A GENETIC STUDY OF AN ESTERASE IN *CULEX PIPIENS QUINQUEFASCIATUS* [1]

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ABSTRACT. Wild populations of *Culex pipiens quinquefasciatus* Wiedemann were chosen for a genetic study of esterase enzyme systems by means of acrylamide gel electrophoresis. Analysis of single individuals from the same population showed a high degree of genetic heterogeneity in esterases of this population. Using the substrate alpha naphthyl acetate, 13 different forms of esterases were observed in adult populations.

Strains of these mosquitoes 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 inhibitors show that these isoenzymes are aliesterases.

Multiple molecular forms of esterases have been demonstrated in a variety of organisms (Shaw, 1965). 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; 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. Studies of esterase isoenzymes in *Aedes aegypti* L. are reported by Townson (1969) and Trebatoski and Craig (1969). 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 pipiens quinquefasciatus* Wiedemann. He presented genetic evidence 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.

MATERIALS AND METHODS. *Culex pipiens quinquefasciatus* larvae were collected in the vicinity of Baton Rouge, Louisiana. 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 cage. Cotton soaked in honey served as food for the adults.

Larvae and adults were reared at 78° F. and 80 percent RH in a room that was lighted from 7:00 a.m. until 10:00 p.m.

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each day. Pigeons were placed in the cages at night to provide a blood meal for the females.

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 pupae were isolated in small shell vials. After emergence the adults selected for genetic crosses were placed in pint containers for breeding.

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). A tris-borate buffer (pH 8.9) was used in both the gel and in the buffer tank. The buffer contained 0.1 M Tris, 1.5 mM Na_2EDTA, 0.384 gm/1. calcium lactate titrated to pH 8.9 with boric acid. The gel was 5 percent cyanogum-41. Gelation occurred within 30 minutes with the addition of 0.001 percent TMED and 0.001 percent ammonium persulfate. A prerun of 1½ hrs. preceded each run with conditions of the run and prerun being the same.

A miniature mortar and pestle were made by using a 4 mm diameter glass rod to grind ½ milliliter centrifuge tubes at the tip. Fresh, single, whole mosquitoes were homogenized in the ½ ml grinding tubes at 0 C in 40 microliters of 0.1 M phosphate buffer (pH 6.5) containing 10 percent sucrose and bromophenol blue as an indicator. The samples were centrifuged at 27,000 x g at 0° C for 10 minutes. 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. Single live mosquitoes were used in most cases; however, no differences were noted in the electrophoretic patterns between live mosquitoes and those frozen for 1–4 days from stocks homozygous for the esterase genes.

The voltage applied to the gel was approximately 250 volts which resulted in a slightly fluctuating amperage which did not exceed 100 ma. Usually the samples migrated 10 cm toward the anode within 3½ hrs.

To lower the pH of the gel to ca 6.5 the gel was rinsed in 0.5M boric acid at 4 C for 5 min. before a 45 min. preincubation at 4 C in 0.1M phosphate buffer (pH 6.5) containing 4 ml of 1 percent alpha naphthyl acetate (Dajac Company) in acetone. Esterases were detected by incubation of the gels in 200 ml of phosphate buffer (pH 6.5) containing 4 ml of 1 percent alpha naphthyl acetate in acetone and 0.1 gram of Fast Blue BB (4′–amino–2′,5′ diethoxybenzamilide, diazonium salt) for two hours at 25 C.

Inhibitors utilized to characterize the esterases were paroxon (diethyl p-nitrophenyl phosphate) 10^{-5}M (American Cyanamid Company and eserine 10^{-5}M (Sigma Company). After electrophoresis the gel was cut into four sections each having identical samples. Each of the three sections was preincubated in one of the test inhibitor-buffer solutions for 30 min. 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. Adult *Culex pipiens quinquefasciatus* from the isolated population used in this study demonstrated a total of 13 zones of esterase activity (Fig. 1). In any given individual, however, only certain of the zones were detectable. Bands 1, 2 and 6 were observed in all adult females examined, where bands 1 and 2 were not found in adult males. A weak zone of esterase activity, band 2.5, which was found in adult males was 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 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.

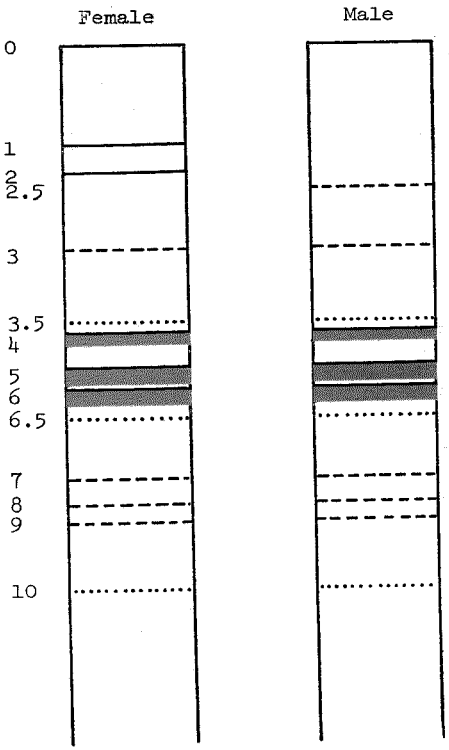

FIG. 1.—Schematic drawing showing the esterase isoenzyme pattern in adult male and female *Culex pipiens quinquefasciatus*. o—origin; solid lines—intense staining; dashed lines—moderate staining; dotted lines—faintly staining.

Three patterns of occurrence of esterase bands 4 and 5 were observed on the electropherogram. 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 (Table 1). After eggs were produced from these crosses, parents were examined electrophoretically to verify their genotype and subsequently the genotype of the progeny. The following discussion concerns only bands 4 and 5.

Individuals homozygous for allele E-4 produced progeny showing only band 4 on the electropherogram. 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 bands 4 and 5. The resolution of these isoenzymes by our electrophoretic system is shown in Fig. 2. Results of reciprocal crosses between individuals homozygous for allele E-5 and heterozygous for allele E-4 and E-5 show 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. Results of crosses between individuals homozygous for E-4 and individuals heterozygous for alleles E-4 and E-5 show esterase activity either at zone 4 alone or at zones 4 and 5 in approximately a 1:1 ratio (Table 1).

These crosses 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 Cross No. 6. 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 produce progeny

TABLE 1.—The occurrence of bands 4 and 5 in progeny of crosses between individual male and female *Culex pipiens quinquefasciatus*

Cross No.	Female Parent	Male Parent	No. of Crosses	Progeny: Females with bands 4	5	4,5	Males with bands 4	5	4,5	Total Individuals with bands 4	5	4,5
1	E–4/E–4	E–4/E–4	8	105			86			191		
2	E–5/E–5	E–5/E–5	10		80			55			135	
3	E–4/E–4	E–5/E–5	5			54			59			113
4	E–5/E–5	E–4/E–4	2			10			6			16
*5	E–5/E–5	E–4/E–5	2		5	11		6	10		11	21
6	E–4/E–5	E–5/E–5	9		42	31		27	35		69	66
**7	E–4/E–5	E–5/E–5	3		5	8		4	5		9	13
8	E–5/E–5	E–4/E–5	5		18	31		12	21		30	52
9	E–4/E–4	E–4/E–5	2	7		5	4		5	11		10
10	E–4/E–5	E–4/E–5	3	1	7	8	5	5	11	6	12	19

* Backcross of F_1 males from Cross No. 4 to females homozygous for E–5.
** Cross between F_1 males and females of Cross No. 6 showing F_2 progeny.

that upon electrophoretic analysis yield the three basic patterns, band 4 alone, band 5 alone, and both bands 4 and 5. 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.

Analysis of F_2 and backcross data indicate that the two alleles are assorting independently and that the gene for these isoenzymes is most probably located on one of the autosomes.

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

The esterases were 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.

Figure 3 shows the effects on the electropherogram following inhibition of the esterases by eserine and paroxon on adult females and males. 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. According to this classification bands 1 and 2 are cholinesterases; bands 3, 4, 5 and area 7 are aliesterases; bands 2.5 and 6 are aromatic esters. The area including bands 7, 8, and 9 were not clearly resolved in the inhibitior studies, and are referred to as area 7.

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 either of the two inhibitors and is therefore classified as an aromatic ester. Bands 4 and 5 that are studied genetically are inhibited by paroxon but not by eserine and therefore, are aliesterases.

DISCUSSION. *Culex pipiens 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. No changes were detectable in esterase patterns of specific stocks after adulthood was reached. The esterase patterns of adult female and male *C. pipiens quinquefasciatus* were characteristically distinguished by three bands of esterase activity. Bands 1 and 2 were

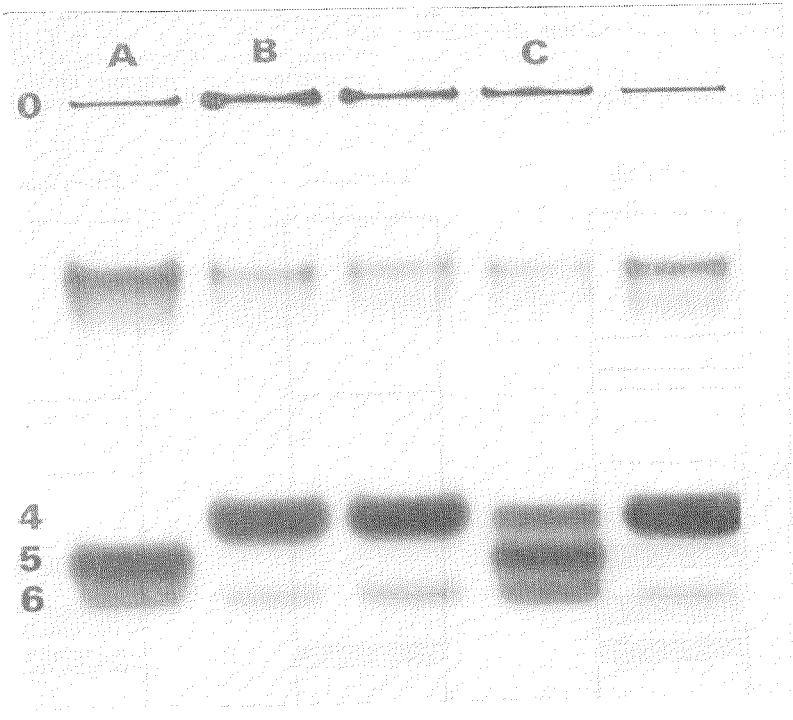

FIG. 2.—Electropherogram showing the three patterns of occurrence of bands 4 and 5. o—origin; A—individual female homozygous for E–5; B—individual female homozygous for E–4; C—individual female heterozygous for E–4/E–5.

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 showed that bands 1 and 2 are cholinesterases whereas band 2.5 is an aromatic esterase.

Simon (1969) reported four weak zones of activity in *C. pipiens quinquefasciatus* 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 work on a population of *C. pipiens 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 were completely inhibited by paroxon and eserine, band 2.5 was still detected in males but not in females. In this study, precise differences distinguish the esterase patterns between adult males and females.

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

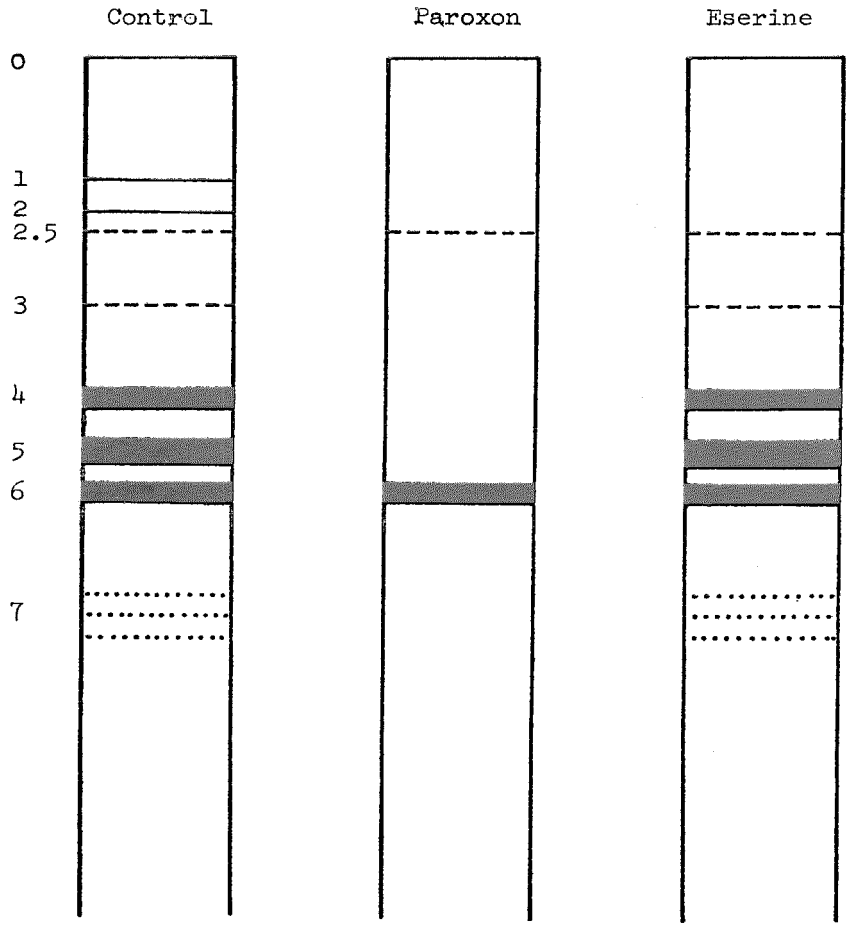

FIG. 3.—Schematic drawing showing the effect of inhibitors on the esterase variations in male and female *Culex pipiens quinquefasciatus*. o—origin; solid lines—intense staining; dashed lines—moderate staining; dotted lines—faintly staining.

the chromosome mapping of esterase genes in *Drosophila* (Wright, 1963; Beckman and Johnson, 1964).

In the present study, homozygous individuals produced either electrophoretically slow-moving (Est. 4) or fast-moving enzymes (Est. 5); heterozygotes showed both parental forms of the enzyme. There was no apparent interaction between the products of the two alleles.

Simon (1969) 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. Simon suggests that the hybrid enzyme exists as a dimer resulting from a random combination of two different Est. E monomeric subunits. The formation of the hybrid enzyme indicates that at least two polypeptide chains are involved in this esterase system.

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 are 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 *C. pipiens 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 1M NaCl and allowed the mixture to incubate at 25 C for 30 min. 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. Through the use of 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.

In experiments where adequate control of the genetic parameters have been exercised, the following classification of genes responsible for isoenzyme formation appears to be 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 a 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 quinquefasciatus* reported by Simon (1969) was classified as a gene with interacting co-dominant alleles. The independent action of non-alleles results in different molecular forms of an esterase in *Tetrahymena pyriformis* (Allen, 1961). Some isoenzymes arise under the joint influence of non-alleles such as the hybrid lactate dehydrogenases (Apella and Markert, 1961).

According to the definition of isoenzymes given by the International Commission on Enzymes, *Culex pipiens 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 all three esterase isoenzyme systems were found.

SUMMARY. Electrophoretic studies were used to examine the inheritance of esterases in *Culex pipiens quinquefasciatus*. A total of 13 sites of esterase activity were found in adult mosquitoes. However, all 13 esterases have not been detected in any

one individual. Adult males and females were differentiated by three zones of esterase activity. Bands 1 and 2 are detected only in females, whereas band 2.5 is found only in males. The heterogenous nature of bands 4 and 5 made them amenable to genetic analysis.

The relationship of bands 4 and 5 demonstrated in the crosses indicates that they are esterases produced by co-dominant alleles. Analysis of F_2 and backcross data indicate that the two alleles are most probably located on one of the autosomes. 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.

By 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 inhibitor studies. Esterases 4 and 5 were shown to be isoenzymes under the control of a single autosomal gene with co-dominant alleles.

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