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A Study of Esterases in Culex Quinquefasciatus.

Phyllis Jane Garnett

Louisiana State University and Agricultural & Mechanical College

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A STUDY OF ESTERASES IN CULEX QUINQUEFASCIATUS

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in

The Department of Zoology and Physiology

by Phyllis Jane Garnett B.A., Harding College, 1962 M.S.E., State College of Arkansas, 1967
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ABSTRACT

Wild populations of *Culex quinquefasciatus* were chosen for a genetic study of esterase enzyme systems by means of acrylamide gel electrophoresis. Electrophoretic analysis of single female mosquitoes yielded several polymorphic forms of esterases. Analysis of single individuals from the same population has shown a high degree of genetic heterogeneity in esterases of this population. Using the substrate alpha napthyl acetate 13 different forms of esterases have been observed in adult populations of *Culex*. Larvae and pupae yield additional forms and electrophoretic patterns of these enzymes.

Populations of *Culex* which are homozygous for certain esterase genes have been established by many generations of inbreeding and selection. A genetic study of adult polymorphism indicates that two zones of esterase activity are specified by a pair of autosomal co-dominant alleles: Esterase 4 and Esterase 5. Electrophoretic analysis shows that homozygotes produce only the slow-moving (Est. 4) or the fast-moving (Est. 5) band. Heterozygotes (E-4/E-5) show esterase activity at bands 4 and 5 with no interaction of the gene products.

Further studies of Est 4 and Est. 5 bands by use of various inhibitors show that these isoenzymes are aliesterases.
INTRODUCTION

As a means of describing enzymes, biochemists give the species and tissue of origin. Enzymes catalysing the same chemical reaction and having the same substrate specificity, but derived from different sources, may differ in other properties. However, due to possible denaturation of proteins or artifacts due to extraction and purification procedures, biochemists have been reluctant to conclude the existence of protein heterogeneity in the native state of the cell.

Acrylamide gel-Histochemistry

In recent years the development and improvement of protein separation by means of electrophoresis has led to the finding of numerous cases of protein polymorphism in a large variety of organisms. The development of zone electrophoresis first by use of starch gel (Smithies, 1955) and later by acrylamide gel (Hubby, 1963) coupled with the development of histochemical staining methods (Hunter, Markert, 1957) provides a simple method for the study of different molecular forms of enzymes.

Raymond (1964) describes the advantages of acrylamide gel over other methods of electrophoresis: "In my opinion it is the 'sieve' or molecular filtration effect, first described by O. Smithies that is the essential characteristic of gel electrophoresis. The combination of
electrophoretic effects and molecular filtration effects produces new and startling resolutions that cannot be obtained by other electrophoretic procedures." Raymond (1964) developed the vertical gel apparatus used in this study and described its advantages, "We prefer a flat slab in contrast to a cylindrical gel for several reasons. First, the flat slab provides maximum surface area for cooling the gel. Second, a large number of samples can be processed in a single gel, making the technique easier to carry out in the laboratory and facilitating the direct intercomparison of specimens processed under identical conditions ...."

The advantage of studying the multiplicity of enzymes by means of electrophoresis instead of in formalin-fixed tissue has been demonstrated by Eranko et al. (1964). They were able to show that only a portion of the total esterase activity of a tissue is revealed by histochemical staining of tissue sections; soluble esterases are not immobilized by the fixative and are not detected. Markert et al. (1959) demonstrated that the protein comprising about 50% of the esterase activity in mouse tissues is either insoluble or electrophoretically immobile in starch gel. Hunter et al. (1964) studying esterases in mouse liver demonstrated that the esterases seen in starch gel zymograms reflect essentially those present in the tissue. These authors, however, state, "we are under no illusions that we have proved that all tissue esterases are accurately reflected in zymograms;
however, we do feel it is useful to assume that most of the esterases are demonstrated.

Certainly, both approaches to the study of enzymes have their advantage. Each method may demonstrate only a fraction of the esterases present in a given tissue with the more soluble enzymes being demonstrated by electrophoresis and the more insoluble enzymes being seen in tissue sections.

**Definition and Occurrence of Isoenzymes**

The advent of zone electrophoresis techniques and the application of histochemical tests for specific enzymes have made it possible to demonstrate the existence of polymorphic forms for many enzymes. Markert and Moller (1959) introduced the term isoenzyme to describe the different molecular forms in which proteins may exist having the same enzymatic specificity. The International Commission on Enzymes has defined the term, isoenzymes, as multiple molecular forms of an enzyme within a single species. Caution must be taken, however, in referring to multiple molecular forms of esterases as isoenzymes because of their non-specific substrate specificity.

The number of enzymes that show multiple forms by electrophoretic separation and the many tissues that have been examined are too large to be covered in this dissertation. Shaw (1965) surveyed the electrophoretic variants of eleven enzymes of various diploid organisms that
have been studied. Agrell and Kjellberg (1965) stated that 30 enzymes
have been shown to have multiple molecular forms.

A number of investigations have demonstrated that not only are
the isoenzyme patterns species specific but also tissue specific. A
major work on the distribution of esterases in mouse tissue by Markert
and Hunter (1959) demonstrated that esterase activity is associated with
particulate fractions in the cell, especially with the microsomes. They
even suggested that esterase activity in the non-microsomal fractions
was due largely to microsomal contamination. Furthermore, they were
able to show that enzymatic activity increased in an orderly fashion in
developing embryonic tissue. "Electrophoretic separation of esterases
from a variety of tissues at successive stages in development clearly
demonstrates the addition of new esterases during development as well
as conspicuous changes in the amount of individual esterases" (Markert
and Hunter, 1959). However, they found that once maturity is reached
that no further significant changes in relative esterase activity occur
even in mice 16 months old.

By comparing electropherograms made from various tissues from
different species these authors were able to show that not only are there
characteristic differences in esterase activity between species but also
striking similarities. They were able to demonstrate "in mouse esterases
so far examined, those occupying the same electrophoretic position have
shown the same substrate and inhibition specificities. This observation
supports the thesis that esterases with the same mobility, from whatever tissue of the mouse, are indeed the same molecular species" (Markert and Hunter, 1959). In order to make comparisons of electrophoretic mobility and resolution of esterases from different tissues, however, it is imperative that the electrophoretic conditions be precisely controlled.

**Molecular Basis of Isoenzymes**

According to the present dogma the deoxyribonucleic acid (DNA) code is transcribed into a ribose nucleic acid (RNA) which specifies the sequence of amino acids in a polypeptide (Spiegelman, 1960). Once the primary polypeptide chain is formed it is subject to various modifications. Disulfide-bridging, hydrogen-bonding and various other types of intramolecular as well as intermolecular reactions between polypeptide chains result in folding of the molecule to produce the secondary, tertiary, and higher order three dimensional structure (Markert, 1968).

At one time it was suggested that polymorphic forms of proteins may be due to mistakes in the protein forming mechanism resulting in slight structural differences in the protein product (Colvin, et al. 1954). However, Markert (1968) views this as improbable and suggests an alternative explanation, i.e., once the primary structure is formed, variations in the tertiary structure result in molecules having different net charges and therefore different electrophoretic mobility. However,
studies by Cook and Koshland (1969) suggest that the folding process occurs either during translation by the ribosome or immediately thereafter. This would indicate that the final three-dimensional structure is formed very rapidly after the formation of the polypeptide chain. These authors suggest, therefore, that intramolecular folding is a very precise process which prevents incorrect aggregation of unfolded chains.

In vitro treatment of esterases of maize with various chemicals (Schwartz, 1967) produced changes in the electrophoretic mobility of certain esterases. Schwartz (1967) concluded that these electrophoretic differences in the isoenzymes result from variable masking of charges on the polypeptide produced by conformational changes of the protein. Moreover, he indicates that the isoenzymes have the same primary structure and that the conformation of the protein is specific or influenced by something other than the amino acid sequence of the polypeptide. Schwarts suggests that in addition to the existence of structural genes, conformational alleles may be responsible for isoenzyme variants.

Likewise, in vivo studies on the effect of insulin on mouse liver esterases indicate slight temporary changes in the mobility of several esterase forms (Ogita and Ogita, 1965). Thus, the polypeptide may conjugate with small molecules to produce slight modifications in electrophoretic mobility.

It is true that various factors influence the electrophoretic mobility of isoenzymes, however, to conclude that environmental factors which alter the structure are responsible for the formation of isoenzymes must
be viewed with considerable skepticism. Such conclusions are not in accord with evidence where adequate genetic and biochemical parameters are established.

Differences in electrophoretic mobility of the many genetic variants of human hemoglobin have been shown to be the result of substitutions of single amino acids in the polypeptide chains. "The altered electrophoretic mobility reflects a change in the net charge of the protein molecule which occurs when the amino acid carries a charge different from that of the one it replaces" (Ingram, 1957). Moreover, the polymorphism of human serum haptoglobin (Smithies et al., 1962) results from duplications or deletions of segments in the primary structure which alter the net charge of the molecule. These studies are representative of the extensive genetic evidence for the changes in electrophoretic mobility brought about by changes in the primary structure of the molecule.

Recent investigations of the genetics of enzyme systems in various organisms indicate that differences in mobility of isoenzymes are under genetic control rather than controlled by the cellular environment. Augustinsson (1961) suggests that certain amino-acid sequences are required for enzymatic activity, however, the sequence of the many other amino acids in the enzyme molecule may vary without altering the specific activity of the enzyme.
Physiological Significance of Isoenzymes

Markert (1968) emphasizes the evidence for tissue specific isoenzymes to indicate that isoenzymes do have biological significance and are not just biochemical curiosities. He has demonstrated that the five principal forms of the lactic dehydrogenase isoenzymes result from the tetrameric association of two distinct polypeptide subunits, A and B. The two genes responsible for the A and B subunits appear to be active in nearly all cells of the mammals and birds examined, but to widely varying degrees. Furthermore, the sensitivity of the two subunits to either lactate or pyruvate appears to determine the proportional occurrence of A and B subunits in various tissues. Enzymes in which subunits A are predominant are found in skeletal muscle where high concentrations of lactate are present, whereas, enzymes composed mainly of subunits B are found in heart and brain tissue, which contain small amounts of lactate.

Markert (1968) also indicates that not only are isoenzymes tissue specific, but that the function of the enzyme is related to the location of the enzyme within the cell. He suggests that the charge of the molecule which influences its electrophoretic mobility is related to the function of the isoenzyme within the cell. Charged molecules within a cell assume positions with reference to other charged molecules that are present. Agostoni et al. (1966) suggest that charge plays an important role in the preferential location of LDH-1 on mitochondria. Likewise,
the cytochemical investigation by J. M. Allen (1961) reveals that certain isoenzymes of LDH are distributed in certain locations within the cell and that they differ somewhat in enzymatic specificity. Moreover, Thorne (1960) after differential centrifugation of rat liver localized one of the two principal isoenzymes of malate dehydrogenase in the mitochondria and the other in the supernant cytoplasm. "This distribution of each isoenzyme to characteristic positions within the cell would enable them to perform distinctive metabolic roles even though their enzymatic properties were very similar" (Markert and Apella, 1961).

"The isozymic forms of an enzyme, though catalyzing the same reaction, do generally have distinct catalytic properties which presumably enables each of them to function in accord with physiological requirements either at different sites within a cell or in distinct metabolic sequences" (Stadtman, 1968). Just what each isoenzyme does that distinguishes it from the others in a series is still a mystery, but their abundance in nature would suggest that they offer selective advantages to the organism.

**Genetics of Isoenzymes**

A review of the literature reveals no adequate genetic classification for isoenzyme systems. The following general genetic classification of isoenzymes developed by this author surveys the reported genetic systems of isoenzymes.
1. **Co-dominant alleles.** Two esterase isoenzymes (or groups of isoenzymes) under the control of alleles at a single locus have been demonstrated in *Tetrahymena pyriformis* (Allen, 1961), *Drosophila melanogaster* (Wright, 1963) (Beckman and Johnson, 1964), *D. simulans* (Wright and MacIntyre, 1963), and *Aedes aegypti* (Trebatoski and Craig, 1969). The alleles are co-dominant, and no hybrid molecule is found in heterozygotes.

2. **Interacting co-dominant alleles.** Some molecular forms arise under the joint action of alleles. Heterozygotes show a hybrid enzyme in addition to both parental forms of the enzyme. Examples of such co-dominant alleles are found in esterases of maize (Schwartz, 1960) and in esterases of *Culex pipiens fatigans* (Simon, 1969). These authors suggest that the hybrid form results from the recombination of two dimer subunits.

3. **Co-dominant genes.** Finally, some molecular forms of enzymes result from the independent action of nonalleles such as in E-1 and E-2 esterases in *T. pyriformis* (Allen, 1961). The relationship of these enzymes would be classed as a family of enzymes rather than isoenzymes.

4. **Interacting co-dominant genes.** Some isoenzymes arise under the joint influence of nonalleles such as the hybrid lactate dehydrogenases (Apella and Markert, 1961). They demonstrated that two LDH dimers under the control of two genes combined randomly to produce five molecular forms of LDH. Analysis of the amino acid composition of the
two dimers and the five tetramers demonstrated that the dimers were encoded by separate genes. Cook and Koshland (1969) have shown that inter-subunit binding sites of various enzymes including LDH are highly specific. Furthermore, they demonstrated that incorrect association of subunits even in mixtures of enzymes from different species did not occur. Rather, the active binding sites are highly specific.

Genetic analysis of the isoenzyme systems in Drosophila gives conclusive evidence that the isoenzyme forms result from mutations in the genetic code. Alleles of isoenzymes in Drosophila which have been mapped include, alkaline phosphatase deficient (Aph^O) at a locus of 46.3 units on the third chromosome, leucine aminopeptidase D-Fast (Lap-D^F) and Lap-A^O. Lap-D^F is located on the third chromosome at 98.3 units, whereas Lap A^O is located near Lap-D^F, but no recombination has yet been observed (Lindsley and Grell, 1967).

Alleles of several esterase isoenzymes in Drosophila have been located on the third chromosome. Wright (1963) described Esterase 6-Fast (Est-6^F) at a locus of 36.8 units. The location of Esterase C-Fast was described by Beckman and Johnson (1964) at a locus of 49 units by means of 2/68 crossovers with Aph and 9/43 crossovers with Est-6.

**Esterase Isoenzymes**

Multiple molecular forms of esterases have been demonstrated in a variety of organisms (Shaw, 1965). However, due to the non-specific
substrate specificity of esterases, caution must be taken in describing
the family of esterases as isoenzymes. In this work esterases are
classified according to the method of Mounter and Whittaker (1953),
in which cholinesterases are inhibited by both eserine and paroxon,
aliesterases by paroxon but not by eserine and aromatic esterases by
neither. (Aliesterases hydrolyze esters of N-free alcohols and phenols,
and cholinesterases attack almost any choline ester including acetyl-
choline.) This system of classification is not definitive, but it allows
for some means of separating the esterases. Using this method of
classification the esterases are divided into a series of isoenzyme
systems. That is, any one strain of mosquitoes could have a cholin-
esterase isoenzyme series, an aliesterase isoenzyme series and an
aromatic isoenzyme series.

Several genetic studies have been made of esterase isoenzymes
in insects. Laufer (1961) includes a study of esterases in silkmoths in
a survey of enzyme systems. There are several reports on the genetics
of esterases in Drosophila (Beckman and Johnson, 1964; Wright, 1963;
Wright et al., 1963; Johnson et al., 1968). Reports on the genetics of
esterases in Musca include Velthius et al. (1963), Ogita (1962), and
Menzel et al. (1963).

Recently published reports of esterase isoenzymes in mosquitoes
appeared. Freyvogel et al. (1968) describe esterase zymograms for 14
species and strains of mosquitoes in the family Culicidae. They showed
that the esterase patterns were species and strain specific. Trebatoski and Hayes (1969) surveyed enzymes in 12 species of mosquitoes and found species-specific esterase patterns for all 12 species, as well as interspecific relationships. Simon (1969) reported an electrophoretic analysis of esterases in the development of *Culex pипiens fatigans* Wiedemann. This species was first described by Say in 1823 and named *Culex quinquefasciatus*. He described an increase in esterase activity in larval development, and females and males could be differentiated by four weak zones of activity. He presented genetic evidence showing that the polymorphism found is controlled by a pair of autosomal, interacting co-dominant alleles. Homozygotes were characterized by either a slow-moving or fast-moving band, whereas heterozygotes had both parental bands and an additional intermediate band. It was suggested that the esterase variants in this case exist as dimers.

**Classification of Mosquitoes**

Wild populations of *Culex quinquefasciatus* Say (Plate 1) were chosen for a genetic study of esterase isoenzymes. This species is commonly referred to as the southern house mosquito and is found in abundance in all southern states. For this study *Culex* larvae were collected in Baton Rouge, Louisiana and classified as *C. quinquefasciatus* according to Ross (1947).
Class: Insecta  
Order: Diptera  
Family: Culicidae  
Subfamily: Culicinae  
Genus: Culex  
Species: quinquefasciatus

In some literature, *Culex quinquefasciatus* has been recorded under the subspecific name, *Culex pipiens fatigans* and sometimes as *Culex fatigans*. *Culex quinquefasciatus* was first described in 1823, whereas *C. fatigans* was described in 1828.

**Purpose of the Study**

The purposes of this study were to investigate by means of gel electrophoresis the esterase systems of *Culex quinquefasciatus*; to determine the genetic nature of the esterase isoenzymes; and to characterize the esterases by their reaction with various substrates and inhibitors.
MATERIALS AND METHODS

Materials

The animal chosen for study was *Culex quinquefasciatus*. *Culex* larvae were collected in the vicinity of Baton Rouge, Louisiana, brought into the laboratory and classified as *Culex quinquefasciatus*. Some stocks of *Culex* referred to in this study were originally collected in Paris, France and Hamburg, Germany and were obtained from Dr. A. Ralph Barr, University of California, Los Angeles. First instar larvae hatched from single egg masses were reared to adulthood in quart milk bottles. The larval food was a 1:1 mixture of wheat germ and Kellogg’s Concentrate. After emergence the adults were removed and placed in cages made from round, half gallon ice cream cartons. A very fine net sleeve covered the cages to retain the adults and to prevent any contamination with mosquitoes that might have been in the room. The netting permitted convenient access for removal of eggs and adults from the cages. Cotton soaked in honey which served as food for the adults and a dish of water were placed in each cage.

After a minimum of five days to ensure insemination, a pigeon was placed in the cage as a blood meal source for the females. The pigeon was restrained in a small box with both feet and wings tied to prevent excessive movement. The immobilized bird with feathers removed from
the pectoral area was placed breast up in the cage and held overnight with the room darkened to facilitate blood feeding. All stocks were found to be anautogenous, that is, a blood meal was necessary to initiate ovarian development.

Egg rafts were deposited in the water dishes four to five days after a blood meal. Each egg raft was removed and placed in a separate shell vial for hatching. The eggs hatched 24-32 hours after deposition. A small amount of food was then added to each vial. One day old larvae were placed in quart milk bottles containing about one inch of water. Each day small amounts of food were added and the water level raised one to two inches until pupation occurred which was from 10-15 days.

To establish stocks homozygous for esterase genes, all the adults from single egg rafts were continuously inbred for at least fourteen generations. The genetic constitution of each stock was tested periodically to select for and to maintain genes homozygous for the esterases.

To ensure the collection of virgins, single pupa were isolated in small shell vials. After emergence the adults selected for genetic crosses were placed in pint containers for breeding. When working with stocks homozygous for esterase genes, several males from one stock and virgin females from another stock were placed together for breeding. However, when dealing with heterogenous stocks, single pair matings were made in pint containers. In both cases after egg laying the esterase patterns of the parents were determined or verified electrophoretically.
All mosquitoes were reared in a temperature, humidity and light controlled room. The temperature and relative humidity were maintained at 78-80°F and 70-80 per cent, respectively. By means of fluorescent lights the room was lighted from 7:00 a.m. until 10:00 p.m. each day; from 10:00 p.m. until 7:00 a.m. the room was in total darkness.

Methods

The vertical gel apparatus was from E. C. Company model No. 470. The procedure for assembly followed the steps outlined in the E. C. Manual (Technical Bulletin 128).

Buffer System

Several continuous as well as discontinuous buffer systems were tested to determine which gave maximum resolution of the esterase banding patterns. Among the discontinuous systems tested were:

1. Running buffer ----Tris/HCl (pH 8.9)
   Tank buffer ----Tris/Glycine (pH 8.3)

2. Running buffer ----Tris/Citric Acid (pH 8.6)
   Tank buffer ----Boric Acid/Sodium hydroxide (pH 8.6)

3. Running buffer ----Tris/Citric Acid (pH 8.6)
   Tank buffer ----Boric acid/Sodium hydroxide (pH 8.6)

In the third discontinuous system a prerun for one and one-half hours with the running buffer preceded the actual run with the tank buffer.

In resolving the esterase patterns the above buffer systems gave results inferior to the continuous buffer system selected for this study.
The continuous buffer system using Tris/Borate (pH 8.9) gave the most distinct and well stained esterase patterns. Several modifications in this system were tested, and it was found that the addition of Na$_2$EDTA (Ethylene-dinitrilo tetraacetic acid disodium salt) and calcium lactate increased the staining intensity and the resolution of the esterases (McCombs, 1969). The buffer used in both the gel and the tank was as follows:

- Tris (Hydroxymethyl aminomethane) ---- 0.1 M
- Na$_2$EDTA ------ 1.5 mM
- Calcium lactate ------- 0.384 gm/l
- Boric acid ------ Tritate to pH 8.9

The pH was found to be very critical for the resolution of the esterases. It was found that temperature, electrolysis during the run, and storage altered the pH. Therefore, all buffers were made and reused several times after the pH was stabilized.

**Preparation of Acrylamide Gel**

The most satisfactory acrylamide gel concentration was found to be 5% (Cyanogum 41, Fisher Company). Lower concentrations gave weak gels which were difficult to handle and higher concentrations hindered the migration of the esterases through the gel. Eleven grams of Cyanogum 41 were added to 220 milliliters of tris-borate buffer and the solution filtered. In order to give uniform gelation, 0.22 ml. of TMED (N,N,N',N'-tetramethylenediamine) and 0.2 grams of ammonium persulfate were added, and the mixture was immediately poured into the
electrophoretic apparatus. The slot former was washed with distilled water and immediately placed in position. Gelation occurred within 10-30 minutes with the cooling system in operation.

After gelation the apparatus was placed in a vertical position, the tank buffer was added, and the excess gel and slot former were removed. A prerun of 1-1/2 hours preceded each run with the conditions of the prerun and the run being the same, i.e., the buffer was circulated between the upper and lower tanks, and 0°C water was circulated through the cooling chamber. The power supply delivered approximately 200 volts at 100 milliamps during both the prerun and the actual run. "During the prerun there is established a steady state flux of buffer ions through the gel that remains substantially unaltered throughout the duration of the experiment" (Raymond, 1964).

Preparation of Samples

A miniature mortar and pestle were made by using a 4 mm. diameter glass rod to grind 1/2 milliliter centrifuge tubes at the tip. Fresh, single, whole mosquitoes were homogenized in the 1/2 ml. grinding tubes at 0°C in 40 microliters of phosphate buffer (pH 6.5) containing 10% sucrose and bromo phenol blue as an indicator. The sucrose was used to give density to the samples, thus allowing them to "settle" into the slots of the gel. Variations in the preparation of samples were tested, but none of the following procedures improved the resolution of the esterase patterns.
1. After grinding samples were frozen for several minutes.

2. Single mosquitoes were ground with a small pestle on filter paper using phenylthiourea.

4. Samples were subjected to sonification.

5. Samples were ground in distilled water.

6. Samples were ground in tris buffer (pH 8.9).

7. Samples were ground in amounts of phosphate buffer varying from 20 to 50 microliters.

Single live mosquitoes were used in most cases, but no differences were noted in the electropherogram patterns of mosquitoes frozen for 1-4 days. Therefore, some of the mosquitoes were frozen for a maximum of four days before being subjected to electrophoretic analysis.

In the experimental design mosquitoes were of varying ages before their esterase patterns were determined electrophoretically. Therefore, it was necessary to determine the effect of age on esterase patterns. Mosquitoes varying in age from 1 to 21 days from stocks homozygous for the esterase genes showed no significant variations in the electropherograms.

After grinding the samples were centrifuged at 27,000 × g at 0°C for 10 minutes to remove cell debris and layer the lipid component.

Twenty microliters of the clear supernatant were carefully applied with a micropipette into the gel slots. Care was taken to minimize the amount of cellular debris and lipids included in the sample. Sample
amounts of 10, 20, and 30 microliters were applied to each slot. It was determined that 20 microliters of the extract gave the best resolution.

Protein concentrations of the sample were estimated spectrophotometrically by the method of Layne (1955). Using this estimate approximately 0.026 mg. of protein were applied to each slot.

**Electrophoretic Run**

In the equilibrated gel the samples were allowed to "settle" for about 10 minutes before initiating the electrophoretic run. It was necessary to keep the voltage constant during the run to prevent distortion of the banding patterns. The voltage applied to the gel was approximately 250 volts which resulted in a slightly fluctuating amperage which did not exceed 100 ma. The duration of the run depended on the gel concentration and the voltage applied. Usually the samples migrated 10 cm. toward the anode within 3-3 1/2 hours. When the samples had migrated 10 cm. the current was turned off and the gel removed.

**Staining Procedure**

It was essential that the hydrogen ion concentration of the gel be lowered to approximately pH 6.5 before coupling of the dye and substrate could occur. A number of techniques were employed to lower the pH of the gel to ensure maximum staining. Phosphate buffer (pH 6.5),
monobasic sodium phosphate (pH around 4.5) and boric acid were each used to rinse the gel in order to lower the pH. A five minute rinse in boric acid at 4°C gave maximum staining. When the gels remained in boric acid longer than five minutes the intensity of the staining was decreased.

The gels were removed from the boric acid and rinsed in phosphate buffer (pH 6.5) to remove any excess boric acid. The gels were then preincubated in 200 ml. of phosphate buffer (pH 6.5) containing 4 ml. of a 1% alpha naphthyl acetate in acetone. The preincubation took place at 4°C for 45 minutes. If the preincubation step was eliminated the staining was very light, whereas if the preincubation period was longer than 45 minutes the bands appeared diffuse after staining.

The gels were incubated in 200 ml. of phosphate buffer (pH 6.5), 4 ml. of 1% alpha naphthyl acetate in acetone, and 0.1 gram of Fast Blue BB (4'-amino-2',5' diethoxybenzamidine, diazonium salt) for two hours at 25°C. Gels incubated at 37°C and at 5°C gave less satisfactory results.

In the study of over 200 runs on acrylamide gels techniques were developed which yielded reproducible patterns on the electropherogram for each adult stock and for each stage of development investigated.

Characterization of Esterases

Other substrates tested for the characterization of the esterases were alpha naphthyl butyrate, beta naphthyl acetate, alpha naphthyl
caprylate, alpha naphthyl myristate, alpha naphthyl propionate and alpha naphthyl laurate. These were quantitatively substituted for alpha naphthyl acetate in the staining solution. All substrates were dissolved in acetone (1% solution) and then rapidly blown from a 2 ml. volumetric pipette into the buffer solution. Since a greater number of esterase-active bands were demonstrated by alpha naphthyl acetate, it was the substrate used in all of the experiments reported in this study unless otherwise stated. In comparing the reaction of the various substrates with the esterases, a number of mosquitoes were homogenized, and 20 microliter samples were applied to each slot. After the run the gel was cut into five equal sections and each section treated with one of the test substrates. Alpha naphthyl acetate served as a control.

Additional stains tested included Fast Blue RR (4-benzoylamino-2,5-dimethyloxyaniline diazonium salt), Fast Red TRN (p-chloro-o-toluiline, diazonium salt), and Fast Blue B (O-Dianisidine, tetrazotized) (Dajac Company). Fast Blue BB gave the most distinct, intense staining patterns and was used throughout this study.

Inhibitors utilized to characterize the esterases were paroxon (diethyl p-nitrophenyl phosphate) $10^{-5}$M (American Cyanamid Company), eserine $10^{-5}$M (Sigma Company) and dylox (dimethyl 2,2,2-trichloro-1-hydroxyethyl phosphonate) $10^{-5}$M (Chemagro Chemical Corporation). After electrophoresis the gel was cut into four sections each having identical samples. Each of the three sections were preincubated in
one of three test inhibitor-buffer solution for 30 minutes at 4°C. The fourth section, a control, was preincubated for a similar time in buffer. The gels were then transferred to the substrate-inhibitor solution for 30 minutes of preincubation and finally transferred to the substrate-dye solution for staining (Salkeld, 1965).
RESULTS

Esterases in Developing Stages of Culex

Esterase electropherograms were examined at several stages in the development of *Culex* (Fig. 1). Slow-moving zones are not detectable in first instar larvae; however, the fast-moving zones of activity correspond to those found in the 4th instar larvae. Many of the fast moving zones seen in larvae are not detectable in the pupae and adult stages (Plate 2).

The highest level of esterase activity appears in the 4th instar larvae. Bands 3, 5, and 6 observed in the adult stage correspond to zones of esterase activity in individual 4th instar larvae, however, there are a number of zones of esterase activity in individual 4th instar larvae that are absent from individual adults. Due to the wealth of esterase activity in the larval stages, direct comparisons for most of the bands are difficult.

There are no additional bands present in the pupae that are not seen in the adult stage. However, in pupae, band 9 is a major zone of activity, whereas, in the adult this area shows only slight activity.

Esterases in Adult Culex

Adult *Culex quinquefasciatus* from the isolated population used in this study demonstrate a total of 13 zones of esterase activity. In
Fig. 1. Schematic drawing showing the esterase isoenzyme pattern in stages of development in *Culex quinquefasciatus*. 
*o* = origin; solid lines = intense staining; dashed lines = moderate staining; dotted lines = faintly staining.
any given individual, however, only certain of the zones are detectable. Bands 1, 2 and 6 are observed in all adult females examined, whereas bands 1 and 2 are not found in adult males. A weak zone of esterase activity, band 2.5, which is found in adult males, is not detected in adult females. In the local population, with the exception of the zones of high esterase activity which were analyzed genetically, all other zones of activity were not readily amenable to genetic analysis with the techniques employed.

Bands 1, 2, 4, 5, and 6 stain very darkly compared to the other bands. These five bands when present were consistently easily resolved, whereas the lighter bands were often difficult to detect (Plate 3; Plate 4). Bands 4 and 5 were chosen for a genetic analysis due to their reproducibility and their pattern of occurrence in single individuals in the unselected population. Stocks homozygous for the alleles responsible for the occurrence of bands 4 and 5 were established by many generations of inbreeding and selection. Hereafter, these alleles will be designated E-4 and E-5. Allele E-5 was found in most of the individuals in the unselected population, whereas E-4 occurred only rarely. Twenty generations of inbreeding and selection were required to isolate and obtain a healthy stock of individuals homozygous for allele E-4, whereas isolation of progeny of single pair matings readily yielded strains homozygous for E-5.
Initial crosses were made between males homozygous for allele E-5 with individual virgin females taken from the unselected population. After eggs were produced from this cross, each female was examined electrophoretically to determine her genotype and subsequently the genotype of the progeny. These initial crosses provided information facilitating the genetic analysis of this esterase isoenzyme system. More precise analysis was possible after the establishment of strains homozygous for allele E-4.

Experimental Crosses

Three patterns of occurrence of esterase bands 4 and 5 are observed on the electropherogram (Plate 5). These are (1) band 4 alone; (2) band 5 alone; (3) bands 4 and 5 together. Genetic crosses and reciprocal crosses were made using all possible combinations of these patterns:

1. Band 4 x Band 4
2. Band 5 x Band 5
3. Band 4 x Band 5
4. Band 4 x Bands 4 and 5
5. Band 5 x Bands 4 and 5
6. Bands 4 and 5 x Bands 4 and 5

The results of these crosses are shown in Tables I-IX. The following discussion concerns only bands 4 and 5.

Individuals homozygous for allele E-4 produced progeny showing only band 4 on the electropherogram (Table I, Plate 6). Individuals homozygous for allele E-5 produced progeny showing only band 5
Reciprocal crosses between individuals homozygous for allele E-4 and E-5, produced heterozygous offspring showing esterase activity at both band 4 and 5 (Plate 8; Tables III, IV). A backcross between F₁ males heterozygous for alleles E-4/E-5 and females homozygous for allele E-5 (Table V) produced offspring homozygous for E-5 and others that are heterozygous for E-4/E-5.

Results of reciprocal crosses between individuals homozygous for allele E-5 and heterozygous for alleles E-4 and E-5 are presented in Tables VI and VII. Table VI shows essentially a 1:1 ratio for the occurrence of allele E-5 alone to the occurrence of heterozygotes expressing activity at zones 4 and 5 (Plate 9).

Results of crosses between individuals homozygous for E-4 and individuals heterozygous for alleles E-4 and E-5 are shown in Table VIII. These individuals show esterase activity either at zone 4 alone or at zones 4 and 5 in approximately a 1:1 ratio (Plate 10).

The figures in Tables V-VIII showed no significant difference for a 1:1 ratio of homozygous individuals to heterozygous individuals. However, there seems to be a trend in favoring the heterozygous individual in Tables V and VII. This trend might be explained by frequency-dependent selection described in *Drosophila* by Kojima and Yarbrough (1967).

Crosses between known heterozygotes for alleles E-4 and E-5 (Table IX) produce progeny that upon electrophoretic analysis yield the three basic patterns, band 4 alone, band 5 alone, and both bands 4 and 5 (Plate 11).
In heterozygotes bands 4 and 5 show equal staining intensity, however, in an individual homozygous for either E-4 or E-5 the single band has much darker staining intensity than in the heterozygous condition (Plate 5).

These results are consistent with the hypothesis that the enzymes responsible for the occurrence of esterases activity in zones 4 and 5 are inherited as autosomal co-dominant alleles.

Characterization of Esterases

Use of Various Substrates

After separation by electrophoresis, the maximum number of zones of esterase activity were found when alpha napthyl acetate was used as the substrate. Other substrates were utilized in order to further characterize these esterases (Wright, 1963). Three of the substrates investigated showed no detectable reaction with any of the esterases present. These were alpha napthyl laurate, alpha napthyl caprylate, and alpha napthyl myristate. Inconsistent results were obtained using alpha napthyl propionate and alpha napthyl butyrate (Fig. 2) due primarily to the precipitation of the substrate in the reaction mixture.

The use of beta napthyl acetate resulted in light purple staining of the esterase bands which contrasted to a brown color produced by the other substrates utilized. This substrate reacted well with the enzymes in zones 1, 2, 4, 5, and 6, but did not react with the faster migrating esterases.
Fig. 2. Schematic representation of the reaction of esterases with various substrates on the electrophoretic variations in homogenates from female *Culex quinquefasciatus*. o = origin; solid lines = intense staining; dashed lines = moderate staining; dotted lines = faintly staining.
The staining properties of alpha napthyl propionate were essentially the same as alpha napthyl acetate except that reproducibility was impaired by the precipitation of this substrate.

The staining properties of alpha napthyl butyrate were essentially the same as alpha napthyl acetate except that it did not stain the esterases in zones 1 and 2.

Esterases 4 and 5 were more intensely stained by alpha napthyl propionate and alpha napthyl butyrate than by alpha napthyl acetate and were only lightly stained by beta napthyl acetate.

Use of Inhibitors

The esterases were further characterized by the method suggested by Mounter and Whittaker (1953). According to their classification cholinesterases are inhibited by both eserine and paroxon, aliesterases by paroxon but not by eserine, and aromatic esters by neither. Dylox (an organophosphate) was also used in this study. These compounds are commonly used as insecticides.

Figures 3 and 4 show the effects on the electropherogram following inhibition of the esterases by eserine, paroxon and dylox on adult females and males. The concentration of the inhibitors were: paroxon $10^{-5}M$, eserine $10^{-5}M$, dylox $10^{-5}M$. In adult females (4-6 days old) paroxon inhibited all zones of activity with the exception of band 6, whereas eserine inhibited only bands 1 and 2. The following tabulation gives the classification of the seven major esterase bands in females.
Fig. 3. Schematic drawing showing the effect of various inhibitors on the esterase variations in female Culex quinquefasciatus. 

$\circ$ = origin; solid lines = intense staining; dashed lines = moderate staining; dotted lines = faintly staining.
Fig. 4. Schematic drawing showing the effect of various inhibitors on the esterase variations in male *Culex quinquefasciatus*.  
-o = origin; solid lines = intense staining; dashed lines = moderate staining; dotted lines = faintly staining.
Band 1. Inhibited by Paroxon and Eserine --Cholinesterase
Band 2. Inhibited by Paroxon and Eserine --Cholinesterase
Band 3. Inhibited by Paroxon --Aliesterase
Band 4. Inhibited by Paroxon --Aliesterase
Band 5. Inhibited by Paroxon --Aliesterase
Band 6. Inhibited by Neither --Aromatic esterase
Area 7. Inhibited by Paroxon --Aliesterase

The area including bands 7, 8 and 9 are not clearly resolved in the inhibitor studies, and will be referred to as area 7.

Dylox totally inhibited bands 1, 2, 5, and area 7 in adult females and partially inhibited band 5. The significance of this inhibition has not yet been determined.

The effect of the inhibitors on adult males (4-6 days old) is the same as on the adult females with the exception of band 2.5 which is found exclusively in males. This band was not inhibited by any of the inhibitors and is therefore classified as an aromatic ester.

Bands 4 and 5 that were studied genetically are inhibited by paroxon, but not by eserine and therefore, are aliesterases.
**DISCUSSION**

*Culex quinquefasciatus* exhibits esterase polymorphism throughout the life cycle. Distinct differences in esterase patterns were observed in each stage of development that was examined. Several of the larval esterases were not present in the adult stage; the converse was also true. The absence of certain esterase bands may be due to low concentrations and/or low activity during a particular developmental stage. No changes were detectable in esterase patterns of specific stocks after adulthood was reached.

The esterase patterns of adult females and males were characteristically distinguished by three bands of esterase activity. Bands 1 and 2 were found only in adult females; these bands were absent in adult males. A weak zone of activity, band 2.5, was present only in males. The inhibitor studies show that bands 1 and 2 are cholinesterases, whereas band 2.5 is an aromatic esterase.

Simon (1969) reported four weak zones of activity in *Culex pipiens fatigans* which differentiated between the esterase patterns of adult females and males. Two were found only in females and the other two only in males. Simon suggested that due to the low activity of the four zones the differentiation between the sexes may be of a quantitative order.

In the present study on a population of *Culex quinquefasciatus*, bands 1 and 2 stain very darkly and were never detected in males.
Due to similar electrophoretic mobility of band 2 in females and band 2.5 in males, it may be suggested that the intense staining of band 2 might interfere with the detection of the lightly stained band 2.5 in females. However, when bands 1 and 2 are completely inhibited by paroxon and eserine, band 2.5 is still detectable in males but not in females. In this study, precise differences distinguish the esterase patterns between males and females.

Velthuis and Van Asperen (1963), in their work on the housefly, suggested that when an esterase is present in all individuals of a population that the enzyme is essential for biological existence of the species. This assumption has been found to be erroneous. In this study all adults examined from the local populations had band 6, which was the only detectable aromatic esterase in females. However, when the esterase patterns of other strains of C. quinquefasciatus were examined band 6 was found to be variable. Adults of the Hamburg strain that were examined had no esterase activity at band 6. Furthermore, some individuals from the Paris strain had band 6 whereas, others did not. These results indicate the fallacy of assuming an essential biological function for a given form of an enzyme because of its uniform presence in a population. Much more logically one would assume that the genes responsible for a given enzyme are homozygous in that particular population.

In the same way, care must be taken in taxonomic classification of species by isoenzyme patterns. Trebatoski et al. (1969) utilized
isoenzyme patterns to study the taxonomic relationships that exist between 12 species of mosquitoes. He reported species-specific esterase and alkaline phosphatase isoenzyme patterns for all 12 species. In this study a wide range of genetic variability was found within single populations and to an even greater extent between populations.

It appears that the isoenzyme patterns reflect the genetic heterogeneity of a given population at a limited number of loci and that taxonomic relationships gleaned from these enzyme patterns must be viewed with the same skepticism as other taxonomic schemes which rely on variable alleles at a limited number of loci.

Evidence that esterase isoenzymes are the products of co-dominant alleles have been reported in *Tetrahymena* (Allen, 1961), *Drosophila* (Wright, 1963; Beckman and Johnson, 1964; Wright and MacIntyre, 1963), in *Aedes aegypti* (Trebatoski and Craig, 1969), and in *Culex quinquefasciatus* (Simon, 1969). Conclusive evidence that isoenzymes are the products of different alleles has been shown by the chromosome mapping of esterase genes in *Drosophila* (Wright, 1963; Beckman and Johnson, 1964).

In the present study, homozygous individuals produce either electrophoretically slow-moving (Est. 4) or fast-moving enzymes (Est. 5); heterozygotes show both parental forms of the enzyme. There is no apparent interaction between the products of the two alleles.
Simon (1969) in his study of *Culex pipiens fatigans* reports the occurrence of certain esterase isoenzymes in 4th instar larvae resulting from the interaction of autosomal co-dominant alleles. Homozygotes produce either the slow-moving (Est. 4) band or a fast-moving (Est. 5) band. Individuals heterozygous for the two alleles show, in addition to the parental components, a hybrid enzyme band with electrophoretic mobility intermediate between the slow- and fast-moving forms.

"The formation of the hybrid enzyme in the heterozygote suggests that the Est. E variants in *Culex* exist as a dimer and that the hybrid enzyme may be the result of random combination of two different Est. E monomeric subunits" (Simon, 1969). The formation of the hybrid enzyme indicates that at least two polypeptide chains are involved in this esterase system.

Electrophoresis has been shown to be a very useful tool in resolving isoenzyme systems. Recent work (Cook and Koshland, 1969) has demonstrated that the component polypeptides of a number of enzymes can be separated and recombined *in vitro*. This work coupled with the technique of acrylamide gel electrophoresis should make possible the resolution of the genetic basis of the isoenzyme systems at the molecular level. No longer will the study of isoenzymes be a biochemical curiosity but a fruitful area of genetic research.

The electrophoretic procedure of Simon (1969) differs somewhat from that used in the present study. Therefore, direct comparisons
between the enzyme system that he reports to that in the present study is difficult, even though superficially the enzyme studied seems to be similar in electrophoretic mobility. The evidence clearly shows that the two similar esterase isoenzyme systems studied in the two populations of *Culex quinquefasciatus* are genetically different.

Simon (1969) demonstrated the formation *in vitro* of a hybrid between two electrophoretically distinct esterases. He macerated larvae known to be homozygous for each allele in 1 M NaCl and allowed the mixture to incubate at 25°C for 30 minutes. Electrophoretic analysis showed a pattern electrophoretically indistinguishable from the pattern obtained from known genetic heterozygotes. In these cases three Est. E bands appeared on the electropherogram.

Utilizing this technique of *in vitro* hybridization in the present study esterases 4 and 5 could not be induced to form a hybrid enzyme. The genetic evidence presented is consistent with the results of these *in vitro* studies.

From a review of the literature it is apparent that the genetic classification of isoenzyme systems is inadequate. Ogita (1968) proposed a genetic nomenclature for the classification of isoenzyme systems. His classification appears to be based primarily on biochemical evidence where genetic parameters were not precisely controlled. The classification is neither clear nor precise and was considered inadequate for this study.
In experiments where adequate control of the genetic parameters have been exercised, the following classification of genes responsible for isoenzyme formation appears to be quite adequate:

1. Co-dominant alleles
2. Interacting co-dominant alleles
3. Co-dominant genes
4. Interacting co-dominant genes

The esterase isoenzymes 4 and 5, subject of analysis in the present work, are classified as the product of autosomal co-dominant alleles since there is no detectable interaction of the gene products. The gene responsible for the esterase isoenzymes in C. pipiens fatigans reported by Simon (1969) was classified as a gene with interacting co-dominant alleles.

According to the definition of isoenzymes given by the International Commission on Enzymes, Culex quinquefasciatus has a number of isoenzymes which can be clearly resolved by acrylamide gel electrophoresis. Because of the differential specificity of the isoenzymes to various substrates, esterase isoenzymes should be further classified. Such a classification of isoenzyme systems has been proposed by Holmes and Masters (1967). They suggest that the esterases be further classified as cholinesterase isoenzymes, aliesterase isoenzymes and aromatic esterase isoenzymes. In the present work with C. quinquefasciatus, all three esterase isoenzyme systems were found.
SUMMARY

By use of electrophoretic and histochemical techniques, it is now possible to detect gene-induced enzyme modifications in which the enzyme activity is not altered. Prior to the development of these techniques, mutations which altered the three dimensional configuration of enzymes were generally not detected. Electrophoretic analysis has demonstrated gene controlled polymorphic protein systems in all organisms thus far examined.

*Culex quinquefasciatus* exhibits a series of polymorphic esterases throughout the life cycle. Distinct differences in the esterase patterns were observed in each stage of development studied. Esterase activity appears to increase during larval development and to reach a peak in the 4th instar. Many of the larval esterases stain very lightly and it is difficult to equate these enzymes to the esterases found in adults.

A total of 12 sites of esterase activity were found in adult females. However, all 12 esterases have not been detected in any one individual.

A classification of the genes controlling isoenzyme systems is proposed. The relationship of bands 4 and 5 demonstrated in the crosses indicate that they are esterases produced by autosomal co-dominant alleles at the same locus. From the genetic crosses it was shown that the alleles may occur in either a homozygous (E-4), (E-5) or a heterozygous (E-4/E-5) state. Upon electrophoretic analysis homozygotes
produce only the slow-moving (Est. 4) or the fast-moving (Est. 5) band. Heterozygotes (E-4/E-5) show esterase activity at bands 4 and 5 with no interaction of the gene products. Reciprocal crosses gave no indication that these genes are sex-linked.

By the use of inhibitors (insecticides) the esterases were further subdivided. The esterases were classified as: bands 1 and 2 as cholinesterase; bands 3, 4, 5, and area 7 as aliesterases, and; band 2.5 and 6 as aromatic esterases.

The genetic relationship between esterases 4 and 5 is further substantiated by substrate and inhibitor studies. Esterases 4 and 5 were shown to be isoenzymes under the control of a single autosomal gene with co-dominant alleles.
Table I. The occurrence of bands 4 in progeny of crosses between individual females and males from different stocks homo- 

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<th>Number of individual male progeny with band</th>
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Table II. The occurrence of band 5 in progeny of crosses between individual females and males from different stocks homo- genous for E-5.

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<td>17</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>80</td>
</tr>
</tbody>
</table>
Table III. The occurrence of bands 4 and 5 in progeny of crosses between individual females homozygous for E-4 and individual males homozygous for E-5.

<table>
<thead>
<tr>
<th>Cross</th>
<th>Number of individual female progeny with band</th>
<th>Number of individual male progeny with band</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>5</td>
<td>54</td>
</tr>
</tbody>
</table>
Table IV. The occurrence of bands 4 and 5 in progeny of crosses between individual females homozygous for E-5 and individual males homozygous for E-4.

<table>
<thead>
<tr>
<th>Cross</th>
<th>Number of individual female progeny with band</th>
<th>Number of individual male progeny with band</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>
Table V. The occurrence of bands 4 and 5 in progeny of a backcross between F₁ males heterozygous for E-4/E-5 recorded in Table IV and females homozygous for allele E-5.

<table>
<thead>
<tr>
<th>Cross</th>
<th>Number of individual female progeny with band</th>
<th>Number of individual male progeny with band</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>2</td>
<td>5</td>
</tr>
</tbody>
</table>
Table VI. The occurrence of bands 4 and 5 in progeny of crosses between individual females heterozygous for E-4/E-5 and individual males homozygous for E-5.

<table>
<thead>
<tr>
<th>Cross</th>
<th>Number of individual female progeny with band</th>
<th>Number of individual male progeny with band</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>4</td>
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<tr>
<td>3</td>
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<tr>
<td>7</td>
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<td>3</td>
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<tr>
<td>8</td>
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<td>7</td>
</tr>
<tr>
<td>9</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>9</td>
<td>42</td>
</tr>
</tbody>
</table>
Table VII. The occurrence of bands 4 and 5 in progeny of crosses between individual females homozygous for E-5 and individual males heterozygous for E-4/E-5.

<table>
<thead>
<tr>
<th>Cross</th>
<th>Number of individual female progeny with band</th>
<th>Number of individual male progeny with band</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>5</td>
<td>18</td>
</tr>
</tbody>
</table>
Table VIII. The occurrence of bands 4 and 5 in progeny of crosses between individual females homozygous for E-4 and individual males heterozygous for E-4/E-5.

<table>
<thead>
<tr>
<th>Cross</th>
<th>Number of individual female progeny with band</th>
<th>Number of individual male progeny with band</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4, 5</td>
<td>4, 5</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>7</td>
<td>5</td>
</tr>
</tbody>
</table>
Table IX. The occurrence of bands 4 and 5 in progeny of crosses between individual females and males from different stocks heterozygous for E-4/E-5.

<table>
<thead>
<tr>
<th>Cross</th>
<th>Number of individual female progeny with band</th>
<th>Number of individual male progeny with band</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
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<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>
Plate 1. Adult male and female *Culex quinquefasciatus*
female

male
Plate 2. Electropherogram of developmental stages of *C. quinquefasciatus*.

1-7 Adult female
8-9 Pupa
10-12 4th instar larvae
13-14 3rd instar larvae
15 1st instar larvae
Plate 3. Electropherogram of adult female *C. quinquefasciatus* intensely stained to resolve the enzymes of high electrophoretic mobility.

Plate 4. Electropherogram of adult female *C. quinquefasciatus* stained to resolve the enzymes of high electrophoretic mobility.
Plate 5. Electropherogram showing the three patterns of occurrence of bands 4 and 5.

1. Individual with band 4
2. Individual with band 5
3. Individual with band 4 and 5
4. Band 4
5. Band 5
Plate 6. Electropherogram showing the pattern of occurrence of band 4 in progeny of parents homozygous for allele E-4.

1. Individual adult male
2. Individual adult female

Plate 7. Electropherogram showing the pattern of occurrence of band 5 in progeny of parents homozygous for allele E-5.

1. Individual adult female
2. Individual adult male
Plate 8. Electropherogram showing the pattern of occurrence of bands 4 and 5 in progeny of parents homozygous for allele E-4 and E-5, respectively.
Plate 9. Electropherogram showing the pattern of occurrence of bands 4 and 5 in progeny of a cross between individuals homozygous for allele E-5 and heterozygous for allele E-4/E-5.

Plate 10. Electropherogram showing the pattern of occurrence of bands 4 and 5 in progeny of a cross between individuals homozygous for allele E-4 and heterozygous for allele E-4/E-5.
Plate 11. Electropherogram showing the pattern of occurrence of bands 4 and 5 in progeny of a cross between individuals heterozygous for alleles E-4/E-5.
BIBLIOGRAPHY


Phyllis Jane Garnett was born on February 28, 1942 in Conway, Arkansas. She was graduated from Ozark High School, Ozark, Arkansas in 1959. She attended Lubbock Christian College and was graduated cum laude from Harding College, Searcy, Arkansas in 1962. While at Harding she was elected to Alpha Chi.

From September, 1962 until May, 1964 she taught biology and applied science at Monterey High School in Lubbock, Texas.

In January, 1965 she entered graduate school at State College of Arkansas in Conway, Arkansas and received a M.S.E. in May, 1967. The fall of 1967 she entered into a Ph.D. program in the Zoology Department at L.S.U. In February, 1968 she was named the recipient of a two year predoctoral research grant from National Institute of Health. While at L.S.U. she was elected to Phi Kappa Phi.

In 1960 she married Don Garnett and is now the mother of two children, Kerry and Karen.

Phillis is presently a candidate for a Doctor of Philosophy degree in the Department of Zoology and Physiology at Louisiana State University.
EXAMINATION AND THESIS REPORT

Candidate: Phyllis Jane Garnett

Major Field: Vertebrate Zoology

Title of Thesis: A Study of Esterases in Culex quinquefasciatus

Approved:

[Signatures]

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

Date of Examination:

20 July 1970