1969

Esterase Activity In Strains Of Aedes Aegypti Selected With Organophosphorus Insecticides

Meir Ziv

CANADA

NATIONAL LIBRARY OF CANADA

CANADIAN THESES

ON MICROFILM

BIBLIOTHÈQUE NATIONALE DU CANADA

THÈSES CANADIENNES

SUR MICROFILM

NL-101(1/66)

ESTERASE ACTIVITY IN STRAINS OF AEDES AEGYPTI SELECTED WITH ORGANOPHOSPHORUS INSECTICIDES.

by

Meir Ziv

Department of Zoology

Submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Canada
November 1968

ABSTRACT

Strains of the yellow fever mosquito Aedes aegypti originating from Kongolikan (Upper Volta), Penang (Malaysia) and Trinidad (West Indies) which had already been intermittently selected were subjected to further larval selection with malathion or parathion in order to determine the upper limits of resistance that they could attain. It was found that the LC_{50} to malathion could not be increased beyond 1.60 ppm, and in the terminal stages of the experiment the LC_{50} actually decreased while the strains were under selection. Making hybrid crosses between the different malathion-selected strains and selecting their progeny led to no further increase in malathion-resistance. Only a slight vigor tolerance could be induced to parathion. In addition, two colonies, one from Montego Bay (Jamaica) and the other from Albina (Surinam), which had already developed tolerance to malathion in the field, were selected with malathion or with Abate; here it was found that no further tolerance could be developed.

The esterase activity of the Kongolikan-Malathion strain which had become the most resistant to malathion and

its unselected parent stock was then assessed in homogenates *in vitro* colorimetrically using acetylcholine and other esters as substrates, and was differentiated by means of the specific inhibitors eserine and paraoxon. It was found that this malathion-selected strain did not differ from the normal in cholinesterase activity, but was characterized by reduced aliesterase and greater arylesterase activities.

The esterases and phosphomonoesterases present in the various strains were separated by zone electrophoresis and visualized by staining techniques. Differences in esterase patterns were found between the 5 stocks mentioned from different origins, both in electrophoretic mobility of the zones of activity and in their intensity. None of these esterase bands resulted from cholinesterase activity, and all of them can be classified as aliesterases or B types, except for the slowest moving one which was paraoxon-insensitive and can therefore be classified as an arylesterase or A type. Only a single band of acid phosphomonoesterase activity was found in each of the 5 stocks and it had the same electrophoretic mobility as the A-type esterase band. A single band of alkaline phosphomonoesterase activity was also discerned. There were differences between the stocks in the intensity of the bands for either phosphatase.

When the esterase patterns of the selected strains were compared with those of their original unselected stocks, no

differences were found in the Albina and Montego Bay material. However, differences were found in the esterase patterns of the Kongolikan, Penang and Trinidad malathion- and parathion-selected strains when compared with those of their unselected counterparts. While the differences in the malathion-selected strains were not consistent, in the parathion-selected strains the second most mobile band was much more pronounced than in the unselected material. However, since this band is paraoxon-sensitive, it cannot be concerned with OP-insecticide degradation.

The Trinidad- and Penang-Parathion strains showed more than, and the Kongolikan-Parathion strain the same paraoxon-insensitive esterase activity as the unselected stock colonies. In contrast, only the Penang-Malathion strain displayed greater paraoxon-insensitive activity than the normal among the malathion-selected strains, although the *in vitro* esterase assay had indicated the Kongolikan-Malathion to be richer in arylesterase than normal.

Spraying acetylcholinesterase (AChE) on agar-gel plates incorporated with malaoxon or paraoxon, followed by the detection of uninhibited AChE, was used as a device to discover zones where the OP compound had been hydrolyzed. A single zone of OP hydrolysis did appear, and this was congruent with the position of the arylesterase band and of the acid phosphomonoesterase band. However, the OP-selected

strains did not display greater hydrolytic activity than the unselected stock colonies.

Zymograms from larvae which had been pre-exposed to a sublethal concentration of malathion or parathion were found to show no difference from those of non-treated larvae. Therefore these insecticides had no inductive or activating effects on the esterases and phosphomonoesterases studied in this research.

ACKNOWLEDGEMENTS

I would like to express my most sincere thanks to Dr. A.W.A. Brown, Head of the Department of Zoology, for his constant encouragement and advice during the entire course of the research and in the preparation of this manuscript.

I am also most grateful to Drs. J.E. Steele and J. Purko of the Department of Zoology, E.H. Colhoun of the Department of Pharmacology and R.M. Krupka of the Agricultural Research Institute, London, for their most helpful suggestions at various stages of this research.

Special thanks are due to Mrs. G. Reikhoff for her assistance in raising the mosquito cultures, and to Mrs. H. Kyle, Mr. A. Gage and especially Mr. S. Pluzak, also of the Department of Zoology, for their helpfulness.

It is also a pleasure to acknowledge the personal instruction received from Dr. Z. Ogita, Department of Genetics, Osaka Medical School, Osaka, Japan, in the use of his thin-layer agar-gel electrophoretic technique.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Human existence in tropical countries was until two decades ago, characterized by high infant mortality and low life expectancy caused by diseases, the most prevalent being transmitted by insects. Mosquitoes have been the most serious of the insect vectors, transmitting malaria, filariasis, yellow fever, dengue and encephalitis. The introduction of the use of synthetic insecticides in the 1940's for the control of insect vectors, resulted in a dramatic drop in the incidence of insect transmitted diseases and a consequent sharp decline in the death rate in underdeveloped countries.

Aedes aegypti, the yellow fever mosquito, may be of greater economic importance to more people in more places than any other insect species (Craig and Hickey, 1957). It is the principal vector of yellow fever in Africa and the Americas, the primary vector of dengue in the tropics and recently has been found to be the vector of haemorrhagic fever in South-East Asia.

The use of the chlorinated hydrocarbon insecticides to control Aedes aegypti was countered by this insect

developing resistance to both DDT and its subsequent substitute dieldrin in many regions of the world. This necessitated the introduction of organophosphorus (OP) compounds, particularly malathion and others of low mammalian toxicity such as Abate (Schoof, 1967).

The use of OP compounds against mosquitoes also can lead in turn to control failures due to the development of OP-resistance. In California a population of Culex tarsalis developed resistance to malathion applied as a larvicide (Gjullin and Isaak, 1957), and Aedes nigromaculis developed a nonspecific OP-resistance involving parathion, methyl parathion, fenthion and malathion, each of which could induce this general resistance (Brown et al., 1963).

Some field populations of A. aegypti have already developed a tolerance to malathion. A Puerto Rican population became 10 times more tolerant to malathion than the normal (Fox, 1961). The LC_{50} of malathion (concentration causing 50 per cent mortality) for larvae in several regions in Surinam was found to be 3-4 times that for susceptible populations (Kerr et al., 1964). At a concentration of malathion which was lethal to 75 per cent of the larvae of a susceptible strain, larvae from several localities in Texas showed only 16-48 per cent mortality, and in Florida the mortalities at this concentration ranged down to zero (Flynn and Schoof, 1965).

Brown and Abedi (1960) had anticipated the possible development of resistance to malathion when they subjected a colony of A. aegypti to larval selection pressure with malathion for 8 generations. They obtained a 5-fold increase in the LC_{50} level. Matsumura and Brown (1963a) selected two additional strains with malathion and parathion for 5 generations, the outcome being again a 5-fold increase in tolerance to malathion, and a lower increase in parathion tolerance. This malathion-tolerance was subsequently found to be due to multiple factors present in about equal proportions on chromosomes 2 and 3 (Pillai and Brown, 1965).

A biochemical comparison of the selected and parent strains (Matsumura and Brown, 1961a, 1963a) showed no difference in degradation of malathion by either phosphatase or carboxysterase activity after the malathion selection, but rather a significant decrease in larval absorption of the toxicant. No significant difference in either detoxication by phosphatase hydrolysis or in larval absorption was found after the parathion selection. Plapp et al. (1965) found no difference from the normal in aliesterase activity, nor in cholinesterase or other esterase activity in a malathion selected strain of A. aegypti as compared to a susceptible one.

Nevertheless, A. aegypti larvae are capable of degrading these insecticides to some extent. In contrast to

the rapid increase in resistance to chlorinated hydrocarbons, organophosphorus resistance may develop much more slowly (Brown, 1966, 1967a). Consequently it is important to investigate the effects of long-continued organophosphate selection of A. aegypti on its content of those enzymes capable of hydrolyzing these compounds (Brown, 1967b).

Therefore the present study was undertaken to produce OP-resistant strains of A. aegypti by long continued selection, and to assess the activity of the detoxication enzymes present in these strains and characterize them by means of substrate and inhibitor studies. These enzymes were then separated by zone electrophoresis and visualized by staining techniques.

REVIEW OF THE LITERATURE

Toxic organophosphorus compounds are highly specific inhibitors of those enzymes which have esterase activity, namely the cholinesterases, carboxylesterases and certain lipases, as well as trypsin and chymotrypsin (Dixon and Webb, 1964). The OP insecticides derive their toxicity from inhibiting the enzyme acetylcholinesterase. This enzyme plays a vital role in the nervous system by removing the acetylcholine which is released in the synaptic transmission of nerve impulses, permitting subsequent transmissions (Gilmour, 1965).

Most of these organophosphorus compounds can be considered as esters of alcohols with a phosphorus acid, or as anhydrides of a phosphorus acid with some other acid. The general formula for the majority of organophosphates is $(RO)_2P(O \text{ or } S)X$, where R is usually CH_3 or C_2H_5, and X can vary enormously. Those compounds containing the P(S) group are poor inhibitors *in vitro*, but are converted *in vivo* by desulfuration into the P(O) form and thus becoming much more potent inhibitors. The metabolic processes to which OP insecticides are subject include not only

activation by desulfuration, but also degradation principally by hydrolysis. All the organophosphates can be hydrolyzed by phosphatases, the general name given to enzymes capable of cleaving any phosphorus ester or anhydride bond. Generally the phosphate-side group (P-X) bond is attacked, but the alkyl-phosphate (RO-P) bond can also be hydrolyzed. When the OP insecticide contains a carboxyester (-C(O)OR) or a carboxyamide ($-C(O)NR_2$) group, carboxyesterase or carboxyamidase activity may effect hydrolytic changes which detoxify the compound. There is a great deal of variety in the manner in which different species of insects and higher animals can degrade any given OP compound (O'Brien, 1967).

The development of insecticide resistance, characteristic of treated populations within a susceptible species, is a perfect example of micro-evolution in the Darwinian sense (Brown, 1967a). Natural selection was defined by Darwin as the preservation in nature of favorable variations and the destruction of those that are injurious. Selection does not create new genes, it only changes gene frequency (Brewbaker, 1964). The effect of selection on gene frequency depends not only on the intensity of selection, but also on the initial gene frequency. The response to selection does not continue indefinitely, but rather diminishes in rate as the genetic variance declines, and finally ceases as the strain or population becomes uniformly homozygous for the

favorable alleles. Any further response to selection will depend on the production of new alleles by mutation (Falconer, 1964).

The appearance of OP-resistant populations of houseflies in Denmark (Keiding, 1956) accelerated the study of possible mechanisms of resistance to OP compounds. Oppenoorth (1958) and March (1959) showed that OP-resistance in houseflies was not related to decreased insecticide penetration or thiophosphate activation, nor was it related to higher levels of cholinesterase activity or a cholinesterase less sensitive to inhibition, but rather resulted from more rapid insecticide degradation.

The most coherent theory for OP-resistance in houseflies was advanced by Oppenoorth and van Asperen (1960) who found that in OP-resistant strains of Musca domestica the appearance of detoxifying enzymes is due to a mutant gene allele which produces an altered form of aliesterase enzyme. This modified esterase is no longer inhibited by the OP compound to which the housefly is resistant, but instead slowly degrades it.

Another case where it has been possible to correlate OP-resistance in an insect of public health importance with a specific gene-controlled detoxifying enzyme is that of malathion-resistance in the western encephalitis mosquito Culex tarsalis. Matsumura and Brown (1961a) found its

malathion-resistance to be due partly to a higher phosphatase activity, but mainly to a higher carboxyesterase activity, which was subsequently found to be due to a 13-fold increase in enzyme quantity (Matsumura and Brown, 1963b), under the control of a single partially dominant gene allele.

A beginning has been made in the study of gene-enzyme-OP-resistance relationship in the tropical house mosquito Culex fatigans, in which Stone (1968a) has found that a strain selected to 9-fold fenthion-resistance (Tadano and Brown, 1966) can degrade significantly more fenthion than its susceptible parent strain by means of a phosphatase-type hydrolysis.

In the Acarina an additional OP-resistance mechanism has been found, deriving from a less sensitive cholinesterase target enzyme. Whereas most OP-resistant strains of the two-spotted mite Tetranychus urticae are characterized by increased detoxication (Matsumura and Voss, 1964; Herne, 1967), the Leverkusen strain selected initially with demeton and subsequently with parathion is characterized by a cholinesterase less sensitive to OP inhibition and less active than normal (Smissaert, 1964). A similar insensitive cholinesterase was found in a dioxathion-resistant strain of the cattle tick Boophilus microplus (Lee and Batham, 1966; Stone, 1968b).

The direct relationship between genes and enzymes was first suggested at the beginning of the century from a study of certain congenital metabolic diseases in man. However, the modern concept of genetic control over the presence of enzymes was formulated only in the early 1940's by Beadle and Tatum (1941). Their "one gene, one enzyme" theory postulates a separate gene for the formation of every enzyme; a given enzyme can only be formed if the corresponding gene is present in the cell. Subsequent research has not only verified Beadle and Tatum, but also has expanded their original concept of "one gene, one enzyme" into its more precise form, "one gene, one polypeptide chain". This modification resulted from the discovery that the structure of each type of polypeptide chain, in a protein composed of more than one type, is under the synthetic control of a separate gene (Hartman and Suskind, 1965; Watson, 1965).

In many cases, the presence of the gene alone is insufficient to ensure enzyme synthesis, which is also under an additional control mechanism executed by metabolites, usually the substrate or the end product. Metabolite control ensures that enzymes are produced in the required amounts when necessary, by controlling gene action through enzyme induction, which increases the formation of deficient enzymes, or end product repression, which decreases the formation of those enzymes that are present in excess. Enzyme activity can also be under metabolite control, which

ensures that pre-existing proteins are transformed into an active state whenever needed, by means of enzyme activation or inactivation (Staehelin, 1965; Wilson and Pardee, 1964). While metabolic control of enzyme production is common in microorganisms, Pardee and Wilson (1963) conclude that induction and repression are much less important in higher animals, because in them the rates of enzyme synthesis do not vary greatly under different nutritional or metabolic conditions, and therefore consider the activation and inactivation of the pre-existing enzymes to be the major metabolic control mechanism in more complex organisms.

One of the most useful tools in the study of enzymes is electrophoresis. This process, which is the migration of a charged particle in an electric field, furnishes one of the best methods for separating proteins which occur in tissues as complex mixtures (Dessauer and Fox, 1964). Zone electrophoresis is the separation of a complex mixture into definite zones or bands in a buffer stabilized by a supporting medium such as paper (Plaisted, 1964). The substitution of gels as support media (Smithies, 1955) has led to a great improvement in zone electrophoresis techniques, and thus has aided in the study of proteins in general and enzymes in particular. While the separation of proteins by paper electrophoresis is limited because many proteins adsorb onto the paper, methods utilizing gels are characterized by a high resolving power and speed of separation,

and avoid the adsorption of the protein to the gel matrix (Plaisted, ibid.).

Hydrolytic enzymes can be visualized histochemically by using a reagent that forms an insoluble colored compound with the hydrolyzed product of the substrate. The reagent can be added either together with the substrate and thus capture the hydrolytic product as it is produced, or after the hydrolysis has taken place in a post-incubation coupling reaction (Barka and Anderson, 1965).

Hydrolases were first demonstrated histochemically by Gomori (1939) and Takamatsu (1939), who independently developed a staining technique for detecting phosphatase activity. Their method was based on the final conversion of the released phosphate to a silver phosphate which precipitates and then turns brown when exposed to light.

Menten et al. (1944) made an important contribution to histochemistry by introducing a new principle, the azo dye method, in a test for alkaline phosphatase. Their method detects the organic instead of the phosphate moiety of a phosphate ester, and uses a mono-aryl (naphthyl) phosphate as substrate for the phosphatase. After hydrolysis, the released aryl molecule is directly coupled with a diazotized amine, and precipitates as an insoluble dye at the site of enzyme activity. By substituting naphthyl acetate instead of naphthyl phosphate as the substrate, Nachlas and Seligman (1949) could utilize the azo dye method for the

histochemical demonstration of esterase activity in general.

Hunter and Markert (1957) combined the more advanced techniques of zone electrophoresis with the histochemical staining methods for identifying enzymes in order to analyze the enzymatic composition of biological material. They proposed the term zymogram to refer to strips in which the location of enzymes is demonstrated by histochemical methods. Each organ studied showed characteristically distinct zymograms, and the substrate specificities of some of the esterases were found to be different.

One of the first results of the zymogram technique was the demonstration that enzymes exist in multiple molecular forms, not only within a single organism, but even within a single tissue. Markert and Moller (1959) proposed the term isozyme for the different molecular forms in which proteins may exist with the same enzymatic specificity. Laufer (1961), a pioneer in the electrophoretic study of insect proteins, found multiple forms of insect enzymes, including esterases. Enzyme polymorphism provides markers enabling the determination of genetic control over specific enzymes (Shaw, 1965).

Multiple molecular forms of esterases and phosphatases and the genes controlling them have been studied in 3 species of insects to date. In M. domestica Ogita and Kasai (1965a, 1965b) found on chromosome 5 (now called chromosome II on the basis of length) two loci controlling esterase

activity and a pair of codominant alleles controlling acid phosphatase activity. In Drosophila melanogaster, Wright (1963) showed the existence of two forms of a non-specific esterase to be under the genetic control of a pair of codominant alleles on the third chromosome, while Beckman and Johnson (1964) found that electrophoretic variations in alkaline phosphatase activity also were controlled by a pair of codominant alleles on this chromosome. MacIntyre (1966) discovered the same situation with variants of an acid phosphatase in D. melanogaster and D. simulans.

Since many enzymes that hydrolyze carboxyl esters show a wide substrate specificity, basing their classification on that criterion alone would be of limited value (Oosterbaan and Jansz, 1965). The first clear differentiation between enzymes hydrolyzing aliphatic esters was shown by Richter and Croft (1942) in their study of blood esterases. They found that eserine sulphate, at a concentration of 10^{-5}M, completely inhibits cholinesterase, but does not inhibit the hydrolysis of aliphatic esters by any other esterase. They therefore introduced the term aliesterase to characterize those enzymes which preferentially hydrolyze the simple aliphatic esters and glycerides.

The next step in the discrimination between esterases by means of selective inhibitors was based on the reaction of esterases with organophosphates. Aldridge (1953a), using p-nitrophenyl esters as substrate and diethyl p-nitrophenyl

phosphate (paraoxon) as inhibitor, found that the esterases present in many sera can be divided into two distinct types. One, like cholinesterase, is inhibited by concentrations of paraoxon as low as 10^{-8}M, while the other is not inhibited by concentrations up to 10^{-3}M. The insensitive esterase hydrolyzed p-nitrophenyl acetate more rapidly than p-nitrophenyl butyrate, and was called A-esterase. The sensitive esterase hydrolyzed the butyrate at the same or a higher rate than the acetate, and was named B-esterase. Letters were used as the prefix in order to avoid placing any emphasis on substrate specificity.

In a subsequent paper, Aldridge (1953b) showed that his A-esterase was identical with the enzyme in mammalian sera which is capable of hydrolyzing diethyl p-nitrophenyl phosphate. That A-esterase is not only insensitive to paraoxon, but also capable of hydrolyzing it, prompted Bergmann et al. (1957) to introduce the term C-esterase for an esterase which they found in hog kidney for which diisopropyl phosphorofluoridate (DFP) was neither inhibitor nor substrate. However, the introduction of this new term is unnecessary, since Aldridge (1954) himself had discovered an A-type esterase in rat pancreas which also does not hydrolyze OP inhibitors such as DFP and paraoxon. Mounter (1963) emphasizes the fact that not all esterases classified as A-esterases are capable of hydrolyzing toxic phosphorus esters.

Mounter and Whittaker (1953) studied the hydrolysis of aromatic esters by human blood cholinesterases. Not only were the p-substituted phenyl acetates rapidly hydrolyzed by the cholinesterases, but they also were attacked by two other esterases in the blood, both insensitive to eserine, but differing in inhibition by organophosphates. One of these two latter enzymes, sensitive to both DFP and paraoxon, hydrolyzed aromatic esters generally more slowly than tributyrin; it appeared to be identical with aliesterase, and possibly can be identified with Aldridge's B-esterase. The other esterase, highly resistant to organophosphorus inhibitors, was called aromatic esterase; it attacked aliphatic esters slowly, and probably was identical to A-esterase.

The most extensive studies dealing with the electrophoretic separation and classification of blood plasma esterases were made by Augustinsson (1959, 1961). He detected 3 groups of esterases, namely cholinesterases, aliesterases and arylesterases (aromatic esterases), on the basis of their electrophoretic mobility relative to known protein fractions, supplemented by their substrate specificity and their sensitivity to selective inhibitors. All 3 types were not found in the plasma of every species of animal studied, and multiple forms of each type were found, differing not only between species, but also within the same species.

The cholinesterases had the slowest electrophoretic mobility, and normally migrated between the α_2- and β-globulin fractions. They were the only esterases to hydrolyze choline esters, which were cleaved at a higher rate than aliphatic or aromatic esters, and they hydrolyzed the aromatic esters either very slowly or not at all. Since they are sensitive to inhibition by OP compounds, cholinesterases can be considered as B-type esterases.

The aliesterases moved more rapidly than the cholinesterases, and were found in or close to the α_1-globulin fraction. They split both aliphatic and aromatic esters, some even hydrolyzing aromatic esters faster than the corresponding aliphatic ester. Nevertheless the term aliesterase still can be used, as its definition has not been invalidated. Aldridge's OP-sensitive B-esterase was found to be identical with aliesterase. The aliesterases are differentiated from cholinesterase in not being inhibited by eserine.

The arylesterases had the greatest mobility of the three groups of esterases, and moved close to or with the albumins. They are typical aromatic esterases, hydrolyzing phenyl acetate more rapidly than phenyl butyrate, and normally do not attack aliphatic esters. Aldridge's paraoxon-insensitive A-esterase was found to be identical with arylesterase.

Insects and other invertebrates, like the vertebrates, contain numerous enzymes that hydrolyze esters, and the general similarity of esterases in insects and mammals is more striking than their differences (Chadwick, 1963). In insects, cholinesterase was first reported by Gautrelet (1938), who discovered the enzyme in the brain of honey-bees. Metcalf et al. (1955) studied the substrate specificity of insect cholinesterases. They found an enzyme with all the properties of an acetylcholinesterase in the heads of 18 species and in the whole bodies of 6 more. The enzyme activity ranged over at least a factor of 10, and the highest activity was present in Diptera, where it equalled, per unit weight of brain tissue, that of any known natural source of acetylcholinesterase. A wide variety of aliphatic esters, and phenyl acetate also, were hydrolyzed.

While studying the mechanism of action of OP compounds as insecticides, Lord and Potter (1950) found that extracts of 7 species of insects hydrolyzed ethyl butyrate and nitrophenyl acetate but not acetylcholine, and were inhibited by tetraethyl pyrophosphate (TEPP). On continuing their studies (Lord and Potter, 1954a), they found two types of esterases that were inhibited by low concentrations of organophosphates, one capable of hydrolyzing acetylcholine, and the other splitting phenyl acetate but not acetylcholine which they called "general esterase" (Lord and Potter, 1954b). From its sensitivity to OP compounds, their

"general esterase" can be considered as a B-type esterase or aliesterase. These findings prompted their suggestion that the inhibition of esterases other than cholinesterase might play an important part in the mechanism of action of the OP compounds. The debate over the major biochemical lesion was finally settled when Stegwee (1959) found that houseflies, in which the aliesterase or "general esterase" activity had been almost completely inhibited by tri-o-cresyl phosphate (TOCP) without any appreciable reduction in cholinesterase activity, showed no symptoms of organophosphorus poisoning.

The presence of an aromatic esterase in insects was first noted by Metcalf et al. (1955) while studying cholinesterase activity in M. domestica and Apis mellifera. They found that homogenates of either species hydrolyzed phenyl acetate more rapidly than choline esters and aliphatic esters. In further studies using the selective inhibitors eserine and paraoxon, Metcalf et al. (1956) found the insect tissues to contain, besides a cholinesterase and an aliphatic esterase, an aromatic esterase which hydrolyzed phenyl acetate faster than phenyl propionate or butyrate, or substituted phenyl acetates. This aromatic esterase also hydrolyzed a number of aromatic phosphorus esters 40-70% as fast as the corresponding acetates. Paraoxon and parathion were included among these phosphorus esters, thus demonstrating a possible detoxication mechanism for these

toxicants.

Housefly esterases have been studied in detail, especially in connection with resistance to OP insecticides, by van Asperen (1958, 1959, 1962) and Stegwee (1960). Acetylcholinesterase hydrolyzed, besides choline esters, other aliphatic and aromatic esters such as triacetin, methyl and ethyl acetate and phenyl, nitrophenyl and naphthyl acetate. Methyl butyrate and tributyrin were not attacked by the cholinesterase. Aliesterase hydrolyzed the same non-choline aliphatic and aromatic esters as the cholinesterase did, and methyl butyrate and tributyrin as well. Aromatic esterase activity, splitting phenyl and nitrophenyl acetate and triacetin, was also detected.

Van Asperen and Oppenoorth (1959) compared the esterase activity of OP-resistant houseflies with that of susceptible ones. While there was no difference in the cholinesterase activity, they found that the resistant strains had much lower aliesterase activity than the susceptible ones, the difference being 2-fold when tributyrin was the substrate, and nearly 6-fold when methyl or ethyl butyrate were used as substrates. The lack of interstrain difference in cholinesterase activity and the great difference in aliesterase activity were confirmed by Bigley and Plapp (1960).

Van Asperen (1962) found that the results were the same with α-napthyl acetate as substrate as with methyl butyrate,

the activity in resistant strains being about 15% of that in susceptible flies. He subsequently found (van Asperen, 1964) that a high percentage of the total non-cholinesterase hydrolysis of certain substrates,--namely methyl and ethyl butyrate, phenyl acetate, propionate and butyrate, and α-napthyl acetate--, resulted from the activity of a single aliesterase, which he named AliE-a to differentiate it from other aliesterases. Van Asperen (ibid.) concluded that it was the absence of this enzyme from the resistant strains he studied that was the cause of their aliesterase activity being much lower than in susceptible strains.

With the aid of agar-gel electrophoresis and the zymogram technique, van Asperen (1962) studied the esterase patterns in centrifuged homogenates of OP-resistant and susceptible strains. He found up to 7 bands of esterase activity, with strain-specific patterns. No clear correlation of esterase pattern with resistance was observed, and this was found to be due to the AliE-a being mainly in the particulate cell fraction. When van Asperen and van Mazijk (1965) treated the homogenates with 5×10^{-3}M deoxycholate to solubilize the AliE-a, they found a very active anodically-moving esterase in zymograms of the susceptible flies, and this was absent in those of resistant strains. Semi-quantitative determinations indicated that this band was responsible for about 80% of the total aliesterase activity, confirming the biochemical studies.

Ogita and Kasai (1965a) also submitted housefly homogenates to thin-layer agar-gel electrophoresis, and found as many as 24 esterase bands. Pronounced differences were observed between the various strains, but there were no characteristic differences between diazinon-resistant and susceptible strains. The lack of a clear difference is possibly the result of their using β-naphthyl acetate, for AliE-a has little activity on this substrate (van Asperen, 1962) and therefore would not display the presence of the enzyme in the zymogram.

Menzel et al. (1963), using starch-gel electrophoresis, also studied the properties of esterases from susceptible and resistant strains. They demonstrated the presence of cholinesterases, aliesterases and arylesterases with the aid of selective inhibitors. While a detailed comparison between a susceptible and a malathion-resistant strain revealed no esterase pattern characteristic of resistance (also probably due to their use of β-naphthyl acetate), the enzymes hydrolyzing naphthyl acid phosphate at pH 6.8 were both more numerous and active in the malathion-resistant strain than in the susceptible one.

More recent work has paid special attention to the characterization and classification of the enzyme bands. Salkeld (1965) used starch-gel electrophoresis to separate the soluble esterases of the large milkweed bug, Oncopeltus fasciatus, and found 14 esterase bands in the egg and 19 in

the young nymphs. Utilizing eserine and paraoxon to differentiate between the different types of esterases, she determined that most of them were arylesterases; the remainder were aliesterases, while 2 bands could not be classified. No cholinesterases were detected.

Arurkar and Knowles (1968) studied the soluble esterases of 6 species of insects,--namely the black blister beetle Epicauta pennsylvanica, the American cockroach Periplaneta americana, the house cricket Acheta domesticus, the fall webworm Hyphantria cunea, the yellow-striped armyworm Prodenia ornithogalli and the bollworm Heliothis zea--, by means of acrylamide-gel zone electrophoresis, and classified them with the aid of inhibitors. They discriminated up to 10 bands of esterase activity in each species. Again none of the bands were cholinesterase, and all of them were of the aliesterase type, except for 2 in the bollworm, which were classified as arylesterases.

Using starch-gel electrophoresis followed by the zymogram method, Cook and Forgash (1965) studied the esterases in P. americana. They found 12 zones of esterase activity which exhibited definite substrate preferences. Tests with inhibitors differentiated the bands into 6 aliesterases and 5 arylesterases, while the remaining band was a cholinesterase. Matsumura and Sakai (1968) used agar-gel electrophoresis and selective inhibitors to differentiate the esterases of this species and found 13 bands. They classified 6 of them as A-type and 7 as B-type

esterases, with one of the B-type being a cholinesterase. They were able to establish that 3 of the A-type esterases could hydrolyze a number of organophosphorus compounds, while 2 of the B-type esterases could attack the carboxylic ester site in malathion.

Kasai and Ogita (1965), using thin-layer agar-gel electrophoresis, studied the relationship between malathion-resistance and esterase activity in the green rice leaf-hopper Nephotettix cincticeps. Among the 12 bands of esterase activity which appeared, they found that one of them (E_9) was more intense in the malathion-resistant strains. One of the malathion-resistant strains showed cross-resistance to all the OP compounds tested, and therefore its phosphatase activity was also assessed. No difference from the normal was found in its acid phosphatase zymograms. But with alkaline phosphatase activity, this malathion-resistant strain revealed 3 bands whereas the susceptible strain showed no detectable activity.

MATERIALS AND METHODS

Biological Material

Stock colonies of *Aedes aegypti* had been obtained from Kongolikan, Penang and Trinidad, and from each of these a malathion-tolerant strain and a parathion-tolerant strain had been produced by continuous selection with malathion or parathion. The history of each colony and strain is as follows:

A. Kongolikan (Rennes): originated from a collection made in 1960 in Kongolikan, Upper Volta, in the interior of West Africa. It was colonized at the University of Rennes, France, and Prof. J. R. Doby supplied a sub-colony to this laboratory in 1961.

Kongolikan-Malathion: a malathion selected strain developed from the Kongolikan stock by Matsumura and Brown (1963a), the larval LC_{50} level rising from 0.06 ppm (parts per million) to 0.30 ppm in 5 generations of selection. Subsequent selection (Kimura, unpublished data 1964) for 5 generations further increased the LC_{50} to 0.58 ppm.

Kongolikan-Parathion: a parathion-selected strain

developed by Matsumura and Brown (ibid.) from the Kongolikan stock. Five generations of larval selection increased the LC_{50} from 0.008 ppm to 0.027 ppm. A second regime of selection (Kimura, ibid.) for 6 generations raised the LC_{50} to 0.046.

B. Penang: originally collected in 1956 on Penang Island, Malaysia, from a district where insecticides had not been applied. It was first cultured in the Institute for Medical Research at Kuala Lumpur. In 1957, a sub-colony was sent to the Army Chemical Center, Maryland, USA, which in turn supplied this laboratory with a sub-colony in 1958.

Penang-Malathion: a malathion-selected strain developed by Brown and Abedi (1960) from the Penang colony. After 8 generations the LC_{50} increased from 0.30 ppm to 1.40 ppm. Four more generations of larval selection (Pillai, 1964) raised it to 1.60 ppm. Subsequent selection for 6 generations (Kimura, ibid.) after relaxation of selection which resulted in a decrease in the LC_{50} to 1.05 ppm did not increase the larval LC_{50} from that level.

Penang-Parathion: a parathion-selected strain developed by Kimura (ibid.) from the Penang stock. Five generations of larval selection failed to increase the LC_{50} above that of the parental colony (0.035 ppm).

C. Trinidad: originated from a field population showing DDT-resistance collected at Aranguez, Trinidad, West

Indies in 1954 and cultured at Port-of-Spain. A subculture commenced at the Technical Development Laboratories, Savannah, Georgia, USA, was the source of a second subculture which was maintained at the Army Chemical Center, Maryland, USA, which in turn supplied a subcolony to this laboratory in 1958.

Trinidad-Malathion: a malathion-selected strain developed from the Trinidad stock by Matsumura and Brown (1963a), who raised the larval LC_{50} in 5 generations of selection from 0.12 ppm to 0.73 ppm. After selection had been relaxed and the tolerance had reverted, a second regime of selection (Kimura, ibid.) for 3 generations brought the LC_{50} level back to 0.60 ppm.

Trinidad-Parathion: a parathion-selected strain developed from the Trinidad colony by Matsumura and Brown (ibid.). Five generations of larval selection increased the LC_{50} from 0.016 ppm to 0.030 ppm. A subsequent selection (Kimura, ibid.) for 6 generations did not raise the larval tolerance.

In addition, two strains were studied which had already developed tolerance to malathion by the time they were collected in the field, namely:

D. Montego Bay: originated from a malathion-tolerant field collection conducted in 1964 by the Pan American Health Organization (PAHO) in Montego Bay, Jamaica. A subculture was supplied by PAHO at the end of 1964.

E. Albina: originally field-collected in Surinam in 1964 by PAHO and found to be malathion-tolerant. A subculture was forwarded to this laboratory by PAHO in the same year.

Rearing Methods

The colonies and strains of mosquitoes were maintained in plastic-screened cages 18 inches square fitted with cloth sleeves, which were kept in an environment-controlled insectary at 25°C and a relative humidity between 75 and 80%. Females were given blood meals 3 times a week by placing a guinea pig, tightly rolled in a wire screen, in each cage for 30 minutes. Raisins suspended on a string provided food for the males. Eggs were collected on strips of paper towelling 1.5 inches wide lining the inside of enamel pans, 11 x 8 x 1.5 inches deep, half-filled with distilled water. The oviposition papers were removed and replaced once a week, allowed to dry and were stored in glass jars. Under these conditions, the eggs remained viable for about 3 months.

The eggs were caused to hatch by placing the oviposition papers in stoppered Erlenmeyer flasks containing deoxygenated water, prepared by boiling the water and stoppering the flask before cooling it. This ensured that virtually all the larvae had hatched at the same time. The larvae were reared in enamel pans, 16 x 9.5 x 2 inches deep, half-filled with distilled water, and covered with aluminum screening to prevent escaped females from ovipositing in the

pans and contaminating the cultures. About 250 larvae were placed in each pan and were fed every second day on a diet of 5 parts brewers yeast powder, 2 parts powdered albumin and 1 part RNA. The pupae obtained were then placed in the adult cages for emergence. When the adults were to be used in crosses, the pupae were placed in stoppered vials, 14 x 46 mm in size; the adults which emerged were divided according to sex, and admitted to the cages for the appropriate mass crosses.

Toxicological Methods

Larval susceptibility tests were conducted on early 4th instar larvae by means of the WHO standard method for mosquito larvae (WHO, 1963). Batches of 25 larvae were placed in 450-ml glass jars containing 1-ml aliquots of ethanolic solutions of the desired concentration of insecticide and 249 ml of distilled water. Tests were made in duplicate at each of a number of concentrations in a geometric series. The percentage mortalities, based on the dead and moribund larvae counted together after 24-hr exposure, were plotted on a probability scale against insecticide concentration on a logarithmic scale. The dosage-mortality lines were fitted by eye and yielded the LC_{50} values.

Insecticide larval selection was performed on 4th instar larvae by exposing 3000-5000 larvae for 24 hr to a concentration of insecticide that was expected to cause 90%

mortality as determined by the LC_{50} tests, the dosage being adjusted in each generation when necessary. The survivors were rinsed with distilled water and reared for pupation and emergence.

The following insecticides in ethanolic solutions were used:

Malathion: diethyl mercaptosuccinate, S-ester with O,O-dimethyl phosphorodithioate, 99.6% pure, obtained from City Chemical Corp., New York, N.Y.

Parathion: O,O-diethyl O-p-nitrophenyl phosphorothioate, 98.76% pure, obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.

Abate: O,O,O',O'-tetramethyl O,O'-thiodi-p-phenylene phosphorothioate, 93.7% pure, supplied by American Cyanamid Co., Princeton, N.J.

Biochemical Assay of Esterase Activity

The esterase activity of the Kongolikan-Malathion strain, which showed the highest tolerance, and its susceptible parent stock, Kongolikan (Rennes) was assessed by the quantitative colorimetric test of Hestrin (1949) as modified by Bigley and Plapp (1960) and Plapp et al. (1965). The test is based on the reaction of carboxylic esters with alkaline hydroxylamine to yield acethydroxamic acids, which form a soluble red-purple complex with ferric ions in acid

solution, the intensity of the color being proportional to the concentration of the ester.

Fourth-instar larvae were homogenized immediately before assay in refrigerated 0.134M sodium phosphate-potassium phosphate buffer at pH 7.2 (prepared by mixing 7 parts (v/v) $Na_2HPO_4.7H_2O$, 35.761 g/liter and 3 parts KH_2PO_4, 18.156 g/liter). Lots of 1 g each were homogenized in 10 ml of buffer for 90 seconds at 750 rpm in a Potter-Elvehjem type glass homogenizer with a power-driven teflon pestle. 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 phosphate buffer until 25 ml were collected in a measuring cylinder, resulting in a concentration of 40 mg larvae/ml buffer. Whenever necessary the homogenate concentration was further diluted.

The substrates and concentrations used were $2x10^{-3}M$ acetylcholine bromide (Eastman Organic Chemicals, Rochester, N.Y.), $4x10^{-3}M$ methyl butyrate (British Drug Houses Ltd., Poole, England), $4x10^{-3}M$ phenyl acetate (Eastman Organic Chemicals, Rochester, N.Y.) and $2x10^{-3}M$ tributyrin (Fisher Scientific Co., Fair Lawn, N.J.). All the substrates were prepared in the phosphate buffer described above except for acetylcholine bromide; this was first dissolved in $1x10^{-3}M$ sodium acetate-acetic acid buffer at pH 4.5 to a concentration of $2x10^{-2}M$ and diluted ten times with

phosphate buffer prior to use.

Analyses were performed by pipetting 1-ml aliquots of the homogenates and 1 ml of the substrate into 18 x 150 mm test tubes and incubating the samples in a hot water bath at 37°C for 15 min. The enzymatic hydrolysis was then stopped by the addition of 2 ml alkaline hydroxylamine, prepared by mixing equal volumes of 2M hydroxylamine hydrochloride (Fisher Scientific Co., Fair Lawn, N.J.) and 3.5M sodium hydroxide shortly before use, which reacts with the unhydrolyzed substrate. Two minutes later, 1 ml hydrochloric acid (concentrated acid diluted three times) and 1 ml 0.37M ferric chloride in 0.1N hydrochloric acid were added, and the solution was filtered through Whatman No. 40 filter paper. The optical density of the brown color formed was determined 10 min after the addition of the ferric chloride, by means of a Beckman DU spectrophotometer set at 540 millimicrons wavelength and a 1-cm light path. A blank correction for non-specific color was obtained using the same procedure, except that the order of addition of the alkaline hydroxylamine and the hydrochloric acid was reversed. The amount of unhydrolyzed substrate remaining in each sample was calculated from a standard curve of optical densities plotted against micromoles of substrate, determined from a series of concentrations of substrate incubated for 15 min at 37°C in 2 ml phosphate buffer and then treated with alkaline hydroxylamine, hydrochloric acid

and ferric chloride as described above.

The esterase activity was differentiated into cholinesterase, aliesterase and arylesterase types on the basis of its inhibition by 10^{-5}M eserine and 10^{-5}M paraoxon. The enzyme inhibition was determined by incubating 0.9-ml aliquots of homogenates with 0.1 ml amounts of aqueous solutions of $1x10^{-4}$ eserine sulphate (Nutritional Biochemicals Corp., Cleveland, Ohio) or $1x10^{-4}$M paraoxon (American Cyanamid Co., Princeton, N.J.) for 15 min at 37°C; then 1 ml substrate was added, the samples were incubated for another 15 min and the remaining unhydrolyzed substrate was determined as previously described.

Electrophoretic Separation and Visualization of Esterases

The esterases were separated by means of thin-layer agar-gel electrophoresis (Fig 1) on glass plates, according to the method of Ogita (1964). The following discontinuous buffer systems (i.e. the buffer in the electrode vessel differs in some way from that in the agar layer) and agar-gel media were used:

Potassium phosphate buffer, pH 6.8:

Constituents	Buffer in electrode vessel	Buffer in agar-gel layer
Monobasic potassium phosphate	17.01 g	2.552 g
Dibasic potassium phosphate	22.27 g	3.341 g
Deionized water to	10 liters	3 liters

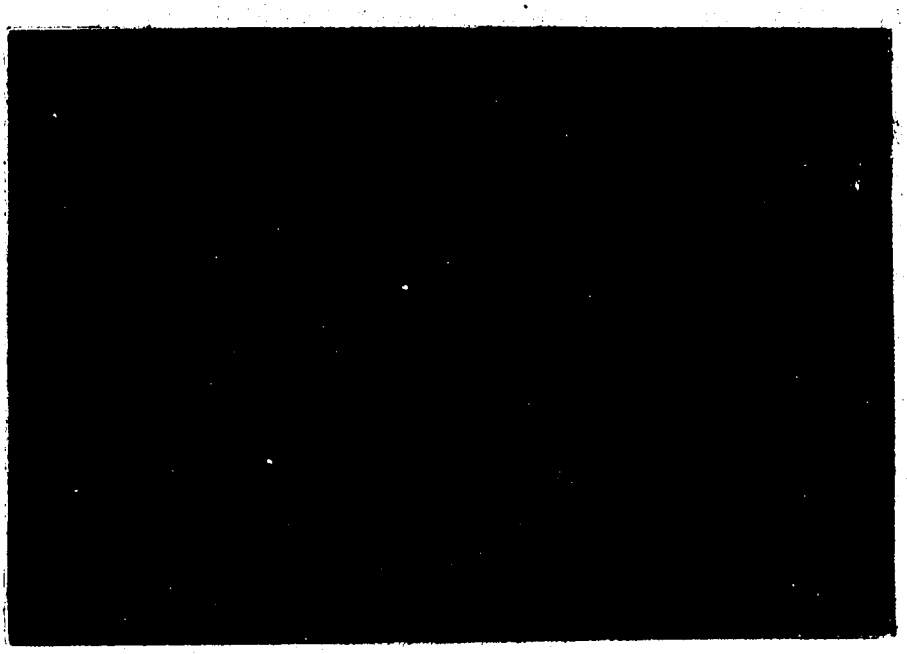


Fig. 1. Voltage regulators and refrigerated cabinet containing thin-layer agar-gel electrophoresis units (Ogita, 1964).

Fig. 1. Voltage regulators and refrigerated cabinet containing thin-layer agar-gel electrophoresis units (Ogita, 1964).

PVP (polyvinyl pyrrolidone)-phosphate agar medium:
20 g PVP (Luviskol K-90, molecular weight 700,000, Badische Anilin und Soda Fabric, Baden, West Germany) and 7 g Difco Bacto-Agar (Difco Laboratories, Detroit, Mich.) were added to 1 liter potassium phosphate buffer.

Calcium lactate-barbital buffer, pH 8.6:

Constituents	Buffer in electrode vessel	Buffer in agar-gel layer
Barbital	13.80 g	1.660 g
Sodium barbital	87.60 g	10.510 g
Calcium lactate	3.84 g	1.536 g
Deionized water to	10 liters	3 liters

PVP-barbital agar medium: 7 g PVP and 7 g Difco Special Agar-Noble (Difco Laboratories, Detroit, Mich.) were added to 1 liter calcium lactate-barbital buffer.

The agar media were made in 1-liter amounts by boiling the mixtures in a hot water bath until they had become completely clear, and were then poured in 50-ml amounts into 125-ml Erlenmeyer flasks; these were covered with aluminum foil and kept at 5°C until used. Thin-layer plates were prepared by liquefying the stored agar-gel and applying it by pipette onto glass plates, usually 15 x 20 or 15 x 10 cm in size, which had been placed on a levelled slide warmer at 60°C. When a uniform layer 0.7-0.9 mm in thickness had been produced, the coated plates were placed to solidify in the refrigerated cabinet maintained at 5-10°C.

Fourth-instar larvae of all the selected strains and stock colonies were homogenized in 1 g batches with a Potter-Elvehjem homogenizer in 1 ml ice-cold 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 Whatman No. 1 filter paper on it for 5 min prior to application. A 20 x 5 cm strip of Whatman No. 1 filter paper formed the bridge between the buffer vessel and the agar-gel layer. The electrophoresis was performed at a constant potential of 300 V between the electrodes, giving a current of 1.5-2 mA/cm width, for 1.5-2 hr at 5-10°C.

Esterase activity was assessed according to the method of Ogita (1964). After the electrophoresis had been completed the plates were sprayed with 1% α-naphthyl acetate or 1% β-naphthyl acetate (Nutritional Biochemicals Corp., Cleveland, Ohio) in acetone solution, and were incubated at 37°C for 5-15 min with the α isomer and for 20-40 min with the β isomer. The esterase bands were then visualized by spraying the plates with a 1% aqueous solution of Fast Blue B Salt (Sigma Chemical Co., St. Louis, Mo.). The developed zymograms were fixed by immersing the plates in 5% acetic acid for 20-30 min, washing them in distilled water for 4-6 hr, and drying them in a stream of warm air.

The non-specific esterases were differentiated into A and B types on the basis of their susceptibility to

inhibition by paraoxon, by adding 0.5 ml aliquots of paraoxon in ethanolic solution to 50 ml quantities of the warm liquid agar gel before it was pipetted onto the glass plates. After electrophoresis, the paraoxon-treated and control plates were incubated with substrate for 1 hr at 37°C, and then the bands were visualized and fixed in the usual manner.

In an attempt to identify cholinesterase activity, aqueous solutions of eserine sulphate were incorporated in the agar gel. In addition, 2 specific cholinesterase staining tests based on the hydrolysis of acetylthiocholine as substrate were applied. In the test of Uriel (1961, cited in Wieme 1965), the thiol groups set free reduce a tetrazolium salt to give the corresponding insoluble formazan; here 5 mg INT (p-iodonitrotetrazolium violet, Mann Research Laboratories, New York, N.Y.) dissolved in 1 ml ethanol were added to 19 ml of a solution of 20 mg acetylthiocholine iodide (Nutritional Biochemicals Corp., Cleveland, Ohio) in 0.2M sodium barbital-hydrochloric acid buffer at pH 8.2, and the substrate-tetrazolium mixture was incubated for 4-6 hr at 24°C. In the test of Bunyon and Taylor (1966), the thiocholine set free reduces ferricyanide to ferrocyanide, which combines with divalent copper ions to form the insoluble copper ferrocyanide; here the substrate-ferricyanide mixture consisted of 5 mg acetylthiocholine iodide dissolved in 16 ml 0.1M sodium

maleate, 1 ml 0.1M sodium citrate, 2 ml 0.03M copper sulphate and 4 ml 2.5mM potassium ferricyanide, and incubation was allowed to proceed for 4-6 hr at 24°C.

Acid and alkaline phosphomonoesterase 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% sodium α-naphthyl acid phosphate (Dejac Laboratories, Philadelphia, Penn.) in 0.2M acetic acid-sodium acetate buffer, pH 5.0, containing 0.1M magnesium sulphate. For alkaline phosphatase activity, the substrate solution was 0.1% sodium β-naphthyl acid phosphate (Dejac Laboratories, Philadelphia, Penn.) in 0.2M sodium barbital-hydrochloric acid 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 and fixed in the same manner as were the esterases.

Protein staining was carried out according to Wieme (1965) and Bunyan and Taylor (1966). First the plates after electrophoresis were placed for 1-2 hr in an incubator heated to 90-100°C both to dry the agar-gel layer and to fix the separated proteins. The dry plates were then incubated for 2 hr at 24°C with a 0.4% solution of Amido Black 10-B (British Drug Houses (Canada) Ltd., Toronto, Ont.) in methanol, water and acetic acid in a

5:5:1 ratio, after which the excess dye was removed by continuous washing with this solvent mixture and the plates were dried again in a stream of warm air.

To determine whether sublethal concentrations of insecticide might have any inductive or activating effects on the esterases or phosphomonoesterases, batches of 4th-instar larvae were exposed to the maximum sublethal concentrations of malathion or parathion for 24 hr, followed by rinsing in water and immediate homogenization. The homogenates of these treated larvae were then submitted to agar-gel electrophoresis.

In an attempt to detect whether any of the enzyme bands could hydrolyze the organophosphorus toxicants, ethanolic solutions of malaoxon (American Cyanamid Co., Princeton, N.J.) and paraoxon were incorporated in agar-gel coatings as described for the inhibitor studies. After the homogenates had been applied and submitted to electrophoresis, the plates were incubated for 3 hr at 24°C and high humidity to allow any hydrolytic degradation of the oxonated forms of malathion and parathion to occur. The plates were then sprayed with a 0.25 mg/ml solution of bovine-erythrocyte acetylcholinesterase (Nutritional Biochemicals Corp., Cleveland, Ohio), and incubated for 1 hr to permit the unhydrolyzed insecticide to inhibit the acetylcholinesterase. The uninhibited cholinesterase was detected by the tetrazolium method previously described,

red areas appearing where the inhibitor had been hydrolyzed by certain enzymes in the zymogram.

The zymograms figured in the thesis were the clearest out of 4 to 5 plates run on different occasions in each of the tests. All zymograms for the same test and material showed the same bands, only their resolution differing. Conditions such as age of larvae and degree of homogenization were kept rigidly uniform, so that the comparison between strains was direct in all cases. Attempts to quantitate the band intensities by means of chromatographic scanning apparatus were unsuccessful.

RESULTS

Insecticide Selection

A. Malathion: when malathion selection was continued on the Trinidad-Malathion strain (Table 1) the LC_{50} slowly increased for 5 generations to reach a level of 0.79 ppm, it having increased from 0.45 ppm up to 0.60 ppm in the 3 generations of selection before this study was commenced. However, 3 further generations of selection had the unexpected effect of reducing the LC_{50} from 0.79 ppm down to about 0.55 ppm.

The continuation of malathion selection on the Kongolikan-Malathion strain (Table 1) resulted in a doubling of the 0.58 ppm LC_{50} to 1.20 ppm in the first generation, it having already increased from 0.29 ppm in the 5 generations of selection previous to this study. Subsequent selection for the remaining 7 generations in this study witnessed a gradual decrease in LC_{50} to about 0.75 ppm.

On the other hand, continued selection of the Penang-Malathion strain (Table 1) for 8 generations did not increase the LC_{50} above 1.05 ppm as was the case in the

Table 1. Larval LC_{50} levels in ppm malathion of the Kongolikan-, Penang- and Trinidad-Malathion strains of Aedes aegypti subjected to further selection with malathion.

Generation	KONGOLIKAN-MALATHION	PENANG-MALATHION	TRINIDAD-MALATHION
0	0.58	0.05	0.60
1	1.20	1.05	0.58
2	0.94	0.90	0.72
3	0.86	0.81	0.72
4	1.00	0.89	0.78
5	0.64	0.64	0.79
6	0.70	0.74	0.55
7	0.76	0.88	0.54
8	0.79		0.57

This regime of malathion selection in these strains therefore consisted of the following number of generations:

Previous to this study	5	6	3
This study	8	7	8
Total	13	13	11

previous 6 generations of selection, but after the first selection the LC_{50} of the successive generations steadily decreased to about 0.80 ppm.

Thus the LC_{50} for the Kongolikan-Malathion strain never exceeded 1.20 ppm, that of the Penang-Malathion strain 1.05 ppm and that of the Trinidad-Malathion strain never rose above 0.79 ppm. On crossing these malathion-selected strains and subjecting their progeny to malathion selection (Table 2), the malathion-resistance levels could not be increased above those peaks already reached by the parental strains.

When the somewhat malathion-tolerant field strains from Albina and Montego Bay were submitted to 7 generations of laboratory selection with malathion (Table 3), very little changes in the LC_{50} were effected. Whereas the LC_{50} of the Albina strain slightly increased from 0.23 ppm to 0.32 ppm, the Montego Bay strain even lost some of its field-acquired tolerance with its LC_{50} decreasing from 0.45 ppm to 0.37 ppm.

B. Parathion: subjecting the Kongolikan-, Penang- and Trinidad-Parathion strains to 6-7 generations of further parathion selection (Table 4), after the previous lengthy regimes of intensive insecticide selection, failed to materially increase the LC_{50} above that of the base generation for the Kongolikan- and Trinidad-Parathion strains, which was 0.046 ppm and 0.030 ppm respectively.

Table 2. Larval LC_{50} levels in ppm malathion of the offspring of crosses between the Kongolikan-, Penang- and Trinidad-Malathion strains and of subsequent generations after selection with malathion.

Cross	KONGOLIKAN ♂ X PENANG ♀		PENANG ♂ X KONGOLIKAN ♀		(KONGOLIKAN X PENANG)♂ X TRINIDAD ♀	
Parental strains	1.20	1.05	1.05	1.20	0.98	0.58
F_1 hybrid	0.98		0.92		1.10	
Subsequent generations after selection:						
F_2	0.86		1.10		0.85	
F_3	0.75		1.15		0.92	
F_4	0.88		1.20		0.80	
F_5			0.90			
F_6			0.74			

Table 3. Larval LC_{50} levels in ppm malathion of the Montego Bay and Albina colonies of *Aedes aegypti* subjected to selection with malathion.

Generation	MONTEGO BAY	ALBINA
P	0.45	0.23
F_1	0.33	0.20
F_2	0.30	0.28
F_3	0.28	0.26
F_4	0.32	0.37
F_5	0.40	0.35
F_6	0.39	0.34
F_7	0.37	0.32

Table 4. Larval LC_{50} levels in ppm parathion of the Kongolikan-, Penang- and Trinidad-Parathion strains of Aedes aegypti subjected to further selection with parathion.

Generation	KONGOLIKAN-PARATHION	PENANG-PARATHION	TRINIDAD-PARATHION
0	0.046	0.023	0.030
1	0.045	0.024	0.030
2	0.044	0.025	0.028
3	0.044	0.030	0.032
4	0.042	0.028	0.030
5	0.035	0.029	0.029
6	0.038	0.028	0.030
7	0.042	0.030	

This regime of parathion selection in these strains therefore consisted of the following number of generations:

Previous to this study	6	5	6
This study	7	7	6
Total	13	12	12

In the Penang-Parathion strain, however, the LC_{50} did slightly increase, namely from 0.023 ppm to 0.030 ppm; but it had not even then returned to its LC_{50} of 0.035 ppm shown by the Penang stock of its first receipt in this laboratory.

C. Abate: subjecting the Montego Bay and Albina field-tolerant colonies to 7 generations of Abate selection (Table 5) failed to produce any significant change in the LC_{50}'s of the strains to this insecticide. While the LC_{50} levels of the Albina strain fluctuated between 0.0050 ppm and 0.0060 ppm, those of the Montego Bay strain increased from 0.0045 ppm to 0.0068 ppm, but subsequently decreased and finally settled at a level of 0.0055 ppm in the same range as the Albina strain.

Biochemical Assay of Esterase Activity

Standard curves were determined for the esters acetylcholine bromide, methyl butyrate, phenyl acetate and tributyrin (Fig. 2); it is seen that the relationships at the concentrations used were linear. The role of enzyme concentration in determining the rate of hydrolysis was studied in larval homogenates of the Kongolikan stock (Fig. 3); with all the substrates the relationship was linear over a definite range before it fell off at the higher enzyme concentrations, leaving room for choice among the lower non-limiting concentrations for use in the subsequent studies.

Table 5. Larval LC_{50} levels in ppm Abate of the Montego Bay and Albina colonies of Aedes aegypti subjected to selection with Abate.

Generation	MONTEGO BAY	ALBINA
P	0.0045	0.0054
F_1	0.0060	0.0050
F_2	0.0068	0.0052
F_3	0.0060	0.0058
F_4	0.0051	0.0054
F_5	0.0050	0.0058
F_6	0.0052	0.0062
F_7	0.0055	0.0050

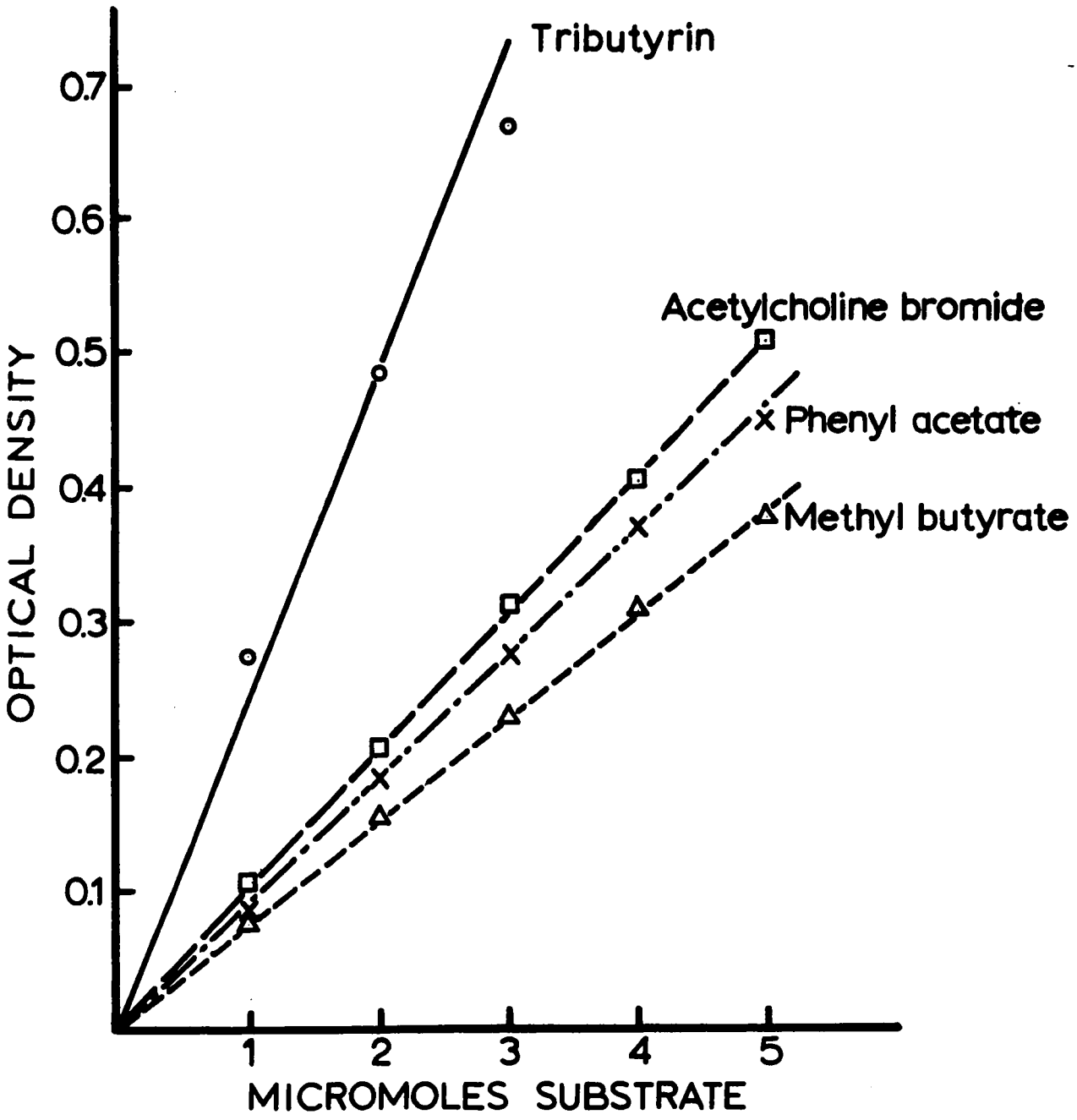


Fig. 2. Standard curve for acetylcholine bromide, methyl butyrate, phenyl acetate and tributyrin; Beckman DU spectrophotometer, 540 millimicrons, 1.0-cm light path. Average of 3-5 determinations. (For original values, see Appendix Table 2.)

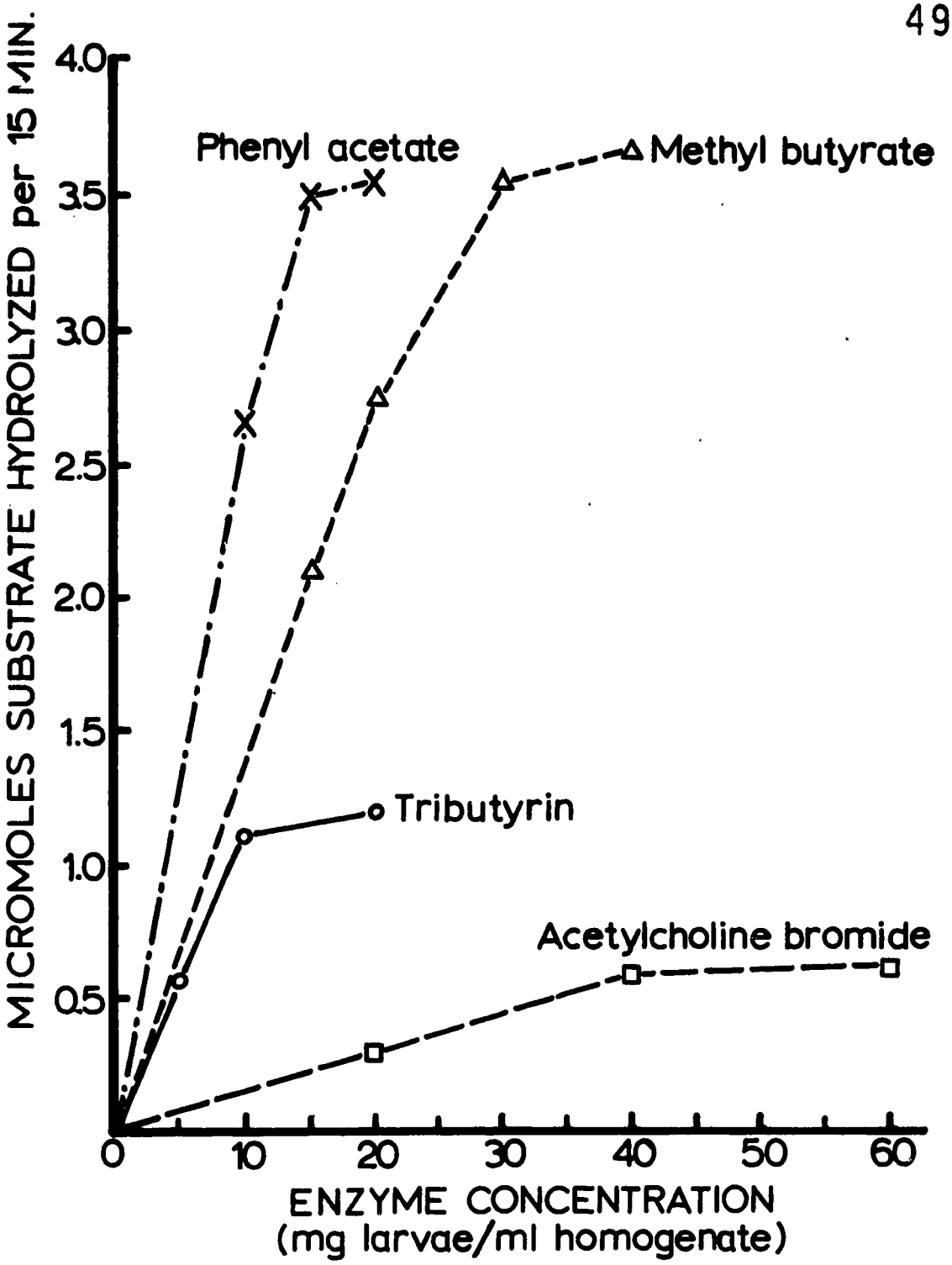


Fig. 3. Rate of substrate hydrolysis as a function of enzyme concentration in larval homogenates of the Kongolikan stock of Aedes aegypti; average of 2-3 experiments.

When the Kongolikan-Malathion strain was compared with the unselected Kongolikan stock for esterase activity in their larval homogenates (Table 6), no difference was found in the hydrolysis of acetylcholine bromide and tributyrin. But this malathion-selected strain was 14% less active in hydrolyzing methyl butyrate, and as much as 40% less active in hydrolyzing phenyl acetate, than its susceptible parent stock.

Inhibition studies (Table 7) indicated that in these A. aegypti larvae acetylcholine bromide was hydrolyzed only by cholinesterase-type activity, since the hydrolysis of this substrate was completely inhibited by 10^{-5}M eserine sulphate. This eserine-sensitive esterase activity was evidently responsible for a portion of the hydrolysis of the non-choline esters, constituting in the Kongolikan stock 16% of the activity with methyl butyrate, 32% with tributyrin and 48% with phenyl acetate as substrate. In the Kongolikan-Malathion strain the proportion of this eserine-sensitive esterase activity in the hydrolysis of these non-choline esters was less than in the unselected stock and amounted to 10%, 16% and 32% for methyl butyrate, tributyrin and phenyl acetate respectively.

Since 10^{-5}M paraoxon inhibits all B-type esterases, whereas 10^{-5}M eserine sulphate inhibits only cholinesterases, an indication of the aliesterase activity may be obtained by

Table 6. Esterase activity in the larvae of the Kongolikan stock and the Kongolikan-Malathion strain of Aedes aegypti; number of experiments in parentheses.*

	ACETYLCHOLINE BROMIDE	METHYL BUTYRATE	PHENYL ACETATE	TRIBUTYRIN
Substrate	$2x10^{-3}M$	$4x10^{-3}M$	$4x10^{-3}M$	$2x10^{-3}M$
Enzyme Concentration (mg larvae/ml homogenate)	40	20	10	10
Micromoles substrate hydrolyzed/15 min:				
KONGOLIKAN	0.53 (3)	2.66 (7)[a]	2.67 (2)	1.14 (2)
KONGOLIKAN-MALATHION	0.51 (3)	2.30 (5)	1.91 (2)	1.22 (2)

[a] $t_{10} = 3.41$

*For original values, see Appendix Table 3.

Table 7. Per cent inhibition of larval esterase activity of the Kongolikan-Malathion strain and the original Kongolikan stock by $1x10^{-5}$M eserine sulphate and paraoxon.

Substrate	ACETYLCHOLINE BROMIDE		METHYL BUTYRATE		PHENYL ACETATE		TRIBUTYRIN	
Inhibitor	Eserine	Paraoxon	Eserine	Paraoxon	Eserine	Paraoxon	Eserine	Paraoxon
KONGOLIKAN	100	100	16	100	48	100	32	53
KONGOLIKAN-MALATHION	100	100	10	90	32	91	16	31

subtracting the proportion of esterase activity inhibited by eserine from that inhibited by paraoxon. When this calculation was completed, it was found that there was no interstrain difference between the Kongolikan-Malathion strain and the unselected Kongolikan stock in aliesterase-type activity on methyl butyrate and phenyl acetate as substrate, both colonies averaging 82% and 55% respectively in the proportion of this esterase. But with tributyrin as substrate, this proportion was only 15% in the malathion-selected strain as compared with 21% for the unselected parent stock. OP-insensitive esterase activity, uninhibited by 10^{-5}M paraoxon, was not found in the unselected Kongolikan stock with methyl butyrate and phenyl acetate as substrates. It was however present in the selected Kongolikan-Malathion strain to the extent of 10% of the activity on these substrates. Moreover, with tributyrin as substrate, it amounted to 69% for the Kongolikan-Malathion strain as compared to 47% for the unselected stock.

Electrophoretic Separation and Visualization of Esterases

A. General: the exploration of appropriate substrates and protein staining methods was performed on material from the Kongolikan stock. While the use of α-naphthyl acetate as substrate displayed the zones of esterase activity as brown bands, with β-naphthyl acetate as substrate the bands were purple in color at pH 8.6 and

red at pH 6.8 (Fig. 4). Since use of the β-isomer as substrate resulted in the appearance of more zones of esterase activity than with the α-isomer, it was employed in all the subsequent esterase studies.

In electropherograms which were stained with Amido-Black to reveal the presence of soluble proteins in general (not illustrated), only one protein band was observed, and it did not correspond in its electrophoretic mobility with any of the zones of esterase activity.

B. Stock Colonies: the esterase zymograms of the 5 stock colonies (Fig. 5) showed interstock differences both in the electrophoretic mobility of the zones of activity and in their intensity. Electrophoretic separation in the pH 8.6 buffer system resulted in zymograms with better resolution than in the pH 6.8 buffer system.

Cholinesterase activity could not be detected by using specific cholinesterase staining techniques. The incorporation of eserine sulphate in the agar-gel layer (Fig. 6) did not inhibit any specific band or bands of esterase activity of a larval homogenate, while completely inhibiting a sample of bovine erythrocyte acetylcholinesterase. These results indicate that cholinesterase was not responsible for the appearance of any of the esterase bands displayed by the larval homogenate.

The incorporation of paraoxon in the agar-gel layer

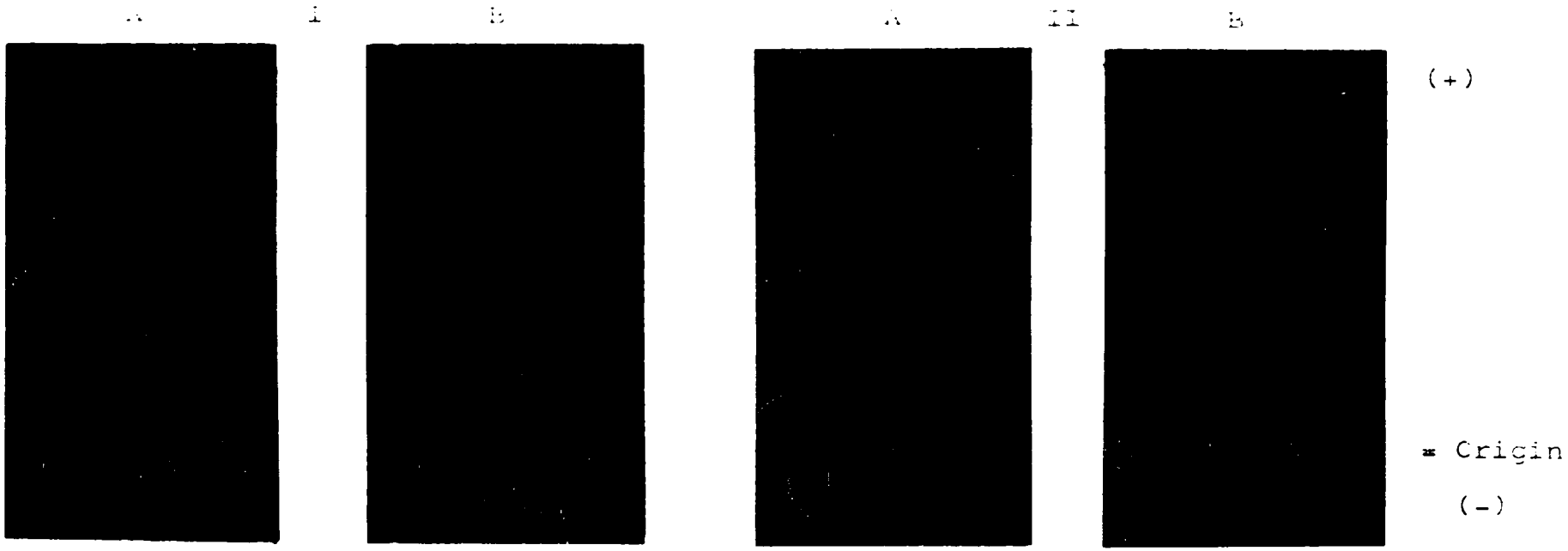


Fig. 4. Comparison of esterase patterns of Aedes aegypti (Rengolikan stock) after electrophoresis at pH 8.6 (I) and pH 6.8 (II), using α-naphthyl acetate (A) and β-naphthyl acetate (B) as substrate.

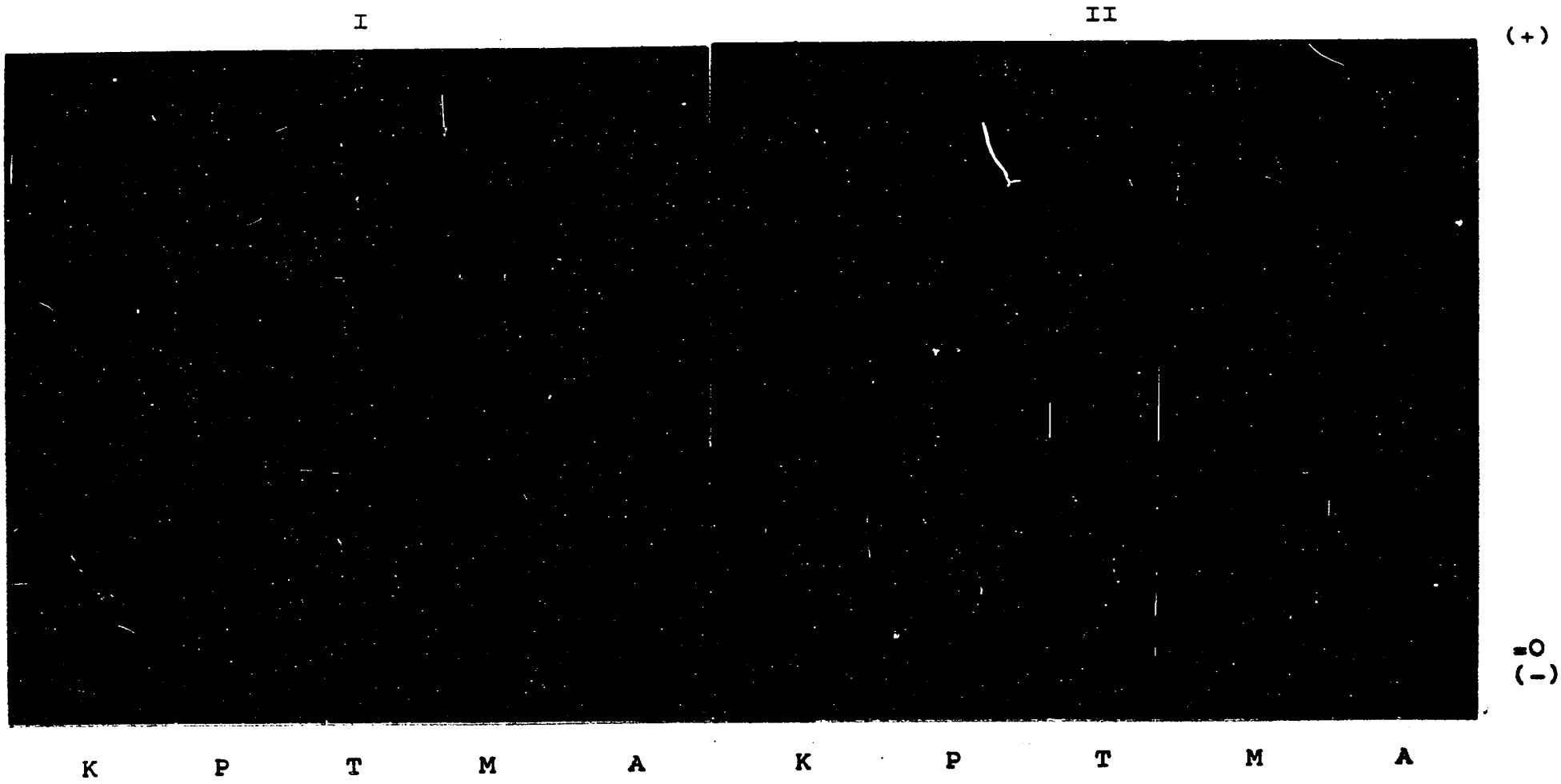
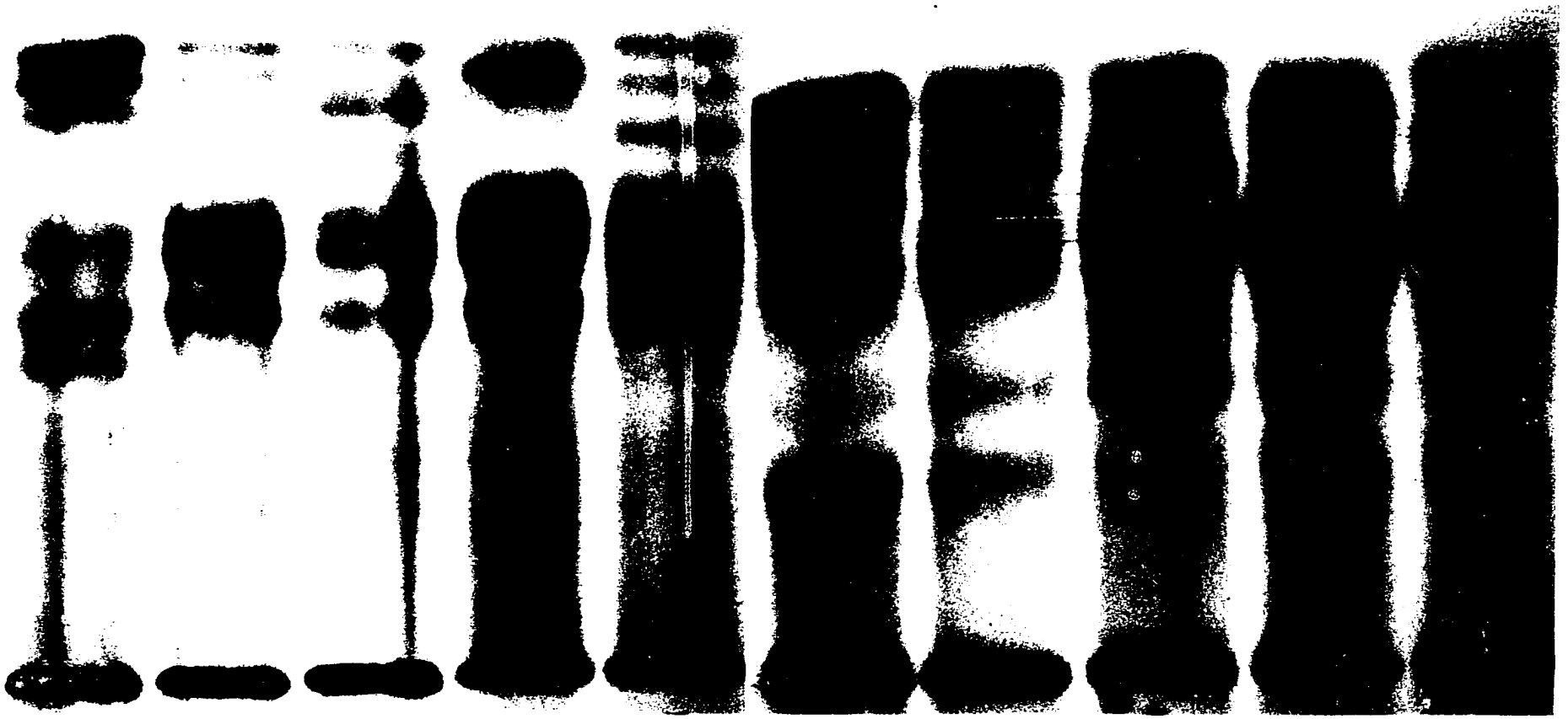


Fig. 5. Esterase patterns of Kongolikan (K), Penang (P), Trinidad (T), Montego Bay (M) and Albina (A) colonies of Aedes aegypti after electrophoresis at pH 8.6 (I) and pH 6.8 (II). (O) Origin.



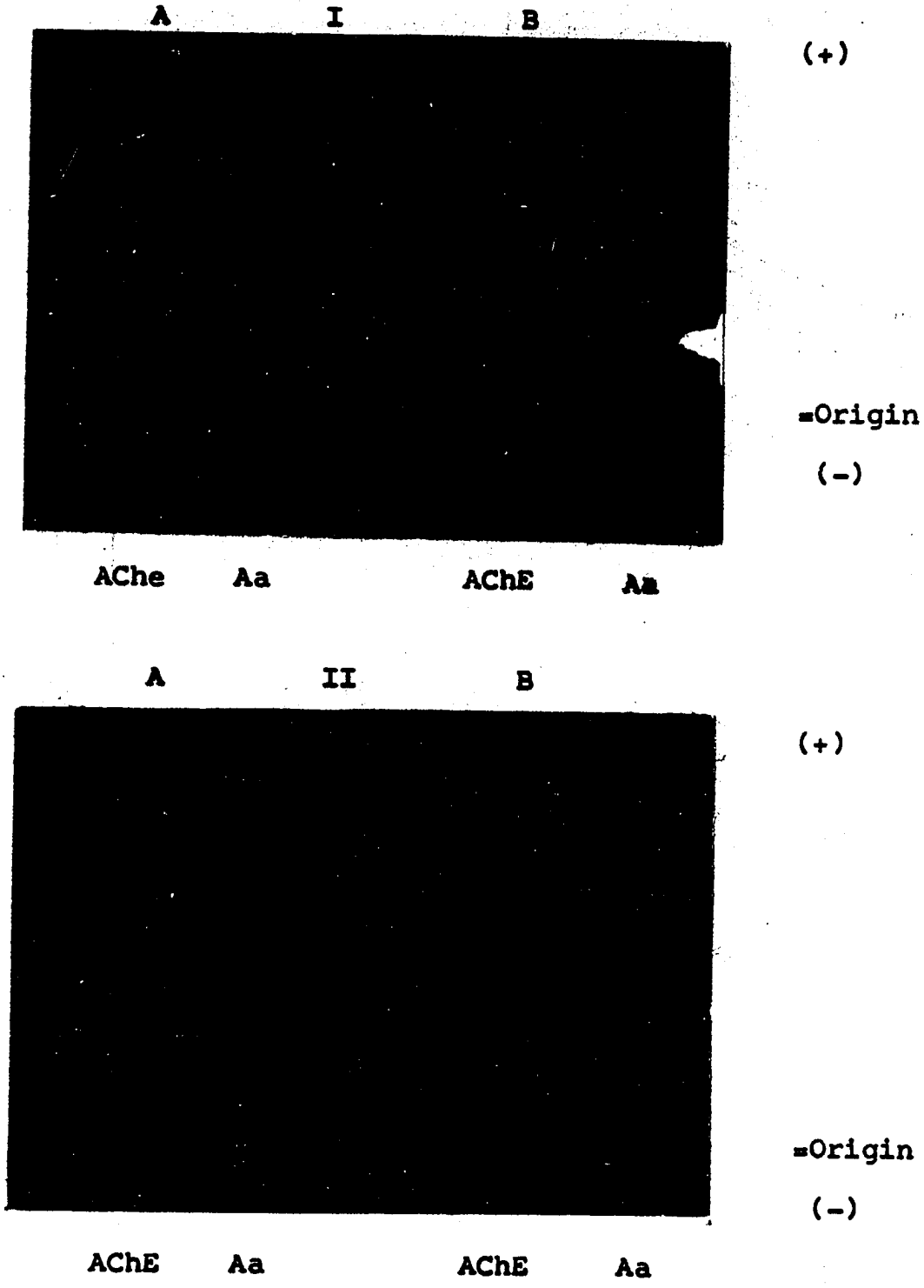
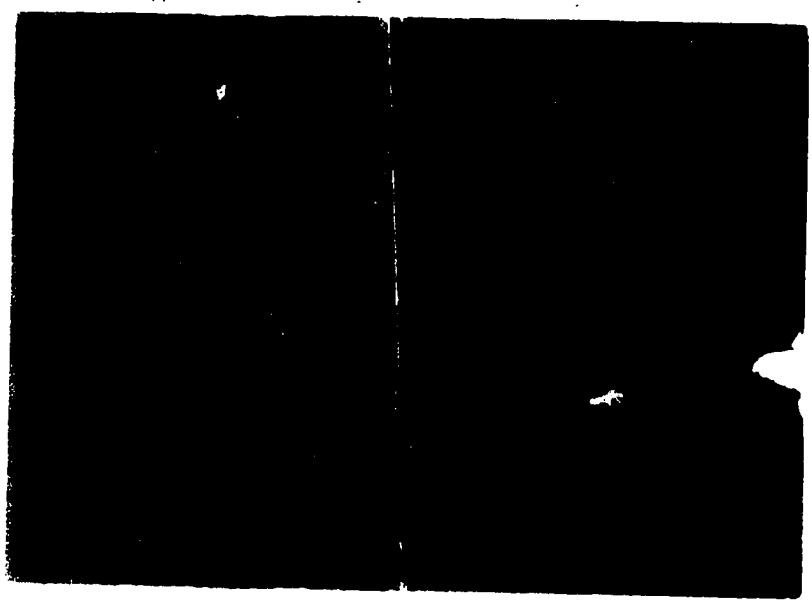
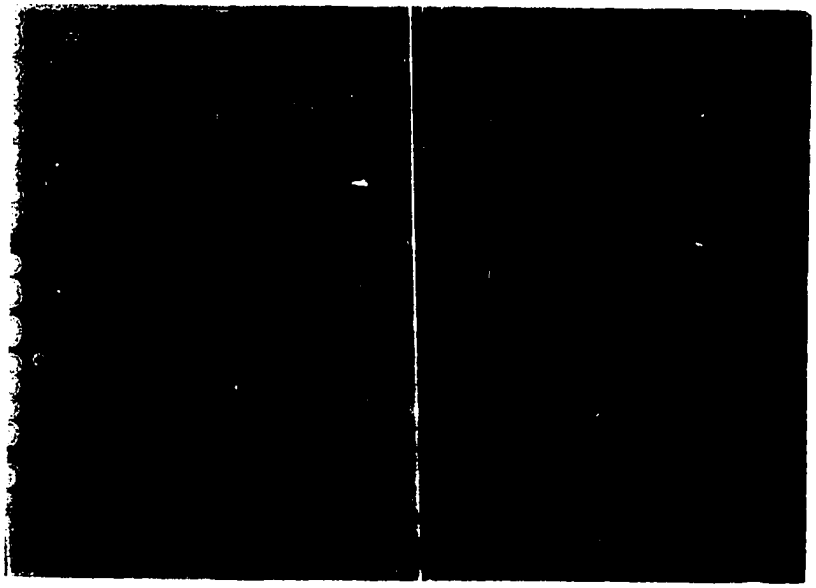


Fig. 6. The effect of 10^{-5}M eserine sulphate on the esterase pattern of Aedes aegypti (Aa) as compared with bovine erythrocyte acetylcholinesterase (AChE) after electrophoresis at pH 8.6 (I) and pH 6.8 (II): (A) control, (B) agar-gel containing 10^{-5}M eserine sulphate.





(Fig. 7) resulted in the inhibition of all the esterase bands of the stock colonies except for the slowest moving one. This paraoxon-insensitive band can therefore be classified as an A-type or arylesterase, while all the rest are B-types or aliesterases. The Kongolikan stock displayed the highest activity in this A-type esterase, and the Penang and Trinidad stocks the lowest.

Only a single band of acid phosphomonoesterase activity was found in each of the 5 stocks (Fig. 8), and it had the same electrophoretic mobility as the A-type esterase band. Here also, the Kongolikan stock displayed the highest activity and the Penang and Trinidad stocks the lowest. There was also a single band of alkaline phosphomonoesterase activity (Fig. 8), but it was electrophoretically faster-moving than that of the acid phosphatase. Except for the Kongolikan stock which was slightly more active, the stocks were about equal in activity. Since electrophoretic separation in the pH 8.6 barbital buffer system resulted in a more pronounced zone of alkaline phosphatase activity than in the pH 6.8 phosphate buffer system, it was used as the buffer system in all the subsequent alkaline phosphatase studies.

C. Selected Strains as compared to Stock Colonies: no differences were found between the esterase patterns of the Montego Bay and Albina strains after selection with

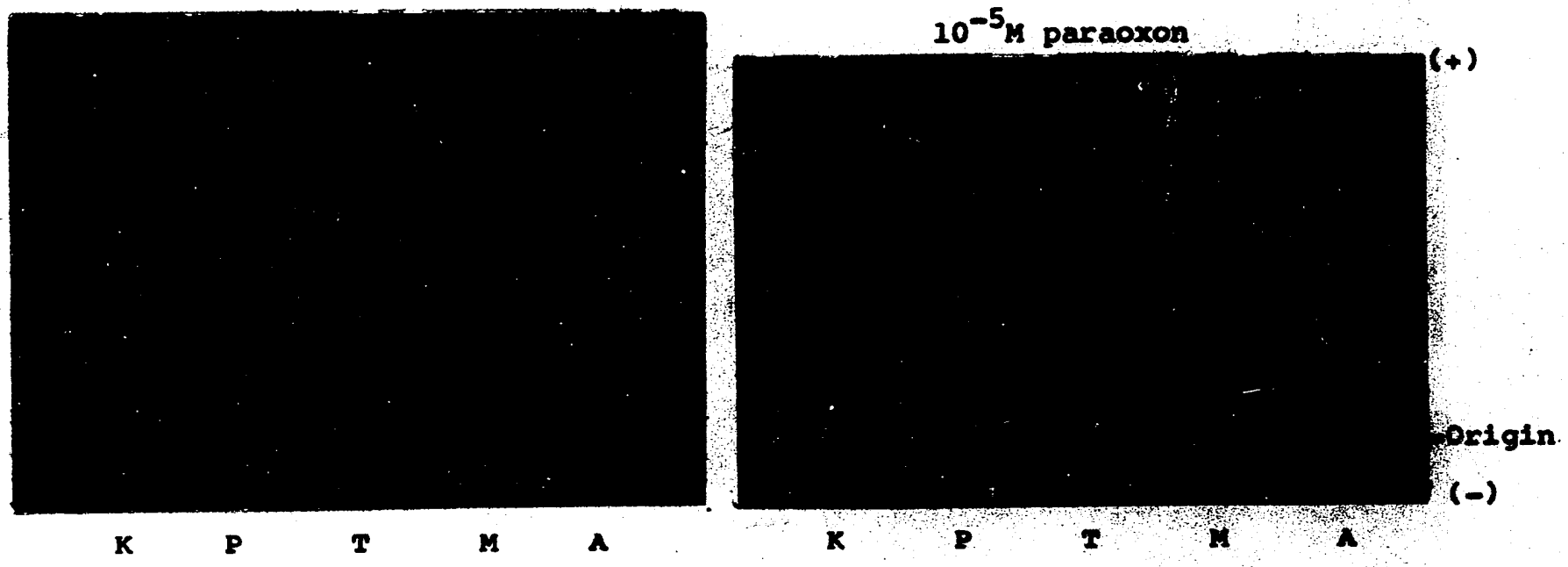


Fig. 7. The effect of 10^{-5}M paraoxon on the esterase patterns of Kongolikan (K), Penang (P), Trinidad (T), Montego Bay (M) and Albina (A) colonies of Aedes aegypti after electrophoresis at pH 6.8; (Fig. 5II included for comparison).

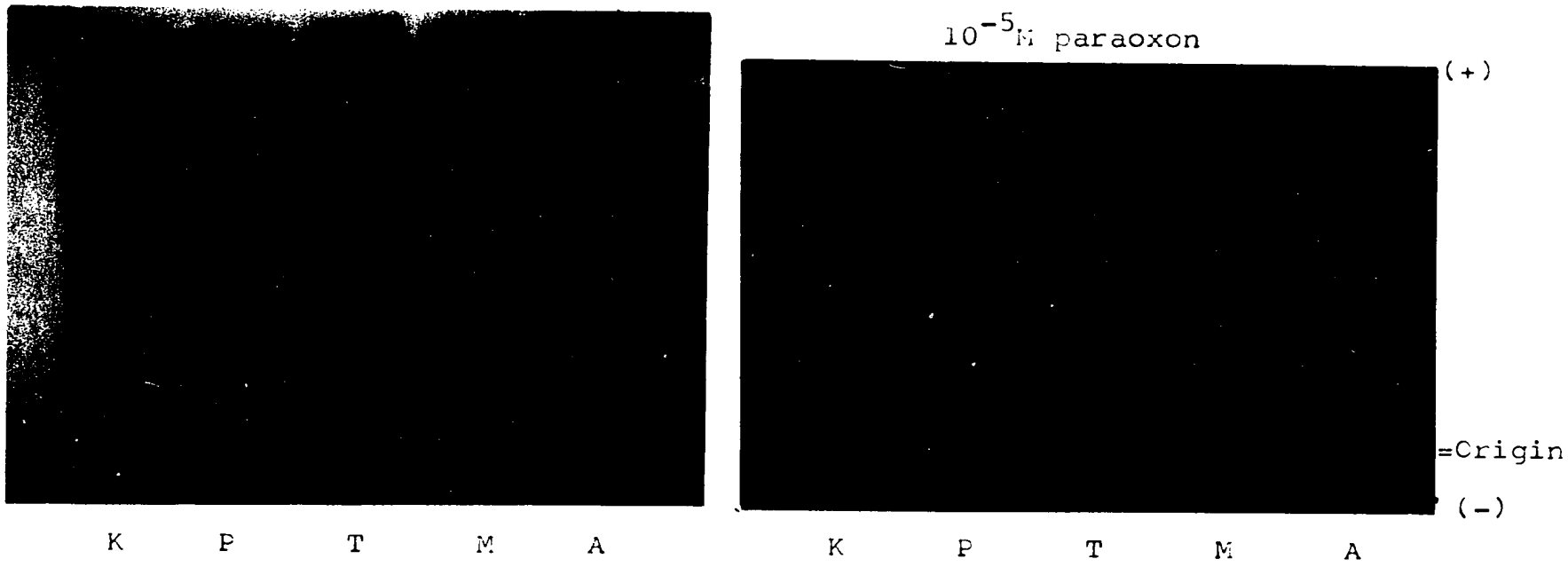


Fig. 7. The effect of 10^{-5}M paraoxon on the esterase patterns of Kongolikan (K), Penang (P), Trinidad (T), Montego Bay (M) and Albina (A) colonies of Aedes aegypti after electrophoresis at pH 6.8; (Fig. 5II included for comparison).

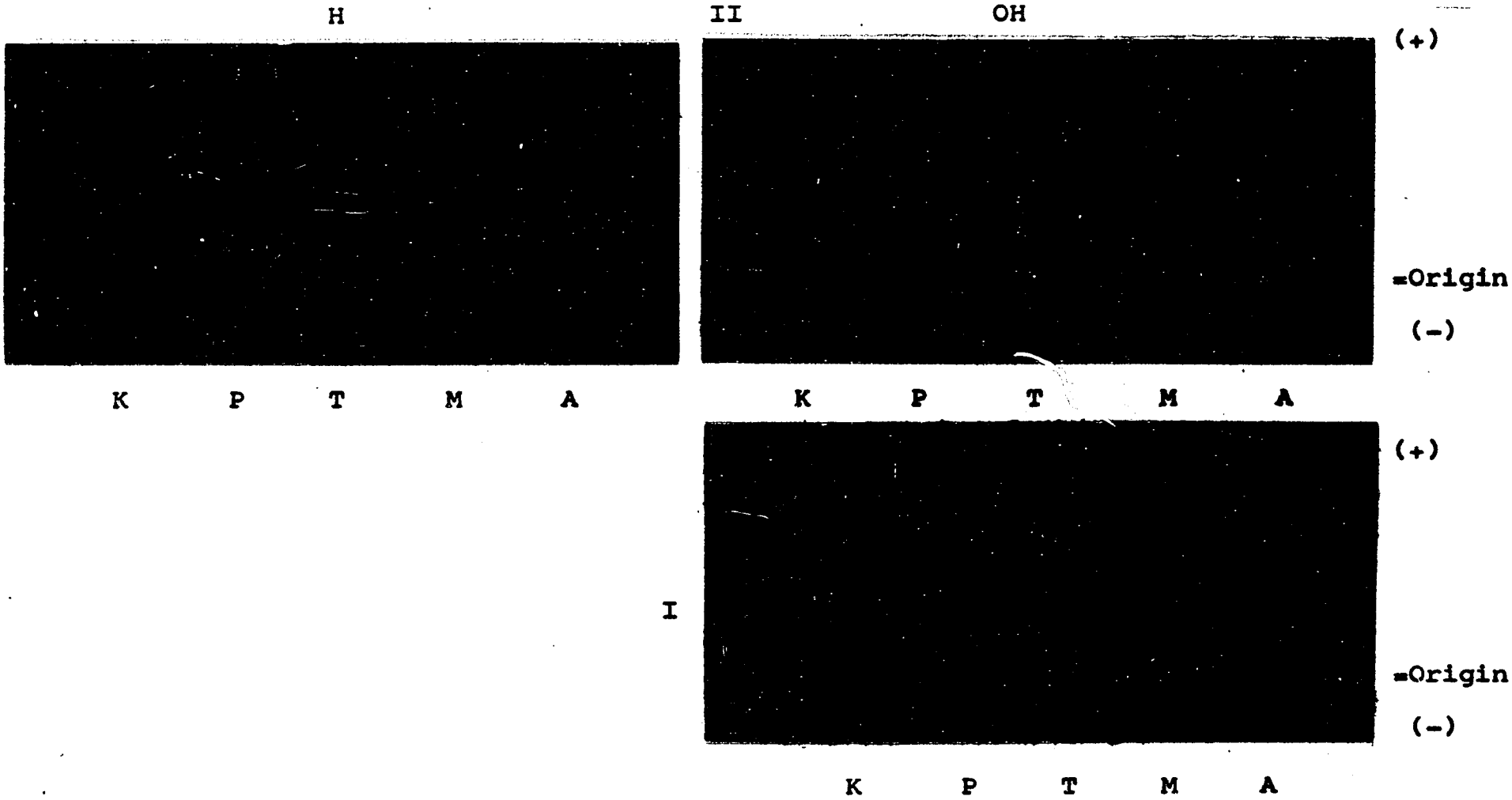


Fig. 8. Zymograms of acid (H) and alkaline (OH) phosphomonoesterase activity of Kongolikan (K), Penang (P), Trinidad (T), Montego Bay (M) and Albina (A) colonies of Aedes aegypti after electrophoresis at pH 8.6 (I) and pH 6.8 (II).

malathion or Abate and the unselected stock colonies.

When the esterase patterns of the selected Kongolikan-Malathion and Kongolikan-Parathion strains were compared with those of the unselected Kongolikan stock (Fig. 9), it was found that the zones corresponding to the two most electrophoretically mobile bands of the Kongolikan stock were almost completely absent in the malathion-selected strain and in the parathion-selected strain the second most mobile band was very active. These differences were also shown when α-naphthyl acetate was used as the substrate (Fig. 10).

The incorporation of paraoxon in the agar-gel layer (Fig. 11) revealed that the A-type esterase band of the Kongolikan-Parathion strain was equal in activity to that of the unselected Kongolikan stock, but that of the Kongolikan-Malathion strain was less active. Esterase inhibition with 10^{-7}M paraoxon showed that not all of the B-type esterase bands were equally affected by the toxicant. While no change whatsoever was seen in the intensity of the 3 bands possessing the greatest electrophoretic mobility, the remaining B-type zones of esterase activity were completely inhibited by this concentration.

The esterase bands of both the Penang-Malathion and the Penang-Parathion strains were in general more active than those of the original Penang stock colony (Fig. 12).

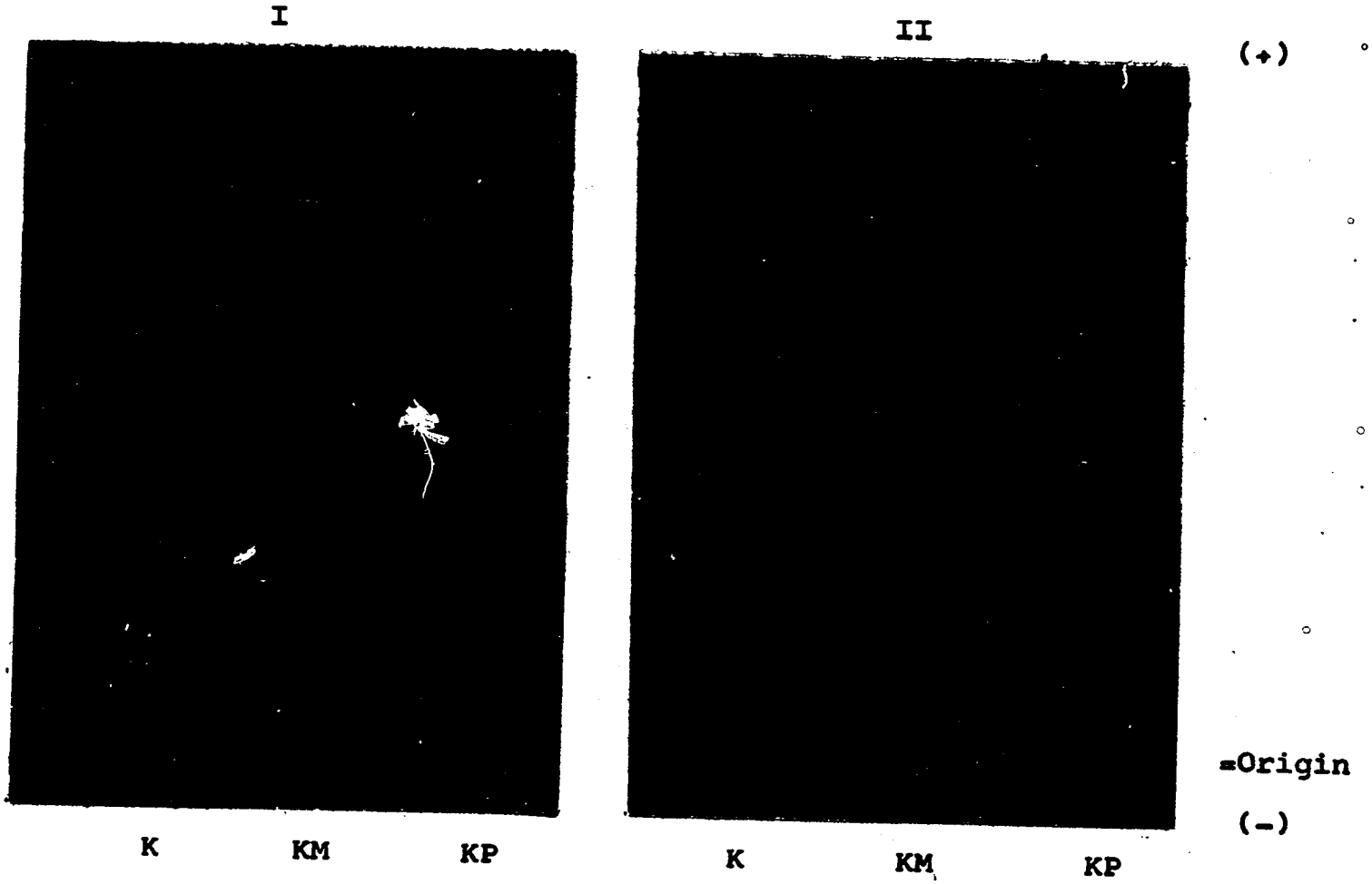


Fig. 9. Comparison of the esterase patterns of the malathion-selected (KM) and parathion-selected (KP) strains with that of the unselected Kongolikan (K) stock after electrophoresis at pH 8.6 (I) and pH 6.8 (II).

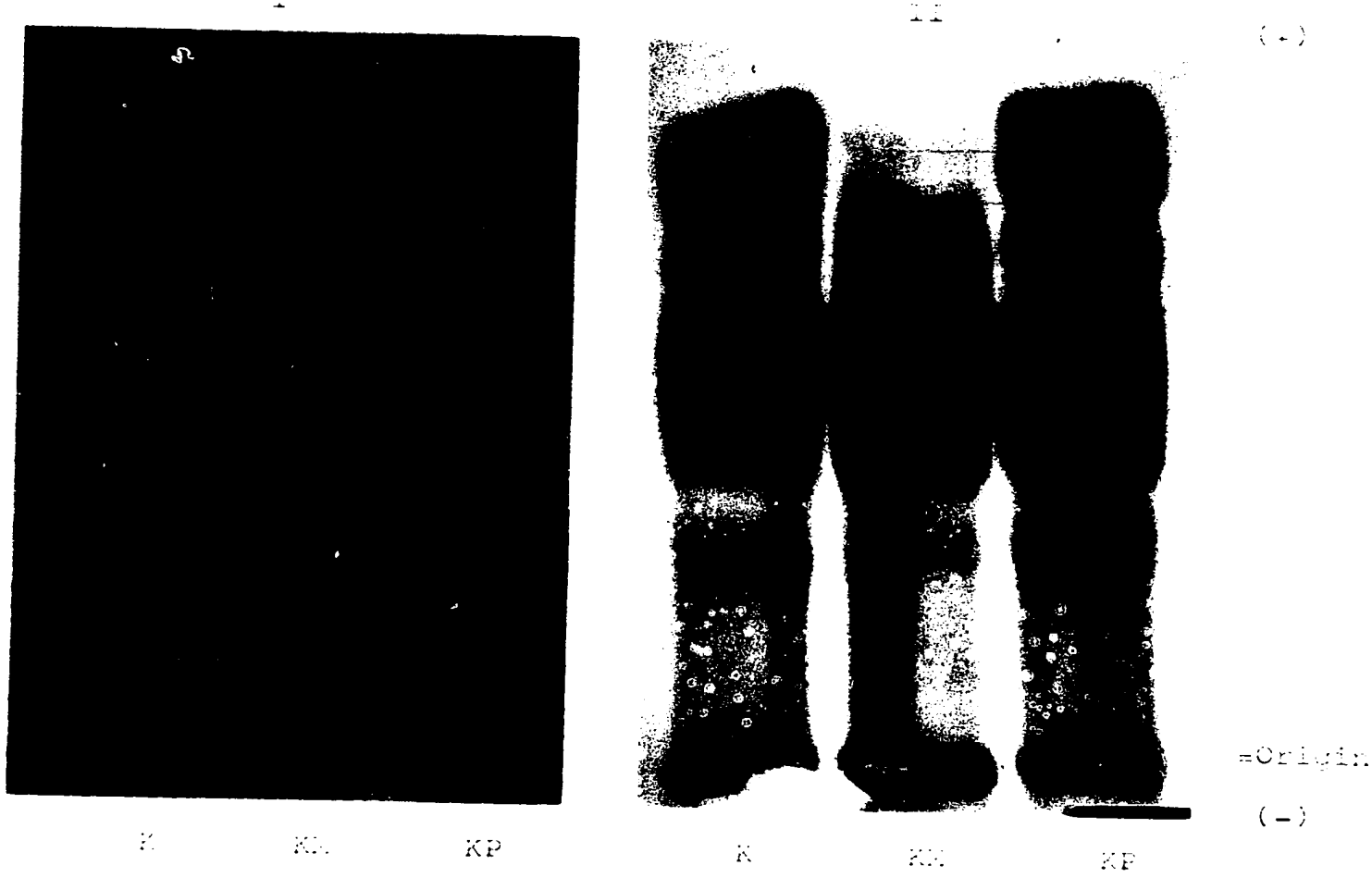


Fig. 9. Comparison of the esterase patterns of the malathion-selected (KM) and parathion-selected (KP) strains with that of the unselected Kongolikan (K) stock after electrophoresis at pH 8.6 (I) and pH 6.8 (II).

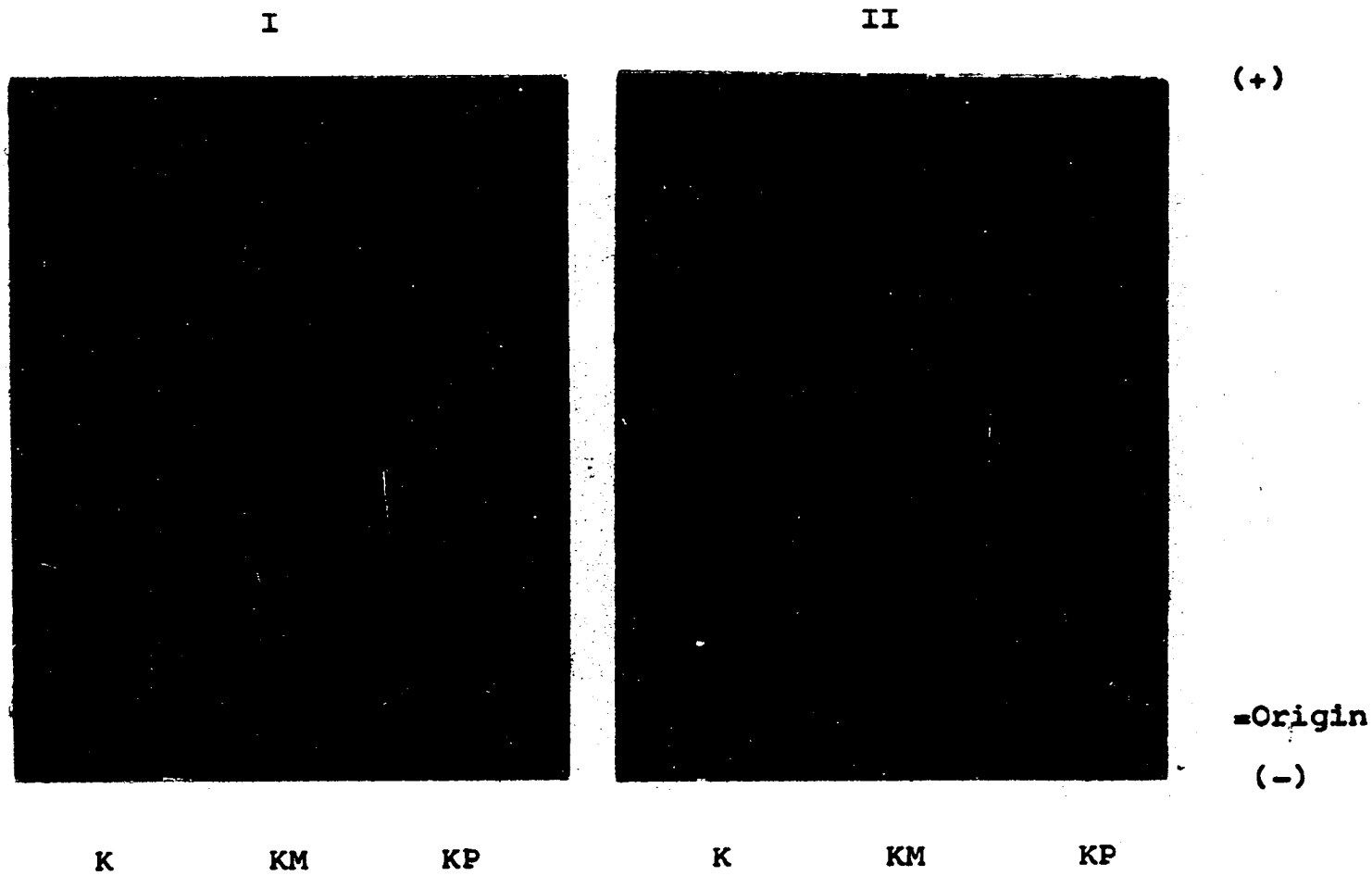
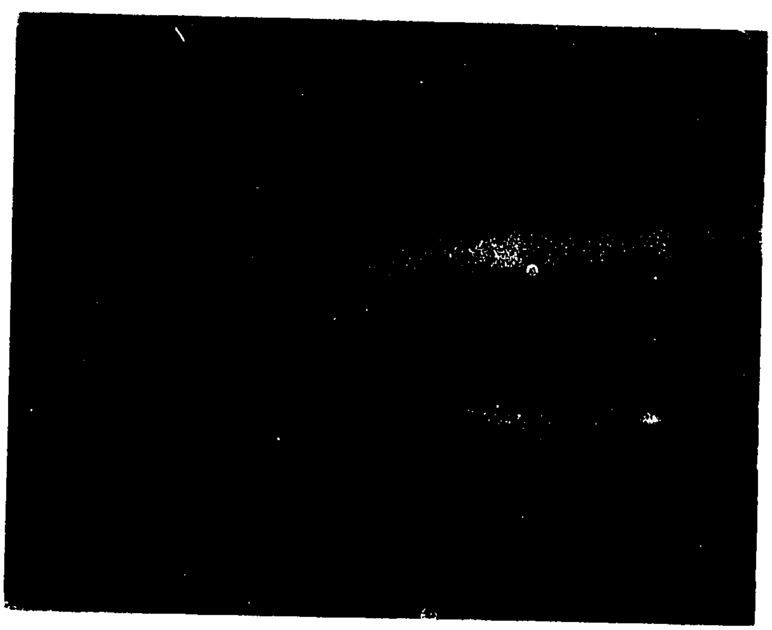


Fig. 10. Comparison of the esterase patterns of the malathion-selected (KM) and parathion-selected (KP) strains with that of the unselected Kongolikan (K) stock after electrophoresis at pH 8.6 (I) and pH 6.8 (II) using α-naphthyl acetate as substrate.



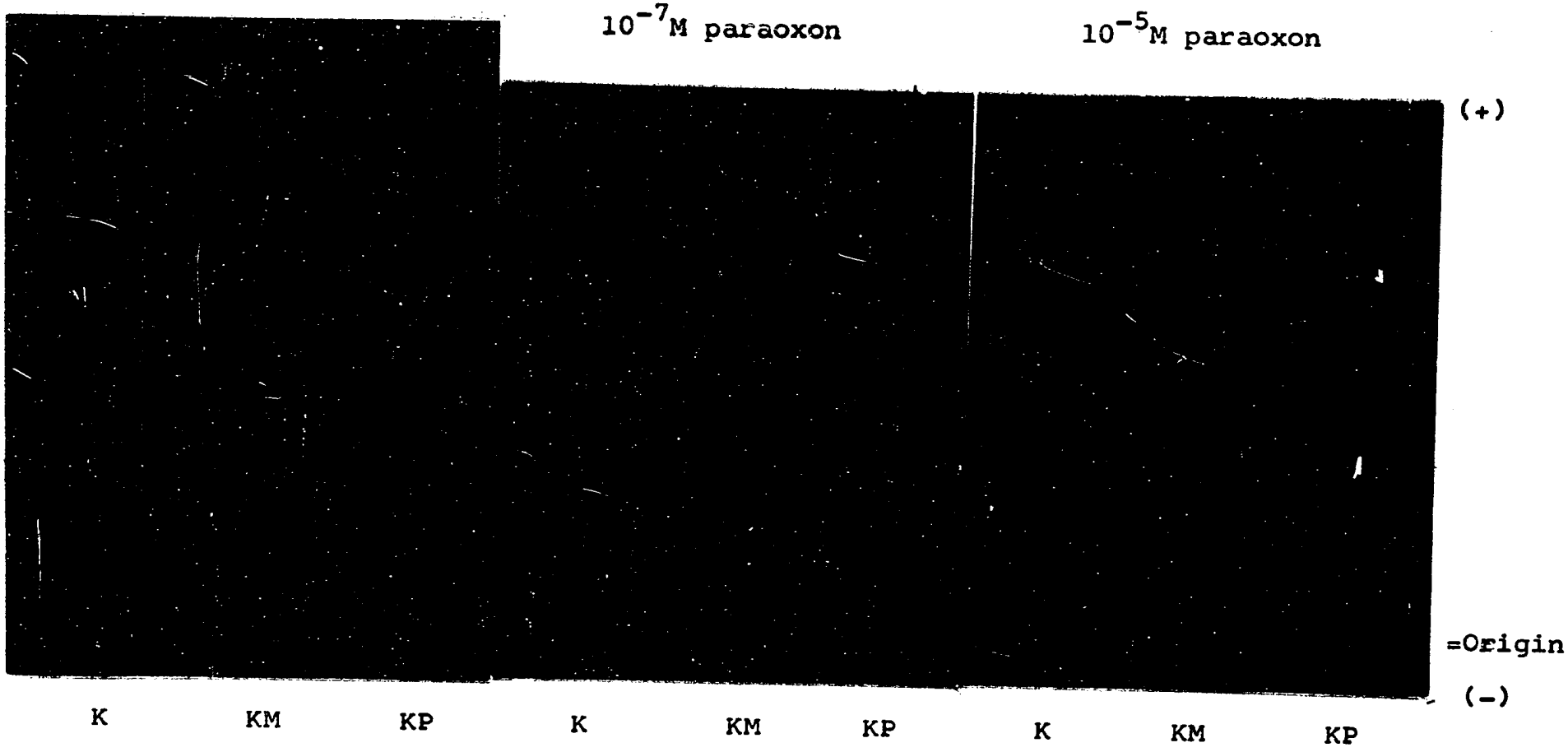


Fig. 11. The effect of 10^{-7}M and 10^{-5}M paraoxon on the esterase patterns of Kongolikan (K), Kongolikan-Malathion (KM) and Kongolikan-Parathion (KP) after electrophoresis at pH 6.8; (Fig. 9II included for comparison).

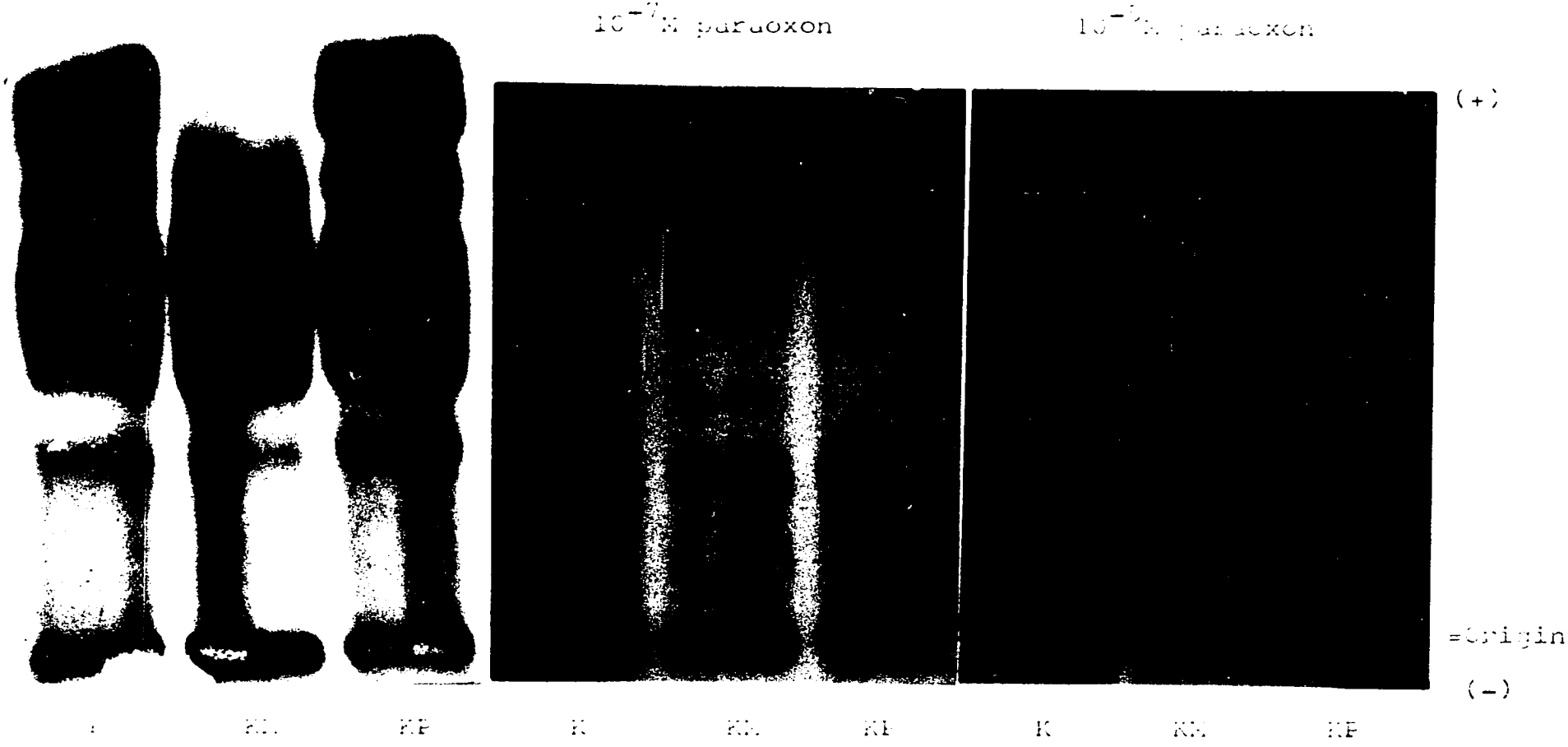


Fig. 11. The effect of 10^{-7}M and 10^{-5}M paraoxon on the esterase patterns of Kongolikan (K), Kongolikan-Malathion (KM) and Kongolikan-Parathion (KP) after electrophoresis at pH 8.6; (Fig. 9A included for comparison).

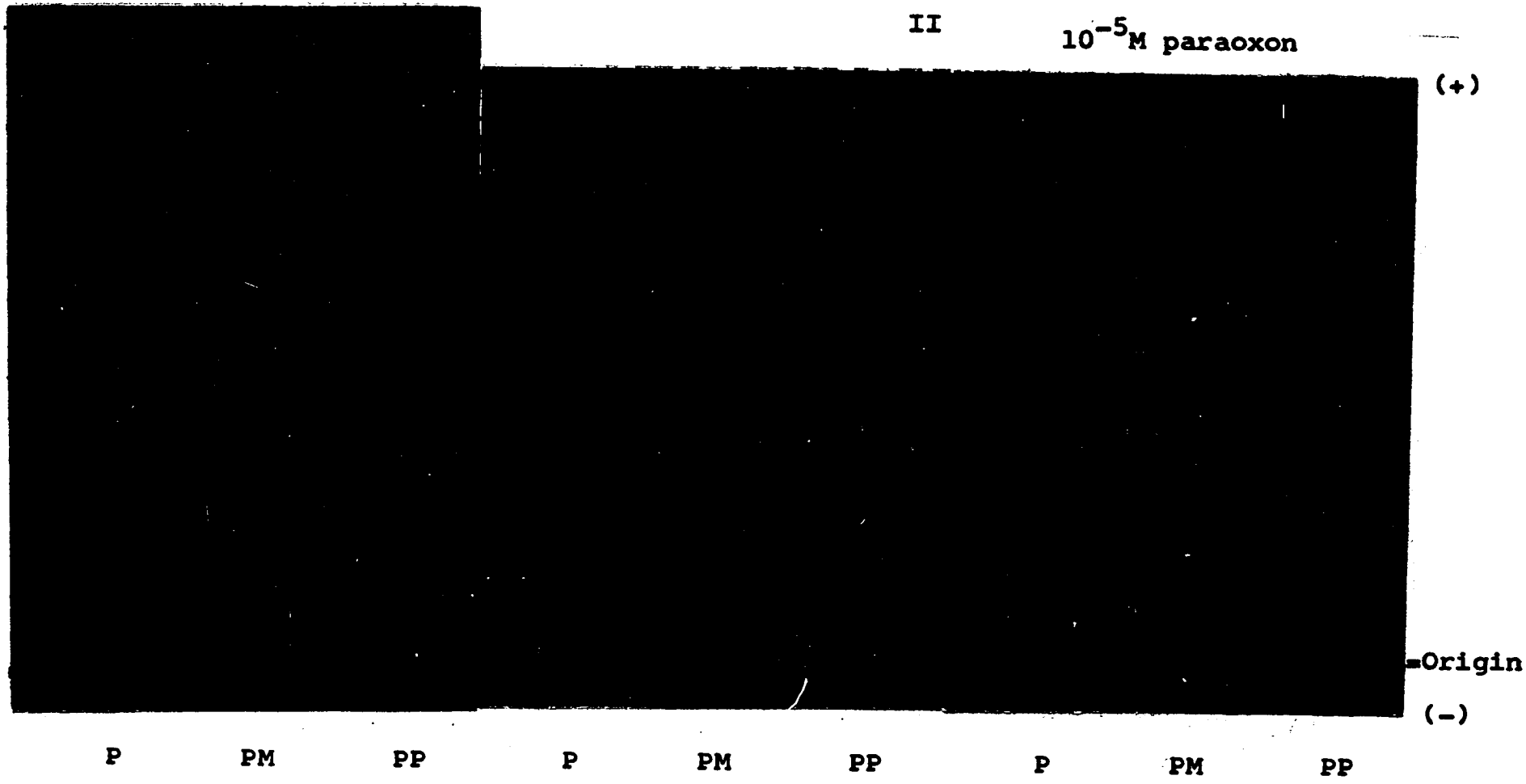


Fig. 12. Comparison of the esterase patterns of the malathion-selected (PM) and parathion-selected (PP) strains with that of the unselected Penang (P) stock after electrophoresis at pH 8.6 (I) and pH 6.8 (II), and the effect of 10^{-5}M paraoxon on these esterase patterns after electrophoresis at pH 6.8.

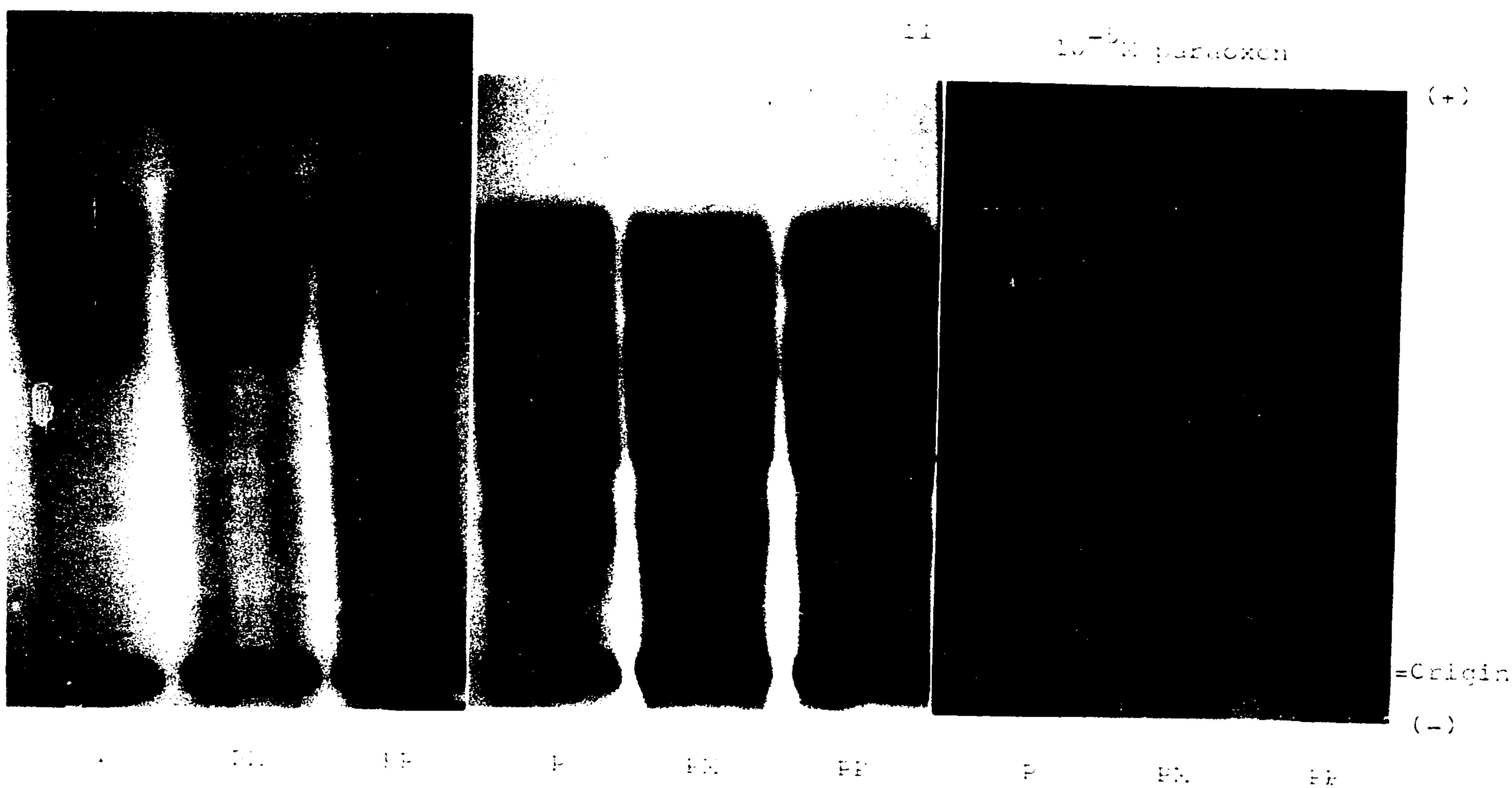


Fig. [illegible] Comparison of the esterase patterns of the malathion-selected ([illegible]) and parathion-selected (II) strains with that of the unselected [illegible] ([illegible]) stock after electrophoresis at pH [illegible] ([illegible]) and pH 6.8 ([illegible]), and the effect of 10^{-5}M paraoxon on these [illegible] patterns after electrophoresis at pH [illegible].

Esterase inhibition with paraoxon revealed that while the unselected colony had very little A-type esterase activity, both selected strains displayed much more, that of Penang-Parathion being the more intense.

Comparing the esterase activity of the Trinidad-Malathion and Trinidad-Parathion selected strains to that of the unselected stock colony (Fig. 13) resulted in zymograms which were similar to those obtained for the Kongolikan material in that the malathion-selected strain lacked a zone of esterase activity which corresponds to the most electrophoretically mobile band of the parent stock. As in the 2 other parathion-selected strains, the second most mobile band was very active in the Trinidad-Parathion strain. The incorporation of paraoxon in the agar-gel layer revealed a more intense A-type esterase band in the parathion-selected strain than in the unselected stock, while that of the malathion-strain was weaker than in the unselected stock.

The zymograms of acid and alkaline phosphomonoesterase activity showed no difference between the Montego Bay and Albina strains selected with malathion or Abate and their unselected stock colonies, just as had been found for the esterase activity. The Kongolikan, Penang and Trinidad OP-selected strains differed slightly in acid phosphatase activity from their respective

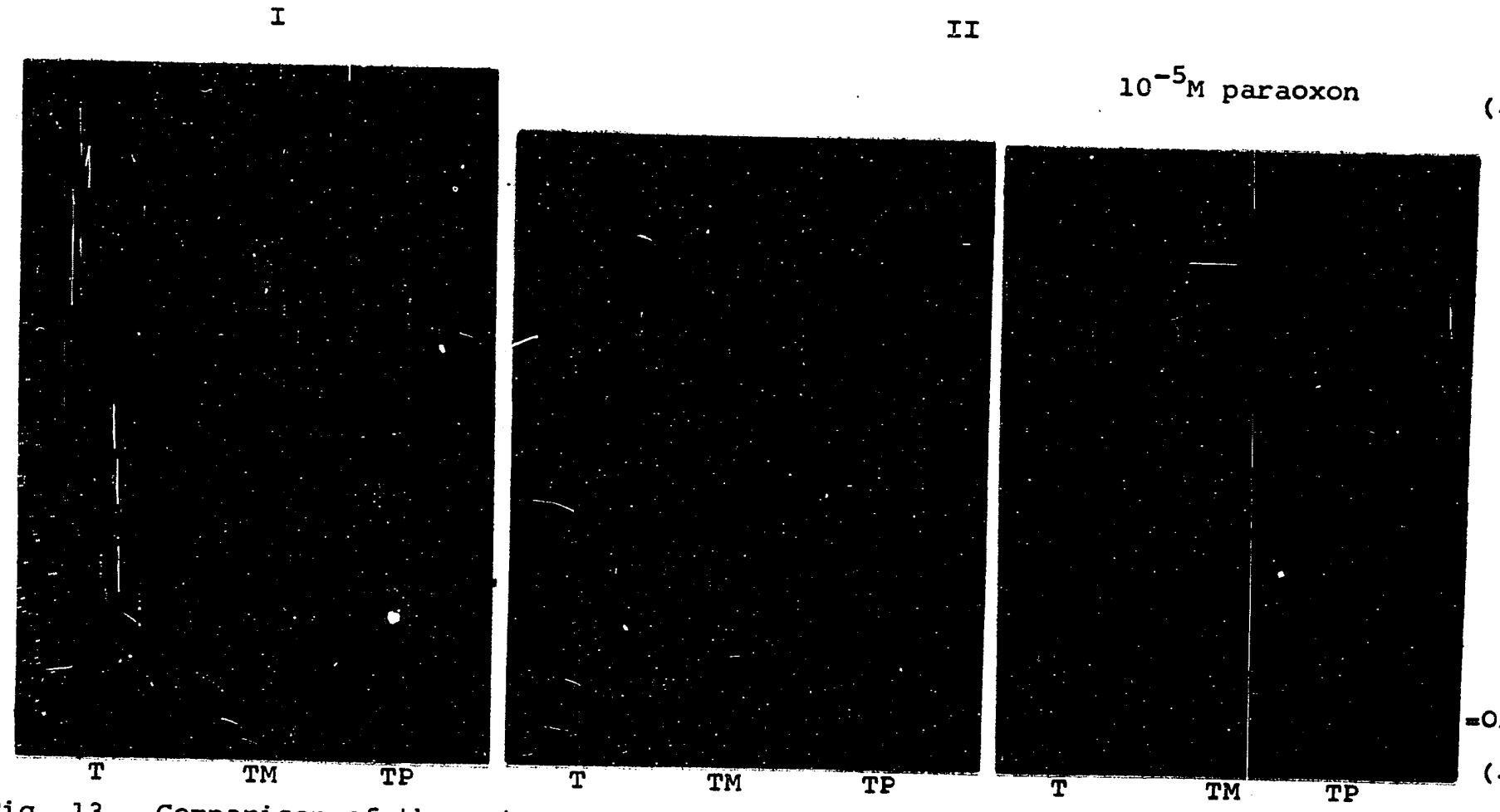


Fig. 13. Comparison of the esterase patterns of the malathion-selected (TM) and parathion-selected (TP) strains with that of the unselected Trinidad (T) stock after electrophoresis at pH 8.6 (I) and pH 6.8 (II), and the effect of 10^{-5}M paraoxon on these esterase patterns after electrophoresis at pH 6.8.

[illegible] Comparison of the esterase patterns of the malathion-selected (TM) and [illegible]
[illegible]

unselected stock colonies (Fig. 14), but the differences were not consistent. Whereas the Kongolikan and Trinidad strains selected with malathion or parathion displayed less activity than their original stock colonies, in the Penang material the malathion-selected strain was equal, and the parathion-selected strain was superior, in acid phosphatase activity as compared with the original stock.

Different results were obtained for alkaline phosphatase activity. Here, only the malathion-selected strains of Kongolikan and Trinidad showed less activity than their respective original colonies, while the parathion-selected strains were either the equal (Kongolikan) or the superior (Trinidad) of their unselected stock (Fig. 15). The Penang material showed no difference in the alkaline phosphatase band between the OP-selected strains and the unselected parent colony.

Zymograms of esterase and phosphomonoesterase activity obtained from larvae which had been treated with sublethal concentrations of malathion or parathion did not display any new zones of activity, nor was there any increase in the activity of those bands already displayed. There was no evidence that these insecticides had any inductive or activating effects on these enzymes in either the stock colonies or the OP-selected strains.

In the experiments to detect whether any of these

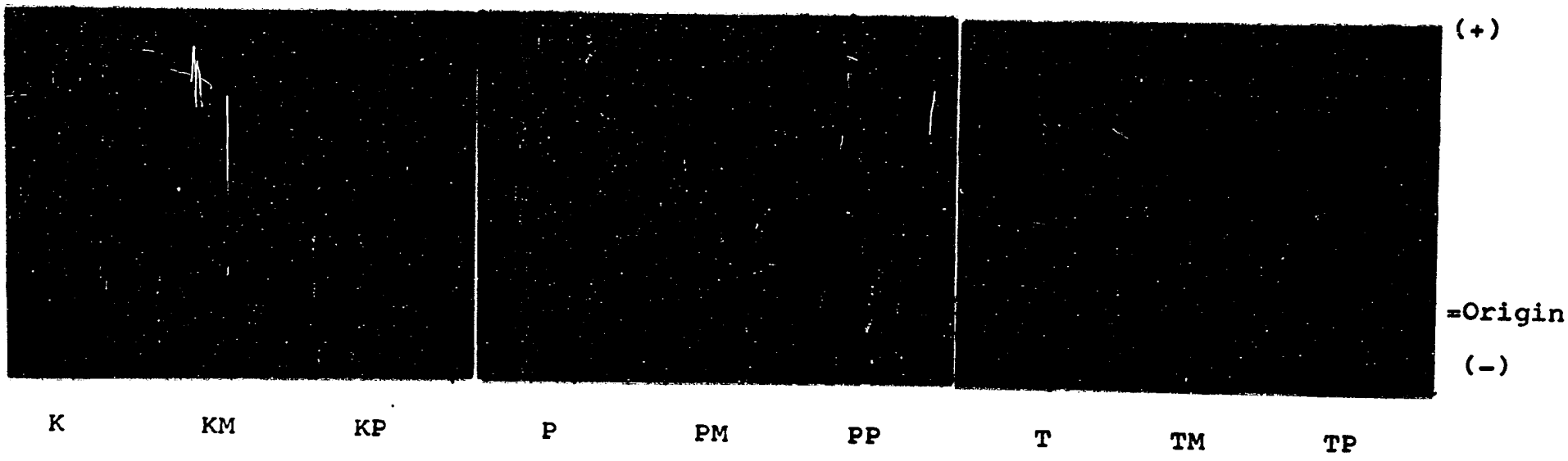


Fig. 14. Comparison of the acid phosphomonoesterase activity of the malathion-selected (KM, PM, TM) and parathion-selected (KP, PP, TP) strains of Kongolikan (K), Penang (P) and Trinidad (T) to that of their respective unselected original stock after electrophoresis at pH 6.8.

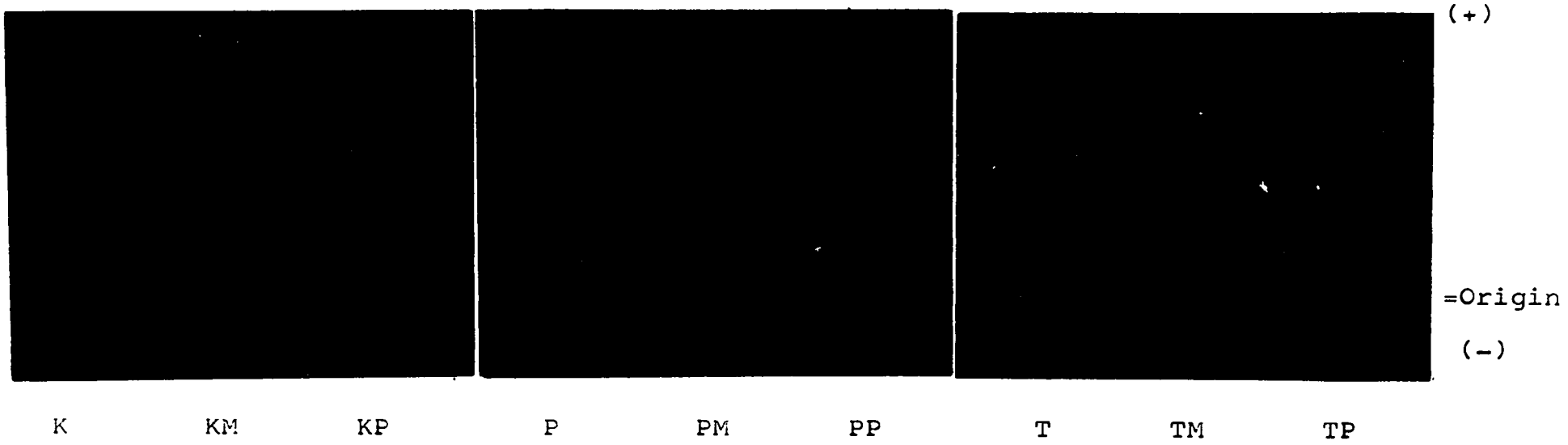


Fig. 14. Comparison of the acid phosphomonoesterase activity of the malathion-selected (KM, PM, TM) and parathion-selected (KP, PP, TP) strains of Kongolikan (K), Penang (P) and Trinidad (T) to that of their respective unselected original stock after electrophoresis at pH 6.8.

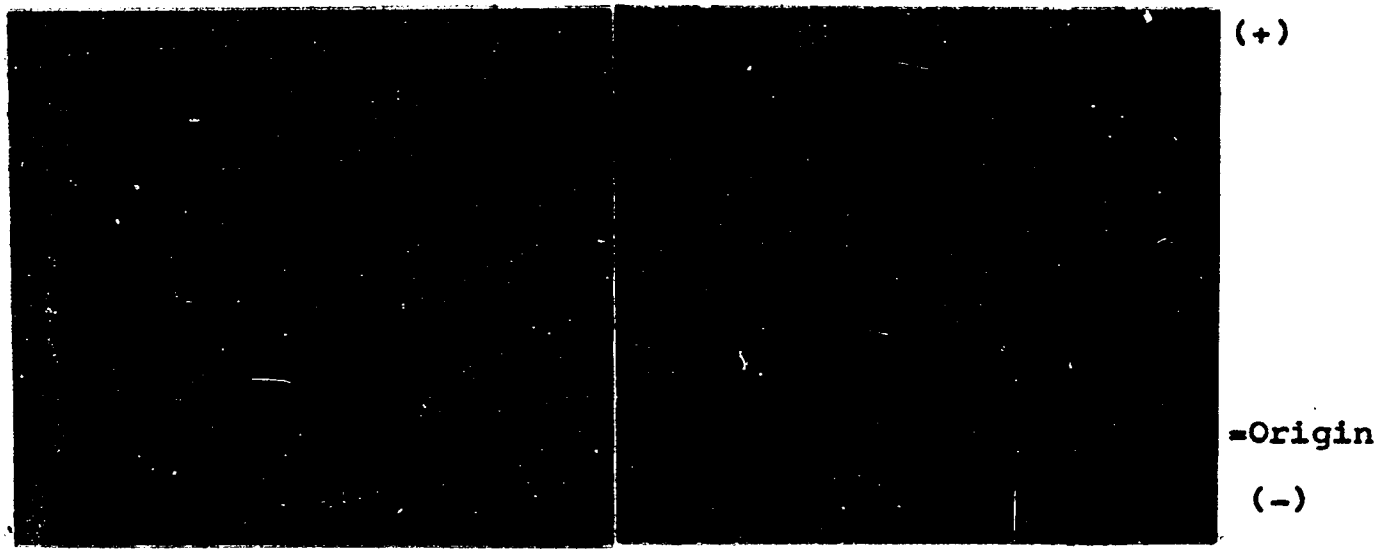


Fig. 15. Comparison of the alkaline phosphomonoesterase activity of the malathion-selected (KM, TM) and parathion-selected (KP, TP) strains of Kongolikan (K) and Trinidad (T) to that of their respective unselected original stock after electrophoresis at pH 8.6.

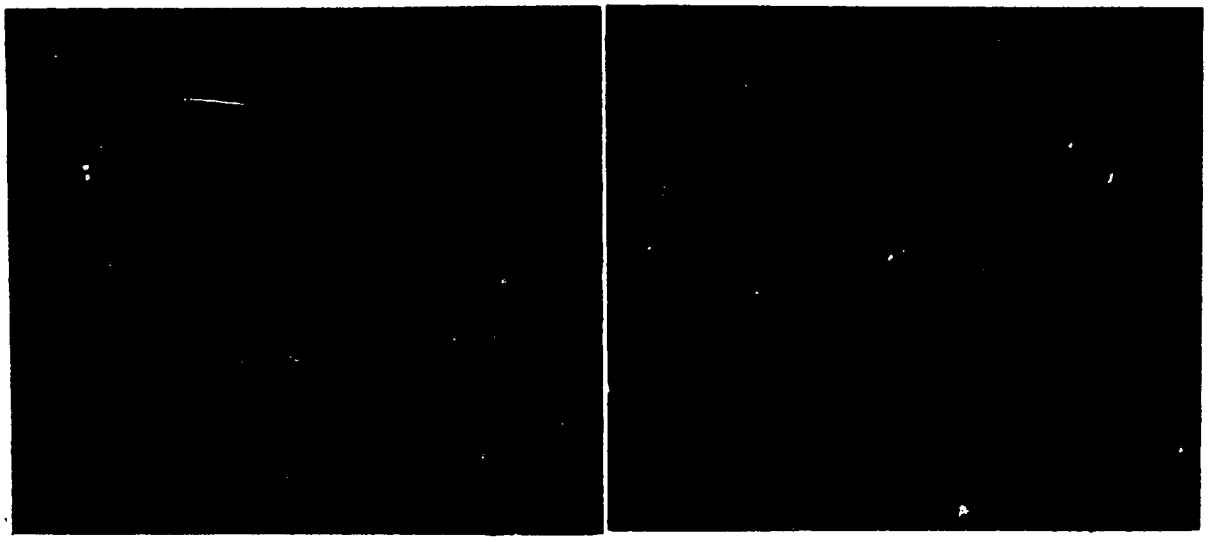


Fig. 15. Comparison of the alkaline phosphomonoesterase activity of the malathion-selected (KM, TM) and parathion-selected (KP, TP) strains of Kongolikan (K) and Trinidad (T) to that of their respective unselected original stock after electrophoresis at pH 8.6.

enzyme bands could hydrolyze the organophosphorus toxicants malaoxon and paraoxon, the developed zymograms displayed one very faint red area where the inhibitor had been hydrolyzed. Its position corresponded to that of the A-type esterase and the acid phosphatase. This OP-hydrolytic activity was no higher in the insecticide-selected strains than in the stock colonies. Here also, larval pre-treatment with sublethal concentrations of malathion or parathion did not produce any other zones of OP-hydrolytic activity, nor did it increase the intensity of the faint red area already displayed in either the OP-selected strains or the stock colonies.

DISCUSSION

The continuation of malathion selection of the Kongolikan- and Trinidad-Malathion strains of Aedes aegypti undertaken in this study raised the LC_{50} levels to maxima which were not very different from those obtained in the first sequence of malathion selection applied soon after the original colonies were originally received in this laboratory and before they had been allowed to revert without insecticide selection. The maximum LC_{50} of 0.79 ppm induced in the Trinidad-Malathion strain is virtually equivalent to that of 0.73 ppm obtained in the first regime of selection by Matsumura and Brown (1963a). The maximum LC_{50} of 1.20 ppm attained by the Kongolikan-Malathion strain is comparable to the 1.40 ppm and 1.60 ppm peaks reached in the first and second malathion selections of the Penang stock by Brown and Abedi (1960) and by Pillai (1964).

The failure of the progeny of crosses between the malathion-selected strains to respond to malathion selection by attaining higher peaks than those reached by the individual strains suggests that these differently selected strains possessed the same factors affecting malathion-

resistance, since there was no additive effect.

It would therefore seem that an LC_{50} level in the 1.20-1.60 ppm range is the upper limit which A. aegypti larvae can attain under laboratory selection pressure with malathion. This is supported by data from the study of Inwang (1966) on the frequency distribution of susceptibility in the Kongolikan material before and after relaxation of selection pressure from malathion or parathion. He found that the variation of response in the Kongolikan-Malathion strain before relaxation was low, indicating a homogeneous population.

An LC_{50} level to malathion in the 1.20-1.60 ppm range cannot be considered as low, as other OP-resistant mosquitoes did not attain higher levels. Larvae of A. nigromaculis which had become field-resistant to organophosphorus insecticides in general were found by Brown et al. (1963) to have an LC_{50} to malathion of only 0.12 ppm and were only 10 times more resistant to malathion than normal. However, it must also be mentioned that the selecting agents had been parathion and methyl parathion rather than malathion. Even the malathion-resistant strain of Culex tarsalis which was 40-45 times more resistant to malathion than normal had a larval LC_{50} to malathion of only 1.20 ppm after 3 months maintenance in this laboratory (Matsumura and Brown, 1961b).

The failure of the Albina and Montego Bay strains to develop resistance to malathion under laboratory selection after having become tolerant to it in the field is most probably due to their lacking any specific factor for malathion-resistance. The surprising decrease in LC_{50} in the Montego Bay strain when it was under laboratory selection could have been due to the lack of an unknown selecting agent which was operating only under the field conditions, and in its absence in laboratory selection the strain reverted to a lower level similar to that of the Albina strain.

The phenomenon of the Penang-Malathion strain not increasing its larval LC_{50} under continued malathion selection above that level to which it had reverted on relaxation of selection pressure may have resulted from genetic drift independent of the selection. In such a manner it could have lost the genetic factors involved in malathion-resistance, which have been found by Pillai and Brown (1965) in A. aegypti to be due to multiple genes on chromosomes 2 and 3. The surprising decrease in larval LC_{50} on continuing the malathion selection of the Kongolikan, Penang and Trinidad material after their peaks had been attained was also probably the result of a severely reduced gene pool after about 5-8 years of intermittent selection in the laboratory.

The parathion-selected strains of Kongolikan, Penang and Trinidad never attained under continued selection a larval LC_{50} to parathion above the range of 0.030-0.045 ppm.

These strains are probably only vigor tolerant, but they lack the mechanisms for true parathion resistance. Inwang (1966) reported that the variation of response to parathion by the Kongolikan-Parathion strain was very high; it would therefore be concluded that whatever heterozygosity was present in the gene pool, none of it was for alleles conferring true resistance.

The Kongolikan-Malathion strain was characterized by a reduced aliesterase activity since it hydrolyzed significantly less methyl butyrate than its unselected susceptible parent stock. In this respect it resembles the OP-resistant houseflies studied by van Asperen and Oppenoorth (1959). This finding was corroborated by the electrophoretic study of the Kongolikan material which showed the almost total absence of 2 zones of aliesterase activity in the malathion-selected strain as compared with the original stock. However, these findings differ from those of Plapp et al. (1965), who found no difference in aliesterase activity on methyl butyrate when they compared the Penang-Malathion strain to a susceptible one.

In the present study, although the esterase zymograms showed that the Trinidad-Malathion strain also lacked a zone of aliesterase activity present in the unselected stock, there was no such absence of bands in the Penang-Malathion strain, which displayed even more esterase activity than its unselected original stock. This increase was shown even

after the first selection, in which Matsumura and Brown (1961a) found that the aliesterase activity of the Penang-Malathion strain was higher than normal, although this difference was not significant.

The inhibition studies revealed that 10% of the esterase activity on methyl butyrate and phenyl acetate in the Kongolikan-Malathion strain was paraoxon-insensitive, whereas none of it was found to be insensitive in the unselected Kongolikan stock. However in the esterase zymograms the picture was the opposite, in that the paraoxon-insensitive band was less active in the malathion-selected strain than in the unselected stock, but here β-naphthyl acetate was the substrate. In the malathion-resistant strain of Culex tarsalis, whereas the activity on α-naphthyl acetate was greater than in the susceptible strain, the activity on β-naphthyl acetate was no different from the normal; on β-naphthyl benzoate as substrate, however, the resistant strain was significantly less active than the normal (Matsumura and Brown, 1963b).

The paraoxon-insensitive esterase band of the Trinidad-Malathion strain, like that of Kongolikan-Malathion, was less active than that of the unselected stock, and the acid and alkaline phosphomonoesterase activities of both of these malathion-selected strains were less than that of their respective stock colonies. In contrast, the Penang-Malathion strain had more paraoxon-insensitive activity

and the same amount of acid and alkaline phosphatase activity as the unselected original material. This inconsistency in results of the malathion-resistant strains when they were compared to their unselected stock colonies indicate that such comparisons cannot be used as an indication of malathion-resistance in A. aegypti.

One of the most conspicuous esterase bands was that which was second from the anodal end of the zymogram, i.e. the second most electrophoretically mobile band. In all 3 parathion-selected strains it was more pronounced than in the unselected original stocks, while in the malathion-selected strains it was almost totally absent in Kongolikan-Malathion, equal in activity in Trinidad-Malathion, but more active in Penang-Malathion, than in their respective original stocks. However, since this band was inhibited by 10^{-5}M paraoxon, it therefore cannot be considered a candidate for an OP-hydrolyzing enzyme.

On the other hand, the paraoxon-insensitive or A-type esterase band is a potential candidate for OP detoxication. This band was the slowest moving one and had the same electrophoretic mobility as the acid phosphatase band.

In the biochemical studies conducted on larval homogenates of the Kongolikan-Malathion strain and the unselected Kongolikan stock, the malathion-selected strain had more paraoxon-insensitive esterase activity on all the substrates than the normal. However in the zymograms of

esterase activity with β-naphthyl acetate as substrate, the malathion-selected strains had less paraoxon-insensitive activity in 2 out of 3 cases. In the parathion-selected strains the results were the opposite, with paraoxon-insensitive activity being higher than normal in the Penang- and Trinidad-Parathion strains and equal in the Kongolikan-Parathion strain.

The experiments to detect the possible hydrolysis of malaoxon or paraoxon showed that degradative hydrolysis did occur, and the faint zone of OP-hydrolytic activity which was detected had the electrophoretic mobility of the A-type esterase and the acid phosphatase. This insecticide degradation would therefore be classified as a phosphatase type. However, the organophosphorus insecticide-selected strains did not display greater activity than the unselected stock colonies. This agrees with the results of Matsumura and Brown (1961a, 1963a) who found that malathion-selected strains of A. aegypti were no different from the normal in OP-insecticide degradation, producing 4-6 times as much phosphatase products as carboxyesterase products from malathion and only phosphatase products from parathion.

SUMMARY AND CONCLUSIONS

The continuation of malathion-selection on the Penang- and Trinidad-Malathion strains of Aedes aegypti did not increase the larval LC_{50} levels to malathion above those already reached in the previous regimes of selection (i.e. 1.60 ppm and 0.73 ppm respectively), and on the Kongolikan-Malathion strain could not raise it above the 1.20 ppm level. Crossing these malathion-selected strains and subjecting their progeny to malathion selection failed to raise the malathion-resistance levels above those peaks already reached by the parental strains. This absence of any additive effect suggests that these different malathion-selected strains possess the same factors affecting malathion-resistance in A. aegypti. It appears that an LC_{50} level of 1.20-1.60 ppm malathion is the upper limit which A. aegypti larvae can attain under laboratory selection. The surprising decrease in larval LC_{50} levels under continued selection after the maxima were reached was probably the result of a severe reduction in the gene pool consequent on 5-8 years of intermittent selection.

The parathion-selected strains from Kongolikan, Penang and Trinidad never attained under continued selection a larval LC_{50} above 0.045 ppm parathion; therefore all they probably gained was vigor tolerance, without any mechanism for true parathion-resistance. The fact that the Albina and Montego Bay strains which had become field-tolerant to malathion did not develop resistance to malathion or Abate under laboratory selection indicates their lack of any specific factors for resistance to these insecticides.

The in vitro assay of esterase activity in larval homogenates of the Kongolikan-Malathion strain and the unselected Kongolikan stock revealed no interstrain difference in cholinesterase activity. However, the malathion-selected strain was characterized by reduced aliesterase activity, hydrolyzing methyl butyrate 14% less, and phenyl acetate 40% less, than its parent stock. But it had a higher arylesterase activity showing 10% paraoxon-insensitive activity instead of 0% on methyl butyrate and phenyl acetate, and 69% instead of 47% on tributyrin.

The esterase zymograms of the 5 different stock colonies showed constant differences in esterase patterns. None of the bands were due to cholinesterase, and all were B types except for the slowest-moving band which was an A type. All the stock colonies showed a single band of acid phosphomonoesterase activity, and this coincided with the

A-type esterase band. Only one band of alkaline phosphomonoesterase activity appeared, and this materialized in the middle of the zymogram. The different stocks showed differences in the intensities of their acid and their alkaline phosphatase bands.

When the esterase patterns of the selected strains were compared with those of the unselected stocks, no differences were found in the Albina and Montego Bay material. But in the 3 other stocks, the two most mobile bands were almost totally absent in the Kongolikan-Malathion strain and the first band was absent in the Trinidad-Malathion strain; moreover the Penang-Malathion strain displayed in general more esterase activity than its normal counterpart. In the 3 parathion-selected strains the second most mobile band was much more pronounced than in the unselected material, but being paraoxon-sensitive it cannot be involved in OP-insecticide degradation.

Although in these 3 stocks the OP-selected strains differed in phosphatase activity from their respective unselected original colonies, the differences were not consistent. No differences were found in the Albina and Montego Bay material.

In two of these 3 stocks the paraoxon-insensitive arylesterase band was more pronounced in the parathion-selected strain, and no less in the third. Of the malathion-selected strains, only the Penang-Malathion

showed more arylesterase, the other two showing less than normal, although the *in vitro* esterase assay had indicated the Kongolikan-Malathion strain to be richer in arylesterases than normal. Malaoxon and paraoxon were hydrolyzed in a zone coinciding with that of the A-type esterase and the acid phosphomonoesterase, indicating a phosphatase type degradation. However, since this zone of OP-hydrolytic activity in the selected strains was no more intense than in the original stocks, it must be concluded that OP-insecticide hydrolytic degradation is not the mechanism for the increased tolerance to malathion or to parathion.

Since the zymograms obtained from larvae of both selected and unselected colonies which had been pre-treated with sublethal concentrations of malathion er parathion were no different from those not pre-treated, evidently these insecticides have no inductive or activating effect on the esterases or phosphomonoesterases.

REFERENCES

Aldridge, W.N. 1953a. Serum esterases. 1. Two types of esterases (A and B) hydrolysing p-nitrophenyl acetate, propionate and butyrate, and a method for their determination. Biochem. J. 53: 110-117.

Aldridge, W.N. 1953b. Serum esterases. 2. An enzyme hydrolysing diethyl p-nitrophenyl phosphate (E 600) and its identity with the A-esterase of mammalian sera. Biochem. J. 53: 117-124.

Aldridge, W.N. 1954. Some esterases of the rat. Biochem. J. 57: 692-702.

Arurkar, S.K. and Knowles, C.O. 1968. Electrophoretic studies of insect esterases. Ann. Entomol. Soc. Amer. 61: 686-690.

Asperen, K. van. 1958. Mode of action of organophosphorus insecticides. Nature (London). 181: 355-356.

Asperen, K. van. 1959. Distribution and substrate specificity of esterases in the housefly, Musca domestica L. J. Insect Physiol. 3: 306-322.

Asperen, K. van. 1962. A study of housefly esterases by means of a sensitive colorimetric method. J. Insect Physiol. 8: 401-416.

Asperen, K. van. 1964. Biochemistry and genetics of esterases in houseflies (Musca domestica) with special reference to the development of resistance to organophosphorus compounds. Entomol. Exp. Appl. 7: 205-214.

Asperen, K. van, and Mazijk, M.E. van. 1965. Agar gel-electrophoretic esterase patterns in houseflies. Nature (London). 205: 1291-1292.

Asperen, K. van, and Oppenoorth, F.J. 1959. Organophosphate resistance and esterase activity in houseflies. Entomol. Exp. Appl. 2: 48-57.

Augustinsson, K.-B. 1959. Electrophoresis studies on blood plasma esterases. I. Mammalian plasmata. Acta. Chem. Scand. 13: 571-592.

Augustinsson, K.-B. 1961. Multiple forms of esterase in vertebrate blood plasma. Ann. N.Y. Acad. Sci. 94: 844-860.

Barka, T. and Anderson, P.J. 1965. Histochemistry. Harper and Row Publishers, Inc. N.Y. 660 p.

Beadle, G.W. and Tatum, E.L. 1941. Genetic control of biochemical reactions in Neurospora. Proc. Nat. Acad. Sci. USA. 27: 499-506.

Beckman, L. and Johnson, F.M. 1964. Variations in larval alkaline phosphatase controlled by Aph alleles in Drosophila melanogaster. Genetics. 49: 829-835.

Bergmann, F., Segal, R., and Rimon, S. 1957. A new type of esterase in hog kidney extract. Biochem. J. 67: 481-486.

Bigley, W.S. and Plapp, F.W., Jr. 1960. Cholinesterase and ali-esterase activity in organophosphorus-susceptible and -resistant house flies. Ann. Entomol. Soc. Amer. 53: 360-364.

Brewbaker, J.L. 1964. Agricultural Genetics. Prentice-Hall, Inc. Englewood Cliffs, N.J. 156 p.

Brown, A.W.A. 1966. Summary of symposium. Mosquito News. 26: 337-340.

Brown, A.W.A. 1967a. Insecticide resistance-genetic implications and applications. World Rev. Pest Contr. 6: 104-114.

Brown, A.W.A. 1967b. Research needs and priorities: biochemistry. Bull. Wld. Hlth. Org. 36: 650-562.

Brown, A.W.A. and Abedi, Z.H. 1960. Cross-resistance characteristics of a malathion-tolerant strain developed in Aedes aegypti. Mosquito News. 20: 118-124.

Brown, A.W.A., Lewallen, L.L. and Gillies, P.A. 1963. Organophosphorus resistance in Aedes nigromaculis in California. Mosquito News. 23: 341-345.

Bunyan, P.J. and Taylor, A. 1966. Esterase inhibition in pheasants poisoned by O,O-diethyl S-(ethylthiomethyl)-phosphorodithioate (Thimet) (phorate). J. Agr. Food Chem. 14: 132-137.

Chadwick, L.E. 1963. Action on insects and other invertebrates. Handb. Exp. Pharmak. 15: 741-798.

Cook, B.J. and Forgash, A.J. 1965. The identification and distribution of the carboxylic esterasesin the American cockroach, Periplaneta americana (L.). J. Insect Physiol. 11: 237-250.

Craig, G.B. Jr. and Hickey, W.A. 1967. Genetics of Aedes aegypti. In Wright, J.W. and Pal, R. (Eds.). Genetics of Insect Vectors of Disease. Elsevier Publishing Co. Amsterdam. pp. 67-131.

Dessauer, H.C. and Fox, W. 1964. Electrophoresis in taxonomic studies illustrated by analyses of blood proteins. In Leone, C.A. (Ed.). Taxonomic Biochemistry and Serology. Ronald Press Co. N.Y. pp. 625-647.

Dixon, M. and Webb, E.C. 1964. Enzymes. Academic Press. N.Y. 950 p.

Falconer, D.S. 1964. Introduction to Quantitative Genetics. Oliver and Boyd. Edinburgh. 365 p.

Flynn, A.D. and Schoof, H.F. 1965. Susceptibility of Aedes aegypti from Florida and Texas to DDT, dieldrin and malathion. Mosquito News. 25: 411-414.

Fox, I. 1961. Resistance of Aedes aegypti to certain chlorinated hydrocarbon and organophosphorus insecticides in Puerto Rico. Bull. Wld. Hlth. Org. 24: 489-494.

Gautrelet, J. 1938. Existence d'un complexe d'acétylcholine dans le cerveau et divers organes, ses caractères, sa répartition. Bull. Acad. Med. (Paris). 120: 285-291.

Gilmour, D. 1965. The Metabolism of Insects. W.H. Freeman and Co. San Francisco. 195 p.

Cjullin, C.M. and Isaak, L.W. 1957. Present status of mosquito resistance to insecticides in the San Joaquin valley in California. Mosquito News. 17: 67-70.

Gomori, G. 1939. Microtechnical demonstration of phosphatase in tissue sections. Proc. Soc. Exp. Biol. Med. 42: 23-26.

Hartman, P.E. and Suskind, S.R. 1965. Gene Action. Prentice-Hall, Inc. Englewood Cliffs, N.J. 158 p.

Herne, D.H.C. 1967. Organophosphorus resistance in the two-spotted spider mite Tetranychus urticae Koch. Ph.D. Thesis. University of Western Ontario. 164 p.

Hestrin, S. 1949. The reaction of acetylcholine and carboxylic acid derivatives with hydroxylamine, and its analytical application. J. Biol. Chem. 180: 249-261.

Hunter, R.L. Markert, C.L. 1957. Histochemical demonstration of enzymes separated by zone electrophoresis in starch gels. Science. 125: 1294-1295.

Inwang, E.E. 1966. Regional characterization of DDT-resistance in Aedes aegypti (L.). M.Sc. Thesis. University of Western Ontario. 126 p.

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

Keiding, J. 1956. Resistance to organic phosphorus insecticides of the house fly. Science. 123: 1173-1174.

Kerr, J.A., Camargo, S. de and Abedi, Z.H. 1964. Eradication of Aedes aegypti in Latin America. Mosquito News. 24: 276-282.

Lambremont, E.N. 1959. A study of acid phosphatase of the mosquito Aedes aegypti L. J. Insect Physiol. 3: 325-334.

Laufer, H. 1941. Forms of enzymes in insect development. Ann. N.Y. Acad. Sci. 94: 825-835.

Lee, R.M. and Batham, P. 1966. The activity and organophosphate inhibition of cholinesterases from susceptible and resistant ticks (Acari). Entomol. Exp. Appl. 9: 13-24.

Lord, K.A. and Potter, C. 1950. Mechanism of action of organo-phosphorus compounds as insecticides. Nature (London). 166: 893-894.

Lord, K.A. and Potter, C. 1954a. Differences in esterases from insect species: toxicity of organo-phosphorus compounds and *in vitro* anti-esterase activity. J. Sci. Food Agr. 5: 490-498.

Lord, K.A. and Potter, C. 1954b. Organo-phosphorus insecticides, insecticidal and anti-esterase activity of organo-phosphorus compounds. Chem. Ind. (London). pp. 1214-1217.

MacIntyre, R. 1966. The genetics of an acid phosphatase in *Drosophila melanogaster* and *Drosophila simulans*. Genetics. 53: 461-474.

March, R.B. 1959. Resistance to organo-phosphorus insecticides. Misc. Publ. Entomol. Soc. Amer. 1: 13-19.

Markert, C.L. and Moller, F. 1959. Multiple forms of enzymes: tissue, ontogenetic and species specific patterns. Proc. Nat. Acad. Sci. USA. 45: 753-763.

Matsumura, F. and Brown, A.W.A. 1961a. Biochemical study of a malathion-tolerant strain of *Aedes aegypti*. Mosquito News. 21: 192-194.

Matsumura, F. and Brown, A.W.A. 1961b. Biochemistry of malathion resistance in *Culex tarsalis*. J. Econ. Entomol. 54: 1176-1185.

Matsumura, F. and Brown, A.W.A. 1963a. Studies on organophosphorus-tolerance in *Aedes aegypti*. Mosquito News. 23: 26-31.

Matsumura, F. and Brown, A.W.A. 1963b. Studies on carboxyesterase in malathion-resistant Culex tarsalis. J. Econ. Entomol. 56: 381-388.

Matsumura, F. and Sakai, K. 1968. Degradation of insecticides by esterases of the American cockroach. J. Econ. Entomol. 61: 598-605.

Matsumura, F. and Voss, G. 1964. Mechanism of malathion and parathion resistance in the two-spotted spider mite, Tetranychus urticae. J. Econ. Entomol. 57: 911-917.

Menten, M.L., Junge, J. and Green, M.H. 1944. A coupling histochemical azo dye test for alkaline phosphatase in the kidney. J. Biol. Chem. 153: 471-477.

Menzel, D.B., Craig, R. and Hoskins, W.M. 1963. Electrophoretic properties of esterases from susceptible and resistant strains of the housefly, Musca domestica L. J. Insect Physiol. 9: 479-493.

Metcalf, R.L., March, R.B. and Maxon, M.G. 1955. Substrate preferences of insect cholinesterases. Ann. Entomol. Soc. Amer. 48: 222-228.

Metcalf, R.L., Maxon, M., Fukuto, T.R. and March, R.B. 1956. Aromatic esterase in insects. Ann. Entomol. Soc. Amer. 49: 274-279.

Mounter, L.A. 1963. Metabolism of organophosphorus anticholinesterase agents. Handb. Exp. Pharmak. 15: 486-504.

Mounter, L.A. and Whittaker, V.P. 1953. The hydrolysis of esters of phenol by cholinesterases and other esterases. Biochem. J. 54: 551-559.

Nachlas, M.M. and Seligman, A.M. 1949. The histochemical demonstration of esterase. J. Nat. Cancer Inst. 9: 415-425.

O'Brien, R.D. 1967. Insecticides, Action and Metabolism. Academic Press. N.Y. 332 p.

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

Ogita, Z. and Kasai, T. 1965a. Genetic control of multiple esterases in Musca domestica. Japan. J. Genetics. 40: 1-14.

Ogita, Z. and Kasai, T. 1965b. Genetic control of multiple molecular forms of the acid phosphomonoesterases in the house fly, Musca domestica. Japan. J. Genetics. 40: 185-197.

Oosterbaan, R.A. and Jansz, H.S. 1965. Cholinesterases, esterases and lipases. In Florkin, M. and Stotz, E.H. (Eds.). Comprehensive Biochemistry. Elsevier Publishing Co. Amsterdam. Vol. 16. pp. 1-54.

Oppenoorth, F.J. 1958. A mechanism of resistance to parathion in Musca domestica (L.). Nature (London). 181: 425-426.

Oppenoorth, F.J. and Asperen, K. van. 1960. Allelic genes in the housefly producing modified enzymes that cause organophosphate resistance. Science. 132: 298-299.

Pardee, A.B. and Wilson, A.C. 1963. Control of enzyme activity in higher animals. Cancer Res. 23: 1483-1490.

Pillai, M.K.K. 1964. Studies on DDT-resistance in Aedes aegypti L. Ph.D. Thesis. University of Western Ontario. 175 p.

Pillai, M.K.K. and Brown, A.W.A. 1965. Physiological and genetical studies on resistance to DDT substitutes in Aedes aegypti. J. Econ. Entomol. 58: 255-266.

Plaisted, P.H. 1964. Zone electrophoresis. In Newman, D.W. (Ed.). Instrumental Methods of Experimental Biology. Macmillan Co. N.Y. pp. 86-112.

Plapp, F.W. Jr., Bigley, W.S., Darrow, D.I. and Hoyer, R.F. 1965. Esterase activity in mosquitoes and its possible relationship to organophosphate and carbamate resistance. Mosquito News. 25: 30-35.

Richter, D. and Croft, P.G. 1942. Blood esterases. Biochem. J. 36: 746-757.

Salkeld, E.H. 1965. Electrophoretic separation and identification of esterases in eggs and young nymphs of the large milkweed bug, Oncopeltus fasciatus (Dallas). Can. J. Zool. 43: 593-602.

Schoof, H.F. 1967. Insecticides for use against Aedes aegypti. Bull. Wld. Hlth. Org. 36: 618-622.

Shaw, C.R. 1965. Electrophoretic variation in enzymes. Science. 149: 936-943.

Smissaert, H.R. 1964. Cholinesterase inhibition in spider mites susceptible and resistant to organophosphate. Science. 143: 129-131.

Smithies, O. 1955. Zone electrophoresis in starch gels: group variations in the serum proteins of normal human adults. Biochem. J. 61: 629-641.

Staehelin, M. 1965. Biochemical mechanism of information transfer. In Weber, R. (Ed.). The Biochemistry of Animal Development. Academic Press. N.Y. Vol. I. pp. 441-482.

Stegwee, D. 1959. Esterase inhibition and organophosphorus poisoning in the house-fly. Nature (London). 184: 1253-1254.

Stegwee, D. 1960. The role of esterase inhibition in tetraethylpyrophosphate poisoning in the housefly, Musca domestica L. Can. J. Biochem. Physiol. 38: 1417-1430.

Stone, B.F. 1968a. Mechanisms of fenthion-resistance in Culex fatigans Wied. Ph.D. Thesis. University of Western Ontario. 116 p.

Stone, B.F. 1968b. Brain cholinesterase activity and its inheritance in cattle tick (Boophilus microplus) strains resistant and susceptible to organophosphorus acaricides. Aust. J. Biol. Sci. 21: 321-330.

Tadano, T. and Brown, A.W.A. 1966. Development of resistance to various insecticides in Culex pipiens fatigans Wiedemann. Bull. Wld. Hlth. Org. 35: 189-201.

Takamatsu, H. 1939. Histochemische untersuchungen der phosphatase und deren verteilung in verschiedenen organen und gewebe. Trans. Soc. Path. Japan. 29: 492-498.

Uriel, J. 1961. Caractérisation des cholinesterases et d'autres estérases carboxyliques après electrophorèse et immunelectrophorese en gelose. I. Application à l'étude des estérases du serum humain normal. Ann. Inst. Pasteur. 101: 104-110.

Watson, J.D. 1965. Molecular Biology of the Gene. W.A. Benjamin, Inc. N.Y. 494 p.

W.H.O. 1963. Instructions for determining the susceptibility or resistance of mosquito larvae to insecticides. WHO Tech. Rep. Ser. No. 265: 51-56.

Wieme, R.J. 1965. Agar Gel Electrophoresis. Elsevier Publishing Co. Amsterdam. 425 p.

Wilson, A.C. and Pardee, A.B. 1964. Comparative aspects of metabolic control. In Florkin, M. and Mason, H.S. (Eds.). Comparative Biochemistry. Academic Press. N.Y. Vol. 6. pp. 73-118.

Wright, T.R.F. 1963. The genetics of an esterase in Drosophila melanogaster. Genetics. 48: 787-801.

Appendix Table 1. Classification of A- and B-esterases.

The enzymes described in the thesis as A-esterase (or arylesterase) and B-esterases (or aliesterases) are characterized by the International Union of Biochemistry as follows:

Classification of Aldridge	Trivial or Other Names	International Union of Biochemistry
A-esterase	Arylesterase	Aryl-ester hydrolase (3.1.1.2)
B-esterase	Aliesterase	Carboxylic ester hydrolase (3.1.1.1)

Appendix Table 2. Optical density values of the substrates acetylcholine bromide, phenyl acetate, tributyrin and methyl butyrate.

Substrate Concentration	ACETYLCHOLINE BROMIDE Optical Density	PHENYL ACETATE Optical Density	TRIBUTYRIN Optical Density	METHYL BUTYRATE Optical Density
$1x10^{-3}M$	.100, .102, .110;	.070, .095, .100;	.265, .270, .278, .283;	.055, .062, .070, .075, .075, .082;
$2x10^{-3}M$	.186, .208, .225;	.173, .185, .195;	.470, .480, .485, .500;	.140, .148, .152, .161, .170, .184;
$3x10^{-3}M$	.297, .325;	.250, .280, .288;	.650, .670, .675, .680;	.222, .230, .232, .237;
$4x10^{-3}M$	.385, .398;	.350, .380, .385;		.288, .288, .294, .311, .325, .336;
$5x10^{-3}M$	.499, .521;	.450;		.367, .370, .377, .402;
$6x10^{-3}M$				.462, .484, .510, .539;

Appendix Table 3. Values of the esterase activity in larvae of the Kongolikan stock and Kongolikan-Malathion strain.

Substrate	ACETYLCHOLINE BROMIDE	PHENYL ACETATE	TRIBUTYRIN	METHYL BUTYRATE
Micromoles substrate hydrolyzed:				
KONGOLIKAN	0.50, 0.54, 0.55;	2.78, 2.56;	1.09, 1.19;	2.40, 2.46, 2.48, 2.64, 2.69, 2.80, 2.87, 2.93;
KONGOLIKAN-MALATHION	0.49, 0.51, 0.53;	1.87, 1.95;	1.18, 1.26;	2.18, 2.30, 2.30, 2.40;