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TOXIC ACTION OF ORGANOPHOSPHORUS COMPOUNDS AND ESTERASE INHIBITION IN HOUSEFLIES

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Abstract—The paper deals with investigations on the inhibition *in vivo* of the cholinesterase and the aliesterase in houseflies poisoned by treatment with organophosphorus insecticides. The kinetics of the inhibition of esterases by DDVP, paraoxon and diazoxon in the presence and in the absence of substrate were closely studied. It was found that the inhibition is strongly retarded by the presence of acetylcholine in the case of the cholinesterase, and of phenylbutyrate in the case of the aliesterase. It could thus be shown that the addition of these substrates at the time of homogenization lends appropriate protection to the enzymes against free inhibitor present in the body of the insect. The results obtained with this "protective method" show that cholinesterase inhibition at the time of knock-down was generally less than 50 per cent, whereas the aliesterase inhibition at this time proved to be about 90 per cent. It could further be shown that the *in vivo* aliesterase inhibition increased rapidly from the beginning of exposure of the flies to the insecticide, and reached values of more than 50 per cent even long before knock-down occurred. This inhibition was found to be not readily reversible *in vivo*.

ORGANOPHOSPHORUS compounds have been shown to be potent inhibitors of cholinesterases,[1, 2, 3, 4] a number of other esterases and lipases[5] and also of some proteolytic enzymes.[6, 7] Further investigations on the action of these substances *in vitro* and *in vivo* left little doubt that their toxicity in mammals is due to the inhibition of the cholinesterase in the nervous system. Strong arguments have been put forward that the toxic action of the insecticidal organophosphates is also due to the inhibition of the cholinesterase in the insect,[8] although the correctness of this view has also been doubted by a number of authors (see Spencer and O'Brien[9]).

Houseflies contain two enzymes that are highly sensitive to organophosphates: a cholinesterase and an enzyme indicated as aliesterase.[10] The latter exerts strong hydrolytic activity on methyl- and ethylbutyrate and some other aliphatic esters, and also on phenylbutyrate and other aromatic esters. The present paper deals with investigations on the reaction of organophosphates with these fly-esterases *in vitro* and *in vivo*, in which special attention is given to the difficulties encountered in obtaining reliable *in vivo* inhibition data. The purpose of this study is mainly to establish a relation between the biochemical symptoms, i.e. the esterase inhibition, and the externally visible symptoms of intoxication.

METHODS AND MATERIAL

Measurement of esterase-activity was performed by the Warburg manometric method. The reaction medium contained 0·025 M $NaHCO_3$, the gas phase consisted

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of 95% N_2 and 5% CO_2, pH was about 7·5. Cholinesterase activity was measured at 37 °C and aliesterase activity at 27 °C. In view of the lability of the aliesterase, especially in alkaline media, homogenates for the estimation of this enzyme were prepared in ice-cold saline, and bicarbonate was added at the very last moment before measurement started. The substrates used were acetylcholine (0·015 M) for cholinesterase and methylbutyrate and phenylbutyrate for aliesterase determinations. Because of its low water-solubility, phenylbutyrate was always used in the form of an emulsion (0·025 ml phenylbutyrate per Warburg flask in most cases; a small amount of emulsifier was added).

The organophosphorus compounds used were DDVP = *O* : *O*-dimethyl *O*-2 : 2 dichlorovinyl phosphate; Parathion = *O* : *O*-diethyl-*O*-*p*-nitrophenyl phosphorothionate; Paraoxon = *O* : *O*-diethyl-*O*-*p*-nitrophenyl phosphate; Diazinon = *O* : *O*-diethyl-*O*-(2-*iso*propyl-4-methyl-6-pyrimidinyl) phosphorothionate; Diazoxon = *O*-*O*-diethyl-*O*-(2-*iso*propyl-4-methyl-6-pyrimidinyl) phosphate; Co-ral ≡ Bayer 21/199 = *O* : *O*-diethyl-*O*-(3-chloro-4-methyl-umbelliferone) phosphorothionate.

Insect material. The experiments were done with a normal insecticide susceptible strain of the housefly, *Musca domestica* L. The flies were reared on a milk powder–yeast diet at 25 °C and *c.* 70 per cent relative humidity. The flies were poisoned by exposing them to a residue of the toxicant on glass, or by exposing them to the vapour of the poison (in the case of DDVP and diazinon). The doses used varied a great deal and were chosen in such a way that knock-down (paralysis) of the flies occurred after 0·5–2 hr in most cases. Further particulars about exposure and the time course of the intoxication will be given where they are relevant.

RESULTS

1. *The inhibition of esterases by organophosphates* in vitro

The organophosphates studied were found to be potent inhibitors of both the cholinesterase and the aliesterase present in housefly homogenates. Table 1 shows some approximate figures with respect to the rate constants observed. An important

TABLE 1. BIMOLECULAR RATE CONSTANTS OF INHIBITION*
(l. moles^{-1} min^{-1})

	Cholinesterase† 37 °C	Aliesterase‡ 27 °C
DDVP	3×10^7	1×10^7
Paraoxon	6×10^6	8×10^7
Diazoxon	2×10^8	4×10^7

* The bimolecular rate constants have been calculated from the equation $v = k'.[E].[I]$, assuming that $Ki \gg [I]$, since then $[E] = [E_{tot}]$.

† Data obtained with fly head homogenates, made up in bicarbonate–Ringer.

‡ Data obtained with whole fly homogenates, made up in 1% NaCl.

point which deserves consideration is the specificity of the inhibitors for the esterases studied. This is reflected in the I_{50}-values (concentrations of the inhibitor giving 50 per cent inhibition after an "infinite" time of incubation), shown in Table 2. The

TABLE 2. CONCENTRATIONS AND AMOUNTS OF ORGANOPHOSPHATE GIVING 50 PER CENT INHIBITION OF ESTERASE ACTIVITY IN WHOLE FLY HOMOGENATES (ONE FLY PER FLASK)

	Cholinesterase		Aliesterase	
	(moles/l.)	(ng/fly)*	(moles/l.)	(ng/fly)*
DDVP	$4{\cdot}5 \times 10^{-9}$	2·0	3×10^{-9}	1·3
Paraoxon	6×10^{-9}	3·3	3×10^{-9}	1·7
Diazoxon	2×10^{-9}	1·2	$3{\cdot}5 \times 10^{-9}$	2·0

* 1 ng = 1×10^{-9} g.

data in the Tables 1 and 2 indicate that the three organophosphates studied react rapidly and rather specifically with the esterases under investigation.

The phosphorothionates do not inhibit esterase-activity *in vitro*, but according to the generally accepted opinion they are converted to their oxygen analogues in the animal and insect body and will thus cause inhibition *in vivo*.

2. *The measurement of* in vivo *inhibition*

If houseflies are exposed to an organophosphate the toxicant will penetrate into the body. Phosphorothionates will, after penetration, be oxidized to the corresponding phosphates. A part of the inhibitor thus absorbed or formed will react with and thus inactivate the esterases, another part will possibly react with other compounds in the body or be enzymically broken down, but in most cases free inhibitor will also be present. In our experiments the amount of free inhibitor generally does not exceed 10 ng per fly, and in most cases is considerably less. However, an amount of 5 ng per fly gives a concentration of about 10^{-6} M in the insect body and of approximately 10^{-8} M in the homogenates prepared for the manometric determination of the esterase activity. Such concentrations are likely to cause quick and strong inhibition. It must be assumed that structural barriers in the insect prevent or at least slow down the reaction between esterases and organophosphates. No such barriers, however, exist after homogenization and inhibition will thus rapidly proceed under these conditions. The presence of free inhibitor thus constitutes a serious difficulty in these measurements.

Kewitz,[11, 12] investigating the *in vivo* cholinesterase inhibition in mouse brain, extracted the tissue with choloroform in order to remove any free organophosphate present. The inhibition values so obtained were indeed considerably lower than those obtained without chloroform-extraction. Another method of preventing the reaction between free enzyme and free inhibitor during and after homogenization, consisting of the addition of substrate to the homogenizing medium,[13, 14, 15] will be discussed below.

The inhibitory reaction may be depicted as occurring in two steps:
a reversible one

$$E + I \rightleftharpoons EI, \quad K_i = \frac{[E] \times [I]}{[EI]} \tag{1}$$

and an irreversible one

$$EI \longrightarrow \text{phosphorylated enzyme} \quad v = k.\,[EI] \tag{2}$$

The rate at which irreversible inactivation of the enzyme occurs (v) will be proportional to the concentration of *EI*. Added substrate will compete with the inhibitor for the enzyme by the reaction

$$E + S \rightleftarrows ES, \quad K_m = \frac{[E] \times [S]}{[ES]} \tag{3}$$

and thus lower the concentration of *EI*. From Lineweaver and Burk's equations[16] for the case of competitive inhibition it can be derived that

$$\frac{1}{v_{is}} = \frac{K_i}{V_{im}} \cdot \left(1 + \frac{[S]}{K_m}\right) \cdot \frac{1}{[I]} + \frac{1}{V_{im}} \tag{4}$$

and

$$\frac{1}{v_i} = \frac{K_i}{V_{im}} \cdot \frac{1}{[I]} + \frac{1}{V_{im}} \tag{5}$$

where v_{is} = velocity of inhibitory reaction in the presence of substrate;
v_i = velocity of inhibitory reaction in the absence of substrate;
K_i = dissociation constant of enzyme–inhibitor complex;
K_m = dissociation constant of enzyme–substrate complex;
V_{im} = maximal rate of inhibitory reaction, i.e. if all the non-phosphorylated enzyme is present as *EI*;
$[S]$ = substrate concentration;
$[I]$ = inhibitor concentration.

From equations (4) and (5) it follows that

$$\frac{v_{is}}{v_i} = \frac{K_i + [I]}{K_i([S] + K_m)/K_m + [I]} \tag{6}$$

No data are available on the value of K_i. Two borderline cases may be considered: the first, where

$$K_i \gg [I], \quad \text{then} \quad \frac{v_{is}}{v_i} \simeq \frac{K_m}{[S] + K_m} \tag{7}$$

$$\text{or} \quad \frac{v_{is}}{v_i} \simeq 1 - \frac{v}{V_{\max}} \tag{8}$$

where v = velocity of enzymic reaction in the absence of inhibitor, and $V_{\max}$ = maximal rate of enzymic reaction (at infinite substrate concentration); and the second, where

$$K_i \ll [I], \quad \text{then, unless } [S]/K_m \text{ is very high,}$$

$$\frac{v_{is}}{v_i} \simeq 1$$

Three enzyme–substrate systems, used in our studies on inhibition *in vivo*, will be briefly considered below. In the calculations it will be assumed that $K_i \gg [I]$, so that the equations (7) or (8) can be used. It is, however, not at all certain that this assumption is right. The values for v_{is}/v_i should therefore be taken as minimum values.

Housefly cholinesterase with acetylcholine as a substrate. The value of K_m for this system is not exactly known. According to some data on enzyme activity obtained with low acetylcholine concentrations and using the colorimetric method described by Hestrin[17], $K_m \leqq 10^{-4}$. This means that the presence of 10^{-2}M acetylcholine will strongly decrease the rate of the inhibitory reaction with organophosphates. If $K_m = 10^{-4}$ we obtain:

$$\frac{v_{is}}{v_i} = \frac{K_m}{[S] + K_m} = \frac{10^{-4}}{10^{-2} + 10^{-4}} = \frac{1}{101}$$

Housefly aliesterase with methylbutyrate as a substrate. Fig. 1 shows the pS-activity curve obtained for this system. According to this v/V_{max} at a methylbutyrate concentration of 0·12 M (vertical dotted line P) is approximately 0·85 and $v_{is}/v_i = 1 - (v/V_{max}) = 0{\cdot}15$. The rate of the inhibitory reaction will thus be only moderately reduced by the addition of substrate. It is impossible to improve the protection of the enzyme by increasing the methylbutyrate concentration because of the limited solubility of this compound in water (vertical dotted line Q).

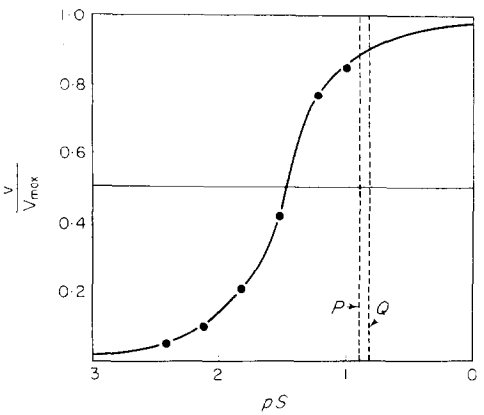

FIG. 1. Activity-pS curve for the hydrolysis of methylbutyrate by housefly-aliesterase. P indicates the pS at which *in vivo* measurements were performed. Q indicates the limit of solubility of methylbutyrate in water.

Housefly aliesterase with phenylbutyrate as a substrate. It has been shown[18] by substrate summation experiments that phenylbutyrate is hydrolysed by the same enzyme as methylbutyrate. Although this enzyme proved to be highly active against phenylbutyrate and other aromatic esters, the name aliesterase, chosen on account of its hydrolytic activity to methylbutyrate and other aliphatic esters, was maintained.

As it was found that the addition of methylbutyrate did not appropriately protect the aliesterase from inhibition by paraoxon and diazoxon (see below), some experiments were performed in which phenylbutyrate was used as the protecting substrate. The prospects of getting convenient results with this substrate were rather bad. As a consequence of its low solubility in water, $[S]$ was necessarily low. Only if the value of K_m would be extremely low, so that $[S]/K_m$ would still be high, good protection could be expected. No data on K_m were, however, available.

Whether sufficient protection of the enzyme against free inhibitor will be obtained by the addition of a substrate is dependent on many factors, viz. the $[S]/K_m$ value of the enzyme–substrate system, the $[I]/K_i$ value of the enzyme–inhibitor system, and the value of the reaction constant k of equation (2) (it should be emphasized that this k-value is not the bimolecular rate constant k' given in Table 1; actually $k = k' \cdot K_i$). Many of these factors are not or inexactly known. Therefore, experiments were performed to determine the extent to which protection occurs.

The esterases under investigation were mixed with their substrates and their activity measured by the manometric method. After about 20 min the inhibitor was added from a side arm of the Warburg flask, and measurement of enzyme activity continued for about 2 hr. The results are shown in the Figs. 2, 3 and 4.

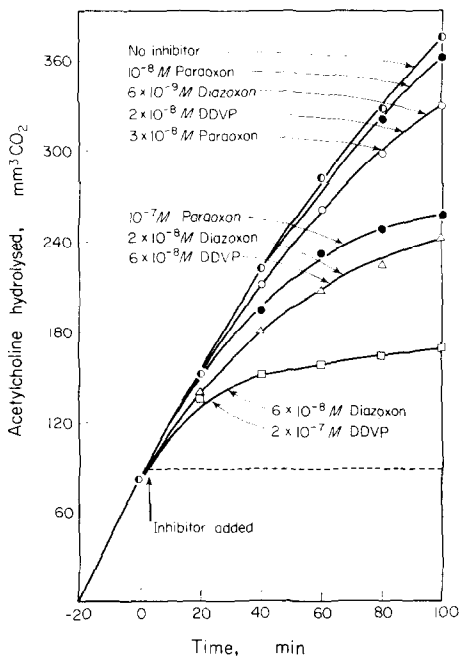

FIG. 2. Inhibition of housefly cholinesterase in the presence of the substrate acetylcholine (ACh). Initial ACh-concentration: 0·015 M; homogenate: two fly heads/flask; temp. 37 °C.

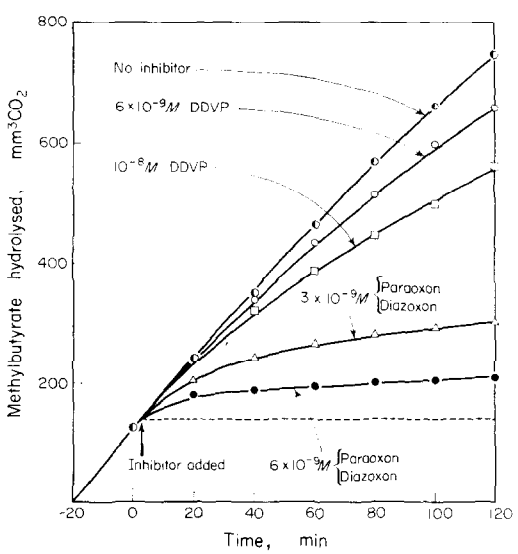

FIG. 3. Inhibition of housefly aliesterase in the presence of the substrate methylbutyrate (MB). Initial MB-concentration: 0·12 M; homogenate: 0·5 flies/flask; temp. 27 °C.

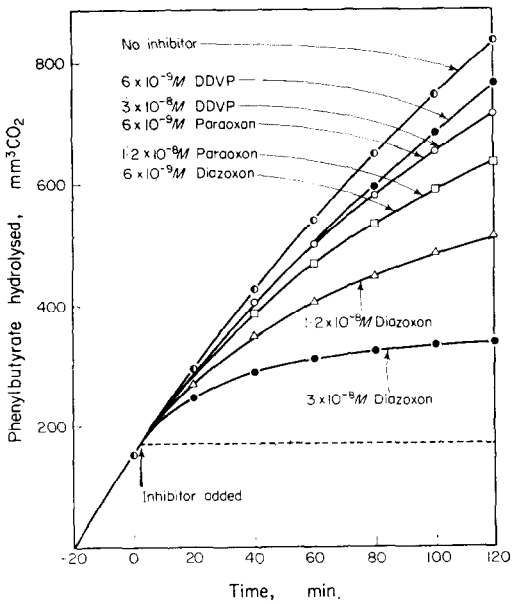

FIG. 4. Inhibition of housefly aliesterase in the presence of the substrate phenylbutyrate (PB). PB-emulsion (1 ml/flask): 1 ml PB + 0·2 ml emulsifier + 50 ml NaCl 1 per cent. Homogenate: 0·5 flies/flask; temp. 27 °C.

The following conclusions can be drawn:

(*a*) According to the data given in the Tables 1 and 2, DDVP, paraoxon and diazoxon in all concentrations used in these experiments would rapidly cause nearly complete inhibition of esterase activity in the absence of substrate.

(b) The addition of acetylcholine yielded very good protection of the cholinesterase against DDVP and paraoxon; much poorer protection was obtained against diazoxon (Fig. 2). This is in agreement with the very high rate constant for the reaction between cholinesterase and diazoxon. In the experiments on *in vivo* inhibition of the cholinesterase the conditions were such that the presence of 5 ng of organophosphate per fly would cause a concentration of about $1{\cdot}5 \times 10^{-8}$ M (1·5 flies per Warburg flask).

(c) Methylbutyrate did not appropriately protect the aliesterase from inhibition by paraoxon and diazoxon, but did so quite well in the case of DDVP (Fig. 3). Although some difference was anticipated from the difference in the bimolecular rate constants (Table 1), it was so striking that this experiment was repeated several times. Similar results were obtained throughout. *In vivo* aliesterase inhibition was measured under such conditions that 5 ng per fly would give rise to a concentration of about 5×10^{-9} M (0·5 flies per Warburg flask). In the case of DDVP the protection by methylbutyrate is probably sufficient.

(d) Very satisfactory results were obtained with phenylbutyrate (Fig. 4). The protection obtained decreased in the order DDVP > paraoxon > diazoxon. The interpretation of these experiments is complicated by the fact that approximately 10 per cent of the hydrolytic activity of a homogenate to this substrate is due to the cholinesterase. Since phenylbutyrate probably does not protect the cholinesterase very well, the protection of the aliesterase may be still better than would be concluded from Fig. 4. The following experiment confirmed this view.

Flies were homogenized in phenylbutyrate emulsions containing $1{\cdot}2 \times 10^{-8}$ M diazoxon or paraoxon, and the homogenate transferred to Warburg flasks, so that each flask contained 2·2 ml of the reaction mixture with 0·5 flies and a final concentration of 6×10^{-9} M of the inhibitor. The hydrolytic activity was compared with that of controls obtained in a corresponding way but without inhibitor. As eserine eliminates all cholinesterase activity, each measurement was performed with and without eserine. Table 3 shows the results.

TABLE 3 INHIBITION OF ALIESTERASE BY PARAOXON AND DIAZOXON

(Substrate and inhibitor simultaneously added)

	Percentage inhibition by			
	Paraoxon		Diazoxon	
Eserine	−	+	−	+
After 30 min	17	11	23	13
After 60 min	21	17	29	19
After 120 min	27	25	37	29

The inhibitor and the substrate were both present at the time of homogenization and it may thus be assumed that substrate and inhibitor get the same chance of reacting with the enzyme as they do in the *in vivo* inhibition measurements.

From the experimental results reported in this section it can be concluded that the addition of a suitable substrate at the time of homogenization is a simple method for obtaining reliable data on *in vivo* inhibition of the cholinesterase and the aliesterase. Since protection of the enzyme by its substrate is the essential feature of this method it will be referred to as the "protective method".

3. *Results obtained with the "protective method"*

The method finally adopted may be briefly described:

(a) *Inhibition*, in vivo, *at the time of knock-down*. If exposed to the insecticides studied each individual fly passes through a stage of hyperactivity which is followed by a state of prostration (knock-down). As soon as a fly reached that state of paralysis it was removed from the jar in which exposure took place (using a funnel–trap-door system) to a test-tube standing in a mixture of ether and solid carbon dioxide, in which it was kept till further treatment. As soon as all flies were collected in this way they were homogenized. One millilitre of the homogenate obtained was then quickly transferred to a Warburg flask containing 1·2 ml of additional fluid, so that the reaction took place in a final volume of 2·2 ml. Flies for cholinesterase measurement were homogenized in 3% NaCl containing 0·025 M bicarbonate and 0·033 M acetylcholine. The additional fluid contained 3% NaCl and 0·025 M bicarbonate. Each flask contained 1·5 homogenized flies. In the case of aliesterase determinations homogenization took place in a phenylbutyrate emulsion in 1% NaCl and the additional fluid contained 1% NaCl, 0·046 M $NaHCO_3$, and 10^{-5} M eserine. In these experiments each flask contained 0·5 homogenized flies.

The activities measured (μl. CO_2 evolved in the first 30 min) were compared with those obtained in control experiments with flies that were not exposed to insecticides but otherwise treated in exactly the same way. The percentage inhibition could then be calculated.

(b) *Inhibition*, in vivo, *at other stages of poisoning*. Groups of flies were exposed to the insecticides for a certain period and the development of poisoning symptoms noted. At the end of the exposure period the flies were narcotized by CO_2-gas and then homogenized in media containing acetylcholine or phenylbutyrate. Further treatment was similar to that described above.

Table 4 shows the data on *in vivo* inhibition at the time of knock-down. It is clear that cholinesterase inhibition, as measured for whole flies, was low. On the contrary aliesterase inhibition approached 100 per cent in several cases and was always distinctly higher than cholinesterase inhibition.

It seemed useful to investigate what relation exists between the time course of *in vivo* inhibition and the time course of the poisoning. In a previous study[14] we

TABLE 4. INHIBITION *in vivo* AT THE TIME OF KNOCK-DOWN

	Cholinesterase* (%)	Aliesterase† (%)
DDVP	27 (6)	76 (5)
Paraoxon	51 (6)	95 (2)
Parathion	49 (6)	95 (1)
Diazoxon	—	91 (1)
Diazinon	32 (3)	90 (3)
Co-ral	44 (5)	75 (3)

Mean values are given; number of experiments in brackets.

*Protection by acetylcholine 0·015 M
†Protection by phenylbutyrate-emulsion

reported on low *in vivo* inhibition values for the cholinesterase activity in the earlier phases of poisoning by parathion and DDVP. This is in accordance with the low inhibition values at the time of knock-down. Experiments on aliesterase inhibition with DDVP, diazinon and paraoxon showed that soon after exposure to the insecticide the inhibition rises sharply.

Experiments with DDVP. Flies were exposed to DDVP vapour. At the time of knock-down the *in vivo* aliesterase inhibition amounted to about 80 per cent. In an experiment where the first symptoms (hyperactivity) occurred after 38 min it was found that after 10 min the *in vivo* inhibition was 54 per cent. If in this case phenylbutyrate was added after the homogenate had been incubated for 1·5 hr at 27 °C, the inhibition was 75 per cent. The increase from 54 per cent to 75 per cent by incubation in the absence of substrate proves the presence of a small amount of free inhibitor in this case. In another experiment flies were exposed to DDVP vapour for 15 min. At the end of the exposure period the first symptoms were observed. The flies were then divided into three groups of ten flies each, which were further kept in clean jars, where the intoxication progressed. The inhibition value immediately at the end of the exposure period was 84 per cent; after 30 and 120 min values of 87 per cent and 87 per cent were measured; almost complete knock-down occurred in the latter two groups soon after they had been transferred to clean jars. Little or no recovery was observed, neither towards normal behaviour nor towards normal aliesterase activity. In a third experiment flies were exposed for 15 min to a smaller amount of DDVP vapour. No symptoms were observed. At the end of the exposure period the flies were divided into five groups of ten flies each, which were kept in clean jars. These groups were used for *in vivo*-inhibition measurement after 0, 30, 60, 90 and 120 min. The inhibition values found were 35, 23, 44, 35 and 51 per cent, respectively. Although there is a great deal of variation it may be concluded that there is little or no recovery of the aliesterase activity.

Experiments with diazinon. Flies were exposed to diazinon vapour. At the time of knock-down the *in vivo* aliesterase inhibition amounted to about 90 per cent. In an experiment where symptoms occurred after 45 min and 50 per cent knock-down after about 60 min, the *in vivo* inhibition was 28, 90, 92 and 87 per cent after 30, 60, 90 and 120 min, respectively. In a second experiment under similar conditions the *in vivo* inhibition after 30 min exposure amounted to 66 per cent. If the homogenate was incubated before adding substrate the inhibition was 81 per cent. This again points to the occurrence of a small amount of free inhibitor. In a third experiment the flies were withdrawn from the diazinon vapour after 30 min and transferred to clean jars. The inhibition found after 0, 30, 60, 90 and 120 min was 29, 31, 45, 44 and 60 per cent, respectively. The tendency to increase may be explained by the progressive conversion of diazinon into diazoxon. A possible recovery could have been masked by this process.

Experiments with paraoxon. Flies were exposed to a residue of paraoxon on glass. At the time of knock-down *in vivo* inhibition was about 90 per cent. In a first experiment four groups of ten flies each were exposed to paraoxon for 5, 15, 30 and 60 min. The first visible symptoms occurred after 30 min, 50 per cent knock-down after about 60 min. The *in vivo* inhibition values were 63, 66, 83 and 96 per cent, respectively. In a second experiment flies were exposed for 5 min only, to a smaller residue. A control group was continually exposed and showed symptoms of intoxication after 40 min and 50 per cent knock-down after about 3 hr. After 5 min exposure the *in vivo* inhibition amounted to 54 per cent. If phenylbutyrate was added *immediately after* homogenization an inhibition value of 71 per cent was found and if the addition of substrate occurred after 0·5 hr of incubation the inhibition found was 94 per cent. This proves the presence of a large amount of free paraoxon on and/or in the flies at the time of homogenization. This conclusion was confirmed in several other experiments, in which the homogenates were diluted with homogenates of unexposed flies. After 5 min exposure the amount of free paraoxon was approximately 7 ng per fly. It was found that after 30 min exposure the amount of free paraoxon did certainly not exceed this amount. This has not been further investigated.

DISCUSSION

In a previous paper[13] experiments on the *in vivo* inhibition of esterases by DDVP have been described and discussed. The present paper deals with further investigations into the same subject, in which other poisons have also been used. The results obtained in these investigations confirm the observations previously made that at the time of knock-down by organophosphates the cholinesterase activity in the housefly is only little inhibited. This is especially surprising in view of the general belief that the cholinesterase is present in great excess[19] and that cholinesterase inhibition will only interfere with the normal function of the nervous system if the percentage inhibition approaches 100%. This discrepancy may be explained in one of several ways.

(1) It is possible that the data in the literature on the excess of cholinesterase are incorrect. These data are all based on *in vivo* inhibition experiments in which the enzyme is not protected against free inhibitor, and could, therefore, be too high.

(2) Although the overall inhibition values found in our experiments are low, high percentage inhibition at some essential part of the nervous system cannot be excluded. Actually it has been found that the inhibition of the cholinesterase in the thoraxes of poisoned flies is somewhat higher than that found for whole flies.[14]

(3) It could be that cholinesterase inhibition is less important for the mechanism of intoxication than it is generally thought to be.

Recently Mengle and Casida[20] studied the *in vivo* cholinesterase inhibition of brain cholinesterase after treatment of houseflies with a number of organophosphorus compounds. The occurrence of paralytic symptoms proved to be associated with high inhibition of the enzyme, i.e. 80 per cent inhibition or more in most cases. We are unable to explain the striking difference between these and our own results.

As the organophosphates are also potent inhibitors of the aliesterase present in houseflies it seemed useful to study the *in vivo* inhibition of this enzyme as well. The "protective method" using phenylbutyrate as a substrate made it possible to obtain reliable data for this enzyme. At the time of knock-down invariably high *in vivo* inhibition was observed. It has been suggested[13] that organophosphates might exert their toxic action by the inhibition of the aliesterase. This hypothesis is strongly invalidated by the observation that flies which are resistant to organophosphates all show a very low aliesterase activity.[21] Moreover, nothing is known about the natural substrate and the normal function of the aliesterase present in organophosphate susceptible houseflies, and any theory that ascribes the toxic action of these compounds to aliesterase inhibition must, therefore, be highly speculative.

If an organophosphate is applied to the living insect, inhibition of both the cholinesterase and the aliesterase will occur and the degree of inhibition will depend on the total amount of the inhibitor picked up or formed, its distribution and the rate of reaction with the two enzymes. The cholinesterase and the aliesterase will compete for the organophosphate present, and it may be expected that the presence of the aliesterase will lower the amount of free inhibitor and thus protect the cholinesterase from inhibition. It seems difficult to reconcile this view with the low aliesterase level in organophosphate resistant flies. Recent work, however, has shown that, at least in some strains, resistance is probably due to a change in the nature of the enzyme protein, which results in a loss of aliesterase activity but enhances the capacity of eliminating (probably hydrolysing) organophosphates.

The data presented in this paper clearly show that the inhibition of the aliesterase precedes that of the cholinesterase in the living insect. Corresponding investigations on the reaction of organophosphates with the two enzymes in homogenates will be published shortly. If the results obtained in these *in vitro* experiments are compared with those of the present paper, it appears likely that structural barriers play an important role in the living fly, the aliesterase being more accessible to the inhibitor than the cholinesterase.

The experimental results on *in vivo* inhibition in earlier stages of intoxication and after withdrawal of the flies from the toxicants indicate that aliesterase inhibition is not readily reversible *in vivo*.

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