larval surveys to control efficiency in Salt Lake County. Proceedings of the 46th Annual Meeting of the New Jersey Mosquito Extermination Association:119–121.

GRAHAM, JAY E., and ANDERSON, R. D. 1958. A seasonal history of mosquito larval populations in Salt Lake County, Utah. Unpublished paper, 6th Annual Meeting Entomological Society of America.

GRAHAM, JAY E., BRADLEY, I. E., and COLLETT, G. C. 1960. Some factors influencing larval populations of *Culex tarsalis* and western equine encephalitis in Utah. Mosq. News 20(2):100–103.

GRAHAM, JAY E., and BRADLEY, I. E. 1962. The effects of species on density of mosquito larval populations in Salt Lake County, Utah. Mosq. News 22(3):239–247.

HESS, A. D., and HAYES, R. O. 1967. Seasonal dynamics of western encephalitis virus. American Journal of the Medical Sciences, 253(3):333–348.

JENKINS, A. A., and DONATH, R. 1959. The 1958 encephalitis outbreak in northern Utah. I. Human aspects. Mosq. News 19(4):221–222.

LONGSHORE, W. A. 1953. The epidemiology and public health significance of encephalitis in California. Proceedings and Papers 21st Annual Conference California Mosquito Control Association:55–59.

LOOMIS, EDMUND C. 1953. Population trends of *Culex tarsalis* in the Central Valley of California. Proceedings and Papers California Mosquito Control Association:73–76.

LOOMIS, EDMUND C. 1959. The function of larval surveys in the California encephalitis surveillance program. Proceedings and Papers 27th Annual Conference California Mosquito Control Association:66–67.

REES, DON M., and COLLETT, G. C. 1959. Factors influencing the encephalitis outbreak in Utah in 1958. Proceedings and Papers 27th Annual Conference California Mosquito Control Association:88–97.

REES, DON, OGDEN, L. T., COLLETT, G. C., and GRAHAM, JAY E. 1959. The 1958 encephalitis outbreak in northern Utah. 3. Mosquito populations in relation to the outbreak. Mosq. News 19(4):227–231.

REEVES, WILLIAM C. 1968. A review of developments associated with the control of western equine and St. Louis encephalitis in California during 1967. Proceedings and Papers 36th Annual Conference California Mosquito Control Association:65–70.

ESTERASE ACTIVITY IN ORGANOPHOSPHORUS-TOLERANT STRAINS OF *AEDES AEGYPTI*

M. ZIV AND A. W. A. BROWN

University of Western Ontario, London, Canada

Larval selection in the laboratory has indicated that strains of *A. aegypti* from Africa, Asia and the Caribbean area do not develop any more than a moderate tolerance to OP compounds such as malathion and parathion. Moreover, strains selected with either of these compounds did not show any increased production of phosphatase or carboxyesterase detoxication products, but only a decreased uptake which was significant in the case of malathion (Matsumura and Brown, 1961; *Ibid.*, 1963). Arylesterase activity had been demonstrated by Plapp *et al.* (1965) in *Aedes aegypti* as in *Culex* and *Anopheles* larvae, particularly on the substrate tributyrin, but the malathion-selected Penang strain showed less paraoxon-insensitive arylesterase activity than the susceptible Orlando strain. Moreover it showed an increased aliesterase activity on phenyl acetate and no less than the normal on methyl butyrate.

Therefore it seemed necessary to assess the aliesterase and arylesterase activities of the malathion- and parathion-selected strains of African, Asian and Caribbean origin in our laboratory. This was performed by means of agar-gel electrophoresis of the esterases, supplemented by an enzyme assay performed on the malathion-selected African strain by the same

method that Plapp *et al.* had applied to the Asian strain.

MATERIALS AND METHODS. The stocks of *A. aegypti* employed came from Kongolikan (Upper Volta), Penang (Malaysia) and Trinidad in the West Indies; and by means of prolonged selection, a malathion-tolerant and a parathion-tolerant strain had been developed from each of these stocks. The LC_{50} levels in p.p.m. and the resistance ratios were as follows:

	Malathion			Parathion		
	Normal	Selected	Ratio	Normal	Selected	Ratio
Kongolikan	.08	.75	9.3	.010	.042	4.2
Penang	.15	.88	5.9	.014	.030	2.1
Trinidad	.16	.60	3.8	.014	.029	2.1

Esterase assessments were performed according to the method described by Bigley and Plapp (1960) and Plapp *et al.* (1965) on homogenates of fourth instar larvae prepared immediately before use. Lots of 1 g. each were homogenized in 10 ml. of refrigerated pH 7.2 phosphate buffer with a Potter-Elvehjem homogenizer. To remove the cell debris the homogenates were filtered through a Buchner funnel lined with a single layer of glass wool, the particulate residue being rinsed with additional buffer until 25 ml. were collected, resulting in a concentration of 40 mg. larvae/ml. buffer. Whenever necessary, the homogenate concentration was further diluted with buffer. The substrates and concentrations used were 2×10^{-3}M acetylcholine bromide, 4×10^{-3}M methyl butyrate, 4×10^{-3}M phenyl acetate and 2×10^{-3}M tributyrin. All esterase assessments were made at least 2–3 times.

The esterase activity was differentiated into cholinesterase, aliesterase and arylesterase types on the basis of its inhibition by 10^{-5}M eserine and paraoxon. The enzyme inhibition was determined by incubating 0.9 ml. aliquots of homogenates with 0.1 ml. amounts of aqueous solutions of 1×10^{-4}M eserine sulphate or paraoxon for 15 min. at 37° C; then 1 ml. substrate was added, the samples were incubated for another 15 min. and the uninhibited esterase activity was assessed.

The esterases were separated by means of thin-layer agar-gel electrophoresis on glass plates 15 x 20 or 15 x 10 cm. in size and visualized by histochemical staining techniques according to the method of Ogita (1964). The non-specific esterases were differentiated into aliesterase and arylesterase zones of activity by incorporating paraoxon into the agar-gel layer.

Fourth instar larvae were homogenized in 1 g. batches with a Potter-Elvehjem homogenizer in 1 ml. refrigerated phosphate buffer at pH 6.8 and ionic strength 0.025. Ten-microliter samples of the homogenates were applied to depressions made in the agar surface by leaving 2 x 18 mm. strips of filter paper prior to application. The electrophoresis was performed at a constant potential of 300 V, giving a current of 1.5–2 mA/cm width, for 1.5–2 hr. at 5–10° C. Each experiment was repeated 4–5 times.

Acid and alkaline phosphatases were similarly separated; their activities were assessed according to methods derived from Lambremont (1959), Ogita (1964), Kasai and Ogita (1965) and Wieme (1965). For acid phosphatase activity, the plates after electrophoresis were covered with a substrate solution consisting of 0.5 percent sodium alpha-naphthyl acid phosphate in 0.2M acetate buffer, pH 5.0, containing 0.1M magnesium sulphate. For alkaline phosphatase activity, the substrate solution was 0.1 percent sodium beta-naphthyl acid phosphate in 0.2M barbitol buffer at pH 8.6. In both cases the plates were incubated for 2–3 hr. at 24° C. The bands of phosphatase activity were visualized in the same manner as were the esterases.

Esterases degrading malaoxon and paraoxon were detected by incorporating these toxicants in the agar-gel layer prior to application of the homogenate samples

and electrophoresis. After separation of the enzymes, the plates were incubated for 3 hr. at 24° C. and high humidity to allow any hydrolytic degradation to occur. The plates were then sprayed with a 0.25 mg./ml. solution of bovine erythrocyte acetylcholinesterase, and incubated for 1 hr. to permit the unhydrolyzed toxicant to inhibit the acetylcholinesterase. Then the plates were covered with a mixture of acetylthiocholine iodide substrate and INT (iodonitrotetrazolium violet) chromogen, according to the method of Uriel (1961), cited in Wieme (1965); where the cholinesterase had remained inhibited due to hydrolysis of the paraoxon, there it showed the red zone due to the reduction of the tetrazolium salt by its released thiol groups.

RESULTS. Comparison of the malathion-selected with the normal Kongolikan strain (Table 1) showed that it had a lower esterase activity as judged by the substrates phenyl acetate and methyl butyrate. Moreover, it had a paraoxon-insensitive arylesterase component which the normal strain lacked on these substrates. On tributyrin, where the normal strain showed this evidence of arylesterase, the paraoxon-insensitive activity was considerably higher in the selected strain as compared to the normal.

Comparisons of the zymograms produced at pH 8.6 of the malathion- and parathion-selected strains with the normal strains for the three stocks (Fig. 1) show differences in the region of the three farthest-moving bands. In the parathion-selected strains there is an intensification in the middle band of the three. On the other hand, in the malathion-selected strains there is a diminution in the most distal band, and in the Kongolikan strain in the middle band also.

The zymograms produced at pH 6.8 (Fig. 2) reveal a slowly moving band with about one-third of the mobility of the most distal band. In parallel zymograms pre-treated with paraoxon it was found that this slowest-moving band was the only paraoxon-insensitive band, and this proved true not only in the three strains under investigation, but also in two strains from Albina, Surinam and from Montego Bay, Jamaica that had developed malathion-tolerance in the field. Direct comparison of this arylesterase band in the three selected strains as against the three unselected stocks (Fig. 3) show that it is more intense in all the parathion-selected strains, although not in the malathion-selected strains.

When the paraoxon-treated plates were incubated after electrophoresis for toxicant degradation, the region where the paraoxon had evidently been hydrolyzed coincided with that of the arylesterase band; the same result was obtained with the malaoxon-treated plates. Zymograms produced with alpha-naphthyl phosphate as substrate revealed a single acid phosphatase band which was in the same region as the

TABLE 1.—Esterase activity and sensitivity in larval homogenates of the normal and malathion-selected Kongolikan strains of *Aedes aegypti*.

Substrate	Acetylcholine bromide 2×10^{-3}M	Phenyl acetate 4×10^{-3}M	Methyl butyrate 4×10^{-3}M	Tributyrin 2×10^{-3}M
Enzyme Concentration (mg. larvae/ml. homogenate)	40	10	20	10
Normal strain				
Activity, micromoles/15 min.	0.53	2.67	2.66	1.14
% inhibited by eserine	100	48	16	32
% inhibited by paraoxon	100	100	100	53
Malathion-selected strain				
Activity, micromoles/15 min.	0.51	1.91	2.30	1.22
% inhibited by eserine	100	32	10	16
% inhibited by paraoxon	100	91	90	31

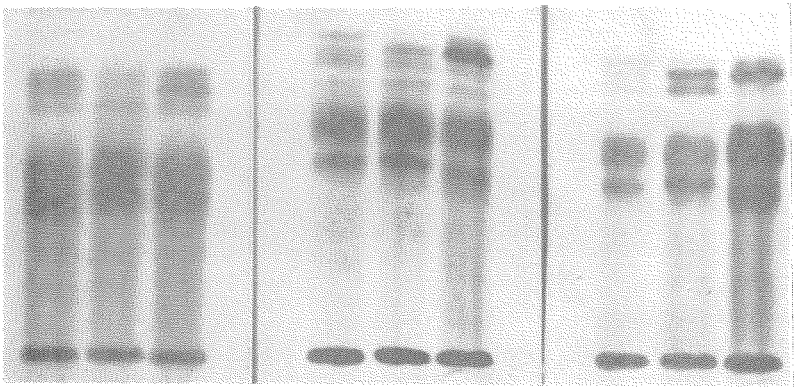

FIG. 1.—Esterase zymograms (pH 8.6) for normal, malathion-selected and parathion-selected strains (in this order) for the Kongolikan, Trinidad and Penang stocks of *A. aegypti*.

arylesterase; a single band was also obtained for alkaline phosphatase with beta-naphthyl phosphate, but this band was more distal. Although the parathion-selected strains showed more intense acid phosphatase bands than normal, the paraoxon hydrolysis was not detectably greater.

DISCUSSION. The multiplicity of esterase bands, numbering about a dozen, found in the *Aedes aegypti* zymograms contrasts with the single broad band found for *Culex fatigans* by Stone and Brown (1969). The greater versatility of the *Aedes aegypti* esterase activity had been suggested by Plapp *et al.* (1965) consequent on their finding that the homogenate of *Culex fatigans, C. peus* and *C. tarsalis* had no esterase activity on methyl butyrate whereas *A. aegypti* had.

In *Culex fatigans,* the single broad band also contained the acid phosphatase activity, and was insensitive to inhibition by fenoxon since it could hydrolyze it (Stone and Brown, 1969). When homogenate of *A. aegypti* and *C. fatigans* were subjected to electrophoresis on the same plate, the broad band of *C. fatigans* was found to coincide with the arylesterase band of *A. aegypti.* The partial sensitivity to paraoxon

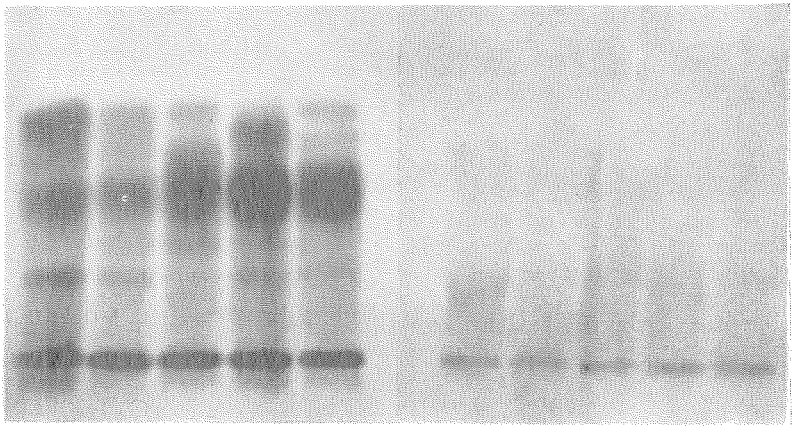

FIG. 2.—Esterase zymograms (pH 6.8) for the Kongolikan, Penang, Trinidad, Montego Bay and Albina stocks on plates untreated (left) and treated (right) with 10^{-5}M paraoxon.

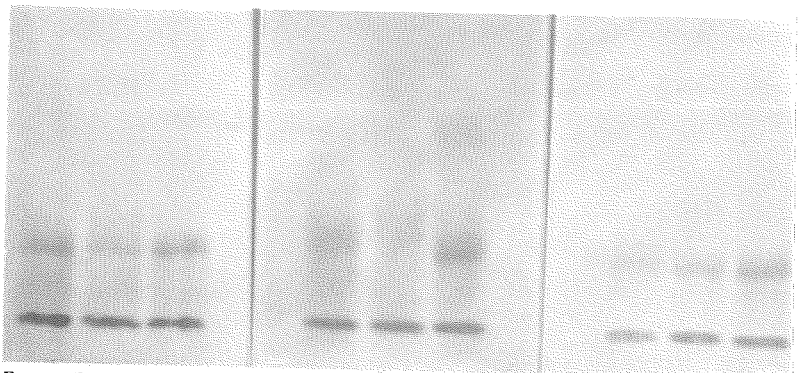

FIG. 3.—Esterase zymograms (pH 6.8) for normal, malathion-selected and parathion-selected strains for the Kongolikan, Trinidad and Penang stocks on plates treated with 10^{-5}M paraoxon.

observed in the *C. fatigans* band, in contrast to its insensitivity to fenoxon, may be due to its containing some aliesterases as well as the OP-insensitive arylesterase.

The results suggest that the *A. aegypti* strains responded to selection with parathion by a modest increase in arylesterase activity, in contrast to *C. fatigans* which had responded to fenthion selection by a decisive increase in such activity. In the malathion-selected *A. aegypti,* there was no detectable increase in arylesterase activity, although the most distal of the aliesterase bands diminished in intensity. In the malathion-selected strains studied by Matsumura and Brown (1963) the only mechanism developed was a decrease in the larval absorption of malathion. In the parathion-selected strains, on the other hand, the decrease in parathion uptake was not statistically significant. It is therefore possible that the slight increase in arylesterase activity observed in this investigation may have contributed to the parathion-tolerance developed, although Matsumura and Brown (1963) could not demonstrate a consistent increase in phosphatase hydrolysis of parathion.

SUMMARY. By means of agar-gel electrophoresis, an arylesterase band has been detected in homogenates of *Aedes aegypti* larvae which is not inhibited by paraoxon but hydrolyzes it. This band was found to be slightly more intense than normal in three parathion-selected strains, but not in three malathion-selected strains. It coincides in position with the acid phosphatase band, as had previously been found in *Culex fatigans.*

This work was performed on a grant-in-aid from the National Institutes of Health and the U.S. Public Health Service. The authors express their profound gratitude to Dr. Z. Ogita of Osaka University for having launched these investigations by demonstrating his method of electrophoresis in person.

References Cited

BIGLEY, W. S., and PLAPP, F. W. 1962. Cholinesterase and ali-esterase activity in organophosphorus-susceptible and -resistant houseflies. Ann. Entomol. Soc. Amer. 53:360–364.

KASAI, T., and OGITA, Z. 1965. Studies on malathion-resistance and esterase activity in green rice leafhoppers. SABCO J. 1:130–140.

LAMBREMONT, E. N. 1959. A study of acid phosphatase of the mosquito *Aedes aegypti* L. J. Ins. Physiol. 3:325–334.

MATSUMURA, F., and BROWN, A. W. A. 1961. Biochemical study of a malathion-tolerant strain of *Aedes aegypti.* Mosq. News 21:192–194.

MATSUMURA, F., and BROWN, A. W. A. 1963. Studies on organophosphorus-tolerance in *Aedes aegypti.* Mosq. News 23:26–31.

OGITA, Z. 1964. Improved agar-gel media for thin layer electrophoresis. Med. J. Osaka Univ. 15:141–153.

PLAPP, F. W., BIGLEY, W. S., DARROW, D. I., and HOYER, R. F. 1965. Esterase activity in mosquitoes and its possible relationship to organophosphate and carbamate resistance. Mosq. News 25:30–35.

STONE, B. F., and BROWN, A. W. A. 1969.

Mechanisms of resistance to fenthion in *Culex fatigans* Wied. Bull. Wld. Hlth. Org. 40:401–408.

URIEL, J. 1961. Characterisation des cholinesterases et d'autres esterases carboxyliques apres electrophorese et immunelectrophorese en gelose. Ann. Inst. Pasteur 101:104–110.

WIEME, R. J. 1965. Agar Gel Electrophoresis. Elsevier Publishing Co., Amsterdam. 425 pp.

EPIDEMIOLOGICAL NOTES: TWO BLUETONGUE EPIZOOTICS

R. H. JONES [1] AND A. J. LUEDKE [1]

In addition to its primary goal of laboratory research on bluetongue (BT) disease of sheep, the Denver Animal Disease Laboratory serves as a diagnostic center for BT in the United States. Because of the cooperative involvement of the laboratory's veterinarians and entomologist in assisting local, state, and federal authorities in the diagnosis of the disease, some epidemiological data have been collected. This paper presents some of these data for two epizootics—one at Hudson, Colorado, and one at Billings, Montana.

HUDSON, COLORADO. This epizootic occurred in a flock of sheep in the fall of 1963 (Jones, 1965; Bowne *et al.*, 1966). Additional data are presented here because the Hudson area was selected for further study the following spring. It was selected not only because BT had occurred there the past season in both sheep and cattle, but also because the suspected primary vector, the small biting midge *Culicoides variipennis* (Coquillett), continued to breed extensively throughout the area.

This heavy breeding (Jones, 1967) continued through at least the 1966 season and indicated that *C. variipennis* was the most common biting fly in the area. This supposition was supported by data collected from an animal-bait trap (Jones, 1961) that was operated occasionally during the 1964 season. The following female biting flies were collected: 6,719 *C. variipennis*, 352 *Culicoides* spp., 549 *Leptoconops* spp., 882 Culicidae, 79 Tabanidae, and 6 Simuliidae. Of the 6,719 female *C. variipennis*, 1,348 (20 percent) had recently fed on blood, presumably from the bait sheep because the trap was the open type that was closed at intervals to collect the flies on and about the animal.

An important step in setting up the Hudson study area was to determine whether the BT infection was still present in 1964. This was accomplished by establishing a sentinel flock of sheep there from April 16 to November 17. These sheep were western white-faced ewes and their week-old lambs from a flock that had overwintered at Wellington, Colorado. The animals were considered susceptible because no serum-neutralization (SN) antibodies were detected in their serum when it was tested against the standard American BT 8 virus passed 10 times in lamb kidney. Blood for subsequent SN tests was taken from each sheep every 4 weeks. Only four sheep remained in test at the end of the season. At that time, two had developed a solid immunity against a challenge inoculation with virulent BT 8 virus, and two remained susceptible. (At 3 months, all of the 10 sheep still in test had failed to show antibodies against the BT 8 virus.) At the end of the test, the two sentinel sheep that had acquired immunity had significant SN indexes against BT 8 virus (Table 1).

[1] U. S. Department of Agriculture, Agricultural Research Service, Animal Disease Research Laboratory, Denver, Colo. 80225. R. H. Jones is in the Entomology Research Division and A. J. Luedke is in the Animal Disease and Parasite Research Division.