INTERACTION OF SOME ORGANOPHOSPHORUS COMPOUNDS IN SUSCEPTIBLE AND RESISTANT HOUSEFLIES (*MUSCA DOMESTICA* L.)

M. D. ABDALLAH

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THESIS

SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF AGRICULTURAL SCIENCES AT THE AGRICULTURAL UNIVERSITY OF WAGENINGEN, THE NETHERLANDS, ON FRIDAY OCTOBER 4TH 1963 AT 4 P.M.

BY

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H. VEENMAN EN ZONEN N.V. - WAGENINGEN - 1963

THEOREMS

I

Although the hypothesis that the toxic effect of parathion is due to the production of the oxygen analogue paraoxon seems reasonable, it fails to explain many observations satisfactorily.

II

The presence of high levels of resistance as tested by injection, comparable to those found by topical application, does not necessarily mean that the cuticle is not a site of resistance factors.

III

A contradiction exists in the conception of a simultaneous positive action of the hormone ecdyson on the processes of moulting and of growth and development of insect larvae.

IV

It is uncertain whether 2-methyl-4-bromopyrimidine is an intermediate in the reaction of 2,6-dibromopyridine with potassium amide in liquid ammonia to yield 2-methyl-4-aminopyrimidine.

H. J. DEN HERTOG. Abstr. A, 19th Intern. Congr. Pure Appl. Chem. p. 279 (A 5 – 133, 1963).

V

The 9-oxodec-2-enoic acid produced by the queens of honey bees is not necessary functioning *per se*, but may be transformed into a more volatile compound which subsequently exerts the effect.

VI

In searching for compounds with a therapeutic effect on plant viruses, it is necessary to study their action on the growth and development of the plant in addition to their inhibitory activity against virus multiplication.

VII

An administrative reform as planned aiming at the introduction of local government officials and trained personnel for the social services in the Egyptian villages will be confronted with the problem of the creation of the basic conditions for this change.

VIII

The successful introduction of the blue green algae in irrigated rice fields is limited to a few countries.

IX

Both Dutch and Egyptian housewives should be enabled to be more engaged in professional or social activities.

M. D. ABDALLAH
Wageningen, oktober 1963

VOORWOORD

Nu ik het grote genoegen smaak mijn vijf-jarig studieverblijf in Nederland met de voltooiing van dit proefschrift af te sluiten, wil ik gaarne de gelegenheid gebruiken om op deze plaats allen te bedanken die ertoe hebben bijgedragen dat deze jaren bij mij in dankbare herinnering zullen voortleven.

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INTERACTION OF SOME ORGANOPHOSPHORUS COMPOUNDS IN SUSCEPTIBLE AND RESISTANT HOUSEFLIES (*MUSCA DOMESTICA* L.)

(met een samenvatting in het Nederlands)

by/*door*

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CONTENTS

GENERAL INTRODUCTION

The first demonstrations of the potency of organophosphorus compounds to inhibit mammalian cholinesterase (ChE), were apparently those of ADRIAN, FELDBERG and KILBY (1941), and DIXON, MACKWORTH and WEBB (1942).

In insects, abundant evidence for the powerful inhibitory properties of the insecticidal organophosphorus compounds or their metabolites against ChE and Aliesterases (AliE) have been reported at a much later date (CHADWICK and HILL 1947; METCALF and MARCH 1949). Since the function of the latter esterases in the normal physiology of the organism is so far unknown, little can be said about the significance of their inhibition in relation to organophosphorus poisoning. The inhibition of ChE, on the other hand, has most dramatic physiological consequences.

The reason why these compounds specifically inhibit cholinesterases and other hydrolytic enzymes has been explained by BARNES (1954). ChE hydrolyses these toxic phosphorus esters, just as it does with its natural substrate acetylcholine (ACh). After hydrolysis, however, the phosphate group remains combined with the "esteratic site" of ChE, thus preventing the hydrolytic action of the enzyme upon its natural substrate. This process is called phosphorylation by analogy with acetylation by ACh. The affinity of the free enzyme for its natural substrate is greater than that for the toxic phosphorus ester. This property had led VAN ASPEREN (1957, 1958) to develop the "substrate protection technique" in determining the *in vivo* inhibition of ChE in intoxicated insects, by adding ACh to the homogenizing medium, and thus preventing the free inhibitor, if present, from coming into contact with the enzyme during and after homogenization.

There is no doubt that a major biochemical lesion in insects exposed to lethal doses of organophosphorus compounds is the inhibition of ChE, leading to accumulation of ACh in conductive tissues, and subsequent malfunction of conductive processes (SMALLMAN and FISHER 1958; SMALLMAN 1956; COLHOUN 1959; METCALF 1959; WINTERINGHAM and LEWIS 1959).

That nerve cholinesterase inhibition is the mechanism of insect poisoning by organophosphorus compounds is concluded from the following considerations (SPENCER and O'BRIEN 1957):

a. The correlation between insect mortality and the degree of *in vivo* ChE inhibition for varying doses of insecticides.

b. The correlation between toxicity and *in vitro* anticholinesterase activity.

c. Analogy with organophosphate poisoning of mammals.

d. The failure to demonstrate other systems affected at equally low concentrations and of comparable physiological significance.

Some serious objections against the "ChE hypothesis" have been raised (HOPF 1952, 1954; LORD and POTTER 1951, 1954 a, b; VAN ASPEREN 1957, 1958; HOPF and TAYLOR 1958). Some of the anomalies on which these objections were based were later corrected or resolved; there still remain some principal objections which make one reluctant to raise the hypothesis to the status of a theory (O'BRIEN 1960).

In mammals, the chain of events in organophosphorus poisoning leading to death is known with almost complete certainty (BARNES 1954). In insects, on the other hand, ChE inhibition is by far the major lesion, but there might be

other secondary lesions operating with the major defect to cause death (WINTERINGHAM and LEWIS 1959; O'BRIEN 1960). It is interesting to note that WINTERINGHAM and HARRISON (1956) have suggested that the fatal lesion may be ascribed to some disturbances in amino-acid metabolism.

It goes without saying that a full understanding of the physiological action of an insecticide would eventually contribute to our understanding of the mechanisms of resistance of insects against such insecticide. On the other hand, studies of the physiological properties of resistant strains, as compared with normal strains, may yield valuable data on the mode of action of insecticides.

In the living insect, poisoning by organophosphorus insecticides is a function of many processes, including penetration, distribution, storage, activation, enzyme inhibition, detoxication and elimination. The ultimate fate of a poisoned insect depends upon the interrelationships of the rates of these various processes, and the balance between those contributing to mortality and those contributing to survival. In seeking for resistance mechanisms, it is correct to assume that in a resistant insect the balance between the above processes is so adjusted as to promote survival.

The WHO Expert Committee defined resistance to insecticides as follows (BROWN 1958): Resistance to insecticides is the development of an ability in a strain of insects to tolerate doses of toxicant which would prove lethal to the majority of individuals in a normal population of the same species. The term "behaviouristic resistance" describes the development of the ability to avoid a dose which would prove lethal.

Extensive research work is being done to disclose the genetical and physiological nature of the resistance phenomenon. An insect may tolerate higher doses of insecticides either because it has developed a real resistance under insecticidal pressure or because the environment has changed in favour of the insect to confer the so called "vigor tolerance". HOSKINS and GORDON (1956) define the two terms as follows: resistance is the added ability to withstand an insecticide, acquired by breeding from those individuals which survive exposure to that particular toxicant.

Vigor tolerance is the added ability to withstand a toxicant which appears to stem from improved nutrition, extra weight, or any other factor usually associated with what may be called extra vigor. Resistance conferred by high vigor will be relatively low and unspecific.

It was hoped that organophosphorus insecticides would not induce resistance (BROWN 1961), but after the increasing use of these compounds, a steady increase in insect resistance against organophosphorus insecticides has become evident (KEIDING 1956, 1959; LA BREQUE and WILSON 1957; MORGAN and ANDERSON 1958; STERN 1962).

In spite of the great mass of information concerning resistance, at present we do not know with precision why insects do tolerate higher doses of insecticides. The main reason for this ignorance is lack of knowledge of their mode of action.

In the present study, the mode of action of three organophosphorus compounds, *i.e.* parathion (0,0-diethyl O-*p*-nitrophenyl phosphorothionate), paraoxon (0,0-diethyl O-*p*-nitrophenyl phosphate) and tri-*o*-cresylphosphate (TOCP), was investigated in some detail. These studies were initiated in an attempt to explain a clear-cut difference noted between results of STEGWEE (1960) and of COLHOUN (1960). Although both authors found that TOCP

could selectively inhibit AliE, without affecting ChE, in STEGWEE's experiments it antagonized the toxicity of TEP to houseflies, whereas in COLHOUN's experiments synergism was evident in the American cockroach. The study of these contradictory results has opened various lines of investigations. One of these pertains to the resistance phenomenon. Comparative studies were carried out with an organophosphorus-resistant strain, which revealed distinct physiological differences as compared with the susceptible strain.

MATERIALS AND METHODS

A. STRAINS OF HOUSEFLIES

Stock cultures of the following two strains were maintained in separate rooms under identical conditions.

S = Susceptible strain which has been reared in the laboratory for many years. It has not significantly changed in susceptibility to parathion during the past two years. It showed, however, some fluctuations in sensitivity towards paraoxon.

C = Resistant strain collected in Italy after the use of Diazinon, and kept under light Diazinon pressure in the Laboratorium voor Insekticidenonderzoek (L.I.O.), Wageningen, the Netherlands. In our laboratory it has been kept under light parathion pressure throughout the course of comparative experiments. It is identical with the Italian strain used by BUSVINE (1959).

Both strains were kindly supplied by Dr. F. J. OPPENOORTH from the L.I.O., Wageningen, the Netherlands.

B. REARING OF HOUSEFLIES

In rearing houseflies there are two main steps to be carried out; (1) maintaining an egg-producing stock and (2) breeding the larvae in a proper medium, which results in the production of good quality pupae and consequently good quality test flies.

The method of FISHER and JURSIC (1958) has been adopted largely for maintaining an egg-producing stock. Holding cages and stock cages were made according to the author's description.

The larval medium consists of 800 g of whole milk powder plus 800 g of yeast powder mixed thoroughly with 4 liters of water and 160 g of agar in 4 liters of boiling water.

The viable eggs were measured in a 1 ml. graduated pipette, and by using air suction 0.12 ml. could be measured, transferred quantitatively to a rearing jar, and covered with sawdust to a depth of two inches for pupation.

When the puparia had become brown, usually at the seventh day of postembryonic life, they were removed from the sawdust, allowed to dry and transferred to half liter milk bottles, 15 g of puparia per bottle. Within a few hours before emergence when the puparia have turned black, the milk bottles – provided with a strip of filter paper – were either attached to the holding cages in the rearing room or kept in a thermostat at 12° C for a few days when not directly needed. After about 8 hours the milk bottles were detached from the holding cages and the remaining unemerged pupae were placed in a clean stock cage.

By doing this, it was certain that the differences in age between the oldest and youngest flies were not more than 8 hours. Transferring pupae from low temperature storage to the holding cages ensures a rapid emergence and a continuous supply of adequate numbers of test flies. The test flies in the holding cages were fed skim milk diluted with an equal volume of water. About 5 % *(w/v)* of sucrose was added to the diluted milk. This food was placed in vials covered with cheesecloth and inverted over the holding cages.

The temperature in the rearing room was maintained at 25 ± 1°C and the relative humidity was 65 ± 5 %.

In this connection, it is worth mentioning that we have been able to obtain a homogeneous population, having a definite age. The average weight of a 2-day-old adult female house fly was about 34 mg. In gross appearance and structure the resistant flies were not noticeably different from the susceptible-flies. The average weight for a 2-day-old adult female does not deviate significantly from the above mentioned figure for the corresponding susceptible fly.

C. Organophosphorus compounds used

Samples of the following insecticides were obtained from Farbenfabriken Bayer AG, Leverkusen, German Federal Republic, as pure compounds:

Parathion: O,O-diethyl O-*p*-nitrophenyl phosphorothionate.
Paraoxon: O,O-diethyl O-*p*-nitrophenyl phosphate.
S-ethyl parathion: O,S-diethyl O-*p*-nitrophenyl thiophosphate.
S-phenyl parathion: O,O-diethyl S-*p*-nitrophenyl thiophosphate.

TOTP, tri-*o*-tolyl phosphate, called also TOCP, tri-*o*-cresyl phosphate, Eastman Kodak, Rochester, N.Y., practical grade, was used without further purification.

D. Testing method

Two-day-old adult female houseflies were used in all experiments. For topical application, batches of 25 flies were treated with acetone solutions by applying one µl per fly either to the dorsal thorax or to the ventral tip of the abdomen, as indicated in the text. The flies were treated during stupefaction with carbon dioxide. A Burroughs Wellcome "Agla" micrometer syringe was employed, with an attachement for simplifying repeated delivery of a standard volume. For injection experiments, the flies were injected into the thorax through the cervical membrane. Solutions in olive oil (1 or 0.5 µl), absolute or 50 % ethanol (0.5 µl), as indicated in the text were used. After treatment, each batch was confined in a milk bottle. A filter paper disc was provided to take up excess moisture together with a piece of cottonwool soaked in 5 % sucrose solution for food. At least four replicates were made for each experiment. The bottles were closed with cotton wool, and kept in the rearing room. After 24 hours, mortality counts were made. The mortalities occurring in controls treated with solvent alone were 0 % at 24 hours after topical application and did not exceed 5 % at 24 hours after injection, except in the case of absolute ethanol when injected into flies of the resistant strain, which will be discussed later.

Colhoun (1960) injected TOCP dissolved in propylene glycol into the abdomen of cockroaches. He preferred this technique to that of Stegwee (1960),

who dissolved TOCP in olive oil for injection into houseflies, for in the roach greater amounts of TOCP dissolved in olive oil were required to bring about the same inhibition found when propylene glycol was used as the solvent. However, propylene glycol proved to be very toxic when injected into houseflies in amounts varied from 0.5 to 1 μl per fly. It caused immediate convulsions and rapid prostration. This has also been observed by KRUEGER *et al.* (1960).

E. MEASUREMENTS OF CHOLINESTERASE AND ALIESTERASE ACTIVITY

The colorimetric method of HESTRIN (1949), modified by ROBBINS *et al.* (1958) as adopted by BIGLY and PLAPP (1960) and PLAPP and BIGLY (1961), was used. Cholinesterase and aliesterase activities were determined in duplicate for each experiment. At least two experiments were carried out in each case.

The most interesting findings of MENGLE and CASIDA (1960) that decapitated and intact flies when poisoned with eight anticholinesterases showed similar symptoms of poisoning and similar LD_{50} values after 4 hours led O'BRIEN (1961) to conclude that inhibition of housefly brain ChE is not likely to be important in organophosphorus poisoning. In our experiments accordingly ChE measurments were made on thorax breis, one thorax per ml for *in vivo* studies, and two thoraces for *in vitro* experiments. Because housefly abdomens contain the highest amount of AliE (STEGWEE 1960), they were used in most of the experiments as the source of this enzyme. A corresponding number of abdomens was used. In a number of experiments using TOCP only, was the AliE activity measured in thoraces. At 30, 120, 240, 360 and 1440 minutes after the application of the toxicant, the flies were quickly frozen in dry ice plus acetone until assayed for ChE and AliE activities. Prior to freezing the number of survivors was recorded, and at 1440 minutes dead flies as well as survivors were analysed separately. With the exception of a few 30 minute-samples, all determinations of enzyme activity were made within 2 hours from the time the insects were sampled to avoid any marked decrease in AliE activity (PLAPP and BIGLEY 1961).

CHAPTER I

THE EFFECT OF TOCP UPON THE TOXICITY OF PARATHION AND PARAOXON

I.1. INTRODUCTION AND REVIEW OF LITERATURE

The term synergism or potentiation is used in this paper when the joint effect of the two compounds is significantly higher than their additive effect. The non-toxic compound (TOCP in our case) is designated as the synergist or potentiator. If the joint action of the two compounds is less than that of the toxicant alone – at equal dosages of the toxicant – antagonism is said to occur.

As early as 1930, TOCP drew the attention of many investigators, because it was suspected to be the causative agent of a peculiar form of paralysis called "ginger paralysis" (SMITH *et al.* 1930; SMITH and LILLIE 1931). Shortly after this discovery, HARTZELL (1934) studied the effect of TOCP on insects, and demonstrated the lesions it caused in the ventral ganglia of mealworm larvae. This was later confirmed by RICHARDS and CUTKOMP (1945).

Few studies have dealt with the joint action of TOCP and organophosphorus insecticides. MURPHY *et al.* (1959) demonstrated that the toxicity of malathion for rats is markedly enhanced by prior or simultaneous administration of TOCP. This was ascribed to the inhibition of malathion detoxifying enzymes. The degree of potentiation is greater when TOCP is given prior to malathion, because presumably inhibition of the detoxifying enzyme (s) is maximal before the latter compound is administered. SEUME and O'BRIEN (1960a) showed that TOCP and TPT (tri-*o*-cresyl phosphorothionate) potentiate the toxicity of many organophosphorus compounds to the mouse, particularly those containing carboxyester or carboxyamide groups. EPN (O-ethyl O-*p*-nitrophenyl phenylphosphonothionate) had a similar effect in the mouse, the cockroach, and the housefly.

O'BRIEN (1959) pointed out that degrading enzymes are more effective in reducing the toxicity of phosphorothionates than of phosphates, since they have an opportunity for longer action because of the time involved in oxidizing phosphorothionates to their actual toxicants. Accordingly, SEUME and O'BRIEN concluded that the inhibition of degrading enzymes should have a more profound potentiating effect with phosphorothionates than with phosphates. COLHOUN (1960) injected male roaches with 100 μg TOCP dissolved in 10μl propylene glycol. After 24 h the TOCP-injected roaches were further treated with a lethal dose of parathion or TEP, applied topically to the dorsal abdomen. It was found that the roaches were more sensitive to TEP or parathion when first treated with TOCP. In contrast, STEGWEE (1960) reported that TOCP antagonized the toxicity of TEP to adult female houseflies. He applied topically 100 μg TOCP dissolved in acetone to the thorax. After 24 h the TOCP-treated flies were further treated with a sublethal dose of TEP applied topically to the thorax. TOCP/TEP-treated flies showed a lower rate of prostration and a lower mortality than those treated with TEP alone. Treatment of roaches and houseflies with TOCP resulted in variable symptoms of lethargy, but no other external signs of poisoning.

I.2. RESULTS AND DISCUSSION

In order to disclose the nature of the antagonistic action of TOCP in houseflies, detailed studies were carried out along the lines already begun by STEGWEE (1960), and COLHOUN (1960).

TABLE 1. Effect of relative time of administration of TOCP (100 μg/fly) on susceptibility of normal houseflies to Parathion (0.04 μg/fly) expressed in mortality percentage.

Parathion alone	Time of parathion application after TOCP..... hours						
	0	*1*	*2*	*4*	*6*	*18*	*24*
65	10	15	15	8	10	8	11

In the first place, the antagonistic effect of TOCP with respect to parathion was confirmed at different time intervals following simultaneous administration (Table 1). Subsequently, experiments were designed in order to see whether or not this antagonistic effect is a function of dosage. Various TOCP doses ranging from 0.5 μg to 100 μg per fly were applied simultaneously with either parathion or paraoxon. Antagonism was most marked with parathion even

at the 0.5 μg level. With paraoxon, however, the effect was obvious only with the higher doses (Table 2). In this connection, it is interesting to report that parathion gave invariably consistent results, whereas with paraoxon occasionally

TABLE 2. Mortality percentage after simultaneous application of various TOCP doses and Parathion (0.03 μg/fly) or Paraoxon (0.065 μg/fly) to normal houseflies.

Insecticide	TOCP μg/fly						
	0	*0.5*	*1*	*5*	*10*	*50*	*100*
Parathion	25	15	8	0	0	0	0
Paraoxon	40	40	41	31	11	12	20

a synergistic effect or no effect at all were observed. Thus the size of the TOCP dose, to certain limits, has no appreciable effect on antagonism; 0.5 μg equals 100 μg in its effect in case of parathion, while with paraoxon the most effective dose was the highest (100 μg). By varying the paraoxon doses in combination with 20 μg TOCP, similar results were obtained (Table 3), except in the case of the highest paraoxon dose where TOCP was almost ineffective.

TABLE 3. Mortality percentage after simultaneous application of various Paraoxon doses and TOCP (20 μg/fly) to normal houseflies.

Treatment	Paraoxon μg/fly					
	0.065	*0.07*	*0.075*	*0.08*	*0.085*	*0.09*
Paraoxon alone	36	47	69	74	79	80
Paraoxon + TOCP	15	27	36	41	47	76

More informative results were obtained when the per cent of knockdown (KD) was recorded. This experiment was carried out with paraoxon because of its rapid action. It was found that % KD is a function of TOCP dosage, if lethal paraoxon doses were applied. The higher the TOCP dosage, the longer the time needed for the toxicant to exert its action (Table 4).

TABLE 4. Knockdown percentage after simultaneous application of various TOCP doses and Paraoxon (0.09 μg/fly) to normal houseflies.

Treatment	Knockdown percentage after..... min.				
	30	*60*	*120*	*180*	*240*
Paraoxon alone	20	38	84	88	88
Paraoxon + TOCP *2* μg/fly	0	2	30	46	60
5	0	0	4	18	35
10	0	0	2	5	22
20	0	0	0	2	8
50	0	0	0	0	2
75	0	0	0	0	0
100	0	0	0	0	0

The above facts gave rise to the following hypothesis: TOCP acts by preventing the entry of the insecticide, or at least by delaying its access to the insect body.

To test this hypothesis experiments were set up to investigate the effect of various routes of administration on the interaction between TOCP and parathion. TOCP was applied either topically to the abdomen or to the thorax, or by injection. Parathion was applied topically six hours after TOCP administration. In injection, one microliter olive oil was used as the vehicle. In spite of this seemingly large volume of olive oil, injected houseflies could survive normally. If the above hypothesis is correct, the entry of the insecticide would not be barred (as deduced from mortality counts) when the two agents were administered externally to two different parts of the insect body, or when one of them was injected. Table 5 reveals, at first sight, that the above hypothesis

TABLE 5. Effect of various routes of administration on the interaction of TOCP and Parathion in normal houseflies, expressed as mortality percentage.

Olive oil (1 µl inj.)	Oil (inj.) P (th)	TOCP (inj.)	TOCP (inj.) P (th)	TOCP (top. abd) P (th)	TOCP (top. th) P (th)
2	35	6	75	90	15
TOCP (top. th) P (abd)	Acetone (th) P (th)	Acetone (abd) P (th)	Acetone (th) P (abd)	P (th)	P (abd)
62	49	44	27	45	22

TOCP = 20 and 100 µg/fly, for injection and topical application respectively
P = Parathion (0.032 µg/fly) applied topically 6 h after TOCP
inj. = injected top. = topically applied
th = thorax abd = abdomen

explains the foregoing results satisfactorily. When houseflies pre-treated with TOCP either by injection or by topical application received a dose of parathion, such that the insecticide was not administered to the site of TOCP application, synergism was brought about. When the two agents were applied topically to the same site, antagonism occurred. The degree of synergism depends upon the site of application of TOCP and the insecticide. For example, parathion administered to the thorax is more effective than when applied to the abdomen. This might be due to the proximity to the site of action (BALL and BECK, 1951) or to a reduced rate of penetration when parathion was applied to the abdomen, since the abdominal cuticle is more difficult to penetrate than the thoracic cuticle (TAHORI and HOSKINS, 1953a). The effect of TOCP was found to depend upon its site of application. Lethargic flies were more frequently seen when TOCP was applied to the abdomen. Consequently, pronounced synergism was brought about when TOCP was applied to the abdomen and parathion to the thorax, as compared with the less marked synergism in the reverse case.

It is obvious then, that susceptible houseflies react to TOCP as do the other animals reported previously. This reaction (synergism) is masked in flies, because of the unique property of TOCP which had not been previously

reported, namely, that it is capable of barring or delaying the entry of the insecticide.

In view of the above mentioned findings, one would expect a synergistic action when a mixture of TOCP and the insecticide is injected. The results of such experiments are shown in Table 6. The degree of synergism increases

TABLE 6. Effect of simultaneous injection of various TOCP doses on susceptibility of normal houseflies to Parathion (0.025 μg/fly) or Paraoxon (0.015 μg/fly) expressed as mortality percentage.

Treatment	Parathion				Paraoxon			
	TOCP μg/fly							
	30	*20*	*10*	*1*	*30*	*20*	*10*	*1*
TOCP + insecticide	75	55	44	26	88	70	54	22
Insecticide alone	30				20			
TOCP 30 μg	4							
Olive oil (1 μl)	0							

steadily with a corresponding increase in TOCP dosage. It is interesting to note that the magnitude of synergism is more conspicuous with the phosphate than with the phosphorothionate (Table 6 and Figs. 2, 3, 4). This is in sharp contrast to the previously cited conclusion of SEUME and O'BRIEN (1960a).

Thus it is now evident that the cuticle is merely the site where TOCP exerts its antagonistic action by preventing or delaying the entry of the toxicant. When the cuticle is by-passed, TOCP is no longer an antagonist, it is a synergist instead. Being aware of this interesting effect of TOCP, we performed a similar experiment to that of COLHOUN's. He injected American cockroaches with TOCP, and after 24h applied the toxicant topically. We designed a more detailed experiment. Houseflies were injected with 30 μg TOCP per fly, and after various intervals were treated topically with parathion. The insecticide was applied to the abdomen to keep as far away as possible from the site of TOCP administration, since any traces of TOCP would affect penetration. By analogy with Colhoun's results, one might anticipate a synergistic effect. Unexpectedly, antagonism was quite marked after 30 h (Fig. 1). Mortality was found to reach its maximum after 4 h, and declined steadily in spite of the peculiar progressive increase in susceptibility of the two controls. The effect of the injected olive oil in decreasing susceptibility (WIESMANN and REIFF, 1956), was obvious after 30 h. The tremendous decrease in the tolerance of S-houseflies to parathion, and not to paraoxon, is striking. The effect was even produced by merely confining untreated houseflies in milkbottles for 24 h, or even less (Fig. 4; Table 7). The results recorded in Table 7 were obtained with

TABLE 7. Effect of confining normal houseflies in milkbottles for 24 h on their susceptibility to Parathion and to Paraoxon.

Treatment		Mortality percentage	
		normal test flies	confined flies
Parathion (μg/fly)	*0.028*	15	66
	0.033	50	90
Paraoxon	*0.06*	27	32

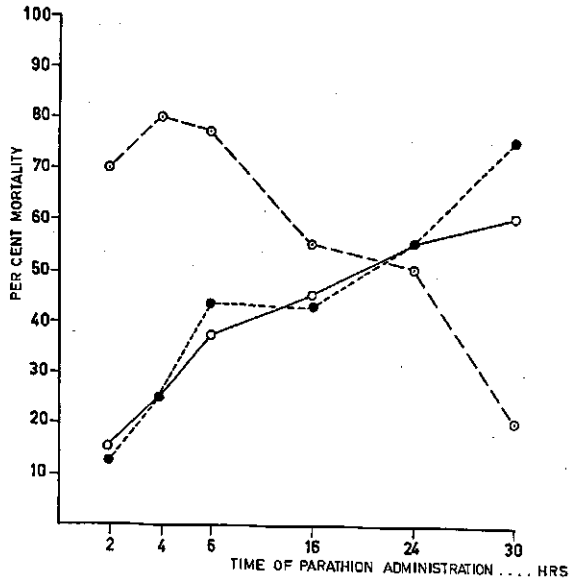


FIG. 1.

Effect of relative time of administration of parathion (0.03 μg/fly) to the abdomen after injection of TOCP (30 μg/fly) on susceptibility of normal houseflies. Injection vehicle = 1 μl olive oil.

⊙- - - -⊙ = TOCP/ parathion

○————○ = injected oil/ parathion

●- - - -● = untreated/ parathion

one batch of houseflies taken from the same holding cage to eliminate any interference from unknown factors.

Instead of applying by injection, a wide range of TOCP dosages was applied topically to the abdomen. Parathion was also applied topically, but to the thorax. Results are recorded in Fig. 2. Synergism was evident when TOCP was applied 2 h prior to parathion administration, and thereafter a steady decrease in susceptibility was obvious. It is quite plain from Fig. 2, that synergism is a function of the size of TOCP doses, whereas antagonism is not affected.

Apart from the most marked variation previously reported, that lower doses of TOCP are more effective in potentiating the toxicity of paraoxon than of parathion, another distinct difference was observed. At the 5 min time

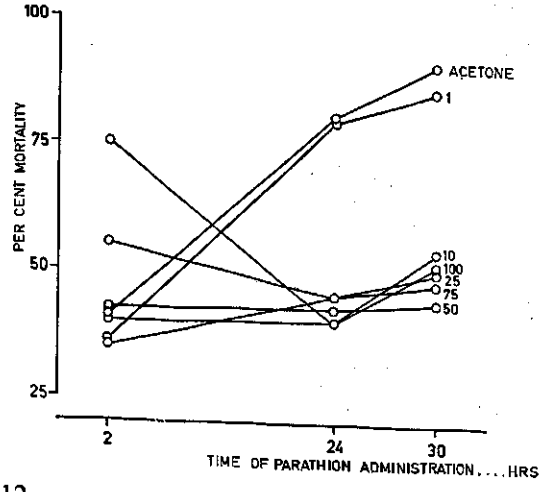


FIG. 2.

Effect of relative time of administration of parathion (0.032 μg/fly) to the thorax after TOCP application to the abdomen, on susceptibility of normal houseflies. TOCP dosages (μg/fly) are indicated in the figure.

interval, higher TOCP dosages were found to potentiate the toxicity of paraoxon, and did not appreciably alter the toxicity of parathion (Figs. 3, 4). For this striking difference two explanations are offered. Firstly, TOCP penetration and distribution inside the insect body may have been so rapid that it reached the site of insecticidal application (the cuticle of the thorax) within 5 min after application. Alternatively, TOCP might have protected, to some extent, the site of action (presumably the central nervous system) against parathion. The latter view is less likely, for simultaneous injection resulted in synergism of the same order of magnitude. Moreover, if this is the case, TOCP would have protected the site of action against paraoxon.

If the first explanation is true, why then was a synergistic effect brought about after 2 and 4 h? One would in fact anticipate an antagonistic effect. It is obvious that other factors intervene which could overshadow the antagonistic

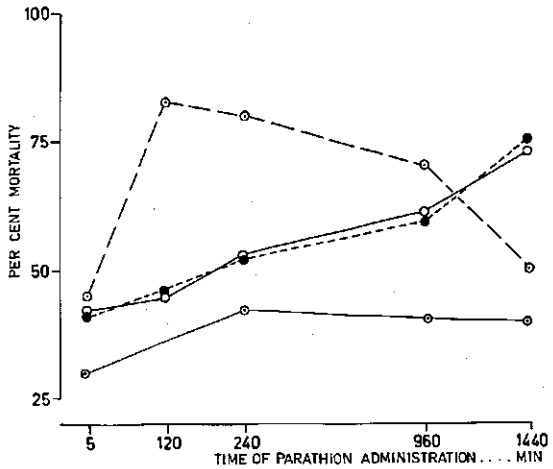


FIG. 3.
Effect of relative time of administration of parathion (0.032 μg/fly) to the thorax after TOCP application to the abdomen, on susceptibility of normal houseflies.

⊙ - - - - ⊙ = 100 μg TOCP/parathion
⊙ ——— ⊙ = 10 μg TOCP/parathion
○ ——— ○ = untreated/parathion
● ——— ● = acetone/parathion

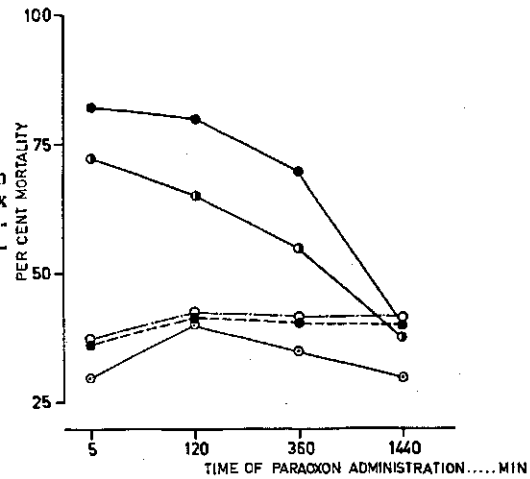


FIG. 4.
Effect of relative time of administration of paraoxon (0.065 μg/fly) to the thorax after TOCP application to the abdomen, on susceptibility of normal houseflies.

● ——— ● = 100 μg TOCP/paraoxon
◑ ——— ◑ = 50 μg TOCP/paraoxon
⊙ ——— ⊙ = 10 μg TOCP/paraoxon
○ —·—·— ○ = acetone/paraoxon
● - - - - ● = untreated/paraoxon

activity of TOCP. It can be assumed, that TOCP causes a certain lesion inside the insect body, which might be responsible for its synergistic effect. In order to cause this lesion, time is necessary, and once it occurs synergism takes place if the insecticide is not barred from entry. Apparently, at the 5 min time interval, owing to the presence of TOCP at the site of insecticidal application, even at very low concentrations, the entry of parathion is interfered with, while paraoxon requires higher concentrations of TOCP to affect its penetration. This might also be due to the low frequency of the occurrence of the lesion. As time proceeds, TOCP is operating to cause this lesion, so that after 2 to 4 h, its synergistic effect outweighs its antagonistic effect, despite the presence of TOCP at the site of insecticidal application. After 4 h, however, the reverse appears to be true. As more TOCP reaches and accumulates in the thoracic cuticle (the site of insecticidal application), its antagonistic effect comes into play in a more dominant way.

This interpretation was put to the test, by repeating the experiments of Figs. 2, 3, and 4. Instead of applying the insecticide topically after 24 h, it was injected. It is evident that instead of the marked antagonism seen in the foregoing figures, synergism was brought about (Table 8). Again, synergism is most marked with the phosphate. The order of magnitude of synergism does not appear to be affected by the time of TOCP administration.

TABLE 8. Effect of various TOCP doses applied topically to the thorax, 24 h prior to the injection of either Parathion (0.025 μg/fly) or Paraoxon (0.015 μg/fly), on susceptibility of normal houseflies. Each insecticide dissolved in 1 μl olive oil.

Pre-treatment	Mortality percentage	
	Parathion	Paraoxon
Acetone	36	27
TOCP (μg/fly) *100*	80	94
50	60	77
10	35	49

The point which is of crucial importance – just how TOCP acts as an antagonist – has not been critically studied. Conclusive evidence has been provided indicating that the antagonistic activity of TOCP is exerted within the cuticle.

TOCP is not the only synergist which possesses antagonistic activity, but it is the most striking compound in this respect, since traces of TOCP could successfully prevent or delay the penetration of the insecticide, most notably parathion. PERRY and HOSKINS (1950; 1951a) found that larger amounts of the synergist piperonyl cyclonene (5 to 10 μg per fly) used jointly with DDT appear to retard penetration and decrease mortality of susceptible houseflies. TAHORI and HOSKINS (1953b) confirmed this observation, and suggested that because of the larger molecules of piperonyl cyclonene, it enters slowly and meanwhile holds DDT in solution from which it escapes less readily than when present as the free solid. The most interesting synergist is di-(*p*-Chlorophenyl)-(trifluoromethyl)-carbinol (DTC). Its antagonistic action is due to the interference with DDT penetration (COHEN and TAHORI 1957). As the compound counteracts its own synergistic activity, an optimum ratio of synergist to DDT exists for maximum affect. Such an optimum ratio does not exist, however, for TOCP

since lower TOCP dosages which do not affect penetration, are also without any synergistic activity. The authors gave an explanation for the adverse effect of DTC on DDT penetration. It is well known that DDT affinity for chitin and its solubility in the cuticular lipids are important features of its insecticidal properties (RICHARDS and CUTKOMP 1946; LORD 1948). Accordingly, the authors proposed the following mechanism: as DTC enters the fly cuticle much more quickly than DDT it is evidently preferentially adsorbed on the chitin micells thus blocking, at least partly, the entry of DDT. Moreover, DTC is an oily liquid which may serve as a solvent for DDT.

TOCP is also an oily liquid, and it was suggested that it enters the cuticle very readily. The affinity of TOCP for chitin is not known. On the other hand, it is known that parathion and paraoxon penetrate very rapidly, as has been demonstrated with the American cockroach (METCALF and MARCH 1949; CHAMBERLAIN and HOSKINS 1951), although parathion penetrates relatively slower than paraoxon (FERNANDO *et al.* 1951). Similarly, it was found that both toxicants are rapidly absorbed by houseflies; paraoxon being absorbed more rapidly than parathion (PLAPP *et al.* 1961). Hence, we are faced with a phenomenon which is likely to be different from the above example of DDT and DTC. It is almost certain that TOCP interferes with the penetration of parathion or paraoxon, either mechanically, physically, or by a physico-chemical process. To determine which one predominates, or is the actual mechanism, further studies are required. In this respect the size of the dose appears to be an important factor. Massive doses may prevent the penetration in a mechanical way. It is inconceivable, however, that very low doses of TOCP act in this manner; a physical and/or a physico-chemical process is likely to be involved.

An alternative mechanism of antagonism has been suggested which is not attributed to a penetration phenomenon, but to the inhibition of certain biological oxidations which activate the compounds. Therefore, when activation of the phosphorothionates and several chlorinated hydrocarbons – being necessary for this type of compounds to exert their lethal action – is inhibited, antagonism takes place (SUN and JOHNSON 1960). The authors found that sesamex significantly reduced the toxicity of various thionophosphorus insecticides, such as chlorthion, parathion, methyl parathion, EPN, and some cyclodiene insecticides. HEWLETT *et al.* (1961) demonstrated that SKF (525 A), 2-diethylaminoethyl 2,2-diphenyl pentanoate, depressed the toxicity of malathion to lesser mealworm beetles *(Alphitobius laevigatus)* and to houseflies. The antagonism of the action of malathion by some known synergists of other insecticides, was first reported in the housefly by RAI *et al.* (1956) and later in *Anopheles stephensi* (HADAWAY *et al.* 1963).

In no case did the above authors express a clear view as to the mechanism of the antagonistic effects. In fact, both HEWLETT *et al.*, and HADAWAY *et al.* were inclined to accept the view of SUN and JOHNSON, stressing the importance of the inhibition of certain biological oxidations.

I.3. THE RELATIVE TOXICITY OF PARATHION AND ANALOGUES

During the course of the present investigations, paraoxon proved to be invariably less toxic than parathion when applied topically. Paraoxon was found to be more toxic than parathion when injected (Figs. 5, 6; Table 9).

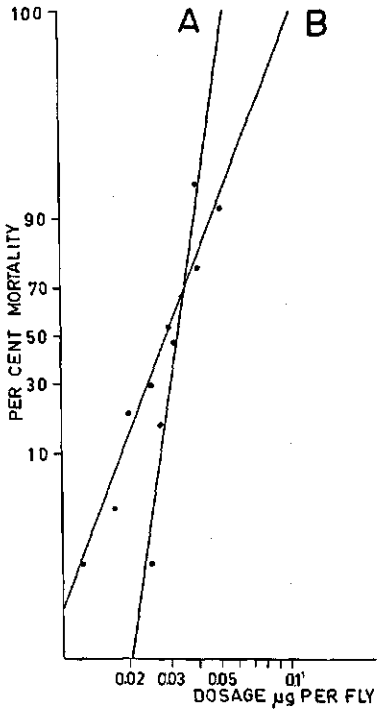


FIG. 5.

Dosage – mortality curves for 2-day-old adult females of susceptible houseflies treated with parathion either topically (A), or by injection (B). Injection vehicle = 1 µl olive oil.

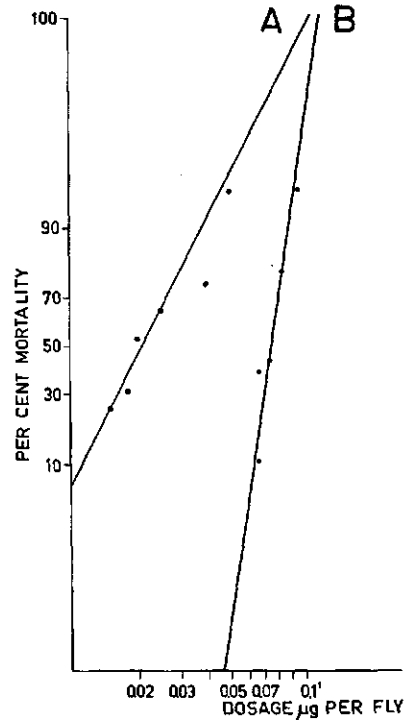


FIG. 6.

Dosage – mortality curves for 2-day-old adult females of susceptible houseflies treated with paraoxon either topically (B) or by injection (A). Injection vehicle = 1 µl olive oil.

Reviewing the literature on this matter reveals inconsistent results particularly with the topical application and injection methods. This would indicate that the route of administration is of primary importance in determining the relative toxicity of the two compounds.

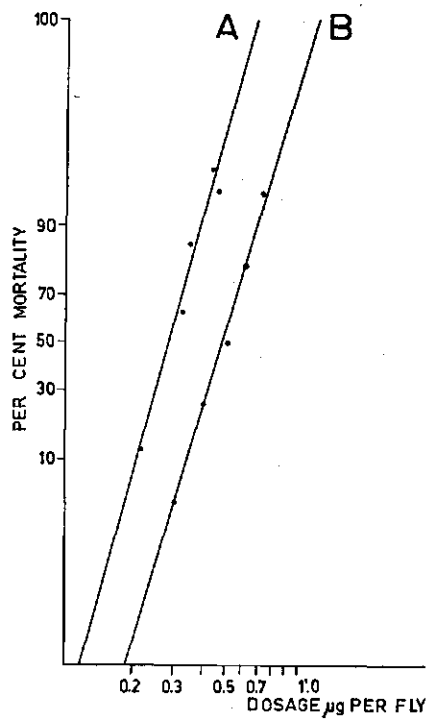
Parathion was less toxic than paraoxon when applied in the same way, either topically or by injection, into the American cockroach. No difference in the toxicity of either insecticide was observed between topical application and injection (METCALF and MARCH 1949). The LD_{50} values for houseflies treated topically with parathion or paraoxon were 0.9 and 0.55 µg/gram body weight

TABLE 9. LD_{50} values (µg/fly) for 2-day-old female normal houseflies*.

Compound	Topical application	Injection (1 µl olive oil)
Parathion	0.033	0.029
Paraoxon	0.07	0.021
S-phenyl isomer	0.28	–
S-ethyl isomer	0.49	–

* The LD_{50}'s were derived from the log dosage-probit lines.

FIG. 7.
Dosage-mortality curves for 2-day-old adult females of susceptible houseflies treated topically with S-phenyl parathion (A) or with S-ethyl parathion (B).



(g.b.w.) respectively (METCALF and MARCH 1949, 1950). Both compounds were equally toxic when injected into the American cockroach (CHAMBERLAIN and HOSKINS 1951). The insecticidal activity of topically applied paraoxon with respect to the adult males of the migratory locust was significantly greater than that of parathion, and the toxicity of injected paraoxon was of the same order as that applied topically (STRINGER 1956; STRINGER and FIELDING 1956). Paraoxon was more toxic than parathion when applied topically to houseflies. Upon injection, lower LD_{50} values were obtained, retaining the same order of toxicity as that found for topical application (BUSVINE 1959). Paraoxon was more toxic than parathion when injected into houseflies (OPPENOORTH and VAN ASPEREN 1960a). The data of PLAPP *et al.* (1961) showed, however, that paraoxon is somewhat less toxic than parathion when applied topically. HADAWAY *et al.* (1963) found that parathion applied topically is only slightly less toxic to the mosquitoes *Anopheles stephensi* and *Aedes aegypti*, and the two compounds were about equitoxic to houseflies.

In general, there is a reasonable agreement between our results and those of PLAPP *et al.* (1961) with topical application, and those of BUSVINE (1959), and of OPPENOORTH and VAN ASPEREN (1960a) with injection. It must be recalled that the houseflies used in the present studies were much larger – $\bar{x}$ = 34 mg per fly – than those commonly used – $\bar{x}$ = 20 mg per fly – (MARCH and METCALF 1949; KRUEGER and CASIDA 1957; SHEPARD 1958).

In contrast, consistent results have been obtained by the contact method of assessing the toxicity of insecticides, making use of residual films. Paraoxon was found to be invariably far less toxic than parathion. The relative insecticidal

potency of parathion and paraoxon against grain weevils was nearly 10 to 1, respectively (WOODCOCK and STRINGER 1951). Parathion was more than 4 times as toxic as paraoxon to houseflies (OPPENOORTH and VAN ASPEREN 1960a). Almost equal results have been reported by PLAPP *et al.* (1961).

It was of great interest to investigate the relative toxicities of the two isomers of parathion, *i.e.* the S-ethyl isomer and the S-phenyl isomer, after topical application. Fig. 7 and Table 9 reveal that the two isomers were far less toxic than either parathion or paraoxon. When compared with each other, the S-phenyl isomer was more toxic than the S-ethyl isomer.

The reduced toxicity of the two isomers has been also found by BENNETT *et al.* (1948), MARTIN (1950), WOODCOCK and STRINGER (1951), METCALF and MARCH (1953c).

CHAPTER II

THE SIGNIFICANCE OF THORAX CHOLINESTERASE IN ORGANOPHOSPHORUS POISONING; THE SYNERGISTIC AND ANTAGONISTIC EFFECTS OF TOCP IN TERMS OF ChE AND AliE INHIBITION

II.1. INTRODUCTION AND REVIEW OF LITERATURE

In most of the experiments in this chapter the same treatments were applied as described in chapter I. In addition to assessing the mortality, the percentage knockdown was recorded, and ChE and AliE activities were determined.

The foregoing experiments revealed that TOCP operates in two opposed (*i.e.* antagonistic and synergistic) processes. It was anticipated that ChE and AliE measurements would yield further evidence as to their possible nature. It is worth mentioning that AliE inhibition has been suspected to be involved in synergism (MURPHY *et al.* 1959; COLHOUN 1960; OPPENOORTH and VAN ASPEREN 1961).

TOCP is of considerable interest to pharmacologists because of its ability to produce flaccid paralysis in some mammalian species (THOMPSON 1954; see also p. 7). HOTTINGER and BLOCH (1934) found that TOCP inhibited ChE in rabbits. One year later, MENDEL and RUDNEY (1944) clearly distinguished between true and pseudo-ChE and showed that TOCP selectively inhibited pseudo-ChE in the rat after an oral administration of 5 gm/kg. Treated rats did not produce any symptoms. With human tissues, concentrations of TOCP which caused 75–99 % inhibition of pseudo-ChE, inhibited the true ChE by only 7–10 %. Similar results were obtained with rabbit and chicken tissues (EARL and THOMPSON 1952a). After feeding TOCP to hens, the true ChE was found to be relatively unaffected, whereas pseudo-ChE activity was markedly diminished (EARL and THOMPSON 1952b). Paralysis and demyelination have also been observed (BARNES and DENZ 1953). True ChE of chickens was only slightly inhibited by TOCP, while pseudo-ChE was selectively inhibited *in vivo* and *in vitro* (DAVISON 1953a).

In contrast, true ChE has been found by some other authors to be considerably inhibited *in vivo*. ALDRIDGE (1954) injected rabbits intravenously with 6.8 mg/kg, and reported 81 % inhibition of pseudo-ChE, and 58 % inhibition of true ChE.

COURSY *et al.* (1957) injected rats intraperitoneally with a large dose of TOCP (2 ml/kg) and found, in contradistinction to all above reports even Aldridge's, that TOCP was an active inhibitor of true ChE, pseudo-ChE was less inhibited. Cholinergic symptoms were noted when activity of the true ChE in erythrocytes and brain fell to less than 20 % of normal. Using smaller dosages, ChE measurements on brain, submaxillary glands and serum of the rat, implied that no inhibition of the enzyme activity occurred after three intramuscular injections of 110 mg/kg of TOCP or after a single cutaneous application (MURPHY *et al.* 1959). Only two publications on this matter are found in the literature concerning insects, the results of which are in good agreement. STEGWEE (1960) has shown that TOCP at a concentration of 10^{-4} M did not affect the ChE activity of houseflies *in vitro*. The ChE activity of flies treated topically with 200 μg TOCP/fly was unimpaired, and only slightly inhibited (11 %) when 20 μg/fly was injected. COLHOUN (1960) reported neither *in vitro* inhibition of ChE of the nerve cord of the roach at 10^{-4} M TOCP, nor did he find ChE to be affected *in vivo* after injection with 100 μg TOCP/roach.

Investigations on both mammals and insects have shown that TOCP is a selective inhibitor of AliE. The AliE of rats was selectively inhibited *in vivo* by the intramuscular injection of TOCP (MYERS and MENDEL 1953). The hydrolysis of tributyrin by rat serum was about 50 % inhibited by 300 μl TOCP/l after a 30 min incubation period and by 0.6 μl/l after incubation for 20 h. After the intramuscular injection of about 50 μl TOCP/kg into rats, the serum tributyrinase was almost completely inhibited (MENDEL and MYERS 1953). The tributyrinase activity of the spinal cords of hens poisoned with TOCP has been found to be moderately reduced (EARL *et al.* 1953). The I_{50} (the molar concentration of inhibitor that gives 50 % enzyme inhibition) of housefly AliE was 2.5×10^{-5} M of TOCP; at a dosage of 100 μg TOCP/fly the AliE activity was completely inhibited after 24 h (STEGWEE 1960). The I_{50} of roach AliE was 10^{-5} M of TOCP; 24 h after treatment, AliE was found to be completely inhibited (COLHOUN 1960).

The relative importance of ChE and AliE in organophosphorus poisoning has been a subject of considerable controversy for some years. VAN ASPEREN (1957, 1958a), using DDVP (O,O-dimethyl-O-2,2-dichlorovinyl phosphate) and adopting the substrate protection technique, found that housefly ChE at time of knockdown was invariably far less inhibited than AliE. Accordingly he did not favour the idea of an essential role of ChE inhibition in the insecticidal action of DDVP, but rather thought that AliE inhibition was of vital importance in organophosphorus intoxication. In another publication, VAN ASPEREN (1958b) was not only inclined to reject an essential role of ChE inhibition but also of that of AliE. Later VAN ASPEREN and OPPENOORTH (1959) concluded that AliE could not be the primary site of attack in organophosphorus poisoning. Two further pieces of evidence against the importance of AliE inhibition in the intoxication process were provided by STEGWEE (1960) and COLHOUN (1960) who found that the complete inhibition of AliE activity in the housefly and in the American cockroach was not necessarily associated with poisoning symptoms, and did not result in appreciable mortality.

II.2. Results and discussion

II.2.1. *The in vivo effect of TOCP on ChE and AliE activities*

It was of considerable interest to determine the time course of inhibition of AliE and ChE activities after treating houseflies with TOCP. We had in mind that TOCP might inhibit AliE, without affecting ChE, and this inhibition probably played a dominant role in its synergistic action. Various TOCP dosages in acetone solutions applied topically to different body parts of *Musca* could selectively, but not completely inhibit AliE (Fig. 8). In no case did TOCP inhibit ChE to any appreciable degree; the maximum inhibition observed was 6 %, 24 h after the application of the massive dose of 100 μg/fly. Confining untreated houseflies in milkbottles during the indicated time intervals did not affect ChE and AliE activities. The same was equally true when the flies were treated topically with the solvent only.

A glance at Fig. 8 shows that TOCP was a very slowly acting inhibitor. In the first stages inhibition proceeds rather rapidly and then relatively slowly during the last stages. It is interesting to note that the higher doses of TOCP failed to produce more than 50 % inhibition. This is in contrast to the findings of Stegwee (1960) who showed with an equal dosage the complete inhibition of the enzyme after 24 h of TOCP administration.

Occasionally a number of flies died after treatment with massive TOCP doses, particularly when administered to the abdomen. Although they died in a manner distinct from that found with parathion or paraoxon poisoning, it was suspected that their ChE activity might be inhibited. Determinations of thorax ChE and abdomen AliE in dead flies after 24 h of TOCP administration to the abdomen showed that ChE was uninhibited. The curious fact was, that their AliE activity was inhibited to essentially the same degree as in the living flies receiving equal amounts (50 % of normal).

The results summarized in Fig. 8 might still be influenced by a possible "homogenization artifact", in spite of adopting the substrate protection technique. This would probably be due to applying massive doses of TOCP (50 to 200 μg/fly), in addition to using the site of application or an adjacent part as the source of AliE. The above argument can almost be disproved by two observations: firstly, TOCP is not a powerful anti-AliE agent, and secondly, the difference between AliE inhibition in the thorax and in the abdomen is not considerable when TOCP is applied to either site. It would appear, therefore, that this difference may be attributable to the proximity of the enzyme to the site of application, rather than to a "homogenization artifact".

II.2.2. *ChE and AliE measurements after parathion and paraoxon poisoning*

Being now aware of the effect of TOCP on ChE and AliE activities and the pattern of AliE inhibition, it was necessary to determine the effect of topically applied parathion and paraoxon on both enzymes, before going a step further to determine the joint action of either insecticide and TOCP.

The size of the dose in the present series of experiments as well as throughout the coming experiments, was chosen such that the observed final mortality after 24 h was somewhat lower than 50 %. These dosages were 0.032 and 0.065 μg/fly for parathion and paraoxon respectively. Applying such amounts enabled us to study the possible correlation between knockdown (K.D) percentage and degree of esterase inhibition, which was of great help in evaluating the relative

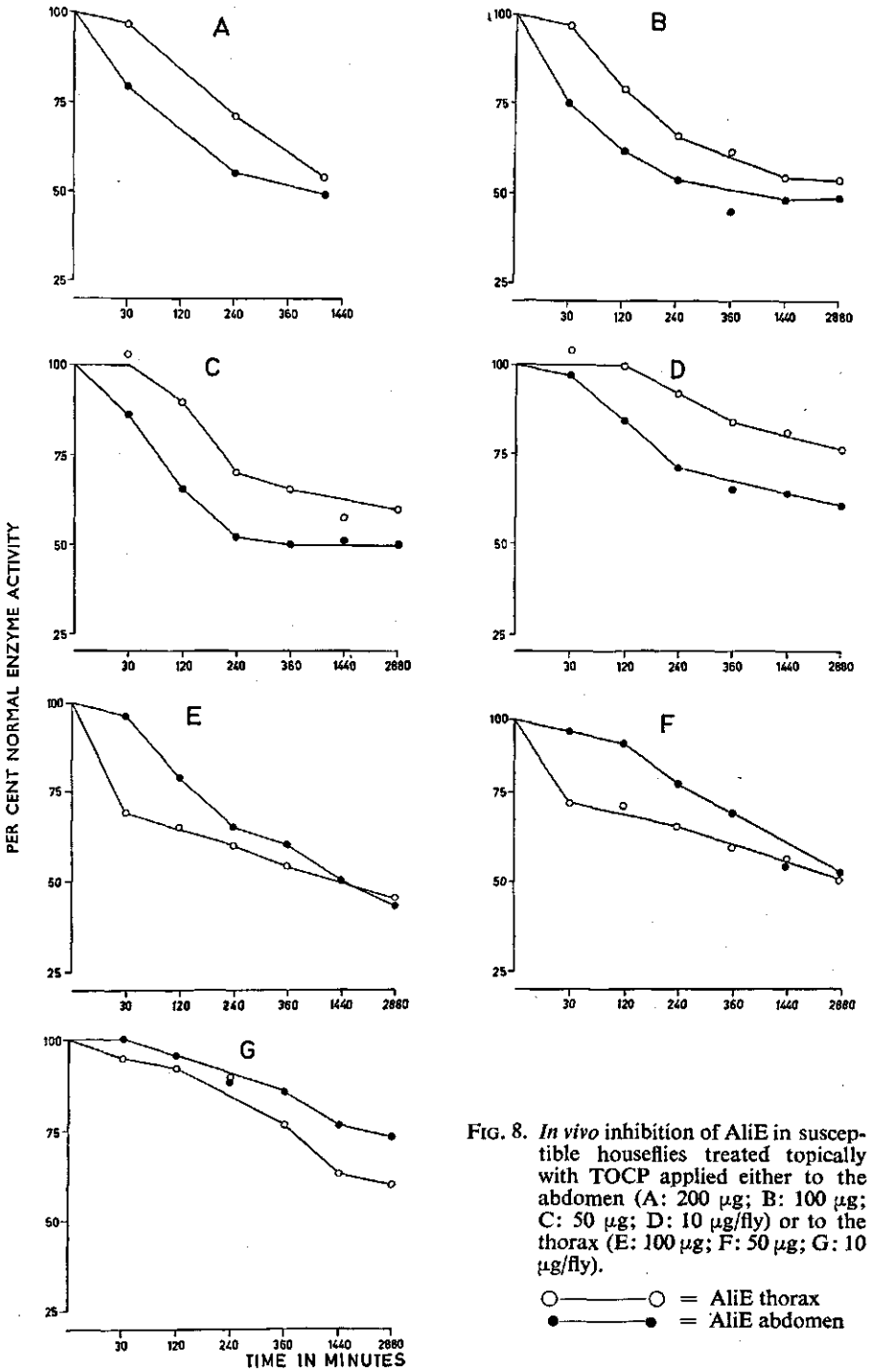


FIG. 8. *In vivo* inhibition of AliE in susceptible houseflies treated topically with TOCP applied either to the abdomen (A: 200 μg; B: 100 μg; C: 50 μg; D: 10 μg/fly) or to the thorax (E: 100 μg; F: 50 μg; G: 10 μg/fly).

○———○ = AliE thorax
●———● = AliE abdomen

physiological importance of the two studied esterases in organophosphorus poisoning. Moreover, applying these doses of either insecticide in studying their joint action with TOCP, could reveal distinctly the antagonistic and synergistic activities of the latter compound. The per cent of knockdown is a more reliable estimate than observations of the earlier symptoms of poisoning, since the latter are liable to greater personal error.

For the determinations of ChE and AliE [defined as the methylbutyrate hydrolyzing enzyme(s)], all treated flies were used up to 6 h after poisoning. After 24 h dead as well as surviving flies were analyzed separately. Taking dead flies into consideration was indispensible in assessing the contribution of each esterase in relation to death.

A special comment should be made regarding housefly ChE. The properties of housefly ChE are more or less comparable to those of the true ChE as found in the nervous system and red blood cells of vertebrates (VAN ASPEREN 1959; KANEHISA 1961). Because housefly ChE is located mainly in the head (VAN ASPEREN 1959; STEGWEE 1960), which contains comparatively little tissue other than the nervous system, it was customary for most workers to use heads as the source of the enzyme. Recently, it has been demonstrated that inhibition of housefly brain ChE is not likely to be important in organophosphorus poisoning (see section on Materials and Methods). Although the thorax contains much less ChE (VAN ASPEREN; STEGWEE, *loc. cit.*) it could be measured with great ease and accuracy.

The distribution of AliE in houseflies is distinctly different from that of ChE. VAN ASPEREN (1959) found equal amounts of the enzyme in the abdomens and thoraces, and much less in the head. STEGWEE (1960) and KANEHISA (1961) reported that the abdomen contains the highest activity of the enzyme. In nearly all experiments described in the present study, ChE and AliE activities were determined in the same flies; ChE in thorax breis and AliE in abdomen breis. This allowed a direct comparison of the inhibition of both enzymes in the same sample of insects.

In Chapter I, it was observed that susceptibility to parathion increased to a great extent when untreated houseflies were merely confined in milkbottles. This is closely associated with severe ChE inhibition, abrupt and high KD percentage, and a shorter latent period (Fig. 9; A, B). By contrast, susceptibility towards paraoxon was not appreciably altered and consequently the pattern of ChE inhibition is almost identical in both cases (Fig. 9; C, D).

The results summarized in Fig. 9 reveal many interesting points. The pattern of AliE inhibition shows no marked variation, nor is there a correlation between AliE inhibition and KD percentage. During the first stages of poisoning, although treated flies were almost normal, AliE was the most inhibited enzyme. ChE inhibition occurred more slowly than AliE inhibition, and was more closely associated with KD percentage. The reason why AliE is so readily inhibited during the first stages of poisoning is probably that 96 % of it is outside the nervous system and therefore is readily available for inhibition; by contrast 91 % of the total ChE of the thorax was found to be present in the ganglia (STEGWEE 1959). Moreover, the affinity of AliE to organophosphorus compounds is higher (VAN ASPEREN and OPPENOORTH 1960).

VAN ASPEREN (1958a) showed that when flies were knocked down by DDVP, whole fly AliE was inhibited by 83 % and the ChE to a lesser degree depending upon its source; (whole fly ChE 27 %; brain ChE 24 %; and thorax plus ab-

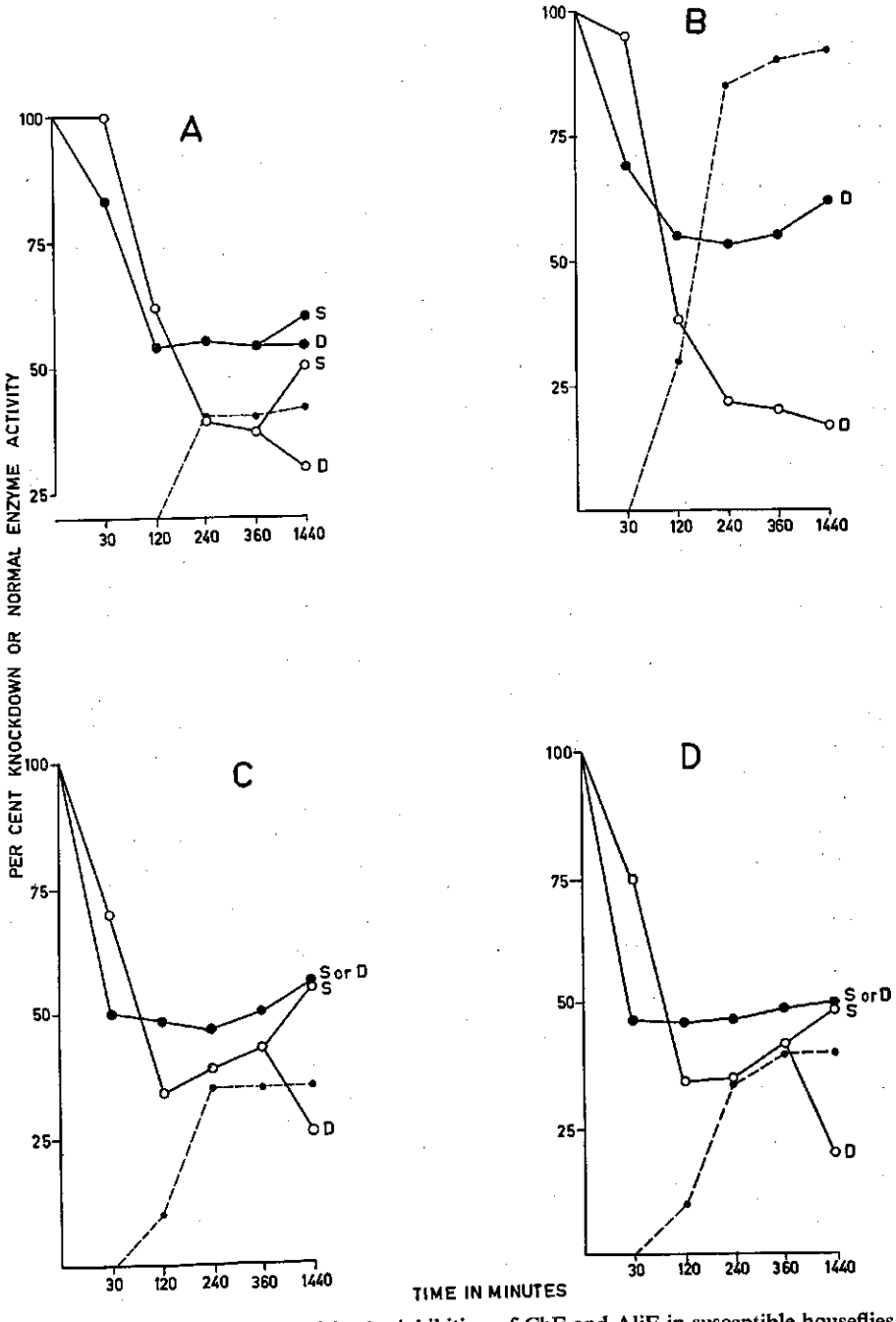


FIG. 9. Per cent knockdown and *in vivo* inhibition of ChE and AliE in susceptible houseflies treated topically with parathion (A, B: 0.032 μg/fly) or with paraoxon (C, D: 0.065 μg/fly).
A, C = flies confined in milkbottles for 24 h before treatment.

●- - - -● = % KD
●————● = AliE
○————○ = ChE
S, D = surviving and dead flies

domen ChE 46 %). Substantially similar results have been found with paraoxon, parathion, Diazinon and Co-ral for whole fly ChE and AliE (VAN ASPEREN 1960). Subsequent work by O'BRIEN (1960) with houseflies treated with the LD_{50} dose of parathion, malathion, Diazinon, and Co-ral, has shown similar results. Maximum inhibition was 60 % for ChE and 85–95 % for AliE. STEGWEE (1960) showed that one hour after treatment with TEP, thorax AliE was inhibited by 97 %, and thorax ChE by 45 % 20 h after treatment, AliE of recovered flies was inhibited by 77 %, but their ChE by only 25 %. PLAPP and BIGLEY (1961) found that inhibition of AliE by an LD_{50} of parathion or malathion was very rapid and did not exceed 50 % of normal. Maximum inhibition of ChE always occurred at or near the time of knockdown and was 69 % 4 h after treatment with parathion. Similar work by O'BRIEN (1961) indicated that inhibition of AliE was always greater than that of ChE.

The degree of ChE inhibition found in our experiments is in general agreement with that of the above mentioned reports. Meanwhile, AliE inhibition values vary to a great extent from most of the foregoing indicated values and agree with those reported by PLAPP and BIGLEY (1961). Some aspects of the results in Fig. 9 are at variance with those of PLAPP and BIGLEY (1961) who found a greater reversibility of AliE inhibition; its activity was always near normal levels 24 h after treatment. The present investigations show that ChE recovery in survivors is evident, whereas no substantial recovery occurs in dead flies. Slight or no recovery was found with AliE regardless of the fate of the fly.

The most interesting findings are that in dead flies ChE is severely inhibited (70–80 % after 24 h) and its maximum inhibition is always greater than that of AliE. Similar results found by O'BRIEN (1961), led him to conclude that this observation radically changes the picture of the relative susceptibilities of ChE and other esterases. The difference between ChE inhibition in surviving and dead flies, ranges from 20 to 30 % or even more, but is seldom less than 20 %. The corresponding difference in AliE inhibition usually does not exceed 5 %, a result which appears to be strong evidence for the cholinesterase hypothesis and rules out the possibility of AliE inhibition as being positively involved in organophosphorus poisoning. This conclusion is in harmony with those of VAN ASPEREN and OPPENOORTH (1959), STEGWEE (1960), COLHOUN (1960), PLAPP and BIGLEY (1961) although the nature of the evidence is quite different, and with that of O'BRIEN (1961).

The widely accepted belief that ChE inhibition is directly related to toxicity does not imply its complete inhibition. In the present study and in other studies as well, making use of the substrate protection technique, complete inhibition has seldom been observed. Histochemical studies of ChE in the central nervous system of houseflies did not show complete inhibition of the enzyme, even after high doses of Diazinon causing over 99 % kill (MOLLOY 1961). The extremely high values of ChE inhibition after organophosphorus poisoning, as reported by earlier workers (CHADWICK and HILL 1947; METCALF and MARCH 1949; CHAMBERLAIN and HOSKINS 1951), were possibly due to "homogenization artifacts". At that time, the substrate protection technique was not known. Their *in vivo* work showed a correlation between symptoms and ChE inhibition; in general, 50 % inhibition led to hyperexcitability; 65 % to knockdown, 90 % to prostration, and 98 % to death. Surprisingly enough, after the development of the protection technique almost identical results have

been shown recently by KANEHISA (1961). It is very probable that this author did not use the protection technique.

II.2.3. *The significance of housefly brain ChE inhibition after poisoning*

A striking difference concerning the pattern of ChE inhibition has been noted between our results and those of MENGLE and CASIDA (1958), and MENGLE and O'BRIEN (1960). MENGLE and CASIDA, working with 17 organophosphates in amounts corresponding to the LD_{50}, showed that housefly brain ChE was usually largely inhibited within a few hours after treatment, but always recovered in the survivors to 90 % or more of normal within 24 h. The validity of their data was questionable owing to the possible occurrence of homogenization artifacts, since the authors did not use the protection technique. Using this technique, MENGLE and O'BRIEN could duplicate the results of MENGLE and CASIDA and concluded that the rapid *in vivo* inhibition and recovery of housefly brain ChE activity is a real phenomenon.

The marked difference observed in the pattern of inhibition between thorax ChE and brain ChE, particularly in the complete recovery of brain ChE, prompted us to perform a similar experiment, using this time housefly heads as the source of the enzyme. The complete recovery of brain ChE is ascertained (Fig. 10), even when the surviving flies were still suffering from poisoning. The

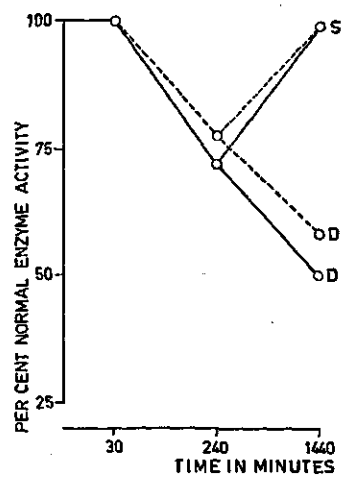


FIG. 10.
In vivo inhibition of brain ChE in susceptible houseflies treated topically with parathion (0.032 µg/fly) or with paraoxon (0.065 µg/fly).
○————○ = parathion treated
○ - - - - ○ = paraoxon treated
S, D = surviving and dead flies

degree of inhibition after 4 h is far less severe than that reported by the above authors. In our experiments, brain ChE is evidently not substantially inhibited as in the case of thorax ChE, although the flies were treated with equal doses of either insecticide. Furthermore, brain ChE inhibition in dead flies is almost equal to that found in the thoraces of surviving flies. Similarly, MENGLE and CASIDA (1958) found that brain ChE was 57 % inhibited in dead flies, after 21 h of poisoning with the LD_{50} of Thimet.

Another interesting feature of Fig. 10 is that the pattern of ChE inhibition after applying the slowly acting insecticide (parathion), or the rapidly acting (paraoxon), is essentially similar. One might anticipate, in view of our observations with thorax ChE, that the enzyme is rapidly inhibited by paraoxon

and slowly by parathion. The pattern of brain ChE inhibition by paraoxon, however, is not consistent with this view. Our results summarized in Figs. 9 and 10 confirm the conclusions of MENGLE and CASIDA (1958) that there is a lack of any general correlation between the degree of brain ChE inhibition and the occurrence of symptoms of organophosphate poisoning. They also agree with the statement of O'BRIEN (1961), that it is improbable that inhibition of the brain ChE is important in poisoning.

We now have an interesting paradox: in survivors brain ChE activity recovers almost completely within one day after poisoning, whereas thorax ChE activity recovers also, but not completely, and remains at nearly 50 % of normal. In an attempt to explain the complete recovery of brain ChE, MENGLE and O'BRIEN (1960) concluded that a factor exists in living flies which can reactivate inhibited ChE. This factor would be very labile after homogenization, and would presumably be of reduced effectiveness in flies killed by organophosphates, since their ChE fails to recover. This hypothesis was evidently brought forward to explain the discrepancy between the very rapid *in vivo* recovery of housefly brain ChE and the slow recovery of mammalian ChE after poisoning. In our experiments a similar discrepancy is observed between brain ChE and thorax ChE inhibition. Thus, if the explanation of MENGLE and O'BRIEN holds true, the reactivation factor should be located mainly in the head, and to a much lesser degree in the thorax.

It is noteworthy to report in this respect, that the complete recovery of brain ChE of houseflies escaping poisoning by an organophosphate was not observed by STEGWEE (1960) and by PLAPP and BIGLEY (1961). The former author found a more or less similar pattern of housefly head and thorax ChE inhibition after receiving a sublethal dose of TEP. The latter authors indicated a slight recovery of head ChE activity in surviving flies after treatment with the LD_{50} of parathion and malathion.

II.2.4. *The joint action of TOCP and organophosphorus insecticides*

It was shown that TOCP is a slow AliE inhibitor; maximum *in vivo* inhibition was about 50 % after 24 h. On the other hand, parathion and paraoxon inhibited AliE very rapidly; maximum inhibition was approximately 50 % after 30 min in paraoxon intoxication and 120 min in parathion poisoning. The AliE activity remained inhibited thereafter with slight or no recovery. The clear difference in the pattern of AliE inhibition, by TOCP and either of the two insecticides, was expected to enable us to assess the contribution of the separate agents when two of them – TOCP was always participating – are applied to the same fly, at least during the first stages of poisoning. In addition, as any of the three agents could halve the AliE activity, then an additive effect could probably be produced.

It was concluded that the antagonistic effect of TOCP is attributable to preventing or delaying the entry of the insecticide. If this is true, and since TOCP does not inhibit ChE, one might expect a low percentage of ChE inhibition when antagonism is revealed. Owing to the extremely rapid penetration of paraoxon, inconsistent results were obtained as mentioned previously. This difficulty could easily be overcome by applying TOCP 5 min prior to paraoxon, to the same site, usually the thorax. The data obtained from this experiment and a corresponding one which merely differed in the site of TOCP administration are presented in Fig. 11. Antagonism is closely associated with a profound

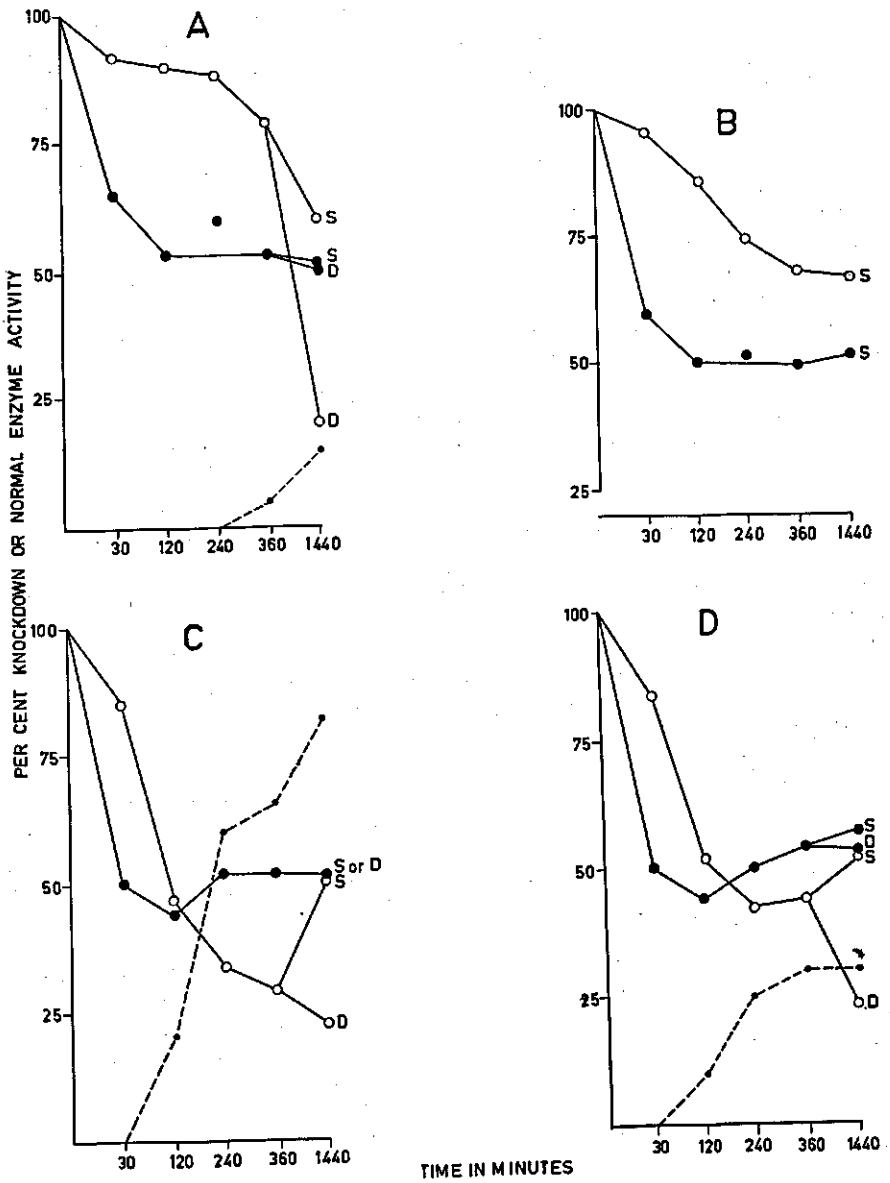


FIG. 11. Per cent knockdown and *in vivo* inhibition of ChE and AliE in susceptible houseflies treated topically with paraoxon (0.065 μg/fly), 5 min. after the administration of TOCP (A, C: 100 μg; B, D: 10 μg/fly).

A, B = TOCP applied to the thorax
C, D = TOCP applied to the abdomen

●- - - -● = % KD
●————● = AliE
○————○ = ChE
S, D = surviving and dead flies

delay in ChE inhibition, being most conspicuous in the case of 100 μg TOCP (Fig. 11; A, B). Few flies of the treated population were knocked down much later than those treated with paraoxon alone. When TOCP was applied to the abdomen, two distinct effects were evident (Fig. 11; C, D). It is obvious that 100 μg TOCP increased the toxicity of paraoxon, while 10 μg resulted in slight antagonism. The degree of ChE inhibition in both cases parallelled the intensity of toxic symptoms.

Although the protection technique has been used throughout the present series of experiments, it was uncertain whether it would lend appropriate protection against the free inhibitor, especially when the thorax (being the site of insecticidal application) was used for analysis. Many authors take some precautions in this respect, *e.g.* by applying the insecticide to the tip of the abdomen and determining ChE in the head. In fact, results summarized in Figs. 11 and 12, prove unquestionably the validity of the protection technique, even when the insecticide was still being held in the integument for some hours after treatment.

More clear results were obtained with parathion when applied simultaneously with TOCP. It has been shown that antagonism was most marked with parathion. Now it can be said that the pattern of ChE inhibition reveals the same phenomenon. Penetration of the insecticide is completely prevented when it is applied simultaneously with 100 μg TOCP. On the other hand, when applied with 10 μg TOCP, the insecticide is barred from entry for at least 6 h. Thereafter it penetrates slowly through the cuticle, so that the final mortality is negligible and ChE is inhibited by only 35 % after 24 h (Fig. 12; A, B). AliE inhibition is almost exclusively attributable to TOCP at the higher dose, while at the lower dose TOCP is almost the sole inhibitor during the first few hours after the administration of the two compounds.

It was supposed that when TOCP is applied to the abdomen, it penetrates very readily, thus reaching the thoracic cuticle within 5 min after treatment. This would affect the penetration of parathion, but not that of paraoxon. Experiments with parathion and TOCP, carried out along the same line as those with paraoxon (see Fig. 11; C, D) substantiated this supposition. ChE inhibition values are slightly higher than in the corresponding experiment with parathion alone (Fig. 12; C, D, E). It is interesting to note the clear difference in AliE activity between flies treated with the three TOCP doses at the 30 min interval; being 60, 73 and 87 % with 100, 50 and 10 μg TOCP respectively. Apparently, these values can not be attributed to either TOCP or parathion when applied separately. The additive effect of both compounds manifests itself only during the first 30 min after their application. After 30 min, however, AliE inhibition values are almost equal in all cases and vary around 50 %.

Results obtained when TOCP was applied 2 h prior to the insecticidal treatment, are given in Figs. 13 and 14. The indicated time intervals are counted after the insecticidal treatment. Fig. 13; A, B reveals the usually encountered relation; severe ChE inhibition is accompanied by abrupt KD and higher mortality. There is no relation whatsoever between AliE inhibition and poisoning symptoms or final mortality. The interesting feature of Fig. 13 (C, D) is the low rate of knockdown due to the antagonistic activity of TOCP, although the final mortality does not greatly differ from the corresponding controls.

It is almost certain that during the first stages of poisoning both agents participate in inhibiting AliE. Since the final degree of inhibition can be brought about by each one of them, it is not clear whether this inhibition is attributable

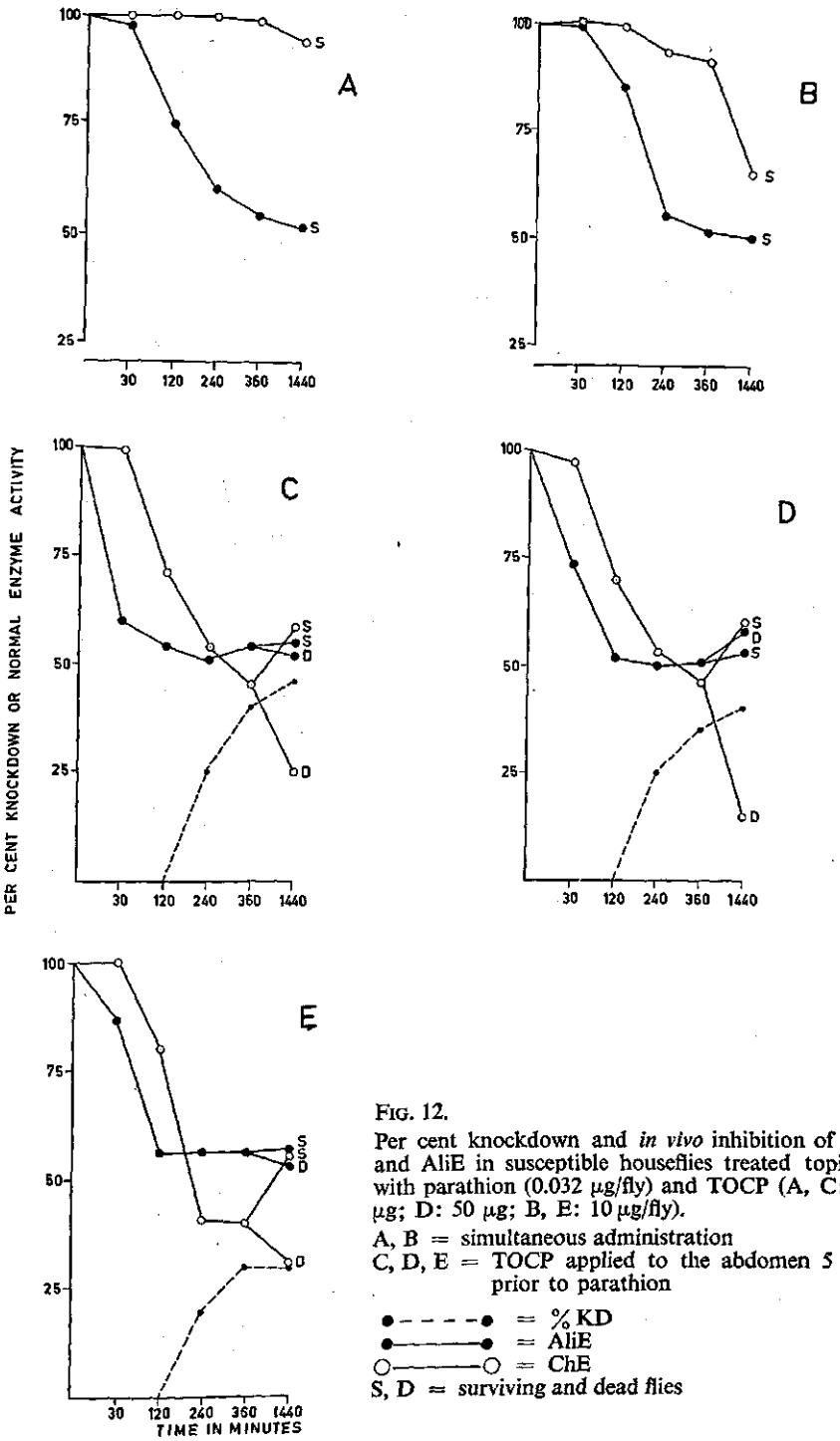


FIG. 12.

Per cent knockdown and *in vivo* inhibition of ChE and AliE in susceptible houseflies treated topically with parathion (0.032 μg/fly) and TOCP (A, C: 100 μg; D: 50 μg; B, E: 10 μg/fly).

A, B = simultaneous administration

C, D, E = TOCP applied to the abdomen 5 min prior to parathion

●- - - -● = % KD

●————● = AliE

○————○ = ChE

S, D = surviving and dead flies

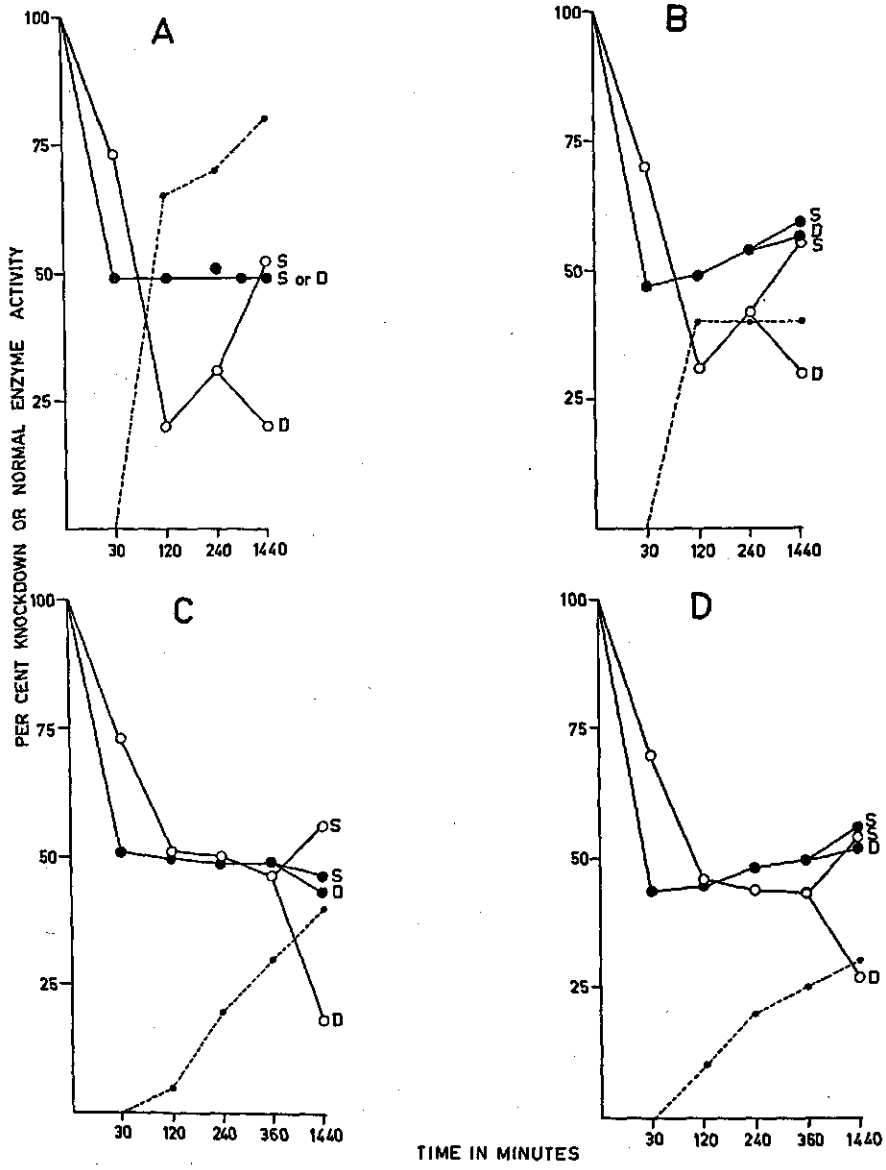


FIG. 13. Per cent knockdown and *in vivo* inhibition of ChE and AliE in susceptible houseflies treated with TOCP (A, C: 100 μg; B, D: 10 μg/fly) applied topically to the abdomen followed by paraoxon (0.065 μg/fly) applied topically to the thorax (A, B: after 2 h; C, D: after 24 h.

●- - - -● = % KD
●————● = AliE
○————○ = ChE
S, D = surviving and dead flies

to one or to both agents. If the affinity for the enzyme plays a dominant part in this connection, it seems safe to conclude that AliE inhibition during the last stages of intoxication may be attributable to the insecticide. This has probably occupied the active centres of the enzyme by a displacement process. Alternatively, one cannot exclude the possibility that the active centres of the enzyme being occupied by TOCP molecules remain blocked by this compound, and those attacked by the insecticide molecules are occupied by the same. Identical experiments with parathion (Fig. 14) are in general harmony with the above described experiments. The most conspicuous evidence of Fig. 14 is that the latent period is not altered when synergism is manifest. This fact is in contrast to COLHOUN's results which indicated that the time to prostration of TOCP plus parathion treated roaches was 112 $\pm$ 28 min compared with 201 $\pm$ 65 min for roaches treated with parathion alone. Probably the size of the dose besides other structural differences, might have accounted for this variation. There is, however, a general agreement with GAINES (1962) who observed that phenothizine derivatives administered to parathion poisoned rats increased its toxicity, but did not alter the time of onset of toxic symptoms.

II.2.5. *Injection Experiments*

The general features of the previous experiments, in which the two agents were applied separately or jointly by topical application, hold true for injection experiments, but the details vary to some degree. The masking effect of TOCP present in the cuticle and interfering with the entry of the insecticide, is eliminated when the two compounds are injected simultaneously. Obviously, houseflies cannot endure the injection of the massive TOCP doses such as were applied topically. The amounts of TOCP injected were 30, 20 and 10 μg/fly, which are almost equally potent to inhibit AliE as 100, 50 and 10 μg/fly (Fig. 15). One microliter olive oil when injected into houseflies did not inhibit either ChE or AliE. The difference between thorax and abdomen AliE inhibition which was observed after topical application of TOCP was less obvious or almost absent when this substance was injected. Again maximum AliE inhibition was not more than 50 %, but it seems that the enzyme was more readily inhibited than in topical application. Injecting TOCP alone did not result in any considerable degree of ChE inhibition.

The behaviour of TOCP after injection, concerning its inhibitory power of AliE, was similar to that after topical application. An entirely different vehicle was sought to investigate the possibility of any interference of olive oil. Absolute ethanol was chosen, although it had the disadvantage that it resulted in locomotory instability for a few hours after injection. Mortality percentage after 24 h was about 5 %. The ChE and AliE activities of the ethanol-injected control flies were not affected.

TOCP was dissolved in ethanol and injected (30 μg in 0.5 μl/fly). The pattern of AliE inhibition was not altered appreciably (Fig. 16). Surprisingly enough, TOCP caused a marked ChE inhibition after injection, associated with high mortality (about 50 %). These events were not observed at all in the foregoing experiments. Obviously, the only difference was the injection vehicle. For injection, olive oil was used by STEGWEE (1960) and by us; propylene glycol was used by COLHOUN (1960). For topical application TOCP was dissolved in acetone. In no case did TOCP materially inhibit ChE activity. Ethanol was found to protect ChE against inhibition by organophosphates *in vitro* (O'BRIEN

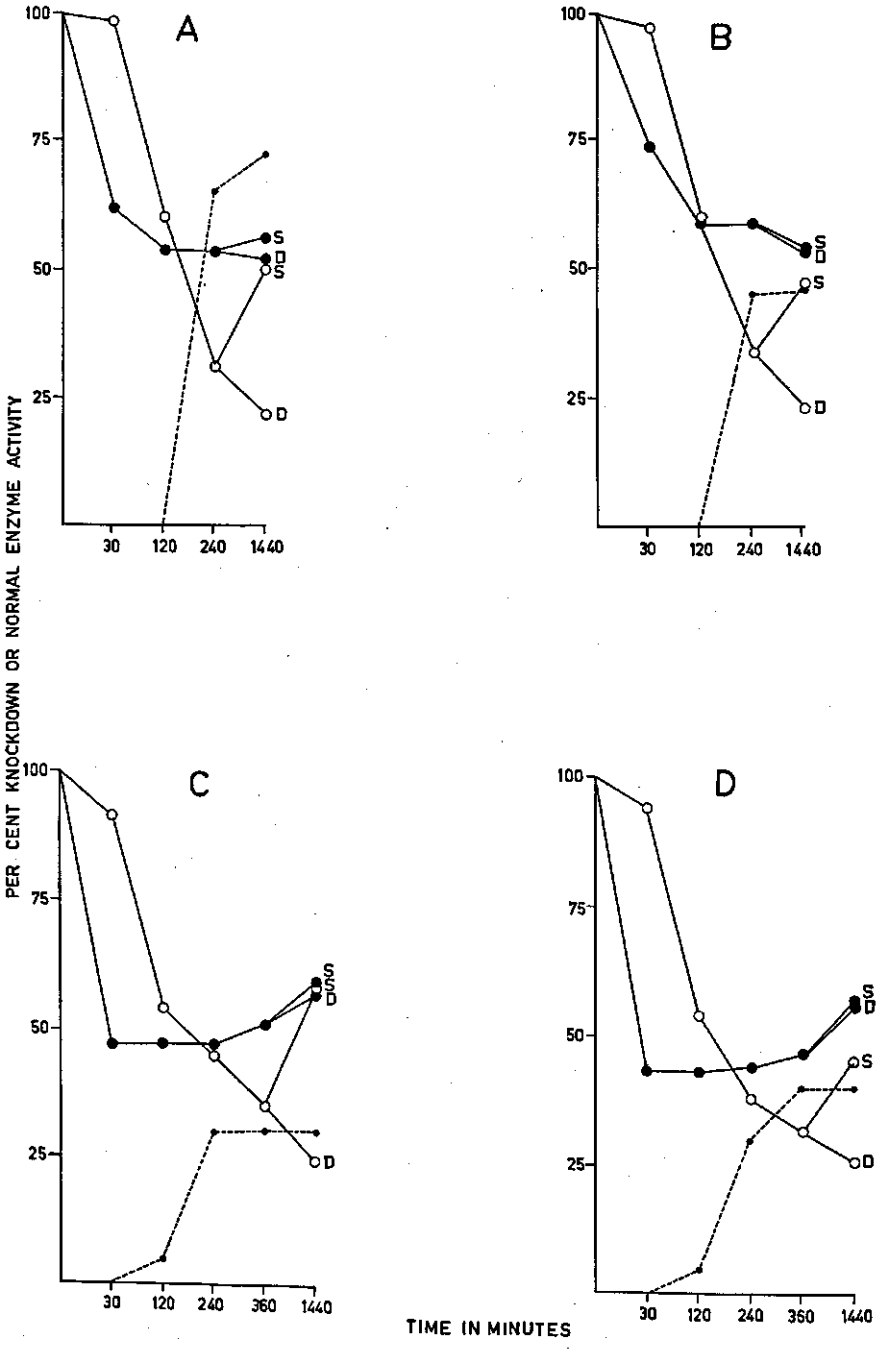


FIG. 14. Per cent knockdown and *in vivo* inhibition of ChE and AliE in susceptible houseflies treated with TOCP (A, C: 100 μg; B, D: 10 μg/fly) applied topically to the abdomen, followed by parathion (0.032 μg/fly) applied topically to the thorax (A, B: after 2 h; C, D: after 24 h).

●- - - -● = % KD
●————● = AliE
○————○ = ChE
S, D = surviving and dead flies

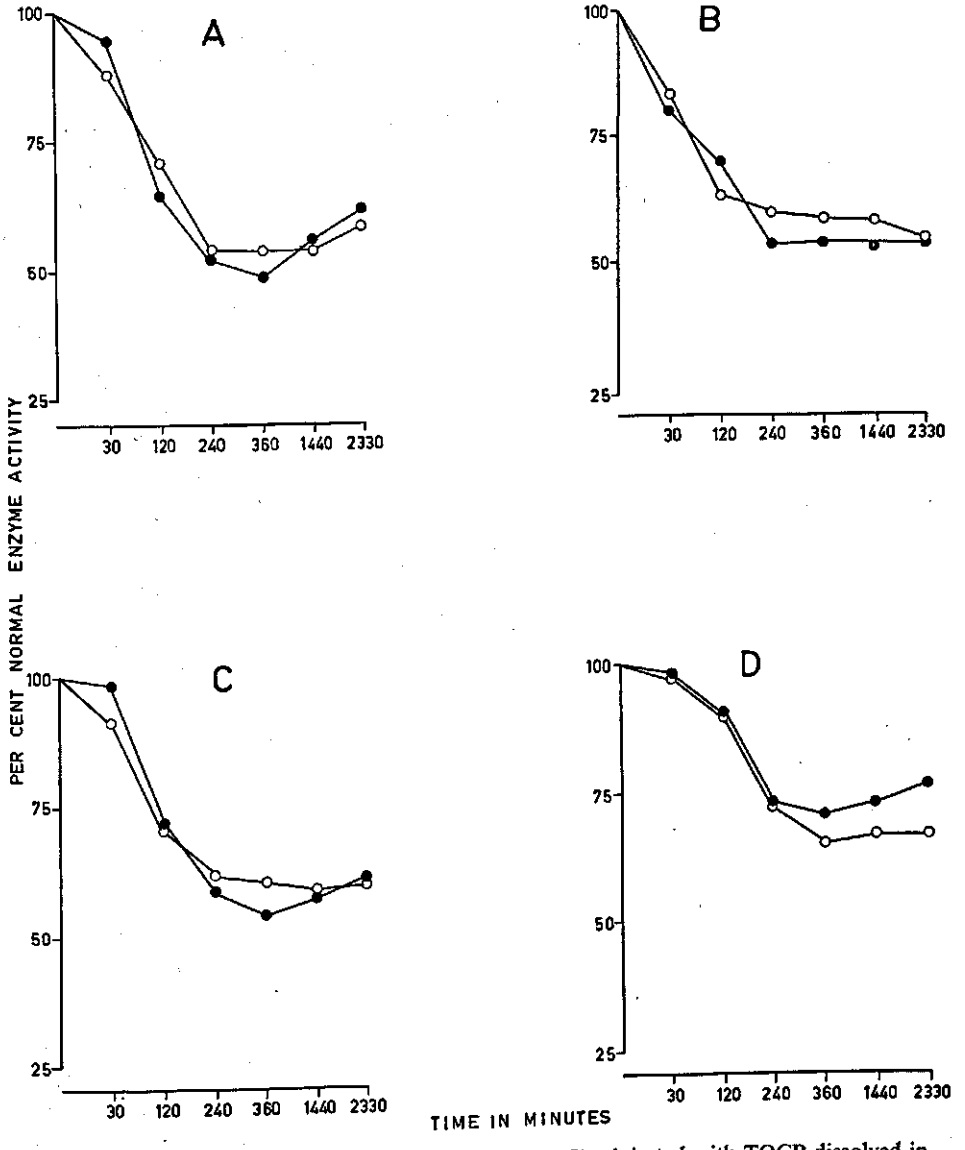


FIG. 15. *In vivo* inhibition of AliE in susceptible houseflies injected with TOCP dissolved in 1 µl olive oil (A: 30 µg; C: 20 µg; D: 10 µg/fly) or in 0.5 µl oil (B: 30 µg/fly).

○——○ = AliE thorax
●——● = AliE abdomen

1960). Nevertheless the same solvent did not protect ChE against inhibition by TOCP *in vivo*.

In fact this result is strong evidence for the "ChE hypothesis". This compound which produced very low or no mortality associated with almost no ChE inhibition, caused higher mortality if the enzyme activity was depressed. In con-

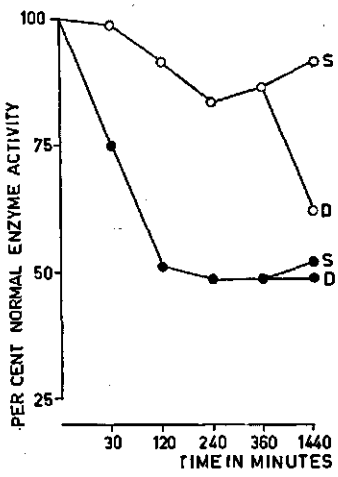


FIG. 16.
In vivo inhibition of ChE and AliE in susceptible houseflies injected with 30 μg TOCP/fly, dissolved in 0.5 μl absolute ethanol.
○———○ = ChE
●———● = AliE
S, D = surviving and dead flies

trast with parathion and paraoxon poisoning, the ChE activity of killed houseflies was inhibited by only 40 %. It would appear that other additional factors are involved in causing death.

Although the pattern of ChE and AliE inhibition was nearly identical when paraoxon was applied either topically or by injection, the time lapse before the toxic symptoms developed was shortened by 15–20 min when the toxicant was injected with 1 μl olive oil (Fig. 17; A). The degree of synergism was substantially higher than in the previous experiments with topical application, although maximum AliE inhibition was essentially the same in both cases (Fig. 17; B. C). It is worth emphasizing that when paraoxon alone was dissolved in 1 μl olive oil and injected, AliE inhibition preceeded that of ChE in the first 30 min. On the other hand, when it was injected simultaneously with TOCP, ChE inhibition was always higher. This is readily explained by assuming that more insecticide is available to the nervous system, resulting in a more severe depression of ChE activity, and consequently a higher rate of KD and higher mortality. The term synergism is applied to such cases. The question as to which mechanism is responsible for making more insecticide available to the site of action, must await further discussion in the next chapter.

Essentially similar experiments were performed with parathion and TOCP. In distinction to internally applied paraoxon, injection of parathion with 1 μl olive oil greatly prolonged the latent period, associated with a slow increase in inhibition of ChE and even of AliE (Fig. 18; A). Again synergism was more pronounced, without altering the length of the latent period (Fig. 18; B, C). In fact, the overall picture of Fig. 18 varies drastically from the corresponding one of paraoxon. AliE is the most inhibited enzyme, except in dead flies. This may indicate the physiological importance of AliE in organophosphorus poisoning. But this picture is not complete. The nature and amount of the solvent have played a major role in changing the pattern of esterase inhibition. It is evident, however, that the latent period was prolonged and maximum KD was reached after 360 min. Maximum ChE inhibition, which was adequately shown to correspond with maximum KD, must have occurred during the period between 360 and 1440 min which is not covered by Fig. 18.

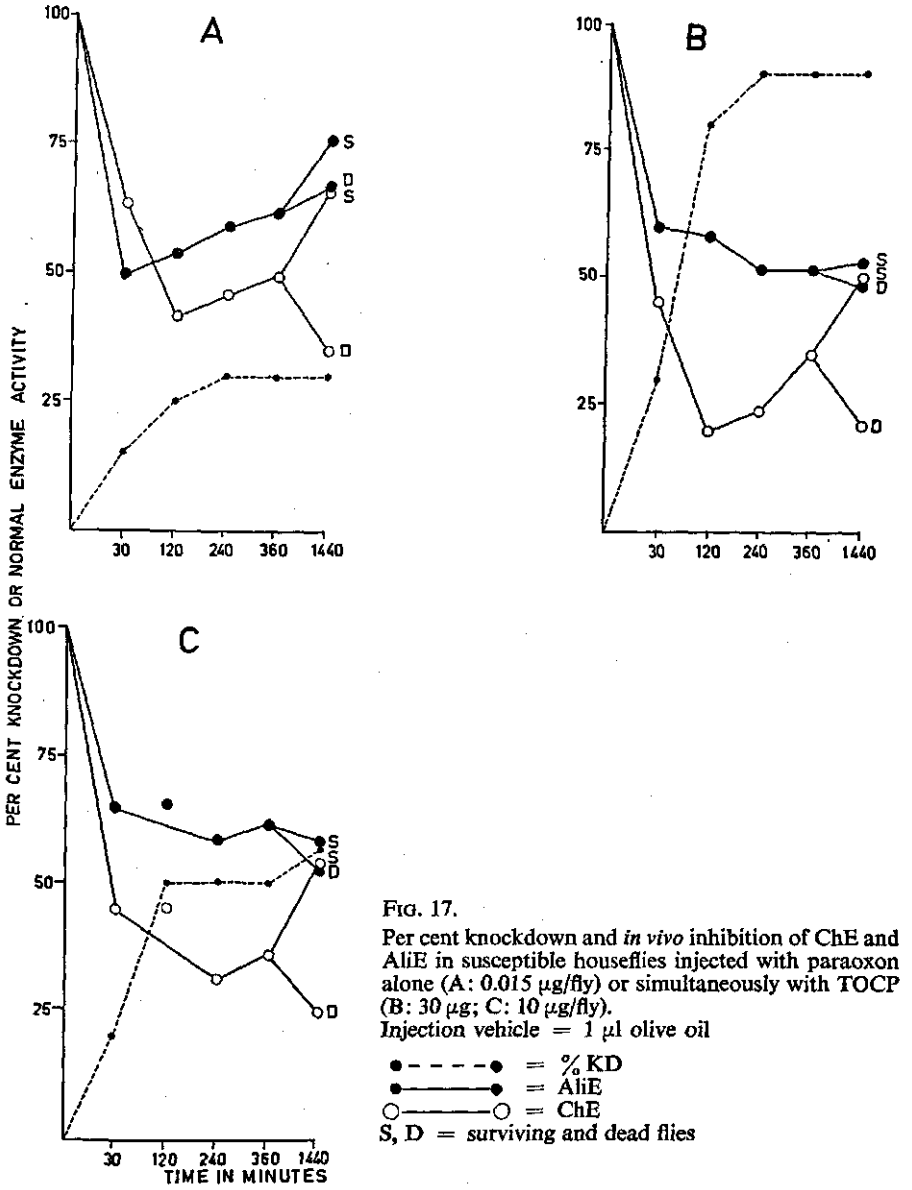


FIG. 17.

Per cent knockdown and *in vivo* inhibition of ChE and AliE in susceptible houseflies injected with paraoxon alone (A: 0.015 μg/fly) or simultaneously with TOCP (B: 30 μg; C: 10 μg/fly).
Injection vehicle = 1 μl olive oil

●- - - -● = % KD
●————● = AliE
○————○ = ChE
S, D = surviving and dead flies

The results described so far appear to indicate conclusively that inhibition of AliE does not account for the poisoning effects. In all cases, AliE was inhibited to nearly the same degree irrespective of the occurrence of synergism or antagonism. The maximum inhibition was invariably 50 %. Higher mortality coincided with stronger ChE inhibition; low or no mortality with stronger AliE inhibition. This points to thorax ChE as essential in the process of intoxication by an organophosphate insecticide.

There may be a certain threshold of ChE inhibition at the vital targets, and

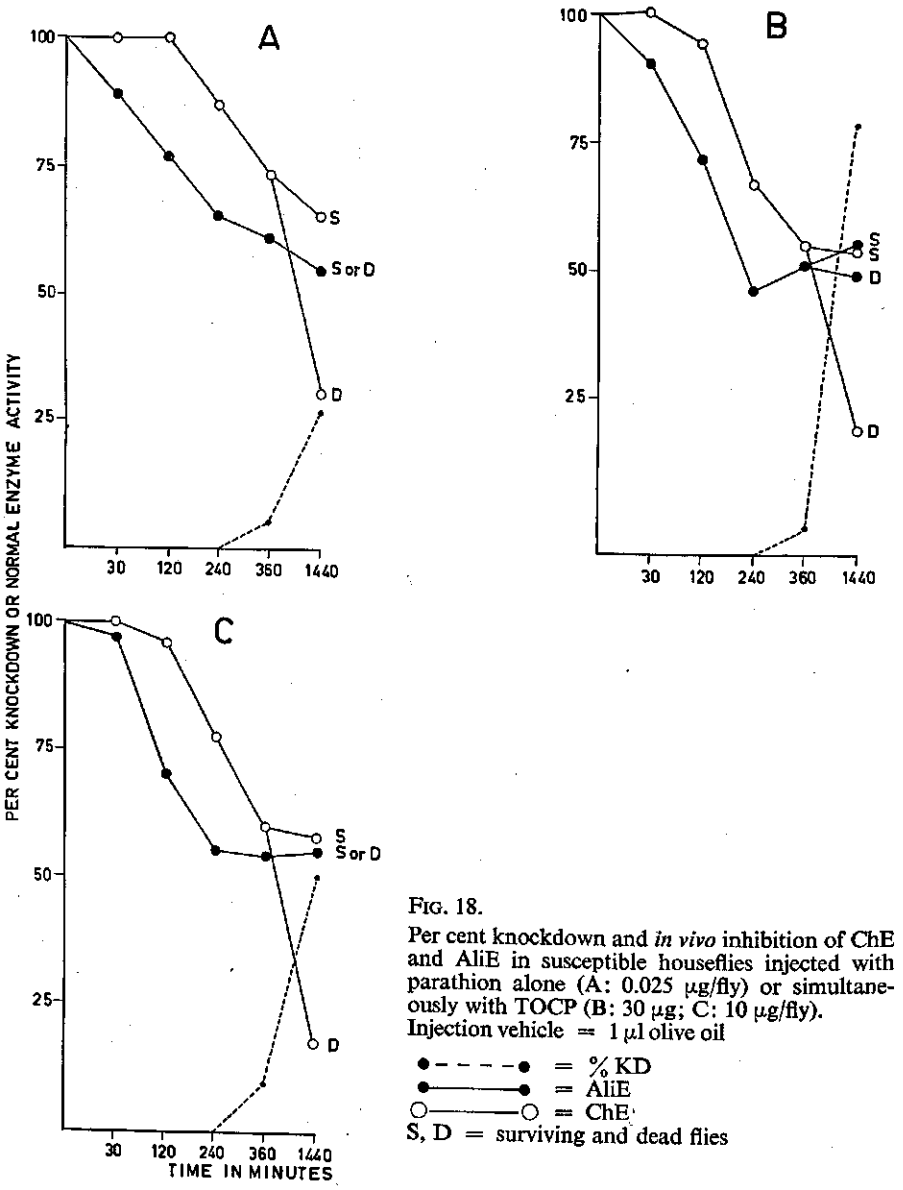


FIG. 18.

Per cent knockdown and *in vivo* inhibition of ChE and AliE in susceptible houseflies injected with parathion alone (A: 0.025 μg/fly) or simultaneously with TOCP (B: 30 μg; C: 10 μg/fly). Injection vehicle = 1 μl olive oil

●- - - -● = % KD
●——● = AliE
○——○ = ChE
S, D = surviving and dead flies

once this threshold is exceeded, death ensues. The present study provides some data on this point. The threshold value for thorax ChE is about 50 %. This value is reached because: (a) during the first stages of poisoning, treated flies were knocked down when their ChE activity levels fell below 50 %; (b) ChE activity was consistently inhibited by 50 %, in surviving flies; and (c) after the application of a lethal dose of parathion, ChE was inhibited in the first 30 min by 54 %. Nevertheless, treated flies were almost normal during that period (Fig. 19; C).

In this connection, it is of interest to note the conclusions of CHADWICK and O'BRIEN (cf. O'BRIEN 1960). CHADWICK stated that "one would expect rather that if ChE inhibition is the sole cause of death, all those having more than a certain amount of their enzyme inhibited would die, the rest would live, yet it seems that some die with only 10 % of their enzyme inhibited, some survive with 90 % inhibited." O'BRIEN concluded that one of the three principal objections to the "Cholinesterase hypothesis" is the fact that ChE need not be substantially inhibited at death. Our results appear to be incompatible with the two conclusions cited above.

Ample evidence has been provided which showed the excellent correlation between *in vivo* ChE inhibition, the onset of toxic symptoms, and mortality percentage. This correlation clearly indicates that ChE inhibition is associated with death. Nevertheless, one might argue that this inhibition *per se* cannot adequately account for the ultimate cause of death. The present study does not furnish any clue to this particular point.

II.2.6. *The number of aliesterases in the susceptible flies.*

Throughout the present series of experiments with normal houseflies, it was shown beyond any question, that the maximum AliE inhibition was not more than 50 %, regardless of mortality percentage. In addition, AliE activity was almost inhibited to the same degree, irrespective of the size of the dose (Fig. 19). Thus when a lethal dose of parathion, or sublethal doses of the two insecticides causing 40 or 0 % mortality, were applied topically, AliE activity was inhibited by nearly 50 %. The inhibition of ChE activity, on the other hand, was always more closely related to the amount of the insecticide (Fig. 19). Similar results have been obtained by PLAPP and BIGLEY (1961).

This new evidence lends further support to the foregoing conclusion regarding the relative physiological importance of ChE and AliE in organophosphorus poisoning.

It seems inadvisable to overlook the striking similarities in AliE inhibition values in almost all the previously described experiments. We have very often encountered the following statement "AliE activity is not inhibited more than 50 % of normal".

This can be easily explained on the basis of the assumption that there are at least two aliesterases involved in the hydrolysis of methylbutyrate, and present in this particular strain in equal amounts. One of them, representing 50 % is very sensitive to parathion, paraoxon and TOCP, whereas the other one is virtually insensitive to the three compounds. This suggestion probably serves to explain the complete inhibition of AliE brought about by TOCP (STEGWEE 1960), and the partial inhibition found in the present study. VAN ASPEREN (1959) and VAN ASPEREN and OPPENOORTH (1959), studying the distribution of esterases and their activity in normal and resistant houseflies, suggested that there are two or even more aliesterases present in these flies. The 50 % of the total hydrolytic activity on methylbutyrate which proved to be organophosphate-insensitive is, however, much higher than the 4 % reported by VAN ASPEREN and OPPENOORTH (1959).

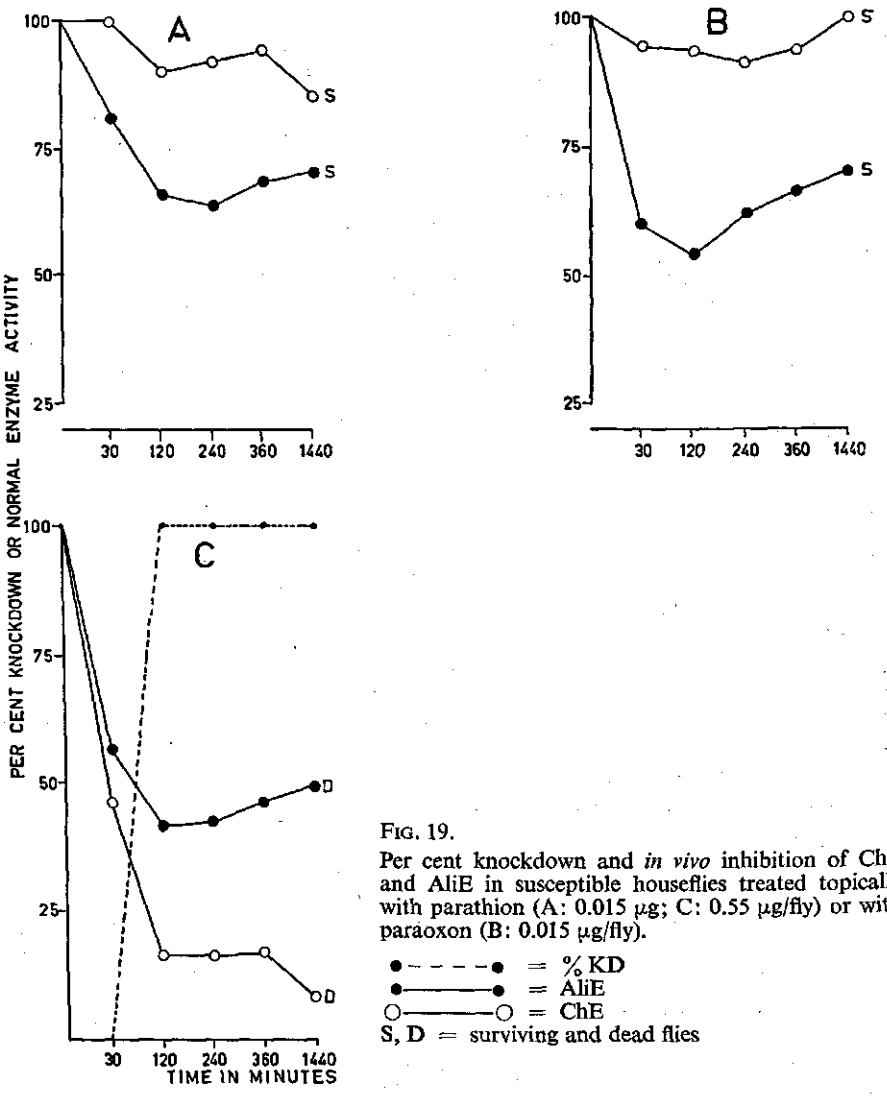


FIG. 19.

Per cent knockdown and *in vivo* inhibition of ChE and AliE in susceptible houseflies treated topically with parathion (A: 0.015 μg; C: 0.55 μg/fly) or with paraoxon (B: 0.015 μg/fly).

●– – – –● = % KD
●————● = AliE
○————○ = ChE
S, D = surviving and dead flies

CHAPTER III

THE RELATION BETWEEN PENETRATION THROUGH THE CUTICLE AND THE LATENT PERIOD IN PARATHION POISONING; MECHANISMS OF SYNERGISM

III.1. INTRODUCTION AND REVIEW OF LITERATURE

The slow toxic action of parathion, as compared with paraoxon is well known and applies to both mammals and insects. In spite of its slow action,

parathion is more recommended as an insecticide than its oxygen analogue, because of its lower mammalian toxicity (MARTIN 1950).

METCALF and MARCH (1949) working with houseflies, and STRINGER (1956) with the migratory locust, observed that the time required to produce comparable toxic effects was about 3 to 5 times longer for parathion than for paraoxon. In the American cockroach, CHAMBERLAIN and HOSKINS (1951) found that parathion and paraoxon were absorbed very rapidly through the back of the thorax and the base of the wings, yet the onset of toxic symptoms occurred much more slowly with the former compound. Our own data fully confirm these findings; at levels producing nearly equal mortality, paraoxon acted about 5 times faster than parathion. As regards the effect on respiration, LORD (1950) observed a lag period after parathion administration.

Because it is generally assumed that both parathion and paraoxon penetrate readily through the insect cuticle (see Chapter I), the cause of the slower action of parathion has been sought inside the insect body. FERNANDO *et al.* (1951), working with the American cockroach, offered an explanation for this phenomenon. They found that parathion accumulated much more slowly in the central nervous system than did paraoxon. The amount of parathion recovered 4 h after application was about three-fourth of the amount of paraoxon found to be present at 30 min after application of an equal dose.

After it had been demonstrated that extremely pure parathion is a weak inhibitor of ChE *in vitro*, many investigators related the slower action of parathion to this phenomenon. They have shown, beyond any doubt, that in the presence of various animal tissues, parathion is converted *in vitro* to a potent anti-ChE agent, most probably the oxygen analogue, paraoxon. It was inferred that inside the living animal, a similar process may take place. Thus, parathion may act slowly mainly because time is needed for oxidizing it to the actual toxicant. At present, this view is widely accepted.

III.2. RESULTS AND DISCUSSION

III.2.1. *Toxicity data*

In the experiments described in Chapters I and II, TOCP was applied prior to or simultaneously with parathion and paraoxon. We now tried to gain more information about the processes causing synergism by reversing the sequence of application, and applying TOCP during the latent period. After topical application with 0.032 μg parathion per fly (resulting in 40–50 % mortality), treated flies remained without obvious ill-effects for approximately 150 min. Thereafter, toxic symptoms began to manifest themselves, thus terminating the latent period. With the onset of the hyperactive-prostrate stage (150 min), few flies (not more than 10 %) were knocked down. Parathion was consistently applied to the thorax, while TOCP was applied in some experiments to the thorax and in parallel ones to the abdomen, at 30, 90 and 150 min time intervals.

Curiously enough, when TOCP was applied to the thorax after parathion, the mortality percentage was substantially lower than in the parallel experiments (Table 10). The most striking difference is found at the 30 min interval; it decreases progressively as the insects approach the hyperactive-prostrate stage. Even when the treated flies reached that stage, about 30 % difference in mortality was obvious between the two parallel experiments, with 100 μg TOCP. On the

TABLE 10. The synergistic and antagonistic action of TOCP after topical application of Parathion (0.032 μg/fly) to the thorax of susceptible houseflies.

TOCP μg/fly	Mortality percentage after 24 hours						
	Time interval Parathion/TOCP min.						Parathion alone
	30		*90*		*150**		
	I	II	I	II	I	II	
100	18	61	42	74	50	79	
50	9	50	38	56	47	54	46
10	13	42	30	47	41	43	

* Onset of hyperactive – prostrate stage
I TOCP applied topically to the thorax
II TOCP applied topically to the abdomen

other hand, synergism occurred when TOCP was applied to the abdomen. Mortality percentage in this case tended to increase with time, most notably with 100 μg TOCP.

Which are the causes for these remarkable differences? It has been adequately shown that when TOCP was applied prior to the administration of the insecticide, antagonism and synergism occurred. Ample evidence was provided to indicate that antagonism occurs because TOCP acts in such a manner that it prevents or retards the entry of the insecticide. Both phenomena were also observed when the sequence of application was reversed. In fact, the antagonistic action of TOCP was entirely unexpected, because it was considered that parathion penetrates readily into the insect body. But still this action did occur and so we are forced to conclude that parathion is retained in the cuticle longer than usually thought.

The results set out in Table 10 may be considered in the following manner. The pronounced antagonism at the 30 min interval is due to the retention of a large proportion, if not all, of the applied dose in the cuticle for as long as 30 min. Therefore, TOCP could effectively exert its antagonistic action. As time elapses, parathion is delivered gradually to the "inside" and accumulates at the site of action. Once its concentration passes a certain threshold, knockdown ensues. Thus, in general, the rate at which this "threshold concentration" is reached is low when antagonism occurs and high when synergism is brought about. In other words, synergism is associated with more insecticide in the "inside", and antagonism with more insecticide in the "outside". Therefore, the degree of antagonism depends mainly on how much parathion is still being retained in the cuticle. Table 10 provides some estimate of this, although it is not quantitative. The effect of 100 μg TOCP (Table 10) suggests that a considerable fraction of the dose of parathion was still present in the cuticle at the 150 min interval. This result, namely, the very slow penetration of parathion, explains neatly the very slow accumulation of parathion in the central nervous system of a treated cockroach, as has been shown by FERNANDO *et al.* (1951).

The relatively weak synergism observed at the 30 min interval can be easily explained on the basis of our previous suggestion that TOCP penetrates very readily, thus reaching the thoracic cuticle within 5 min.

It might be argued that the striking difference between thoracic and abdominal application may be partly due to the fact that TOCP is somewhat toxic when

applied to the abdomen. This, however, would be an unsatisfactory explanation. The slight toxicity of TOCP was observed only with 100μg TOCP, and the difference in mortality did not exceed 10 %. Nevertheless, with the other two doses of TOCP which caused virtually no mortality, this remarkable difference was evident up to 90 min after the application of parathion.

It is conceivable that the presence of parathion in the cuticle after 150 min, as mentioned before, could be detected only after the administration of 100 μg TOCP due to its higher antagonistic activity.

Further supporting experimental data are provided by other experiments in which parathion was injected prior to TOCP treatment. The same TOCP doses were applied. It has been shown (Chapter II) that the latent period after dissolving parathion in 1 μl olive oil and injecting it, was extraordinary long. In this respect, in order to compare data obtained from injection experiments with those from topical application experiments, two requirements must be satisfied: (a) almost equal mortality, and (b) almost equal length of latent period. These requirements were fulfilled when 0.025 μg parathion per fly was dissolved in 0.5 μl olive oil instead of dissolving it in 1 μl.

In view of the foregoing results, one might anticipate a mere synergistic action, since the cuticle is out of the picture. Table 11 reveals at once that only synergism did occur. The interesting feature of Table 11 is the nearly equal mortality obtained, irrespective of whether TOCP is applied to the abdomen or to the thorax. Moreover, synergism is somewhat more marked at the 30 min interval.

TABLE 11. The synergistic action of TOCP after the injection of Parathion (0.025 μg/fly dissolved in 0.5 μl olive oil) into susceptible houseflies.

TOCP μg/fly	Mortality percentage after 24 hours						
	Time interval Parathion/TOCP min.						Parathion alone
	30		*90*		*150**		
	I	II	I	II	I	II	
100	72	73	75	71	69	74	
50	58	62	43	48	44	45	41
10	50	53	38	43	41	42	

* Onset of hyperactive-prostrate stage
I TOCP applied topically to the thorax
II TOCP applied topically to the abdomen

In fact, our findings that parathion is retained in the cuticle for a considerable period after topical application (see Table 10), prompted us to conduct a similar experiment with paraoxon. The flies were treated with TOCP during the latent period of paraoxon poisoning. At the LD_{35}, this period lasted about 30 min. Therefore, two time intervals were chosen to apply TOCP: 5 and 30 min. The latter corresponds with the onset of the hyperactive – prostrate stage.

It is known that paraoxon penetrates extremely rapidly. Accordingly, it was to be anticipated that TOCP applied after paraoxon would have a synergistic effect. This was confirmed by the results recorded in Table 12. Synergism was of the same order of magnitude as with parathion. A marked antagonism was observed only with 100 μg TOCP at the 5 min interval, whereas 10 μg which

TABLE 12. The synergistic and antagonistic action of TOCP after topical application of Paraoxon (0.065 μg/fly) to the thorax of susceptible houseflies.

TOCP μg/fly	Mortality percentage after 24 hours				
	Time interval paraoxon/TOCP min.				Paraoxon alone
	5		*30**		
	I	II	I	II	
100	16	81	76	84	35
10	30	44	45	48	

* Onset of hyperactive – prostrate stage
I TOCP applied topically to the thorax
II TOCP applied topically to the abdomen

was very effective in antagonizing parathion poisoning at the 30 min interval, did not materially antagonize paraoxon poisoning.

Let us now summarize our views on parathion and paraoxon penetration. Parathion is retained for some time in the cuticle, thus preventing the insecticide from accumulating within the nervous system. This accounts for the observed latent period, which is terminated as soon as parathion accumulation carries on to reach the threshold concentration. On the other hand, paraoxon penetrates very rapidly, being retained in the cuticle for about 5 min only. Thereafter it reaches the threshold concentration very rapidly.

III.2.2. *ChE and AliE measurements*

Further evidence regarding the relation between penetration and the latent period was obtained from determinations of ChE and AliE activities in experiments which, for the rest, were identical to those described above. In this case our major interest was to measure the antagonistic activity of TOCP in terms of ChE inhibition. Therefore, both agents were applied topically to the thorax. TOCP was applied at 30, 90 and 150 min after parathion, and at 5 and 30 min after paraoxon.

The results recorded in Fig. 20 denote that the rate of parathion penetration is closely associated with % KD and the degree of ChE inhibition. Clear-cut differences in ChE inhibition values are observed between the three treatments of parathion, particularly with the higher dose of TOCP (Fig. 20, A). These are most obvious at the 240 min interval. It is interesting to note that ChE inhibition in survivors shows a remarkable difference between the three treatments. The usually encountered pattern of AliE inhibition is seen in Table 13. Differences between AliE activities in the three treatments are of such low order of magnitude, that they can be disregarded.

When paraoxon was used instead of parathion, the differences in ChE inhibition between the two treatments are greater than those found in the case of parathion (Fig. 21). This is due to the occurrence of antagonism and synergism. The latter was not observed in case of parathion. The administration of TOCP 5 min after paraoxon resulted in antagonism associated with a less severe depression of ChE. On the other hand, when it was adminstered 30 min after paraoxon, marked synergism was brought about accompanied by severe ChE inhibition. The differences in ChE activity found between the survivors

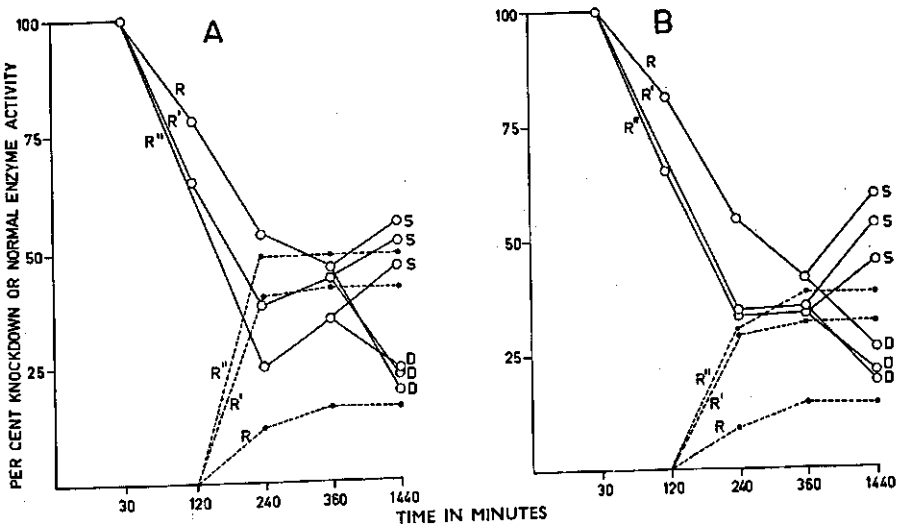


FIG. 20. Per cent knockdown and *in vivo* inhibition of ChE in susceptible houseflies treated topically with parathion (0.032 μg/fly) to the thorax, followed by TOCP (A: 100 μg; B: 10 μg/fly) applied topically to the same site (R: after 30 min; R′: after 90 min; R″: after 150 min).

●- - - -● = % KD ○———○ = ChE
S, D = surviving and dead flies

TABLE 13. Percent of normal AliE activity in susceptible houseflies treated topically with Parathion (0.032 μg/fly) followed by TOCP.*

Time interval Parathion/TOCP min.	TOCP 100 μg						TOCP 10 μg					
	Time after parathion min.											
	30	120	240	360	1440		30	120	240	360	1440	
					S	D					S	D
30	79	60	53	55	58	60	83	60	52	55	60	58
90	–	54	57	59	57	59	–	56	53	58	61	53
150	–	–	55	54	56	54	–	–	50	57	64	55

S, D = surviving and dead flies
* both agents applied to the thorax

escaping parathion intoxication, are not observed with paraoxon. AliE inhibition showed its usual pattern (Table 14).

At least two points must be borne in mind when examining Figs. 20 and 21: (a) the antagonistic activity of TOCP is less marked when the sequence of application is reversed, and (b) TOCP helps, in some way, to make more of the insecticide, which is already present in the "inside", available to the nervous system. These factors both would cause an increase in the inhibition of ChE.

It is interesting to note that the results of Figs. 20 and 21 imply that the amounts of paraoxon which penetrate the cuticle within 5 and 30 min respectively, appear to equal those of parathion penetrating within 30 and 150 min

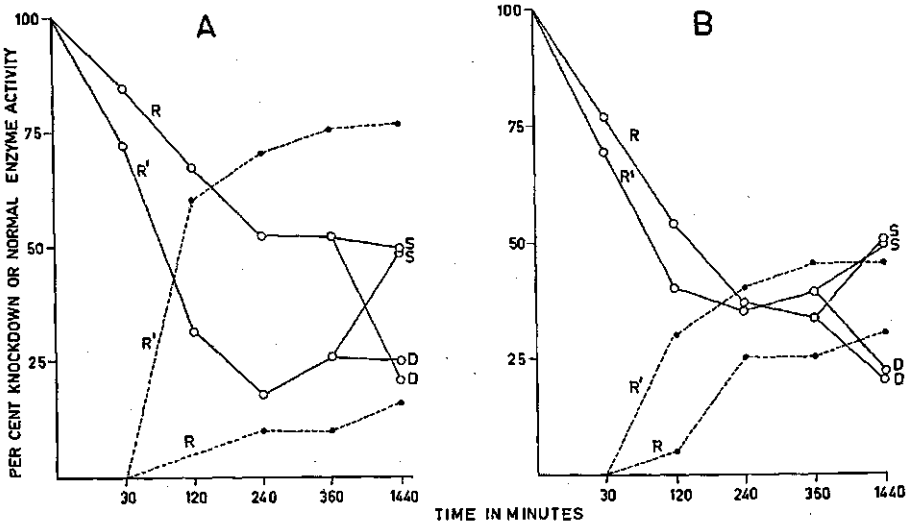


FIG. 21. Per cent knockdown and *in vivo* inhibition of ChE in susceptible houseflies treated topically with paraoxon (0.065 μg/fly) to the thorax, followed by TOCP (A: 100 μg; B: 10 μg/fly) applied topically to the same site (R: after 5 min; R': after 30 min).

●- - - -● = % KD ○——○ = ChE
S, D = surviving and dead flies

TABLE 14. Per cent of normal AliE activity in susceptible houseflies treated topically with Paraoxon (0.065 μg/fly) followed by TOCP*.

Time interval Parathion/TOCP min.	TOCP 100 μg						TOCP 10 μg					
	Time after parathion min.											
	30	120	240	360	1440		30	120	240	360	1440	
					S	D					S	D
5	55	50	48	46	44	43	51	50	52	51	49	45
30	51	46	48	52	52	52	50	52	48	49	48	48

S, D = surviving and dead flies
* both agents applied to the thorax

This would indicate that the rate of penetration of paraoxon is 5 to 6 times higher than that of parathion, as measured by ChE inhibition. A similar value has been observed with regard to the time elapsing between poisoning and onset of toxic symptoms, as mentioned previously.

III.2.3. *Injection experiments*

The fact that the latent period was considerably long when parathion was dissolved in 1 μl olive oil and injected (see Fig. 18, A) would seem to refute the role of the cuticle in controlling the length of the latent period. When the amounts of olive oil were halved, keeping the quantity of parathion constant (0.025 μg/fly), the length of the latent period was markedly reduced, and mortality increased (see Table 10). It is remarkable that in the latter case, the

length of this period and the mortality percentage were almost equal to those recorded after the topical application of parathion. These observations fully show that the solvent used for injection is of primary importance, as was clearly demonstrated by LANGENBUCH (1954), who found that a given amount of DDT acted much more rapidly when dissolved in propylene glycol than when dissolved in olive oil.

Our major interest was to seek for another appropriate vehicle which did not mask or hinder parathion action. After several trials, we found that 50 % aqueous ethanol is an appropriate solvent too, producing very low mortality when 0.5 µl was injected. It has the disadvantage of causing a slight narcotic effect, but this did not last longer than 10 to 15 min. Complete recovery of injected flies occurred thereafter. ChE and AliE activities were unimpaired after injecting houseflies with this solvent.

The same amount of parathion (0.025 µg per fly) was dissolved in aqueous ethanol and injected. We now have three different treatments, namely with, 1 µl and 0.5 µl olive oil, and 5 µl aqueous ethanol. Each amount of solvent contained the same quantity of parathion. The dramatic picture of esterase inhibition by parathion dissolved in 1 µl oil (see Fig. 18, A) is no longer seen when the amount of oil is halved (Fig. 22, A). When the aqueous ethanol vehicle was used, a striking change in the pattern of ChE inhibition could be observed (Fig. 22, B). ChE was the most inhibited enzyme. Two important facts emerge from Fig. (22, B): (a) the latent period is greatly reduced to about 30 min., which is as long as that for topically applied paraoxon, and (b) parathion inhibits ChE activity soon after injection, which was never observed in

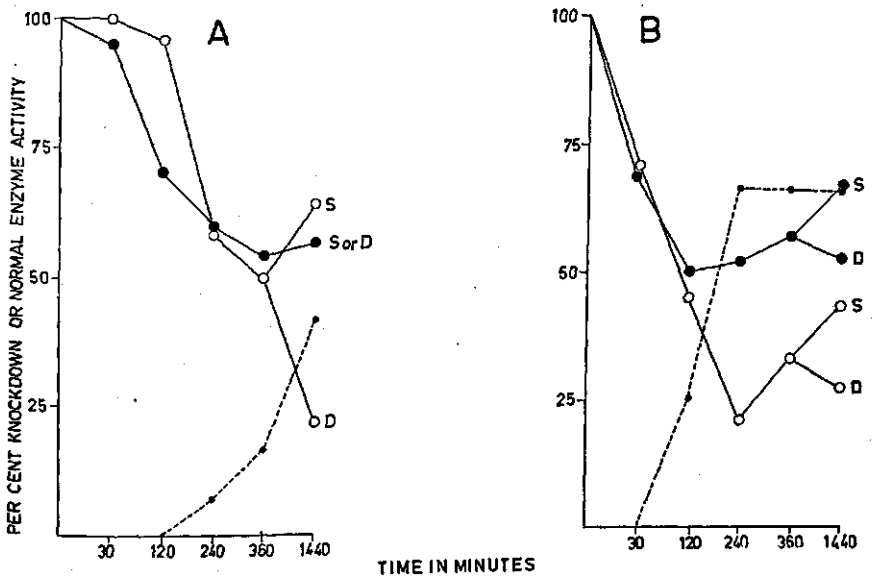


FIG. 22. Per cent knockdown and *in vivo* inhibition of ChE and AliE in susceptible houseflies injected with parathion (0.025 µg/fly) dissolved in 0.5 µl (A: olive oil; B: 50 % ethanol).

●- - - -● = % KD
●——● = AliE
○——○ = ChE
S, D = surviving and dead flies

any other of the experiments carried out with topically applied or injected parathion.

Similar experiments were performed using paraoxon. In contrast, when paraoxon was injected, the time elapsing between administration and onset of toxic symptoms was markedly reduced compared to topical application (Figs. 17, A; and 23). The amount of oil, however, had no appreciable effect in this connection.

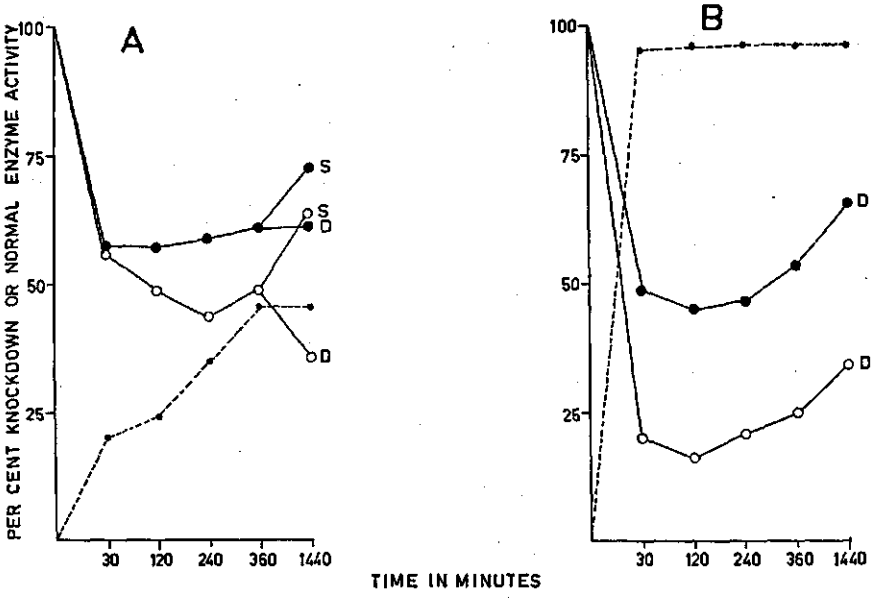


FIG. 23. Per cent knockdown and *in vivo* inhibition of ChE and AliE in susceptible houseflies injected with paraoxon (0.015 µg/fly) dissolved in 0.5 µl (A: olive oil; B: 50 % ethanol).

●- - - -● = % KD ○———○ = ChE
●———● = AliE S, D = surviving and dead flies

The obvious recovery of ChE in dead houseflies (Fig. 23, B) has been shown by histochemical studies of the cholinesterases in the nervous system (MOLLOY 1961). It was found that reactivation of ChE may occur at some stage after poisoning, possibly after death, since samples of flies examined 24 h after Diazinon poisoning showed more enzyme present than samples taken 2 to 3 h after poisoning.

It appears then, that there is a correlation between the amount of oil, knockdown percentage and the subsequent final mortality, most notably with parathion. The larger the amount of injected oil, the lower the rate of knockdown, and the lower the mortality. By decreasing the amount of oil, the latent period following parathion injection is reduced by hours, whereas that following paraoxon injection is reduced by a few minutes.

The behaviour of both insecticides, with respect to their toxic action, revealed another interesting difference. After parathion injection, there is a remarkable reduction in the length of the latent period associated with a steady increase in

mortality. After paraoxon injection, however, there is a negligible reduction in the lag period accompanied by a steady increase in mortality, as long as oil is the vehicle. When aqueous ethanol is used, a sharp increase is observed. This difference is likely to be related to the physico-chemical properties of either insecticide, particularly liposolubility. It would seem, therefore that, as far as the length of the latent period is concerned, olive oil has the effect of limiting the availability of parathion to the site of action, and concerning toxicity, by protecting the insect against injected parathion or paraoxon.

Although the latent period after parathion injection was reduced to 30 min, it appears relatively long. But it seems safe to conclude that the injection experiments do not invalidate the idea that after topical application of parathion retention within the cuticle is the main factor controlling the length of the latent period. It is quite plausible that the length of this period could be further reduced if the physico-chemical properties of the insecticide do not intervene.

After topical application, the S-ethyl and the S-phenyl isomers were far less toxic than either parathion or paraoxon (see Fig. 7). Being phosphates, the two isomers possess high anti-ChE activity (METCALF and MARCH 1953c). It was of considerable interest to investigate their effect on the pattern of ChE and AliE inhibition, in addition to their insecticidal potency after injection. Doses equal to those of parathion (0.025 μg/fly) dissolved in 0.5 μl aqueous ethanol were used. The results of such experiments are presented in Fig. 24.

It is obvious that the S-ethyl isomer caused a rapid inhibition of ChE activity, associated with 25 % KD at the first 30 min after injection. As the

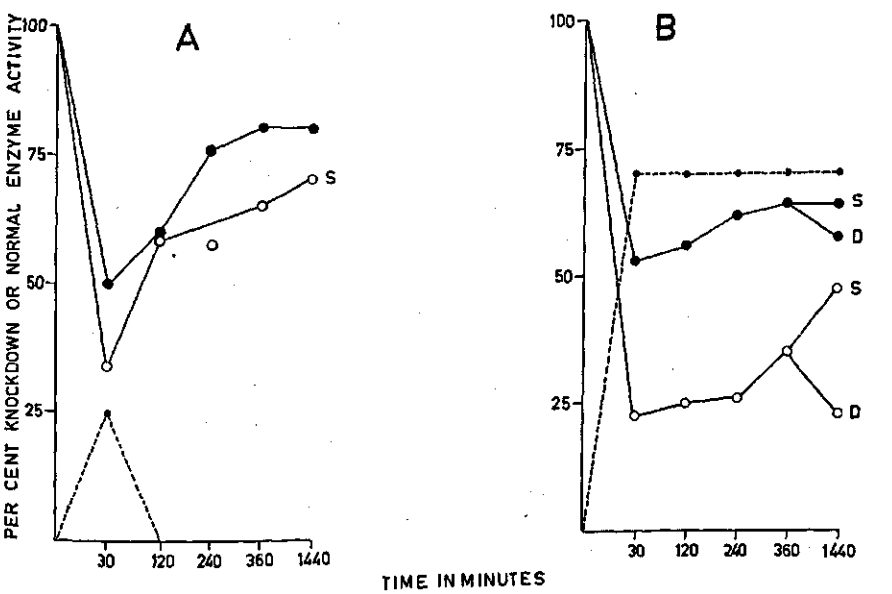


FIG. 24. Per cent knockdown and *in vivo* inhibition of ChE and AliE in susceptible houseflies injected with S-ethyl parathion (A: 0.025 μg/fly) or with S-phenyl parathion (B; 0.025 μg/fly) dissolved in 0.5 μl 50 % ethanol.

●- - - -● = % KD
●————● = AliE
○————○ = ChE
S, D = surviving and dead flies

prostrate flies recovered, there was also a marked recovery of ChE activity. The recovery of AliE activity was more pronounced (Fig. 24, A). It is likely that the observed recovery of the treated flies and of the two enzymes as well, is due to the extremely rapid hydrolysis of the S-ethyl isomer (see Chapter VI). This would cause a rapid hydrolysis of the enzyme phosphate. ALDRIDGE (1953a), studying the inhibition of erythrocyte ChE by tri-esters of phosphoric acid, suggested a similar mechanism. He found that the reversal of inhibition is consistent with a reaction involving a dephosphorylation, and not with a simple reversal of the inhibitory process. Our results are, however, incompatible with an alternative view stated by DAVISON (1953b) who pointed out that recovery from ChE inhibition is due to resynthesis of new enzyme. It is inconceivable that the resynthesis of new enzyme in sufficient proportion occurs within one hour. On the other hand, the S-phenyl isomer produced high mortality associated with less marked recovery of ChE and AliE activities (Fig. 24, B).

By now, we are able to explain some observations reported in the foregoing two chapters. It has been shown that TOCP is a stronger antagonist of parathion than of paraoxon. This might be due, firstly to the very slow penetration of parathion, and secondly to the location of the antagonistic activity of TOCP in the cuticle. Owing to the very rapid penetration of paraoxon, TOCP has little opportunity to prevent its entry.

III.2.4. *The concept of the holding capacity*

Mention was made earlier that normal houseflies showed some fluctuations in their susceptibility towards paraoxon. Although this is otherwise undesirable in research work, we could make use of these fluctuations. A certain batch of flies was more tolerant; application of 0.08 μg paraoxon (instead of the usual 0.065 μg) caused 40 % mortality. Essentially the same experiment with paraoxon described above (Table 12), but on a wider scale, was conducted.

In this case, though mortality was almost equal, the time elapsing between poisoning and onset of toxic symptoms was reduced by 5 to 10 minutes (Table 15). The most striking difference between results in Tables 12 and 15 is seen in the first column under 5 min time interval. The antagonistic action of TOCP was replaced by a marked synergistic effect, but to a lesser degree than in the

TABLE 15. The synergistic action of TOCP after topical application of Paraoxon (0.08 μg/fly) to the thorax of susceptible houseflies.

TOCP μg/fly	Mortality percentage after 24 hours				
	Time interval Paraoxon/TOCP min.				Paraoxon alone
	5		*25**		
	I	II	I	II	
100	77	87	82	92	
50	66	89	74	72	40
10	51	77	68	62	
5	42	46	48	45	

* Onset of hyperactive – prostrate stage
I TOCP applied topically to the thorax
II TOCP applied topically to the abdomen

parallel experiment where TOCP was applied to the abdomen. Moreover, synergism was of higher order of magnitude than reported previously.

In view of the above considerations, these conspicuous differences are not unexpected. It is reasonable to assume that when 0.08 μg paraoxon is applied topically, it penetrates more rapidly than 0.065 μg (admittedly, mortality was almost equal, but it is well known that penetration is but one factor governing insecticidal action). Hence, when TOCP is applied 5 min after paraoxon (0.08 μg) a greater proportion of the dose is already in the inside; synergism is consequently brought about. It would seem then, in terms of the size of the dose, that the cuticle of a given strain has the capacity to retain a certain amount of insecticide for some time, depending on the properties of the cuticle and the insecticide.

This view receives further support from some other pieces of evidence. The length of the latent period in parathion poisoning depends upon the dosage. For example, when lethal doses are applied, the latent period is reduced by about 2 hours (see Fig. 19, C).

Another peculiar phenomenon can also be explained on the basis of the holding capacity concept. When susceptible houseflies were confined in milkbottles for 24 h, then treated topically with parathion, their tolerance had decreased remarkably. The LD_{40} of such flies was 0.022 μg parathion per fly, and the length of the latent period was almost equal to that of the normal population of test flies treated with about the LD_{40} of parathion (0.032 μg/fly). Therefore, a similar pattern of the synergistic and antagonistic activities of TOCP in both populations was expected to occur. In general, there is a similarity between the results recorded in Tables 10 and 16.

TABLE 16. The synergistic and antagonistic action of TOCP after topical application of Parathion (0.022 μg/fly) to the thorax of susceptible houseflies confined in milkbottles for 24 h before treatment.

TOCP μg/fly	Mortality percentage after 24 hours						
	Time interval Parathion/TOCP min.						Parathion alone
	30		*90*		*150**		
	I	II	I	II	I	II	
100	40	77	50	80	49	78	
50	27	42	38	59	40	53	41
10	25	39	40	42	38	36	

* Onset of hyperactive – prostrate stage
I TOCP applied topically to the thorax
II TOCP applied topically to the abdomen

The most impressive difference is the weak antagonism occurring after treating confined flies with TOCP 30 min after parathion poisoning. In view of the above evidence, it is very probable that the rate of parathion penetration in flies kept in milkbottles is higher than in normal test flies, when compared at equal parathion dosages (Table 17). But in this special case, the dose of parathion is reduced to equalize the higher dose of the normal test flies, with respect to the toxic action and the length of the latent period. Thus it is likely that the lower magnitude of the antagonistic activity of TOCP is not due to rapid penetration,

TABLE 17. Effect of TOCP on the toxicity of Parathion (0.032 μg/fly) to milkbottle – confined susceptible houseflies.

TOCP μg/fly	Mortality percentage after 24 hours		
	Time interval Parathion/TOCP min.		Parathion alone
	30	*100**	
100	88	96	93
50	87	96	
10	92	96	

* Onset of hyperactive – prostrate stage
Both agents applied to the thorax

but rather to a reduction in the frequency of occurrence of a certain factor present in the cuticle. This factor in combination with TOCP, particularly at lower concentrations, would be responsible for the antagonistic action in the cuticle. This explanation is further supported by the equal synergistic activity of TOCP when applied to the abdomen after 30 min and 150 min (Table 16).

The relatively lower degree of synergism observed with normal test flies when TOCP was applied to the abdomen after 30 minutes (see Table 10), was explained by assuming the rapid penetration and distribution of TOCP. This allows the substance to reach the thoracic cuticle within 5 minutes. Although present in low concentrations, it exerts in combination with this factor, a marked influence on parathion penetration. As the frequency of occurrence of this factor is reduced in confined flies, it then follows that its combination with TOCP is less effective. This agrees in general with our previous suggestion that physical or physico-chemical factors are probably involved in the antagonistic action of TOCP. It was also suggested that massive TOCP dosages might act in a mechanical way. But it seems inconceivable that TOCP acts in the same way, when the sequence of application is reversed. We cannot exclude the possibility that other factors participate. The decrease in the average weight of milkbottle-confined flies (15 %) as compared with normal test flies may be a contributing factor.

The phenomenon, as a whole, is a strong indication that in the case of parathion, penetration determines the rate of toxic action. This is not valid for paraoxon, since the susceptibility of confined flies to paraoxon was almost identical to that of normal test flies. In terms of the holding capacity concept, we may say that the capacity of the cuticle of milkbottle-confined flies to retain parathion is lower than that of the normal test flies. We may also say that the holding capacity of the cuticle has a greater influence on parathion penetration than on paraoxon penetration in susceptible houseflies.

III.2.5. *The "opportunity factor" concept*

It has adequately been shown that the latent period following parathion application is due mainly to the retention of the insecticide in the cuticle and its very slow penetration. These findings are in sharp contrast to the widely accepted belief that this period is chiefly attributable to the necessity of parathion oxidation.

The concept of the "opportunity factor" has been postulated by O'BRIEN

(1959). After application of phosphorothionates, as opposed to phosphates, there is a lag period during which the actual toxicant, the phosphate, is produced by P = S oxidation. This lag period gives an opportunity for detoxifying systems to operate. This factor has, for this reason, been called the "opportunity factor".

If detoxifying systems were operating mainly in the cuticle, the concept of the "opportunity factor" would be completely applicable. But this is very unlikely, for it is well known that detoxifying systems operate mainly inside the living insect.

III.3. Mechanisms of synergism (potentiation)

Based upon the literature on this subject, which contains abundant information about synergism and its mechanisms, and upon our own observations, a mechanism for the synergistic action of TOCP in susceptible houseflies poisoned with parathion or paraoxon is suggested.

Some of these reports will be discussed here:

III.3.1. *Inhibition of cholinesterase*

Severe depression of ChE activity was found to be always associated with synergism. It might be argued that this severe depression would account for this phenomenon. This seems very unlikely. Results obtained with dogs, rats, and cockroaches have shown the same correlation. Simultaneous feeding of malathion and EPN to dogs caused marked inhibition of pseudo- and true ChE (60–80 %), but when fed individually no inhibition was produced (Frawley *et al.* 1957a). The authors stated that potentiation appeared to be related to increased ChE inhibition. ChE activity was not appreciably affected by doses of malathion or TOCP when given separately to rats, but resulted in profound ChE inhibition when given simultaneously (Murphy *et al.* 1959). After 22.5 minutes nearly three times as much ChE was inhibited in the nerve cords of "TOCP- and TEP-treated" roaches as in TEP treated roaches (Colhoun 1960).

It is of interest to note that Frawley *et al.* (1957b) pointed out that the increased ChE inhibition, which is associated with pronounced potentiation of the acute toxicity of EPN and malathion to rats and dogs, is the result of a prior reaction of the compounds with some other biological system. Similarly, Monroe and Robbins (1959) pointed out that when a synergist is administered jointly with the toxicant, it can be assumed that both compounds are competing for identical "sites of loss" (*vide* Veldstra 1956), and thereby more of the toxicant will remain in the organism for a longer time. If necessary, it may undergo a hypertoxic change, and ultimately more of it will phosphorylate the ChE at the final site of action. Thus a higher mortality would result.

It seems advisable in seeking for mechanisms of synergism, to look for processes contributing to make more of the insecticide available to the site of action, rather than considering the mere inhibition of ChE, which is the result of synergism and not the cause.

III.3.2. *Inhibition of detoxication mechanisms*

The inhibition of detoxication systems by the synergists is the most attractive

explanation of their mode of action. In this concept, synergism is brought about because the synergist (potentiator) inhibits these systems. Many pieces of evidence have been provided to support this view with a wide range of synergists and insecticides.

The observations made by WILSON (1949) on the action of piperonyl butoxide and piperonyl cyclonene with pyrethrum on houseflies, led him to conclude that the synergists damage a detoxifying mechanism. CHAMBERLAIN (1950), using non-toxic concentrations, found that applying piperonyl butoxide to the heads of houseflies and pyrethrum to the abdomens resulted in knockdown similar to that caused by application of equal amounts of both compounds to the abdomens only. He suggested that the synergist acts by inhibiting a detoxifying mechanism. Inhibition of lipase was probably involved. WINTERINGHAM *et al.* (1955) reported that when the synergist piperonyl cyclonene was applied simultaneously with the pyrethroid, its metabolism was substantially inhibited. This suggested that the synergism involved an interference with the natural detoxication mechanisms of the housefly. HEWLETT *et al.* (1961) noted that SKF (525 A) and piperonyl butoxide increased the effectiveness of pyrethrins towards houseflies and the lesser mealworm beetles. They proposed that piperonyl butoxide and other 3,4- methylene- dioxyphenyl compounds synergize pyrethrins in insects by depressing oxidative detoxification.

Numerous studies with DDT and various synergists led essentially to the same conclusion. Piperonyl cyclonene has been found to increase markedly the toxicity of DDT for DDT-resistant houseflies. Little or no effect was observed with normal houseflies. The conversion of DDT to DDE was largely prevented when resistant flies were treated with a DDT – piperonyl cyclonene mixture (PERRY and HOSKINS 1950; 1951 a, b). The synergist DMC, di – (*p*-chlorophenyl) methylcarbinol, which had little or no effect on susceptible flies when applied simultaneously with DDT, enhanced the DDT toxicity to resistant houseflies (PERRY *et al.* 1953). The authors believe that DMC acts by a specific competitive type of inhibition of the DDT-detoxification. The synergist competes with the insecticide for the mechanism of detoxification. The toxicity of DDT to resistant houseflies was greatly increased when applied in an optimum ratio with the synergist di – (*p*-chlorophenyl) – (trifluoromethyl) – carbinol. It was suggested that DDT-dehydrochlorinase could be inhibited by the carbinol (COHEN and TAHORI 1957).

The picture of the carbamates does not substantially differ from those of the pyrethrins and DDT. Piperonyl butoxide acted as a carbamate synergist in resistant houseflies by blocking the carbamate detoxication enzymes(GEORGHIOU and METCALF 1961), and in normal houseflies, apparently by interfering with detoxication systems (FUKOTO *et al.* 1962).

As organophosphorus insecticides are of great concern to us, the synergism and its mechanisms will be subjected to a more detailed discussion. RAI *et al.* (1956), working with susceptible and DDT-resistant houseflies, observed a significant synergistic effect of piperonyl butoxide with Diazinon and Bayer L13/59. MONROE and ROBBINS (1959) found that Co-ral and its phosphate analogue, coroxon, were approximately 2.8 times more toxic to houseflies when administered jointly with piperonyl butoxide. There was no evidence as to the mechanism of synergism, but the authors were in favour of the theory of the "sites of loss" postulated by VELDSTRA (1956). HADAWAY *et al.* (1963) reported that the addition of piperonyl butoxide increased the toxicity of both malathion

and malaoxon to *Aedes aegypti.* It appeared that piperonyl butoxide did not only inhibit the oxidation of malathion but stabilized the oxygen analogue. The addition of piperonyl butoxide did not have any marked influence on the toxicity of parathion or paraoxon to houseflies and the two species of mosquitoes under test. The concept of the inhibition of biological oxidation systems and the stabilization of the oxygen analogue (HEWLETT *et al.*; HADAWAY *et al. loc. cit*) was proposed earlier by SUN and JOHNSON (1960). It was found that pyrethrin synergists such as piperonyl butoxide, sulphoxide, propyl isome and sesamex potentiated the toxicity to houseflies not only of pyrethrins but also of several organophosphates, presumably because the synergists prevented detoxification caused by biological oxidation systems. The low increase in the toxicity of methyl paraoxon induced by sesamex led the authors to conclude that sesamex may not only inhibit the oxidation of methyl parathion, but also stabilizes its oxygen analogue.

Recently it has been shown that certain combinations of organophosphate insecticides, when administered to mammals, are more toxic than anticipated from the "theoretical sum" of their individual effects. The much studied case in this respect is the potentiation of malathion toxicity by EPN. FRAWLEY *et al.* (1957b) reported a 50-fold potentiation in the acute toxicity of EPN and malathion if they were adminstered simultaneously to dogs; a less marked potentiation for rats; and none for houseflies. The authors suggested that the biological system being interfered with by one or both compounds, was more reactive in the dog and rat than in the fly. *In vitro* studies using rat liver homogenates and twelve organic phosphates have shown that only malathion was altered very quickly by an esterase to a more water-soluble compound which was a very poor anticholinesterase agent. Many compounds besides EPN blocked the alteration (COOK *et al.* 1957). The malathion detoxifying enzyme system was found to be inhibited *in vitro* and *in vivo* by EPN. This interference with detoxication enzymes provided an explanation for the potentiation of toxicity which occurred after the simultaneous administration of the two compounds (MURPHY and DU BOIS 1957; 1958). Supporting experimental data have been provided by COOK *et al.* (1958). The esterase enzyme system present in rat liver which detoxified malathion very rapidly (called malathionase by the authors) was extremely sensitive to very small quantities of some organic phosphate chemicals, including EPN. When these compounds were adminstered at the same time as malathion, they inhibited the detoxification of malathion and thus made it available to inhibit ChE.

The data of SEUME and O'BRIEN (1960b) are compatible with an alternative hypothesis in explaining the potentiation of malathion toxicity to rats by EPN. By virtue of its inhibition of hydrolysis and oxidation, EPN causes a greater persistence of malathion in the body tissues. The synergistic effect itself, however, must be due to an increased level or persistence of malaoxon at the target site.

III.3.3. *Sites of loss*

The concept of "sites of loss", as a factor in synergism, has been developed by VELDSTRA (1956). Sites of loss are the loci of secondary actions, *e.g.* adsorption in a harmless way, interaction at sites of secondary importance, reaction with non-vital enzymes, and transport barriers. All of these events imply a waste or loss with respect to the primary action. The synergists function by successfully

competing with an active compound to attain and accumulate at the primary sites. Thus, the co-operation of the compounds in obtaining the desired end-effect is not a direct contribution to the primary activity. Such synergists will generally be structurally related to the highly active compound.

So far, we have dealt with synergism and its mechanisms in insects and in mammals by various types of substances in combination with different insecticides. Let us now approach the question as to whether AliE inhibition by TOCP is an important factor in its synergistic action, or whether one or more of the above mechanisms is involved.

MURPHY *et al.* (1959) demonstrated that the toxicity of malathion to rats was markedly enhanced by TOCP due to the inhibition of enzymatic detoxification of malathion. The authors referred to the data of MYERS and MENDEL (1953) which indicated that TOCP could inhibit AliE, without a clear statement whether its inhibition was involved or not. COLHOUN (1960) likewise suggested that TOCP was able to inhibit some enzyme systems, among them AliE, in tissues of the cockroach and so permitted topically applied TEP to reach the ChE of the thoracic nerve cord more quickly than in roaches treated with TEP alone. OPPENOORTH and VAN ASPEREN (1961) showed that breakdown enzymes present in resistant houseflies could be effectively inhibited *in vitro* by a number of organophosphorus compounds, including n-propyl paraoxon. Topical application of mixtures of this compound with malathion to a susceptible and two malathion resistant strains resulted in pronounced synergism in the two resistant strains, which was attributable to the inhibition of breakdown enzymes. The nature of such enzymes was disclosed by the authors as being altered AliE. The less marked synergism in the susceptible flies was ascribed to the inhibition of AliE and possibly other enzymes.

Our data tend to refute the assumed importance of AliE inhibition as a factor in synergism. It was inhibited to the same degree and showed alsmost the same inhibition pattern in all cases studied, irrespective of the occurrence of antagonism or synergism.

The effect of TOCP on susceptible and resistant houseflies revealed radical differences between the two strains. It will be seen later (Chapter IV), that AliE activity was slightly inhibited, when 100 μg TOCP per fly was applied to the R-flies. Meanwhile, no synergism occurred and the flies were completely normal.

If the inhibition of detoxication enzymes is the mode of action of TOCP as a synergist, and since it is generally believed that such breakdown enzymes are abundant in resistant flies, we might expect a more pronounced synergism in the resistant flies. Therefore, the fact that TOCP is not a synergist for the resistant flies, tends to rule out the inhibition of detoxication enzymes by TOCP as the main cause of its synergistic action.

We are also inclined to discard the theory of the "sites of loss" as an explanation for two reasons: (a) TOCP is not structurally related to either parathion or paraoxon, and (b) it is conceivable that "sites of loss" are present in both strains, and their occurrence is most probably more marked in the resistant strain. Nevertheless, TOCP acts as a synergist only in the susceptible flies.

By now, we have nearly discarded all three above mentioned points. All of them failed to explain the synergistic action of TOCP. The most interesting question still to be answered is: why is TOCP a synergist in the susceptible flies

and not so in the resistant ones? It seems most likely that the following observations may account for this effect. TOCP was without ill effects in resistant flies, even when 100 μg was administered to the abdomen. On the other hand, TOCP-treated susceptible flies showed various degrees of lethargy and a low mortality percentage, particularly when 100 μg was applied to the abdomen. The development of the symptoms of lethargy was very slow.

To ascribe the synergistic action of TOCP to lethargy is a very superficial and descriptive explanation. But the phenomenon itself may provide a clue to this problem. In seeking for such mechanisms, we must think of the processes which are associated with, and possibly cause lethargy. These processes are, of course, located inside the living fly and once they are known, mechanisms of TOCP's synergistic action can be explained.

It was observed that when susceptible flies were treated with 100 μg TOCP/fly, and confined for 24 h in milkbottles provided with filter paper discs, these discs were almost dry with very little amounts of excreted materials, unlike untreated flies or TOCP-treated resistant flies. In the latter cases, the filter paper was completely wet with an abundance of excreted materials. These observations may be explained on the basis of data provided by COLHOUN (1960). After injecting cockroaches with TOCP, he found that the foregut contained copious amounts of watery fluid. The mid-gut and hind-gut contained undigested food although the roaches were deprived of food during the 24-hours period after treatment. Based upon our own observations, it seems likely that TOCP has a similar effect on susceptible houseflies. This is further supported by the greater effectiveness of TOCP when it is applied to the abdomen. Hence, if the gut has a certain function in diminishing the toxic effects of organophosphates, and this function is interfered with, the synergistic action of TOCP is explained.

FERNANDO *et al.* (1951) used topically applied radioactive parathion and paraoxon to determine their uptake within the tissues of the cockroach. Concentration of paraoxon by the crop was extremely rapid and selective, whereas that of parathion was small, although quite marked in comparison with other tissues. Small amounts of the toxicants were recovered from the nerve cord. Accordingly, COLHOUN (1960) suggested another mechanism for the synergistic action of TOCP in the cockroach. Since TOCP was thought to interfere with gut function, it is possible that malfunction of the alimentary tract permitted less uptake of the toxicant by the gut from the blood and hence a greater concentration of the insecticide was available to inhibit the ChE of the nerve cord.

It now appears that TOCP has a similar, if not identical, effect on susceptible houseflies. The difference in the rate of concentrating parathion and paraoxon by the crop may account for the more marked synergism occurring with paraoxon. Thus, it seems safe to add a fourth point to describe another mechanism of synergism.

III.3.4. *Interference with metabolism and excretion.*

TOCP may interfere with the normal metabolism and excretion of susceptible houseflies. Consequently, the metabolism and excretion of the insecticide will be affected, raising its level inside the insect body above the threshold concentration. In this case more of the insecticide is available to the central nervous system, giving rise to severe ChE inhibition and higher mortality.

CHAPTER IV

EXPERIMENTS WITH A RESISTANT STRAIN

IV.1. Introduction

The experiments of the foregoing Chapters, particularly those described in Chapter III, led us to perform comparative studies with an organophosphate-resistant strain. It was thought that TOCP, by virtue of its antagonistic and synergistic activities, might be of value in disclosing some differences between susceptible and resistant houseflies.

IV.2. Review of literature

The experimental data of various authors on the reduced rates of penetration through the cuticle as a contributing factor in resistance of insects to organophosphorus insecticides are fraught with disagreements.

Administering DFP (di-isopropyl fluoro-phosphate) by injection directly into the thoracic hemocoel has been found to reduce the factor of resistance from 10 to about 2 or 3. This result led Chadwick (1954) to conclude that a considerable fraction of resistance must be provided by a less permeable integument in the resistant flies. The remaining low level was ascribed to other barriers (*e.g.* the nerve surface) or processes. Busvine (1957) arrived at essentially the same conclusion when he found that the resistance of houseflies was considerably reduced after the injection of DFP.

Oppenoorth (1958) found that his two resistant strains (U_2 and D) were also resistant to injected parathion. Accordingly, he concluded that decreased penetration cannot be the cause of resistance. After the injection of paraoxon, however, the differences in susceptibility observed between susceptible and resistant houseflies were small. This was attributed to the very rapid action of paraoxon. Similar results have been obtained by Busvine (1959) which led him to reject the idea of a selective cuticular barrier. He ascribed the loss of resistance to injected paraoxon to the excess of this rapidly acting poison swamping the protective mechanism. March (1959) performed similar experiments with malathion- and malaoxon-resistant and-susceptible strains of houseflies. An outstanding difference was observed: whereas resistance to topical application was greater than 150-fold for both malathion and malaoxon, tolerance to injection was only 2 to 3½-fold. At first appraisal the data of March would indicate that penetration through the cuticle is a primary cause of resistance. Nevertheless, he rejected this view and stated that the primary basis for organophosphorus resistance most logically resides in detoxication mechanisms.

Krueger *et al.* (1960) conducted penetration studies on Diazinon-resistant and -susceptible houseflies, and found only 7 % difference in integument permeability. Similar studies by Mengle and Casida (1960) with radiolabeled Diazinon and malathion indicated that both compounds penetrated more rapidly into the susceptible than into the resistant houseflies. The major difference occurred within the first 30 minutes. The authors concluded that their available data do not implicate penetration as the major factor contributing to the resistance. Plapp *et al.* (1961) noted no difference in the absorption of topically applied parathion and paraoxon by susceptible and resistant houseflies.

MATSUMURA and BROWN (1961a) found that malathion was absorbed at a similar rate by susceptible and resistant mosquitoes (*Culex tarsalis*). On the other hand, they (MATSUMURA and BROWN 1961b) demonstrated that the malathion tolerance of the Penang strain of *Aedes aegypti* was caused by decreased absorption. FORGASH *et al.* (1962) applied topically equal amounts of P^{32}-Diazinon to three strains of houseflies (two resistant and one susceptible). In the susceptible strain, Diazinon penetrated the integument considerably more rapidly than in the resistant strains. The differences in penetration between strains were considerably greater than the 7 % observed by KRUEGER *et al.* (1960). Moreover, resistance levels were found to be substantially reduced after the injection of Diazinon or parathion. The authors concluded, therefore, that reduced penetration can be an important factor contributing to the total resistance.

IV.3. RESULTS AND DISCUSSION

By and large, the experiments of this chapter were similar to those described previously. The difference was the insect material. An organophosphate-resistant strain (C-strain) was used instead of the susceptible colony (see Materials and Methods). With topical application in some experiments TOCP was applied prior to either insecticide (parathion or paraoxon). In others the sequence of application was reversed. Furthermore, injection experiments were conducted.

IV.3.1. *Degree of resistance*

The degree of resistance-*i.e.* the 24 hour LD_{50} of the resistant strain (R-strain) divided by the LD_{50} of the susceptible strain (S-strain) – was determined by topical application. The level of resistance against parathion was found to be 17-fold and that against paraoxon 12-fold (Fig. 25; see also Figs. 5 and 6). The degree of resistance was satisfactorily consistent during the course of our experiments.

IV.3.2. *Levels and relative susceptibilities of ChE and AliE*

The ChE activities of the R- and S-strains were identical, and were almost equally inhibited *in vitro* (see Chapter V). The AliE activity (hydrolysis of methylbutyrate) of the R-strain was much lower: 65 % of that of the S-strain. This proportion is, however, much higher than the 20 % found by OPPENOORTH and VAN ASPEREN (1961) using the same substrate.

IV.3.3. *The effect of TOCP*

A decisive difference between the R- and the S-strains as to the toxicity and inhibitory action of TOCP was observed. The symptoms of lethargy occurring with S-flies, most notably after the administration of massive doses to the abdomen, were not noticed with the R-flies. Practically, TOCP-treated R-flies behaved normally for as long as 48 h after treatment. The AliE activity of the R-flies was not inhibited by TOCP after 24 h, and slightly so after 48 h. ChE activity was unimpaired.

An experiment similar to that described in Chapter II (Fig. 16) showed almost the same pattern of ChE inhibition. After injecting TOCP with absolute ethanol, ChE was considerably inhibited, especially in dead flies. AliE activity

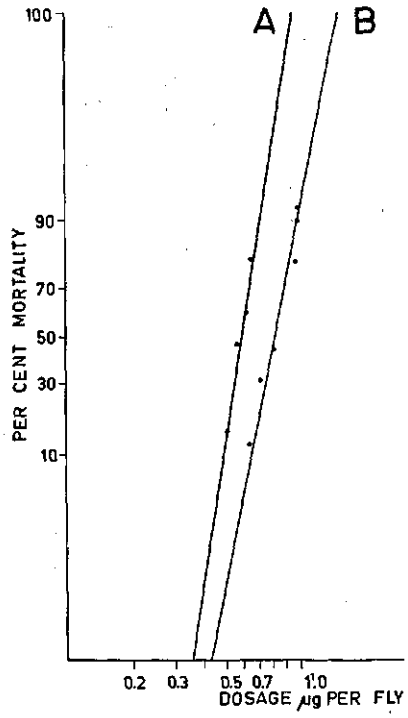


FIG. 25.
Dosage-mortality curves for 2-day-old adult females of resistant houseflies treated topically with parathion (A), or with paraoxon (B).

was slightly inhibited (Fig. 26). The results of the two figures may imply that the central nervous system of both strains is equally susceptible to organophosphorus compounds.

As mentioned previously, after the injection of ethanol into S-flies, mortality usually did not exceed 5 %. Surprisingly, when injected into R-flies, the ethanol resulted in a very high mortality (about 80 %). Apparently the solvent was not the cause of this high mortality. It was observed that R-flies were very sensitive

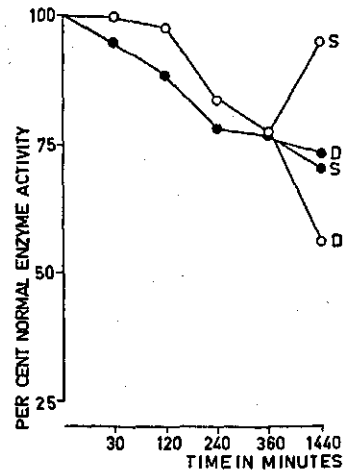


FIG. 26.
In vivo inhibition of ChE and AliE in resistant houseflies injected with 30 µg TOCP/fly dissolved in 0.5 µl absolute ethanol.
○———○ = ChE
●———● = AliE
S, D = surviving and dead flies.

to CO_2 anaesthesia, since after injecting the flies under light anaesthesia, the mortality percentage was substantially reduced (about 15 %). This difference may reveal some interesting physiological variations between the two strains.

IV.3.4. *The effect of topically applied parathion and paraoxon*

In order to obtain data reliably comparable with those of the S-strain, the dosage was chosen such that nearly equal mortality was produced, *i.e.* 0.55 μg of parathion per fly, and 0.75 μg of paraoxon per fly. Either dose resulted in 40 to 50 % mortality.

After the administration of either insecticide, the pattern of ChE inhibition did not greatly differ from that of S-flies (Fig. 27; A, C). With the S-strain, ChE inhibition values at 240 and 360 min intervals were somewhat higher. This might be ascribed to differences in the amount of the insecticide being available to the nervous system. Apparently, defense mechanisms are operating in such a manner that the amount of the toxicant at the site of attack is reduced.

An interesting difference in the pattern of AliE inhibition was observed between the two strains. AliE appeared to be less sensitive to inhibition by either insecticide (Fig. 27; A, C). In view of the above results and in addition to those recorded in Chapter V (which indicate that the degree of AliE inhibition *in vitro* is similar to that found *in vivo*), it may be concluded that the R-strain also contains at least two aliesterases acting on methylbutyrate. One of these, representing about 15 to 20 % of the total hydrolytic activity, is sensitive especially to higher concentrations of parathion or paraoxon. The other is much less sensitive to higher concentrations of either insecticide. Similarly, VAN ASPEREN and OPPENOORTH (1959, 1960) found in homogenates of some R-strains (strains D, A and F), that 13 to 50 % of the total hydrolytic activity proved to be insensitive to lower concentrations of paraoxon, and could be inhibited only by higher concentrations.

Our results on AliE inhibition in the R-flies are in sharp contrast to those reported by VAN ASPEREN and OPPENOORTH (1959, 1960), and OPPENOORTH and VAN ASPEREN (1960b, 1961). Their *in vitro* experiments showed that AliE of the R-strains could be as severely inhibited as the AliE of the S-strains. They pointed out that if the altered AliE (breakdown enzymes; called also phosphatase) which is present in the R-strains is blocked by the insecticide, it would be no longer capable of degrading the insecticide. To explain this discrepancy, het authors stated that the main difference between the two aliesterases found in R- and S-flies, is in the rate of dephosphorylation. This rate is almost zero for the AliE of S-flies, while a slow but definite turnover is present in the AliE of R-flies. This dephosphorylation, in addition to their high affinity for the toxicant (one of the properties the breakdown enzymes still have in common with the parent AliE; DRESDEN *et al.* 1961), may prevent the toxicant from reaching a fatal concentration at the site of action.

Our results also disagree with those of BIGLEY and PLAPP (1960) and PLAPP and BIGLEY (1961). The former reported that the two aliesterases in parathion-susceptible and- resistant houseflies, were equally inhibited *in vitro* by a similar amount of insecticides. The latter found that after treating R-flies with an LD_{50} of parathion, 90 % inhibition of AliE activity was recorded 15 min after treatment. This strong inhibition was similar to that observed with the normal colony and also appeared to be rapidly reversed.

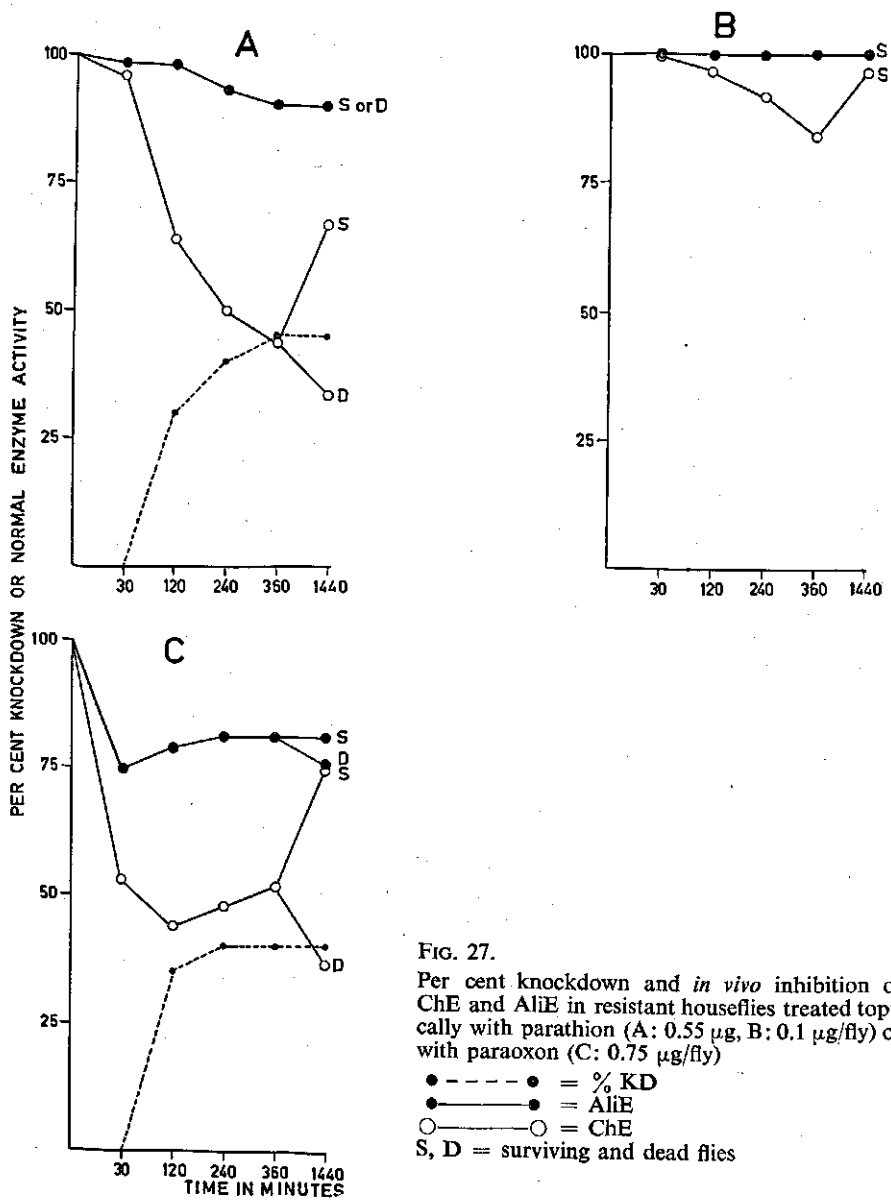


FIG. 27.
Per cent knockdown and *in vivo* inhibition of ChE and AliE in resistant houseflies treated topically with parathion (A: 0.55 μg, B: 0.1 μg/fly) or with paraoxon (C: 0.75 μg/fly)
●- - - -● = % KD
●——● = AliE
○——○ = ChE
S, D = surviving and dead flies

With a sublethal dose of parathion which caused no mortality (about 3 times the LD_{40} of the S-flies). ChE activity was slightly inhibited and reached the normal level after 24 h (Fig. 27; B). In general, it appears that recovery of ChE activity is more marked in R-flies than in S-flies. AliE activity was not affected. This result reflects the relative susceptibilities of the two sensitive fractions being present in both strains. The organophosphate susceptible AliE in the R-flies is less sensitive than that in the S-flies.

IV.3.5. *The joint action of TOCP and parathion or paraoxon*

An interesting clue to the mode of action of TOCP was provided by data obtained with the S-strain. Its synergistic activity was suggested to be due to interference with metabolism and excretion, whereas its antagonistic activity was ascribed to preventing or delaying the entry of the insecticide.

Similar to TOCP action in S-flies, simultaneous application of TOCP plus parathion resulted in a marked antagonism (Fig. 28; A), associated with a very low rate of knockdown. Maximum ChE inhibition was 10 % after 24 h, as compared with 35 % in S-flies (see Fig. 12; B). AliE activity was not inhibited, and slightly so in dead flies.

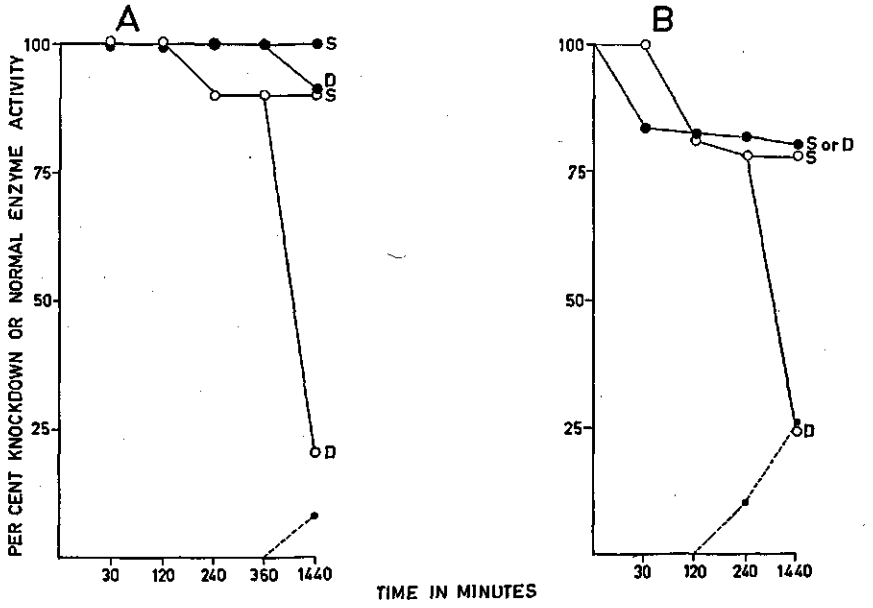


FIG. 28. Per cent knockdown and *in vivo* inhibition of ChE and AliE in resistant houseflies treated with parathion (0.55 μg/fly) and TOCP (A: 10 μg; B: 100 μg/fly).
A = simultaneous application.
B = TOCP applied to the abdomen 2 h prior to parathion.
●- - - -● = % KD ○——○ = ChE
●——● = AliE S, D = surviving and dead flies

An outstanding difference between the two strains was observed when TOCP was applied to the abdomen 2 h prior to parathion administration to the thorax. In an identical experiment with the S-strain, a synergistic effect associated with severe ChE inhibition was evident. With the R-strain, however, an antagonistic effect associated with a low rate of knockdown and mild ChE depression was obvious (Fig. 28; B). This antagonistic effect can be explained in view of our previous suggestion that TOCP penetrates readily and reaches the thoracic cuticle shortly after its application.

In general, the antagonistic action of TOCP in the R-strain is stronger than in the S-strain. The following two factors might contribute to this pronounced effect:

a. synergism is absent in the R-strain. The presence of it in the S-strain could probably overshadow the antagonistic activity of TOCP.

b. antagonism is stronger in the R-strain. The frequency of occurrence of a certain factor, which in combination with TOCP may account for the total antagonism, would be much higher in the cuticle of the R-flies than in that of the S-flies.

The informative experiments described in the previous chapter showed that parathion penetrates very slowly through the cuticle. In these experiments parathion or paraoxon was applied prior to TOCP administration. After treatment with parathion, it was noted that some R-flies were knocked down much faster than S-flies treated with the same insecticide, although the mortality percentage did not greatly differ in both cases (see Figs. 9; A and 27 A). Consequently, TOCP was applied to the thorax only at 30 and 90 min after parathion. Because the antagonistic activity of TOCP was quite marked in the R-strain, even when the compound was applied far from the site of parathion application, a parallel experiment was performed where TOCP was applied to the abdomen. The results of these experiments are presented in Fig. 29 (A, B). Very clear results were obtained with the R-strain. When TOCP was applied 30 min after parathion, the antagonistic effect in terms of ChE inhibition was more pronounced than in the corresponding experiment with the S-strain, although the mortality percentage was almost equal in both cases. The degree of ChE inhibition was closely associated with the amount of insecticide that has already penetrated. Therefore, ChE activity was more inhibited when TOCP

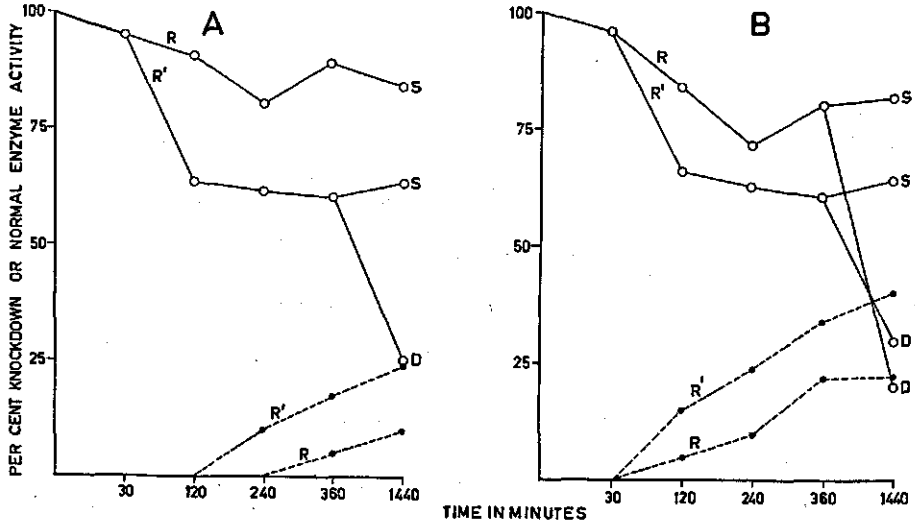


FIG. 29. Per cent knockdown and *in vivo* inhibition of ChE in resistant houseflies treated topically with parathion (0.55 μg/fly) to the thorax, followed by 100 μg TOCP applied topically to the thorax (A) or to the abdomen (B).

R = parathion applied 30 minutes prior to TOCP
R′ = parathion applied 90 minutes prior to TOCP

●- - - -● = % KD ○———○ = ChE
S, D = surviving and dead flies

was applied 90 min after parathion. In the parallel experiments where TOCP was applied to the abdomen, the general picture was similar to that described above. In this case the differences between ChE inhibition values in the two treatments, *viz.* 30 and 90 min time intervals, tend to be smaller (Fig. 29; B). Doubtless the ChE inhibition value at a certain time is an index of the amount of the inhibitor being available for the central nervous system. It was found that this amount is chiefly controlled by the rate of penetration of the insecticide. For example, 30 min after topical application of parathion to either strain, ChE inhibition was almost null. In view of the above results (see also Chapter III), it would appear that this fact is attributable to the retention of parathion in the cuticle during that period.

It is evident then that in the resistant strain, TOCP acts exclusively as an antagonist irrespective of the time and the site of administration. The overall picture of TOCP action in the susceptible strain varies strongly. The time and site of TOCP application are decisive factors in determining its effect, that is, whether it acts as an antagonist or as a synergist.

The differences between AliE inhibition values in these experiments (*i.e.* TOCP applied after parathion) were of such low order that they can be disregarded (Table 18). Obviously, AliE measurements in both strains cannot be relied upon in assessing the rate of penetration of the insecticide.

TABLE 18. Per cent normal AliE activity in resistant houseflies treated topically with Parathion (0.55 μg/fly) followed by TOCP (100 μg/fly).

Time interval Parathion/TOCP min.	I						II					
	Time after parathion min.											
	30	120	240	360	1440		30	120	240	360	1440	
					S	D					S	D
30	94	94	95	95	80	–	98	89	85	88	82	83
90	–	95	100	99	82	90	–	79	80	80	80	76

I TOCP applied topically to the thorax
II TOCP applied topically to the abdomen
S, D = surviving and dead flies

Similar results were obtained with paraoxon. TOCP antagonized the toxicity of paraoxon regardless of the time and the site of its application. Curiously enough, ChE and AliE activities were inhibited to a great extent without showing the usual correlation between ChE inhibition and toxic symptoms. The failure of ChE and AliE measurements to exhibit the usually encountered pattern, was apparently due to "homogenization artifacts". It is interesting to note that this was the first time during the course of our investigations with the two strains to observe such artifacts. Certainly, some factors have contributed to diminish the protective action of the substrates. In the first place, paraoxon is a phosphate, and "homogenization artifacts" have only been observed with phosphates, and never with phosphorothionates (COLHOUN 1959; SEUME *et al.* 1960; PLAPP and BIGLEY 1961; O'BRIEN 1961). In the second place, the presence of the phosphate for longer time in the cuticle due to the antagonistic activity of TOCP, played a major role in revealing this phenomenon. With the S-strain these artifacts were not observed because of the size of the dose.

Similar experiments to those described in Fig. 29 were performed with paraoxon. R-Flies were treated topically with the insecticide and at the indicated time intervals, TOCP was administered to the thorax in some experiments and to the abdomen in parallel ones. Prior to the administration of TOCP, the knock-down percentage (% KD) was recorded. These experiments were so designed that if TOCP bars the insecticide from further entry, the % KD must not greatly differ from the final mortality reached after 24 h, particularly if TOCP is applied to the thorax (*i.e.* the site of insecticidal application). The data recorded in Fig. 30 reveal that in the resistant strain the rate of penetration of paraoxon

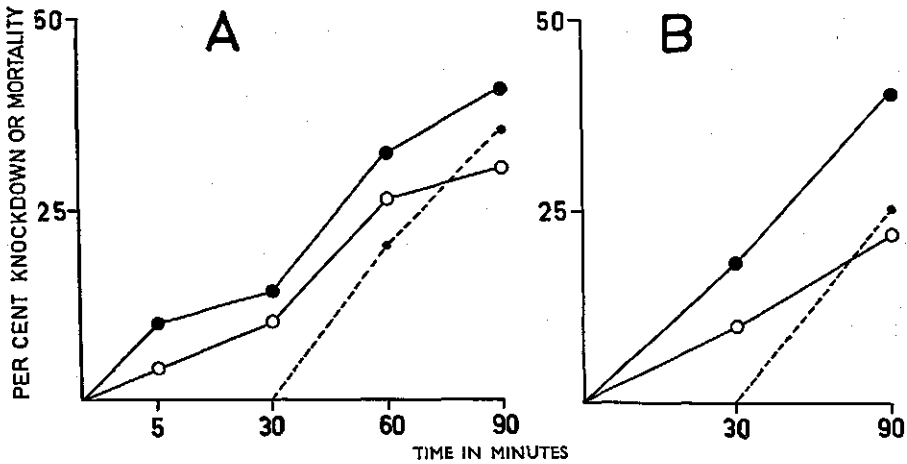


FIG. 30. Per cent knockdown or mortality of resistant houseflies treated topically with paraoxon (A: 0.75 μg/fly) or with parathion (B: 0.55 μg/fly) prior to TOCP (100 μg/fly) treatment. The abscissa designates the time after insecticidal application. TOCP was applied at the times indicated.

●——● = % Mortality, TOCP applied to the abdomen.
○——○ = % Mortality, TOCP applied to the thorax.
●- - - -● = % KD.

was nearly identical to that of parathion. The greatest difference between % KD and that of mortality did not exceed 10 %, which occurred after an interval of 30 min. Thereafter these differences decreased till % KD equalled and even exceeded mortality percentage. Obviously, the antagonistic activity of TOCP was of lower magnitude when it was applied to the abdomen.

The conspicuous features of Fig. 30 indicate that certain alterations occurred in the properties of the cuticle during the development of resistance. These alterations induced a remarkable decrease in the rate of penetration of paraoxon, which was not found with the S-flies. A considerable proportion of the total dose of paraoxon was still present in the cuticle of the R-flies for as long as 90 min and probably longer. This result implies that reduced penetration is a primary cause of resistance to paraoxon. The same conclusion is equally true for parathion, as mentioned previously.

IV.3.6. *Resistance levels after injection*

As in some of the experiments described in Chapter III, either insecticide was

injected prior to TOCP application. Such experiments were carried out to substantiate our conclusion that the antagonistic action of TOCP – when applied after parathion – is attributable to the presence of a considerable proportion of the total dose of the toxicant in the cuticle. Parathion was dissolved in olive oil and injected (0.5 μg in 0.5 μl/fly). The onset of the hyperactive–prostrate stage was after one hour. After the indicated time interval, TOCP was applied either to the abdomen or to the thorax. Thus by eliminating the role of the cuticle, and since TOCP was without any ill effects in the R-flies, we anticipated no interaction. The results of such experiments showed that the joint action of TOCP and parathion was nearly equal to the action of parathion alone (Table 19). These results, in addition to those obtained with the S-strain (see also Table 10), prove beyond any doubt, that after topical application, the toxicant was retained in the cuticle longer than usually thought. This could be demonstrated with parathion in both strains and with paraoxon only in the R-strain.

TABLE 19. Effect of TOCP (100 μg/fly) applied topically after the injection of Parathion (0.5 μg/fly) into resistant houseflies.

Time interval Parathion/TOCP min.	*30*		*90*		Parathion alone
Site of TOCP application	TH	ABD	TH	ABD	
% Mortality	60	58	54	56	54

TH = thorax ABD = abdomen
injection vehicle = 0.5 μl olive oil/fly

The most serious criticism of the foregoing results is that the level of resistance to parathion is not altered after its injection (Table 19). This seems to refute our view that the primary defense mechanism against an externally applied dose of parathion or paraoxon resides in the cuticle. It was suspected that the injection vehicle (olive oil) has played a certain part in this respect. To illustrate the role of the oil, aqueous ethanol was used instead. Hence each toxicant was dissolved in 0.5 μl of 50 % aqueous ethanol and injected. The results of these experiments are presented in Fig. 31. It is at once evident that the 17-fold resistance to injected (with oil) or topically applied parathion has been considerably reduced to only 4-fold (compare with Fig. 22; B). Another important feature of Fig. 31 (A) was the severe ChE inhibition soon after injection. Knockdown percentage and ChE inhibition reached a maximum in the first 30 min while AliE activity showed its customary pattern. Resistance to paraoxon, however, was almost completely eliminated after injection (Fig. 31; B, compare also with Fig. 23; B). It is interesting to note that AliE activity in dead flies was unimpaired. This adds evidence to our previously mentioned conclusions that AliE has no physiological importance in organophosphorus poisoning.

The experiments described in Fig. 31 do indicate that the nature of the injection vehicle is the main cause of the presence of equal levels of resistance after injection and after topical application. They also provide strong evidence that the cuticle is the site of an important defense mechanism against externally applied parathion or paraoxon.

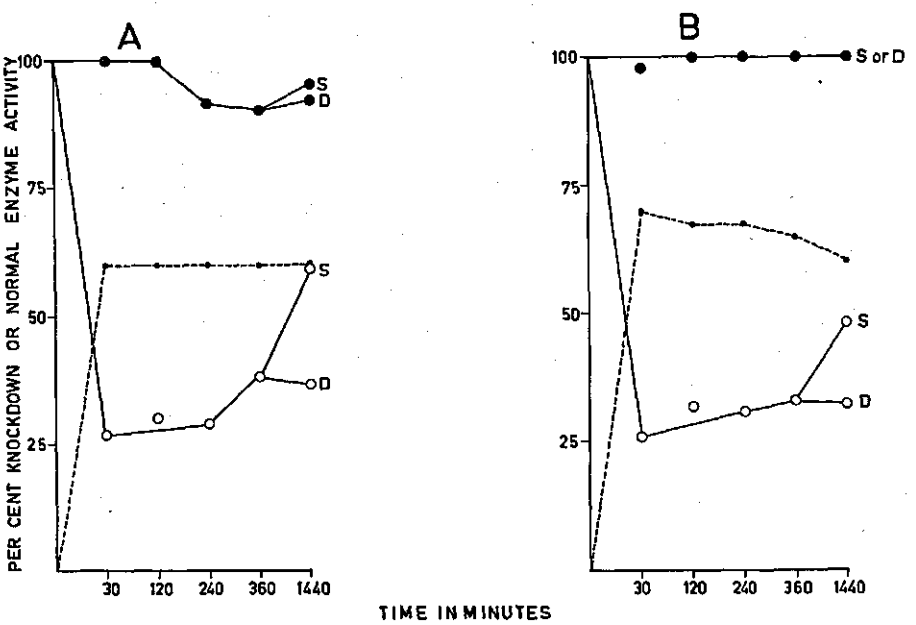


FIG. 31. Per cent knockdown and *in vivo* inhibition of ChE and AliE in resistant houseflies, injected with parathion (A: 0.1 μg/fly) or with paraoxon (B: 0.015 μg/fly) dissolved in 0.5 μl 50 % ethanol.

●- - - -● = % KD ○——○ = ChE
●——● = AliE S, D = surviving and dead flies

IV.3.7. *Discussion*

In the preceeding sections the most relevant problems have been discussed. We shall hereafter deal with some interesting observations which have been made during our investigations.

IV.3.7.1. Reduced penetration as a factor in resistance. It was observed that after the topical application of parathion doses causing comparable mortalities in both R- and S-strains, the latent period in the R-flies was decreased markedly. This would seem to be incompatible with our view that reduced penetration is a primary cause of resistance.

R-flies are called resistant because they endure doses which kill 100 % of S-flies. Hence when such a dose (0.55 μg parathion/fly) was applied to the S-flies, the onset of knockdown occurred after 40 min (see Fig. 19; C). Similarly, when a dose of paraoxon (0.75 μg/fly) was administered to the S-flies, the onset of knockdown was after 10 min. In both cases, obviously extremely rapid penetration took place, and therefore very rapid inhibition of ChE was brought about followed by rapid and complete mortality. These events, however, did not occur with identically treated R-flies (see Fig. 27; A, C).

HOSKINS and GORDON (1956), using six toxicants, found among seven species of insects an unmistakable relation between decrease in susceptibility and increase in heterogeneity towards the toxicant. Thus the R-strain is evidently not homogeneous. The population contains a wide range of susceptibilities. The

most susceptible individuals present in the resistant population would be comparable to the normal flies. Applying the massive dose of parathion for example, enables us to discern between relatively susceptible and resistant flies both present in the R-strain. In the comparatively susceptible individuals, the insecticide penetrates rapidly and consequently knockdown ensues also rapidly. In terms of the holding capacity, those individuals whose cuticles possess a relatively smaller capacity are knocked down much faster than the others.

If we had to assume that the properties of the cuticle are not altered, one would expect no or slight resistance to an externally applied dose. In such a case both toxicants would penetrate extremely rapidly, and a "swamping effect" would definitely result as has been found with the S-flies.

The fact that the susceptibility of the S-flies to parathion greatly increases when they are merely confined in milkbottles reflects the importance of the cuticle in parathion poisoning (see Chapter III). This was suggested to be due to the decrease in the frequency of the occurrence of a certain factor which is responsible for the very slow penetration of parathion. In an identical experiment with the R-flies their susceptibility towards parathion was, however, unaltered. This result furnishes a strong evidence for our previously postulated idea that certain alterations take place in the cuticle of the R-flies. In such case this factor is not easily lost or reduced. This factor is likely to be related to the holding capacity of the cuticle.

IV.3.7.2. Methods of studying penetration. An interesting resistance mechanism has been suggested by MENGLE and CASIDA (1960). Although brain ChE from R- and S-flies is equally susceptible *in vitro* to inhibition by organophosphates, it is much more inhibited *in vivo* by the same organophosphate dosage in susceptible than in resistant strains. Their results on the rates of penetration and detoxication have shown, however, relatively small differences between S- and R-strains. Resistance could not be reliably attributed to such differences. They therefore suggested, that in resistant flies there is probably a higher concentration of some "factor" in the thorax and/or abdomen capable of reducing the rate of ChE inhibition by organophosphates without hydrolyzing the inhibitor. According to our results, it seems likely that this "factor" is the low rate of penetration. If our suggestion is valid, why did they find smaller differences in the rates of penetration between S- and R-flies?

Our experimental approach in studying the rate of penetration was entirely different. Almost all investigators dealing with the penetration phenomenon study the rate of penetration by adopting the following procedure. After topical application and at various time intervals, the flies are rinsed several times with an organic solvent to remove the adhering insecticide. This fraction represents the amount of the insecticide which did not penetrate. The difference between this amount and the total amount applied, is assumed to have been absorbed. Some authors analyse the flies after rinsing and determine both fractions separately, either chemically or by a radiotracer technique.

The serious drawbacks of this procedure are the following:

a. loss usually occurs during the long and elaborate analytical procedure.
b. the fraction described as being "outside" is actually present only in the superficial layers of the epicuticle. ARMSTRONG *et al.* (1952) pointed out that washing the insect with an organic solvent docs not dissolve anything

that has penetrated below the wax layer of the epicuticle. It is well known that the epicuticle constitutes a very small part of the whole cuticle (1–7 %; BROWN 1956). Thus the amount of the toxicant present in the rest of the cuticle, which is presumably greater, is added automatically to the amount which has already penetrated, giving artificially higher estimates of the fraction described as being "inside".

c. personal errors, particularly in the degree and way of washing the insects and the use of different organic solvents by various authors.

Our experimental approach, although of a qualitative nature, has some advantages as compared with the above technique. It depends on the biological response of the insect, without using any elaborate procedures. The actual amount of the insecticide which does not penetrate at a certain time, is retained in the cuticle by the aid of TOCP, or at least is released very slowly so that the biological response is not affected.

IV.3.7.3. The relation between the nature of the injection vehicle and the level of resistance. The interesting result (see Table 19) that the level of resistance is not altered after injecting parathion (in olive oil) has also been obtained by other investigators. OPPENOORTH (1958) injected resistant houseflies with parathion dissolved in peanut oil, and with paraoxon as a solution in insect saline. He found that resistance to parathion was not eliminated, and concluded therefore that reduced penetration cannot be the cause of resistance. With paraoxon, however, resistance was largely overcome and was ascribed to the "swamping effect" of the toxicant. BUSVINE (1959), using olive oil as the injection vehicle, arrived at the same conclusion although he found that the levels of resistance to injected parathion and paraoxon were all lower than the corresponding figures for external treatment.

The use of aqueous ethanol resulted in almost complete elimination of the resistance to paraoxon, and in a very low level of resistance to parathion (see Fig. 31). Thus if aqueous ethanol had not been used as the injection vehicle, we could have arrived at essentially the same conclusions of OPPENOORTH and BUSVINE. But it is now clear that the nature of the solvent plays a major role in the behaviour of the injected parathion. High levels of resistance comparable to those obtained after topical application occur if oil is used as the vehicle.

BUSVINE (1959) found that resistance to paraoxon was depressed much more than to parathion. This may be stated otherwise in terms of liposolubility: resistance to the less liposoluble compound was depressed much more than to the more liposoluble compound (see Chapter VI). Apparently the rate of release of each toxicant from an oil droplet is determined by its degree of liposolubility. Therefore, parathion is released much more slowly than paraoxon.

With the S-strain, we found a small difference in the length of the latent period and in mortality between topical application and injection of similar dosages of parathion, only when it was dissolved in 0.5 µl olive oil. The LD_{50} of injected parathion was 0.83 of the external dose. In fact the same phenomenon was observed with the R-strain. The LD_{50} of injected parathion was 0.91 of the external dose. With paraoxon, however, the differences were much greater, particularly with the R-strain. Thus if a certain phenomenon does exist in S-flies and at the same time in R-flies, it might be infered that it is not related to resistance.

These findings with both strains throw some light on the role of oil. The effect of the minute droplet (0.5 µl) equals more or less the effect of the cuticle. In what respect does an oil droplet resemble the cuticle? Both affectuate the gradual release of parathion. Therefore, it was not surprising to find a similarity between the LD_{50} values of topically applied and injected parathion, regardless of the degree of susceptibility. If our interpretation is valid, the amount of injected oil would affect the degree of susceptibility. If the size of the oil droplet decreases, one might anticipate a rapid release of the toxicant resulting in more depression of resistance. The data of BUSVINE (1959) and MARCH (1959) in addition to our own data provide supporting experimental evidence for this view. Different amounts of oil have been used in the three experiments. In our experiments the use of 0.5 µl olive oil/fly did not materially alter the level of resistance as compared with topical application, *i.e.* about 17-fold in both cases. BUSVINE using 0.35 µl olive oil/fly found that the resistance level of 35-fold to topical application fell to 12-fold when parathion was injected. MARCH using lesser amounts, *viz.* 0.11 µl peanut oil/fly, reported for malathion that whereas resistance to topical application was greater than 150-fold, tolerance to injection was only 3-fold.

When OPPENOORTH and BUSVINE (*loc. cit.*) ascribed the elimination of resistance to injected paraoxon to a "swamping effect", they suggested indirectly that the cuticle has prevented this effect. This holds particularly when we know that the topically applied dose was about 50 times as high as the injected dose.

One might argue that the cuticular barrier is not a factor in resistance since the R-strain is resistant to injected parathion (in 0.5 µl oil), as compared with an identical experiment with the S-strain. In view of the foregoing results, this cannot simply indicate that the cuticle is not of primary importance in the resistance to externally applied parathion or paraoxon, but it points to the existence of some internal defense mechanisms. It would appear that the changes occurring in the properties of the cuticle during the development of resistance, are associated with internal modifications.

IV.3.7.4. The possible relation between the gut and resistance.

In an earlier discussion on the mechanisms of synergism (see Chapter III), it was stated that the application of TOCP could reveal internal physiological differences between the susceptible and the resistant strains. This compound acted as a synergist and as an antagonist in S-flies, whereas it acted exclusively as an antagonist in R-flies. If our previous suggestion is valid, namely, that TOCP potentiates the toxicity of parathion and paraoxon to S-flies because it interferes with the gut function, it then follows that in the R-flies the gut function is not interfered with by TOCP. This internal physiological difference may be related to resistance. It is interesting to note that this suggestion receives experimental support from the work of FAST and BROWN (1962), who found that a DDT-resistant strain of *Aedes aegypti* developed by malathion pressure, retained twice as much DDT in the gut as the susceptible strain.

In conclusion the available data on penetration are adequate to indicate that the low rate of penetration is an important factor in resistance. The cuticle may function as a regulatory membrane, allowing the gradual release of minute amounts of parathion or paraoxon, so that the internal biochemical and physiological processes can cope with such amounts. This would result in the inactivation of these amounts and in diminishing the concentration of the

toxicant at the vital target. If the function of the cuticle is interfered with, larger amounts of the toxicant would penetrate rapidly and thus being beyond the capacity of these processes. In studying resistance mechanisms, one must not deal with them as separate independent entities, but one must rather consider all defense mechanisms, present in a certain strain, as one unit. The loss of one may decrease the level of resistance of the whole complex. Resistance is developed in the field or in the laboratory by exposing the population to the frequent application of insecticides. In nearly all cases the toxicant is applied externally. As the cuticle is the first part of the insect body which receives the toxicant, one might anticipate therefore that it would also be the first part to be adapted to the new conditions.

CHAPTER V

THE IN VITRO INHIBITION OF ChE AND AliE ACTIVITIES BY PARATHION AND PARAOXON IN SUSCEPTIBLE AND RESISTANT HOUSEFLIES

V.1. Introduction

A clear-cut difference was observed between susceptible and resistant houseflies in their *in vivo* reaction either to TOCP alone or with parathion or paraoxon. The question arose as to whether this difference would exist also under *in vitro* conditions. Therefore, the joint action of TOCP and either insecticide in the homogenates of the two strains was investigated.

V.2. Results and discussion

The *in vitro* inhibition of ChE and AliE activities by either insecticide was first determined in the two strains. Paraoxon strongly inhibited the ChE activity of both strains; the I_{50} value was 4×10^{-10} M (Fig. 32). A remarkably high degree of ChE inhibition was brought about by parathion; the I_{50} value was 3×10^{-7} M (Fig. 33).

The interesting feature of Figs. 32 and 33 is that the ChE activities of both strains were equally inhibited by paraoxon or by parathion. This indicates that the sensitivity of ChE activity is not related to resistance. Similar results have been obtained by Chadwick (1954); Lord and Solly (1956); Oppenoorth (1958); Van Asperen and Oppenoorth (1959); March (1959); Mengle and Casida (1960); Bigley and Plapp (1960, 1961); Matsumura and Brown (1961a) and Forgash *et al.* (1962).

The I_{50} value of parathion was found by the early investigators to be in the range of 10^{-6} M (Du Bois *et al.* 1949; Metcalf and March 1950; Aldridge 1950). This was considered later to be a high value due to the contamination of parathion with the S-ethyl isomer (for details see following Chapter). Thus our supposedly pure sample of parathion was in fact contaminated. Although this sample was stored in the refrigerator during the course of our investigations, it is quite likely that some isomerization took place upon storage. This probably resulted in the production of an active inhibitor.

Did this contamination affect our *in vivo* results? This seems very unlikely.

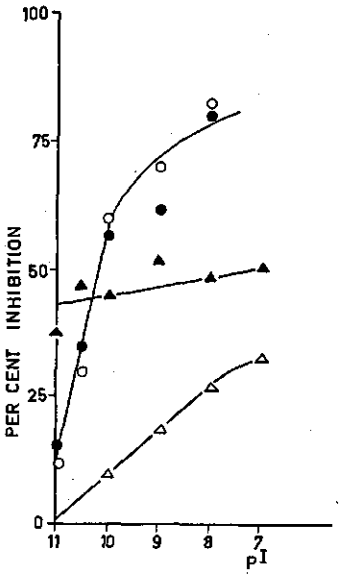


FIG. 32.

In vitro inhibition of ChE and AliE of susceptible (S) and resistant (R) houseflies by paraoxon.

pI = the negative logarithm of the molar concentration of the inhibitor.

○——○ = ChE (R)
△——△ = AliE (R)
●——● = ChE (S)
▲——▲ = AliE (S)

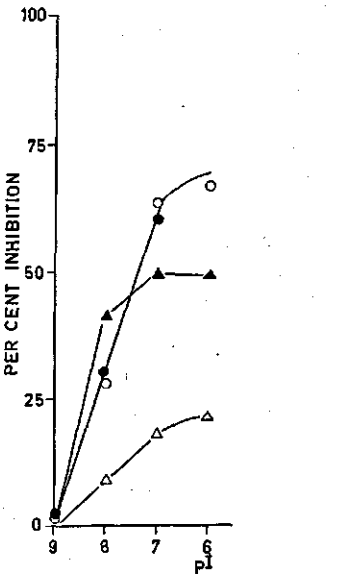


FIG. 33.

In vitro inhibition of ChE and AliE of susceptible (S) and resistant (R) houseflies by parathion.

pI = the negative logarithm of the molar concentration of the inhibitor.

○——○ = ChE (R)
△——△ = AliE (R)
●——● = ChE (S)
▲——▲ = AliE (S)

Histochemical studies revealed comparable areas and degrees of inhibition of ChE in the thoracic ganglion and brain of the housefly treated with a technical grade (85 %) or pure Diazinon (MOLLOY 1961). This would indicate that the active impurities are eliminated *in vivo*. METCALF *et al.* (1956) pointed out that these impurities may completely mask the anti-ChE activity of the less active compounds without, however, altering their *in vivo* toxicity. This is conceivable since the presence of the isomer in the phosphorothionate in traces as low as 0.01 % produces appreciable ChE inhibition (METCALF and MARCH 1953a). If serious transformations took place in storage, one would have expected a noticeable modification in the toxicity of parathion. Mention was made earlier (see Materials and Methods) that the LD_{50} of parathion was almost constant during our investigations. This observation is in harmony with the above reports. In view of our discussion in the following Chapter it might be stated that after the application of a contaminated sample, the active impurities would be hydrolyzed very rapidly due to their extreme instability before having the chance to phosphorylate the ChE. Moreover a considerable part of these impurities would be involved in phosphorylating non-specific groups (BURGEN 1949).

The infra-red absorption spectrum of our parathion showed no clear ab-

a. pure phosphorothionates are poor anticholinesterase agents *in vitro*, yet produce strong ChE inhibition *in vivo*.
b. phosphorothionates and their corresponding phosphates have similar LD_{50}'s to insects.
c. oxidations of phosphorothionates to phosphates occur with insect tissues *in vitro*. We can add one more point *i.e.*:
d. the slow toxic action of parathion as compared with its oxygen analogue.

We shall hereafter discuss these points separately.

VI.3. Discussion

VI.3.1. *The LD_{50} values of parathion and paraoxon*

The evidence of the similarity of the LD_{50} values for parathion and paraoxon stems from the reports of, Chamberlain and Hoskins (1951) and Hadaway *et al.* (1963). Conflicting evidence can, however, be gathered from the work of Metcalf and March (1949; 1950); Woodcock and Stringer (1951); Stringer (1956); Stringer and Fielding (1956); Busvine (1959); Oppenoorth and Van Asperen (1960a); Plapp *et al.* (1961). A more detailed review was given in Chapter I. Our results (see Chapter I) indicate that parathion is almost as twice as toxic as paraoxon after topical application, and less toxic after injection.

It is at once evident that the data on the relative toxicity of both compounds are fraught with sharp variations, indicating the intervention of certain factors which dictate the toxicity of the compound. It is quite obvious that this point (similar LD_{50}'s) is of questionable validity. In fact, it would have been important evidence if it had been shown consistently that paraoxon is far more toxic than parathion, say 10 times or more, irrespective of the route of administration. As the problem of toxicity is complicated by the interference of many factors, we shall discuss the most relevant ones.

VI.3.1.1. Stability to hydrolysis.

The replacement of the thiophosphoryl group (P = S) by the phosphoryl group (P = O) is known to markedly increase the rate of hydrolysis (Martin 1956). The greater stability of parathion is due to the weaker electrophilic effect of = S compared to = O. Therefore the phosphorus atom in phosphorothionates is not expected to carry as large a positive charge as the phosphorus atom does in phosphates (Ketelaar *et al.* 1952; Heath 1956a; Fukoto 1957).

The stability of the two isomers (the S-phenyl isomer and the S-ethyl isomer) to hydrolysis is far less than that of either parathion or paraoxon. Aldridge and Davison (1952b) found that at PH 8, 95 % of the S-ethyl isomer would be hydrolyzed in 6 min, the S-phenyl isomer in 32 min, paraoxon in 18 h and parathion in 122 h. Metcalf and March (1953c) reported that the S-ethyl isomer was hydrolyzed 300 times as fast as parathion and nearly 40 times as fast as paraoxon in alkaline solution. Much higher rates of hydrolysis have been recorded by Heath (1956a, b). The S-ethyl was hydrolyzed 1600 times more rapidly than paraoxon, and several times faster than its S-phenyl isomer. Paraoxon was found to be hydrolyzed nearly 10 times as fast as parathion. The rates of hydrolysis at neutral pH, however, were much lower than in alkali.

A relation between inhibitory power and stability to aqueous hydrolysis of a

series of substituted diethyl phenyl phosphates has been found by ALDRIDGE and DAVISON (1952a). The more stable the inhibitor, the lower is its inhibitory power. Parathion, its oxygen analogue and its two isomers were found to obey this rule (ALDRIDGE and BARNES 1952; ALDRIDGE and DAVISON 1952b; KILBY and Youatt 1952). Thus, the ability of the compounds to inhibit ChE is a result of their instability to hydrolysis (METCALF *et al.* 1956; FUKOTO and METCALF 1956). According to STRINGER and FIELDING (1956), however, the potency of the five compounds to inhibit ChE did not completely follow the rule of ALDRIDGE and DAVISON (1952a). It was in the order: paraoxon >S-ethyl isomer>S-phenyl isomer>parathion technical>parathion pure. Also LORD and POTTER (1954a) found that the two isomers were slightly less potent than paraoxon in their *in vitro* inhibition of ChE activity in four insect species.

The stability of parathion is reflected in its insecticidal potency. LORD and POTTER (1950, 1951) suggested that although TEP is a more potent esterase inhibitor, yet it is less effective as a contact insecticide than parathion because it is unstable and hydrolyzes rapidly. DU BOIS (1953) pointed out that the greater resistance of parathion towards hydrolysis is advantageous from the standpoint of insecticidal action. METCALF and MARCH (1953c), studying the isomerization of parathion, found that the greatly enhanced *in vitro* anticholinesterase activity of the two isomers did not result in high *in vivo* toxicity to the mouse and housefly. This was ascribed to the fact that the two isomers were so unstable to hydrolysis that they were largely destroyed before reaching the site of action. According to the above mentioned reports, the rate of hydrolysis of the four compounds is in the order: parathion<paraoxon< S-phenyl isomer<S-ethyl isomer. Our data on the toxicity of the four compounds tested against houseflies by topical application (see Chapter I), indicate that their relative toxicity is inversely correlated to their rates of hydrolysis: parathion>paraoxon> S-phenyl isomer>S-ethyl isomer. It is evident then that stability to hydrolysis favours parathion as a potent insecticide.

VI.3.1.2. Liposolubility.

The thionophosphates differ from the corresponding phosphates in properties other than stability and anticholinesterase activity; they are more liposoluble (MARTIN 1956). Why is parathion more soluble than the corresponding phosphate and its two isomers? It is well known that the more polar the compound, the less liposoluble it is. The high polarity of the P = O bond induces in phosphates a greater water solubility and poorer lipoid solubility than in the corresponding thionophosphates (KETELAAR 1953). Therefore, on a reversed phase paper chromatogram where the paper is the nonpolar phase, phosphorothionates will move less readily on the paper than their more polar phosphate analogues, for P = S is less polar than P = O (O'BRIEN 1960). The degree of liposolubility of the compound can be deduced then from its rate of flow (R_f) on a reversed phase paper chromatogram. METCALF and MARCH (1953b) found that the S-alkyl isomers appear first, then paraoxon and later parathion.

The order of the liposolubility of the four compounds can be then presented as follows: parathion>paraoxon> the two isomers. Thus, there is also a correlation between the relative toxicity of the four compounds applied topically to houseflies (see Chapter I) and their solubility in lipoid media.

The liposolubility of parathion also favours its insecticidal potency. This

property would result in greater affinity for the central nervous system (GROB 1950; GROB *et al.* 1950).

So far, we have gained some knowledge about the relative stability, liposolubility and potency to inhibit ChE of the four organophosphorus compounds under consideration. In view of this information we shall try to explain the interesting observations which have been reported previously (see Chapers I and III), namely, the variations in the toxicity of the four compounds when the route of administration is altered. It seems probable that each of the three above mentioned factors, *viz.* stability, liposolubility and intrinsic inhibitory power is playing a part, minor or major, in dictating the final mortality, depending upon the route of administration. The latter determines the extent of this part, which may account for variations in toxicity.

It is generally agreed that the potency of the compound to inhibit ChE *in vitro* is not an essential prerequisite for high insecticidal activity. Doubtless, the toxicity of an organophosphorus compound will be profoundly affected by the physico-chemical properties of the compound and the biochemical processes of the insect (MARTIN 1953; LORD and POTTER 1954a; FUKOTO and METCALF 1956; SPENCER and O'BRIEN 1957; FUKOTO 1957).

The order of the insecticidal potency of the four compounds when injected differs from that after topical application. The injection of the compounds with aqueous ethanol (see Chapter III), ranked them in the order: paraoxon> parathion≥S-phenyl isomer>S-ethyl isomer. This would indicate that the factors which are responsible for this difference are not dominating in the case of topical application.

After injection, the whole dose appears at once in the haemolymph, furnishing a steep gradient toward the central nervous system. Thus the high anti-ChE activity is a major factor in this case. This would probably explain why paraoxon is the most potent insecticide among its analogues after injection. With the other three compounds, apparently other factors intervene. It would appear that liposolubility interferes in the case of parathion, and stability with the two isomers. After the injection of parathion a considerable proportion of the injected dose is probably dissolved in the lipoids of the thoracic tissues from which it is slowly released. This would account for the relatively long latent period after injecting parathion with aqueous ethanol into houseflies (see Chapter III).

A reliable comparison can be made between parathion and its two isomers, for identical injection experiments were performed with each compound (see Figs. 22; B and 24). Apparently, the great instability of the S-ethyl isomer was the dominating factor in decreasing its toxicity, and in facilitating the hydrolysis of the ChE-phosphate complex (ALDRIDGE 1953a; DAVISON 1953b; see also Chapter III). When the picture of the S-phenyl isomer is compared with that of parathion, we may notice an equilibrium between stability and anticholinesterase activity. In view of the results with paraoxon (see Fig. 23, B), the S-phenyl isomer could have caused a higher mortality than that produced by parathion, because of its higher anti-ChE activity. The mortality, however, equalled that recorded after parathion injection. It is quite likely that the rapid hydrolysis of the S-phenyl isomer has played a considerable role in this respect. The same reasoning holds, *a fortiori*, for the S-ethyl isomer.

FUKOTO (1957) suggested two requirements for high inhibition of ChE. These requirements are, firstly, rapid reaction between enzyme and phosphate, and

secondly, reasonable stability of the phosphorylated enzyme. The first requirement is satisfied with the three phosphates. The extent to which the second one is met with depends on the relative stability of the three phosphates to hydrolysis.

Because stability of the two isomers interferes with their toxicity after injection, it is quite obvious that this property would interfere even more markedly when the two compounds are applied either topically or by the contact method. Therefore, it seems justified to disregard the two compounds in our further discussion.

It has been found consistently that paraoxon is far less toxic than parathion after application by the contact method. At the best of the author's knowledge, no attempt has been made to explain this phenomenon. It seems that the rate at which the toxicant reaches the site of action is of crucial importance in determining the final mortality. This rate is mainly controlled by the route of administration. In the contact method the toxicant is picked up slowly and in minute amounts. As paraoxon penetrates rapidly and is relatively unstable, as compared with parathion, these minute amounts are easy targets of attack by detoxifying enzymes. On the other hand, the very slow penetration of parathion in addition to its greater stability, render the toxicant less readily available to detoxifying enzymes and less susceptible to their attack. It would seem then, that if houseflies were treated in a certain manner so that each toxicant was applied very slowly and in minute amounts, the difference in toxicity would be even much greater than that found after using the contact method.

Because the injection and the contact methods represent the two extremes with regard to the rate at which the toxicant reaches the site of action, almost always consistent results have been obtained with houseflies. After injection paraoxon is more toxic than parathion, whereas after using the contact method it is far less toxic. The results of the intermediate route of administration, *i.e.* the topical application method, showed some variations. In this case, the whole dose is applied at once (similar to injection), but externally (similar to contact). Although paraoxon penetrates rapidly, it appears in the "inside" only gradually when compared with the injection method.

Furthermore, as the reactivity of the compounds increases, the rate of non-specific reactions such as phosphorylation of random amino-, hydroxyl-, or phenolic groups in the proteins may also increase. These side reactions will divert some of the active agent: an important matter with substances effective at very low concentrations (BURGEN 1949). As groups like those mentioned are present in the cuticle (see RICHARDS 1951; WIGGLESWORTH 1953), their reaction with paraoxon would contribute to decrease the number of molecules which are able to phosphorylate ChE.

Liposolubility properties are also likely to affect the toxicity of the compound. The pattern of the distribution of the toxicant inside the insect body is mainly controlled by these properties. ROAN *et al.* (1950) concluded that the more water soluble the compound, the more rapid it is transported and distributed inside the insect body. LORD and POTTER (1954a, b) pointed out that the differences in the pattern of distribution of two poisons in one insect species are due to differences in their physical properties, so that the relative concentration of each poison at the site of action would not be the same. Accordingly, one might anticipate that paraoxon is more rapidly and widely distributed

inside the insect body as compared with parathion. Consequently, the toxicant will be more exposed to enzymic attack. With parathion, the incidence of the insecticide being "trapped" by the lipoid nerve sheath would be higher than with a comparable amount of paraoxon. The experimental data of RICHARDS and WEYGANDT (1945) indicated that lipoid-soluble materials accumulated in the insect nervous system.

In general, there is a better correlation between anti-ChE activity and toxicity when the injection method is used. With the other two methods (topical application and contact) stability and liposolubility properties are likely to overshadow the anti-ChE activity of the compound. This is most conspicuous with the contact method.

VI.3.2. *Oxidation of parathion in insect tissues*

It has been demonstrated conclusively that such oxidation occurs *in vitro*. It is equally true that this activation also takes place *in vivo* as discussed below. Moreover, parathion undergoes other metabolic changes *in vivo* (GARDOCKI and HAZLETON 1951; PANKANSKI *et al.* 1952; ARTHUR 1960). These changes are likely to occur *in vitro*, and are induced by non-specific enzyme systems widely distributed in the animal body.

As the conclusions about the conversion hypothesis are derived mostly from *in vitro* studies, they are somewhat speculative.

The conclusions are more meaningful when obtained from *in vivo* studies. It is generally agreed that a part of the total dose of the phosphorothionates is converted to the oxygen analogue in the living animal (PLAPP and CASIDA 1958; KRUEGER *et al.* 1960). Attempts have been made to recover paraoxon quantitatively from parathion poisoned animals. Only 0.1 % of the total parathion dose was recoverable as paraoxon in rats (GAGE 1953), and in the American cockroach (BENJAMINI *et al.* 1959).

In view of the above *in vivo* results, let us consider what would occur after the topical application of parathion. The following processes may occur: penetration, metabolism, excretion, storage, transport to and attack on the target, and the physiological consequences of the attacked target (KRUEGER and O'BRIEN 1959). It has been shown that parathion penetrates very slowly (see Chapter III). Since the cuticle is inactive in converting parathion (METCALF and MARCH 1953a; KOK and WALOP 1954), the toxicant may enter without suffering metabolic changes. The amount of parathion in the cuticle is gradually depleted, and its concentration in the "inside" is gradually raised. This process lasts about 150 min (see Chapter III). The "inside" contains sensitive and insensitive sites. The sensitive sites, presumably the central nervous system, constitute a very small part of the whole body. Therefore, the greatest proportion of the total parathion dosage is distributed gradually among the insensitive sites. Some molecules are altered to the oxygen analogue, or suffer hydrolytic attack; the rest may remain intact for a longer time. In this case the oxygen analogue, being gradually and slowly formed, has the opportunity of being rendered inactive by enzymic attack before it ever has a chance to phosphorylate the ChE.

It is of great interest to mention the deduction made by KUBISTOVA (1956), of the approximate *in vivo* activity of several rat tissues. She incubated parathion with liver, kidney and intestine slices. According to her assumption, if the lethal dose of parathion is diluted to the volume of the animal body, the resulting

concentration will lie within the limits used on incubation with tissue slices in her *in vitro* experiments. Under these conditions, the whole liver and kidney together with the intestine would convert in one hour not more than 6 % of the total parathion dose to paraoxon, which would be for the greater part metabolized further. KUBISTOVA concluded that the conversion rate is too slow to explain the rapid intoxication of rats injected with parathion, which seems to be contradictory to the actual ratio of the lethal doses of the two compounds. This discrepancy was explained as follows. In paraoxon poisoning, the substance is very quickly split in the organism not only by the liver and kidney, but also in the blood. Consequently its concentration falls rapidly below the effective levels so that the corresponding enzymatic systems of the organism are exposed to higher concentrations of the drug only for a relatively short period. In parathion poisoning a low but steady concentration of paraoxon is maintained in the blood for a longer period. In view of the above assumptions, it would seem that in parathion poisoning the low and steady concentration of paraoxon in the living organism is always kept below the effective levels. Once its concentration rises to reach these levels, the breakdown enzymes would operate in a similar manner to that after paraoxon intoxication. In addition, the low and steady concentration of paraoxon in the blood being produced after parathion poisoning may suffer a hydrolytic attack as well. ALDRIDGE (1953b) demonstrated the presence of an enzyme capable of hydrolyzing paraoxon in the rat serum. Furthermore, since the catabolic activity of the animal is located mainly in the three organs, namely the whole liver, kidney and the intestine, it would appear that the slow oxidative reactions and the enzymic detoxication of the formed paraoxon may occur concurrently.

The remaining part of the total parathion dose would be in the sensitive sites. If this part is largely and quickly converted to paraoxon, it may adequately account for parathion poisoning. It is unlikely, however, that this is valid. In the first place, it has been shown that the nervous system is moderately active in converting parathion (METCALF and MARCH 1953a; KOK and WALOP 1954). Therefore, the 6 per cent/h calculated by KUBISTOVA for the most active organs may be replaced by 1 per cent/h or even less, for the nervous system. In the second place, the perineurium of the nerve sheath was found to be exceptionally rich in enzymes which may well be responsible for detoxication (WIGGLESWORTH 1956). Thus a rather slow conversion associated with brisk hydrolysis makes it unlikely that the formed paraoxon would ever have the opportunity to phosphorylate the ChE.

The marked low toxicity of paraoxon, as compared to parathion, after using the contact method, was suggested to be attributable to the slow pick-up of the toxicant in small amounts and thus giving an opportunity for detoxifying systems to operate. During the conversion process, paraoxon is also slowly formed in minute amounts. If our explanation is true, one would expect that to produce equal mortality, the parathion dosage should be at least 10 times as much as that of paraoxon.

VI.3.3. *The slow toxic action of parathion*

Various pieces of evidence have been provided, which indicate that the latent period is not due to the time being spent in oxidizing parathion to produce the presumed real toxicant, paraoxon, but rather to the very slow penetration of parathion (see Chapter III).

VI.3.4. *The strong in vivo ChE inhibition produced by parathion*

In order to shed some light upon the point that parathion whilst being a poor anti-ChE *in vitro* produces strong *in vivo* ChE inhibition, we shall first discuss another phosphorus compound, *viz.* TOCP. The inhibiton of mammalian and insect esterases by TOCP has been reviewed in Chapter II. Most investigators have demonstrated that TOCP is a poor inhibitor of true ChE *in vivo* and *in vitro*, and a selective inhibitor of pseudo-ChE and AliE. In contrast, ALDRIDGE (1954) and COURSEY *et al.* (1957) reported that TOCP could inhibit true ChE *in vivo*.

ALDRIDGE (1954) pointed out that since TOCP is a stable substance, it is unlikely that the unaltered molecules would have inhibitory activity. The same reasoning was applied to parathion. He suggested that TOCP is converted to a potent inhibitor on injection into the rabbit or chicken. MYERS *et al.* (1955) obtained some evidence in favour of the conversion of TOCP to an active inhibitor of pseudo-ChE. The authors, however, did not state clearly whether true ChE was inhibited or not.

The results of STEGWEE (1960) with houseflies and COLHOUN (1960) with cockroaches are in general agreement with most of the results obtained with mammals. No evidence of conversion has been found, and our own results agree with those of STEGWEE and COLHOUN. Even with cockroaches (COLHOUN) or houseflies which occasionally died after TOCP treatment, the ChE activity was not appreciably inhibited.

When injected with absolute ethanol TOCP, which is a weak anti-ChE agent *in vitro*, produced moderate inhibition *in vivo*, notably in dead flies (see Figs. 16 and 26). There are two possible explanations for these results. Either TOCP is converted in the housefly to an inhibitor, or TOCP *per se* is a moderate inhibitor, and ethanol acts in a certain manner (probably by facilitating dispersion) to reveal this phenomenon. The former possibility seems unlikely, since all of the experiments conducted with insects failed to demonstrate such conversion (see also COLHOUN; STEGWEE, *loc. cit*).

ALDRIDGE and DAVISON (1952b) pointed out that the chemical structure of parathion makes it unlikely that it would have no inhibitory activity against ChE. CASIDA (1956) found that careful chromatographic purification of phosphorothionates, to remove active phosphate impurities, leaves a definite residual anti-ChE, apparently due to the phosphorothionate *per se*. Accordingly, parathion can inhibit ChE, but under *in vitro* conditions this ability is probably weakened by its stability.

The above arguments may now be applied to the action of parathion. If the conversion hypothesis is invalid, at least two possibilities may exist under *in vivo* conditions:

a. stability of the compound is not of crucial importance in the *in vivo* inhibition process, and in this case parathion is the real inhibitor.
b. parathion undergoes a transition stage where it is made more reactive without altering its chemical structure.

In the foregoing Chapters, we have described some more observations which cannot be explained by the conversion hypothesis satisfactorily. It was found that the ChE activity of the suceptible houseflies was considerably inhibited soon after the injection of parathion with aqueous ethanol (see Fig. 22; B). This inhibition was more pronounced in the resistant flies (see Fig. 31; A). After

the topical application of a lethal dose of parathion, ChE activity was very rapidly and profoundly inhibited immediately after treatment (see Fig. 19; C).

So far no conclusive evidence has been furnished that paraoxon is the actual toxicant in parathion poisoning. The widely held belief that it is indeed so, stems mostly from *in vitro* studies. The arguments presented in this Chapter appear to be in harmony with the conclusion of CASIDA (1956) that "although certain phosphorothionates can be oxidized *in vivo* to the corresponding phosphates, this does not mean that such an oxidation is essential for their anti-esterase activity and toxicity."

GENERAL DISCUSSION

In the following we shall deal with some phenomena which bear a relation to our present investigations and have not been discussed in the foregoing chapters.

A. ANTAGONISM

The antagonistic action of TOCP found in houseflies but not in American cockroaches (COLHOUN 1960) may be due to the size of the applied dose of insecticide. COLHOUN applied lethal doses, whereas in the work of STEGWEE (1960) and in the present investigations sublethal doses were applied to houseflies. It is probable that if sublethal doses were applied to the cockroaches, an antagonistic effect would have resulted.

Alternatively, this phenomenon may reveal an outstanding difference between the two insect species. Since the antagonistic activity of TOCP is located mainly in the cuticle, it would appear that the essential difference between its reaction in the fly and in the cockroach points to some difference in the structure and composition of the cuticle. This has been found by KRUEGER (cf. O'BRIEN 1959). If the nature of such differences could be known, the mechanism of the antagonistic action of TOCP could probably be identified. Moreover, the nonacquisition of appreciable resistance in the American cockroach (ALEXANDER *et al.* 1958; BROWN 1958) may also be related to such differences. One of the well known structural differences between the cuticle of the housefly and that of the American cockroach is the presence of the soft grease in the cuticle of the latter instead of wax (BEAMENT 1945; RICHARDS 1951; WIGGLESWORTH 1953).

The previously mentioned view of SUN and JOHNSON (1960) that the antagonistic action of some pyrethrin synergists might be mainly due to the inhibition of certain biological oxidations, may be interpreted otherwise in view of similar observations reported in the present study. If TOCP and parathion or paraoxon had not been applied by different routes of administration, we most probably would have arrived at a similar conclusion. It is now evident, however, that the antagonistic action of TOCP is related to a penetration phenomenon.

B. PENETRATION AND PERMEABILITY

Penetration is the actual passage of material through a barrier membrane, while permeability is a statement of the amount of penetration that would

be expected under a stated set of conditions (RICHARDS 1951). Penetration of the insecticides through the integument of arthropods is far from being well or even satisfactorily understood. If we are to understand insecticide penetration, unambiguous data are essential, but such data are difficult to obtain (RICHARDS 1951). At present the pitfalls in studying insecticide penetration are not only attributable to the complexity of the problem itself, but also to the paucity of our current knowledge, especially of the insect cuticle.

The cuticle as a whole, far from being a homogeneous phase, includes aqueous and lipoid phases. The lipoid materials, although for a large part present in the epicuticle, may extend far into the exocuticle and probably also occur in the endocuticle (WIGGLESWORTH 1942; WEBB and GREEN 1945).

The excellent paper of HURST (1943), although it was published before most of the modern synthetic organic insecticides were developed, contains valuable information. The most important physico-chemical factors which control the permeability of the functional cuticle are the following:

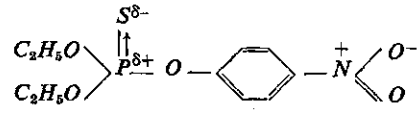
a. mutual cohesion between the lipophilic elements or chains.
b. cross-linkages which normally regulate the stability and elasticity of the lipophilic chains.
c. the presence of labile cuticle components, acting as a "bonding" phase and which regulate the cohesion of the chemically linked lattice elements. Both proteins and lipoid components are enclosed in an elastic lattice framework.
d. viscocity; the bulk lipoid phase is normally of high "functional viscosity".

If the above factors are interfered with by the dispersing action of fat solvent carriers, the permeability of the cuticle increases.

RIDEAL (1945) dealt with the penetration phenomenon at interfaces. Lipoid/protein interfaces, which are fundamentally of the oil/water type, are of universal occurrence in biological systems. The molecules at such interfaces are orientated in such a sense that the polar reactive groups (heads) are situated in the aqueous phase, and the non-polar portions (tails) in the lipoid phase. While penetrating, the diffusing molecules must be free to move under the osmotic gradient and for this purpose, it is essential that neither their polar heads, nor their non-polar tails should be anchored too firmly to immobile groups.

The antagonistic activity of TOCP is likely to be located in the whole cuticle, as mentioned previously, but this activity is probably higher at a certain layer or sublayer. In view of the above considerations, the antagonistic action of TOCP may be explained by interference with the physico-chemical factors which are related to the permeability of the cuticle, in a manner opposite to the dispersing action of the solvent carrier, or by formation of immobile groups with a certain component in the cuticle. Moreover, some of these factors may be modified in resistant houseflies in such a way that the rate of insecticidal entry is lowered.

With normal houseflies, it has been shown that parathion penetrates much slower than paraoxon. One of the factors which may be of importance in this repect, is the relative liposolubility of the two compounds. The electronic formula of parathion may be presented as follows:



An important feature of the nitro group is its strong polar character (see FIESER and FIESER 1958). As this group is present in both compounds, the essential difference between parathion and paraoxon is the presence of the more polar phosphoryl group in the latter as compared with the less polar thiophosphoryl group in the former (see Chapter VI). Assuming that the more polar group (*i.e.* the nitro group) is the polar head and the other portion of the molecule represents the non-polar tail, it then follows that, while penetrating the molecules are orientated at the lipoid/protein interfaces present in the cuticle, in the same sense as described by RIDEAL (*loc. cit*). Since the phosphoryl group is more polar than the thiophosphoryl group, the mutual cohesion between the parathion tails and the lipoid phase is much stronger than that between the paraoxon tails and the same phase. Consequently, the tendency of the paraoxon molecules to escape from the lipoid phase to the aqueous phase is greater.

Such interfaces are also likely to occur in the central nervous system. The penetration of an insecticide into the cells (*e.g.* of the central nervous system) is related to its capacity for permeating through the cuticle (Veldstra 1956). This fact, in addition to the above interpretation, may clarify our previous explanation of the somewhat delayed toxic action of parathion after injection with aqueous ethanol. Factors other than liposolubility are likely to contribute to the whole phenomenon of penetration.

The remarkable general observation of the extremely high levels of resistance developed by DDT pressure, as compared with the low levels of resistance developed by organophosphate pressure (METCALF 1955; BROWN 1958; MARCH 1959, 1960) may be related to the rate of toxic action of both groups. With parathion, for example, its lower rate of toxic action as compared with paraoxon was shown to be related to its lower rate of penetration. In the case of DDT, however, the rates of penetration and toxic action are much lower than in the former example (LINDQUIST *et al.* 1951; HOFFMAN *et al.* 1952; BABERS and PRATT 1953). If this relation proves to be significant, it might open an interesting approach to penetration studies.

C. ENHANCED RATE OF DETOXICATION OF ORGANOPHOSPHORUS INSECTICIDES AS A FACTOR IN RESISTANCE

METCALF (1959) pointed out that organophosphorus resistance is probably the result of either increased rates of detoxication or decreased rates of penetration, or perhaps both factors operating together. CASIDA (1958) and PAL (1958) stated that the development of more effective hydrolytic detoxication systems by the resistant insects is the most probable explanation of resistance. Various authors, led by their experimental data, are in favour of this view (OPPENOORTH 1958; MARCH 1959; VAN ASPEREN and OPPENOORTH 1960; PLAPP *et al.* 1961; MATSUMURA and BROWN 1961 a; BIGLEY and PLAPP 1962). In sharp contrast, other investigators have demonstrated no significant differences between resistant and susceptible strains in their ability to detoxify organophosphorus compounds (CHADWICK 1954; LORD and SOLLY 1956; DARROW and PLAPP 1960; KRUEGER *et al.* 1960; MENGLE and CASIDA 1960; MATSUMURA and BROWN 1961b; FORGASH *et al.* 1962).

A critical evaluation of the evidence of detoxication as a major factor in resistance, was comprehensively given by WINTERINGHAM (1962). Meta-

bolism *in vivo* depends on the well being of the insect. In a comparable insecticidal treatment a normal insect, owing to its very susceptibility, would be expected to metabolize less of the insecticide than a resistant one. The greater metabolism in the resistant strain would thus be a consequence of resistance and not a cause. After examining the data of MARCH (1959), VAN ASPEREN and OPPENOORTH (1960), MATSUMURA and BROWN 1961a, PLAPP *et al.* (1961); WINTERINGHAM pointed out that their data show that the differences in detoxication rates, especially *in vivo*, are smaller than might be expected from the observed levels of resistance and in some cases would not appear to be statistically significant. Accordingly, he concluded that it would be unwise to regard this evidence of the role of detoxication in insect resistance to organophosphorus compounds as other than circumstantial at this stage.

Breakdown enzymes

In the present study the AliE activity of the R-flies was found to be lower by 35 % than that of the S-flies. This is in general agreement with the results of BIGLEY and PLAPP (1960), and in contrast to the results of OPPENOORTH and VAN ASPEREN (1961).

Low AliE activities have been almost consistently found in houseflies resistant to organophosphorus insecticides. VAN ASPEREN and OPPENOORTH (1959) reported that the activity of AliE against methylbutyrate in the resistant strains (strains D, A and F) was less than 25 % of that in the susceptible strains. OPPENOORTH (1959 a, b) suggested that the low AliE activity in the resistant flies is caused by an autosomal gene either identical in all strains or different according to their relative susceptibilities. If the removal of AliE by denaturation enhances the inhibition of ChE activity by organophosphates (VAN ASPEREN and OPPENOORTH 1960), the AliE activity and susceptibility relationships tend to favour the survival of the susceptible rather than the resistant flies. This discrepancy was explained by assuming (with no more than meager correlative evidence; cf MENGLE and CASIDA 1960) that mutation has resulted in the modification of AliE to a phosphatase capable of destroying the organophosphates (VAN ASPEREN and OPPENOORTH 1960; OPPENOORTH and VAN ASPEREN 1960b). The rate of degradation was found to be fairly low as compared with the LD_{50}'s of the applied phosphates. Therefore, VAN ASPEREN and OPPENOORTH (1960) and OPPENOORTH and VAN ASPEREN (1961) concluded that the function of the phosphatase is likely to prevent the toxicant from reaching a fatal concentration at the site of toxic action, whereas most of the total elimination is brought about by other mechanisms present in both susceptible and resistant strains.

The findings of the presence of low AliE activity in organophosphate-resistant houseflies have been reported by other investigators (BIGLEY and PLAPP 1960, 1961; PLAPP and BIGLEY 1961; FORGASH *et al.* 1962).

There is some evidence which casts doubt on the validity of the hypothesis of the altered AliE. A susceptible strain of houseflies (the *bwb* strain) was found by FRANCO and OPPENOORTH (1962) to contain low AliE activity. The authors pointed out that this result, in addition to the unknown function of the enzyme, throws more doubt on its importance. Studies made by FORGASH *et al.* (1962) showed that one of the resistant strains had a high enzyme activity.

So far the low AliE activity has only been found in resistant houseflies and not in other insect species. No significant difference has been demonstrated in

the AliE activity between organophosphate-resistant and -susceptible strains of the mosquitoes *Culex tarsalis* and *Aedes aegypti* (MATSUMURA and BROWN 1961a, b). If the conversion of AliE to phosphatase is the main factor in resistance, houseflies would then form a class of their own. This seems to be improbable were it only because resistance to organophosphorus insecticides is not confined to houseflies.

According to our results (see Chapters II and IV) the enzyme activity of the S-strain, expressed as 100 %, may be considered as a basis for comparison between the AliE activities in the two strains. The two following simple equations may shed some light on the relation between levels of AliE activity and its susceptibility to inhibition by an organophosphorus compound:

a. The S-strain
50 sensitive + 50 insensitive = 100 %

b. The R-strain
10 sensitive + 35 absent + 55 insensitive = 100 %

It would then appear that the origin of the lacking enzyme (supposed to be converted to phosphatase) is the sensitive fraction of the total AliE activity in the S-strain. The 85 % insensitive portion of the AliE activity found in the R-strain is in fact almost equal to the 50 % insensitive fraction found in the S-strain.

An analogous situation has been found with rats. During the life span of the rat changes occur in the level of the testicular AliE, which remains at low and fairly constant levels until age 27 days, when it increases markedly reaching adult levels at puberty, after about 35 days (HUGGINS and MOULTEN 1948). The low AliE activity of the testes of immature rats was found to be fairly insensitive to inhibition by TOCP. On the other hand, the high AliE activity of the mature rats proved to be sensitive to inhibition by TOCP (MYERS and SIMONS 1953). Obviously the resistance phenomenon was not involved in such studies. Either TOCP, parathion or paraoxon were found to inhibit AliE activity to similar degrees in susceptible and resistant houseflies. Thus it is probable that either insecticide would have resulted in a similar degree of AliE inhibition in the rat as has been found with TOCP.

PLAPP and BIGLEY (1961) offered an alternative possible explanation for the low AliE activity in houseflies resistant to organophosphorus compounds. AliE may be involved in poisoning by acting as a temporary storage site for the insecticide, which is thereby protected against detoxication by other enzyme systems. The lower levels of the enzyme in resistant strains could then serve simply as a less effective storage site for the insecticide.

Our results with susceptible and resistant houseflies are incompatible with this explanation, simply because there was no marked recovery in the activity of AliE in either strain after poisoning with parathion or paraoxon.

SUMMARY

Investigations were performed on the effects of tri-*o*-cresyl phosphate (TOCP) upon parathion and paraoxon poisoning in susceptible and resistant houseflies, *Musca domestica* L. The compounds were administered topically or by injection. In topical application the insecticides were usually applied to the

thorax, TOCP either to the thorax or to the abdomen. Injections were performed intrathoracically through the cervical membrane.

The effects were related to mortality or knockdown percentages, as well as to the inhibition of cholinesterase (ChE) and aliesterase (AliE). The activity of the two enzymes was determined colorimetrically, using acetylcholine bromide and methyl-n-butyrate as substrates. In most experiments thoraces and abdomens were used as the sources of ChE and AliE respectively. Substrate protection was used throughout.

CHAPTER I

With susceptible houseflies (S-strain), it was found that TOCP had two opposed actions. Its antagonistic effect was due to its interference with the entry of the insecticides, as was evident when both TOCP and either insecticide were applied topically to the same site (usually the thorax). Antagonism was stronger with parathion than with paraoxon. It was argued that TOCP might act as an antagonist at the whole cuticle and not at a certain layer.

The synergistic effect was evident when TOCP and either insecticide were applied topically to separate sites and by applying one topically and the other by injection.

The experiments also showed that paraoxon was less toxic than parathion after topical application, and more toxic after injection. The S-ethyl and S-phenyl isomers were far less toxic than either parathion or paraoxon after topical application.

CHAPTER II

In susceptible houseflies, the separate and joint action of TOCP and either parathion or paraoxon were studied in terms of ChE and AliE inhibition. As in Chapter I, TOCP was applied prior to the insecticidal treatment. After the administration of TOCP, AliE activity was halved, whereas ChE activity was not appreciably inhibited. Only when TOCP was injected with absolute ethanol, was ChE activity considerably inhibited. After the application of either insecticide, the intensity of ChE depression was found to be correlated with the severity of toxic symptoms. AliE activity was inhibited by 50 % in almost all cases, regardless of the dosage and of the severity of toxic symptoms. It was suggested therefore, that the susceptible flies contained at least two aliesterases, one being very sensitive to inhibition by the three phosphorus compounds, and the other one virtually insensitive.

The joint action of TOCP and parathion or paraoxon caused varying degrees of ChE inhibition which paralleled the increase (synergism) or the decrease (antagonism) of the toxicity.

The inhibition of brain ChE did not show any marked correlation with toxic symptoms.

CHAPTER III

Using susceptible houseflies, the sequence of application was reversed, *viz.* TOCP was applied after the insecticides. In this case synergism and antagonism were also evident. It was found that parathion penetrated through the cuticle very slowly. This result accounts adequately for the latent period usually observed in parathion poisoning. Paraoxon penetrated very rapidly.

The degree of ChE inhibition at a certain time was a good index of the penetration rate of either insecticide. Confining S-flies in milkbottles prior to treatment increased their susceptibility to parathion but not to paraoxon. It was suggested that the "holding capacity" of the cuticle of such flies was smaller than that of the normal test flies.

Mechanisms of synergism were discussed. It was felt that interference with metabolism and excretion by TOCP would account for its synergistic effect.

CHAPTER IV

Experiments were performed with an organophosphate-resistant strain of houseflies (Strain C). Reduced rates of penetration of either parathion or paraoxon were found to be an important factor in resistance. In the R-flies TOCP acted exclusively as an antagonist.

The failure of some injection experiments to eliminate or depress resistance does not necessarily mean that reduced rates of penetration are not involved in resistance. The nature of the injection vehicle is of primary importance in determining the behaviour of the insecticide.

The AliE activity of the R-flies was 65 % of that of the S-flies. It was suggested that the R-flies also contained at least two aliesterases; about 15 % of the total enzyme activity was rather sensitive to inhibition by the three phosphorus compounds, and the remainder much less sensitive.

In studying the joint action of TOCP and paraoxon "homogenization artifacts" were observed.

CHAPTER V

Studies were made on the *in vitro* inhibition of ChE and AliE from susceptible and resistant houseflies by TOCP, parathion or paraoxon. The ChE activities of the two strains were almost equally inhibited. TOCP was a poor anti-ChE agent.

In sharp contrast to *in vivo* results, TOCP protected ChE against inhibition by parathion or paraoxon, especially at lower concentrations of the two inhibitors. The degree of AliE inhibition was nearly identical to that obtained in *in vivo* studies.

CHAPTER VI

Several observations were described which are incompatible with the hypothesis of parathion conversion. The implications of the hypothesis were discussed.

In conclusion, TOCP proved to be an interesting compound for tracing the entry of parathion or paraoxon through the cuticle and for determining the role of the insect cuticle in resistance against either insecticide. Compared with paraoxon, the slow toxic action of parathion could be accounted for by its slow penetration. The reduced rate of penetration was a primary cause of resistance of houseflies against parathion or paraoxon.

Detailed investigations on the action of TOCP may disclose some physiological differences between resistant and susceptible houseflies. Further studies along these lines may be rewarding.

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SAMENVATTING

Een gedetailleerd onderzoek werd ingesteld naar de werking van tri-*o*-cresylfosfaat (TOCP) op de vergiftiging van gevoelige en resistente huisvliegen (*Musca domestica* L.) door parathion of paraoxon. De verbindingen werden òf uitwendig òf per injectie toegediend. Bij uitwendige toediening werden de insekticiden als regel op de thorax gebracht; TOCP hetzij op de thorax, hetzij op het abdomen. De injecties geschiedden intrathoracaal door de cervicale membraan.

De werking werd uitgedrukt in het percentage sterfte of „knock-down", terwijl verder de mate van cholinesterase (ChE)- en ali-esterase (AliE) remming werd vastgesteld. De activiteiten van deze enzymen werden colorimetrisch bepaald met acetyl-cholinebromide en methyl-n-butyraat als substraten. In de meeste experimenten werden thoraces en abdomina gebruikt als respectievelijke bron van ChE en AliE. Bij de bereiding van de enzympreparaten (homogenaten) werd steeds bescherming van de enzymen door toevoeging van substraat toegepast.

HOOFDSTUK I

Het effect van TOCP op een parathion- of paraoxon-vergiftiging bij gevoelige vliegen (stam S) bleek van tweeërlei aard.

Antagonisme werd gevonden wanneer de twee verbindingen uitwendig op dezelfde plaats werden toegediend. Het was het gevolg van een belemmerde penetratie van het insecticide. Antagonisme tegen parathion was sterker dan tegen

paraoxon. TOCP oefent zijn antagonistische werking waarschijnlijk uit in de gehele cuticula en niet in een bepaalde laag hiervan.

Synergisme trad zowel op bij uitwendige toediening van TOCP en insecticide op verschillende plaatsen van het lichaam als bij uitwendige toediening van de ene en injectie van de andere verbinding.

Uit de verrichte proeven kwam ook nog naar voren dat bij uitwendige toediening paraoxon minder giftig was dan parathion, terwijl bij injectie het omgekeerde het geval was. De S-aethyl en S-fenyl isomeren waren na uitwendige toediening veel minder giftig dan parathion en paraoxon.

HOOFDSTUK II

Bij gevoelige vliegen werd de werking van TOCP en parathion of paraoxon, afzonderlijk of in combinatie, op ChE- en AliE-remming *in vivo* onderzocht.

Na toediening van TOCP was de AliE-activiteit gehalveerd, terwijl de ChE-activiteit niet noemenswaardig geremd was. Slechts injectie van TOCP in aethanol resulteerde in een duidelijke ChE-remming.

De insecticiden veroorzaakten een remming van de ChE, die gecorreleerd was met de optredende symptomen. Practisch onafhankelijk van deze symptomen en van de grootte van de dosis bleek steeds ongeveer 50 % AliE-remming op te treden. Op grond hiervan werd verondersteld, dat de gevoelige vliegen ten minste twee ali-esterasen bevatten: één die zeer gevoelig is voor remming door de drie fosfor-verbindingen, een tweede die practisch ongevoelig is.

TOCP samen met parathion of paraoxon gaf ChE-remmingen te zien, die parallel liepen met de verhoogde of verlaagde toxiciteit (vergeleken met de toxiciteit van de insecticiden afzonderlijk).

Er werd geen duidelijk verband gevonden tussen symptomen en de remming van hersen-ChE.

HOOFDSTUK III

In de experimenten op gevoelige vliegen, beschreven in dit hoofdstuk, werd de volgorde van toediening van TOCP en insecticiden omgekeerd, d.w.z. eerst werd parathion of paraoxon aangewend en daarna TOCP. Wederom werd èn antagonisme èn synergisme gevonden, afhankelijk van de proefopzet.

De gevolgtrekking werd gemaakt dat parathion slechts langzaam door de cuticula penetreert. Dit verklaart op afdoende wijze de latente periode die bij parathion-vergiftiging wordt waargenomen. Paraoxon penetreert zeer snel. De mate van ChE-remming leverde een bruikbaar criterium voor de penetratiesnelheid van de twee insecticiden.

Wanneer S-vliegen gedurende enige tijd in met een watteprop afgesloten $\frac{1}{2}$ l-melkflessen werden bewaard, steeg hun gevoeligheid voor parathion bij gelijkblijvende gevoeligheid voor paraoxon. De veronderstelling werd geuit, dat in dergelijke vliegen het vermogen tot retentie van parathion ("holding capacity") verminderd is in vergelijking met de normaal gebruikte proefdieren.

Mogelijke mechanismen van synergisme werden besproken. De synergistische werking van TOCP kan verklaard worden door beïnvloeding van stofwisseling en excretie van de insecticiden.

HOOFDSTUK IV

De proeven in dit hoofdstuk werden verricht met een vliegenstam (stam C) die resistent is voor organofosfaten. Verminderde penetratie van parathion en paraoxon bleek een belangrijke rol te spelen in de resistentie. In de resistente (R)-vliegen vertoonde TOCP uitsluitend een antagonistische werking.

In sommige injectieproeven bleek het onmogelijk om de resistentie geheel of gedeeltelijk te onderdrukken. De conclusie dat derhalve een verminderde penetratie door de cuticula niet verantwoordelijk kan zijn voor resistentie is echter niet dwingend, aangezien de aard van het injectie-medium in hoge mate het gedrag van het insecticide bepaalt.

De AliE-activiteit van de R-vliegen was 65 % van die van de gevoelige stam. Ook de R-vliegen worden verondersteld ten minste twee ali-esterasen te bezitten, waarvan ongeveer 15 % gevoelig voor remming door de drie gebruikte organofosfor-verbindingen.

Het onderzoek naar de gecombineerde werking van TOCP en paraoxon werd bemoeilijkt door homogenisatie-artefacten.

HOOFDSTUK V

Dit hoofdstuk geeft de resultaten van het onderzoek naar de *in vitro* remming van ChE en AliE van gevoelige en resistente vliegen door TOCP, parathion en paraoxon.

ChE uit beide stammen werd vrijwel gelijkelijk geremd. TOCP remde slechts zwak. In tegenstelling tot de resultaten van de *in vivo* proeven, bleek TOCP *in vitro* ChE te kunnen beschermen tegen remming door parathion en paraoxon, vooral wanneer deze in lage concentraties aanwezig waren.

De mate van AliE-remming vertoonde sterke overeenkomst met die gevonden *in vivo*.

HOOFDSTUK VI

Enkele eigen waarnemingen, alsmede enige litteratuurgegevens, die in strijd lijken met de hypothese aangaande de omzetting (toxificatie) van parathion, worden besproken. Een discussie is gewijd aan de implicaties van de omzettingshypothese.

De conclusie is, dat TOCP een waardevol hulpmiddel bleek te zijn voor het bestuderen van het binnendringen van parathion of paraoxon door de cuticula. Hiermee kon worden vastgesteld dat de, in vergelijking met paraoxon, langzame werking van parathion op rekening komt van de langzame penetratie van de laatstgenoemde verbinding. Verminderde penetratie moet ook gezien worden als de eerste oorzaak van resistentie bij de huisvlieg. Een gedetailleerd onderzoek naar de fysiologische werking van TOCP kan wellicht fysiologische verschillen tussen gevoelige en resistente vliegen aan het licht brengen.

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