pyrethroids. Pest. Sci. 8:527-533.

Ramage, P. I. and I. A. Nimmo. 1983. The purification of the hepatic glutathione S-transferases of rainbow trout by gluthione affinity chromatography alters their isoelectric behavior. Biochem. J. 211:523-526.

Rathor, H. R. and R. J. Wood. 1981. In-vivo and in-vitro studies on DDT uptake and metabolism in susceptible and resistant strains of the mosquito Aedes aegypti L. Pestic. Sci. 12:255-264.

Redie, G. P. 1982. Genetics. Macmillian Pub. Co. New York. 736 pp.

Segel, I. H. 1975. Enzyme kinetics. Behavior and analysis of rapid equilibrium and steady state enzyme systems. John Wiley and Sons. New York. 957 pp.

Shen, D.-W., A. Fojo, J. E. Chin, I. B. Roninson, N. Richert, I. Pastan, and M. M. Gottesman. 1986. Human multidrug-resistant cell lines: increased mdr1 expression can precede gene amplification. Science 232:643-645.

Simons, P. C. and D. L Vander Jagt. 1977. Purification of glutathione-S-transferases from human liver by glutathione-affinity chromatography. Anal. Biochem. 82:334-341.

Spector, T. 1978. Refinement of the coomassie blue method of protein quantitation. Anal. Biochem. 86:142-146.

Tomich, P. K., F. Y. An, and D. B. Clewell. 1980. Properties of erythromycin-inducible transposon Tn917 in Streptococcus faecalis. J. Bact. 141:1366-1374.

Usui, K. and Jun-ichi Fukami. 1977. Insect glutathione S-transferase: separation of transferases from fat bodies of American cockroaches active on organophosphorus triesters. Pest. Biochem. Physiol. 7:249-260.

Uyenoyama, M. K. 1986. Pleiotropy and the evolution of genetic systems conferring resistance to pesticides. In: Pesticide Resistance: Strategies and Tactics for Management. National Academy Press, Washington, D.C.

Varshavsky, A. 1981. On the possibility of metabolic control of replicon "misfiring": relationship to emergence of malignant phenotypes in mammalian cell lineages. Proc. Natl. Acad. Sci. USA 78:3673-3677.

Wagner, R. P., B. H. Judd, B. G. Sanders, and R. H. Richardson. 1980. Introduction to modern genetics. John Wiley and Sons, Inc. New York.

Weir, D. M. (ed.) 1978. Handbook of experimental immunology. Application of immunological methods. Vol. 3, third ed. Blackwell Scientific Publications, Oxford.

Wold, F. 1981. In vivo chemical modification of proteins (post-translational modification). Annu. Rev. Biochem. 50:783-814.

Yawetz, A. and B. Koren. 1984. Purification and properties of the Mediterranean fruit fly Ceratitis capitata W. glutathione S-transferase. Insect Biochem. 14:663-670.

Zar, H. J. 1974. Biostatistical Analysis. Prentice-Hall, London. 620 pp.

APPENDICES

APPENDIX 1

Record of Deposition of Voucher Specimens*

The specimens listed on the following sheet(s) have been deposited in the named museum(s) as samples of those species or other taxa which were used in this research. Voucher recognition labels bearing the Voucher No. have been attached or included in fluid-preserved specimens.

Voucher No.: 1986 - 3

Title of thesis or dissertation (or other research projects):

Glutathione S-transferase in Aedes aegypti

Museum(s) where deposited and abbreviations for table on following sheets:

Entomology Museum, Michigan State University (MSU)

Other Museums: none

Investigator's Name (s) (typed)

David F. Grant

Date 24 November 1986

*Reference: Yoshimoto, C. M. 1978. Voucher Specimens for Entomology in North America. Bull. Entomol. Soc. Amer. 24:141-42.

Deposit as follows:

Original: Include as Appendix 1 in ribbon copy of thesis or dissertation.

Copies: Included as Appendix 1 in copies of thesis or dissertation.
Museum(s) files.
Research project files.

This form is available from and the Voucher No. is assigned by the Curator, Michigan State University Entomology Museum.

APPENDIX 1.1

Voucher Specimen Data

Page ____ of ____ Pages

Species or other taxon	Label data for specimens collected or used and deposited	Number of: Eggs	Larvae	Nymphs	Pupae	Adults ♀	Adults ♂	Other	Museum where deposited
Aedes aegypti Rockefeller strain	Lab strain, Notre Dame Univer. Notre Dame, IN 1986 David F. Grant		5		5	5	5		
Aedes aegypti Isla Verde strain	Lab strain, Notre Dame Univer. Notre Dame, IN 1986 David F. Grant		5		5	5	5		

(Use additional sheets if necessary)

Investigator's Name(s) (typed)

David F. Grant

Date 24 November 1986

Voucher No. 1986 - 3

Received the above listed specimens for deposit in the Michigan State University Entomology Museum.

Curator

Date