CHANGES IN ACETYCHOLINESTERASES AND CHOLINESTERASES DURING DEVELOPMENT OF *AEDES AEGYPTI* (L) (DIPTERA, CULICIDAE)

RENUKA SENGUPTA *
Department of Entomology
University of Alberta
Edmonton, Alberta
Canada T6G 2E3

Quaestiones Entomologicae
13: 183-189 1977

Changes in activity of cholinesterases (ChE) and acetycholinesterases (AChE) were studied during the development of Aedes aegypti *(L.). Using starch gel electrophoresis, esterase zymograms showed 5-12 bands (designated esterases A to F in order of decreasing mobility) concentrated in two major zones of mobility. The enzyme bands exhibited changes in activity in different developmental stages, as well as within a particular stage. Significant changes were reflected in the loss of esterase A as development proceeded and in increase in esterase F in the adult. Quantitative studies showed a sharp increase in AChE in adults; the AChE was concentrated in the head. In the thorax and abdomen, ChE equalled or exceeded AChE activity. Eserine sulfate and diisopropylfluorophosphate (DFP) at 10^{-5}M inhibited the AChE completely (colorimetric determination), while on the zymograms 10^{-5}M eserine sulfate inhibited esterases C, E and F, and 10^{-5}M DFP inhibited esterases E and F.*

*Nous avons étudié durant le developpement de l'*Aedes aegypti *le changement de l'activité des cholinestérases (ChE) et des acétylcholinestérases (AChE). A l'aide d'une électrophorèse sur gelée d'amidon nous avons obtenu des zymogrammes d'estérases de 5 à 12 bandes (les estérases sont designés de A à F selon leur ordre de mobilité) concentrées dans deux zones de mobilité majeures. Les bandes enzymatiques demontrent non seulement un changement d'activité dans différents stades du developpement, mais aussi à l'intérieur de certains stades. Nous avons noté des changements significatifs tel que la perte de l'estérase A durant la poursuite du developpement et un accroissement de l'estérase F dans le stade adulte. Des études quantitatives démontrent un accroissement rapide de l'AChE chez l'adulte et une concentration de l'AChE dans la tête. Dans le thorax et l'abdomen, ChE égale ou surpasse l'activité de l'AChE. Le sulphate d'ésérine et le fluorophosphate di-isopropylique (DFP) à 10^{-5}M suspend l'activité de l'AChE complètement (détermination colorimétrique), alors que sur les zymogrammes le sulphate d'ésérine à la même concentration suspend l'activité des estérases C, E et F, et le DFP à cette concentration suspend l'activité des estérases E et F.*

INTRODUCTION

Acetycholinesterases (AChE) and cholinesterases (ChE) constitute an important enzyme system not only physiologically and biochemically but also in pesticidal action (Colhoun, 1963, O'Brien, 1967; Freyvogel, Hunter and Smith, 1968). ChE's are the target enzymes in organophosphate poisoning (O'Brien, 1967; Fest and Schmidt, 1973), are primarily involved in carbamate poisoning (O'Brien, 1967; Corbett, 1974), and are involved in poisoning by cholorinated hydrocarbon pesticides (Sengupta, Sarkar and Ghosh, unpublished results). The esterases also seem to play a significant role in the development of adaptation (Sengupta, Basak, Sarkar and Ghosh, 1975). The positive relationship between cholinergic system and differentiation of the nervous system was reviewed by Smallman and Mansigh (1969).

Esterases during development have been studied electrophoretically by several authors. Though a direct comparison of the results is difficult due to differences in techniques and experimental conditions, certain general inferences can be drawn from these studies. Conspicuous changes in the zymogram and isozyme patterns can indicate important alterations in metabolism during embryogenesis and metamorphosis (Briegel and Freyvogel, 1971; Simon, 1969).

* Present address: Department of Biochemistry, University College of Science, Calcutta University, 35, Ballygunge Circular Road, Calcutta - 700019, INDIA

Electrophoretic variations in esterases due to species, sex, and age of adults have also been reported (Freyvogel *et al.* 1968).

Changes in ChE during metamorphosis have been studied by various workers. However, most studies were restricted to a particular developmental stage of an insect species rather than a comparative investigation involving all developmental stages. The present study is concerned with developmental changes in AChE and ChE, and with the distribution of these in the body regions of larvae, pupae and adults of the Guelph strain of *Aedes aegypti* (L.).

MATERIAL AND METHODS

Experimental insects.

Aedes aegypti (L.) (Guelph strain), originally obtained from British Columbia Research Council, Vancouver, Canada, was maintained in the laboratory during the investigations. A DDT resistant strain of *A. aegypti* has been maintained in the laboratory for several years.

Electrophoretic experiments were conducted with single specimens of second, third, and fourth larval instars; one, two, three, and four-day-old pupae; and four, twelve and 35-day-old adult males and females.

Tissue Preparation.

Homogenates were prepared in a miniature glass homogenizer in 25-30 μl saturated solution of phenylthiourea. The homogenate was then centrifuged at 14,500 g for 30 minutes at 2°C and supernatant used for all experiments.

Electrophoresis.

Esterases were studied electrophoretically using the methods of Smithies (1955) and Smith (1968). The starch gel was prepared using 10% hydrolysed starch (Connaught Medical Laboratories, Toronto, Canada) in 4 mM borate buffer at pH 8.6. Borate buffer (0.01 M) at pH 8.6, was the electrode buffer.

The electrophoresis was run for 3 hours at 400 V (70-75 mamps) at 4°C. Bromophenol blue was the tracking dye. For demonstration of esterase activity the following substrate-stain solution was found to give best results (modified from Beckman and Johnson, 1964): 4.5 ml of a 1:1 mixture of water and 1% α-naphthylacetate in acetone was mixed with 100 ml phosphate buffer (0.1 M, pH 6.0) containing 65 mg of Fast blue RR salt. The gels were incubated with the stain solution for 60 min at room temperature. For inhibition studies the gels were incubated at room temperature with eserine sulfate or diisoprophylfluorophosphate (DFP) at 10^{-5}M for 30 mins before placing in the substrate-stain solution. The gels were then transferred to two wash solutions; the first was ethanol:glycerine:glacial acetic acid: water (1:1:1:2), and the second was methanol:water:glacial acetic acid (50:50:1). The gels were washed at room temperature long enough to wash out the background colour. After washing, the gels were stored in saran wrap.

Colorimetric determination of enzyme activity.

Cholinesterases were assayed, by the method of Ellman, Courtney, Andres and Featherstone (1961), for 5 min at pH 8.0 (in 0.1 M phosphate buffer) at 25°C in a Beckman DU-2 spectrophotometer equipped with dual thermospacers. Acetylthiocholine iodide was used as one of the substrates at 75 mM, which has been found to be the optimal concentration for the present enzyme system (Fig. 1). Propionylthiocholine iodide and butyrylthiocholine iodide were also used at 75 mM, for the comparative studies.

For inhibition experiments the homogenate was incubated with eserine sulfate or DFP (10^{-5}M) for 30 mins at pH 8.0, 25°C, before adding the substrate.

RESULTS AND DISCUSSION

The major stages in the life cycle of *Aedes aegypti* Guelph strain were investigated for AChE and ChE. The results (Fig. 2-5) indicate that, in general, esterase enzymes were concentrated in two major zones of mobility. A total of 5-12 bands were found and may be described as being of intense, intermediate or low activity. The bands were designated A-F, in the order of decreasing mobility (Fig. 2-5).

Esterase A was present in the egg, larval and pupal stages as a single weak band (Fig. 2-4). It was best represented in the egg stage, was gradually lost as development proceeded, and was absent from the adult stage. This may reflect a lessening need for this particular enzyme as adult life approaches. Esterase B was present as a single intense band except in a few males where it showed weak activity. Esterase C was a single band with intermediate to high activity; it was well represented in the egg stage but was absent from some pupae and from adults. Esterase D was an intense band in all stages; in a few specimens it was seen as a double band. Esterase E was weak and invariably present in adults. In a few pupae, esterase E was a double or triple band. Esterase F, the slowest moving band, appeared as one to four bands with varying levels of activity in practically all developmental stages (Fig. 2-5). Esterase F corresponded to cholinesterase enzymes (mobility compared with standard horse serum cholinesterase from Sigma Chemicals) and showed less activity in the egg than in the larval stage (when expressed in terms of activity per individual). Casida (1956) and Dewhurst, McCaman and Kaplan (1970) have demonstrated a many fold increase in house fly AChE activity from egg to larval stage. In the pupal stage of *A. aegypti* a faint additional band (designated as Est ϵ) was observed and this band appeared to be characteristic of this stage.

Freyvogel, Hunter and Smith (1968) observed sex differences in esterase zymograms of *Anopheles stephensi*. However, with *A. aegypti*, I observed no sex differences in the esterase patterns, except that the activity of enzymes from females appeared slightly more intense than that of males.

In the present study, changes in levels of AChE correlated well with the general physical activity of a particular developmental stage. Thus in larvae and adults which are very active, levels of whole body AChE were much higher (6.32 ± 0.705 and 11.12 ± 0.374 μmoles acetylthiocholine hydrolysed/min/gm tissue respectively) than in the pupae (3.01 ± 0.235).

Studies of distribution of AChE and ChE in different body regions of larvae, pupae and adults, revealed that the highest concentration of AChE was in the head (Table 1). On the other hand the activity of ChE's (using propionylthiocholine and butyrylthiocholine as substrates) was usually equal to or greater than the activity of AChE in the thorax and abdomen of each stage.

Using heads of adult *A. aegypti*, activity of AChE in the DDT resistant strain was lower (18.14± 0.75 μmoles/min/gm tissue) than that of the Guelph strain (23.46 μmoles/min/gm tissue). This difference was observed in 3-4 day-old adults, however the difference became insignificant as the adults grew older. Similar observations were made by Babers and Pratt (1950).

In vitro inhibition studies with heads of larvae and adults showed that AChE is totally inhibited by eserine sulfate and DFP at 10^{-5}M. In electrophoretic studies with homogenates of whole bodies, eserine sulfate and DFP at a final concentration of 10^{-5}M inhibited esterases C, E, F and E, F respectively. Esterases A and C were inhibited by n-isopropanol.

Table 1. Acetylcholinesterase and cholinesterase activities (μmoles substrate hydrolysed/min/gm tissue) in the Larvae, Pupae and Adults of *Aedes aegypti* L. (Guelph Strain)

Substrate Conc. - (0.075 M)	LARVA			PUPA		ADULT		
	Head	Thorax	Abdomen	Cephalo Thorax	Abdomen	Head	Thorax	Abdomen
Acetylthiocholine iodide	9.97 ±0.92	4.02 ±0.19	2.67 ±0.28	3.79 ±0.54	3.45 ±0.29	23.46 ±0.00	11.19 ±1.05	3.44 ±0.71
Propionylthiocholine iodide	2.87 ±0.00	4.31 ±0.00	2.15 ±0.39	1.00 ±0.11	2.87 ±0.00	15.37 ±0.53	8.03 ± 0.99	3.73 ±0.71
Butyrlthiocholine iodide	0.70 ±0.00	0.72 ±0.00	1.72 ±0.00	0.50 ±0.00	1.44 ±0.00	1.15 ±0.00	1.73 ±0.36	3.59 ±0.72

ACKNOWLEDGEMENTS

I thank R.H. Gooding, Department of Entomology, University of Alberta, Canada for his help and constructive criticism during the investigations. I thank also D. Cameron, Department of Genetics for his kind help and J. Bererton, B. Rolseth and J.S. Scott for their technical assistance. The research for this paper was supported (in part) by the Defence Research Board of Canada, Grant nos. 6801-41 and 6801-51 awarded to Dr. Gooding and was carried out during the tenure of a University of Alberta Post-doctoral Fellowship, which is also gratefully acknowledged. Facilities for completing the manuscript were provided by the Department of Biochemistry, University of Calcutta.

REFERENCES

Babers, F.H. and J.J. Pratt, Jr. 1950. Studies on the resistance of insects to insecticides I. Cholinesterases in house flies (*Musca domestica* L.) resistant to DDT. Physiological Zoology 22: 59-63.

Beckman, L. and F.M. Johnson. 1964. Esterase variation in Drosophila. Hereditas 51: 212-220.

Briegel, H. and T.A. Freyvogel. 1971. Non-specific esterases during development of culicine mosquitoes. Acta Tropica 28: 291-298.

Casida, J.E. 1956. Comparative enzymology of certain insect acetylesterases in relation to poisoning by organophosphorous insecticides. Biochemical Journal 60: 487-496.

Colhoun, E.H. 1963. The physiological significance of acetylcholine in insects and observations upon other pharmacologically active substances. *In* Advances in Insect Physiology I: 1-46. W.L. Beament, J.E. Treherne, and V.B. Wigglesworth, editors. Academic Press.

Corbett, J.R. 1974. The Biochemical Mode of Action of Pesticides. Academic Press. New York; London. p. 109-149.

Dewhurst, S.A., McCaman, R.E. and W.D. Kaplan. 1970. The time course of development of acetylcholinesterase and choline acetyltransferase in *Drosophila melanogaster*. Biochemical Genetics 4: 499-508.

Ellman, G.L., Courtney, K.D., Andres, V. Jr. and R.M. Featherstone. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology 7: 88-95.

Fest, C. and K.J. Schmidt. 1973. The chemistry of organophosphorus pesticides - reactivity, synthesis, mode of action, toxicology. Springer-Verlag Berlin Heidlberg, New York p. 164.

Freyvogel, Theirry, A., Hunter, R.L. and E.M. Smith. 1968. Non-specific esterases in mosquitoes. Journal of Histochemistry and Cytochemistry 16: 765-790.

O'Brien, R.D. 1967. Insecticides - action and metabolism. Academic Press, New York and London p. 55-58.

Sengupta, R., Basak, S., Sarkar, D. and J.J. Ghosh. 1975. Effect of acute and multiple dose administration of sumithion (0,0-dimethyl 0-(3 methyl-4-nitrophenyl) phosphorothionate) on some enzymes of *Periplaneta americana* (Linn.). Pesticide Biochemistry and Physiology 5: 52-56.

Sengupta, R., Sarkar, A. and J.J. Ghosh. Effects of acute administration of BH C and DDT on blood and brain cholinesterases in rat and toad. (unpublished results)

Smallman, B.N. and A. Mansingh. 1969. The cholinergic system in insect development. Annual Review of Entomology 14: 378-408.

Smith, J. 1968. Chromatographic and electrophoretic techniques Vol. 2. Zone Electrophoresis 2nd Edn. Pitman Press, Great Britain, 217 p.

Smithies, O. 1955. Zone electrophoresis in starch gels. Group variations in the serum proteins of normal human adults. Biochemical Journal 61: 629-641.

Simon, Jean-Pierre. 1969. Esterase isozymes in the mosquito *Culex pipiens fatigens*. Developmental and genetic variations. Annals of the Entomological Society of America 62: 1307-1311.

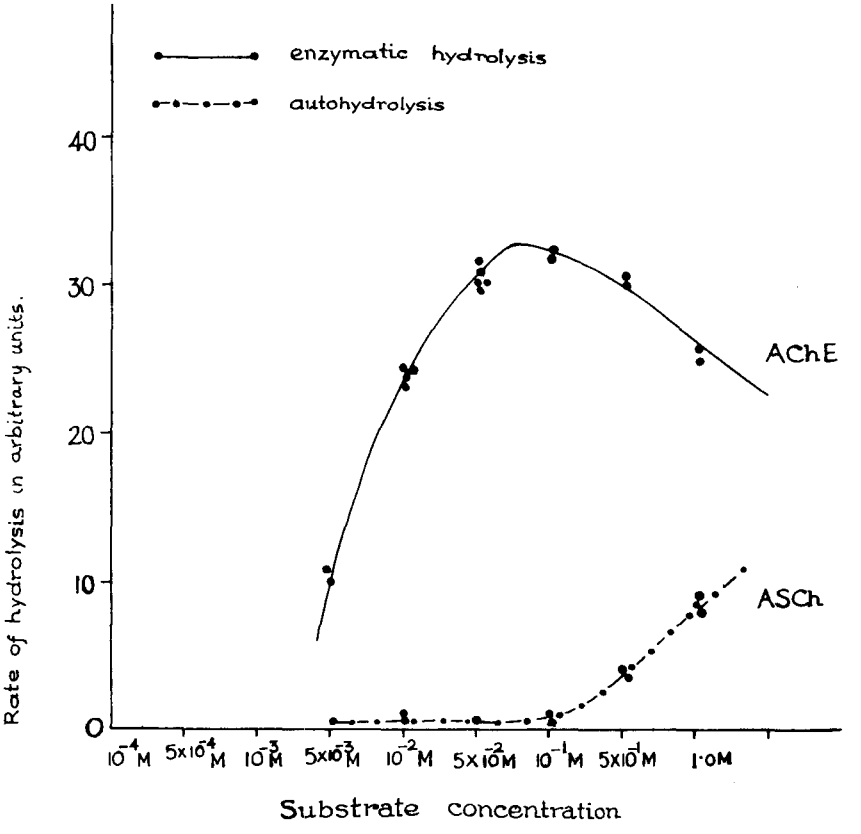

Fig. 1

Fig. 1. Rate of hydrolisis as a function of substrate concentration (AChE-acetylcholinesterase catalyzed hydrolysis; ASCh-autolysis of acetylthiocholine iodide).

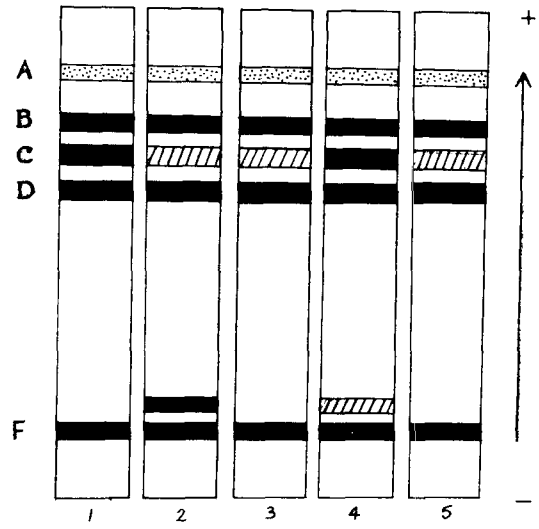

Fig. 2

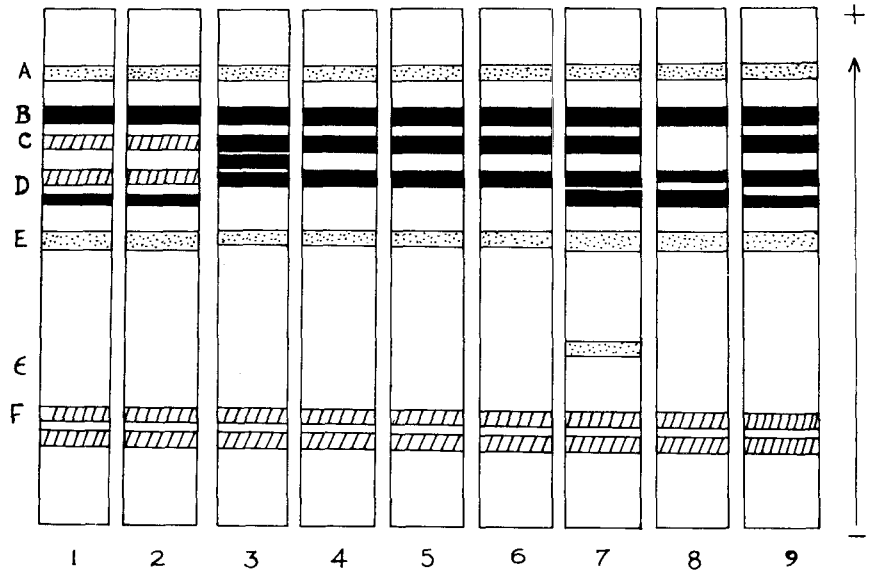

Fig. 3

Fig. 2. Diagrammatic representation of patterns of esterase zymograms of eggs of *Aedes aegypti* L. (A-F=Est A to Est F) high, ▬ medium, ▨ and low, ▭ activity. Fig. 3. Diagram of patterns of esterase zymograms of different larval instars of *Aedes aegypti* L. (1,2-1st instar; 3,4-2nd instar; 5,6-3rd instar; 7,8,9-4th instar). Symbols as in Fig. 2.

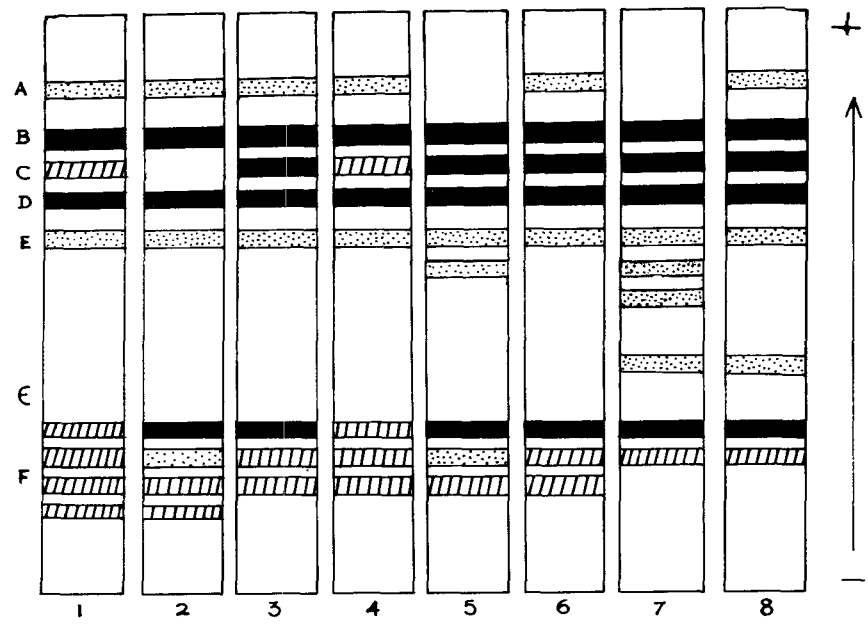

Fig. 4

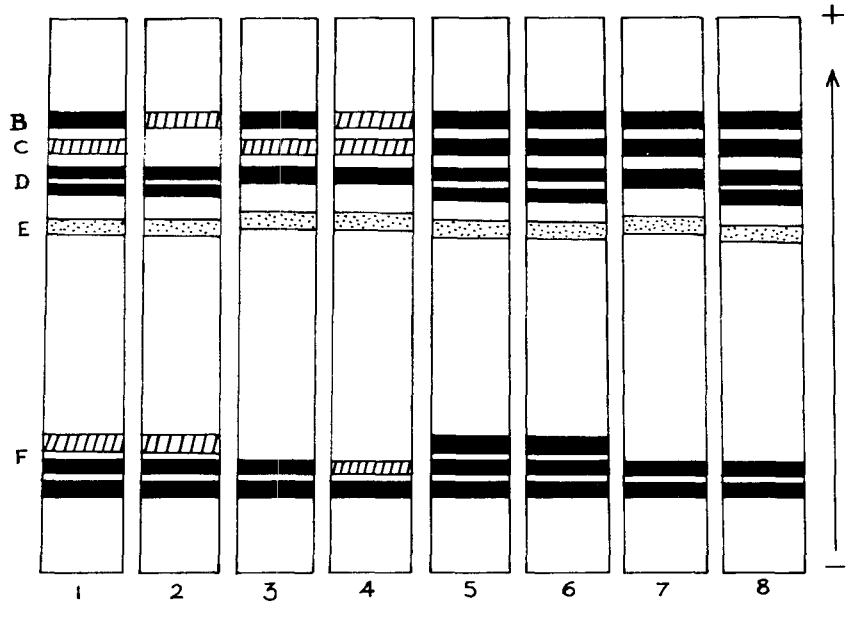

Fig. 5

Fig. 4. Diagram of patterns of esterase zymograms of pupae of *Aedes aegypti* L. (1,2-1 day; 3,4-2 days; 5,6-3 days and 7,8-4 days old). Symbols as in Fig. 2. Fig. 5. Diagram of patterns of esterase zymograms of adult *Aedes aegypti* L. (1-4, males and 5-8, females). Symbols as in Fig. 2.