Lethality and Behavioural Symptoms Produced by Some Organophosphorous Compounds in the Snail (*Helix aspersa*)

by

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Although the chlorinated hydrocarbons are very effective insecticides against a wide range of undesirable insects, their persistence in the environment has necessitated their replacement by the more easily degraded carbamates, and organophosphorus esters. The mode of action of both carbamates and organophosphorus compounds is by inhibition of esterases, particularly cholinesterase. This reaction involves phosphorylation of the serine hydroxyl at the active site. The rate of inhibition of the enzyme is dependent on many parameters, amongst which is the electron density of the phosphorus atom. Thus, other factors being equal, phosphates are found to be more toxic to animals than the phosphorothioates. This can be seen by comparison of the pI_{50} value for fenitro-oxon which is 1000 times less than fenitrothion (MIYAMOTO, et al., 1968). This effect can be explained by the greater polarization of the P=O bond than the P=S which results in larger positive charge on the phosphorus atom. The more potent anticholinesterase activity of the phosphates results from direct inhibition of the enzyme. The phosphorothioates are called latent inhibitors because, although they do cause a small amount of direct inhibition, they are known to be metabolized in vivo in Guinea pig and rat to the more toxic oxygen analogue (MIYAMOTO 1968).

The toxicity of anticholinesterases towards insects is complicated by factors such as the rate of penetration through the cuticle or skin, the ability of the insect to metabolize or excrete the material, and the age and sex of the insects. Also, the phosphorothioates, because of their less polar nature, may be expected to be absorbed more readily by the fatty tissues. HEATH (1968), using the data of other workers, showed that a plot of log LD_{50} vs I_{50} for direct cholinesterase inhibitors with a given animal showed a rough correlation indicating cholinesterase inhibition as the mode of toxic action. There are however, data which do not support the correlation. MENGLE and CASIDA (1958) reported that houseflies topically exposed to malathion, showed the highest mortality and greatest paralysis when the cholinesterase activity had greatly recovered. HOPF and TAYLOR (1958) observed that locusts were capable of surviving 100% inhibition of ganglionic cholinesterase by an organophosphorus compound while houseflies exposed to O,O-dimethyl-O-2,2-dichloro-

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vinyl phosphate suffered mortality with only 24% mean flyhead cholinesterase inhibition (VAN ASPEREN 1958).

The discrepanices in this correlation of cholinesterase inhibition and lethality must assume that another enzyme system is involved in nervous activity or that nerves are affected in some biophysical manner. This paper investigates the effects of the organophosphorus compounds, fenitrothion, fenitro-oxon and diethylphenylphosphate (DEPP) and the carbamate, physostigmine sulphate on the snail in toto. A later paper will discuss the effects of these compounds on the cholinergic neurones of the snail suboesophageal ganglion.

Materials and Methods

Experimental Solutions

Analytical grade samples of fenitrothion and fenitro-oxon were kindly donated by Sumitomo Chemical Co. Ltd. Osaka Japan and American Cyanamide Co. Ltd. Princeton, N.J. The diethylphenyl phosphate was prepared by the action of diethyl phosphorochloridate and phenol in the presence of sodium hydride. The purity of the samples was established by gas chromatography and shown to be > 98% by elemental analysis. The physostigmine sulphate was obtained from the Sigma Chemical Company, St. Louis. Mo.

Experimental solutions were made by mixing the test compounds in Corexit 7664, adding this mixture to the snail Ringer's solution and shaking vigorously. The maximum amount of Corexit 7664 used was 20% by volume. Corexit 7664 is an esterfied polyethylene glycol ether dissolved in isopropanol, manufactured by the Enjay Chemical Co., N.J. It is a non-ionic surfactant that has been used to disperse oil slicks and has low toxicity to fish (ZITKO 1970).

The snail Ringer's solution was that used by KERKUT and GARDNER (1967).

Experimental Procedure

Fresh emulsions of the experimental solutions were added directly to the foot of an estivating snail. The total volume applied was .01 ml per gram of snail. The dose was computed as a given weight of a compound per gram fresh weight of the animal (including the shell). The ratio of shell weights to tissue weights (minus the shell) showed no significant variation.

Initial control experiments demonstrated that both ethanol and acetone solvents exerted behavioural effects on snails; they were therefore not used in the experimental solutions. Experiments performed with 100% Corexit, 20% Corexit by volume and with snail Ringer alone gave identical results.

The measurement of lethality was calculated as a percentage of the number of deaths that occurred within 36 hours of application of an experimental compound. Each of the test groups was composed of 10 snails and all appeared to be physiologically normal (i.e. in normal estivating condition). The behaviour of the snails was observed at 30 minutes, 2 hours

and 12 hours.

Results

1. Lethality

No deaths occurred (20 snails/group) in any of the control experiments (snail Ringer's, Corexit 7664, or a 20% by volume mixture of Corexit and Ringer) within the 36-hour experimental period. The LD_{50} values were obtained from a graph of log percent lethality vs. log dose (expressed as micrograms of compound per gram of total fresh snail weight) and are shown in Table 1.

TABLE 1

Lethality of physostigmine and organophosphorus compounds to *Helix aspersa*.

Compound	LD_{50}
Fenitrothion	175 µg/g
Fenitro-oxon	140 µg/g
Diethylphenylphosphate	160 µg/g
Physostigmine sulphate	135 µg/g

2. Behavioural Symptoms

Corexit 7664 applied either alone or 20% by volume in snail Ringer produced behavioural responses that were indistinguishable from those produced by application of normal snail Ringer.

The responses were of two distinct types. In Type 1 responses the snails were observed to writhe within their shells and exude a green mucous-like substance. In Type 2 responses (at low concentrations of test compounds) the snails emerged from their shells and moved around inside the container (with antennae slightly drooped). At higher concentrations the antennae were drooped; the body appeared flaccid and muscular co-ordination was poor. As the concentration of the test compounds increased the locomotion and physical co-ordination of the snails was reduced and the changes in physical appearance became more pronounced. In some snails the higher doses caused lethality, but in others it was found that the behavioural effects wore off with time so that the snails became indistinguishable from the controls.

The behavioural responses were classified on a subjective scale based on the magnitude of the response and are summarized in Table 2.

TABLE 2

Classification of behavioural symptoms of Helix aspersa.

Classification (Type)	Observations of Type 1 Behavioural Response
1A	Snails writhe inside shell after application and recover within 6 - 8 hours or die.
1B	Snails writhe vigorously inside shell and exude green mucous-like substance and recover within 12 hours or die.

Classification (Type)	Observations of Type 2 Behavioural Response
2A	Snails move around container, antennae are slightly drooped.
2B	Antennae very drooped, muscular co-ordination poor.
2C	Snails immobile, antennae are completely relaxed lying against body.

The concentration of the compounds used and the magnitude of reactions observed at the 3 time intervals are shown in Table 3. All of the test compounds in concentrations above 25 µg/g were observed to elicit behavioural responses in the snail. DEPP only produced Type 1 responses, while physostigmine only produced Type 2 responses. Snail responses changed from Type 1 to Type 2 between 30 mins. and 2 hours after application of ≥50 µg/g of fenitrothion. Fenitro-oxon gave a combination of Type 1 and Type 2 responses up to two hours after application, but only Type 2 after this time.

TABLE 3

Behavioural symptoms of Helix aspersa.

Compound	Concentration	Observations/10 Snails 30 Min.	2 Hours	12 Hours
	µg/g			
Fenitrothion	10	NE	NE	NE
	25	1A	NE	NE
	50	1A	2A	NE
	100	1B	2A	NE*
	150	1B	2A	NE*
	200	1B	2B	NE*
	250	1B	2C	NE*
Fenitro-oxon	10	NE	NE	NE
	25	1A+2A	2A	NE
	50	1A+2A	2A	NE
	100	1B+2B	2B	NE*
	150	1B+2B	2B	NE*
	200	1B+2C	2C	NE*
	250	1B+2C	2C	2A
Diethylphenyl-phosphate	10	NE	NE	NE
	25	NE	NE	NE
	50	1A	1A	NE
	100	1B	1A	NE*
	150	1B	1A	NE*
	200	1B	1B	NE*
	250	1B	1B	NE*
Physostigmine	10	2A	2A	NE
	25	2B	2A	NE
	50	2B	2A	NE
	100	2C	2B	NE*
	150	2C	2C	2A
	200	2C	2C	2A
	250	2C	2C	2B

*Behavioural response of surviving snails

NE = No effect (same as controls).

3. Inhibition of Snail Blood Cholinesterase

The procedure outlined by KERKUT (1968) was used to determine if snail blood cholinesterase was inhibited *in vitro* by any of the test compounds. Fenitrothion and DEPP caused no inhibition of cholinesterase at concentrations up to 10^{-3} g/ml while fenitro-oxon and physostigmine produced over 90% inhibition at 10^{-4} g/ml.

Discussion

Lethality

In some species organophosphates (direct cholinesterase inhibitor) are significantly more toxic than the phosphorothioates. For example, fenitro-oxon was more toxic after oral administration to mice (LD_{50} = 90 µg/g) than fenitrothion (LD_{50} = 870 µg/g) (MIYAMOTO 1969). However, our experiments with *Helix aspersa* showed that topical application of fenitrothion and fenitro-oxon caused similar degrees of lethality. Also, topical application of these compounds to housefly showed little difference in resultant mortality (fenitro-oxon, LD_{50} = 4.3 µg/g, fenitrothion, 3.1 µg/g (HOLLINGWORTH, *et al.*, 1967). These discrepancies in relative toxicity suggest that metabolism of fenitrothion to its more toxic phosphate fenitro-oxon is greater in houseflies and snails than in mice.

Although lethality due to organophosphorus or carbamate exposure is thought to result mainly from cholinesterase inhibition, other factors can modify the toxicity. Thus, the carbamate, physostigmine sulphate, was extremely toxic to mice (oral LD_{50} = 3.0 µg/g (MERCK INDEX 1968)) compared to housefly (topical LD_{50} = 500 µg/g (METCALF and MARCH 1950)). This may be due to a difference in metabolism or in mode of application since the ability of physostigmine to inhibit cholinesterase *in vitro* in fly head and mouse brain extracts was similar I_{50} = 2×10^{-8}M, 3×10^{-7}M, respectively (METCALF and MARCH 1950)). Also fenitro-oxon was much more toxic than physostigmine to housefly although cholinesterase inhibition of fly head extract by fenitro-oxon (I_{50} = 5.6×10^{-8}M (HOLLINGWORTH, *et al.*, 1967)) was similar to physostigmine.

As all the test compounds produced similar LD_{50} values in *Helix aspersa*, lethality cannot simply be accounted for by cholinesterase inhibition. It might be assumed that fenitrothion, fenitro-oxon and physostigmine have similar absorbance rates from the topical application to the snail foot and that fenitrothion undergoes significant metabolic conversion to fenitro-oxon in snail. However it does not account for the similar lethality produced by DEPP which did not inhibit cholinesterase in snail blood and shows relatively poor inhibition of fly head cholinesterase (I_{50} = 1.0×10^{-3}M (MELNIKOV 1971)). As it seems unlikely that DEPP is metabolized to a more toxic compound another mode of toxicity must be assumed.

Behavioural Symptoms

METCALF (1955) found that exposure of cockroach Periplaneta americana to physostigmine produced symptoms indistinguishable from those produced by organophosphorus compounds (i.e. muscular incoordination, convulsions and paralysis). These symptoms (Type 2 responses, see Tables 2 and 3) were observed when high doses of both the direct and latent cholinesterase inhibitors were applied to snail. It is therefore suggested that the Type 2 symptoms were associated with cholinesterase inhibition. The Type 1 responses (writhing within shell) exhibited by snails after exposure to the three organophosphorus compounds (but not physostigmine) suggested that the Type 1 symptoms were due to some common characteristic of fenitrothion, fenitro-oxon and DEPP not associated with cholinesterase inhibition.

Both the lethality and behavioural observations therefore suggest that toxic organophosphorus compounds have more than simply an anticholinesterase effect in H. aspersa. Experiments to investigate the effects of these compounds on snail neurone membrane potentials will be described in a later paper.

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