DDT-RESISTANCE MECHANISMS IN MOSQUITOES AND THEIR SIGNIFICANCE

A Thesis

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ABSTRACT

A research on insecticide resistance and its significance was surveyed on DDT-resistant and susceptible strains of 5 species of mosquito, Culex pipiens fatigans, Aedes aegypti, Anopheles gambiae, An. quadrimaculatus and An. stephensi.

1. The technique of detecting and measuring resistance in adult mosquitoes was studied in relation to problems raised by new insecticides. A wide range of concentrations of malathion, fenthion, fenitrothion and propoxur were tested against C. p. fatigans in the relations of exposure It was shown that time can be used as a dose parameter.

In studies of the storage life of W.H.O. test papers, there was found no evidence of deterioration of malathion and propoxur impregnated papers over a period of a year but a considerable decline is found thereafter.

2. Cross-resistance spectra to various DDT analogues and certain other compounds were determined by larvicide tests on each strain. The effects of two kinds of synergist, MNC and piperonyl butoxide were also investigated in the hope that specific detoxication mechanisms would be revealed. The overall results indicated that DDT-resistance mechanisms in C. p. fatigans, An. quadrimaculatus and An. stephensi. depended largely on dehydrochlorination. In An. gambiae and A. aegypti there is clearly another mechanism responsible for DDT-resistance suggesting a microsomal oxidation.

Since there are probably more than one mechanisms present to different degrees in several strains, one cannot expect these experiments to give very simple, clear-cut results.

3. Radiometric measurements of the ^{14}C marked DDT and malathion

were made on exposed larvae and the residual suspensions, to measure pick-up and penetration of these insecticides. There was no evidence of reduced penetration for DDT; on the contrary, the resistant strain allowed more DDT to penetrate. A definite correlation between the amount of pick-up of DDT in µg per larva and then exposed concentration were observed. With malathion, there was no difference in the percentage penetration between the resistant and susceptible strains.

4. In view of the high levels of DDT-resistance noted, some alternative compounds were tried as larvicides. DDT is a highly potent larvicide with an LC50 value of 0.005 ppm. Prolan and Bulan are less effective with LC50 of 0.005 to 0.12 ppm. The LC50 levels of some biodegradable DDT analogues ranged from 0.02 to 0.2 ppm and showed distinct differences with the species. LC50 of bioallethrin and allethrin were rather high and ranged from 0.015 to 0.4 ppm. Bioallethrin was about 4 times more potent than allethrin and fenthion was also more potent than malathion with LC50 of 0.002 to 0.14 ppm.

New compounds affecting moulting and metamorphosis were tested. The juvenile hormone mimic ZR-515 and the moulting disturbance compound (believed to inhibit chitin synthesis) PH60-40, were the most potent with LC50 value 0.0013 to 0.003 ppm. Non 0585, which interferes with melanisation during pupation, was also moderately potent. Cartap hydrochloride, phenol, aliphatic amines and unsaturated fatty acids were not potent.

5. The resistant strains of An. quadrimaculatus, An. stephensi and C.p. fatigans showed highly specific resistance to DDT and DDD. Presumably these strains depend on dehydrochlorination mechanisms. There was definite evidence of cross resistance to the biodegradable

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analogues and various other compounds (including moulting disturbance compounds) in An. stephensi and A. aegypti. These results suggested enhanced microsomal detoxication besides the dehydrochlorination mechanisms.

6. Among the compounds affecting moulting and metamorphosis, PH6040 showed some ovicidal activity. All of the compounds tested with various stages of larvae were involved in the process of ecdysis and the early IVth instar larvae was the most susceptible stage. The effects were specific in each group of compounds. The hormone mimics ZR-515 and R-20458 exhibited their main effects in the very late pupal stage when the adult form had become very clear.

Non-0585 expressed its activity in unmelanized form pupa prior to darkening of the cuticle. The Duphar compounds PH60-40 and PH60-38 interrupted the development between larval and pupal stage, the pupae being trapped inside. Sometimes, they can split the exuviae but they were unable to free themselves from the larval skin. Ecdysterone had the earliest activity at the larval stage and produced no significant mortality in the surviving larvae. All compounds showed delayed development effect except ecdysterone. PH60-40, PH60-38 and ZR 515 showed some sterilization activity when the adults were fed with sugar solution containing these compounds.

The aliphatic amines can be used as ovicides, larvicides and pupacides but their potency was not high. Delayed development and involvement of emerged adults were also observed. The two fatty acids produced morphological abnormalities and interfered with melanization process, though the activity was not high.

CONTENTS

PART I.

REVIEW OF LITERATURE

PRACTICAL IMPORTANCE OF INSECTICIDE RESISTANCE

1. Synthetic Insecticides and the Growth of Resistance

A new era in pest control was begun with the introduction of DDT, the first and in many ways the most valuable of modern synthetic insecticides. It was first synthesised by an Austrian chemistry student, Othmar Zeidler, in 1873; but its remarkable insecticidal properties were not discerned until 1939, by a research team of the Geigy Chemical Company in Basel, under Paul Müller.

DDT and later synthetic insecticides, because of their relative cheapness and safety in use, achieved remarkable success in protecting man from the pests of growing crops, of forests, of stored food, of domesticated animals and, above all, the arthropod vectors of many serious epidemic diseases. Unfortunately, the use of chemicals to kill a large proportion of insects of certain species has frequently resulted in the development of resistant strains and has become the greatest single barrier to the completion of control programmes. A great deal of research has been carried out on the biochemistry, physiology and genetics of resistance, but although the nature and development of resistance is more fully understood, it has never been possible so far, to reverse, or even to halt the process. Thus, in regions where pests have developed extensive resistance to a given insecticide, so that the latter has had to be abandoned, it has never been possible to re-introduce the insecticide, with success.

The practical response to resistance has been to change to an alternative insecticide, but this has begun to fail by the steady extension of resistance to alternatives. It is only to be hoped that a thorough understanding and a wide background of knowledge of the major resistance mechanisms will enable the use of new alternative insecticides for overcoming the resistance problems.

In the special sense, commonly used by applied entomologists, the word "resistance" usually means that a population of an originally susceptible species has lost its susceptibility and become tolerant of doses of insecticides which would prove lethal to individuals in a normal population of the same species. This phenomenon is a change in the insects themselves, and is brought about by a selection of abnormal individuals, as a result of the use of the insecticide over a period of time. The survivors in the successive generations under treatment become more and more difficult to kill with that insecticide. The mechanisms responsible for most types of resistance are not general ones, to protect the insects against all insecticides, but are specific to a particular group of compounds. There are various groups of such compounds. Thus, insects made resistant by DDT selection pressure are cross-resistant to compounds allied to DDT, but not to cyclodiene derivatives or to gamma BHC (Beard, 1960; Mount, 1965). Insects made resistant by selection with dieldrin or related compounds are cross-resistant to the other chbrinated cyclodiene derivatives and also to YBHC (or lindane) but not to DDT and its relatives.

(Busvine, 1954; Metcalf, 1955b). Organophosphorus resistance is developed only by organophosphorus selection pressure. Insects with resistance to both DDT and dieldrin are not normally cross-resistant to organophosphorus insecticides. But it is noteworthy that selection of houseflies or mosquitoes with organophosphorus compounds sometimes results in a high DDT resistance and high cyclodiene resistance in houseflies (Brown and Abedi, 1960; Winteringham & Harrison, 1959). This phenomenon is not yet fully understood.

Apparently, the earliest example of resistance dates from 1908, when Aspidiotus permicions (San Jose scale) developed resistance to lime sulphur in Wasington State, U.S.A. Over the next 40 years, a small number of cases occurred, involving resistance to HCN, lead arsenate, sodium arsenate, tartar emetic, selenium, cryolite, and retenone in the pre-DDT era. DDT-resistance was first observed in the houseflies in 1946 in the region of Arnas, Sweden, 2 years after its introduction into the area for residual spraying (Wiesmann, 1947). Since then, the problem of resistance among the public health and veterinary pests has continued to grow and has come to involve a large number of species and extended to numerous insecticides and most geographical areas. During that period, DDT was the most widely used of the first group of modern insecticides. When DDT resistance became serious, alternative insecticides were introduced for the control of the resistant strains. Among these, γBHC and dieldrin were playing important roles since they combine high potency with long or fairly long residual action and are not irritant to mosquitoes like DDT, allowing the insects to settle long enough to pick up a lethal dose. They are also reasonably safe to man

and animals. Unfortunately, many insects soon showed high degree of resistance to those compounds dissipating the hope for replacement of DDT. Following the growth of dieldrin resistance, various long-residual organophosphorus compounds were introduced; but these, too, showed early incidence of resistance. By 1960, about 139 species of public health and agriculture pests have developed resistance to insecticides. These species belong to different orders: Diptera, Hemiptera, Coleoptera, Lepidoptera, Thysanoptera, Siphonoptera, Orthroptera, Anoplura and also the Acarina.

At the present, there are about 9 types of resistance, three of them being the most important: DDT-resistance, dieldrin or cyclodiene resistance, and organophosphorus resistance. The known cases of the development of resistance are 98 species with DDT resistance, 141 species with cyclodiene resistance and 54 species with organophosphorus resistance (Brown, 1971). The growth of resistance has increased each year as has been reviewed by Busvine (1970) in Table 1.

Table 1. The growth of reported cases of insecticide resistance.

Number of cases of resistance	Years					
	1946	1956	1958	1960	1967	1969
Public health pests	2	20	50	81	97	102
Agricultural and veterinary pests	8	-	52	-	127	228

The status of insecticide resistance, however, has been reviewed by several scientists: Busvine (1954, 1956a, 1957, 1969, 1970); Brown (1958a, 1961, 1971); Micks (1960); Hamon & Garrett Jones (1963), Bruce-Chwatt (1970); and Schoof (1970). All information indicated that the greatest increase of resistance was in the period of use of the chlorinated hydrocarbon insecticides and particularly the cyclodiene derivatives, during the decade of the 1950s. Since then, the increase in species is not quite so rapid, although the number of resistances per species and their distribution is enlarging. This effect is probably due to the organophosphorus and carbamate insecticides as replacement for DDT and dieldrin.

2. The Impact of Resistance on Agricultural & Veterinary Pest Control

Before turning to a closer examination of the effects of resistance on the control of public health pests, one may note that the Food and Agriculture Organization has tried to make a similar assessment of the status of resistance in regard to agricultural and veterinary pests (F.A.O., 1970). The situation is similarly serious and thought to be steadily deteriorating. Of the primary foods, rice is threatened by resistance of two major pests: the stem borer (Chilo suppressalis) and green rice leaf hopper (Nephotettix cincticeps). Stored cereals are in danger from resistance in several beetle pests, especially Tribolium castaneum. Various field crops are at risk due to resistance in root maggot flies (Hylemya spp.); the Colorado potato beetle (Leptinotarsa decemlineata); the potato tuber moth (Phthorimea operculella); the peach-potato aphid (Myzus persicae) and the codling moth (Laspeyresia pomonella).

Apart from food pests, the main crops of several areas are seriously threatened. For example, cotton, by resistance in cotton leafworm (Spodoptera littoralis), pink bollworm (Pectinophora gossypii) and cotton bollworm (Heliothis spp.). Also cocoa in West Africa, from the capsid (Distantiella theobroma). Likewise, tobacco from resistance in Protoparce sexta.

Fruit and greenhouse crops in many countries are severely troubled by resistance in spider mites (Tetranychus spp.). Among veterinary pests, the most serious resistance is in cattle ticks (Boophilus) and sheep blowflies (Lucilia cuprina). Enough has been said, perhaps, to indicate briefly the magnitude of these problems in agriculture and veterinary practice.

3. The Impact of Resistance on Disease Vector Control

Quantitative information on the numbers of species with strains developing to various types of pesticide is useful, but rather limited in its practical interpreation. Thus, a single "case" of resistance may refer to a species of limited distribution, with mere "nuisance" importance; or it could refer to an important disease vector, with very wide distribution.

Again, resistance to a single group of pesticides may be relatively unimportant if effective, safe and cheap alternatives exist. Therefore it is very desirable to assess the global position in regard to the actual impact of resistance on the use of pesticide to control disease vectors from time to time.

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these vectors, a questionnaire prepared by the World Health Organization was sent out to over 100 health authorities throughout the world. The information has been collected widely and was compiled with the help of vector control experts closely in touch with the field situation in various parts of the world. The attempt was to give a clear picture of the impact of insecticide resistance on control of vector-borne diseases and are summarised by Busvine & Pal (1969). Control is severely limited by resistance to organochlorine insecticides in Culex pipiens fatigans, vector of Bancroftian filariasis; and the housefly, vector of enteric and ophthalmic diseases. Some insecticides are still the best means of controlling these vectors; for instance, anopheline mosquitoes, vector of malaria; Aedes aegypti, vector of yellow fever and haemorrhagic fever; Xenopsylla cheopis, plague vector; human lice, vectors of typhus, bed bugs and various culicine mosquitoes which are serious nuisances from their bites have been moderately handicapped by resistance. Control of other insect-borne disease such as the blackflies of the genus Phlebotomus, vector of various leishmaniasis; the tsetse flies, Glossina spp., vectors of sleeping sickness and Triatomid bugs, vectors pf Chagas' disease have not, so far, been handicapped by resistance; but there is some evidence that this is on the way, since a cyclodiene-type resistance in Rhodnius prolixus has arisen in Venezuela.

4. Resistance in Mosquitoes

By 1970 the number of species of insects that had developed resistant populations had increased to 224. Furthermore, the geo-

graphical area where resistant populations had previously been found, had enlarged to some extent and the number of insecticides had also become greater to embrace all three types, namely, the organochlorines, the organophosphorus compounds and the carbamates. Research workers indifferent disciplines have collected a considerable amount of valuable information in this field. Details of resistance in vectors and vector-borne disease species concerned have been reviewed by Busvine & Pal (1969). All the literature relevant to insecticide resistance in many species of arthropods have been covered extensively by Brown & Pal (1971).

A. Anophelines and Culicines

As a result of the simplicity of detecting emergent resistance and the consequent wide use of the standardised tests, supported by the World Health Organization, a very clear picture of the changing states of resistence in mosquitoes throughout the world can be established. Among the Diptera, not less than 37 species of anopheline mosquitoes have developed resistance, 35 of them to dieldrin and 15 to DDT. On the other hand, 24 species of culicine have become resistant: 16 to DDT, 12 to dieldrin, 10 of them to both classes of chlorinated hydrocarbon and 9 to organophosphorus compounds.

Apart from Aedes aegypti and Culex fatigans, most culicine mosquitoes are troublesome autdoors and have always been attacked by larvicides; whereas adulticides are used against anophelines, which mainly transmit malaria indoors. Larvicides tend to have a greater selective effect because they affect a much larger sample of the wild population. This was especially true of various American

nuisance mosquitoes including the salt marsh breeders: Aedes sollicitans, A. taeniorhynchus and the flood water breeders in California: Aedes nigromaculis, A. melanimon and Culex tarsalis. Both groups have been extensively and vigorously attacked by aerial spraying and have developed through all three main types of resistance.

Some larviciding was practised at the begining of malaria control, with synthetic insecticides. Later, the W.H.O. co-ordinated Programme for Global Malaria Eradication, adopted a house spraying policy. The selective effect of adulticides due to the smaller proportion of the wild population affected, which depends on the degree of endophily and anthropophilly of the species; and with DDT, the selective effect is still further reduced by the irritating property which can expel the mosquitoes before they acquire a lethal dose. It appears from the foregoing results that both factors held back incidence of DDT-resistance in anophelines and in many cases, resistance was ascribed to the larvicidal action of extensively used agricultural insecticides.

The pattern of growth of resistance in anophelines and culicines are similar in that, in both cases, DDT resistance began first and was followed by a big expansion of BHC-dieldrin resistance, especially in anophelines. Finally, resistance to organophosphorus insecticides developed, especially in culicines. The total incidence curve in both types of mosquito is sigmoid and the period of most rapid growth in numbers of species involved has apparently been passed. The order of appearance of different forms of resistance reflects the initiation of wide use of the insecticides concerned. The observations reveal that resistance to dieldrin or BHC could develop to the distinctive high

levels in 2 or 3 years because of the partially dominant genes involved; whereas DDT-resistance is inherited through recessive genes, so has tended to grow slowly over a decade or more. These observations may not explain the growth of organophosphorus resistance, which is nearly always dominant, but is not quick to develop. Deleterious effects of resistant gene in the homozygous state is suggested to be involved, in this case.

The present status of resistance in both types of mosquitoes may be summarised below.

1. No case of resistance extends throughout the overall species concerned.

2. Even when DDT-resistance has developed, DDT may continue to have some effect from its irritation. In contrast, BHC-dieldrin resistance develops to the point that renders dieldrin virtually useless.

3. In most cases, resistance has only developed to either DDT or the BHC-dieldrin group, so that an alternative long residue insecticide is available.

4. In some problem areas where double resistance has occurred, organophosphorus or carbamate compounds such as malathion or propoxur can be used as an alternative.

B. Species used in this investigation

Three species of anophelines, Anopheles gambiae, An. quadrimaculatus, An. stephensi, and two species of culicines, Culex fatigans and Aedes aegypti, were used for various studies. The world distribution of resistance in these species was described as follows.

(i) Anopheles gambiae.

Africa Region. In this area, double resistance of the main

vectors make it more difficult for the control and eradication and is only overcome by a highly effective insecticide, such as HCH or CNS 33. Resistance to HCH and dieldrin in An. gambiae, the serious vector of malaria, has been widespread in Africa for a decade. Dieldrin resistance in this species was first observed in 1955, in Nigeria, and the number of adult An. gambiae returned to its original level within 2 months of insecticide application. In Ambursa (Nigeria) DDT susceptibility was observed but was slightly resistant to malathion while the original dieldrin resistance gave cross-resistance to gamma HCH and aldrin. The first definite case of DDT resistance appeared in large numbers in Senegal and Upper Volta in 1967; these areas had been treated with residual DDT once a year from 1953 to 1960. DDT may be a suitable alternative in the forest regions but could not interrupt malaria transmission in the savana areas and no economic alternative compounds are effective for the control in most countries in the African continent. Even where An. gambiae remained DDT susceptible, nevertheless, its characteristic of being so irritated by DDT when it enters houses to bite and of leaving sprayed surface before receiving a lethal dose of insecticide made it difficult to interrupt malaria transmission by means of DDT residual deposits. An. gambiae is perhaps the most DDT irritable of all anophelines and is more quickly irritated than An. funestus. Neither HCH nor dieldrin has this irritating effect.

In East Africa, until recently, dieldrin resistance in An. gambiae was confined to some parts of Madagascar and in many places

dieldrin residual control continues. Recently, however, both species A and B of the gambiae complex in Kenya have developed resistance.

(ii) Anopheles quadrimaculatus

American Region. In North and Central America, DDT resistant An. quadrimaculatus has been revealed for some time and double resistance to both DDT and dieldrin can occur. This resistance happened after the successful conclusion of the malaria eradication campaign. A population with strong DDT resistance and a considerable dieldrin resistance was discovered in 1959 in Georgia. Another DDT resistance case was reported in Maryland. Double resistance to both DDT and dieldrin was also observed in part of north-eastern Mexico, which had been treated with DDT residual sprays for 2 years. The DDT resistant An. quadrimaculatus proved to be cross-resistant to DDD and methoxychlor, but a purified DDT resistant strain was completely susceptible to dieldrin. On the other hand, a purified dieldrin resistant strain was cross resistant to other cyclodiene derivatives and show in the order aldrin > dieldrin > chlordane > endrin.

(iii) Anopheles stephensi

Eastern Mediterranean Region. An. stephensi is the widely distributed and important malaria vector in the Persian Gulf Region. Double resistance on the part of this species caused serious problems in malaria control especially in the oil-bearing regions of Saudi Arabia and in southern Iran resulting in a recrudescence of malaria. DDT was used successfully until resistance appeared in 1957; dieldrin was substituted and was much more effective. Nevertheless, dieldrin resistance occurred widely after about 2 years and became more serious, causing a recurrence of malaria. DDT was re-introduced in 1963 and was

effective for 3 years. In 1966 and 1967 DDT resistance level was increased especially in certain localities in the south. This double resistance brought back malaria and an alternative insecticide such as propoxur (OMS 33) may be needed.

The emerging of DDT resistance in numerous localities of West Pakistan in An. culicifacies and An. stephensi does not, however, appear to change the situation of malaria control.

South-East Asia Region. In urban districts, An. stephensi developed double resistance to DDT and HCH, and control was continued by oil or Paris Green. In Nepal, DDT resistance appears in several species of non-vector anophelines.

(iv) Culex pipiens fatigans

This species is normally rather more tolerant of DDT than the other culicines. Moreover, it breeds in polluted water, in which it is more difficult to kill the larvae with DDT. Culex p. fatigans develops DDT resistance readily and can become equally resistant to dieldrin and BHC. Resistance to both organochlorine insecticide groups has developed in most parts of the world and various organophosphorus compounds have been substituted for the control of this vector of filariasis, including incipient resistance to these in some places. Certain populations of C.p. fatigans have shown a resistance to organophosphorus compounds in the field, but this disappears when the mosquitoes are colonized in the laboratory. On the contrary, C. tarsalis, the vector of western equine encephalitis, developed a specific malathion resistance that has been thoroughly studied in the laboratory.

American Region. In N. America, insecticides have been mainly

used because of nuisance from bites. Resistance to organochlorine insecticides is common in the U.S.A.; and elsewhere (in Colombia, Brazil and Peru, for example). Incipient organophosphorus resistance has been reported in the U.S.A.

Eastern Mediterranean Region. Double resistance to DDT and dieldrin occurred in the C. pipiens complex species in the United Arab Republic, but had a moderate effect on field control by DDT and a serious effect on field control by dieldrin. However, the number of cases of disease has not increased and no resistance to organophosphorus compounds or carbamates has been observed.

African Region. In the Congo, where DDT and HCH had been applied as residual insecticides for many years, a slight resistance to HCH-dieldrin and a very high resistance to DDT was indicated. On the other hand, a dieldrin resistant but DDT susceptible population of C.p. fatigans was reported in Mali. In Upper Volta and Ivory Coast, there is high HCH-dieldrin resistance with slight DDT-resistance.

Resistance to malathion and diazinon was observed in Cameroon after application of malathion sprays for 2-3 years. In West Africa, there was no correlation between the level of DDT and dieldrin resistance and those of the organophosphorus compounds except trichlorfon. A negative correlation was shown with the tolerance of difenphos. In East Africa strong dieldrin resistance and intermediate DDT resistance were widespread. The number of mosquitoes was increased and the second spray had been less effective than the first in general situation.

South-East Asia Region. Resistance to both organochlorine insecticides has impeded the satisfactory control of C.p. fatigans, according to the National Filaria Control Programme in India. Since

1960 trials of new insecticides and larvicides in oil film have been carried out. The WHO Filariasis Research Unit in Rangoon, Burma, has succeeded well by using fenthion, emulsifiable concentrate for the control of C.p. fatigans larvae.

In Ceylon, DDT resistance had developed first and then been followed by the rapid development of resistance to HCH and dieldrin. In Malaya, high levels of HCH and dieldrin resistance have developed in C.p. fatigans, but its susceptibility to DDT was normal. In Kuala Lumpur, larvae of C.p. fatigans showed an indication of resistance to fenthion although it had not been applied as a larvicide. In China the populations of this species had developed double resistance to DDT and HCH.

(v) Aedes aegypti.

This species is perhaps the most important culicine, as a potential vector of urban yellow fever and a sporadic vector of haemorrhagic fever and similar viruses.

American Region. In the neo-tropics, especially in the Caribbean islands and northern South America, the eradications were obstructed by double resistance to ogranochlorines and these populations have occasionally developed malathion tolerance. In the U.S.A., trials of safe organophosphorus compounds that can be applied to drinking water have indicated promise of difenphos.

African Region. In West Africa, BHC-dieldrin resistance is common in most big cities and DDT resistance in restricted areas. Neither DDT nor HCH could be recommended for controlling yellow fever epidemics. Resistance to diazinon has been observed at Congo.

South-East Asia and Western Pacific Regions. The spread of haemorrhagic fever due to dengue and chickungunza virus types trans-

mitted by A. aegypti first started in the Philippines in 1964. Larger outbreaks followed and the disease has since spread westwards to Vietnam, Cambodia, Malaysia, Thailand, Burma and as far as India. Many control schemes are being tried for controlling dengue viruses. Small scale pilot control projects have been carried out in Bangkok and Singapore, but widespread resistance to DDT is due to the general use of this insecticide. Accordingly, malathion is being employed for example in ultra-low-volume sprays from aircraft.

RESEARCH ON RESISTANCE AND ITS VALUE

The serious consequences of resistance have stimulated many people to investigate the problem. At first the subject seems to concern only applied entomology, but later on, it involved other disciplines, especially genetics and biochemistry. The challenge of the problem attracted the attention of experts in these fields, resulting in contribution to greater knowledge on resistance. In the past three decades, there have been numerous researches on all aspects of resistance, including the detection and measurement of resistance, the physiological, biochemical and genetic bases of resistance, and countermeasures to resistance.

1. Detection and measurement of resistance

In facing the challenge of insecticide resistance in the respective fields of public health and agriculture, the first essential countermeasure is to develop standardised methods for detecting and measuring its presence in field populations.

The first sign that resistance may have developed comes from the failure to control by the insecticide. Such field observations are not

conclusive, since so many factors can be involved; for instance, incorrect application, defective insecticide, unusual climatic conditions or elimination of parasites. In order to exclude all other factors, reliable and accurate tests are needed that will measure solely the susceptibility of insects. Furthermore, to obtain comparable and meaningful results for workers in different countries, internationally standardised tests are very desirable. This is especially true for insect pests with very wide distributions. However, during 1947-1955 there were no standardised tests for resistance and a considerable variety of methods were in use. Early work showed how widely different could be assessments of resistance made by different techniques: (Busvine, 1956b) and this drew attention to the need for standardisation.

The initiative in developing tests for insects of public health importance was taken by the World Health Organization. Methods suitable for different insect vectors and other pests were agreed at meetings of the W.H.O. Committee on Insecticides from 1956 onwards. As regards insect pests of agricultural and veterinary importance, the first moves were made by a committee of the Entomological Society of America, in 1960. From 1965, however, the matter was taken up by the Food and Agriculture Organization, which has similarly approved standardised test methods for international use.

Principles. The usual method of detecting and measuring resistance is to treat batches of a target population with serial doses of insecticide to obtain proportional mortality data and must be compared with a normal or standard population of the same species. The evaluation of results was based on the interpretation of log-dose/probit lines and may be supported by concurrent results from genetics investigations.

As a general rule, the log-dose/probit mortality response for a normal population is established first, with extensive tests. From these data, it is usually possible to choose a single dosage level which may be expected to kill all normal individuals of the species examined, under specified conditions. This critical dose can be used as a monitor to check samples of wild populations for incipient resistance.

When resistance is suspected, more extensive tests are necessary, over the whole dose-response range. If there is a slight change in the susceptibility of the population which is not due to the development of resistance, the line will move slightly without change in slope. The appearance of a plateau of the regression line indicates that a part of the population has become resistant and if genetical data are available, discriminating dosages may be available to distinguish the three genotypes. Furthermore, the different characteristic inflexions of the dosage mortality lines may show whether resistance is due to a single recessive gene, a single dominant gene, a single incomplete dominant gene or two dominant genes, or perhaps multiple genes.

Techniques. In regard to the method of exposing insects to toxicants, two main types of technique have been used. In some cases, especially with the larger or more robust insects, it has been possible to apply doses to individual specimens, by the so-called "topical application" method. In other cases, the insects are merely placed in a treated environment so that they may pick up a dose which is presumably related to the local concentration. This general method includes all tests in which the insects are confined on treated surfaces as well as those in which they are immersed in solutions or suspensions of toxicant, or gaseous concentrations of toxic vapour. The lethal effect is usually

determined by mortality at a standard interval after exposure. In some cases, the proportions paralysed (or "knocked down") may be noted at a given interval of exposure.

In the investigations to be described, two methods of assessing susceptibility or resistance levels in mosquitoes have been used; one for the adults and the other for the larvae.

Adult Mosquito Resistance Test

A method for adult mosquitoes was first developed by Busvine & Nash who used filter papers impregnated with DDT in Risella oil (Busvine & Nash, 1953; WHO Expert Committee on Malaria, 1954). This method made it possible to establish base line susceptibilities of all mosquito species and the W.H.O. standard test method was based on this principle. The test papers were impregnated in such a way as to give the same results as the Busvine & Nash test. This method for adult mosquitoes has been almost universally used in malaria eradication programmes and provided most valuable information, However, in recent years, the introduction of organophosphorus and carbamate insecticides has complicated the detection of resistance by this method. Whereas only two types of test paper (DDT and dieldrin) were required to detect resistance to organochlorine insecticides, it is not possible to choose one or two organophosphorus or carbamate compounds which will indicate resistance to other members of the groups concerned. The prospect of supplying a complete range of all those compounds would have been impossible, especially in the view of their relatively rapid deterioration.

A possible way round this difficulty which was suggested at the 1968 meeting of the W.H.O. Insecticide Committee, would be to use a small number of concentration levels (probably two) and expose for

different periods. This would alter the criterion of toxic action from dosage to exposure time. It had already been shown that, for organochlorine insecticides, over a considerable range, the relations between concentration and exposure time for an equitoxic effect are inverse (Busvine, 1958). This was later confirmed and shown to be due to close relations between exposure time and the dose picked up by mosquitoes exposed to impregnated papers (Pennell et al., 1964; Ariartnam & Brown, 1969; Hamon & Sales, 1970). Accordingly, the W.H.O. Expert Committee on Insecticides prepared standard concentrations of organophosphorus and carbamate compounds which were despatched for field trials and laboratory testing, in regard to lasting powers and the relations between time and concentration for equitoxic effects. Experiments on these matters will be described later.

Mosquito larva resistance test

The W.H.O. standard test for resistance in mosquito larvae was developed rather early on the basis of several rather similar methods of assessing larvicidal potency. These depended on preparing suspensions at different concentrations by adding a small volume of acetone- or alcohol- solution of chemical to a large volume of water. Fourth instar larvae were then exposed for 24 hrs. Brown (1957) mentioned several techniques used in the early 1950s and then discussed the importance of various items in the test. Largely on the basis of this evaluation, the W.H.O. adopted its standard method, which was published in the Report of the Expert Committee for Insecticides (1957). Since then, the test has been very extensively used and many aspects subject to further examination.

In the W.H.O. standard method, the test should be performed in

glass vessels with water depths about 2.5-7.5 cm. Some workers had used disposable waxed paper cups or plastic cups in order to avoid the washing and decontaminating of glass vessels for re-use. This practice does not affect the results obtained with organophosphorus compounds, but with DDT it causes a marked difference (Curtis, 1961; Jones, 1967) owing to flocculation of the colloidal particles by cancellation of the zeta-potential that kept them apart (Hawkins & Kearns, 1956).

With regard to the larval density, if there is an increase in number above the level of 25 larvae/250 ml, the mortality is greatly reduced; but a decrease in larval density below this figure does not make much difference to the mortality obtained (Brown, 1971).

This larval test for resistance is most widely used for culicines (Brown, 1958b) and the effects of variable exposure time have been summarized by Brown (1957). With some sensitive anophelines, a test of shorter exposure time followed by a 24-hr observation period in clean water are often desired to avoid high control mortality and pupation during the period. Details of the techniques and factors involved in testing insecticide are covered extensively by Busvine (1971a).

2. Genetical Research

Genetical investigations may be of practical value in indicating the possibilities of potential resistance in wild populations of vector species, from the prevalence of resistance genes. Also, from the dominance status of such genes, the likely rate of spread of resistance can be forecast. Furthermore, analytical genetics has

helped to learn more about the relative importance of different resistance mechanisms and the possibilities of their being overcome.

The first study of the inheritance of DDT resistance was done by Harrison (1951) in houseflies. At that time the lack of fundamental genetic information on vector species hindered further work in studies of resistance. However, it was clearly revealed that resistance is an innate and inherited character and does not develop by exposure to sublethal doses. These preadaptations are gene alleles which are heredity units carried by chromosomes.

The number of genes responsible for resistance to a certain compound was investigated by various workers in the following decade. The first genetic work on resistance in mosquitoes was done by Davidson (1956; 1958). Nguy & Busvine (1960) were the first to study the inheritance of organophosphorus resistance in the houseflies. In almost all cases, the results were assessed from segregation in progeny of mass crosses and showed that major resistance mechanisms were normally inherited through Mendelian inheritance of single autosomal gene pairs which may be dominant, intermediate or recessive. Thus, DDT resistance was found to vary in different strains, but it is usually recessive in anopheline mosquitoes; dieldrin resistance is nearly always intermediate (Macdonald, 1959); while organophosphorus and carbamate resistance is nearly always dominant.

Great progress followed the isolation of morphological marker genes, which were used to indicate the existence of different types of resistance gene in different linkage groups (Hiroyoshi, 1960) and the possibility of several alleles at some loci (Agaki & Tsuka-

moto, 1953; Crow , 1957). Furthermore, the genes for resistance could now be located in particular positions in the linkage groups. Moreover, by separation of individual recessive marker genes and corresponding resistance genes and recombination, it became possible to assess their individual and joint action (Sawicki and Farnham, 1967). Following, the studies of X-ray induced mutations made it possible to assign the linkage groups to specific chromosomes of houseflies (Wagoner, 1967). Among mosquitoes, marker genes have been used to determine linkage groups in A. aegypti (Kimura and Brown, 1964) and in C. pipiens (Todano and Brown, 1967).

At the present, over 40 examples of monofactorial inheritance of resistance, in different species, have been demonstrated. The outcome of these advances in genetics is that it has been possible to isolate, and study separately, multiplicate resistance genes which are the genetic mechanisms for protection insects against the same toxicant. These various genetic factors for any type of resistance strongly reinforced each other and their combined effect resulting in multiplying rather than additive. These multiple mechanisms involved have been unravelled. Tsukamoto (1969) suggested that detoxication may involve a series of metabolic steps under control of separate genes. If the first step goes at a lower rate than the next step, a preliminary genetical analysis will indicate only single gene inheritance. On the other hand, Busvine (1971b) mentioned that changes in all steps will be cumulative and that conjugation to produce soluble excretion end products might be controlled by a gene. Other genes which may be involved might control the supply of co-factors.

3. Toxicological Research

In an attempt to reveal the mechanisms responsible for insecticide resistance, genetic studies are supported by toxicological investigations, which have become more and more sophisticated with the introduction of advanced techniques and wider application of statistics in the interpretation of data. The various ways to reach understanding of the toxicology of resistance are as follows:-

A. Resistance Spectra

Very early in the study of resistance, it was realised that there were forms of resistance specific to groups of insecticides; e.g. DDT and analogues; YBHC and cyclodiene compounds; organophosphorus compounds. Further useful information could be obtained by examining the relative resistance levels within such main groups. The patterns of such relative levels are known as "resistance spectra".

Strains which have only one single common defence mechanism are expected to show consistent resistant spectra and are likely to appear in inbred laboratory colonies. Resistance that developed in the field due to sustained insecticidal pressure usually involved several multiplicate mechanisms, giving the blurred spectra with specificity obscured.

Using the homogeneous resistance and susceptible strains selected for laboratory investigations, it is possible to obtain further information from "resistance spectra" which may be used

to discover the types of protective mechanism involved. Clues to resistance mechanisms are gained by finding the particular compounds to which high resistance developed. It is also useful to observe how the degree of resistance is affected by molecular changes in analagous compounds. Thus, DDT resistance depending on a dehydrochlorination mechanism, would vary in resistance to a series of DDT analogues according to their ease of metabolism (Busvine, 1951). Analogues with deuterium on the 2-carbon atom (Barker, 1960) and on ortho chlorine on one phenyl ring (Sternburg et al., 1954) were proved to be refractory. This mechanism cannot cope with Prolan or with dianisyl neopentane which appeared to be immune (Busvine, 1953). Resistance to these immune compounds would indicate other types of defence mechanism.

Organophosphorus resistant spectra are more likely to be complicated and difficult to interpret because a variety of defence mechanisms are known to be involved. Only when a single highly specific mechanism is present would one expect a simple consistent pattern: e.g. in malathion-resistance depending on carboxyesterase metabolism. This degradation would not extend to other organophosphorus compounds lacking carboxy radicals (Busvine et al., 1963). Other spectra in this group showed higher levels of resistance to ethyl or methyl esters (Busvine, 1968a) In complete contrast to this highly specific resistance mechanism is the mixed-function microsomal oxidase. Resistance to naphthaline vapour is believed to be due to this oxidation (Schonbrod et al.. 1965).

B. Effects of synergists

In another toxicological test for resistance mechanisms, the

compound affected by resistance is mixed with various synergists known to inhibit specific detoxication enzymes. Allied to inferences drawn from resistance spectra are the indications from the effects of synergists. The effectiveness of different synergists towards resistant strains of insects is likely to vary in a manner that reflects the critical metabolic pathway on which resistance depends. A synergist more active against a resistant strain than a normal one gives evidence of an altered or enhanced detoxication mechanism. The type of mechanism can be predicted from the specificity of synergists. In some cases, the synergist has a molecular form resembling the toxicant, so that it is relatively easy to imagine how it could block specific detoxication enzymes. Such synergists are described as analogue synergists. Examples are DMC (which resembles DDT); this inhibits dehydrochlorination; another type comprises non-toxic phosphorus or carbamate esters which synergise certain organophosphorus compounds.

The action of synergists which do not resemble toxicants is rather more obscure, though it appears that they too block detoxifying enzymes. An example is WARF anti-resistant which appears to inhibit dehydrochlorination. Also, there is a large class of synergists which inhibit microsomal oxidation enzymes. For examples, the methylenedioxy phenyl compounds, the aryloxyalkylamines, the thiocyanates, the propynyl aryl ethers, and the 1, 2, 3 benzothiadiazoles (Casida, 1970). Enhanced synergism in resistant strains indicate a reliance on such mechanisms as a first step in detoxication (Sun and Johnson, 1960) before more extensive biochemical investigations are attempted. Many compounds would become available as insecticides if detoxication could be prevented by the use of synergists. Brooks and Harrison (1964) showed that many cyclodiene analogues are quite toxic if the degradation

is blocked by sesamex.

Since many forms of resistance depend on detoxication mechanisms, it is theoretically possible to overcome resistance by appropriate synergists. Busvine (1972) pointed out the three practical limitations of synergists for this purpose.

1. Synergist-insecticide combinations were proved to be able to overcome high levels of resistance in the laboratory, but resistance eventually re-appeared, probably by virtue of alternative defence mechanisms such as insensitive physiological targets and reduced penetration. Alternatively, if more than one detoxication mechanism were involved, several synergists might be necessary, and this would complicate the detoxication systems.

2. Some safe insecticides which have low toxicity to mammals because of inherent detoxication systems might have these inhibited by the synergists used against insects (for example, anti-carboxyesterase synergists for malathion). This would increase hazard to mammals.

3. Even if a satisfactory synergist-insecticide combination could be found, it would be rather difficult to apply for field use according to its insoluble property.

C. Reduced Penetration

That reduced penetration of insecticides through the cuticle of the insects might be a cause of resistance was suggested in 1947 by Wiesmann. He noted that DDT-resistant houseflies had a thicker tarsal and pulvillar cuticle than the normal strains; this might delay the entry of poison and explain differences in knockdown between susceptible and resistant insects. It did not, however, explain the specific

resistance to DDT at a time when other organochlorine insecticides with very similar physical properties were still effective. Subsequently, it was shown that differences in penetration rate were not of the same order of magnitude as the high resistance levels generally found. A loss of interest in the role of delayed penetration in resistance might be due to the above reasons.

Recently, with more detached knowledge of resistance, the idea of reduced penetration has been reconsidered. An understanding of the physiochemical and biophysical factors involved in penetration of insecticide is not only essential to interpret the comparison of actual doses, but also explain the detoxication mechanisms relevant to resistance. The possible influence of penetration in association with some defence mechanisms on effective resistance was shown by Winteringham and Hewlett (1964) and later on DDT resistance was proved to be linked with delayed penetration (El Basheir, 1967). It was suggested that reduced absorption was a mechanism involved in a DDT-resistant strain of Culex pipiens quinquefasciatus. A mechanism delaying entry of insecticide into the houseflies has been genetically isolated (Sawicki and Farnham, 1968; Plapp and Hoyer, 1968).

More recently, it has been shown that reduced penetration, under orthodox genetic control, may be quite important for magnifying the effect of detoxication mechanisms (Sawicki, 1970). This effect of delayed penetration on entry of insecticides into insects was relatively small and was not important in insects lacking metabolic mechanisms for breaking down insecticides; but it could have large effects in insects that have detoxifying mechanisms; i.e. the penetration delaying factor then acts as a genetical modifier (Sawicki and Lord, 1970). All

investigations, generally, indicate that penetration, metabolism, toxicity and resistance are closely linked. However, the effects seem to be so complex that no attempt has been made to develop a theoretical treatment of these results. Furthermore, the lack of knowledge in this area offers rich dividends to future investigators.

D. Biochemical Studies of Metabolism

The outstanding discovery of DDT dehydrochlorination in houseflies was made with a relatively simple technique. The Schechter-Haller colorimetric method was used to assay the amount of DDT and DDE in treated houseflies. Since then, substantial advance biochemical techniques have become more widely used. The adoption of the new methods was developed step by step especially in radiochemistry and paper chromatography which was improved to thin layer chromatography and later gas chromatography (GLC). From 1960 onwards, advances in understanding of detoxication pathways responsible for resistance involved the detection and measurement of metabolites formed both *in vitro* and *in vivo*. Homogenised tissues are extracted with polar and non-polar solvents to separate the main classes of metabolites. The pH levels are changed where desirable, to alter ionisation and hence partition solubility. Gas chromatography was employed for separation and identification of metabolites, and found convenient for chlorinated compounds (Busvine and Townsend, 1963; Brooks and Harrison, 1964), and later with the more refractory organophosphorus metabolites (Dyte and Rowland, 1968). This technique has the advantage of being able, theoretically, to record all metabolites.

On the other hand, the wider availability of radioactively marked insecticide (e.g. ^{14}C, ^{36}Cl, ^{32}P, ^{3}H and ^{82}Br) and the convenience of

sensitive scintillation counters has tended to re-establish the popularity of radiochemical techniques. In this case, separation of the metabolites has been accomplished by thin layer chromatography or electrophoresis (Feroz, 1971) and the spots identified by their Rf values (Brown, 1968). In addition, information on unknown compounds may be obtained from mass spectrometry and infra-red spectrometry (Sellers and Guthrie, 1972). Furthermore, it is realised that primary metabolites with free hydroxyl groups may be conjugated with sugars or other polar molecules, from which they must be freed by hydrolysis (e.g. treatment with glucosidase, glucuronidase, sulphatase or phosphatase) before identification (Shrivastava et al., 1969; 1970).

The results obtained so far from these investigations have revealed detoxication mechanisms of considerable complexity. Some possible DDT degradation pathways are shown in Figure 1. The commonest defence against DDT seems to be by dehydrochlorination to DDE which was the main metabolite in many resistant fly strains and in several species of culicines (Kimura et al., 1969) and anophelines (Perry, 1960). Subsequently, evidence of resistant spectra and the action of synergists strongly indicate another metabolic pathway is involved, probably depending on microsomal oxidase systems under separate genetic control (Oppenoorth, 1965). Dicofol seems to be the main DDT-metabolite in Drosophila melanogaster in both resistant and normal strains (Tsukamoto, 1961) and may also occur in other species. Degradation to DDD has been detected in larvae of Culex pipiens fatigans in Australia (Hooper, 1967).

E. Biochemistry of Enzymes Involved

Another type of research concerns the enzymes responsible for

Some

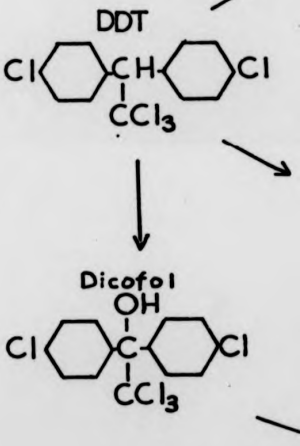
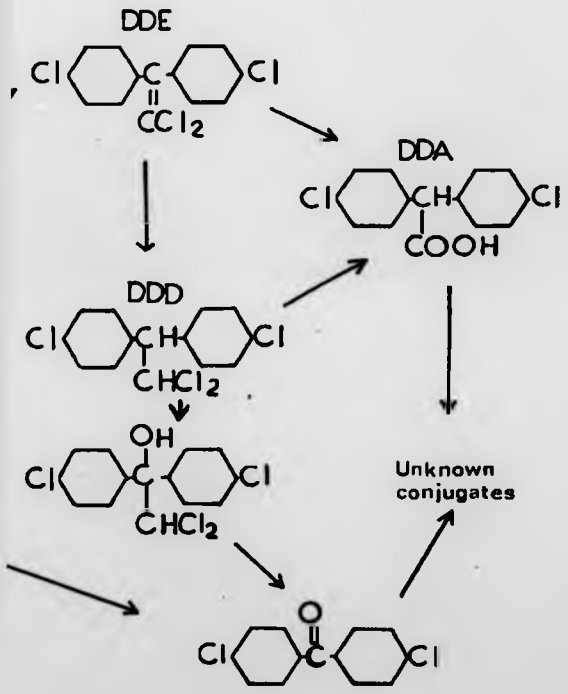


Figure 1

possible DDT degredation pathways



detoxication. Homogenised insects are fractionated by centrifugation. A lot of enzyme activity is usually found to reside in the microsomal fraction; but some soluble enzymes may occur in the supernatent after exposure to 100,000 x g. Much attention has been paid to microsomal mixed-function oxidase enzymes because of their wide capabilities in initiating metabolism of toxicants. Thus, they can oxidise phosphorothionates to phosphates, hydroxlate dimethylphosphoramidates, methyl carbmates, napthalene and DDT and epoxidise aldrin to dieldrin. The first two and the last of these processes result in potetiation of the insecticide, but the others result in detoxication. The importance of these enzymes in resistance was first recognised in 1965 (Schonbrod et al.) and advances in this field are being continued (Oppenoorth and Houx, 1968; Oppenoorth et al., 1971).

These microsomal enzymes require NADP as a co-factor and involve cytochrome P450; they are antagonised by pyrethrum synergists (i.e. methylene dioxyphenyl compounds; Casida, 1970), by SK525A and by non-insecticidal phosphorothionates (Oppenoorth et al., 1971). Other enzymes may also be located in the microsomes including esterases which are not NADP or oxygen dependant and are inhibited by different types of synergist, for example some organophosphates. In addition, there are soluble enzymes demonstrable by agar gel electrophoresis; but these do not seem to be involved in detoxication. The well known DDT dehydrochlorinase in the houseflies appears to be a small globulin (Lipke and Kearns, 1960). Some studies of analogous enzymes have been made in mosquitoes in regard their substrate specificity and glutathione requirements. However, there are distinct qualitative differences in these respects (Kimura and Brown, 1964;

Kalra et al., 1967). DDT-dehydrochlorinase has been found in triatomid bugs though DDT-resistant strains are not known. Agosin (1963) suggested the possible competition of dehydrochlorinase with a hydroxylase system for NADPH. This would explain why DDE and dicofol-type metabolites do not usually co-exist; the balance is usually one way or the other.

The work on enzyme induction began in 1968. It has been observed that certain organochlorine insecticides stimulate the activity of microsomal oxidase systems in mammalian liver, and evidence for similar induction has been obtained in insects. It was suggested that the capacity to respond in this way to DDT might enhance resistance in certain strains of houseflies. Later it has been shown that dieldrin is a more powerful stimulant than DDT (Walker and Terriere, 1970; Plapp and Casida, 1970). It was used on dieldrin-resistant flies and enhanced their metabolism of a variety of quite different insecticides. The effect was apparently due to increased oxidative effect and was accompanied by acceleration of protein synthesis as shown by a more rapid incorporation of ^{14}C-labelled L-isoleucine. Addition of dieldrin to the in vitro preparations had no effect, suggesting that the induction was not merely a direct action of microsomal structure. The practical significance of enzyme induction in relation to resistance is not clear, since the rather massive doses of stimulant are unlikely to be acquired in the field.

All interesting progress researches in biochemical toxicology discussed so far concerns detoxication systems. Early searches for changes in vital enzymes, which might be targets of toxicants, were unsuccessful. Only two well-established cases of changed target

enzyme systems have been discovered. Both involve acetylcholinesterase of reduced sensitivity to organophosphorus compounds and both occur in acarines. Evidence of this was found in one form of resistance of Boophilus macroplus (Wharton and Roulston, (1970). The other case concerned the spider mite, Tetranychus telarius (Smissaert, 1964).

There is some evidence of reduced target sensitivity as a cause of one DDT-resistance mechanism. This is suggested by an observed decrease in the sensitivity of exposed nerves and of labellar taste receptors to DDT. If, in fact, there is a change in the physiological target, it might involve reduction in the formation of a charge-transfer complex between DDT and a component of the nervous system. Possibly the DDT-receptors may have a changed configuration. Further information may develop from studies of the steric properties of DDT, recently reported by Holan (1969, 1971).

4. Research on Ways of Countering Resistance

During the past two decades, there have been extensive researches on resistance in different disciplines. But although the advanced understanding of the nature of resistance has improved diagnosis, prognosis and epidemiology of the trouble, it has never reached the stage of any simple and conveient cure. The rapid development of resistance, together with the potential enviromental hazards of many insecticides need a more enlightened approach to new types of insecticides. Some possible ways that may be helpful to cope with resistance are summarized briefly as follows.

A. Continuing Use of Existing Insecticides

(i) Restricted use of insecticides

It has been known that resistance is provoked by excessive use

of insecticides. So far as the prevention of resistance is concerned, the most hopeful way is to restrict the use of effective insecticides and combine them with alternative methods of control. For endemic diseases, control at a level sufficient to prevent transmission is required.

(ii) Synergists

The possibility of adding appropriate synergists to suppress detoxication enzymes is unlikely to be successful due to multiple mechanisms are involved. As one is blocked, another may be developed. Nevertheless, it can provide a clearer picture of the problems which will be useful for further investigations. In recent years, attention has been focused in microsomal enzymes and their inhibitors. Several reviews have been published dealing with detoxication in insects and with the aspects of synergistic action. Effects of synergists on the metabolism and toxicity of anticholinesterases were reported by Wilkinson (1971). The role of metabolism and the possible use of synergists were also dealt with by Oppenoorth (1971). In relation to DDT, a major interest at the present time is to devise compounds which are effective against insects but will disappear from the environment in a reasonable time. This usually means that chlorine has to be removed from the molecule. There is recent evidence (Focht and Alexander, 1970) that if some of the chlorine atoms can first be removed by anaerobic processes, then aerobic organisms can degrade the simplified structures.

There is a difference between resistance due to DDT dehydrochlorinase and the oxidative resistance, as can be seen by the use of syner-

gists. It was indicated that FDMC, an analogue synergist, can be used to suppress DDT$_A$ resistance in a strain of housefly; but this combination was ineffective against another strain which requires sesamex, an inhibitor of mixed function oxidases, as a synergist for DDT. It is common to use synergists of this type, rather than molecular modification, to prevent oxidations in vivo. One good reason for this is that many insecticides contain several sites that are vulnerable to oxidation and it may not be possible to modify the molecule to prevent attack at all these sites while retaining the overall structural requirements for toxicity. By using a second agent, the synergist, oxidation at many or all sites can be suppressed simultaneously without altering the molecular structure (Brooks, 1973). The commercial use of synergists has so far been economical only with pyrethrins. However, there are a number of problems associated with the practical use of mixtures of compounds, and it remains to be seen whether the use of synergists will find more favour than the use of a single biodegradable compound.

B. Searches for New Insecticides

As each type of resistance began to develop in recent decades, the only alternative available seemed to be a substitution of an alternative not involved in the already developed resistance. In some cases, double or treble resistance has gradually reduced the number of effective alternatives. This is a very serious matter, because at least one alternative insecticide should be available, especially for emergency use against vectors of epidemic diseases.

Clearly, there is a need for new types of effective insecticides not involved in any known type of resistance. So far, extensive empirical searches for such new types has not been outstandingly successful.

The most extensive organised search for new compounds suitable for vector control was organised by the W.H.O. about 10 years ago. Over 2000 compounds were examined, each one being subjected to a series of seven evaluation stages. Three of these are performed in the laboratory and four in the field. Details of each stage were described by Wright (1971). By meeting the criteria for each successive stage, a compound advances to the next higher level of testing, until finally it qualifies for large-scale field evaluation. Seven laboratories were as W.H.O. reference centres and perform the investigations required for stages I-IV. Six W.H.O. field research units working in six different countries are responsible for the studies required at advanced levels of evaluation. The locations of these units are also listed by Wright (1971). No very new compounds emerged from this extensive work, but it did sort out the most suitable of existing insecticides for mosquito control. Malathion and propoxur are the most effective compounds, but they seem to be detoxified by some insects. The new synthetic pyrethroids were proved to be useful to prevent transport of mosquitoes by aircraft especially the infected ones (Brooks and Evans, 1971). They are as effective as the natural product against insects and no obvious hazards to man. They are found to be more stable and superior to the natural pyrethrins in knockdown effects (Nishizawa, 1971). Moreover, these compounds do not need synergists. Resistance to them is possible, but unlikely to develop from this stage.

The development of biodegradable analogues of DDT is of interest (Holan, 1971). Detailed metabolic pathways have been worked out (Metcalf et al., 1971) in flies and mosquitoes. These DDT analogues

have no indication of environmental hazards and insensitive to dehydrochlorination. Their biodegradability depends on liability to oxidative metabolism but can be inhibited by synergists. However, it seems to have a narrower activity and costs more than DDT.

Recently, a biodegradable non-toxic liquid "Inmol G" was shown to spread as a monolayer on water surfaces and suppress mosquito pupae (McMullen and Hill, 1971). Some alternative larvicides which were synthetic from the attraction of garlic (Amonkar and Reeves, 1970) and mucilaginous seeds (Reeves and Garcia, 1969) were also demonstrated. Furthermore, various types of aliphatic amines including ammonium salts, primary alkyl amines, diamines, beta amines and beta diamines have shown promise as larvicides and pupicides (Mulla et al., 1970).

Miller and Maddock (1970) reported some possible new ovicides for mosquitoes. Certain phenols which were particularly effective appear to act as inhibitors of tyrosinase and prevent melanization during embryogenesis, so that the eggs die or only weak larvae hatch. Recently derivatives of petroleum with specific and uniform compositions have been developed for control of mosquitoes (Micks et al., 1967, 1968, 1969). Applications of these control agents can be expected to achieve maximum effectiveness in the shortest time when employed against field populations consisting predominantly of 4th-stage larvae and pupae (Micks et al., 1972). Pathological effects in mosquito larvae exposed to hypoxia and to pertoleum hydrocarbon was found, indicating that these derivatives of petroleum may initiate their larvicidal action by producing irreversible hypoxia. (Berlin and Micks, 1973). Moreover, these compounds markedly retarded the development of all instars of Aedes aegypti and Culex ripiens fatigans (Micks, 1970). Selection with petroleum derivative agents suggests that resistance to these compounds

is not likely to occur (Hicks and Gaddy, 1973).

C. Alternative to Insecticides

The effective use of insecticides has encountered resistance and pollution. It is unlikely that any new insecticide will provide an entirely satisfactory alternative, if it has a long residual action. There are intensive examination of alternative control methods, not involving toxic chemicals, by the co-ordination of W.H.C. and F.A.O. These possibilities were reviewed by Busvine (1968b) and they are grouped as below.

i. Biological Control

a. Arthropod parasites and predators.

The control by parasites and predators is demanding in scientific research and other resources. Its economic success is hard to predict due to the limitation of known types of parasite and predator species. Furthermore, a possibility exists that the target pest might change its biology to become immune to its enemies.

b. Microbial control

Attempts have been made to utilize pathogenic organisms such as Bacillus thuringiensis, B. popilliae and B. lentimorbus for the control. Some viruses, fungi, protozoa and nematodes have also been tried and shown promise, Nevertheless, there are many problems to overcome. Apart from difficulties in dissemination, the possible toxic hazards to mammals must be carefully investigated. Also, a few cases of resistance seem to have appeared already.

c. Pest-resistant varieties

The plants protect themselves by discouraging insects from attacking

them. Physical and chemical means are employed to suppress or destroy the insects. This method of pest control are well established but further success may result from sophisticated studies.

d. Substitution of a vector by a non-vector

Suppression by insecticides on one species can change the environment which make it suitable for another strain or related species to become dominant. This has thrown light on pest replacement as a means of satisfactory elimination of one pest by another. Results envisaged by this idea are not easily forecast.

e. Genetic control

A method which has reached the level of practical field trials is the release of strains of a pest species, genetically incompatible with the local strain, thus producing sterility. Further developments could follow if artificial races were produced with incompatibility due to chromosome inversions, translocations, polyploidy, etc.

ii. Chemical control (other than by toxicants)

a. Repellents and deterrents

Many compounds have at one time or another been investigated as repellents for bloodsucking insects and deterrents for other insects. The ideal compounds have not yet been discovered because after application they are rapidly rubbed off, absorbed, or washed away by perspiration. It has been suggested that repellents could be used to confuse and mislead insects searching for hosts.

b. Pheromones

So far, there are two types of pheromones which could be useful, sex attractants and general aggregating agents. The former could con-

ceivably confuse and nullify mating instincts of pest populations but so far have not been practical in the field.

c. Antibiotics

Mode of action of certain antibiotics have been suggested that they might interfere with the biosynthesis of chitin and moulting process. Reduced fertility and caused sterility has also been observed.

d. Insect hormones

Two types of insect hormone have been intensively studied in recent decades: the moulting hormones (ecdysones) and the juvenile hormone. Carroll Williams has advocated their use for pest control, calling them the "Third Generation of Pesticides" (1967). He demonstrated that treatments with these hormones at critical stages of the life cycle of insects leads to abnormalities and death. Unfortunately, the development of moulting hormones for this purpose has encountered difficulties, notably their lack of penetrating insect cuticle. More success has been obtained with juvenile hormones and mimics (analogous synthetic compounds). For example, it is known that application of juvenile hormone or active analogues to insects will succeed at periods when natural hormone is absent or present at low titres. In this way, intermediate forms in metamorphic moults or abnormalities in embryogenesis are resulted and most of these are ultimately lethal. A low dose of these hormones can also be mixed to break the adult reproductive diapause by stimulating viable egg production, whereas a higher one can cause the disruption of embryonic development.

A large number of injection and implantation studies have been carried out to establish the relationship between the two types of hormone.

(Wigglesworth, 1970). It was been shown that the proportion of one hormone to the other is probably the deciding factor (Schnal, 1971; Loufer and Calvert, 1972). Injection of juvenile hormone displaces the balance in favour of this hormone and the larval life of insect created by this means can be extended by one ot two moults to give so-called "giant" larvae (Wigglesworth, 1954). However, many exceptions have been found. As far as it is known, Dipters cannot produce additional larval instars, and any application of juvenile hormone in the last larval instar acts, in effect, on the adult metamorphosis and not on pupation. The pupa itself is no longer sensitive in mosquitoes. Similarly, injection of moulting hormone causes premature pupation resulting in "Dwarf" adults, usually sexually immature and incapable of breeding. Other functions, such as cuticle tanning and hardening which are dependent on these hormones are usually affected by any change from normal in the blood titre of these hormones (Robbins et al., 1968; Wright and Kaplanis, 1970; Frankel et al., 1972). Research into all the possibilities of these new hormone alternatives to insecticides is not yet very extensive. Nevertheless, at least one type of juvenile hormone mimic has become commercially available in the U.S.A. (ZR 515, or Zoecon).

It was hoped that hormone mimics would provide an answer to the problem of resistance and that resistance could never develop to such compounds, because they were required by the insects for normal development. Unfortunately, it has been found that an insecticide resistant strain of the flour beetle, Tribolium castaneum, also inhibits resistance to strongly active juvenile hormone analogues (Dyte, 1972). Another example of cross resistance to a juvenile hormone analogue ZR-515 in some resistant houseflies has been observed (Cerf and Georghiou, 1972).

Related to the cross resistance which appears to be on the way, is a lack of adequate studies on the degradation of these hormones and their mimics. The complexity of the ecdysone detoxification system makes it an unlikely candidate for practical insect control (Watkinson and Clarke, 1973). It is to be hoped that future research work will continue on this line.

iii. Physical Control

a. Dehydrants

About 1930, a dust which had dehydrating effects on insects was discovered in Germany. Later work in Britain showed that its action depended on abrading the waxy layer of the insect cuticle. In the early 1960s new dusts depending on adsorption of cuticular waxes were developed in the U.S.A. Despite initial promise, this method does not seem to have proved very widely useful, possibly because of the inconvenient nature of dust treatments for residual control.

b. Ionising radiation

Short wave radiations such as x-rays and Y-rays can kill insects but this is not often feasible in practice. Heavy doses of radiation damage cell nuclei and are more or less rapidly lethal. At radiation levels considerably lower, insects can be sterilized, without greatly involving their longevity or vigour.

iv. Combination Control

a. Radiation-induced sterilization

X-rays or Y-rays can sterilize insects without altering their longevity and sexual vigour of the male insects. The sterile males are

released into wild populations, and mate with wild females, which thus produce no offspring; and many female insects will mate only once. Success depends on overwhelming the wild insects with vigorous sterile males. This demands artificial rearing on a vast scale. Despite numerous investigations of the possibilities of this method, it has only been found feasible with one species of insect; the screw-worm fly of Central America.

b. Chemosterilants

The use of chemosterilants is another modern alternative method of sterilizing males prior to release. They can also be used, in baits, to sterilize wild insects; and in this case, the exterminating effect should be more rapid, since both sexes would be sterilized. Nevertheless, one difficulty of this method is that, at doses below those causing actual sterilization, these compounds have mutagenic effects which could be highly dangerous to man and domestic animals.

Chemosterilants can also be used to produce sterile males for release (instead of radiation treatment). But the same difficulties exist in the way of general application, as with radiation sterilization.

c. Trapping

Traps are still marginally useful in pest control; for example, to assess density of wild insect populations. The attractiveness of modern traps has been improved by the use of ultra-violet radiation, release of carbon dioxide, and various attracting chemicals or pheromones.

PART II.

PRESENT INVESTIGATIONS

SUBJECTS INCLUDED IN THE PRESENT INVESTIGATIONS

The research types which have been investigated and which constitute the experimental portion of this thesis all concern aspects of insecticide resistance in mosquitoes. They are as follows.

1. Problems concerned with the resistance of adult mosquitoes to organophosphorus and carbamate insecticides.

A. The use of time as a dosage parameter in the standardised test for resistance inadult mosquitoes. Investigations of the relations between exposure time and concentration for equitoxic effects.

B. Lasting powers of organophosphorus and carbamate impregnated papers during storage.

2. Defence mechanisms against DDT in larvae of resistant strains of mosquitoes, as indicated by the following.

A. Relative resistance levels of DDT analogues varying in liability to degradation by different pathways.

B. The effects of synergists believed to inhibit specific DDT-detoxifying enzyme systems.

C. Radiometric measurements of the pick up of ^{14}C marked DDT and malathion by normal and resistant larvae of different species.

3. New larvicidal compounds for control of resistant strains. These included compounds believed to act on hormone systems concerned with moulting and metamorphosis as well as some miscellaneous new insecticides. The subjects investigated were as follows.

A. Potency of the new compounds and the possible extension of DDT-resistance mechanisms to them.

B. Preliminary investigations on the mode of actions of certain new compounds.

MATERIALS AND METHODS

MATERIALS

1. Mosquitoes

Fourteen strains from five species of mosquitoes were used in these present studies as follows.

Anopheles gambiae Complex species A.

1. UV 19R5 DDT and dieldrin resistant strain.
2. IBAD Susceptible strain

Anopheles stephensi

3. STMAM 2A DDT and dieldrin resistant strain
4. 2Ra " " " " "
5. 2Rb " " " " "
6. STSS DP1 Susceptible strain

Anopheles quadrimaculatus

7. QDTA DDT resistant strain
8. QUA Susceptible strain

Culex pipiens fatigans

9. Lagos R DDT resistant strain
10. Lagos L Susceptible strain
11. Rangoon Resistant strain
12. Tanamarive Resistant strain

Aedes aegypti

13. T_8 DDT resistant strain
14. N Susceptible strain

The UV 19R5, IBAD, STMAM 2A, 2Ra, 2Rb, STSSDP1, QDTA and QUA strains were obtained from Dr. G. Davidson, the Ross Institute of Tropical Hygiene.

A. Anopheles gambiae Complex species A.

UV 19R5. A DDT-resistant strain of species A which also is resistant to dieldrin. This colony was isolated from eggs obtained from DDT-resistant wild caught females from a suburb of Bobo Dioulasso, Upper Volta. The mosquitoes surviving from 4% DDT for 6 hours were used to establish a colony at the Ross Institute in February, 1969. Mosquitoes from this colony continued to show high mortality on 4% DDT for 1-hour exposure for several months after colonisation. However, further selections were made, resulting in a population showing only a low mortality after 1 hour to 4% DDT.

When the colony was first obtained for this study, the LC_{50} of larvae for DDT at 24 hours exposure was only 0.03 ppm. It was decided to select for DDT (as described later). The selections were done for 10 generations when the LC_{50} reached about 5 ppm.

IBAD. The susceptible strain originated from Ibadan, Nigeria, and was colonised at the laboratory of the Ross Institute in 1966. The strain is susceptible to both DDT and dieldrin.

B. Anopheles stephensi

STMAM 2A. This strain, which is resistant to both DDT and dieldrin, originated from Mamlaha, Iraq, and was brought to the Ross Institute in 1966. There, selection pressure was performed in the laboratory by exposing the mosquitoes to 4% DDT for 4 hours. After receiving this strain, I applied further DDT selection for larvae to obtain a homozygous resistant colony reaching an LC_{50} of about 5 ppm.

2RA. This is a selection from the STMAM 2A strain by intraspecific inversion from the basic arrangement of chromosome 2. The inversion

occurred on the arm R of chromosome 2, including zones 12 and 13. This strain is more resistant to DDT than STMAM 2A.

2Rb. This is another selection by chromosome inversion from the STMAM 2A strain, involving a larger segment (zones 13 to 16) of the same chromosome. On investigation of the resistance spectra of these two "inverted" strains, no interesting differences from STMAM 2A were observed. Therefore they were not used in further investigations.

STSSDP1. The original strain was obtained from Delhi and was started in the Malaria Reference Laboratory, Horton Hospital, Epsom, Surrey, in 1947, and has never been in contact with any insecticide. In 1950, a sub-colony was started at the Ross Institute. When the colony was obtained for this study, the larval LC_{50} for DDT was rather high, so "knockdown selection" for susceptibility was applied as described later.

C. Anopheles quadrimaculatus

QDTA. A population selected from a cross between a susceptible strain from South Carolina, acquired in 1955, and a DDT and dieldrin resistant strain from Hartwell Dam, Tennessee, acquired in 1964. The population is homozygous for the marker stripe. Further selections for DDT were done during the initial generations for this investigation.

QUA. An insecticide-susceptible strain selected from the same two populations as QDTA. The larvae and pupae of this strain are unstriped.

D. Culex pipiens fatigans

Lagos. The original strain was collected around Lagos, Nigeria. This strain has been a laboratory colony at the Ross Institute since 1960. A sub-colony of this strain was colonised in the Entomology Department, The London School of Hygiene and Tropical Medicine in 1965. Selections were made in order to get both DDT resistant and susceptible strains for this study. The LC_{50} for resistant strain was 5.4 ppm and for susceptible strain was 0.005 ppm.

Rangoon. A DDT resistant strain derived from Dr. M.I.D. Sharma, National Institute of Communicable Diseases, Delhi, India. The level of larval tolerance was about 10 ppm with 24 hours exposure.

Tananarive. Another DDT-resistant strain of C. fatigans was obtained from Dr. R. Subra, Office De La Recherche Scientifique Et Technique Outre-Mer, (ORSTOM-BP434), Tananarive,(Madagascar). The LC_{50} level of larval tolerance was 1.5 ppm at 24 hours exposure.

E. Aedes aegypti.

T_8 (Trinidad T_8 or Trinidad T). A DDT-resistant strain from Dr. R.J. Wood, Department of Zoology, University of Manchester. The strain derived from a single mating of Trinidad 30 (Wood, 1968). It was used to demonstrate the $RDDT_2$ gene in adults and the $RDDT_1$ gene in larvae. It is very resistant to DDT, the LC_{50} of the larvae was 17.5 ppm.

N. This susceptible strain is of the type form of the species (Mattingly, 1957). It originated in West Africa in 1926 and has been maintained at the Entomology Department, London School of Hygiene and Tropical Medicine since then, without exposure to insecticides.

2. Insecticides, etc.

It will be convenient to group the compounds tested under the following headings. A, DDT and its analogues; B, other conventional insecticides; C, hormone-type compounds; D, miscellaneous substances. The chemical formulae of most of these compounds are shown in Figures 2 .

A. DDT and its analogues

In addition to DDT, tests were made with compounds not susceptible to dehydrochlorination. These included Prolan and Bulan and various biodegradable analogues (as described by Holan, 1971 and Metcalf et al., 1971).

(I) pp DDT: 1,1,1,-trichloro-2,2-di-(4-chlorophenyl) ethane

(II) pp DDD: 1,1,dichloro-2,2-di-(4-chlorophenyl) ethane

(III) 'Prolan'. 1,1-bis(p-chlorophenyl)-2-nitropropane

(IV) 'Bulan' 1,1,bis(p-chlorophenyl)-2-nitrobutane

(V) 1,1-bis(p-ethoxyphenyl)-2-nitropropane

(VI) 1,1-bis(p-ethoxyphenyl)-2-nitrobutane

(VII) 1-(p-ethoxyphenyl)-1-(p-ethylthiophenyl)-2-nitropropane

(VIII) 1-(p-ethoxyphenyl)-1-(3,4-methylenedioxyphenyl)-2-nitropropane

(IX) 1-(p-ethylthiophenyl)-1-(3,4-methylenedioxyphenyl)-2-nitropropane

B. Other Conventional Insecticides

(X) Dieldrin (HEOD)1,2,3,4,10,10-hexachloro-6,7,epoxy-1,4,4a, 5,6,7,8,8a-octahydro-exo-1,4-endo-5,8-dimethanonaphthalene

(XI) Gamma BHC. Y-1,2,3,4,5,6-hexachlorocyclohexane

(XII) Fenthion. Dimethyl 3-methyl-4-methylthiophenyl phosphorothionate.

(XIII) Malathion. S-[1,2-di(ethoxycarbonyl) ethyl]dimethyl phosphorodithioate.

(XIV) Allethrin. (±) 3-allyl-2-methyl-4-oxycyckopent-2-emyl(±)-(cis + trans) chrysanthemum-monocarboxylate

(XV) Bioallethrin. (±)-allethronyl (+) trans [1R,3R]-chrysanthemate

C. Hormone-type Compounds

The substances tested included compounds chemically analogous to natural hormones and certain other new insecticides which appear to have similar action. In the first category, can be placed "Altosid" and "R-20458" which are juvenile hormone mimics and ecdysterone, which is 20-hydroxy ecdysone. The second group comprise "Mon-0585" and two similar compounds, "PH-60:40" and "PH-60:38".

(XVI) "Altosid" or "ZR-515" (marketed in U.S.A. by Zoecon Co.) Isopropyl (2E,4E)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate.

(XVII) "R-20458" (patented by the Stauffer Co., U.S.A.) 4-ethylphenyl-6,7-epoxy geranyl ether.

(XVIII) Ecdysterone. 20-hydroxy ecdysone.

(XIX) Mon-0585 (Discovered by Monsanto Chemical Co.). 2,6-di-t-butyl-4-(a,a-dimethylbenzyl)phenol.

(XX) "PH-60:40" (Patented by Philips-Duphar, Holland) 1-(4-chlorophenyl)-3-(2,6-difluoro-benzoyl) urea.

(XXI) "PH-60:38" (Philips-Duphar, Holland). 1-(4-chlorophenyl)-3-(2,6-dichlorobenzoyl) urea.

D. Miscellaneous Compounds

This group is rather heterogeneous. It includes XXII Cartap

hydrochloride, based on a toxic substance found in a marine annelid, pereistoxin (see Sakai et al., 1967) and various organic compounds suggested for mosquito control in recent years. These fall into the following groups. (1) XXIII, XXIV and XXV, aliphatic amines (see Mulla et al., 1970 and Cline, 1972). (2) XXVI and XXVII unsaturated fatty acids (see Quarashi, 1971). (3) XXVIII, XXIX, XXX and XXXI, phenols and anti-oxidants. (See Miller & Maddock, 1970).

(XXII) Cartap hydrochloride (marketed in Japan by Takeda Chemical Industries). 1,3-di (carbamoylthio)-2-dimethylamino propane hydrochloride.

(XXIII) "Duomeen T1" $R_{n-2}NH\ (CH_3)NH_2$ R_{n-2} is an alkyl chain derived from tallow, with the diamine attached 2 carbons from the end.

(XXIV) "Duomeen L15". $R_{n-2}NH(CH_2)_3NH_2 + OOC_{17}H_{33} - .R_{n-2}$ is a 15 carbon alkyl chain with the diamine attached 2 carbons from the end.

(XXV) Alamine 11. Oleyl amine $C_{18}H_{35}\ H_2$.

(XXVI) Trans-2-octenoic acid

(XXVII) Trans-2-nonenoic acid

(XXVIII) Butylated hydroxyanisole

(XXIX) Cinnamyl alcohol

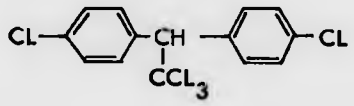
(XXX) 4-Chloro-2-cyclopentyl phenol

(XXXI) Para-phenyl phenol

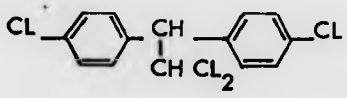
FIGURE 2. Structural formulae of the tested compound

A. DDT and its analogues

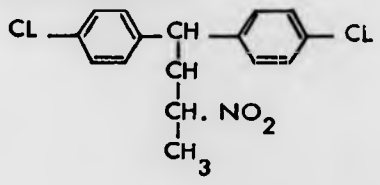
I DDT



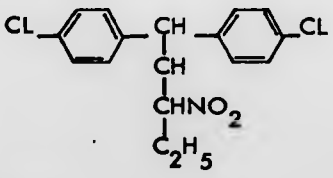
II DDD



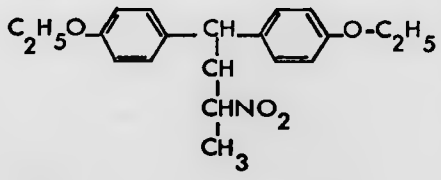
III PROLAN



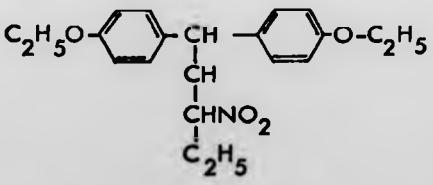
IV BULAN



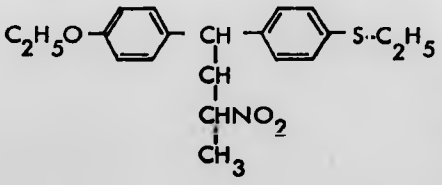
V



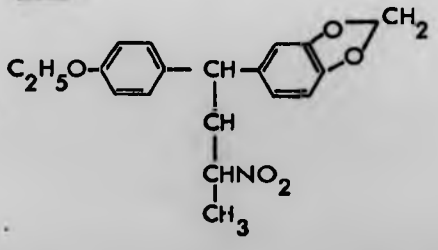
VI



VII



VIII



IX

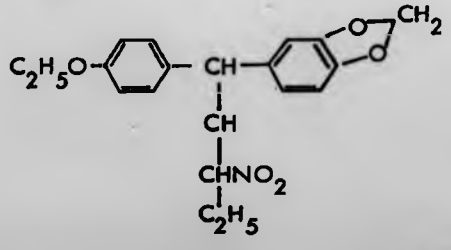
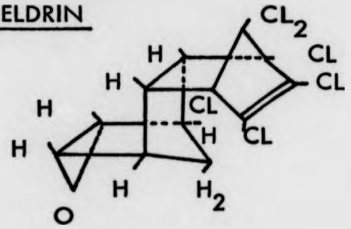


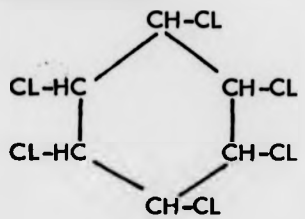
FIGURE 2. Continued.

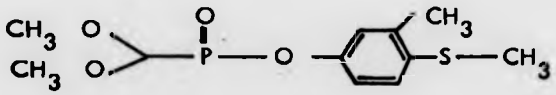
B. Other conventional Insecticides

X DIELDRIN



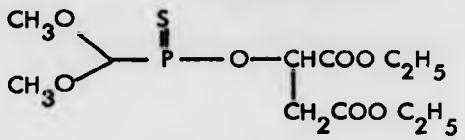
XI GAMMA-BHC



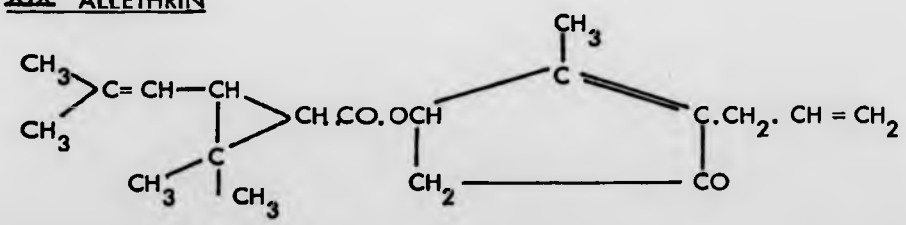


XII FENTHION

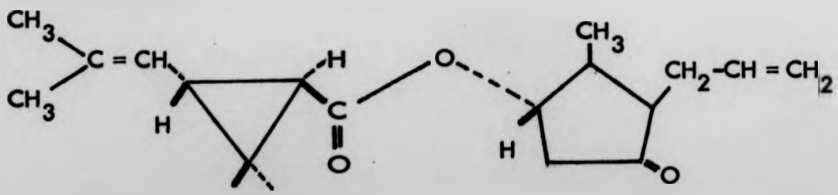
XIII MALATHION



XIX ALLETHRIN



XV BIOALLETHRIN



Miscellaneous Compounds,

XXII CARTAP HYDROCHLORIDE

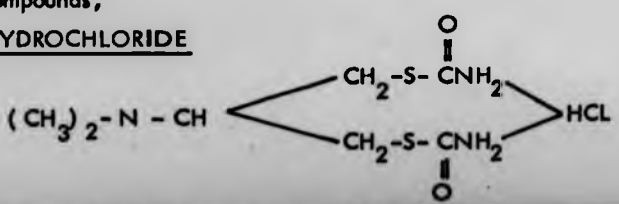
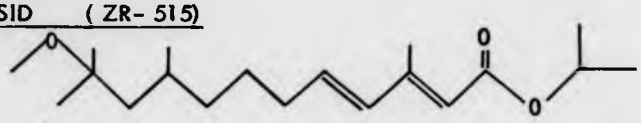


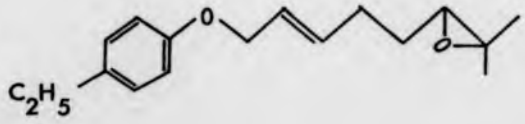
FIGURE 2. Continued.

C. Hormone - type compounds.

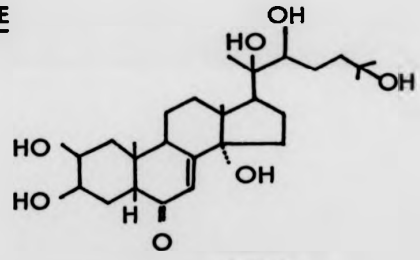
XVI ALTOSID (ZR- 515)



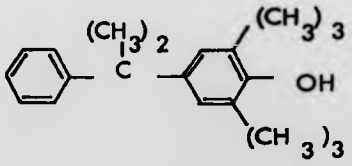
XVII R- 20458



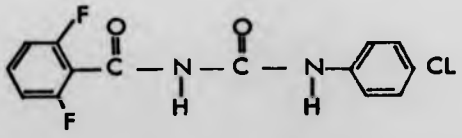
XVIII ECDYSTERONE



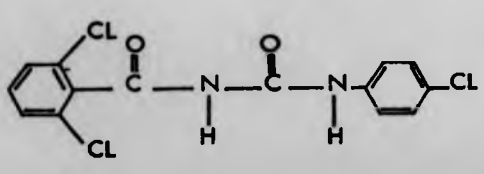
XIX MON - 0585



XX PH 60—40



XXI PH 60— 38



METHODS

Adults and larvae of the mosquitoes employed in these studies were maintained in two insectaries at a temperature of 26°C and a relative humidity of 70-80%. The illumination in each room was from three 24 inch, 20W cool white neon strips, and the period of light in the rooms was controlled by a time switch, set to give 12 hours of light per day.

The adults were kept in cages measuring 12"×12"×12" (approx. 30×30×30 cm). Access to the cage was through an 8 inch circular opening with a 12 inch sleeve attached, which was securely knotted when out of use. This size cage was ideal for maintaining strains of mosquitoes under laboratory conditions. The larvae were reared in polythene bowls 30 cm in diameter and 13 cm deep, containing about 3 litres of tap water.

1. Rearing Methods

A. Anopheles species

Adults were supplied with 20% glucose solution on a lint wick which was changed twice a week. A few days after emergence, mating occurred. Females were blood fed twice a week, by placing on the top of the cage a guinea pig which was anaesthetised with sodium nembutal. Anaesthetic administered intra-peritoneally at the rate of 1 ml for each 5 lb of body weight. The feeding period was about 30 minutes per cage. Newly emerged females sometimes required two blood meals before the first oviposition. Eggs were laid about 3 days after the blood meal. An enamel egg bowl, 11 cm diameter, lined with filter paper (Whatman No. 1, 15 cm diameter) was provided.

It was half filled with water and was placed in the cage 3 days after the blood meal. Adults fed on Monday and Friday produced egg batches on Thursday and Monday. The egg bowl was taken from the cage and was covered by a 14 cm square clear plastic plate. The eggs hatched within 1-2 days. About 200-300 of first instar larvae were reared in the larval bowl containing tap water and a 4 cm square piece of turf was added into the bowl in order to provide nutriment for the larvae. The bowls were then labelled and covered with the bead-weighted "Terylene" netting. The larvae were fed with small quantities of finely ground Farex (a baby food which added vitamins and minerals) twice daily. Over-feeding was avoided, to prevent scum forming and high mortality in early larval stages. The water was changed when necessary.

When the larvae pupated, they were transferred to a plastic drinking cup and put into a cage for emergence. The pupae cup was covered with a perforated zinc cone 17cm height, 14 cm diameter at the base and tapering to a 2.5 cm opening at the top. The cone prevented accidental drowning of adults during mating and also making it difficult for gravid females to lay their eggs in the pupae cup. Newly emerged adults had no trouble finding their way out of the cone. The duration of development from egg to adult was about 10 days. In order to prevent the contamination of the strains, adult cages and the corresponding egg, larval and pupal bowls were all carefully labelled to this end.

B. Culex pipiens fatigans.

The adults emerged about 2 days after pupation and were supplied with 5% sugar solution soaked in cotton wool which was changed twice

a week. The sugar pad was removed about 8 hours before the feeding time in order to let the mosquitoes have a full blood meal. Adults fed once a week on a 3 day old chicken, which was restricted in a small cage and was introduced into the mosquitoes' cage. The chicken was left overnight with the light turned off. A new sugar pad was replaced on removal of the chicken. About 3 days after the blood meal, a plastic bowl measuring 15 cm in diameter with tap water was placed in the cage for oviposition. The egg rafts hatched within 24 hours later and about 300-400 of the first stage larvae were transferred to each larvae bowl. The larvae were fed twice daily on a mixture of dry yeast powder, Bemax and liver powder in the ratio 1:1:1. The larval development lasted about 7 days. When the pupae appeared, they were removed daily and placed in a pupal cup which transferred to the mosquito cage. The pupal cup was covered by the perforated zinc cone as described before.

C. Aedes aegypti

Adults were fed with 5% sugar solution soaked in cotton wool in a sugar cup. Four days after emergence the females were given the blood meal. The sugar cups were removed the night before the females were fed on a guinea pig which was anaesthetised with sodium nembutal. The guinea pig was placed on the top of the cage for 30 minutes and sugar containers were replaced on removal of the pig. Three days after the blood meal the sugar cups were removed and replaced with a 75 ml beaker 1/3 filled with water containing an inverted cone made from an 11 cm diameter Whatman's No. 1 filter paper. The tip of the cone was immersed in the water. Around the side of the beaker, the slips of filter paper were lined for oviposition too.

Eggs laid by stock mosquitoes were kept on the filter paper in the egg bowl container for 3-4 days so that the larvae are ready to hatch when the eggs are immersed in water in order to allow adequate time for embryonic development. The filter papers with the eggs attached were dry in the room. These eggs can be kept for other generations and further experiments.

The eggs were immersed in 5 cm of tap water in a 13 cm diameter plastic bowl. Almost all of these eggs hatched within one hour. About 200-300 of newly emerged larvae were transferred to a larval bowl containing about 3 litres of tap water. The larvae were fed on desiccated mammalian liver powder (Armour Pharmaceutical Company Limited) by sprinkling over the surface of water and mixed well by hand. The air was bubbled gently through the water in order to prevent surface scum forming. The water can be changed if it becomes necessary.

The duration of larval development lasted about 4-7 days. When the pupae appeared, they were sieved and transferred to a paper cup about 5 cm in diameter and about 8 cm deep, with clean water and inserted into the cage. The pupae cup was also covered with the perforated zine cone.

2. Testing Methods

A. Standard test method for adult mosquitoes

In the standard test for relation between time and concentration and in the investigation of storage life of treated papers all the tests were performed with two or three day old unfed females of Culex pipiens fatigans from the laboratory colony. Mosquitoes

were exposed to the impregnated papers at a series of appropriate exposure times ranging from 7.5, 15, 30, 60, 120, 240, 480 to 960 minutes. For each concentration and time four replicates of 25 insects were usually employed. Two replicates of control were used for each performance. After exposure the mosquitoes were transferred to the control tube (W.H.O. test kit) and a piece of cotton wool soaked with sugar solution was placed on the gauze end. Mortalities were recorded after 24 hours, mosquitoes unable to walk being counted as dead. Mortality percentages were corrected by the Abbott's formula. The LT50 and LC50 values were estimated graphically from the log-dosage-probit regression mortality line.

B. Standard test for mosquito larvae

The testing procedures were carried out according to the W.H.O. standard test for larvae (W.H.O. 1963) with some modifications. Before the tests were conducted, the larvae were sieved, rinsed and transferred into a small bowl with clean water. Groups of 25 early fourt instar larvae were exposed in 249 ml of water containing 1 ml of acetone solution of insecticide at desired concentrations. The dose of insecticides supplied provided a series of 2-fold dilution. After preliminary tests, each insecticide at serial dose of 5-7 concentrations producing 5-95% mortalities were chosen for determinating the rank of susceptibility of the available strains. The test containers were [illegible] glass dish[es] measuring 1 cm diameter and 7.5 cm deep. After addition of insecticide solution, the contents of the glass were stirred with a glass rod. No food was provided during treatment. Mortality was assessed 24 hours later, and larvae which pupated during the period of observation were not considered in

calculating. Moribund larvae were also recorded and added to the dead for calculation of percentage mortality. When checking the results with anophelines, the larvae should not be disturbed, because this causes them to dive to the bottom. Larvae persisting at the bottom of the glass were counted as dead and those at the surface were scored as alive. Controls, treated with 1 ml acetone, were maintained in every test and were utilized in correcting the experimental results by Abbott's formula. At least 2 replicates for each concentration were performed and 3-4 such replicate expetiments were repeated on different days. The LC50 values were estimated graphically from the log-dosage-probit-regression mortality line.

C. Assessments for the new types of compounds

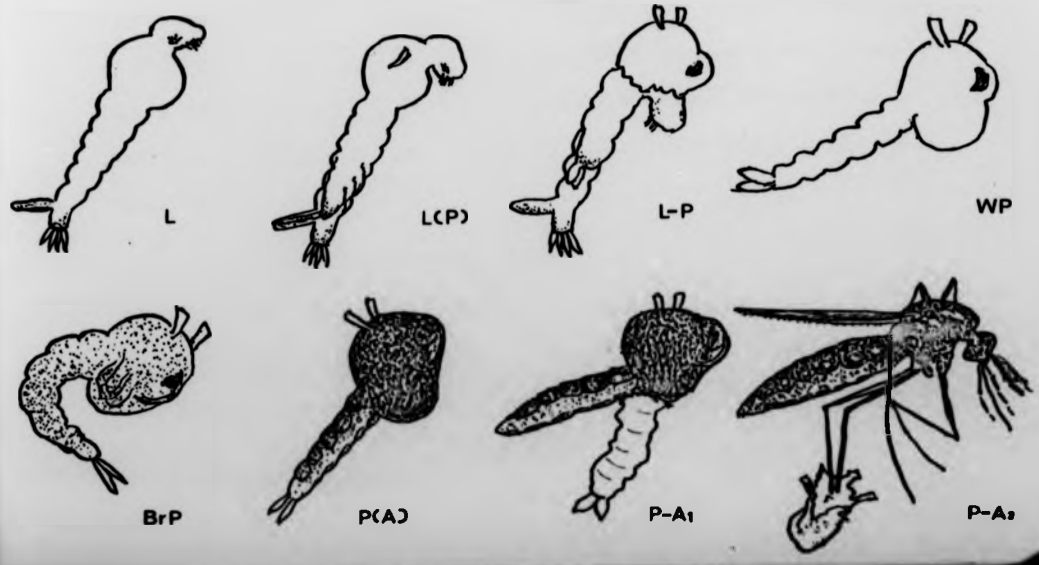
Initially, the standard W.H.O. test procedure for larvae was used to compare the larvicidal activity. The hormone mimics and certain other compounds with analogous activity were tested with exposures longer than 24 hrs; in some cases continuous exposure to low concentration was investigated. The effects on development were classified as described by Spielman & Shaff (1967) and adapted for additional effects, as shown in Figure 3 . Adults which emerged were also counted, and removed daily. Each experiment was concluded when all specimens had died or completed its development. The surviving adults were fed. The number of eggs laid and hatched larvae from each female were counted in order to assess for a sterility effect. Comparative tests were set as controls. Following larvae generations were treated in the same way and observed for further sterile effect and development of resistance.

D. Selection for resistance

The following general procedure was used to select strains of

Figure 3. Different categories of toxic effects of the moulting disturbance compounds on mosquito larvae.

Symbol	Explanation = death at different stages in metamorphosis
L	As larvae.
L(P)	At beginning of pupation, with respiratory trumpets visible and tracheal system disengaged.
L-P	In the process of ecdysis from larvae to pupae.
WP	As white opaque pupae.
BrP	As enclosed adults, showing the beginning of pigmentation.
P(A)	As black adults, inside pupal exuviae.
P-A_1	In the process of adult emergence.
P-A_2	As adults, almost completely free except for the tarsi of the hind legs.
A	As feeble adults dying on the water surface.



high and homogeneous resistance, in all species.

About 100-200 glasses were prepared for each generation. The larvae were allowed to contact with DDT for 24 hours. At the end of the exposure time the number of dead larvae were recorded and all of the survivors were rinsed with tap water, and transferred to clean water in a rearing bowl to continue their development. About 2500-5000 larvae were tested per generation at a selection level of 50-60% mortality. The selections were done every generation until the LC50 of the strain reached a stable resistant level, then the further tests were performed. The selection procedure were also continued in each generation.

E. Selection for susceptibility

Two different methods were used for removing "contaminating" resistant individuals from susceptible colonies.

(i) A simple sib-selection method

This method was feasible with Culex pipiens fatigans. The egg rafts were reared separately in a plastic bowl containing about 1 l. of water. When the larvae become fourt instar, 25 larvae from each bowl was exposed to a discriminating dosage of DDT (.03 ppm) and only batches of larvae from egg rafts showing 100% mortality were used for production of the next generation. By repetition of this procedure, a pure strain of susceptible colony could be established.

(ii) Knock down method

For other mosquitoes such as anopheles species and Aedes aegypti, the eggs laid are scattered so the knock down method was applied for selection. This was more convenient than separating

eggs from individual fed females.

Approximately 150 early fourth instar larvae were introduced into the large enamel bowl (diameter 16 cm and depth 10 cm) containing 1.25 l. of appropriate discriminating dosage of DDT solution. The larvae were exposed for 2 to 4 hours, then the contents of the bowl were poured into a glass funnel (diameter 20 cm). The funnel which is supported by a retort stand, contains a 45 cm glass plunger, occluding the stem with a ground glass joint and rising above the water surface. As larvae are paralysed ("knocked down") by initial DDT action, they fall to the bottom of the funnel and can be removed by gently raising the glass plunger. This does not disturb unaffected larvae at the surface. The knocked down larvae are collected in a net sieve, rinsed several times with clean water and transferred to a rearing bowl for further development.

F. Determination of micro amounts of insecticide picked up by mosquito larvae.

(i) Bioassay test

The basic method of bioassay was to use highly susceptible first instar larvae to assess concentration of DDT in bowls of water after groups of fourth instar larvae had been exposed in them. The pick up by the fourth instar larvae should approximate to the difference from the original concentration to which they had been exposed.

Susceptibility tests of first stage larvae were performed in order to obtain a standard concentration-mortality line. Batches of 50 first instar larvae were exposed at a range of concentrations of DDT in beakers containing 50 ml of water and 0.2 ml of appropriate acetone solution of DDT. Each concentration assessment consisted of

3 replicates and each test was repeated at least 3 times. Mortality was assessed after 4, 8, and 16 hours. Average percentage mortalities were determined and plotted against concentrations.

For the actual bioassay, groups of early fourth instar larvae of resistant and susceptible strains were exposed in beakers containing 50 ml of water and 0.2 ml of DDT solution at varying concentrations from 0.005 up to 0.1 ppm. After 16 hours, the treated larvae were removed and the number of dead larvae was recorded. Batches of 50 of first instar larvae of susceptible strain from the same species were put instead and were exposed for 4, 8 and 16 hours. Each test consisted of 3 replicates and was repeated on different days. Controls, treated with acetone, were maintained in every test. Mortalities were recorded subsequently and the concentrations were determined from the standard concentration curve. Then the pick up by the resistant and susceptible larvae can be calculated.

(ii) Radioactive test

As an alternative (and more precise) way of determining the pick up amounts of insecticide by mosquito larvae, ^{14}C DDT and ^{14}C malathion were used in this study. The object was to obtain radiometric measurement of insecticide (a) externally on larvae, (b) internally in larvae and (c) in the test suspension after removing the larvae.

The actual quantity of insecticides present in the radioactive samples used was not known exactly. Therefore it was necessary to assess them by bioassay. Very exact information was not required, but it was necessary to prepare suspensions giving approximately known expected toxic effects. It was essential to have some results

with exposures producing negligible mortality in the susceptible strain; otherwise difference in pick up might be due to differences in tolerance, rather than the cause of them.

Batches of 20 early fourth instar larvae of resistant and susceptible strains in 99 ml of distilled water to which 1 ml of different concentrations of the radioactive insecticides had been added. After 4, 8 and 16 hours the mortality was recorded. The LC50 values were estimated from the mortality curves and the required doses were then chosen for further experiments.

At the same time tests were run with known concentrations of ordinary insecticides, for comparison. Controls treated with acetone, were maintained every test.

(a) External pick up.

At indicated times after treatments, the larvae were removed from the test solutions using a nylon net sieve, and then transferred to the counting vials. Initially, n-hexane was used to strip off external adsorbed insecticide from the larvae; but it was found inconveniently volatile and methanol was used.instead. They were then rinsed with 3 ml methanol, which was enough to cover them, and after gentle washing for a few seconds, the methanol was removed by pipette and transferred to another counting vial. This process was repeated so that the 20 larvae were washed with a total of 6 ml methanol. The rinsed larvae were transferred to a Hunt ampoule and frozen in liquid nitrogen to facilitate grinding. The counting vial was rinsed once more with 3 ml methanol and the rinse added to the 6 ml previously collected. The total external rinse volume was 9 ml. The methanol rinse was evaporated to dryness in a vacuum

desiccator overnight.

(b) Internal pick up.

In order to avoid loss in a separate homogenizing tube, larvae were put directly into round bottomed centrifuge tubes and homogenized with a ground glass pestle. Four ml of methanol was added and mixed by further homogenizing. The liquid was then centrifuged for 5-10 minutes until the supernatant was clear. It was then transferred to a scintillation counting vial. This process was repeated being added to the same counting vial. The contents were then evaporated to dryness in a vacuum.

(c) Residue in suspension.

A 50 ml aliquot of the water in which the larvae were exposed was pipetted into a separating funnel and extracted 3 times each with 10 ml n-hexane. This process was repeated with the remaining volume of water. In all tests, the containers were rinsed carefully with n-hexane, since control tests showed that considerable amount of insecticide was located on the surface of containers rather than in water solution or suspension. The extracts were kept in the 35 ml vials with a plastic screw cap, containing a piece of aluminium foil. At first, the hexane extractions were evaporated in a rotary evaporator. This procedure was inconvenient, so a Liebig condenser was used with the "quick-fit" equipments and evaporated from a water bath. The residue was transferred to the counting vial by 3 washes of 3 ml diethyl ether and left overnight for evaporation by air. The next day, the counting vials were put into vacuum desiccator and evaporated to dryness.

After the evaporation process, the residues in counting vials

from external and internal larvae and from the water were each dissolved in 10 ml of scintillating solution (0.5% (w/v) butyl PBD in toluene) and shaken well to ensure the completion solution. The counting vials were cooled in the liquid scintillation spectrometer (Packard model 3314) for 1 hour prior to the start of counting. The radioactive samples (^{14}C) were counted for 2 minutes at about 65% efficiency.

RESULTS

1. STUDIES RELATING TO ADAPTATION OF ADULT MOSQUITO RESISTANCE TEST FOR PHOSPHORUS AND CARBAMATE INSECTICIDES

All the tests were performed with two- or three-day-old unfed females of Culex pipiens fatigans from the laboratory colony, at a series of appropriate exposure times. For each concentration and time four replicates of 25 insects were usually employed. After exposure the mosquitoes were transferred to the control tube (W.H.O. test kit) and mortalities were observed after 24 hours. Mortality percentages were corrected by Abbott's formula. The LT50 and LC50 values were estimated graphically from log-dosage-probit regression lines.

A. Concentration-time relations

So far as organochlorine insecticides are concerned, it has long been known that, over a considerable range, the relations between concentration and exposure time for an equitoxic effect, are inverse; i.e. $C \times T$ = constant (Busvine, 1958). This was later confirmed and shown to be due to close relations between exposure time and the dose picked up by mosquitoes exposed to impregnated papers (Pennell et al., 1964; Ariartnam & Brown, 1969). Some preliminary data with organophosphorus and carbamate papers, suggested that exposure time would provide a suitable "dosage" variable, (Hamon & Sales, 1970). Adequate data for the newer compounds is, however, needed and, accordingly, experiments have been undertaken with a wide range of concentrations of malathion, fenthion, fenitrothion and propoxur papers supplied by W.H.O. for this purpose.

The results are set out in Table 2, together with LT50 and LC50

TABLE 2. Results (Percentage Mortalities) of Exposing Batches of Culex P. fatigans to WHO papers, for Different Periods, at Various Concentrations

(Estimates of LC50, LT50 or C×T values based on a very few points, are given in brackets. In such cases, a line was drawn with a slope parallel to other comparable ones).

	Conc. %	Exposure times (minutes) 7.5	15	30	60	120	240	480	960	LT 50	CT
Fenitrothion	0.1				0	20	80	99	100	168	16.8
	0.4		0	13	88	99	100			42	16.8
	1.6	22	79	100						10.5	16.8
	LC50	(2.3)	(1.2)	(.56)	(.28)	.145	(.07)			means	16.8
	CT	(17.3)	(18.0)	(16.8)	(16.8)	17.4	(16.8)			17.2	
Fenthion	0.1					0	64	100		222	22.2
	0.2				0	85	100			108	21.6
	0.4			12	81	100				48	19.2
	0.8		25	83	100					21	16.8
	1.6	31	87	99	100					10.7	17.1
	3.2	92	100							(4.8)	(15.6)
	LC50	1.9	1.1	.62	.36	.18	(.09)			means	18.8
	CT	14.3	16.5	18.6	21.6	21.6	(21.6)			19.0	
Malathion	0.1					0	3	33	99	500	50.0
	0.2				0	6	79			192	38.4
	0.5				11	34	100			108	54.0
	0.8			0	30	99				66	52.8
	1.6	0	2	21	99	100				32.4	41.8
	3.2	2	44	98	100					16.0	51.2
	5.0	36	82	100						9.0	45.0
	LC50	(6.2)	3.8	1.9	.85	.48	.17			means	47.7
	CT	(46.5)	57	57	51	58	41			51.8	
Propoxur	0.01			0	3	4	32	73	97	300	3.0
	0.04		0	7	40	90	100			72	2.88
	0.16	5	.14	64	96	100				22.8	3.64
	0.80	82	99	100						(4.8)	(3.84)
	1.6	97	100							(3.0)	(4.80)
	3.2	100									
	LC50	.48	.28	.11	.046	.023	(.012)			means	3.63
	CT	3.6	4.2	3.3	2.8	2.8	(2.9)			3.22	

values estimated from them. It is possible to calculate C × T values in two ways: from LT50 × concentration, or from LC50 × time. The values estimated in these two different ways were not found to be substantially different. It therefore seems likely that effect is related to both variables in the same way.

In order to test this statistically, it was assumed that $Y = a + \beta_1 \log C + \beta_2 \log T$ where Y = kill in probits (or logits) and β_1 and β_2 are slope constants. These were calculated from the data and compared with a joint slope constant, β_3 where $Y = a + \beta_3 \log (C.T)$.

Slope coefficients found

Insecticide	β_1	β_2	β_3
Fenitrothion	5.28	5.22	5.21
Fenthion	6.56	5.80	5.55
Malathion	5.26	5.20	5.21
Propoxur	3.35	3.56	3.36

It will be seen that slope values within each insecticide group, were reasonably consistent, suggesting that a joint slope value would fit the data.

The various sets of data were tested for goodness of fit to the concentration × time hypothesis and evidence of heterogeneity was found in all cases, except for fenitrothion. Examination of the results showed, however, that the discrepancies responsible were random, without indications of a systematic trend, except perhaps in the data for fenthion. Here there was evidence of lower CT values for short exposures to high concentrations, than for long exposures to low concentrations.

In short, it can be said that the lethal effect was related to CT^n, where n = 1; except for fenthion, where n = 0.91.

Comparative results of other workers

Comparable estimations of concentration × time values have been made by other workers who have very kindly allowed me to quote some of their data, most of which are unpublished. These are assembled in Table 3. In most cases, it will be found that the values obtained by keeping time constant and varying concentrations are not too different from those got by varying the exposure to one or two standard concentrations. Also, there is reasonably good agreement in the estimates of different investigators for respective mosquito-insecticide combinations, when it is remembered that some differences in experimental conditions are inevitable. Thus, the data came from widely different localities and in most cases the temperature was not given (and probably not controlled).

Conclusion regarding concentration-time relations

Assembled results showed some evidence that mortality is equally dependent on concentration and exposure time in the mosquito resistance test. This does not necessarily mean that in future assessment of resistance should be based on a concentration-time product, since however good the evidence mentioned, this brings in an extra variable.

Resistance checks for organophosphorus and carbamate insecticides should be made on the basis of equilethal exposures to standard concentrations, as proposed by the W.H.O. Insecticide Committee. The evidence I have adduced should tend to establish the validity of this procedure and its equivalence to the equitoxic concentration basis of the

TABLE 3. Concentration-Time Values for Various Mosquitoes Exposed to Different Insecticides

Insecticide	Species	Locality	Ref.*	Mean CT: With constant	
				Conc.	Time
Fenitrothion	Culex p. fatigans	London	1	16.8	17.2
	Culex p. fatigans	U. Volta	2	26	39
	Culex p. fatigans	Thailand	3	19.4	16.2
	Culex p. fatigans	Taiwan	4	19.0	36
	Aedes aegypti	U. Volta	2	12.0	12.0
	Aedes aegypti	USA	5	12.0	11.8
Fenthion	Culex p. fatigans	London	1	18.8	19.0
	C. tritaeniorhynchus	Korea	4	29	24
	Aedes aegypti	USA	5	16.2	15.8
Malathion	Culex p. fatigans	London	1	48	52
	C. tritaeniorhynchus	Taiwan	4	47	66
	C. tritaeniorhynchus	Korea	4	29	24
	C. annulus	Taiwan	4	93	28.2
	Aedes aegypti	U. Volta	2.6	61	69
	Aedes aegypti	USA	5	36	35
Propoxur	Culex p. fatigans	London	1	3.6	3.2
	Culex p. fatigans	U. Volta	2	3.2	4.1
	C.t. summorosus	Taiwan	4	10.8	10.0
	Aedes aegypti	U. Volta	2	7.2	5.5
	Aedes aegypti	USA	5	6.3	5.5

1*This thesis (Table 2)

2. Sales and Mouchet (1973)

3. WHO ARU (Aedes Research Unit) Thailand

4. WHO JEVRU (Japanese Encephalitis Virus Research Units) Taiwan and Korea

5. Dr. H.F. Schoof and Dr. A.D. Flynn, U.S.A.

6. Hamon and Sales (1970)

I wish to thank various workers for permission to quote unpublished data of references 2,3,4,5.

earlier tests with organochlorine insecticides. Furthermore, the general orders of magnitude of the C×T products found for various insecticides could provide a guide for initial tests on susceptible strains of mosquitoes. It seems that malathion values range mostly from 25 to 70 (the constant $\frac{\text{concentration}}{\wedge}$ figure for C. annulus is dubious); values for fenthion and fenitrothion range mostly from 15 to 40; values for propoxur range from 3 to 11. Results giving values far outside these limits should be somewhat suspect, possibly due to deterioration of the impregnated papers.

B. Storage life of malathion and propoxur papers

One convenient aspect of the adult mosquito resistance test for organochlorine insecticides, is the long persistence of the papers used. This may not apply with some newer organophosphorus and carbamate papers. The experiments to be described were intended to evaluate the shelf life of papers impregnated with malathion or propoxur. This was an ad hoc study of practical value to W.H.O.; but certain basic principles of testing procedure were involved. WHO arranged for the preparation of large batches of papers impregnated with either malathion or propoxur in February 1971. Half of these were stored under normal room conditions (say about 20°C) and half were kept in a refrigerator. At intervals of two to three months, samples were supplied for determination of insecticidal potency.

Each series of papers was tested over a range of two to five exposure periods (as appropriate), with four to seven replicates of 25 mosquitoes for each period. The results were obtained as LT50 values; but they have been converted to C×T indices and set out in Table 4. Several comments may be made.

1) There is no evidence of extensive deterioration in the potency of either type of paper.

2) Unfortunately there is considerable variety in the results of different assays, which must be ascribed to variations in tolerance of the mosquitoes. This seems to have effected the whole generation of mosquitoes used for each assay, since all the estimates on one occasion (e.g. after 7 months) tend to be high, while those on another occasion (i.e. 13 months) tend to be low.

3) All the results with the 0.01% propoxur papers of the original batch gave abnormally low C×T values. This would suggest a faulty impregnation, at too high a rate; and the interpretation was confirmed by the results of tests on additional batches, which gave more reasonable results. Since no very large changes in potency could be detected in storage up to 13 months, other tests were made with propoxur papers which had been kept in storage (room conditions) up to six years. These results are given in Table 5.

The values for freshly impregnated papers were calculated from all propoxur data in the tests of C×T relation plotted as C × T values against mortality, with expected kills read off from a regression line fitted to them. It will be seen that there is not much evident loss in potency after one year, but a substantial fall thereafter.

The general conclusion from these results is that decline in potency of malathion and propoxur papers over a period of a year is not excessive, with storage under European room conditions. It is true that Brengues & Sales (1967) found significant difference in

TABLE 4. Concentration-Time Values for 50% Mortality of C.p. fatigans Exposed to Malathion or Propoxur Papers Stored for Various Periods

Insecticide	Conc. %	Stored*	Concentration × Time values						
			Original batch stored (months)					New batches stored (months)	
			2	4	7	10	13	1.5	1
Malathion	5.0	R	70	30	60	55	55	50	50
	5.0	F	65	35	63	45	50	-	-
	0.5	R	45	50	70	65	35	60	48
	0.5	F	48	52	73	58	48	-	-
Propoxur	0.1	R	3.0	2.0	2.1	3.6	2.1	4.8	1.6
	0.1	F	2.4	2.1	2.5	3.9	2.8	-	-
	0.01	R	0.38	0.33	0.37	0.60	.30	1.3	2.2

*Stored in: R = room
F = refrigerator

TABLE 5. Percentage Kills of C.p. fatigans by Propoxur Papers, Stored for Various Times

(Numbers used per entry: 125 for 0.1% and 60 minutes; 100 for 0.025% and 60 minutes; 50 for 0.025 and 180 minutes)

Concentration (%)	0.1	0.025	0.025
Exposure (minutes)	60	180	60
Storage			
Nil	87*	48*	11*
1 year	92	36	13
2 years	66	17	10
3 years	18	14	4
4 years	13	14	6
5 years	13	4	6
6 years	2	3	5

*Expected kills, based on data in Table 2.

mortality of Aedes aegypti exposed to propoxur and fenitrothion papers with different lengths of storage. Their most striking difference was in nine-month-old propoxur papers even though the papers had been kept in a refrigerator. Nevertheless, these investigators found several discrepancies in their results, which they suggest might have been due to unsatisfactory standardization or faulty impregnation of the papers.

2. DEFENCE MECHANISMS AGAINST DDT IN LARVAE OF RESISTANT STRAINS

A. Species and Strains used for this part of the investigations

The sub-colonies of susceptible and DDT-resistant strains of 5 species of mosquito were established from the original colonies as described earlier and further selections were applied to obtain homozygous resistant and susceptible colonies by the methods described. As a result, the susceptibilities of some of the strains, as determined in fourth instar larvae by the standard W.H.O. method (WHO, 1963) changed. Table 6 gives the initial LC_{50} values when the strains were obtained, the LC_{50} values at the time of this investigation (test LC_{50}), and the number of generations of selection for each strain.

Table 6. DDT LC_{50} values for the various strains of mosquito at the time of colonization and at the time of these studies.

Species	Strain	Initial LC50 ppm.	Generations of selection	Test LC50 ppm.
An. gambiae	UV19R5	0.03	10	6.0
	IBAD	0.009	7	0.004
An. stephensi	STMAM2A	2.8	6	4.6
	STSSDP1	0.3	6	0.08
An. quadrimaculatus	QDTA	0.21	6	3.6
	QUA	0.005	3	0.004
C.p. fatigans	Lagos R	0.04	12	5.5
	Lagos S	0.04	12	0.005
	Rangoon	8.0	3	11.0
	Tananarive	1.0	5	1.5
A. aegypti	T8	12.3	3	17.5
	N	0.02	3	0.0175

B. Cross-Resistance Studies

(i) Presentation of Results

A very large number of tests was needed to establish the patterns of cross-resistance in different strains. For each assessment of a resistance level to a particular compound, it was necessary to establish a regression line (and from it an LC_{50} value) for both normal and resistant strains. By the conclusion of the investigation the following numbers of such comparisons were available for consideration. With Culex pipiens fatigans (3 strains) 25, 19, and 13, total 57; with Anopheles quadrimaculatus, 23; with An. stephensi, 12; with An. gambiae, 25; with Aedes aegypti, 25. In all, this amounts to 142. measurements of resistance levels.

Clearly it is not desirable to reproduce all the experimental data accumulated for this purpose. Two examples will be quoted as illustrations: the tests with DDT and with Prolan against C.p. fatigans. The summarised results of the tests are set out in Tables 7 and 8; and they are shown graphically in Figures 4 and 5. In nearly all cases, the regression lines for resistant strains were straight (except during the course of selection to derive a more homogeneous resistant strain). Accordingly, it seems justifiable to use the LC_{50} values for assessing resistance levels. These are shown in Tables 9 to 13. For ease of interpretation, the resistance patterns have been shown as histograms in Figures 7 to 13.

(ii) Interpretation of Results

It will be useful to consider the results from two standpoints: (a) according to the characters of the various resistant strains, and (b) in relation to the various compounds examined.

Table 7. LC50 of 4 strains of Culex pipiens fatigans larvae exposed with DDT for 24 hours.

Strain	Concentration ppm	Number of larvae tested	Number of dead larvae	Mortality %	LC50 ppm
Lagos R	20.	200	194	97.0	
	16	200	180	90.0	
	10	182	152	79.17	
	8	172	120	69.7	6.0
	4	152	56	36.8	
	2	200	16	8.0	
	1	200	4	2.0	
Rangoon	40	200	196	98.0	
	20	200	160	80.0	
	10	200	102	25.3	11.0
	5	200	24	12.0	
	2	200	2	1.0	
Tananarive	8	200	198	99.0	
	4	200	191	95.5	
	2	200	109	54.5	
	1	200	41	20.5	1.5
	0.5	200	5	2.5	
	0.25	200	2	1.0	
Lagos S	0.05	200	200	100.0	
	0.025	200	180	99.0	
	0.0125	250	220	88.0	
	0.00625	250	184	73.8	0.005
	0.00312	250	68	26.7	
	0.00156	200	13	6.5	

Table 8. LC50 of 4 strains of Culex pipiens fatigans larvae exposed with Prolan for 24 hours.

Strain	Concentration ppm	Number of larvae tested	Number of dead larvae	Mortality %	LC50 ppm
Lagos R	0.025	150	150	100.0	
	0.0125	196	158	89.7	
	0.00625	200	89	44.5	0.007
	0.00312	200	6	3.0	
	0.00156	150	0	0	
Rangoon	0.1	150	0	100.0	
	0.05	198	167	89.4	
	0.025	200	70	35.0	0.031
	0.0125	200	5	2.5	
	0.00625	0	0	0	
Tananarive	0.2	200	200	100.0	
	0.1	200	160	80.0	
	0.05	200	79	34.5	0.07
	0.025	200	8	4.0	
	0.0125	200		0	
Lagos S	0.02	150	150	100.0	
	0.01	144	136	94.4	
	0.005	136	66	48.5	
	0.0025	135	20	14.8	0.0048
	0.00125	150	1	1.3	
	0.00625	150	0	0	

Figure 4. Dosage mortality regression lines of 4 strains of Culex pipiens fatigans larvae exposed to DDT

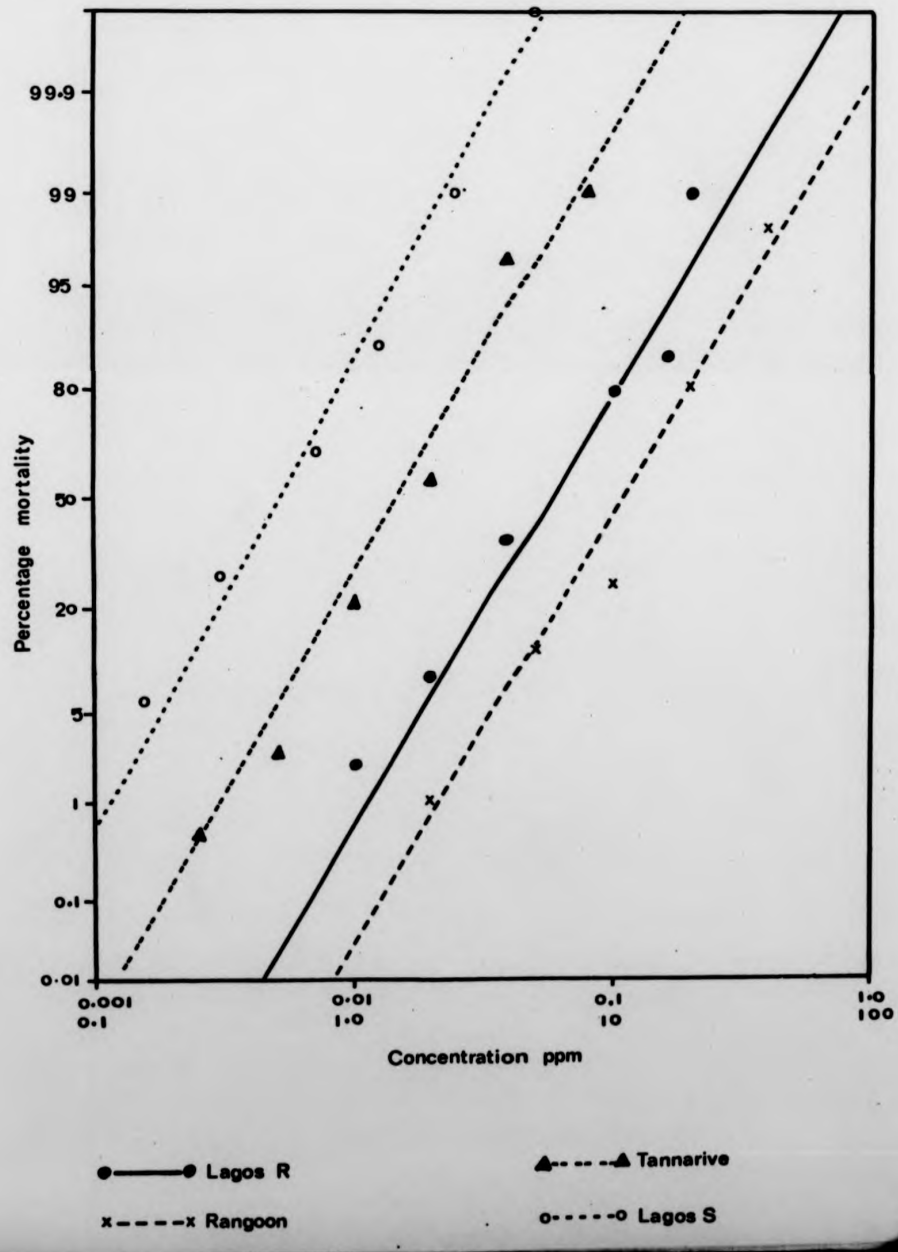
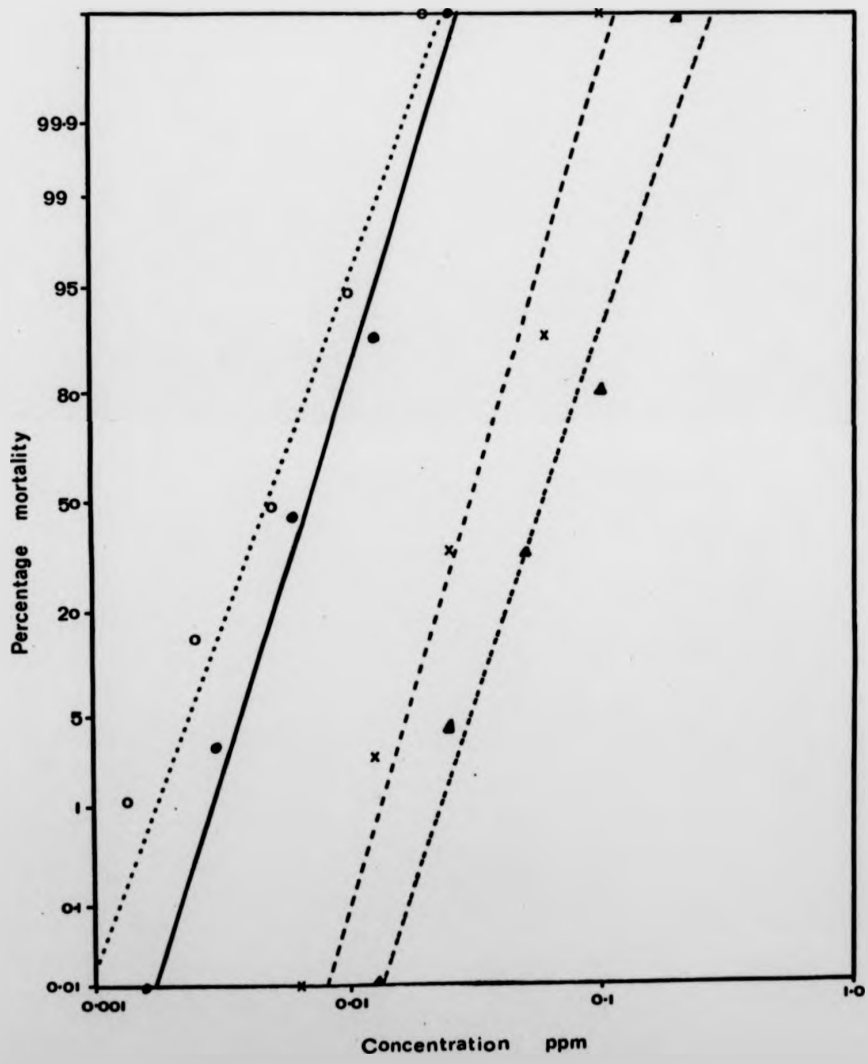


Figure 5. Dosage mortality regression lines of 4 strains of Culex pipiens fatigans larvae exposed to Prolan



(a) Characteristics of Strains

Culex pipiens fatigans

Three distinct strains were examined, respectively, from Lagos R, Tananarive and Rangoon. Their resistance spectra are shown in Figures 7 to 9. Their resistance levels to DDT were all high, being × 1100, × 300 and × 2200 respectively. (Accuracy of values at these high levels is questionable, because thephysical constitution of high concentrations of DDT suspensions is difficult to standardise). Moderate cross-resistance to DDD (about × 40) was noted in the Lagos R colony, which was the only strain tested.

Cross-resistance to the biodegradable DDT-analogues was low in all cases; and usually also to Prolan and Bulan. The highest level in this group was × 5.8 resistance to Prolan by the Rangoon strain. The inference of these facts is that these strains depend for DDT-resistance on the dehydrochlorination mechanism, since they do not show high tolerance to compounds which cannot be metabolised in this way.

There is, however, evidence that an alternative mechanism can exist in C.p. fatigans as proved by results of Kalra (1973) with a resistant strain from Delhi. He obtained evidence of resistance to non-dehydrochlorinatable compounds. Unfortunately, several efforts to obtain a sub-colony of this strain from India were unsuccessful.

In an attempt to develop a strain with this mechanism, selection with Prolan was undertaken with each of the strains and also with progeny of a cross between Lagos R and Rangoon. Selection pressure was maintained on each generation at the original estimated LC_{90} 0.002 ppm for Lagos R and 0.025 ppm for Rangoon. Although there were fluctuation

Figure 7. Resistance spectra of DDT-resistant strain of Culex pipiens fatigans (Lagos R)

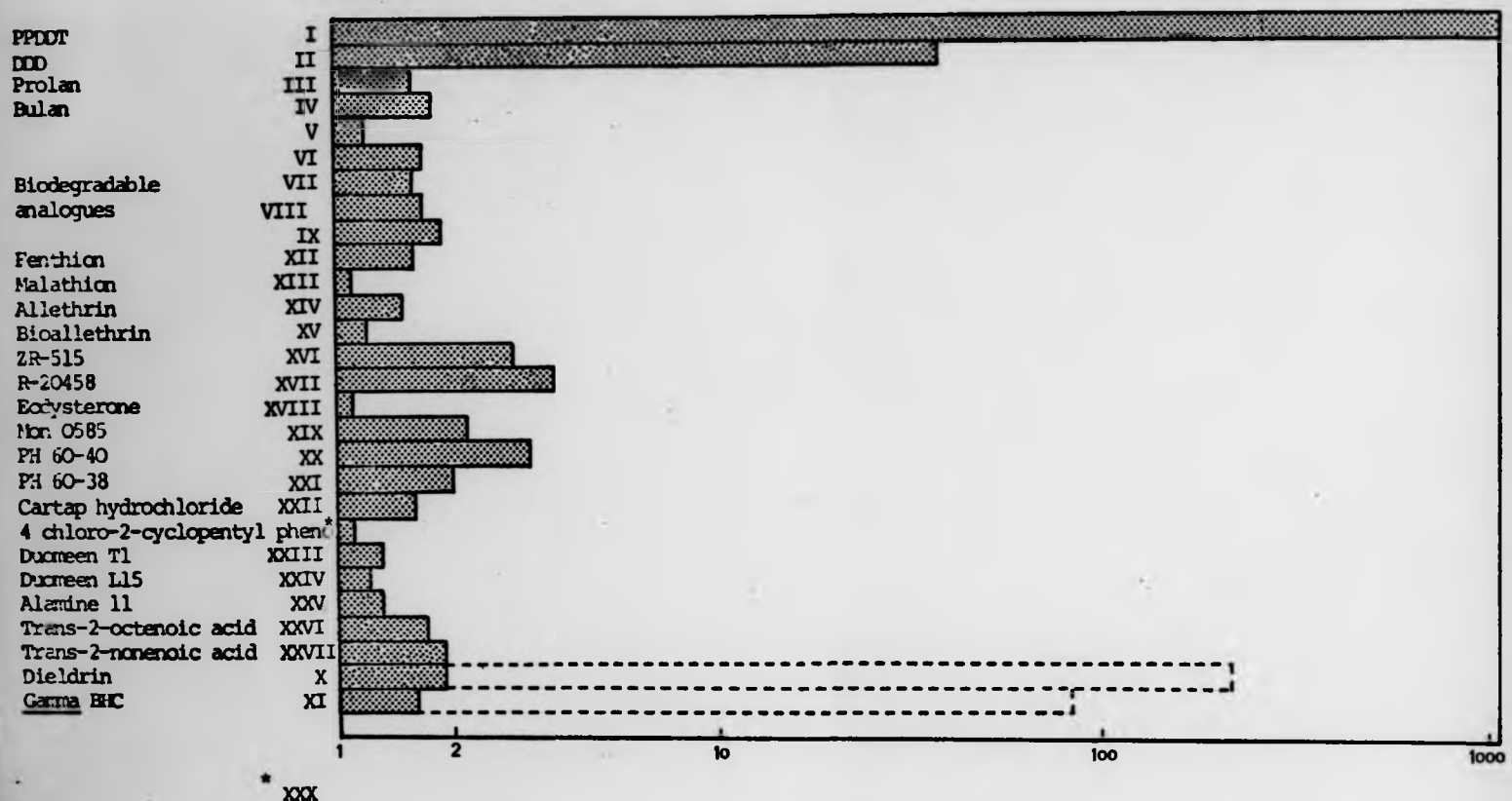


Figure 8. Resistance pattern of DDT-resistant strain of Culex fatigan (Rangoon)

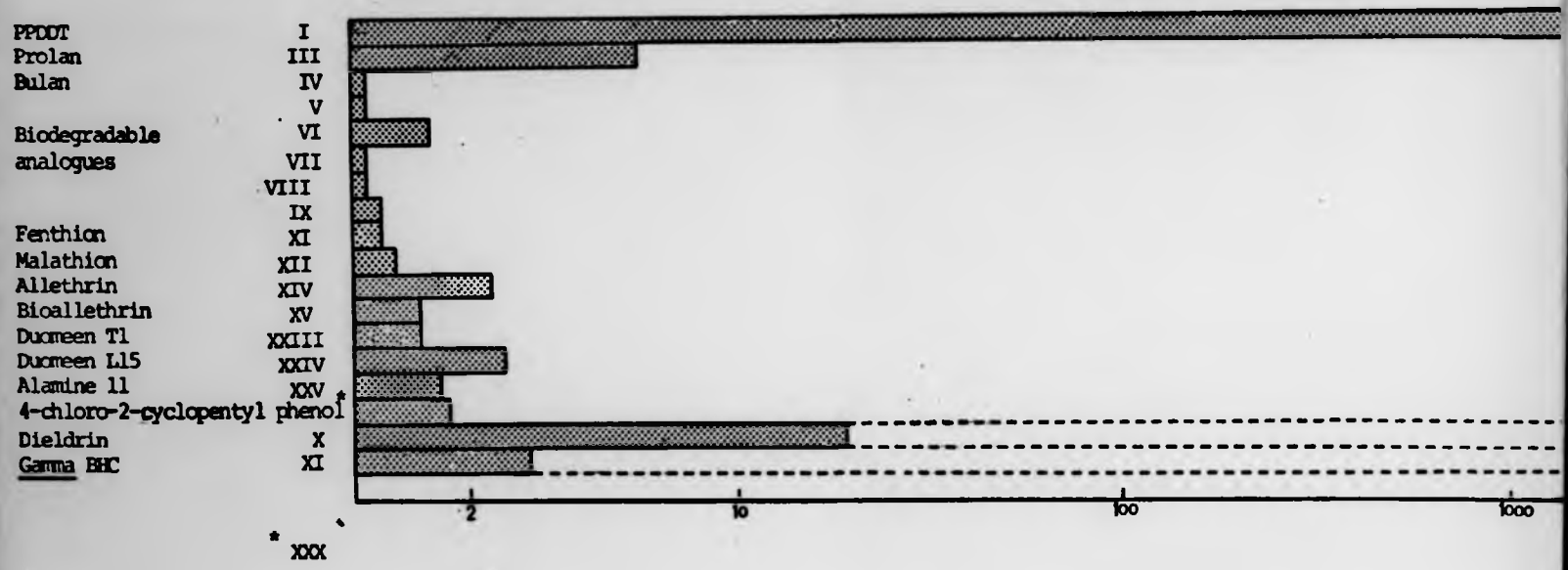


Figure 9. Resistance spectra of DDT-resistant strain of Culex pipiens fatigans (Tananarive)

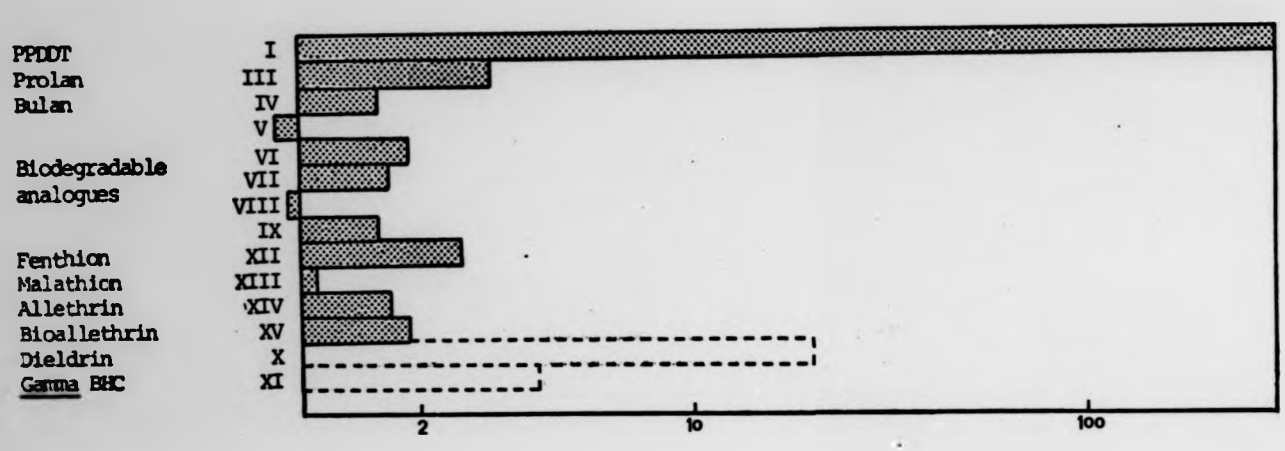


Figure 10. . Resistance spectra of DDT-resistant strain of Anopheles quadrimaculatus

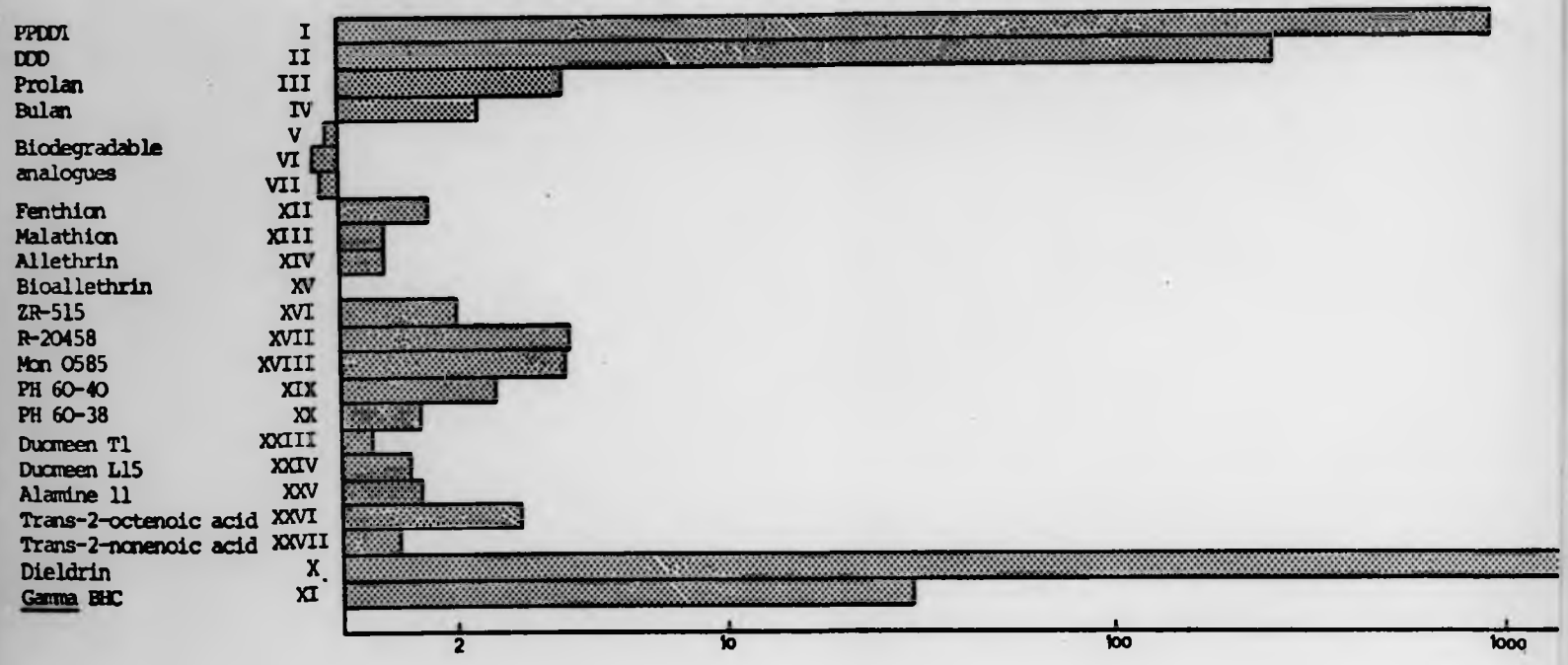


Figure 11. Resistance pattern of DDT-resistant strains of Anopheles stephensi

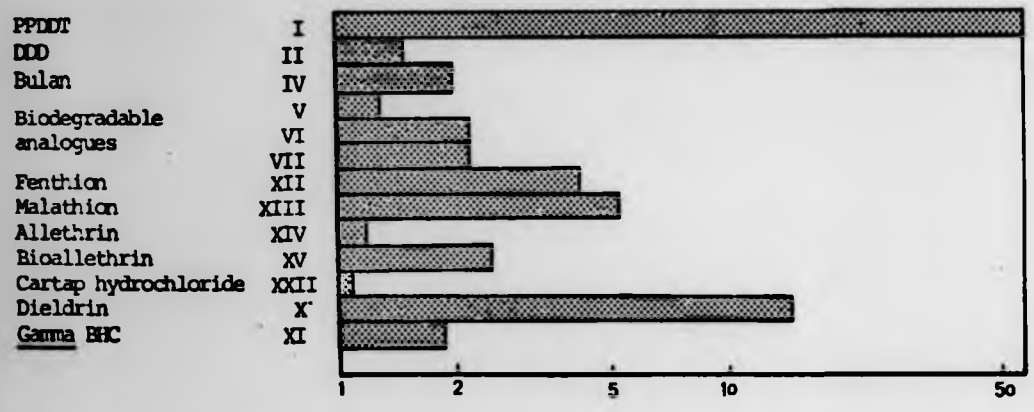


Figure 12. Resistance spectra of DDT-resistant strain of Anopheles gambiae

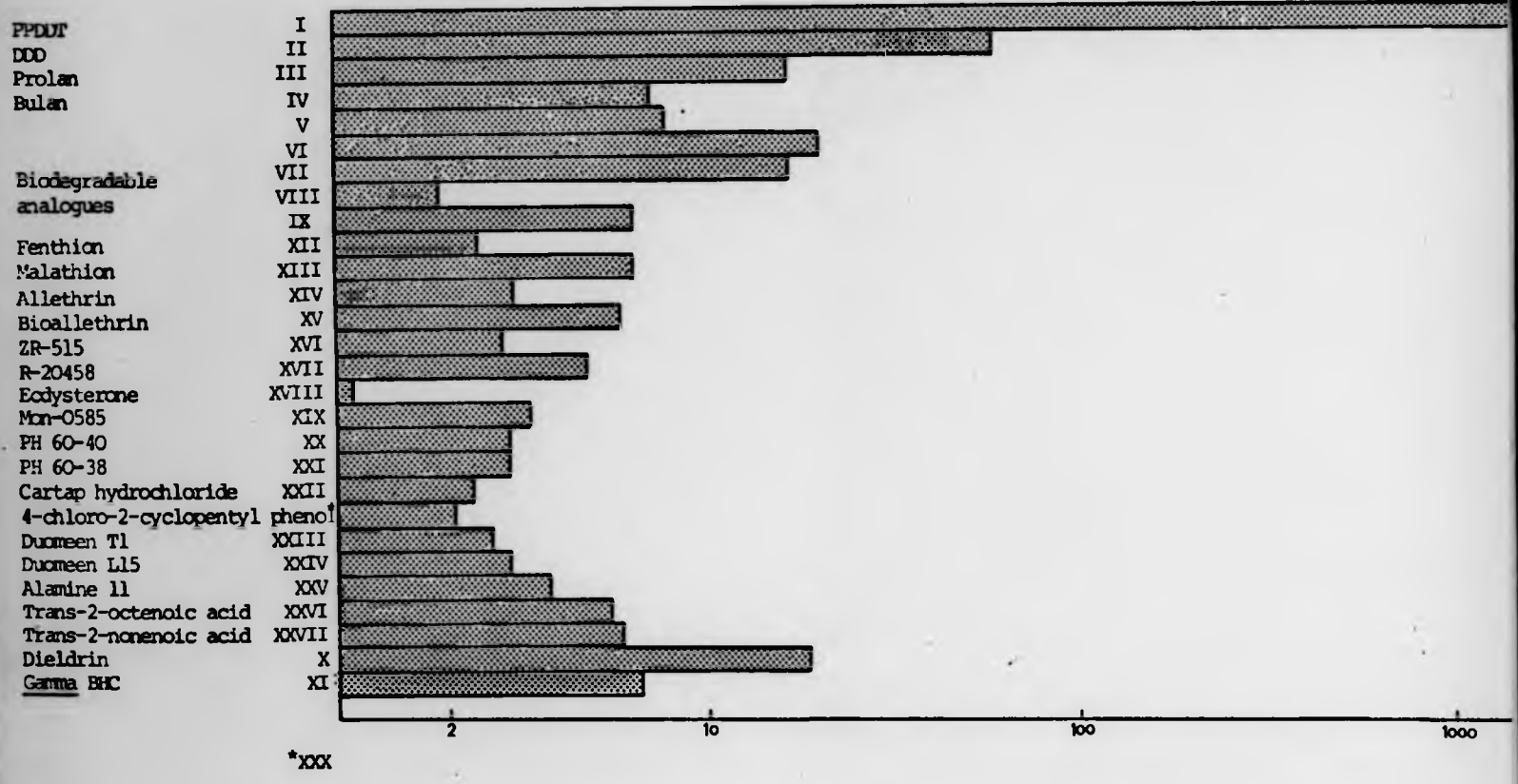


Figure 13. Resistance pattern of DDT-resistant strain of Aedes aegypti

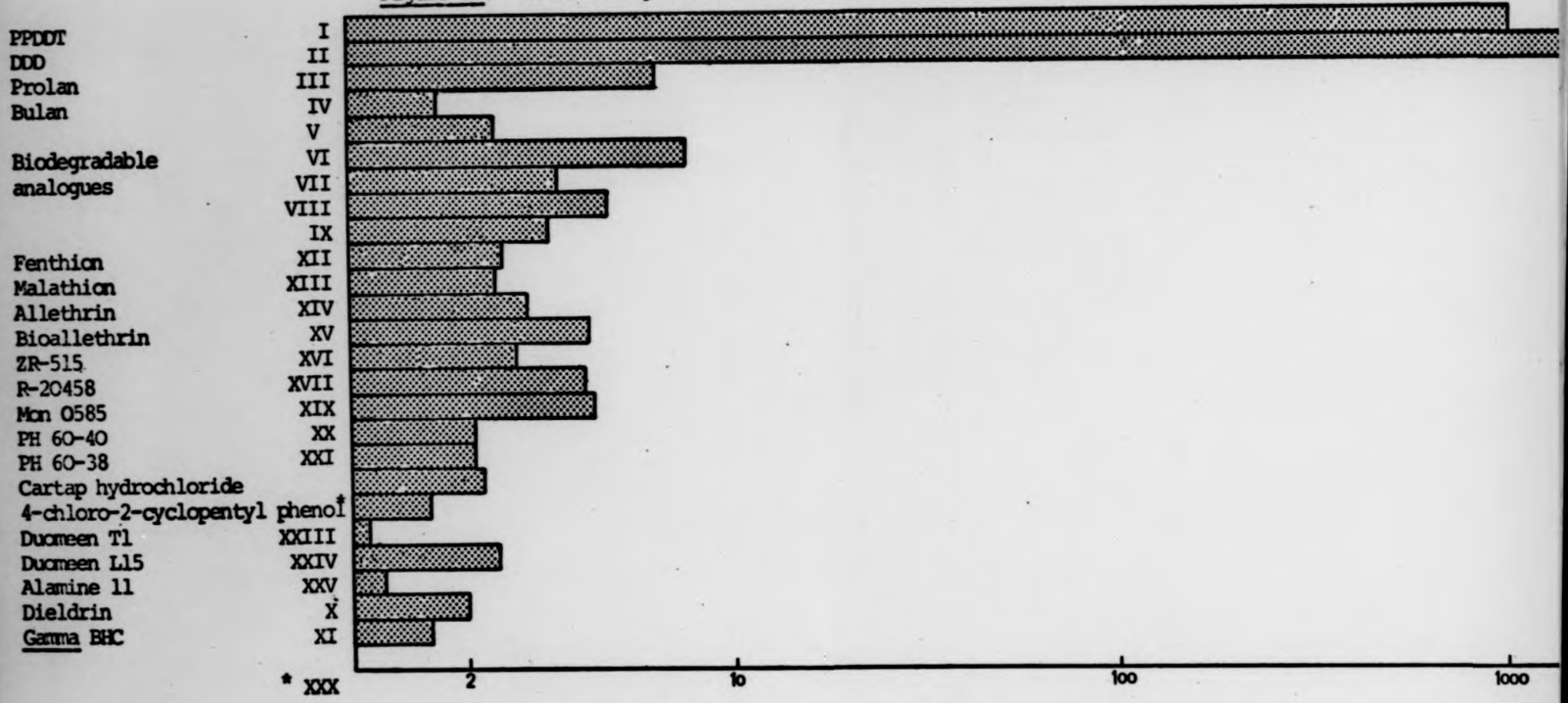


Table 9. Cross-resistance between DDT-resistant strains: Lagos R, Rangoon, Tananarive and susceptible Lagos S strains of Culex pipiens fatigans.

Type	Insecticides	LC50 ppm				Resistance ratio		
	Sample	Lagos S	Lagos R	Rangoon	Tananarive	Lagos R	Rangoon	Tananarive
pp DDT	I	0.005	5.5	11.00	1.50	1100	2200	300
pp DDD	II	0.014	0.53	-	-	37.9	-	-
Prolan	III	0.0048	0.007	0.031	0.015	1.6	5.8	3.1
Bulan	IV	0.033	0.061	0.035	0.054	1.8	1.1	1.6
	V	0.037	0.044	0.039	0.01	1.2	1.1	0.3
Biodegradable	VI	0.018	0.03	0.019	0.035	1.7	1.6	1.9
analogues	VII	0.021	0.033	0.023	0.036	1.6	1.1	1.7
	VIII	0.064	0.11	0.070	0.052	1.7	1.1	0.8
	IX	0.027	0.051	0.033	0.042	1.9	1.2	1.6
Dieldrin	X	0.48	0.90	9.40	0.08	1.9	19.5	0.7
Gamma BHC	XI	0.42	0.68	1.20	0.035	1.6	2.9	0.1
Fenthion	XII	0.0025	0.004	0.003	0.0066	1.6	1.2	2.6
Malathion	XIII	0.08	0.09	0.10	0.09	1.1	1.3	1.1
Allethrin	XIV	0.06	0.09	0.14	0.10	1.5	2.3	1.7
Bioallethrin	XV	0.0105	0.018	0.021	0.027	1.2	1.5	1.9
ZR-515	XVI	0.0014	0.004	-	-	2.9	-	-
R-20458	XVII	0.027	0.1	-	-	3.7	-	-
Ecdysterone	XVIII	128.0	140.0	-	-	1.1	-	-
HON-0585	XIX	0.0045	0.01	-	-	2.2	-	-
PH 60-40	XX	0.0013	0.0042	-	-	3.2	-	-
PH 60-38	XXI	0.005	0.01	-	-	2.0	-	-
Cartap hydrochloride	XXII	0.62	0.98	0.80	-	1.6	1.3	-
Duomeen T1	XXIII	1.2	1.6	1.8	-	1.3	1.5	-
Duomeen L15	XXIV	0.38	0.46	0.96	-	1.2	2.5	-
Alamine 11	XXV	1.5	1.9	2.5	-	1.3	1.7	-
Trans-2-octanoic acid	XXVI	14.0	29.0	-	-	1.7	-	-
Trans-2-nonenoic acid	XXVII	7.5	9.5	-	-	1.9	-	-
4-chloro-2 cyclopentyl phenol	XXX	6.90	7.60	12.6	-	1.1	1.8	-

Table 10. Cross-resistance between DDT-resistant and susceptible strains of Anopheles quadrimaculatus

Insecticides		LC50 ppm		Resistance ratio
Type	Sample	QDTA	QUA	
pp DDT	I	3.60	0.004	900.0
pp DDD	II	30.0	0.12	250.0
Prolan	III	0.019	0.005	3.80
Bulan	IV	0.068	0.03	2.3
Biodegradable analogues	V	0.022	0.025	0.9
	VI	0.037	0.076	0.5
	VII	0.11	0.133	0.8
Dieldrin	X	10.0	0.005	2000.0
Gamma BHC	XI	0.18	0.006	30.0
Fenthion	XII	0.0029	0.0017	1.7
Malathion	XIII	0.10	0.075	1.3
Allethrin	XIV	0.043	0.033	1.3
Bioallethrin	XV	0.029	0.030	1.0
ZR-515	XVI	0.003	0.0015	2.0
R-20458	XVII	0.027	0.007	3.9
Mon-0585	XIX	0.016	0.0042	3.8
PH 60-40	XX	0.0028	0.0011	2.5
PH 60-38	XXI	0.004	0.0025	1.6
Duomeen T1	XXIII	1.40	1.20	1.2
Duomeen L15	XXIV	0.58	0.39	1.5
Alamine 11	XXV	1.70	1.1	1.6
Trans-2-octenoic acid	XXVI	2.3	0.8	2.9
Trans-2-nonenoic acid	XXVII	0.27	0.19	1.4

Table 11. Cross-resistance between DDT-resistant and susceptible strains of Anopheles stephensi.

Insecticides		LC50 ppm		Resistance ratio
Type	Sample	STMAM 2A	STSSD01	
ppDDT	I	4.60	0.08	57.5
Prolan	III	0.12	0.08	1.5
Bulan	IV	0.28	0.14	2.0
Biodegradable	V	0.28	0.21	1.3
analogues	VI	0.39	0.18	2.2
	VII	1.10	0.50	2.2
Dieldrin	X	4.70	0.32	14.7
gamma BHC	XI	0.15	0.08	1.9
Fenthion	XII	0.014	0.0033	4.2
Malathion	XIII	0.032	0.006	5.3
Allethrin	XIV	0.52	0.45	1.2
Bioallethrin	XV	0.27	0.11	2.5
cartap hydrochloride	XXII	2.70	2.50	1.1

Table 12. Cross-resistance between DDT-resistant and susceptible strain of *Aedes aegypti*.

Insecticides		LC50		Resistance ratio
Type	Sample	T8	N	
ppDDT	I	17.5	0.0175	1000
ppDDD	II	300.0	0.12	2500
Prolan	III	0.25	0.04	6.3
Bulan	IV	0.20	0.12	1.7
	V	0.12	0.034	2.4
Biodegradable	VI	0.15	0.02	7.5
analogues	VII	0.11	0.034	3.5
	VIII	0.25	0.054	4.7
	IX	0.16	0.048	3.3
Dieldrin	X	0.012	0.006	2.0
Gamma BHC	XI	0.018	0.011	1.6
Fenthion	XII	0.0033	0.0013	2.5
Malathion	XIII	0.32	0.135	2.4
Allethrin	XIV	0.29	0.10	2.9
Bioallethrin	XV	0.063	0.015	4.2
ZR-515	XVI	0.008	0.003	2.7
R-20458	XVII	0.061	0.015	4.1
Mon-0585	XIX	0.02	0.0046	4.3
PH-60-40	XX	0.006	0.0029	2.1
PH-60-38	XXI	0.009	0.0044	2.1
Cartap hydrochloride	XXII	1.10	0.5	2.2
Duomeen T_1	XXIII	1.0	0.90	1.1
Duomeen L_{15}	XXIV	0.8	0.34	2.4
Alamine 11	XXV	1.35	1.1	1.2
4-Chloro-2-cyclopentyl phenol	XXX	12.8	8.2	1.6

Table 13. Cross-resistance between DDT-resistant and susceptible strain of *Anopheles gambiae*.

Insecticides		LC50		Resistance ratio
Type	Sample	UV19R5	EBAD	
ppDDT	I	6.0	0.004	1500
ppDDD	II	0.29	0.005	58.0
Prolan	III	0.11	0.007	16.2
Bulan	IV	0.50	0.07	7.1
	V	0.54	0.07	7.7
	VI	1.5	0.075	20.0
Biodegradable analogues	VII	0.99	0.054	16.5
	VIII	0.35	0.19	1.9
	IX	0.34	0.054	6.3
Dieldrin	X	2.8	0.15	18.7
Gamma BHC	XI	1.3	0.2	6.5
Fenthion	XII	0.017	0.0072	2.4
Malathion	XIII	0.38	0.06	6.3
Allethrin	XIV	0.54	0.18	3.0
Bioallethrin	XV	0.32	0.055	5.8
ZR-515	XVI	0.0044	0.0016	2.8
R-20458	XVII	0.07	0.015	4.7
Ecdysterone	XVIII	150.0	140.0	1.1
Mon-0585	XIX	0.02	0.006	3.3
PH 60-40	XX	0.01	0.0034	2.9
PH 60-38	XXI	0.013	0.0046	2.9
Cartap hydrochloride	XXII	1.40	0.60	2.33
Duomeen T	XXIII	0.23	0.09	2.6
Duomeen LT5	XXIV	0.19	0.065	2.9
Alamine 11	XXV	0.56	0.15	3.7
Trans-2-octanoic acid	XXVI	10.2	1.9	5.4
Trans-2-nonenoic acid	XXVII	6.0	1.1	5.8
4-chloro-2 cyclopentyl phenol	XXX	10.4	4.5	2.08

from one generation to the next, 40 generations of selection with Prolan on Lagos R resulted in only small increases of the LC_{50} value. Selection for 15 generations of Rangoon strain with this compound revealed a similar response. The results are shown in Tables 14 and 15, and Figure 14. Furthermore, the cross between Lagos R and Rangoon indicated no increase in resistance level (Table 16). In conclusion, the results obtained indicate that both of the strains increased their resistance level to Prolan by about 4-fold, as a result of selection at LC_{90} level. It would seem that only minor factors affecting resistance are available in the colonies used and that no major gene, involving an important mechanism, is present.

To a variety of other compounds tested (pyrethroids, organophosphorus, hormone-like compounds, aliphatic amines, etc.) very low levels of resistance were noted (about ×2). It is difficult to account for these low levels of tolerance, which might be described as "vigour tolerance" if that phrase has any meaning.

The resistance measurements of C.p. fatigans strains to dieldrin and YBHC were complicated by the fact that the susceptible strain used had evidently been contaminated with this type of resistance. The LC_{50} values for dieldrin and YBHC were 0.004 and 0.008 respectively (WHO, 1970). Accordingly, theoretical resistance levels were calculated on the basis of the WHO data (and are shown, dotted, in Figure 7-9). These calculations indicate very high resistance in the India strain, followed by the Lagos and Tananarive colonies, thus:

Resistance in colonies	India	Lagos R	Tananarive
To dieldrin	× 2300	× 220	× 20
To YBHC	× 1500	× 85	× 4

Table 14. Larval mortality in Lagos R substrain of Culex pipiens fatigans during 40 generations of Prolan selection

Generation	Number of larvae tested	Selection conc. ppm	Mortality %	LC50 ppm	Generation	Number of larvae tested	Selection conc. ppm	Mortality %	LC50 ppm
P	2050	0.0025	69.75	0.002	F_{21}	1000	0.025	50.6	0.025
F_1	2250	"	67.65	0.002	F_{22}	2250	0.05	90.0	0.022
F_2	2675	"	63.90	0.002	F_{23}	3000	"	95.0	0.018
F_3	3350	"	74.00	0.0012	F_{24}	1750	0.025	46.25	0.021
F_4	3600	"	67.6	0.0023	F_{25}	2150	"	50.5	0.025
F_5	3575	"	69.4	0.0025	F_{26}	2000	"	59.6	0.02
F_6	3225	"	49.5	0.0026	F_{27}	1750	"	46.5	0.03
F_7	2675	0.005	87.4	0.002	F_{28}	2500	0.05	87.5	0.03
F_8	2650	"	53.1	0.003	F_{29}	2250	"	80.0	0.031
F_9	4900	"	47.6	0.006	F_{30}	2150	"	68.5	0.038
F_{10}	2200	0.0125	56.6	0.012	F_{31}	2550	"	62.0	0.04
F_{11}	2000	0.025	86.0	0.012	F_{32}	1950	0.1	89.7	0.035
F_{12}	1900	"	78.0	0.021	F_{33}	1700	"	71.0	0.038
F_{13}	2475	"	67.0	0.0135	F_{34}	1650	"	89.4	0.036
F_{14}	2775	"	62.5	0.015	F_{35}	1250	"	88.6	0.035
F_{15}	1525	"	62.7	0.012	F_{36}	1500	"	91.4	0.035
F_{16}	1250	"	78.4	0.018	F_{37}	2100	0.05	2.4	0.054
F_{17}	1500	"	78.3	0.018	F_{38}	2000	"	40.6	0.05
F_{18}	1950	"	51.6	0.024	F_{39}	2025	"	45.2	0.051
F_{19}	1350	"	62.5	0.021	F_{40}	1750	"	48.5	0.055
F_{20}	1500	"	58.4	0.022	Average LC50 = 0.022 ± 0.0156				

Table 15. Larval mortality in Rangoon substrain of Culex pipiens fatigans during 15 generations of Prolan selection

Generation	Number of larvae tested	Selection conc. ppm	Mortality %	LC50 ppm
P	2000	0.025	54.6	0.025
F_1	1550	"	44.0	0.026
F_2	1200	"	37.0	0.026
F_3	2500	0.05	98.4	0.031
F_4	2700	"	92.0	0.034
F_5	2775	"	91.5	0.034
F_6	2750	"	85.9	0.035
F_7	1800	"	83.5	0.035
F_8	2150	"	94.2	0.029
F_9	2000	"	98.0	0.025
F_{10}	1600	0.025	65.8	0.024
F_{11}	1500	"	70.5	0.030
F_{12}	1800	0.05	76.4	0.032
F_{13}	2000	"	72.6	0.033
F_{14}	1675	"	68.4	0.036
F_{15}	2125	"	65.9	0.037
Average LC50 = 0.0307 ± 0.0044				

Figure 14. LC 50 values in Lagos R and Rangoon strains of C.p.fatigans during laboratory selection by Prolan.

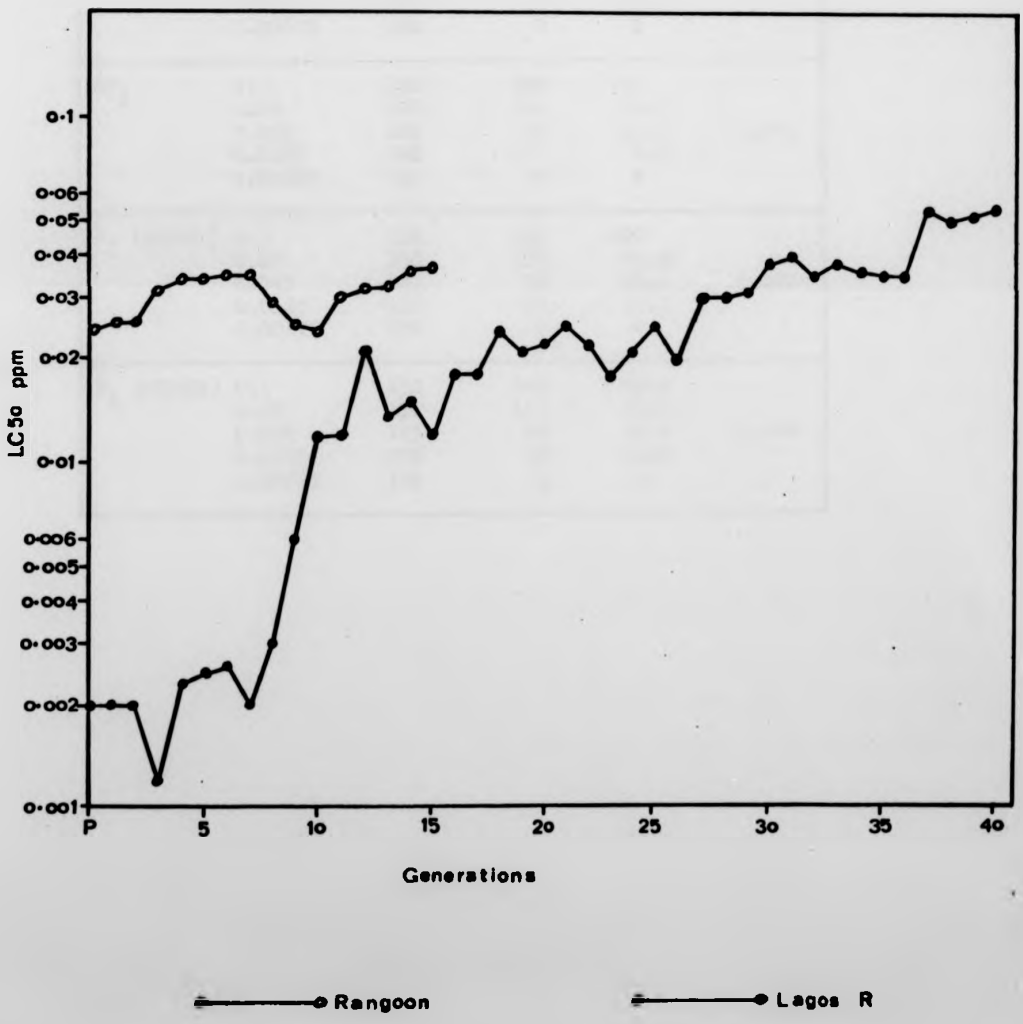


Table 16. Resistance levels in crosses between strains used to select for Prolan resistance

Strain	Concentration ppm	Number of larvae treated	Number of larvae treated	Mortality %	LC50 ppm
LF_{25}	0.1	200	200	100	
	0.05	200	175	87.5	
	0.025	200	119	59.5	0.026
	0.0125	200	32	16.0	
	0.00625	100	0	0	
RF_9	0.1	100	100	100	
	0.05	200	191	95.5	
	0.025	200	95	47.5	0.024
	0.0125	200	17	8.5	
	0.00625	100	0	0	
F_1 (δL×♀R)	0.1	150	150	100	
	0.05	150	137	91.4	
	0.025	149	95	63.8	0.024
	0.0125	150	19	12.7	
	0.00625	150	0	0	
F_1 (♀L×δR)	0.1	150	149	99.4	
	0.05	150	121	85.2	
	0.025	125	66	52.8	0.028
	0.0125	150	18	12.0	
	0.00625	150	0	0	

Anopheles quadrimaculatus

The resistance spectrum obtained with the QDIA strain shows some similarities with those of the C.p. fatigans strains (Figure 10). DDT-resistance is high (× 900) with ×250 resistance to DDD; but there is no cross-resistance to the three biodegradable analogues tested and only low level tolerance (×4, ×2) to Prolan and Bulan. Again it must be concluded that resistance is almost exclusively due to dehydrochlorination.

The same rather low tolerance levels are shown to the pyrethroids, organophosphorus, hormone type compounds and aliphatic amines.

Towards dieldrin, very high resistance (×2000) is present and a moderate level to YBHC (×30).

Anopheles stephensi

Relatively few compounds were used in the determination of the cross-resistance pattern of the STEAN2A strain (Figure 11). The results produced a picture very similar to those of C.p. fatigans and A. quadrimaculatus resistance; so that similar conclusions apply.

Anopheles gambiae

A full spectrum was obtained for the resistant strain of this species and is shown in Figure 12. DDT-resistance is high (×1500), with moderate resistance to DDD (about ×60). In this case, however, there is rather more cross-resistance to the biodegradable analogues and to Prolan and Bulan (×6 to ×16). There are similar levels noted in the miscellaneous group of compounds (pyrethroids, organophorus, hormone type compounds, aliphatic amines).

Tests on the resistant colony with dieldrin and YBHC, gave LC50 values of 2.8 and 1.3 p.p.m. respectively, indicating rather high resis-

tance. Unfortunately, the susceptible colony used appeared to be contaminated, since the LC50 values for these compounds (at 0.15 and 0.2 ppm) were well above expectations, based on other species. No relevant data for known susceptible colonies of *An. gambiae* could be found.

The quite definite resistance observed to a wide range of compounds suggest the existence of a generalised resistance mechanism. From information in literature reviews, one would suspect the mixed-function oxidase system.

Aedes aegypti.

The resistance spectrum for the T8 strain of *A. aegypti* is shown in Figure 13. DDT-resistance is very high (about ×1000); and this time DDD-resistance is very high, perhaps even higher at about ×2500. As regards other DDT analogues, the picture is similar to that for *An. gambiae*, though the cross-resistance to these compounds is slightly lower. Nevertheless, there seems evidence of a mechanism other than dehydrochlorination.

It may be noted that resistance to dieldrin and γBHC is almost absent in this strain.

(b) Resistance affecting different compounds

DDT and its analogues

Resistance to DDT is rather high in all strains and in three species (*An. quadrimaculatus*, *An. stephensi* and *C.p. fatigans*) it does not convey appreciable cross-resistance to Prolan, Bulan or the biodegradable DDT analogues. This suggests that a major component of the mechanism consists in dehydrochlorination.

Resistance to DDD is moderate or high; and it is of interest to note the higher level in A. aegypti than in C.p. fatigans, which is consistent with the biochemical observations of Kimura et al. (1965). These workers found that the dehydrochlorinase of A. aegypti was more effective on DDD than on DDT; whereas that of C.p. fatigans was only one tenth as active on DDD as on DDT.

The other DDT analogues comprise:-

(i) biodegradable analogues which contain no chlorine and (ii) Prolan and Bulan which retain the p-chlorophenyl moities of DDT.

According to the theories of Holan (1971) and Metcalf et al. (1971) the existence of the p-chlorine in Prolan and Bulan would be expected to inhibit microsomal oxidation as compared to the biodegradable compounds. It does not appear, however, that resistance to the latter can develop much more easily than to Prolan and Bulan; the levels are of the same order.

So far as Prolan and Bulan are concerned, it seems that resistance to Prolan usually reaches higher levels than to Bulan, as noted by Perry (1959) for houseflies.

The variability of the results with the non-dehydrochlorinatable DDT-analogues makes it difficult to visualise any simple mechanism responsible; e.g. an oxidative enzyme system. There are almost certainly various degradation pathways for this group of compounds, as indicated in Figure 6 .

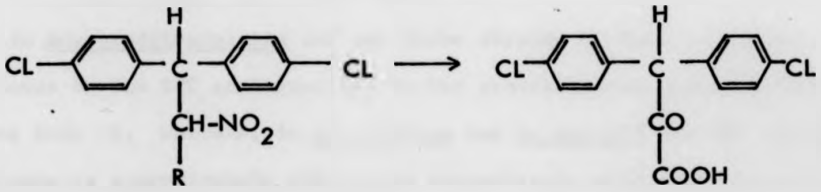
Miscellaneous Compounds

Careful examination of the figures for the various strains reveals that modest levels of resistance to the biodegradable DDT-analogues

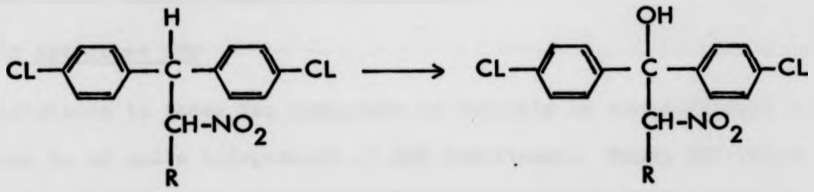
FIGURE 6. Some possible oxidative degradation pathways for DDT analogues not liable to dehydrochlorination,

A,B relevant to Prolan and Bulan.
C,D relevant to biodegradable analogues.

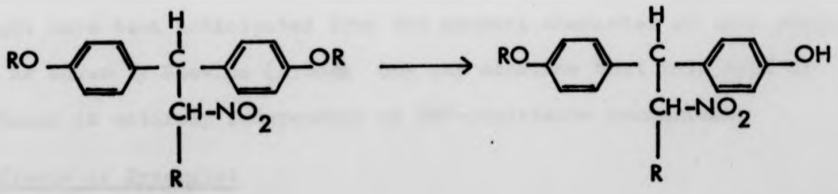
A. Metabolism to 3,3 - bis (p - chlorophenyl) pyruvic acid, as tentatively suggested by Perry (1959).



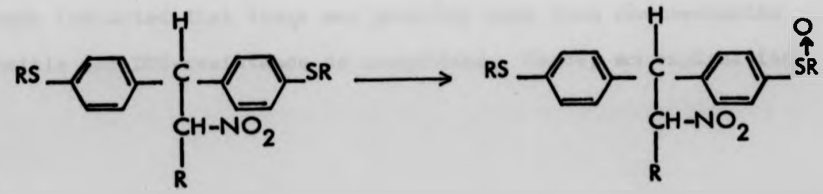
B. Metabolism to an analogue of dicofol.



C. D. Pathways among these suggested by Metcalf et al (1971).



D.



and to Prolan and Bulan, seems to confer cross-resistance to the following miscellaneous group:

(i) pyrethroids

(ii) organophosphorus compounds

(iii) hormone-like compounds

(iv) aliphatic amines.

Thus, in An. quadrimaculatus and the three strains of C.p. fatigans, resistance to the DDT analogues and to the miscellaneous compounds above is less than ×2. Whereas, in An. gambiae and A. aegypti the DDT analogue resistance is approximately ×10 and ×4 respectively with about ×4 resistance to the miscellaneous group. These facts suggest the possibility of a low-level, common resistance mechanism.

Dieldrin and gamma BHC

Resistance to these two compounds is variable in the different strains and seems to be quite independent of DDT resistance. Thus, DDT resistance is very high in both A. aegypti and An. quadrimaculatus; but in the former, dieldrin resistance is low and in the latter it is very high. In all cases, the dieldrin-resistance was higher than BHC-resistance, as might have been anticipated from the general character of this resistance as shown by Busvine (1968a). One may conclude that this type of resistance is entirely independent of DDT-resistance mechanisms.

C. Effects of Synergist

Results obtained from resistance spectra or different groups of compounds indicated that there was possibly more than one mechanism responsible for DDT-resistance in mosquitoes. Hence, an explanation

was sought for the type of mechanism involved by the effects of synergists believed to inhibit specific DDT detoxifying enzyme systems. In this regard, insecticide-synergist combinations were tested, using two of the well known synergists, DMC and piperonyl butoxide, with the different insecticides. The former would be expected to inhibit DDT-dehydrochlorinase; the latter should inhibit mixed function microsomal oxidation enzymes.

Synergists have not generally been used in aqueous insecticide tests and there was some doubt whether they would be effective in this medium. In order to give them every chance of acting, high constant concentrations were used in all tests: 2 ppm of DMC or 5 ppm piperonyl butoxide. These concentrations did not injure the mosquito larvae.

(i) Presentation of results

The interaction was measured by "synergistic ratio" which was given by measuring the value of LC50 of insecticide alone/LC50 of mixture. If this value is greater than one, synergism has occurred, if this value is less than one, antagonism has occurred. The results of the effectiveness of the compounds alone and in combination with synergists are given in full in Tables 17 to 22. The Overall findings are summarised in Table 23.

(ii) Results

(a) Effects of DMC

It will be noted that addition of DMC to suspensions of DDT or its analogues has an antagonistic effect on the potency of all compounds to the susceptible strains (except for An. gambiae, with DDT-analogues). This may be due to some physical effect, possibly reducing pick up of

Table 17. Effects of synergists on the toxicity of various insecticides to DDT-resistant and susceptible larvae of Anopheles arabiae.

Insecticides		LC50 ppm									
		UV19R5 (Resistant)					LBAD (Susceptible)				
Type	Sample	Alone	+PB	SR	+DMC	SR	Alone	+PB	SR	+DMC	SR
ppDDT	I	6.00	4.00	1.5	4.00	1.5	0.004	0.006	0.7	0.005	0.8
ppDDD	II	0.29	0.04	7.3	-	-	0.005	0.004	1.3	-	-
Prolan	III	0.11	0.014	7.9	0.10	1.0	0.007	0.003	2.3	0.006	1.2
Bulan	IV	0.50	0.08	6.3	0.55	0.9	0.07	0.015	4.7	0.08	0.9
	V	0.54	0.027	20.0	0.17	3.2	0.07	0.003	23.3	0.028	2.6
Biodegradable	VI	1.5	0.016	93.8	0.43	3.5	0.075	0.004	18.8	0.022	3.4
analogues	VII	0.99	0.01	99.0	0.50	2.0	0.054	0.002	27.0	0.025	2.4
	VIII	0.35	0.10	3.5	0.37	1.0	0.19	0.018	10.6	0.04	4.8
	IX	0.34	0.06	5.7	0.21	1.7	0.054	0.015	3.6	0.037	1.5
Fenthion	XII	0.017	0.036	0.4	-	-	0.007	0.015	0.5	-	-
Allethrin	XIV	0.54	0.03	18.0	-	-	0.18	0.01	18.0	-	-
Bioallethrin	XV	0.32	0.013	24.6	-	-	0.055	0.005	11.0	-	-
Duomeen T	XXIII	0.23	0.08	2.9	-	-	0.09	0.06	1.5	-	-
Duomeen LI5	XXIV	0.19	0.064	3.0	-	-	0.065	0.042	1.6	-	-
Alamine 11	XXV	0.36	0.15	3.7	-	-	0.15	0.12	1.3	-	-

PB = Piperonyl butoxide
SR = synergistic ratio

Table 18. Effects of synergists on the toxicity of various insecticides to DDT-resistant and susceptible larvae of Aedes aegypti.

Insecticides		LC50 ppm									
		T8 (Resistant)					N (Susceptible)				
Type	Sample	Alone	+PB	SR	+DMC	SR	Alone	+PB	SR	+DMC	SR
ppDDT	I	17.50	8.00	2.2	8.50	2.06	0.018	0.022	0.8	0.027	0.7
ppDDD	II	300.	250.	1.2	-	-	0.12	0.150	0.8	-	-
Prolan	III	0.25	0.15	1.7	0.41	0.61	0.04	0.024	1.7	0.034	1.8
Bulan	IV	0.20	0.11	1.8	0.74	0.27	0.12	0.041	2.9	0.10	1.2
	V	0.12	0.05	2.4	0.22	0.56	0.034	0.011	3.1	0.05	0.8
Biodegradable	VI	0.15	0.03	5.0	0.11	0.49	0.02	0.015	1.3	0.038	0.5
analogues	VII	0.11	0.013	8.5	0.27	0.41	0.024	0.004	6.7	0.04	0.6
	VIII	0.25	0.06	4.2	0.21	1.19	0.054	0.025	2.2	0.04	1.4
	IX	0.16	0.09	1.8	0.37	0.43	0.048	0.013	3.5	0.11	0.4
Allethrin	XIV	0.29	0.054	5.4	-	-	0.10	0.08	1.3	-	-
Bioallethrin	XV	0.06	0.022	2.9	-	-	0.015	0.018	0.8	-	-
Duomeen T	XXIII	1.00	0.30	3.3	-	-	0.90	0.30	1.7	-	-
Duomeen LT5	XXIV	0.80	0.16	5.0	-	-	0.34	0.19	1.8	-	-
Alamine 11	XXV	1.35	0.48	2.8	-	-	1.10	0.70	1.5	-	-

PB = Piperonyl butoxide
SR = Synergistic ratio

Table 19. Effects of synergists on the toxicity of various insecticides to DDT-resistant and susceptible larvae of Culex pipiens fatigans (Lagos)

Insecticides		LC50 ppm									
Type	Sample	LAGOS R (Resistant)					LAGOS S (Susceptible)				
		Alone	+PB	SR	+DMC	SR	Alone	+PB	SR	+DMC	SR
ppDDT	I	5.50	8.00	0.7	0.50	10.8	0.005	0.007	0.7	0.018	0.3
ppDDD	II	0.53	0.47	1.2	-	-	0.014	0.036	0.4	-	-
Prolan	III	0.0075	0.007	1.1	0.01	0.8	0.0048	0.0025	1.9	0.021	0.2
Bulan	IV	0.061	0.043	1.3	0.094	0.7	0.033	0.016	2.1	0.049	0.7
	V	0.044	0.017	2.6	0.056	0.8	0.037	0.036	1.0	0.14	0.3
Biodegradable	VI	0.03	0.0056	5.4	0.026	1.2	0.018	0.0074	2.5	0.026	0.3
analogues	VII	0.033	0.0064	5.2	0.027	1.2	0.021	0.004	5.3	0.021	1.0
	VIII	0.11	0.03	3.7	0.037	2.9	0.064	0.032	2.0	0.048	1.3
	IX	0.051	0.014	3.6	0.038	1.3	0.027	0.011	2.5	0.042	0.6
Fenthion	XII	0.004	0.006	0.7	-	-	0.0025	0.005	0.5	-	-
Allethrin	XIV	0.09	0.019	4.7	-	-	0.06	0.007	8.6	-	-
Bioallethrin	XV	0.018	0.0033	5.5	-	-	0.015	0.002	7.5	-	-
Duomeen T_1	XXIII	1.60	0.75	2.1	-	-	1.20	0.72	1.7	-	-
Duomeen LT5	XXIV	0.46	0.22	2.1	-	-	0.38	0.20	1.9	-	-
Alamine 11	XXV	1.90	1.20	1.6	-	-	1.50	0.90	1.7	-	-

PB = Piperonyl butoxide
SR = Synergistic ratio

Table 20. Effects of synergists on the toxicity of various insecticides to DDT-resistant and susceptible larvae of Culex pipiens fatigans (Rangoon).

Insecticides		LC50 ppm									
		Rangoon (Resistant)					Lagos S (susceptible)				
Type	Sample	Alone	+PB	SR	+DMC	SR	Alone	+PB	ST	+DMC	SR
ppDDT	I	11.00	11.00	1.0	1.00	1.1	0.005	0.007	0.7	0.018	0.3
Prolan	III	0.031	0.012	2.6	0.023	1.3	0.0048	0.0025	1.9	0.021	0.2
Bulan	IV	0.035	0.017	2.1	0.05	0.7	0.033	0.016	2.1	0.49	0.7
Biodegradable	V	0.039	0.018	2.2	0.047	0.9	0.037	0.036	1.0	0.14	0.3
analogues	VI	0.019	0.006	3.2	0.017	1.1	0.018	0.0074	2.5	0.026	0.3
	VII	0.023	0.007	3.3	0.018	1.9	0.021	0.004	5.3	0.021	1.0
Fenthion	XII	0.003	0.0025	1.2	-	-	0.0025	0.0049	0.5	-	-
Bioallethrin	XV	0.021	0.0018	11.7	-	-	0.015	0.002	7.5	-	-

PB = Piperonyl butoxide
SR = Synergistic ratio

Table 21. Effects of synergists on the toxicity of various insecticides to DDT-resistant and susceptible larvae of Culex pipiens fatigans (Tananarive).

Insecticides		LC50 ppm									
Type	Sample	Tananarive (Resistant)					Lagos S (Susceptible)				
		Alone	+PB	SR	+DMC	SR	Alone	+PB	SR	+DMC	SR
ppDDT	I	1.50	1.40	1.1	0.072	20.8	0.005	0.007	0.7	0.018	0.3
Prolan	II	0.015	0.033	0.5	0.056	0.3	0.0048	0.0025	1.9	0.021	0.2
Bulan	III	0.054	0.048	1.2	0.100	0.6	0.033	0.016	2.1	0.049	0.7
Biodegradable	V	0.010	0.005	2.0	0.066	0.2	0.037	0.036	1.0	0.140	0.3
analogues	VI	0.035	0.006	5.8	0.040	0.9	0.018	0.0074	2.5	0.026	0.3
	VII	0.036	0.006	6.0	0.037	1.0	0.021	0.004	5.3	0.021	1.0

PB = Piperonyl butoxide
SR = Synergistic ratio

Table 22. Effects of synergists on the toxicity of various insecticides to DDT-resistant and susceptible larvae of Anopheles quadrimaculatus.

Insecticides		LC50 ppm									
		QDTA (Resistant)					QUA (Susceptible)				
Type	Sample	Alone	+PB	SR	+DMC	SR	Alone	+PB	SR	+DMC	SR
ppDDT	I	3.6	3.8	1.0	1.1	3.3	0.004	0.007	0.6	0.005	0.8
ppDDD	II	30.0	27.0	1.1	-	-	0.12	0.05	2.4	-	-
Prolan	III	0.019	0.011	1.7	0.02	0.9	0.005	0.0042	1.2	0.006	0.8
Bulan	IV	0.068	0.04	1.7	0.07	1.0	0.03	0.025	1.2	0.031	1.0
Biodegradable	V	0.022	0.012	1.8	0.015	1.5	0.025	0.26	1.0	0.04	0.5
analogues	VI	0.037	0.025	1.5	0.035	1.1	0.076	0.07	1.1	0.08	1.0
	VII	0.11	0.06	1.8	0.05	2.2	0.133	0.10	1.3	0.14	1.0
Malathion	XIII	0.10	0.08	1.3	-	-	0.075	0.07	1.1	-	-
Allethrin	XIV	0.043	0.01	4.3	-	-	0.033	0.007	4.9	-	-
Bioallethrin	XV	0.029	0.006	4.8	-	-	0.033	0.006	5.0	-	-
Duomeen T	XXIII	1.4	0.68	2.1	-	-	1.20	0.81	1.5	-	-
Duomeen L_1	XXIV	0.58	0.25	2.3	-	-	0.39	0.20	2.0	-	-
Alamine 11^5	XXV	1.7	1.1	1.6	-	-	1.1	0.65	1.7	-	-

Table 25. Influence of DMC and piperonyl butoxide (PB) on resistant and susceptible strains of 4 species of mosquito larvae to various groups of compounds

Insecticide	Species	Resistance factor with			
		DMC		PB	
		Resistant	Susceptible	Resistant	Susceptible
pp DDT	An. quadrimaculatus	3.27	0.80	0.95	0.57
	C.p. fatigans	14.00*	0.27	0.88*	0.71
	An. gambiae	1.45	0.80	1.45	0.69
	A. aegypti	2.06	0.65	2.19	0.80
Prolan and Bulan	An. quadrimaculatus	0.95	0.90	1.69	1.20
	C.p. fatigans	0.70*	0.46	1.40*	2.00
	An. gambiae	0.96	1.02	7.05	3.49
	A. aegypti	0.44	1.20	1.80	2.30
Biodegradable analogues	An. quadrimaculatus	1.62	1.12	1.70	0.61
	C.p. fatigans	1.07*	0.70	3.84*	2.63
	An. gambiae	2.30	3.00	44.0	17.0
	A. aegypti	0.62	0.78	4.36	3.7
Pyrethroid	An. quadrimaculatus	-	-	4.60	4.90
	C.p. fatigans	-	-	7.28*	8.16
	An. gambiae	-	-	21.30	14.5
	A. aegypti	-	-	4.14	1.04
Aliphatic amines	An. quadrimaculatus	-	-	2.0	1.7
	C.p. fatigans	-	-	1.9*	1.8
	An. gambiae	-	-	3.2	1.5
	A. aegypti	-	-	3.7	1.7

*Average resistance factor of 3 strains of C.p. fatigans

insecticide. This antagonistic effect must mitigate against the synergistic action to be expected when a DDT-dehydrochlorinase mechanism exists.

The overall results are, to some extent, consistent with expectations from the cross-resistance studies. Thus, DMC synergism is highest, for DDT, in resistant Culex p. fatigans and Anopheles quadrimaculatus resistant strains which, from the cross-resistance data, would be expected to rely largely on dehydrochlorination. No very distinct difference in DMC-synergism of DDT-analogues was noted as between the resistant and normal strains. This is reasonable, since the analogues tested were not amenable to degradation by this route.

(b) Effects of piperonyl butoxide

As with DMC, there was a distinct antagonistic effect of this synergist on DDT toxicity to susceptible strains, possibly for a similar reason. Its action was also antagonistic to DDT on resistant strains of A. quadrimaculatus and C.p. fatigans as might be expected, since these strains probably rely on dehydrochlorination only. The slight positive synergism with resistant strains of A. gambiae and A. aegypti may indicate some oxidative degradation of DDT in these colonies.

On the non-dehydrochlorinatable DDT-analogues and on the pyrethroids, piperonyl butoxide usually had a distinct synergistic effect. This was evident in both susceptible and resistant strains; but the effect was generally greater in the latter. The highest levels of synergism were noted in A. gambiae resistant colony. These observations are consistent with the broad resistance spectrum noted in this strain.

In comparing effects of piperonyl butoxide on the different com-

pounds, it will be observed that the biodegradable analogues were most highly synergised, followed by the pyrethroids, Prolan and Bulan and (least affected) DDT.

Among the biodegradable analogues, compounds VI, VII and V were most easily synergised (Table 24). The lower synergistic ratios of compounds VIII and IX is consistent with the suggestion of Metcalf et al. (1971) that DDT-analogues with a methylene dioxyphenyl grouping would be "self-synergising" and therefore less amenable to further potentiation by piperonyl butoxide.

The "self-synergising" principle does not appear to have been very extensively investigated; but it could depend on a blocking of detoxifying enzymes by part of a dose, allowing unhampered toxic action by the remainder. This dual action, however, may well be obtained at the expense of deviation from the optimum DDT shape. The results shown in Table 17 are consistent with these suggestions, in that the "self-synergising" compounds VIII and IX are more effective (than VI and VII) against resistant larvae when used alone, but distinctly less effective than the others when in the presence of piperonyl butoxide. Rather similar results are shown for the resistant strain of C.p. fatigans.

D. Radiometric.

Investigation of Pick-up of Insecticide.

Reduced penetration of insecticides through insect cuticles has been reported on numerous occasions as a possible cause of resistance.

Table 24. Effects of piperonyl butoxide on resistant strains of 4 species of mosquito larvae to the biodegradable analogue compounds

Species	Resistance factor with piperonyl butoxide				
	Biodegradable analogues				
	V	VI	VII	VIII	IX
An. quadrimaculatus	1.8	1.5	1.8	-	-
C. p. fatigans	2.4*	4.8*	4.8*	3.7	3.6
An. gambiae	20.0	93.8	99.0	3.5	5.7
A. aegypti	2.4	5.0	8.5	4.2	1.8

*Average resistance factor of 3 strains of C. p. fatigans

An attempt has been made to investigate whether this occurs with the resistant larvae being tested. The results obtained from the bioassay method, using highly susceptible first instar to assess the amount of DDT which was picked up by the fourth instar of the same species indicated that there was a slight difference of the pick up amount of DDT between DDT-resistant and susceptible strains. This slight, but consistent difference indicated a greater pick-up by resistant larvae, which could not explain resistance. It seemed, however, with a more precise experimental investigation, using radioactive tracer technique.

(i) Establishment of technique

^{14}C-labelled samples of DDT and malathion were available for these experiments. The quantities were very small and it was not feasible to weigh out portions. Accordingly, stock solutions were prepared by dissolving the whole samples in acetone and making dilutions as follows. Stock solution = S, with dilutions 0.1S, 0.033S, 0.01S, 0.003S, and so forth. The actual concentrations of insecticide in these standard dilutions were estimated by bioassay, using 1 ml aliquots of each to prepare suspension in water and adding 4th instar Aedes aegypti larvae, as in the usual larvicide test. The 24-hour mortalities were compared to those obtained with standard normal insecticide solutions. This provided estimates of the strengths of the standard radioactive solutions.

These preliminary bioassay tests also gave information on the con-

centration levels likely to be convenient for estimating pick-up. With DDT, the 0.0033S standard, giving an aqueous suspension equivalent to 0.013 ppm DDT, seemed adequately radioactive. It gave 41% kill of susceptible A. aegypti larvae (and only 2% of resistant ones) after 24 hrs. During the shorter exposures in the pick-up tests the percentages of paralysed susceptible larvae were 4% after 8 hrs and 29% after 16 hrs.

With malathion, the standards chosen and the expected 24 hr kills were as follows:-

0.01S (0.045 ppm) less than 1% kill
0.03S (0.150 ") " " 60% "
0.10S (0.45 ") " " 99% "

The higher concentrations were used to determine whether the initiation of toxic action would reduce pick-up or penetration of insecticide.

The next step was to determine the radioactivity, as measured in the scintillation counter, of the quantities of insecticides used in the tests. First, the counts per minute were determined for 1 ml quantities of standard solutions put directly into the counting vials. The solvent was removed by evaporation and replaced by scintillation fluid.

From these assays it was found that 1 µg DDT (estimated by bioassay) gave 41,000 cpm and 1 µg malathion, 14,000 c.p.m. The higher count with DDT was referable to the greater activity of the sample: 15mC per mMol, as compared to 4.6mC per mMol with malathion. Calculating from the respective molecular weights, these correspond to 960,000 and 1 million c.p.m. per microcurie respectively, a remarkably good agreement.

Following these tests, the efficiency of extraction of insecticide from aqueous solution was determined by comparing the counts from radioactive acetone solution put directly into the counting vessel, with an extract from an aqueous suspension prepared from the same quantity of solution added to water. It was found that DDT extraction was 82% efficient while the malathion extractions ranged from 95 to 98% efficiency.

These extractions were made immediately after preparation of the suspensions. Extractions made at different time intervals afterwards showed gradual losses, presumably due to loss of insecticide from the suspension. The rate of loss was of the same order for both insecticides. After 8 hours, 0.0135 ppm DDT had lost 13.5%, while the malathion losses at this time were: 18% at 0.045 ppm; 26% at 0.15 ppm; and 15.5% at 0.5 ppm.

(ii) Results of Pick-up and Penetration Tests

Table 25 shows the results of the investigation, with the quantities of insecticide determined by radiometric counts converted to µg, or ppm.

Pick-up and depletion of suspension. It will be noted that the larvae steadily picked up insecticide from the suspension, which was accordingly depleted below the concentrations found in suspensions without larvae. When the total pick up quantities are added to the residue in the suspensions, the amounts range from 95 to 99% of those for suspensions without larvae.

(a) Relation between pick-up per larva and concentration. At 16 hours, the total pick-up of DDT per larva averaged 0.0175 µg.

Table 25. Total, external and internal amounts of C^{14}DDT and C^{14}malathion in larvae of susceptible (N) and resistant (T8) strains of Aedes aegypti at different exposure periods after treatments.

Insecticide	Estimated conc. ppm	Hour	Strain	Pick up (µg/20 larvae) External	Internal	Total	% of internal pick up	Residue in water	Total pick up & water residue	Residue in water without larvae
C^{14}DDT	0.0135	0		-	-	-	-	-	-	1.11
	0.0127	4	N	0.021	0.088	0.109	80.73	0.881	0.990	1.04
			T8	0.021	0.101	0.122	82.78	0.868	0.989	
	0.0117	8	N	0.027	0.117	0.144	81.23	0.800	0.944	0.96
			T8	0.027	0.165	0.192	85.93	0.759	0.951	
	0.0104	16	N	0.029	0.269	0.298	90.40	0.546	0.844	0.85
			T8	0.031	0.383	0.414	92.51	0.434	0.848	
C^{14} malathion	0.045	0	-	-	-	-	-	-	-	4.43
	0.0421	4	N	0.0052	0.0148	0.0200	76.59	3.903	3.923	4.048
			T8	0.0056	0.0173	0.0229	75.33	3.751	3.774	
	0.0371	8	N	0.0068	0.0274	0.0342	81.91	3.597	3.631	3.613
			T8	0.0075	0.0298	0.0373	79.92	3.593	3.630	
	0.150	0	-	-	-	-	-	-	-	13.8
	0.111	8	N	0.0099	0.0426	0.0525	81.25	10.588	10.641	10.671
			T8	0.0091	0.0382	0.0473	80.69	10.607	10.654	
	0.45	0	-	-	-	-	-	-	-	43.5
	0.300	8	N	0.0253	0.131	0.1563	83.73	35.951	36.107	36.134
			T8	0.0291	0.124	0.1531	80.54	35.956	36.109	

Since the estimated initial concentration was 0.0135 ppm, this agrees fairly well with the relationship pointed out by Busvine (1968). That is, if larvae are exposed to x ppm for 24 hours, they will pick up y μg/larva; and, for DDT, x = y.

(b) Penetration. The percentage penetration was estimated by comparing the amount extracted from larvae by maceration (after washing off the external insecticide) and comparing this with the total pick-up. The following points were noted.

With DDT, internal insecticide was 80 to 82% at 4 hours, increasing to 90 to 92% at 16 hours. With malathion, internal insecticide was 75 to 76% at 4 hours, rising to 79-81% at 8 hours.

Penetration of malathion at 8 hrs did not differ much over a considerable range of concentrations (0.045 to 0.45 ppm).

In the DDT tests, both percentage and actual penetration was less in the susceptible strain than in the resistant strain. This disposes of the possibility of a resistance due to decreased pick-up. The reason for the lower pick-up in susceptible larvae could possibly be due to incipient intoxication. Penetration of topically applied DDT in houseflies has been found to decline with the intoxication of the flies (Sternburg et al. 1950).

With the malathion tests, the percentage penetration in the susceptible strain was always higher than in the resistant one; though in some cases, the actual amount was lower.

The percentage penetration of malathion did not show a consistent change with increasing concentration. Thus, at the highest level, where some intoxication might be expected, there was no evidence of reduced penetration. However, the physical properties of DDT and malathion

as well as their toxic effects, are rather different; so that the two situations cannot well be compared.

3. POSSIBLE ALTERNATIVE LARVICIDAL COMPOUNDS

A. Relative Potency

(i) DDT and analogues

DDT is a highly potent larvicide with LC50 values (by the standard WHO test) of around .005 ppm for many mosquitoes. Prolan and Bulan are less effective against normal strains with LC50 values of 0.05 to 0.04 and 0.03 to 0.12 ppm respectively. These compounds have been known for a long time; and Metcalf (1955b) summarised early work as follows. Prolan and Bulan "were stated to be 5 times as toxic as DDT to the bean thrips and okra aphid, and were 2.2 and 0.8 as toxic respectively to Calandra granaria and 0.3 to 0.2 times as toxic to Musca domestica." Despite the potency to some insects equal to (or even greater than) DDT, neither compound nor the mixture of them known as Dilan, has challenged the use of DDT to any great extent. It is, however, possible that their immunity to dehydrochlorination resistance may alter this situation, as will be considered in the next section.

The biodegradable DDT-analogues show a general level of potency rather similar to that of Prolan and Bulan, with LC50 values in the range 0.02 to 0.2 ppm. Comparisons of their potencies relative to DDT showed distinct differences with the species. For A. aegypti, C.p. fatigans and A. stephensi, they were about a half to a sixth as active; but for A. gambiae and A. quadrimaculatus their potencies were nearer to a twentieth that of DDT. Holan (1971) working with houseflies, found the potencies of this group to be about a half to one and a half times as potent as DDT.

These compounds have been introduced comparatively recently and little is known of their practical potentialities. Since they are likely to be considerably more expensive than DDT, rather less potent and with less residual action, they need solid advantages (in immunity to high resistance and reduced pollution hazard) to challenge the established insecticide.

(ii) Other conventional insecticides

The two pyrethroids were moderately potent. Allethrin had LC50 values of 0.06 to 0.4 ppm and bioallethrin was about four times more potent (LC50 .015 to .11). Though new synthetic pyrethroids have shown great promise for several uses, they do not appear to be very practical as larvicides, due to cost.

The LC50 values for dieldrin and gamma BHC were low with A. aegypti and An. quadrimaculatus (0.005 to 0.01 ppm). The measurements with the other so-called susceptible strains were suspiciously high as already mentioned. Fenthion was the more potent of the two organophosphorus compounds, with LC50 values of 0.002 to 0.013 ppm; malathion levels were considerably higher, at 0.06 to 0.14 ppm.

Both the alternative organochlorines (dieldrin and gamma BHC) and various organophosphorus compounds have been utilized as larvicides and both are liable to resistance. In addition, the organochlorine compounds are suspect from the environmental contamination aspect.

(iii) Hormone mimics and moulting disturbance compounds

The hormone-type compounds were defined as compounds having biological activity which mimic that of natural insect juvenile hormones.

These compounds exhibit morphogenetic effects against many stages in the life cycle of insects. In recent years, several of these compounds have been evaluated against aquatic stages of mosquitoes and found to be quite effective in inhibit growth and emergence. (Jakob, 1973; Mulder & Gejswijt, 1973; Schaefer & Wilder, 1972).

In addition to these compounds apparently acting as hormone mimics, others have been introduced which act at the time of moulting and metamorphosis, though not resembling hormones.(e.g. Mon 585; Sacher, 1971a 1971b; Mulla , 1974. Also Duphar PH60:40 and PH60:38; Wellinga et al., 1973).

As part of the search for new, safe methods to control both DDT-resistant and susceptible mosquitoes, 5 of the outstanding compounds were investigated in this study. Of the more obvious hormone mimics, Altosid or ZR-515 (XVI) was most potent (see Table 32) with LC50 values in the range 0.0014 to 0.003 ppm; it was about 10 times more active than R-20458 (XVII). Ecdysterone was of very low activity, as expected, probably because of lack of penetrating power.

The other compounds are chemically unrelated to the insect hormones, but have action at times of moulting and metamorphosis. They were all fairly potent, especially PH60-40 (XX), with LC50 values ranging from 0.0011 to 0.0034 ppm, which can be considered promising in comparison with conventional larvicides. PH60-38 (XXI) and Mon-0585 were shown to be almost equally effective.

(iv) Miscellaneous compounds

Cartap hydrochloride was only tested on four species (not An. quadrimaculatus). It was not very promising, with LC50 values

about 0.5 to 2.5 ppm. This is, perhaps, not surprising as the most useful field for this novel compound appears to be as a stomach poison for lepidopterous pests (Saki et al., 1967).

The range of aliphatic amines was tested against a wider range of strains (largely because of the interest of their involvement in resistance, as discussed below). The LC50 values were all rather high, in the range of 0.07 to 1.5 ppm, which agrees with expectations from results published by Mulla et al. (1970).

Recent work on fatty acids as insecticides has been reported by Quraishi & Thorsteinson (1965) and by Quraishi (1971). The interest of that work, however, centres on the unusual teratogenic mode of action of the compounds, rather than their high potency. The two unsaturated fatty acids tested in the present investigation were found to have very low potency with LC50 values in the range of 1-15 ppm for trans-2-octanoic acid of 1-8 ppm for trans-2-nonenoic acid. The latter compound was more effective in all cases. In their immediate effects the compounds appeared to be less dramatic. The quantities used were large between tens of ppm to a few hundred ppm in some cases. In the long run they may prove more beneficial for the regulation of insect populations.

Miller & Maddock (1970) called attention to the ovicidal effects of certain phenols and anti-oxidising agents on mosquito eggs and it was thought worth determining their possible larvicidal action. Of the 4 samples of phenols and anti-oxidising agents, the compound XXIX (4-chloro-2-cyclopentyl) phenol was most effective; but even so, the LC50 values were about 5 to 7 ppm.

B. Involvement in DDT-resistance

It has already been pointed out that neither Prolan and Bulan nor

the biodegradable DDT-analogues were so greatly vitiated by resistance as DDT and DDD. It is therrfore tempting tc suppose that this observation is due to the much greater efficiency of the dehydrochlorination mechanism. Indeed, certain strains (as the DDT-resistant An. quadriculatus) show highly specific resistance to analogues which can be dehydrochlorinated and to no other larvicides, and the fact that these show high resistance levels supports this view.

The resistant strain of A. aegypti and, even more, that of An. gambiae, show low-level, generalised cross-resistance. This reaches as high as about ×20 for one or two biodegradable DDT analogues; but in most cases amounts to about ×2 to ×5. The enhanced tolerance of hormone mimics by strains resistant to conventional insecticides, has already been pointed out by Cerf & Georghiou (1972) for Musca domestica, and by Dyte (1972) for Tribolium castaneum. In the present results for A. gambiae, a cross-tolerance was observed to aliphatic amines and to fatty acids. Previous work with resistant strains of C.p. fatigans and Anopheles albimanus. did not find cross-resistance to aliphatic amines. It seemed possible that the mechanism involved in this cross resistance was the mixed function microsomal oxidase system (Brooks, 1973). Tests with the addition of piperonyl butoxide gave some support to this theory, by showing high synergistic ratios with the resistant strains.

C. Investigation of mode of action of compounds affecting moulting and metamorphosis.

(i) Hormone-type compounds

The chemicals discussed in this section cause harmful effects to the insects during moulting, especially at the time of metamorphosis.

These investigated compounds included orthodox hormone mimics (such as ecdysterone, Altosid and R-20458). For some of them the similarity of molecular configuration to natural insect juvenile hormone, strongly suggests that the action is as a hormone mimic. In addition, the pest control agents introduced by commercial firms (Hon-0585, PH60-40 and PH60-38) not resembling known insect hormones; but definitely affect insects at the time of metamorphosis. It is not clear whether this is due to mimicing a natural hormone, to blocking hormone degradation or to some other physiological interference at these vital joints. The mode of action may, for the moment, be left unspecified.

Research on hormone mimics by various workers during the past decade, has shown that these may be active at different stages in the life cycle, but that their greatest effects were often during a critical period. For example, juvenile hormone mimic may be most active when applied shortly before metamorphosis, when the natural J.H. hormone titre is falling in preparation for the change to the adult state. This is liable to prevent proper metamorphosis and even cause the appearance of extra juvenile instars. On the other hand, moulting hormone treating at this time is likely to accelerate metamorphosis and produce premature, dwarf adults.

Various functions occuring during moulting and metamorphosis may be affected, such as cuticle tanning and hardening. To discover the actual modes of action is likely to prove a highly difficult piece of biochemical research. At this stage, what has been attempted is merely to distinguish types of toxic action of the various compounds on the basis of visible effects produced and their timing.

The techniques involved in treatment were all simple (as described

earlier) most of the inferences will depend on description of effects, their timing of occurrence and the proportions affected. In order to gain some insight into each type of compound, their effects were investigated on (a) the eggs, (b) 1st and 11nd instar larvae, (c) early IVth instar larvae, (d) late IVth instar larvae, (e) adults.

(a) Tests on eggs

In these experiments, batches of eggs of known age were put into water containing various concentrations of the chemicals for periods of 6, 12, 24 and 48 hours. After treatments, they were removed to clean water and kept until all eggs would be hatched in untreated batches.

The tests were, in most cases, done with very young eggs about 1 to 2 hours old and the results are set out in Table 27. It will be seen that none of the compounds showed much evidence of ovicidal activity except PH60-40 (XX). Further tests with older eggs (12 to 16 hours old) showed that this effect was limited to the very young eggs. With the older eggs, there was 63 to 85% hatch after 48 hours exposure to 10 and 1 ppm of this substance. There was no marked difference between eggs of C. fatigans and An. gambiae.

In order to compare the susceptibility of eggs obtained from resistant and susceptible strains, the test was carried out with young eggs (1-2 hours old). Results of these experiments (Table 28) indicated that there was no difference between the two strains against many compound tested.

Observation under themicroscope showed that some larvae were unable to break the egg shell but tried to rupture at the side of egg and attempted to free themselves about half way out (Plate 1A) Some of these

Table 27. Effectiveness of various compounds against eggs of Culex pipiens fatigans and Anopheles gambiae

Species	Compounds	Conc. (ppm)	Exposure period (hr) 6		12		24		48	
			No. treated	% hatch	No. treated	% hatch	No. treated	% hatch	No. treated	% hatch
C.p. fatigans	ZR-515	10.0	456	94	437	92	353	90	360	82
		1.0	389	97	496	95	313	92	396	90
		0.1	392	98	420	95	331	92	385	92
	R-20458	10.0	351	75	404	50	318	28	365	4
		1.0	346	98	364	94	382	88	405	70
		0.1	316	99	391	98	294	96	458	95
	Ecdysterone	500.0	319	96	303	95	261	95	385	95
		100.0	305	98	231	95	320	94	361	86
	Mon-0585	10.0	504	98	522	93	524	90	465	88
		1.0	515	98	542	98	497	97	502	98
	PH60-40	10.0	516	0	508	0	462	0	495	0
		1.0	504	0	526	0	481	0	514	0
		0.1	539	2	517	0	513	0	522	0
		0.01	501	94	495	93	487	85	492	78
An. gambiae	ZR-515	10.0	164	54	184	45	120	27	106	13
		1.0	105	95	124	90	118	91	104	84
	R-20458	10.0	95	0	110	0	132	0	130	0
		1.0	92	64	132	52	102	37	132	0
		0.1	104	94	117	92	100	89	141	78
	Mon-0585	10.0	111	86	108	83	109	70	117	54
		1.0	122	95	131	94	122	94	109	92
	PH60-40	10.0	105	0	114	0	98	0	107	0
		1.0	119	0	130	0	150	0	89	0
		0.1	88	36	76	10	108	0	110	0
		.01	158	75	133	60	113	56	125	38

Table 28. Effect of various compounds on the hatch of Culex pipiens fatigans eggs from resistant and susceptible strains.

Compounds	Concentration (ppm)	% of larvae hatch from eggs exposed for 48 hours			
		Susceptible		Resistant	
		No. treated	% hatch	No. treated	% hatch
ZR515	10.0	720	82	548	92
	1.0	792	90	666	96
	0.1	770	92	635	95
R-20458	10.0	730	6	418	22
	1.0	405	78	465	89
	0.1	458	95	304	98
Ecdysterone	2000	562	96	473	98
	1000	472	98	461	98
Mon 0585	10.0	539	88	427	98
	1.0	444	96	594	99
	0.1	465	98	438	98
PH 60-40	10.0	695	0	578	0
	1.0	514	0	492	0
	0.1	522	0	575	4
	0.01	1909	74	1730	81
	0.001	1505	95	1152	98
PH 60-38	10.0	498	0	517	4
	1.0	874	51	481	69
	0.1	860	86	464	94

larvae can survive and continue their development if they were helped to come out from the egg shell. It is possible that PH60-40 may have ovicidal activity associated with damage to the egg shell membrane.

(b) Tests with Ist and IInd instar larvae

These experiments were all done with the susceptible strain of C.p. fatigans and An. gambiae. Batches of Ist instar larvae were exposed to various concentrations of the different compounds for 21 hrs. At the end of this period, no larvae were usually dead. They were transferred to clean water and allowed to continue development up to the adult stage (unless mortality survened). Food was added as required. Observations were made of the proportions dying in different instars and in the pupal stage.

It was very clear that the toxic action of all the compounds tested consisted in some type of interference with ecdysis. Larvae dying in the early stages were unable to escape from the old cuticle. Sometimes the head was able to emerge without the rest of the body (Plate 1B); in other cases, most of the body became free except for the terminal portion (Plate 1C). In many cases, gross anatomical distortions were evident: for example, greatly swollen heads, probably due to excessive hydrostatic pressure during the attempt to complete moulting.

The results, considered numerically, are shown in Tables 29-30. It will be seen that, at all concentrations which eventually produced a high kill (>90%), the compounds were most toxic to 1st and 2nd instars. With ecdysterone (XVIII) the effect was mainly on 1st instar and with Mon-0585 (XIC) on early pupae stage; but with the others, the highest mortality occurred in the 2nd instar.

PLATE 1.

A. Effects of PH60-40 on eggs of C.p. fatigans (see pp. 130 and 133).

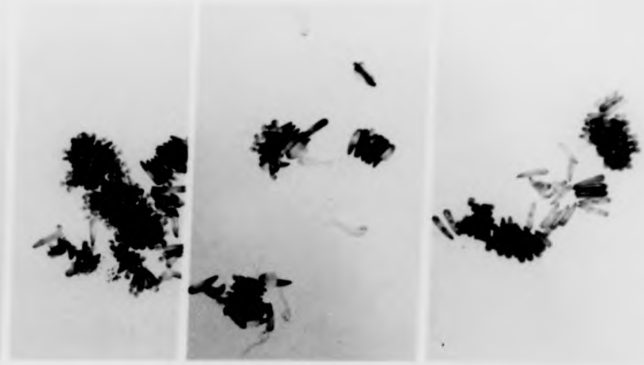
B. & C. Effects of moulting disturbance compounds on I & II instar larvae of C.p. fatigans (see p.133)

A

B

C

Plate 1



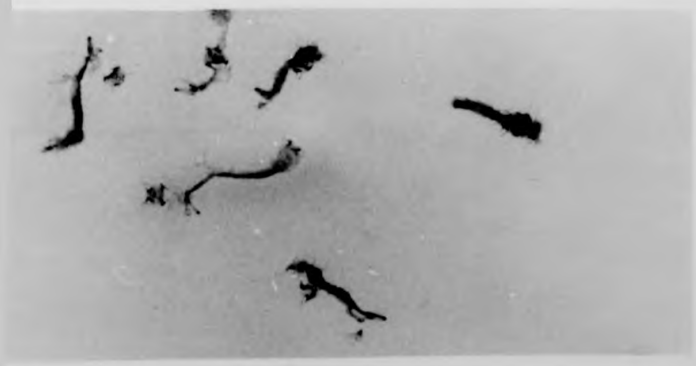


Table 29. Activity of the test compounds against Ist instar larvae of Culex pipiens fatigans (susceptible strain)

Compounds	Sample No.	Conc. ppm.	% kill at various stages I	II	III	IV	P	Total % kill	LC50
ZR-515	XVI	0.1	18	56	4	0	8	86	
		0.02	14	26	4	4	22	70	
		0.004	0	12	2	4	16	34	0.007
		0.0008	0	2	4	0	14	20	
		0.00016	0	0	0	0	10	10	
R-20458	XVII	1.0	28	52	6	2	10	98	
		0.2	20	20	4	0	36	80	
		0.04	10	30	2	0	14	56	0.04
		0.008	6	4	0	0	8	18	
		0.0016	0	6	0	0	4	10	
Ecdysterone	XVIII	200	76	12	6	0	0	94.0	
		100	70	4.5	8	0	0	82.5	
		50	48	8	8	0	4	68.0	30.0
		25	4	8	8	0	20	40.0	
		10	2	4	0	0	10	16.0	
		5	0	3	1.5	0	0	4.5	
Mon 0585	XIX	0.5	30	10	0	0	60	100	
		0.1	12	18	0	2	58	90	
		0.02	8	22	2	0	42	74	0.005
		0.004	4	4	0	6	30	44	
		0.0008	0	0	0	2	20	22	
PH60-40	XX	0.05	16	70	8	0	0	94	
		0.01	12	53	6	8	0	79	
		0.002	8	24	12	4	4	52	0.002
		0.0004	0	2	2	0	18	22	
		0.00008	0	2	0	0	6	8	
PH60-38	XXI	0.25	30	58	10	0	0	98	
		0.05	26	46	6	0	8	86	
		0.01	6	30	8	0	16	60	0.006
		0.002	0	4	4	2	22	32	
		0.0004	0	0	2	0	8	10	

Table 30. Activity of the test compounds against IInd instar larvae of *Culex pipiens fatigans* (susceptible strain).

Compounds	Sample No.	Conc. ppm.	% kill at various stages				Total % kill	LC50
			II	III	IV	P		
ZR-515	XVI	0.5	100	0	0	0	100-	
		0.1	44	32	0	18	94	
		0.02	36	30	0	6	72	0.009
		0.004	12	10	0	4	26	
		0.0008	2	0	0	4	6	
R-20458	XVII	1.0	39	43	4	4	90	
		0.2	25	31	2	12	70	
		0.04	16	10	4	8	38	0.07
		0.008	2	4	0	12	18	
		0.0016	0	0	0	7	7	
Non-0585	XIX	0.5	10	4	2	84	100	
		0.1	12	10	0	66	88	
		0.02	6	4	2	60	72	0.007
		0.004	0	6	0	30	36	
		0.0008	0	0	1	13	14	
PH60-40	XX	0.05	72	16	4	0	92	
		0.01	38	24	4	6	72	0.0025
		0.002	27	12	11	2	52	
		0.0004	0	4	2	8	15	
PH60-38	XXI	0.25	48	44	2	2	96	
		0.05	26	46	4	2	78	
		0.01	6	28	10	10	54	0.01
		0.002	2	6	4	8	20	
		0.0004	0	0	0	8	8	

As the dose was reduced to a level resulting in overall mortality of 50% or less, the deaths in the early instars declined sharply, but a delayed effect occurred during pupation. There was little effect during the 3rd or 4th instars, in any test.

(c) Exposure in the early IVth Instar

Treatments in the early IVth instar were made with four species of mosquito and included normal and DDT-resistant strains. In all cases, the exposure was for 24 hrs, after which the larvae were transferred to clean water and examined periodically until the end of the pupation period.

A considerable variety of toxic effects was observed and recorded in different categories, according to the stage of metamorphosis reached. when death occurred. These will be described, in order to interpret the comparative results obtained.

L (Death as larvae). This category represents death during the larval stage, with no evident initiation of pupation.

L (P) (Larval cuticle with pupa inside). Death in this category has occurred at an early stage of pupation. The pupal abdomen can be seen to be withdrawn from the terminal part of the abdomen and the pupal tracheal system has become disengaged from the larval tracheae, which can be seen between the larval and pupal spiracles. In the thorax, respiratory trumpets are visible (Plates 2A & 2B).

L-P (Larvae with pupae partly emerged). At this stage the larval skin has been ruptured and the pupal body has partly emerged from the thoracic split. The abdomen has retracted to at least half way along the larval abdominal skin and has adopted the characteristic pupal shape. (Plates 2C & 3)

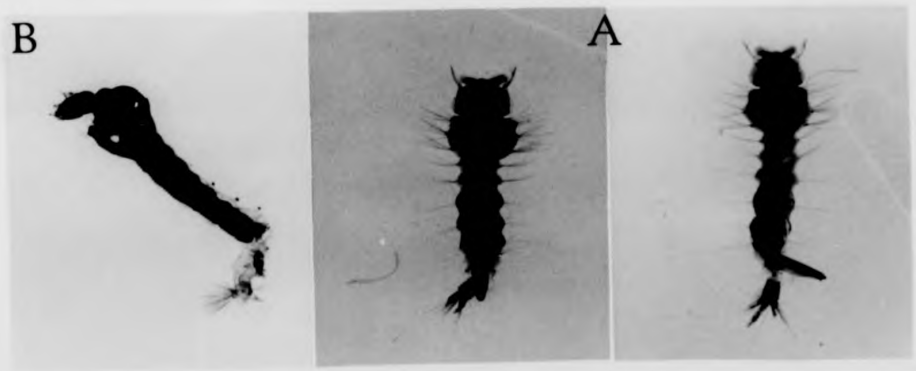
PLATE 2.

A. Treated IV instar larvae of C.p. fatigans showing death in stage L(P) (see p. 138).

B. Range of the same effect on An. quadrimaculatus (see p. 138)

C. Treated larvae of C.p. fatigans showing death in stage L-P (see p. 138).

Plate 2



A. & C.

B.

PLATE 3.

Partially emerged pupae of C.p. fatigans in stage L-P. Note air bubbles in A. (See p. 138).

Range of partial emergence of pupae of An. quadrimaculatus. One of them has completely withdrawn the abdomen but the head remained enclosed (See p. 138).

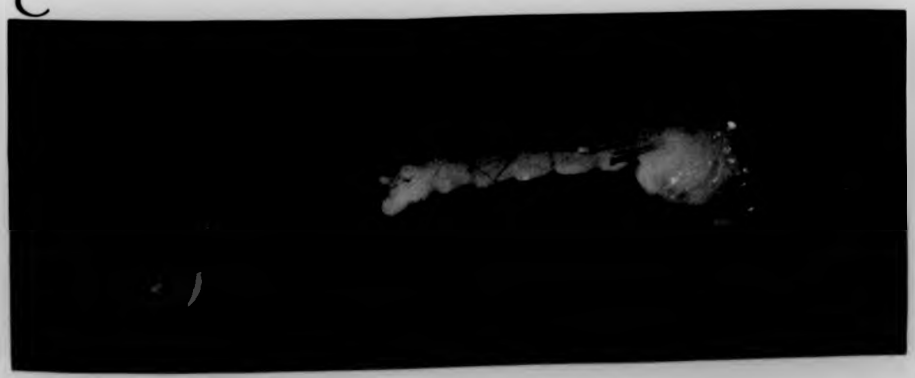
Plate 3

A

B

A

C



WP (White pupae). "White pupae" have completely escaped from the larval cuticle but have remained completely un-melanised, except for eye pigment. The abdomen is held in a stiff, abnormal position; either straight or recurved dorsally (Plates 4A & 4C).

BrP. (Brown pupae). "Brown pupae" show some melanisation and the abdomen is held in the normal, ventrally curved position (Plate 4B).

P(A) (Pupae with adults visible inside). In this stage, most of the adult anatomy can be distinguished and appears to be normally pigmented (e.g. the abdominal tergites can be clearly distinguished). The pupal skin has not split, however, and the abdomen is straight or recurved dorsally. (Plate 5). Unlike the previous categories, the dead insects normally float, presumably because the internal air bubble is preserved.

P-A1 (Pupae with adults beginning emergence). In this category are placed adults which have begun to escape from the pupal skin but have been unable to free themselves very far. Sometimes head and thorax are freed, but the abdomen remains enclosed (Plate 6). Alternatively, the abdomen may be free and the head and thorax stuck fast. Occasionally the whole body is nearly free, except for the legs (Plate 7.).

P-A2 (Pupae with adults almost completely free). This stage represents complete emergence from the pupalskin, except for the tarsi of the hind legs (Plate 8).

A (Feeble adults). This category is reserved for adults which have freed themselves completely from the pupal skin, but cannot escape from the water film.

(d) Late IVth instar larvae

The larvae were exposed for 24 hours and mortality was based on

PLATE 4.

A, C. Unmelanised dead pupae of C. p. fatigans (Stage WP; see p. 143). (A with light background; C with dark background)

B. Enclosed adult, dying with the beginnings of pigmentation (stage Br.P; see p. 143).

Plate 4

A

B

C

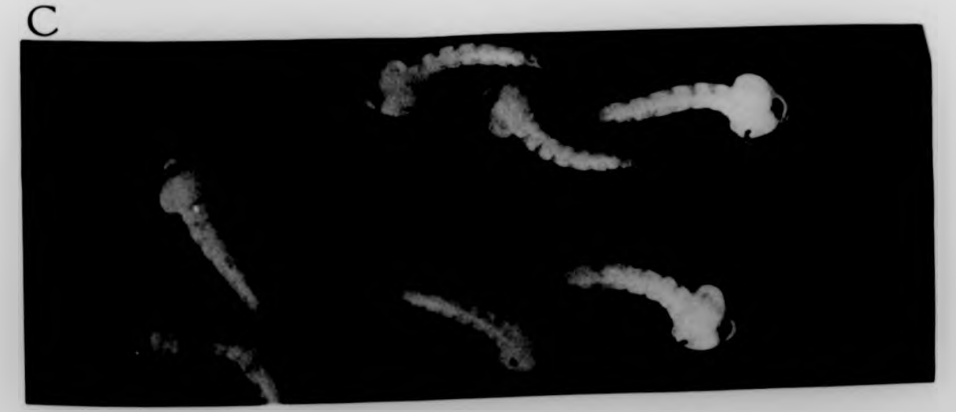


PLATE 5.

Death at P(A) stage of treated larvae of *A. aegypti* showing black adult within pupal exuvium. (See p. 143).

A. Dorsal view

B. lateral view

C. Group


A
B
C

Plate 5

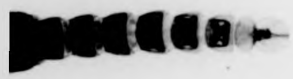


PLATE 6.

A. Failure of emergence of adults of A. aegypti (stage P-Al; see p. 143).

B & C. Similar effect with C. p. fatigans.

Plate 6

A

B

B

C

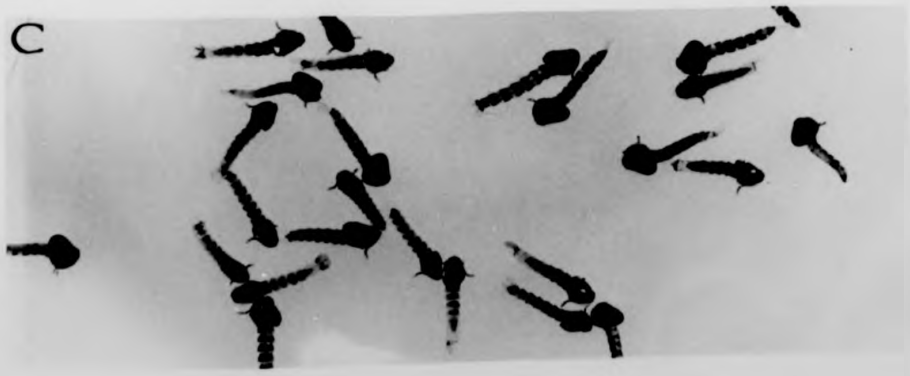


PLATE 7.

Incomplete eclosion of adults (stage P-A1; see p. 143).

A. C.p. fatigans. Eclosion split on pupal abdomen; head and thorax partly free, with body twisted.

B. C.p. fatigans. Eclosion split on pupal thorax; abdomen free, with head and thorax stuck.

C. Half-emerged adult of A. aegypti.

Plate 7

A

B

C

PLATE 8.

Death at stage P-A2 (see p. 143).

A. A. aegypti.

B. & C. C.p. fatigans

Plate 8

A

B

C

the same characteristic effects as described before in treatments with early IVth instar. The results obtained indicated that late IV instar were generally less affected than the early IV instar. The qualitative difference in activity between type of compounds was nearly the same as with treatments of early IV instar larvae; for example, the lower dose trea ments gave a more dispersed action. The percentage mortality of each deleterious effect was high in the late metamorphosis (between pupa and adult) especially in P(A) and P-B2. It is interesting to note that activities of PH60-40, PH60-38 and Mon 0585, which are expressed in the death in early metamorphosis (between larvae and pupae, and newly formed pupae, respectively) were also delayed and appeared in the late metamorphosis. There is a considerable probability (with only scanty evidence yet) that these varied effects of all compounds depended on the age of treatment of larvae.

(e) Summary of effects of larval treatments

To illustrate the qualitative and quantitative differences between the effects of the various compounds used, the results have been shown as a series of histograms. For each of the species used, the data illustrated are those for the normal susceptible strain. (The data for resistant strains were substantially similar, though the dosage levels for particular effects were slightly higher).

The effects of each compound are shown in relation to the time of metamorphosis, at which they occurred, according to the schedule of effects just described. In eachcase, a histogram is provided to illustrate effects of doses which would eventually produce high mortality (> 90%) and another histogram to show the distribution of effects by a dose

causing 50% kill. Nearly always, the dose producing a high kill would cause its toxic effects over a more restricted period and at a characteristic point in metamorphosis; whereas effects from the lower dose level gave more dispersed action. On this account, the qualitative differences in toxic action between the different types of compound are more easily appreciated from the high dosage data.

If, then, the histograms for high kill doses are examined, it will be seen that the groups of compounds compare as follows.

(i) The two orthodox hormone mimics (xvi and xvii) produce their main effects relatively late; usually when the adult form has become visible. This agrees with the observations of Schaefer & Wilder (1972) who state that "most mortality occurred in the pupal stage; with most compounds in the late pupal stage ....". In a later paper (1973) they also refer to "large numbers of newly emerged adults that were unable to leave the water surface" after field treatments with Altosid. This corresponds to the stage "A" mortality in this present account.

Jakob & Schoof (1971) mention a "small proportion of larvae which gave rise to anomalous pupal forms" after treatment with JH mimics. These aberrant forms, which were most commonly found with Anopheles albimanus, "usually remained unmelanised for considerable periods (sometimes as long as 24 hours), usually on the water surface in a horizontal position, rather than in the vertical position of a normal pupa." This effect does not correspond exactly with any of the stages described above; it appears to be a form of unmelanised "P-A".

(ii) Mon 0585 (xix) characteristically causes death in the "White Pupa" stage, a fact which has been pointed out by earlier workers (Sacher, 1971;

Jakob & Schoof, 1972). Schaefer & Wilder (1972) also note that its action occurs earlier than that of other compounds affecting metamorphosis.

Sacher (1971) speculation on the mode of action of Mon 0585, suggested that, since melanisation was inhibited, an effect on tyrosinase might be expected; but he found no evidence of this. On the other hand, the effects of the compound in causing unmelanised pupae was partly reversed by continuous bubbling of oxygen. It therefore seems that intoxication is due to some interference with oxygen utilization.

(iii) The two Duphar compounds (XX and XXI) tend to be most active rather early in metamorphosis, so that many insects die between larval and pupal stages. A few, however, die at a later stage and this is especially noticeable at the lower dose level.

Mulder & Gijswijt (1973) show that compounds of this type interfere with cuticle formation in insect larvae during the process of ecdysis. Post & Vincent (1973) present interesting evidence to suggest that the physiological process involved is chitin synthesis.

(iv) Ecdysterone has the earliest activity in the series of compounds tested and, especially at high doses, kills many insects in the larval stage. On the other hand, a proportion of the larvae which survived tended to die later during metamorphosis.

Experiments with moulting hormone mimics are usually done by injection, to avoid the difficulty of penetrating the insect cuticle. Robbins et al. (1968) did, in fact, demonstrate interference with moulting and metamorphosis when such compounds were added to insect diet. There appear, however, to be no published data of this kind for mosquito larvae.

Table VI. Variety of toxic effects of the compounds against early fourth instar larvae of susceptible strain of Culex pipiens fatigans.

Compounds	Conc. ppm.	% dying at each stage									Total % kill	LC50
		L	L(P)	L-P	WP	BrP	P(..)	$P\text{-}A_1$	$P\text{-}A_2$	A		
ZR-515	1.0	-	-	-	8	84	8	-	-	-	100	
	0.25	-	-	-	12	60	8	4	-	4	88	
	0.05	-	-	4	12	36	16	4	-	4	76	
	0.01	-	-	-	4	26	14	4	12	12	72	0.0014
	0.002	-	-	-	4	28	12	8	4	12	68	
	0.0004	-	-	-	8	20	4	8	12	-	52	
	0.00008	-	-	-	-	4	4	4	8	-	20	
R-20458	1.0	-	-	2.2	6.5	30.3	54.4	2.2	-	-	95.6	
	0.5	-	-	-	2.1	28.0	58.2	2.1	2.1	-	92.5	
	0.25	-	-	-	7.1	13.1	54.8	1.2	2.4	-	78.6	
	0.125	-	-	0.8	6.5	11.5	39.4	0.8	0.8	1.6	71.4	
	0.0625	-	-	0.8	9.8	2.8	33.8	2.1	2.1	1.4	52.8	0.061
	0.0312	-	-	-	6.0	4.0	14.0	2.0	4.0	4.0	34.0	
	0.0156	-	-	-	-	1.0	9.0	3.0	4.0	2.0	19.0	
	0.0078	-	-	-	-	6.0	0.8	1.0	1.0	-	8.8	
Ecdysterone	500	44	12	-	-	12	8	-	-	8	84	
	200	8	24	12	8	9	4	-	-	4	69	
	100	-	20	4	4	8	8	-	-	-	44	
	50	-	8	8	8	4	2	-	-	4	34	128
	25	-	8	-	-	-	8	-	-	-	16	
	10	-	-	-	-	4	-	-	-	2	6	
Mon-0585	2.0	-	-	4	92	4	-	-	-	-	100	
	1.0	-	-	-	92	4	4	-	-	-	100	
	0.25	-	-	-	90	2	-	4	4	-	100	
	0.05	-	-	-	76	4	4	-	-	-	84	
	0.01	-	-	-	36	8	4	4	4	-	56	0.0045
	0.002	-	-	-	4	4	8	8	12	4	40	
	0.0004	-	-	-	-	4	4	8	4	-	20	
	0.00008	-	-	-	-	4	4	-	-	-	8	
PH 60-40	1.0	-	64	24	-	12	-	-	-	-	100	
	0.25	-	76	15	-	8	-	-	-	-	99	
	0.05	-	56	36	-	4	-	-	-	-	96	
	0.01	-	4	40	8	8	32	-	-	-	92	0.0013
	0.002	-	-	-	-	4	28	4	10	4	50	
	0.0004	-	-	-	4	16	4	-	8	-	32	
	0.00008	-	-	-	-	-	-	4	8	-	12	
PH 60-38	0.1	-	78	22	-	-	-	-	-	-	100	
	0.05	-	65	33	-	-	-	-	-	-	98	
	0.025	-	11	58	8	14	-	-	-	-	91	
	0.0125	-	-	51	12	21	-	-	-	-	84	
	0.00625	-	-	22	16	18	3	-	-	-	59	0.005
	0.0031	-	-	2	1	4	11	-	7	1	26	
	0.00156	-	-	-	-	-	8.7	-	6.5	4.3	19.5	
	0.00078	-	-	-	-	-	1	2	4	2	9	

Figure 15. Effects of the compounds in different categories against fourth instar of Culex pipiens fatigans

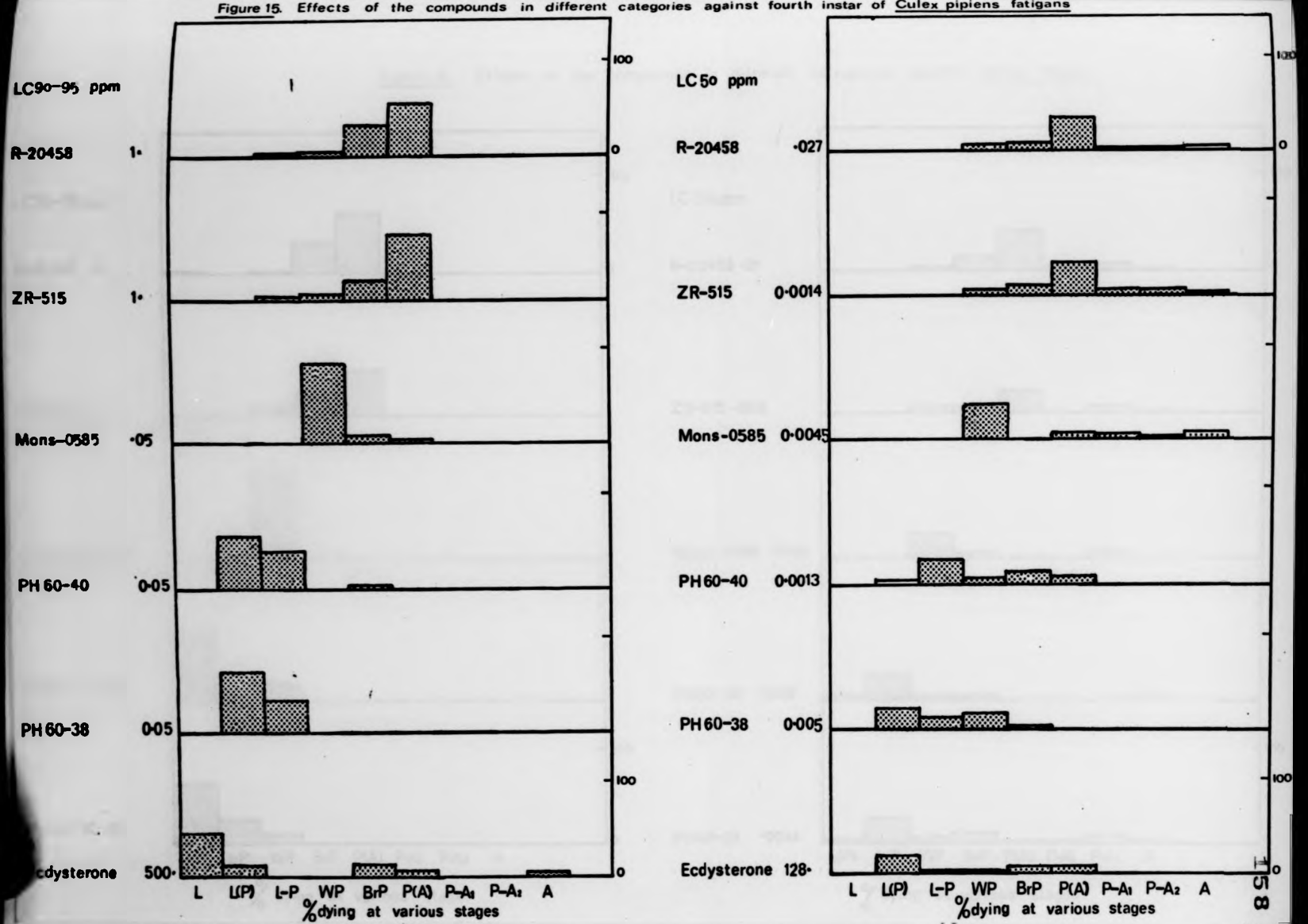


Figure 15. Effects of the compounds in different categories against fourth instar of Culex pipiens fatigans

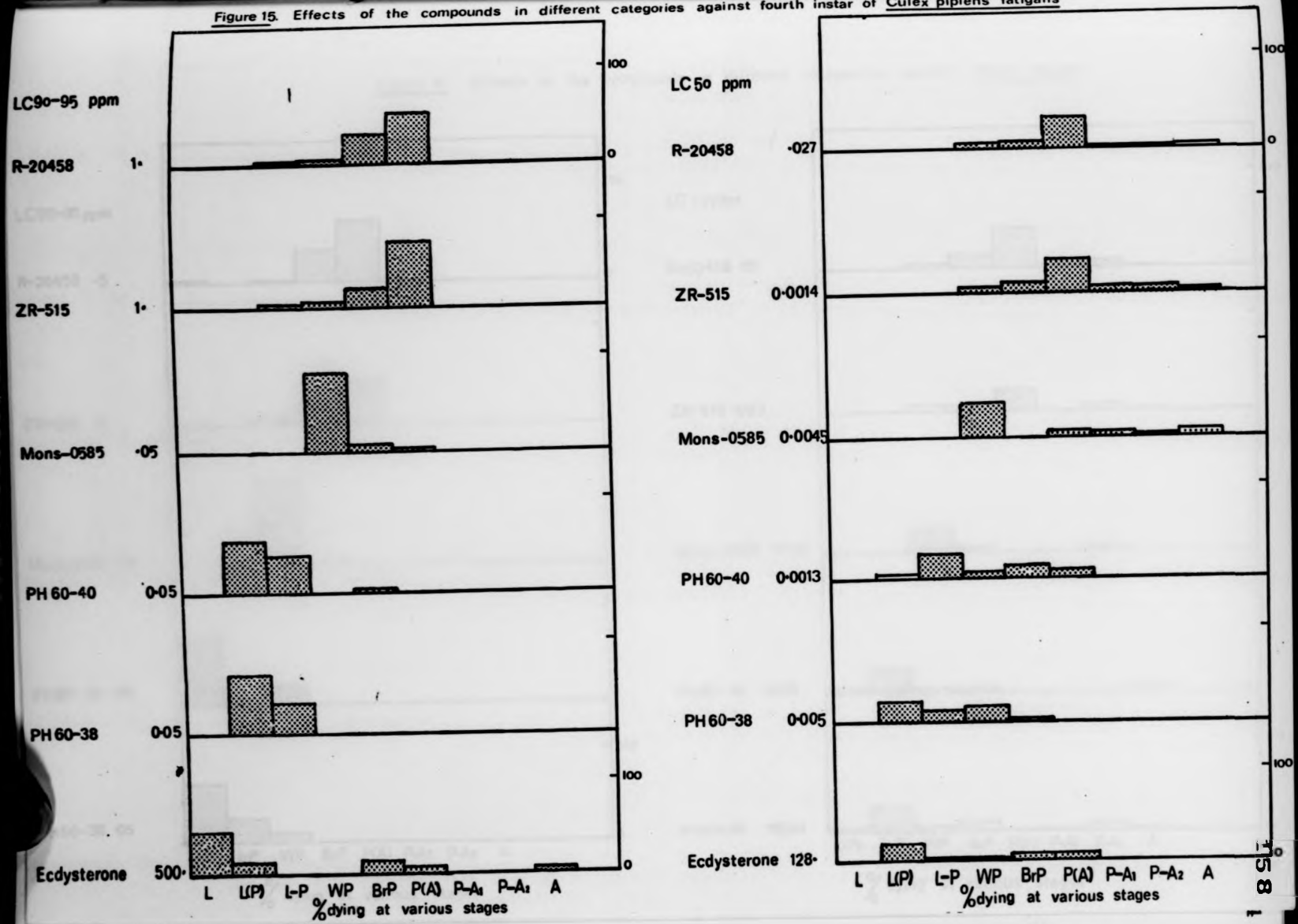


Figure 16. Effects of the compounds in different categories against Aedes aegypti

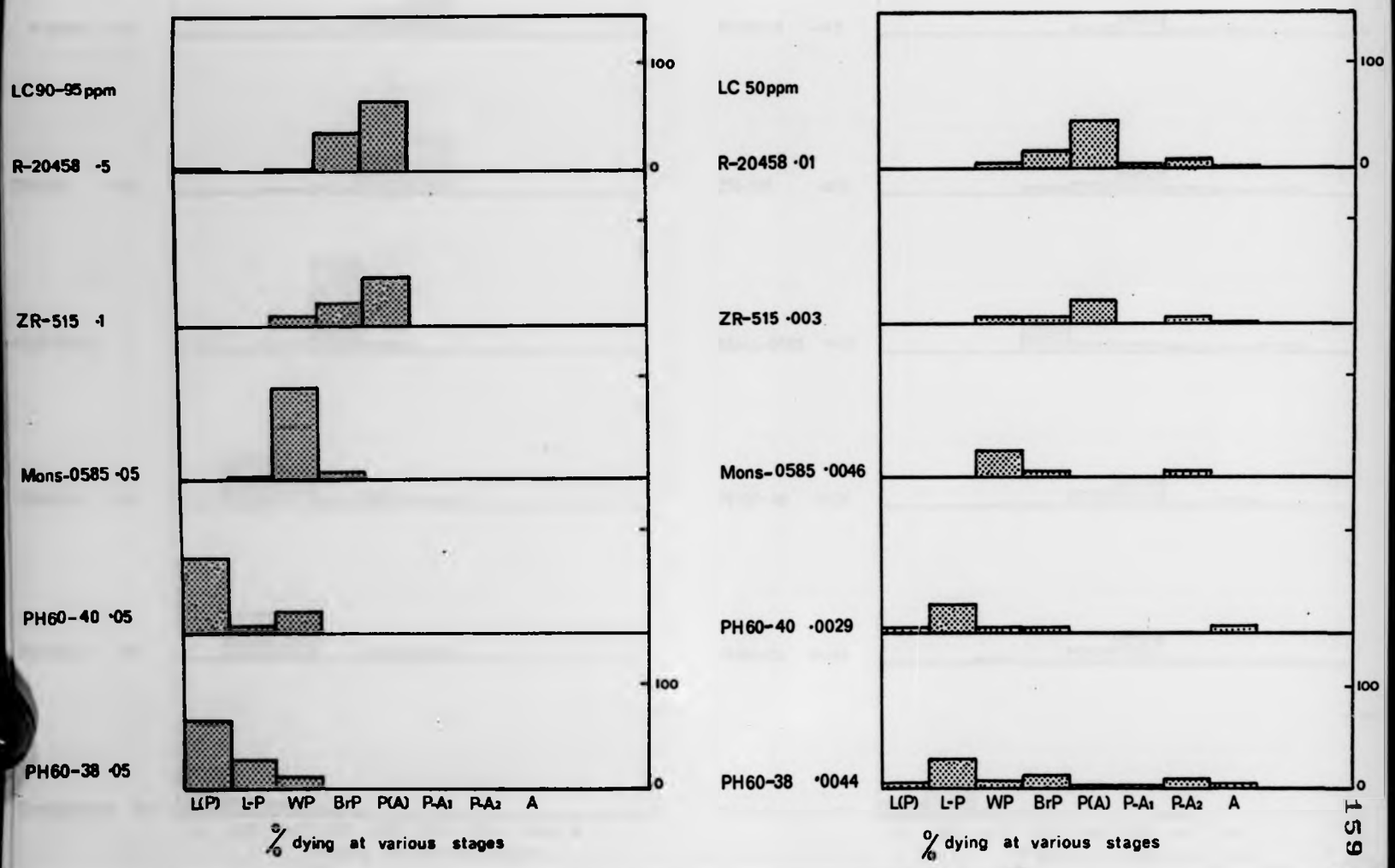


Figure 17. Effects of the compounds in different categories against fourth-instar larvae of Anopheles gambiae

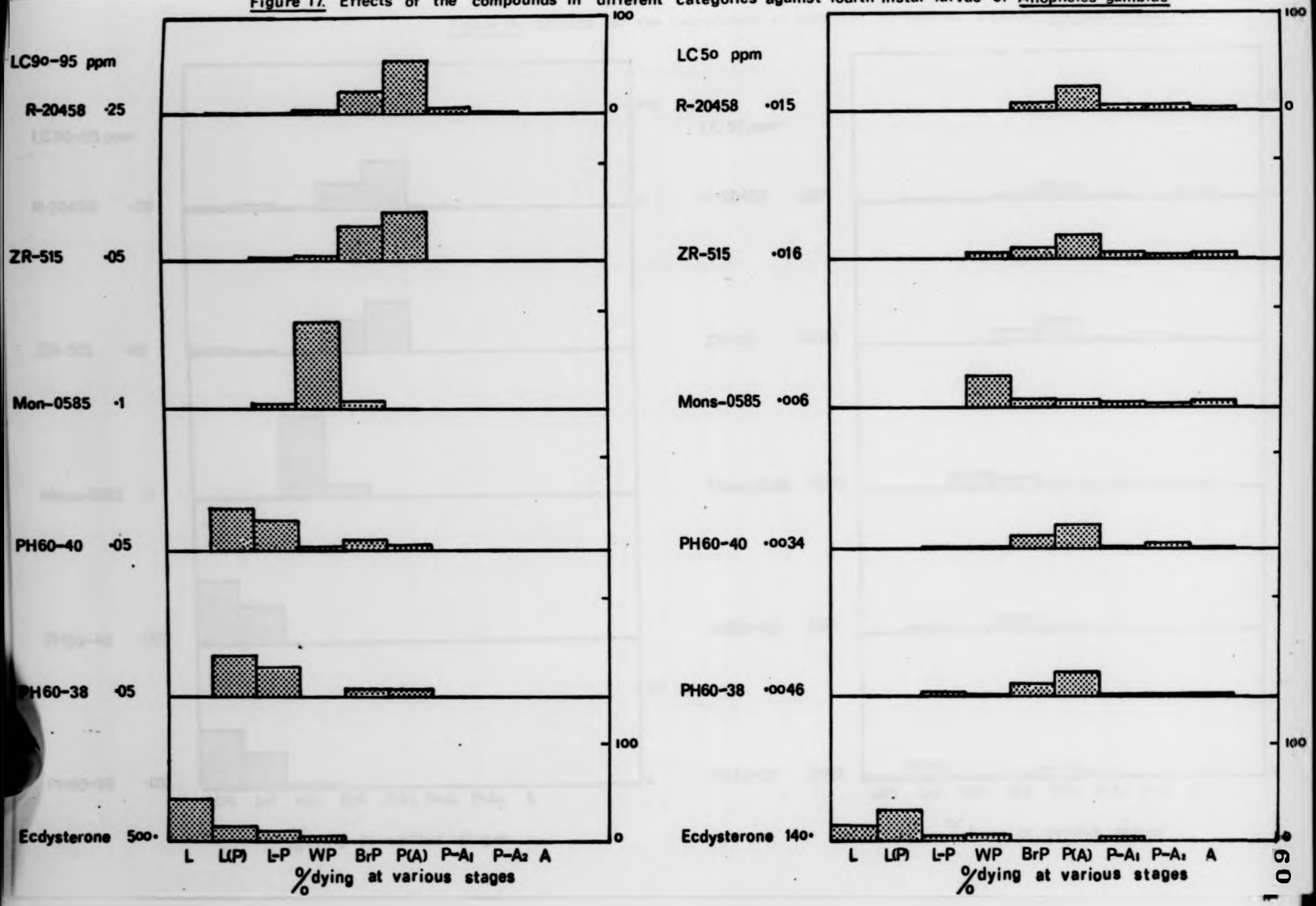


Figure 18. Effects of the compounds in different categories against An.quadrimaculatus

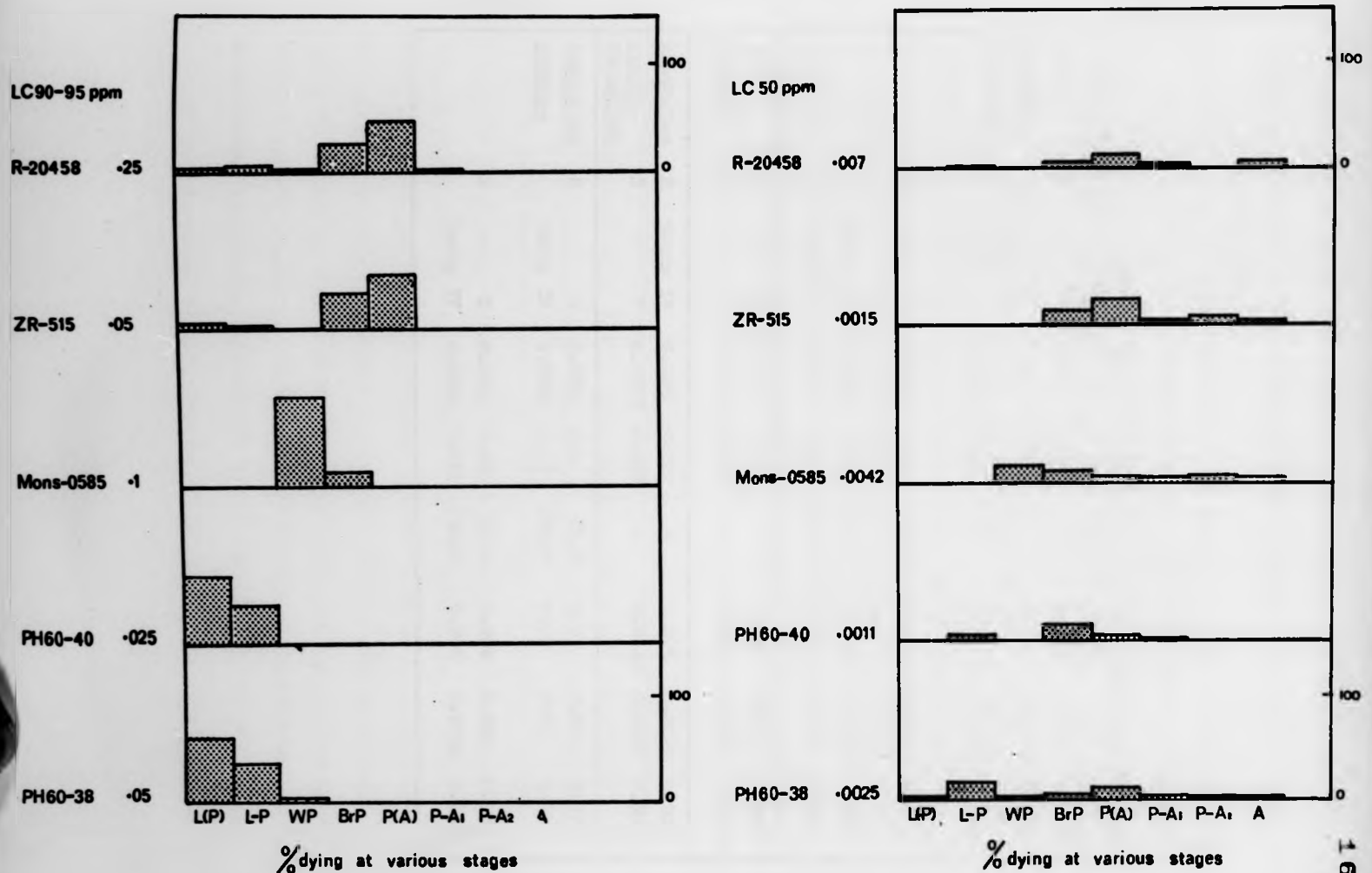


Table 12. Activity of the compounds against various stages of 4 species of mosquito.

Species	Strain	Larval instar	LC50 (ppm) ZR-515 (XVI)	R-20458 (XVII)	Ecdysterone (XVIII)	Non 585 (XIX)	PH60-40 (XX)	PH60-38 (XXI)
Culex	R	I	0.009	0.06	52.0	0.006	0.005	0.023
pipiens		II	0.012	0.11	-	0.06	0.0075	0.025
fatigans		Early IV	0.002	0.1	140.0	0.01	0.0042	0.01
		Late IV	0.0025	0.18	-	0.025	0.006	0.025
	S	I	0.007	0.04	30.0	0.005	0.002	0.006
		II	0.009	0.07	-	0.007	0.0025	0.01
		Early IV	0.0014	0.061	128.0	0.0045	0.0013	0.005
		Late IV	0.001	0.1	-	0.01	0.002	0.01
Aedes	R	Early IV	0.008	0.061	-	0.02	0.006	0.009
aegypti	S	" "	0.003	0.015	-	0.0046	0.0029	0.0044
Anopheles	R	Early IV	0.003	0.027	-	0.016	0.0028	0.004
quadri-maculatus	S	" "	0.0015	0.007	-	0.0042	0.0011	0.0025
Anopheles	R	I	0.008	0.1	32.0	0.03	0.02	0.028
gambiae		Early IV	0.0044	0.07	150.0	0.02	0.01	0.013
	S	I	0.003	0.031	26.0	0.008	0.005	0.007
		Early IV	0.0016	0.015	140.0	0.006	0.0034	0.0046

From these modest experiments it is difficult to speculate on the actual mode of action of the different compounds; but at least it is interesting to note the characteristic differences in time and nature of deleterious effects, which show similarities in related compounds.

(f) Adult treatments

Sterilisation of adult insects with juvenile hormone mimics has been reported by various workers (Ellis et al., 1970). The feeding of natural hormone, ecdysterone, was reported to inhibit the ovarian development in housefly (Robbins et al., 1966). Recently, adults of A. aegypti treated with juvenile hormone mimics showed some sterilisation in reduction of egg fertility and female fecundity; also, a large number of abnormal eggs were produced (Patterson, 1971).

In this study, 5 of the moulting disturbance compounds and ecdysterone were tested with adults by feeding them with sugar solution containing these compounds. In each test 5 ml 0.1% of the compounds in acetone were applied to 1/4" × 3" absorb lint strips and the solvent allowed to evaporate at room temperature. The treated lint strips were put into small tubes and 5 ml of 5% sugar solution was applied to each. Groups of 20 of newly emerged adult male and female of equal number of C. p. fatigans were fed continuously on these treated sugar solution. Blood meals were provided 3-5 days after treatments. Both of the number and hatchability of laid eggs were recorded. The results were summarised in Table 33. There is some evidence of reduced fertility due to feeding on these compounds especially ecdysterone and PH60-40, PH60-38 and ZR515, have similar sterilising activity but is not very high.

It is interesting to note that female adults treated with ecdysterone

Table 23. Activity of tested compounds in sugar solution with adult of Culex pipiens fatigans.

Compounds		Concentration %	No. of eggs laid	No. of eggs laid per female	% hatch
Type	Sample No.				
ZR 515	XVI	0.1	1442	160.2	71.6
R-20458	XVII	0.1	1752	175.2	85.9
Ecdysterone	XVIII	0.1	463	154.3	27.0
Mon-0585	XIX	0.1	1637	163.7	91.1
PH 60-40	XX	0.1	1578	175.3	42.5
		0.5	1452	161.3	38.4
PH 60-38	XXI	0.1	1476	147.6	8.3
Control	-	-	1825	182.5	98.0

lose their blood feeding activities. Many efforts had been made for the feeding when compared with adults treated with another compound. From a close observation this difficulty of feeding is possibly due to a malfunction of the proboscis, but no evidence of this was observed under the microscope. However, this preliminary investigation needs further evidence and confirmation with approved techniques and details of observations. The results reported here do not provide conclusive evidence about sterilising activity of the compounds. More extensive experiments are needed, preferably with improved technique. In the method used, the availability of the test compounds in the sugar solution was somewhat doubtful.

(g) Effect on speed of development

Practically all studies on the biological activity of chemicals against mosquitoes are concerned with the assessment of mortality occurring within 24-48 hours after treatment. It is, however, well established the delayed development and inhibition of ecdysis can be caused by insect hormone mimics and certain other compounds (Lewallen, 1963; Spielman & Skaff, 1967). Delayed pupated varied with the concentration of farnesol and ziram (Lewallen, 1964). Similar effects have been observed by exposing young larvae of mosquitoes to petroleum oils (Micks et al., 1969). Delayed development when exposing mosquitoes pupae to organophosphorus insecticides and the longevity of adults emerging from treated pupae were also reported (Roberts et al., 1969).

The results of the present investigation showed the obvious significance in delayed and incomplete development as related to the moulting disturbance compounds. Table 34 shows the results obtained from

Table 34. Influence of the moulting disturbance compounds on duration of development of mosquito larvae

Compounds		Delayed days of development										
		C. f. fatigans				A. aegypti		An. sinensis			An. quad.	
Type	Sample No.	I	II	EIV	LIV	EIV	LIV	I	EIV	LIV	EIV	LIV
ZR-515	XVI	10	8	3	2	3	2	10	4	2	4	3
R-20458	XVII	9	8	3	2	3	1	10	3	2	4	2
Ecdysterone	XVIII	1	-	-1*	-	-	-	2	-1*	-	-	-
Non-0585	XIX	9	7	3	2	4	3	9	4	3	4	2
PH60-40	XX	10	8	4	3	5	3	12	5	4	5	3
PH60-38	XXI	11	8	4	3	5	4	10	5	4	4	4
Duomeen T1	XXIII	-	-	2	-	3	-	-	3	-	4	-
Duomeen L15	XXIV	-	-	3	-	3	-	-	4	-	5	-
Aldmine 11	XXV	-	-	2	-	2	-	-	4	-	4	-
Trans-2-octanoic acid	XXVI	-	-	3	-	-	-	-	5	-	6	-
Trans-2 nonenoic acid	XXVII	-	-	3	-	-	-	-	6	-	6	-

*Accelerated development

A species of mosquito with a progressively slower larval development. Data obtained from the replicates of each experimental series were averaged and compared with the untreated control larvae.

All of the compounds have delayed effect of the development of all species tested, except ecdysterone when applied to early IV stage larvae. It accelerated the development only 1 day when compared with the control larvae. Maximum retardation of development was obtained at the lower concentrations.

The results generally showed maximum delayed effect, when the compounds were used early in development (to 1st instars) and the effect became gradually less pronounced with later applications.

There was no obvious difference in effectiveness between the various compounds used, in this delaying effect.

(ii) Aliphatic amine compounds

In some respects, the aliphatic amines seem to have a similar type of activity against pre-imaginal mosquitoes as the hormone-type compounds, but some of them also have direct toxic effects on eggs, larvae and pupae. Although the biological activity of the three aliphatic amines against fourth instar larvae is much lower than that of the other type of larvicides, the amine compounds offer additional advantages of being capable of effecting morphogenesis and causing delayed development of immature stages. Therefore, their potential use as mosquito larvicides, pupacides and ovicides were explored.

a. Eggs test.

Three aliphatic amines were tested against young eggs and some older eggs of C. p. fatigans and An. barbiae (Table 35). Aliphatic 11

Table 39. Ovicidal action of aliphatic amines against eggs of Culex pipiens fatigans and Anopheles gambiae

Species	Compounds	Conc. (ppm)	Exposure period (hr)							
			6		12		24		48	
			No. treated	% hatch	No. treated	% hatch	No. treated	% hatch	No. treated	% hatch
C.p. fatigans	Duomeen T1	10.0	518	97	390	98	483	83	389	19
		1.0	459	98	415	96	504	95	494	71
	Duomeen L15	10.0	489	96	459	95	397	51	426	22
		1.0	520	98	504	98	429	92	398	81
	Alamine 11	10.0	422	96	408	94	387	28	354	0
		1.0	375	97	486	96	359	43	373	18
An. gambiae	Duomeen T1	10.0	135	64	101	18	132	0	215	0
		1.0	120	89	108	44	128	32	146	41
	Duomeen L15	10.0	182	94	138	93	135	90	110	86
		1.0	135	95	150	93	121	87	116	70
	Alamine 11	10.0	108	62	112	50	118	36	109	0
		1.0	116	94	132	94	124	89	125	68

(XXV) showed some activity at 1 to 10 ppm; but 48 hours' exposure was necessary for complete suppression of hatching. Duomeen L15 (XXIV) was even less ovicidal, though it was one of the most promising of these groups against larvae and pupae. Age of eggs as well as duration of treatment period influenced level of susceptibility of eggs. The younger eggs were more susceptible than older eggs and longer exposure time (48 hours) gave higher kill of the eggs than a shorter treatment period (24 hours). Exposure periods of shorter than 24 hours produced no marked effects. Mulla and Chaudhury (1968) studied the ovicidal activity of alamine 11 against eggs of Culex pipiens quinquefasciatus and An. albimanus. They found that the toxic effect decreased as the age of eggs increased and duration of exposure period also influenced viability of eggs, especially in An. albimanus. They also noted that lethal treatments with these compounds arrested embryonic development in the eggs; this contrasts with the effects of ovicidal treatments with organophosphorus compounds, as recorded by Sharma & Kalra (1962).

In some treatments of eggs of C.p. fatigans with Duomeen L15 at concentration 10 ppm. the treated egg rafts sank to the bottom and did not hatch. This sinking effect observed in eggs of the same species treated with petroleum oil (Mulla and Chaudhury, 1968). It is obvious that submerging of egg rafts is detrimental to the eggs, but the mode of action of aliphatic amines as ovicides is not clearly understood. Some evidence shows that they seem to produce a quantitative change in the water permeability of the egg shell and allowing penetration of the compounds (Wilton and Fay, 1969) or attack layers of the egg shell which resist water permeability. This attack causes the eggs of Aedes aegypti (which are not permanently in water) to dehydrate and collapse (Cline, 1972). Embryonic development of C.p. fatigans was retarded

and most of them showed no differentiation or were found at the earlier stages of differentiation. On the other hand, some embryos in treated eggs can reach full maturity but were not able to emerge (Mulla and Chaudhury, 1968). The developing embryo of Culex tarsalis takes about 7-9 hours to reach the stage of superficial segmentation (Rosay, 1959). The effective amine would diffuse into the ovum when the embryos reach this stage. In this study Alamine 11 produced the highest mortality in eggs probably penetrated the chorion easier and faster than the other compounds.

b. Pupae and larvae test

The tests against pupae and larvae gave rather similar level of activity although one of these compounds, Duomeen L15, proved most effective against all tested species of mosquitoes. (Table 36). This, however, is especially promising because pupae are very tolerant of many mosquito larvicides. Comparative effectiveness of aliphatic amines showed that all materials proved to be more active against the larvae and pupae of An. barbiae than the other species. The trend of toxicity against the larvae and pupae of anopheline and culicine was not consistent. Some compounds were more active against the pupae of the two groups, while the other were more active against the larvae. In all cases, longer exposure (48 hours) for pupae gave better effect than the shorter period. However, the overall range of dosage was too high for practical use of control.

It was expected that anopheline larvae would show higher susceptibility than culicine larvae to these materials, since the anopheles larvae remain in a near-horizontal position at the water surface which

Table 36. Activity of aliphatic amines against fourth instar larvae and pupae of 4 species of mosquito

Species	Compounds	LC50 (ppm)					
		Susceptible			Resistant		
		Larvae 24*	Pupae 24	Pupae 48*	Larvae 24	Pupae 24	Pupae 48
Culex pipiens fatigans	Duomeen T1	1.2	1.8	1.6	1.6	2.5	1.9
	Duomeen L15	0.38	0.46	0.29	0.46	0.64	0.38
	Alamine 11	1.5	1.5	0.5	1.9	1.9	1.2
Aedes aegypti	Duomeen T1	0.9	0.6	0.26	1.0	0.9	0.5
	Duomeen L15	0.34	0.17	0.056	0.8	0.5	0.29
	Alamine 11	1.1	0.63	0.32	1.35	1.2	0.7
Anopheles gambiae	Duomeen T1	0.09	0.12	0.06	0.23	0.32	0.1
	Duomeen L15	0.065	0.07	0.04	0.19	0.19	0.09
	Alamine 11	0.15	0.17	0.11	0.36	0.58	0.25
Anopheles quadrimaculatus	Duomeen T1	1.2	1.1	0.75	1.4	2.1	1.7
	Duomeen L15	0.39	0.58	0.25	0.58	0.9	0.7
	Alamine 11	1.1	1.1	0.48	1.7	1.7	1.0

* Exposure time for 24 and 48 hours

rovides a better contact or exposure with the amine films than the culicines. Results reported here seem to confirm this with An. gambiae but not with An. quadrimaculatus. In both larval and pupal stage the susceptibility levels of various species ranked: An. gambiae, A. aegypti, An. quadrimaculatus, C.p. fatigans. Mulla et al. (1970) found the order of susceptibility of the larvae of the species tested to rank in susceptibility: An. albimanus, C.p. quinquefasciatus, A. aegypti. For the pupae A. aegypti was equal to or more susceptible than C.p. quinquefasciatus.

In the present investigation, both pupae and larvae exposed to sublethal concentrations showed delayed development; the adults were unable to emerge completely. The half-emerged adults remained on the surface of the water for 1-2 days, then died. In some treatments, where larvae and pupae were exposed to sublethal concentrations of aliphatic amines, the adults were able to eclose completely, but soon after eclosion, they drowned or fell flat on the surface of the water, incapable of flying. It was interesting to note that there was a great deal of the abdominal, wing and leg scales fallout from these adults which covered the water surface, giving a peppery appearance. Similar observations were also first noted by Mulla (1966, 1967 and 1970b). However, the type of physical action seems to be complex and may be due to interference in hormonal balance as indicated by the abnormal eclosion of adults, appearance of abnormal structures and shedding of scales in the emerging of adults. The metabolic and chemical changes result in the death, delayed development, or morphogenic changes in the immature stages of mosquitoes. On the other hand, some other plausible suggestions from Mulla (1967) are that the amines dissolve in or disrupt the epidermal layer of larvae or pupae resulting in nutrient, chemical,

and water imbalance. It may interfere with the membrane of anal gills, change the function of tracheae or the characteristics of cuticle. This was also noted by Cline (1972) who suggested that the attack on the larval cuticle is similar to the attack on the egg shell. Much more work is needed to elaborate on the mode of action of these compounds.

(iii) Unsaturated fatty acids

Earlier works on the toxicity of fatty acids have been reported by various workers (Quraishi and Thorsteinson, 1965; Quraishi, 1971). Some of the unsaturated fatty acids have been found to be more toxic than the corresponding saturated acids. In an effort to find chemicals for control of resistant mosquitoes, the two unsaturated fatty acids, trans 2-octenoic acid (XLVI) and trans-2 nonenoic acid (XLVII), were selected for this study and tested against 3 species of mosquito larvae. The studies just mentioned refer to teratogenic effects or interference with ecdysis or eclosion rather than selective toxicity. The compounds had low direct toxicity to the mosquito larvae but produced morphological abnormalities in adults emerging from treated larvae. Pupation and subsequent emergence were delayed by 2 to 5 days after early IV instar larvae were treated. In addition, mortality occurred in pre-imaginal stages and the imagoes failed to complete eclosion. Some adults obtained from treated larvae, at all concentrations, show morphological deformities. The emerging adults managed in some cases, to withdraw the first pair of legs, while the others stuck inside the pupae skin. There was no hardening in the legs which showed abnormalities. The wings were crumpled, twisted, folded, and sometimes fused. Adults had lost their characteristic stripes on the abdomen, some small black and grey patches were noticed. Irregular spots also appeared on one side of the abdomen in some pupae. The

remaining parts of the pupae were unmelanized (Plates 9 and 10). Earlier work (Quraishi, 1971) on these unsaturated fatty acids in the housefly confirm these observations. He also stated that marked melanogenic action and teratogenic effects were apparent when immature stages of insects are treated with these compounds.

The unusual effects of these compounds might be useful for the control of mosquitoes if the quantities used can be reduced.

PLATE 9.

Effects of unsaturated fatty acids on treated larvae of C. p. fatigans (see p. 173).

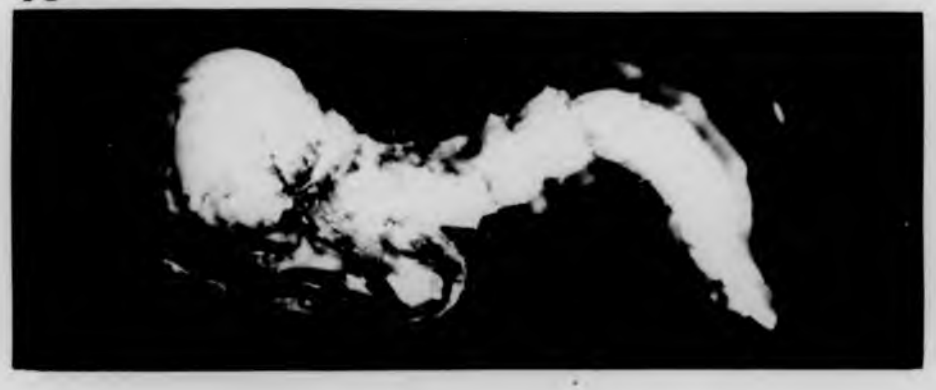
A. Half emerged and unmelanized adult.

B. Irregular melanization on pupae abdomen

C. Nearly completely emerged adult with fused wings.

Plate 9

A



B

C

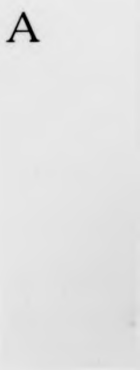
Plate 10.

Abnormal adults of C. p. fatigans emerging from larvae exposed to unsaturated fatty acids. (see p. 173).

A. Half emerged adult.

B, Deformities in wings and legs.

C. Partial melanization, swollen abdomen.


A


B


C

Plate 10

SUMMARY & CONCLUSIONS

1. The problem of insecticide resistance is outlined, with special reference to insects of medical importance and, in particular, mosquito disease vectors.

2. The methods and findings of research on insecticide resistance are surveyed, under the main headings of (a) detection and measurement, (b) genetical research, (c) toxicological research and (d) ways of countering resistance.

The subjects comprised in the investigations described in this thesis, fall mainly in category (c), though some aspects of (a) were investigated and (so far as alternatives to conventional insecticides were examined) in (d). Most of the work (categories (c) and (d)) was done with larval mosquitoes; but adult mosquitoes were used for topic (a).

3. Normal and insecticide-resistant strains of the following 5 species of mosquitoes were obtained from various sources and maintained as laboratory colonies. Culex p. fatigans, Aedes aegypti, Anopheles gambiae, An. quadrimaculatus and An. stephensi.

4. As part of a refinement of the standard W.H.O. test for detecting resistance in adult mosquitoes, a study was made of the relations between time of exposure and concentration of insecticide. A wide range of concentrations of malathion, fenthion, fenitrothion and propoxur were tested against C.p. fatigans by the method recommended by the Expert Committee. The results indicated that mortality is equally dependent on concentration and exposure time. The CT values obtained from LT50 × concentration and LC50 × time are not much different.

The storage life of malathion and propoxur impregnated papers were also investigated. There is no evidence of deterioration in the potency of either type of paper over a period of a year under European room conditions but a substantial decline is found thereafter.

5. The pattern of cross resistance was established by comparison of the LC50 values for normal and resistant strains in each mosquito species. For C.p. fatigans three resistant strains from Lagos, Tananarive and Rangoon were examined. The LC50 value for each compound were determined by the W.H.O. standard method for mosquito larvae and altogether 142 measurements were obtained for cross resistance spectra.

Resistance spectrum obtained with C.p. fatigans, An. quadrimaculatus and An. stephensi shows some similarities. Resistance to DDT is rather high and moderate to DDD. There is no cross tolerance to Prolan, Bulan or the biodegradable analogues. Therefore the major mechanism responsible for this is probably dehydrochlorination. Resistance to pyrethroids, organophosphorus, hormone-like compounds and aliphatic amines is low (less than × 2).

DDT resistance in A. aegypti and An. gambiae is very high and shows some cross resistance to the biodegradable analogues approximately ×4 and ×10 respectively. This indicates that there is also another mechanism responsible for DDT-resistance in addition to dehydrochlorination. For miscellaneous compounds the resistance level is about ×4 from which a low-level common resistance mechanism is expected.

Resistance to dieldrin and gamma BHC is variable in different strains and seems to be independent of DDT-resistance.

6. The type of mechanism involved in eachstrain was investigated by the effects of two synergists, DMC and piperonyl butoxide, with different insecticides. The former is known to inhibit DDT-dehydrochlorination and the latter would inhibit the mixed function microsomal oxidase. The interaction of synergist and insecticide was measured by synergistic ratio which obtained from the value of LC50 of insecticide alone/LC of mixture.

The overall results with DMC synergism for DDT resistant strains of C.p. fatigans and An. quadrimaculatus combined with the information from cross resistance studies indicated that DDT resistance mechanism for both strains is dependent largely on dehydrochlorination.

The results with C.p. fatigans and An. quadrimaculatus showed distinct antagonist effects with most compounds in the susceptible strains, for unknown reasons. Therefore, the presence of a synergistic effect with DDT (together with the cross-resistance data) indicated that the principle resistance mechanism was dehydrochlorination, in both species. The action of piperonyl butoxide on DDT for these two species, was also antagonistic, both in normal and resistant strains, thus supporting the above argument. (An. quadrimaculatus showed particularly specific DDTresistance).

So far as An. gambiae and A. aegypti were concerned, DMC have slight, variable effects; it was somewhat more synergistic to DDT in the resistant strain. Piperonyl butoxide again had an antagonistic effect on DDT in the susceptible strains but was slightly synergistic in the resistant colonies. It was more obviously synergistic to biodegradable analogues and pyrethroids, especially in the resistant

strains. (These levels were highest in An. [illegible]). All these facts, together with the resistance spectra, suggest that microsomal oxidative mechanisms are important in these two species.

7. Investigation of pick-up of insecticide was carried out by bioassay method, using susceptible first instar Aedes aegypti larvae to assess the amount of DDT which was picked up by the fourth instar larvae. The results showed a slight indication of greater pick-up by resistant than by susceptible larvae. This could not explain resistance, therefore the radiometric techniques were applied to obtain more accurate results.

Isotopically labelled ^{14}C DDT and ^{14}C malathion were used to examine the pick up of insecticide by A. aegypti larvae. Since the quantities were very small, stock solutions were prepared by dissolving the whole samples in acetone and a series of 3-fold dilutions were made. The actual concentrations of insecticide in these standard dilutions were estimated by bioassay, using the 4th instar larvae and the same procedure as in the usual larvicide test. The 24-hour mortalities were compared with those obtained with standard normal insecticide solutions. From this, the concentrations of standard radioactive solutions were estimated and suitable concentrations for pick-up tests were also selected. Standard stock solutions for DDT and malathion were prepared, giving an aqueous suspension equivalent to 4.05 ppm and 4.50 ppm respectively.

The radioactivity in various tests were measured by a scintillation counter. First, the counts per minute were determined for 1 ml of standard solutions put directly into the counting vials. It was found that 1 µg DDT gave 41,000 c.p.m. and 1 µg malathion, 14,000 c.p.m. The activity of the samples were 15 microcurie per mMol. for DDT and 4.6 microcurie per mMol for malathion. Results obtained for molecular

weights calculation of DDT and malathion gave 96,000 and 1 million c.p.m. per microcurie respectively, showing good agreement.

The efficiency of immediate extraction of insecticides from aqueous solution of DDT was 82% and 95-98% for malathion. Extractions made at different time intervals, after preparing the suspensions, showed the same gradual losses of both insecticides. After 8 hours, 0.0135 ppm DDT lost 13.5%; while malathion losses were 18% at 0.045 ppm; 26% at 0.15 ppm and 15.5% at 0.5 ppm.

The quantities picked up by mosquito larvae were determined separately for external and internal insecticide. When the internal and external insecticide amounts were added to the amount to depletion from the suspensions, the total ranged from 95 to 9?% of the original value.

There was a definite correlation between the amount of pick-up per larvae and concentration to which they were exposed. This followed the rule suggested by Busvine (1968) relating pick-up and concentration. For DDT, x-y where **y** is pick-up in µg per larvae and **x** is the initial concentration in ppm. Thus, from a suspension containing 0.0135 ppm DDT, in 16 hr, the susceptible larvae acquired 0.0149 µg and the resistant 0.0204; average 0.0175 µg, a fair approximation.

The percentages of insecticides penetrating into the larvae were estimated by comparing the amount extracted from the larvae with the total pick-up. With DDT, internal insecticide was 80 to 82% at 4 hours, increasing to 90 to 92% at 16 hours. With malathion, internal insecticide was 75 to 76% at 4 hours, rising to 79-81% at 8 hours. Malathion penetration at 8 hours did not differ much over a considerable range of concentrations.

Percentage and actual penetration of DDT was less in the susceptible strain than in the resistant strain. This difference might be expected to lead to a progressive reduction of pick-up in the susceptible larvae, possibly due to incipient intoxication.

With malathion tests, the percentage penetration in the susceptible strain was always higher than in the resistant one, though in some cases the actual amount was lower. It did not show a consistent change with increasing concentration, so that there was no evidence of reduced penetration even at the highest level.

It must, therefore, be concluded that there is no definite correlation of the rate of absorption between resistant and susceptible strains studied here.

3. The relative potencies of all tested compounds were examined. DDT is a highly potent larvicide against many normal strains of mosquito with LC50 of 0.005 ppm. Prolan and Bulan are less effective, with LC50 values of 0.005 to 0.04 and 0.03 to 0.12 ppm., respectively. In all cases, Prolan is better than Bulan.

The LC50 levels of the biodegradable DDT-analogues ranged from 0.02 to 0.2 ppm. Their relative potencies compared with DDT showed distinct differences with the species. They were about a half to a sixth as active as DDT in A. aegypti, C. fatigans and An. stephensi; but for An. gambiae and An. quadrimaculatus their potencies were nearer to a twentieth that of DDT.

Bioallethrin was about four times more potent than allethrin, with LC50 values of 0.015 to 0.11 and 0.06 to 0.4 ppm respectively. They

are not considered to be very practical as larvicides.

LC50 values for dieldrin and gamma BHC were low with A. aegypti and An. quadrimaculatus. Fenthion was more potent than malathion with LC50 values of 0.002 to 0.013 and 0.06 to 0.14 ppm, respectively.

For the hormone-type compounds ZR-515 was most potent with LC50 values 0.0014 to 0.003 ppm, and was about 10 times more active than R-20458. Ecdysterone had very low activity.

Of the two Duphar compounds, PH60-40 was as potent as ZR-515, followed by PH60-38 and Non-0585 which were almost equally effective.

Cartap hydrochloride, aliphatic amines, unsaturated fatty acids and phenol compounds were not promising, their LC50 values being all rather high.

9. Involvement in DDT-resistance of each group of compounds was considered. Some of the resistant strains showed highly specific resistance to DDT and DDD (for example, An. quadrimaculatus, An. stephensi and C.p. fatigans). Presumably these strains depend largely on dehydrochlorination mechanisms.

With the resistant strain of An. gambiae there was definite evidence of cross-resistance to biodegradable analogues, pyrethroids and various other compounds. The high synergistic ratios with piperonyl butoxide, combined with these facts, suggest the presence of an enhanced microsomal detoxication mechanism.

The resistant strain of A. aegypti was intermediate in that it showed some incipient evidence of a cross-resistance pattern like that of An. gambiae (and similarly, some raising of synergist ratios with piperonyl butoxide).

In both An. gambiae and A. aegypti, however, the very high levels of DDT and DDD resistance indicate the importance of the dehydrochlorination system.

10. The mode of action of compounds affecting moulting and metamorphosis (ZR-515, R-20458, ecdysterone, Hon-0585, PH60-40 and PH60-38) were investigated on eggs, Ist and IInd instar larvae, early IVth instar and late IVth instar and adults.

Tests on eggs were carried out with C. p. fatigans and An. gambiae. PH60-40 (XX) showed some ovicidal activity but this effect was limited to the young eggs and long exposure period. Minimum lethal concentration required for both species was above 0.1 ppm. There was no marked difference between eggs of tested species or strains. Evidence from abnormal half-emerged larvae indicated that PH60-40 may have ovicidal activity.

Results of all compounds tested with 1st and 2nd instar larvae showed some type of interference with ecdysis. All compounds were more effective to 1st instar than 2nd instar. With ecdysterone (XVIII) the effect was mainly on 1st instar and with Hon-0585 rather than on early pupae stage; but with the others the highest mortality occurred in the 2nd instar when 1st instar was treated. When the dose was reduced, the mortalities in early instars decreased sharply and delayed development occurred.

In treatments with early IVth instar larvae, a variety of toxic effects was observed and recorded in 9 categories, according to the stage of metamorphosis reached when death occurred.

The two orthodox hormone mimics (ZR-515, and R-20458) exhibited

their main effects in the very late pupal stage when the adult form had become visible. ZR-515 was the most effective in all species tested with LC50 values 0.0014 to 0.009 ppm and was about 2 to 10 times better than R-20458.

Metamorphosis of the fourth instar larvae, treated with Non-0585, was often blocked in the early stage of pupation prior to darkening of the cuticle. This resulted in the pupa dying in a characteristic unmelanised form. According to the literature, its effect may be caused through interference in oxygen utilization of the tyrosine metabolism pathway involved in cuticle darkening. Activity of this compound ranged from LC50 of 0.0042 to 0.006 ppm.

The Duphar compounds PH60-40 and PH6038 expressed their activity early in metamorphosis between larval and pupal stages. The pupae with their new cuticle appeared to be trapped inside the larval skin, but they were unable to split the exuviae and free themselves. At marginally effective dosages, some larvae having succeeded in pupation, died as pale pupae, black pupae or as adults that during emergence became stuck in the pupal skin. According to published data, the effects of these compounds may be due to inhibition of chitin synthesis. On the whole, PH60-40 was more active than PH60-38, With LC50 0.0011 to 0.0034 ppm.

Ecdysterone had the earliest activity in the series of compounds tested, especially at high doses, by killing at the larval stage. It produced no significant mortality in the surviving treated larvae. In this respect, it showed sharp contrast to the juvenile hormone mimics and the other types. Its activity was low, probably due to the difficulty of penetrating through the insect cuticle.

Late IVth instar larvae treated with any of these compounds were less affected than the early IVth instar larvae. The deleterious effects occurred in the later stages of metamorphosis, even with those compounds which caused earlier effects, when applied to younger larvae.

Adults of C.p. fatigans were tested with juvenile hormone-type compounds in order to investigate the sterilising effects. Newly emerged adults were fed with sugar solution containing these compounds. After 3-5 days of treatment, blood meals were provided and the number of eggs laid and hatches were recorded. PH60-4C, PH60-38, and ZR-515 caused some sterilization, but their activity was not very high.

Effects on speed of development of all compounds were examined. With all the compounds, the effect was a delay in development, with the exception of ecdysterone, when applied to early IVth instar larvae (which caused a slight acceleration of pupation). No obvious difference in the delaying effects between other compounds was noticed.

11. The aliphatic amines Duomeen T1, Duomeen L15 and alamine 11 were tested as ovicides, pupacides and larvicides against C.p. fatigans and An. gambiae.

The ovicidal activity was not high, since the most potent compound (Alamine 11) required 1 to 10 ppm and a long exposure (48 hrs). At the high concentration of 10 ppm, Duomeen L15 caused the eggs to sink.

The activity of these compounds against larvae and pupae were of the same general order of potency, though some of them were more active against larvae and others against pupae. The general level of potency was not very high, however, and even against the most susceptible species (An. gambiae) the series did not seem likely to be of practical value.

The aliphatic amines were found to cause delayed development; and at high doses adults were unable to emerge completely, resulting in death soon after. At sublethal concentrations the adults were able to emerge completely, but remained on the water surface and could not fly away. The abdominal, wing and leg scales fell out and covered the water surface, giving a dusty appearance.

Though not of high potency the amines seem to provide a better chance for the control in that they can be used as ovicides, larvicides and pupacides.

12. The two unsaturated fatty acids, trans-2-octanoic acid and trans-2-monenoic acid had low activity with LC50 values, ranging from 0.2 to 14.0 p.p.m. The activity of the monenoic acid was better than the octenoic acid, against every species tested; but their effects in producing morphological abnormalities were similar. Larvae treated with these compounds often resulted in adults which could not emerge completely; and some of those which did emerge had deformities in the wings. These compounds also interfered with melanisation, which was restricted to small areas of cuticle. Some of the adults also lost their characteristic stripes on the abdomen. The unusual effects ot these compounds appeared to be promising and efforts are being made to synthesize a better compound in this series.

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