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Biochemical and behavioural effects of some halo-substituted vinyl phosphorus esters

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ANIMAL behavioural studies with anticholinesterase agents have so far indicated that significant changes in behaviour can only be detected following doses of those agents which produce considerable reductions in both blood and brain cholinesterase activity. For example, Russell *et al.*[1] using Demeton-S (*O,O*-diethyl S-2-ethylthioethyl phosphorothiolate) showed that it increased the rate of extinction of a conditioned response in rats but only when there was a 60–65 per cent reduction in brain cholinesterase activity. Goldberg *et al.*[2] investigated three carbamates and found similarly that they all disrupted discrete avoidance behaviour in the rat but only at doses which produced significant reductions in brain cholinesterase activity. Pan'shina[3] found that 40 mg/kg of the organophosphorus insecticide phosphamide, a dose which reduced serum and erythrocyte cholinesterase levels by 40 per cent or more, modified conditioned responses in the cat.

The work reported in this communication extends the knowledge in the field by examining some halogen-containing vinyl phosphorus esters of a type which have been studied as potential pesticides. Their activities in inhibiting various cholinesterase preparations have been studied and attempts made to relate their anti-enzyme activity to their activity in modifying behaviour. Some related saturated esters were also included in the study.

Methods and Materials

Rates of hydrolysis. All hydrolyses were carried out in stirred solutions at 25° in an atmosphere of nitrogen. A known weight of the compound (approximately 5×10^{-5} mole) was added to de-ionized water (25 ml) the pH of which had been adjusted to the required value by addition of 0·1 N sodium hydroxide. The rate of addition of 0·1 N sodium hydroxide which was required to maintain the pH of the solution at the predetermined value was recorded by a Radiometer SBR 2c Autotitrator. The first-order rate coefficients and second-order rate constants were calculated from the results in the usual way and are shown in Table 1.

Rates of enzyme inhibition. An aliquot (0·5 ml) of the enzyme solution (containing approximately 1 μM unit/ml) was added to a 4×10^{-2} M solution of acetylcholine chloride in normal saline (25 ml) at pH 7·4 and 25°. The rate of addition of 10^{-2} M sodium hydroxide required to maintain the pH at 7·4 was recorded using a Radiometer SBR 2c Autotitrator. This rate of addition of sodium hydroxide was taken to represent 100 per cent enzyme activity. After 2 min, a known amount of inhibitor was added and the subsequent rate of addition of 10^{-2} M sodium hydroxide was recorded until it reached less than 5 per cent of the original rate. The rate of addition of sodium hydroxide every 2 min was expressed as a percentage of the rate in the absence of the inhibitor.[4] The first-order rate coefficients obtained from these results were converted into second-order rate constants by dividing by the concentration of the inhibitor used. It should be noted that these were, for practical purposes, measured in the presence of substrate. The true inhibition rate constants can be obtained from the values given in Table 1 by dividing by the factor

$$\frac{1}{1 + \frac{[S]}{K_m}} \equiv \frac{1}{150} \text{ (for AcChE), } \frac{1}{25} \text{ (for ChE).}$$

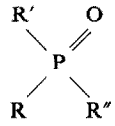
Preparation of compounds. Compounds 5, 6, 7 and 8 were prepared by published methods.[5–7]

2-*Chloroethyl* 2,2-*dichlorovinyl ethylphosphonate* (*Compound* 1). Ethylphosphonous dichloride (1 mole) in petroleum ether (b.p. 40–60°, 200 ml) was added dropwise, with stirring under nitrogen to a mixture of 2-chloroethanol (2 moles) and *N,N*-diethylaniline (2 moles) in petroleum ether (b.p. 40–60°, 1 l.). The mixture was stirred for 1 hr, amine hydrochloride removed by filtration under nitrogen, and the solvent removed under reduced pressure.

Anhydrous chloral (1 mole) was added slowly to the residue, in an atmosphere of dry nitrogen, with stirring, and stirring continued at room temperature for 1 hr. 1,2-Dichloroethane formed in the reaction was removed under reduced pressure, and the residue fractionated *in vacuo* to give (I) as a colourless liquid, b.p. 104°/0·2 mm, $n_D^{25} = 1{\cdot}4810$. Yield 52 per cent of theory. (Found: C, 27·5; H, 3·8%. $C_6H_{10}Cl_3O_3P$ requires C = 27·0; H = 3·8%.)

2-*Fluoroethyl* 2,2-*dichlorovinyl methylphosphonate* (*Compound* 2). Prepared as for compound 1, using 2-fluoroethanol and methylphosphonous dichloride in place of 2-chloroethanol and ethyl-

TABLE 1. LIST OF COMPOUNDS WITH THEIR RATES OF INHIBITION OF ACETYLCHOLINESTERASE (AChE) AND CHOLINESTERASE (ChE), IN THE PRESENCE OF 2×10^{-2} M SUBSTRATE. RATES OF HYDROLYSIS FOR THE COMPOUNDS ARE ALSO SHOWN

General formula 

Compound no.	R	R′	R″	k_{OH} (l. moles^{-1} min^{-1})	AChE; k_{In} (l. moles^{-1} min^{-1})		ChE k_{In} (l. moles^{-1} min^{-1})
					Bovine erythrocyte	Rat brain	
1	C_2H_5	$ClCH_2CH_2O$	$Cl_2C{=}CHO$	$9{\cdot}0 \times 10^{1}$	$3{\cdot}7 \times 10^{3}$	$4{\cdot}2 \times 10^{3}$	$3{\cdot}1 \times 10^{5}$
2	CH_3	FCH_2CH_2O	$Cl_2C{=}CHO$	$2{\cdot}1 \times 10^{2}$	$4{\cdot}6 \times 10^{3}$	$3{\cdot}2 \times 10^{3}$	$1{\cdot}5 \times 10^{5}$
3	CH_3	$ClCH_2CH_2O$	$Cl_2C{=}CHO$	$3{\cdot}2 \times 10^{2}$	7×10^{3}	$4{\cdot}7 \times 10^{3}$	$2{\cdot}3 \times 10^{5}$
4	CH_3	C_2H_5O	$Cl_2C{=}CHO$	$1{\cdot}6 \times 10^{2}$	$1{\cdot}2 \times 10^{3}$	3×10^{3}	4×10^{4}
5	CH_3	C_2H_5O	$ClCH_2CH_2O$	0·35	$6{\cdot}9 \times 10^{1}$	$7{\cdot}5 \times 10^{1}$	$2{\cdot}8 \times 10^{2}$
6	C_2H_5O	C_2H_5O	$Cl_2C{=}CHO$	2·5	$1{\cdot}3 \times 10^{2}$	2×10^{2}	$3{\cdot}5 \times 10^{5}$
7	C_2H_5O	C_2H_5O	$ClCH_2CH_2O$	1·0	$1{\cdot}0 \times 10^{2}$	8×10^{1}	$5{\cdot}6 \times 10^{4}$
8	CH_3O	CH_3O	$Cl_2C{=}CHO$	$4{\cdot}0 \times 10^{1}$	$1{\cdot}3 \times 10^{2}$	3×10^{2}	$3{\cdot}4 \times 10^{4}$

phosphonous dichloride. It had b.p. 96–98°/0·2 mm, n_D^{25} = 1·4688. Yield 12 per cent of theory. (Found: C, 25·7: H, 2·4%. $C_5H_8Cl_2FO_3P$ requires C, 25·3; H, 2·4%.)

2-*Chloroethyl* 2,2-*dichlorovinyl methylphosphonate* (*Compound* 3). Prepared as for compound 1, using methylphosphonous dichloride in place of ethylphosphonous dichloride. It had b.p. 104°/0·1 mm, n_D^{25} = 1·4889. Yield 88 per cent of theory. (Found: C, 24·0; H, 3·4%. $C_5H_2Cl_3O_3P$ requires C, 23·75; H, 3·2%.)

Ethyl 2,2-*dichlorovinyl methylphosphonate* (*Compound* 4). Prepared as for compound 1, using ethanol and methylphosphonous dichloride in place of 2-chloroethanol and ethylphosphonous dichloride. It had b.p. 70°/0·1 mm, n_D^{25} = 1·5040. Yield 69 per cent of theory. (Found: C, 27·6; H, 3·9%. $C_5H_9Cl_2O_3P$ requires C, 27·4; H, 4·1%.)

Biological methods

Toxicity. The LD_{50} for each compound administered subcutaneously to make albino Wistar rats was determined using four groups of five animals each and with a ratio between doses of 1·5. LD_{50}'s were computed by probit analysis.[8]

Behavioural—Open-field test. This procedure is used to test the effects of drugs upon the response of rats to a novel situation. Details of the apparatus and its use have been described previously.[9] Male albino rats were used and the behaviour of placebo-injected animals was compared with that of drug-injected animals, 1½ hr after subcutaneous injection of drug or placebo.

Cholinesterase estimations. The Michel method[10] was used to measure brain cholinesterase activity. The rats were killed by breaking the neck, and the brain removed and homogenised as quickly as possible. For blood cholinesterase activity the method of Fleisher *et al.*[11] was used, the blood being taken from the heart immediately after death.

Enzyme preparations. The acetylcholinesterase (EC 3.1.1.7) preparation was bovine erythrocyte acetylcholinesterase (Winthrop Labs. Inc. New York) and the cholinesterase (EC 3.1.1.8) was prepared from horse serum and obtained from Sigma Chemical Co. (London) Ltd. Rat brain acetylcholinesterase was prepared by homogenisation of fresh whole brain. Partial purification was achieved using DEAE Sephadex.

RESULTS

The compounds used in this study are listed in Table 1, together with their rates of inhibition (k_{In}) of bovine erythrocyte acetylcholinesterase, rat brain cholinesterase and horse serum cholinesterase. Also given are the second-order rate constants (k_{OH^-}) for the alkaline hydrolysis of the compounds.

In Table 2 are given the LD_{50}'s for all the compounds and their approximate minimal effective doses (M.E.D.'s) in the open-field test. The LD_{50}/M.E.D. ratios are also shown.

TABLE 2. TOXICITIES AND ACTIVITIES OF THE COMPOUNDS IN THE OPEN-FIELD TEST

Compound no.	LD_{50} (mg/kg s.c. rats)	Approx. M.E.D. Open-field test (mg/kg) s.c. rats	Effect on behaviour at M.E.D.	Ratio LD_{50}: M.E.D.
1	1·25	0·02	DEC P, R, S	62
2	2·69	0·1	DEC R, D	27
3	0·79	0·1	DEC D; INC R, S	8
4	1·01	0·1	DEC P	10
5	> 50	0·01	DEC %	> 100
6	15·5	0·2	INC S	77
7	13·0	> 5	—	< 3
8	35	0·2	INC S, %	175

DEC = decrease, INC = increase, P = No. of times preening, R = No. of times rearing, D = No. of times defaecating, F = No. of faecal boluses, S = No. of squares traversed, % = percentage of central squares crossed.

Coefficients of correlation between log k_{OH^-}, log M.E.D., log LD_{50} and the log k_{In} values for the three enzyme preparations were calculated and are given in Table 3. These indicate a significant degree of correlation between log k_{OH^-} and log k_{In} for the acetylcholinesterase preparation but not for cholinesterase. There were also significant correlations between log LD_{50} and log k_{In} for acetylcholinesterase but no correlations between any of the log k_{In} values and log M.E.D.'s in the open-field test.

TABLE 3. COEFFICIENTS OF CORRELATION (r)

	log k_{In}	log k_{OH^-}	log LD_{50}	log M.E.D.
Bovine erythrocyte acetylcholinesterase	+0·879	−0·916	−0·282	
	P < 0·01	P < 0·01	P > 0·1	
Rat brain acetylcholinesterase	+0·946	−0·920	−0·327	
	P < 0·001	P < 0·01	P > 0·1	
Cholinesterase	+0·585	−0·596	−0·346	
	P > 0·1	P > 0·1	P > 0·1	

The results of experiments in which the inhibitors were given by subcutaneous injection to rats are given in Table 4. With all the compounds tested, significant inhibition of blood cholinesterase occurred after lower doses than those which affected brain cholinesterase levels. In no case was the brain cholinesterase affected by the minimum dose of the compound which had a significant effect on open-field behaviour.

TABLE 4. INHIBITION OF BLOOD AND BRAIN CHOLINESTERASE *in vivo* BY SOME OF THE ESTERS

Compound no.	Dose (mg/kg)	% Inhibition of cholinesterase			Remarks
		Brain	Plasma	Erythrocytes	
3	0·05	1	0	12	
	0·1*	0	17	31	
	0·2	5	42	62	
	0·4	31	50	73	
4	0·05	0	25	24	
	0·1*	0	24	31	
	0·2	9	55	66	
	0·4	39	78	88	
1	0·003	1	0	0	
	0·02*	2	8	0	
	0·12	5	15	23	
	0·72	58	79	86	3/8 rats died
6	0·125	0	7	2	
	0·5 (0·2*)	20	31	24	
	2·0	72	67	71	
	8·0	88	88	92	All rats died
5	400	2	0 (whole blood)		

Blood and brain samples taken at 90 min after injection or at death when this occurred earlier All values represent the mean of eight estimations.

* Denotes M.E.D. in open-field test.

Discussion

All these halogen-containing phosphorus esters are inhibitors of both acetylcholinesterase and cholinesterase. A significant correlation was demonstrated between the rates of inhibition of the acetylcholinesterase preparations used and the rates of hydroxide ion catalysed hydrolysis of the phosphorus esters. This suggests that the rate controlling step in both the process of enzyme inhibition and of hydrolysis is the same, presumably P—X bond fission.[12] A similar correlation could not be demonstrated, however, with cholinesterase. The animals in the toxicity experiments showed signs typical of poisoning by anticholinesterase agents and the fact that the lethal effects of the compounds were due to their antiacetylcholinesterase activity is borne out by the correlation between LD_{50}'s and activities in inhibiting acetylcholinesterase.

The behavioural effects of the compounds in the open-field test seemed, however, not to be related to their enzyme-inhibiting activity. In addition, the results of the *in vivo* studies indicated that brain acetylcholinesterase levels were virtually unaffected by doses of the compounds which were effective in modifying open-field behaviour. The acetylcholinesterase determinations were made 90 min after injection at which time the open-field tests were carried out. The most obvious explanation of these findings is that the phosphorus esters are producing their effects on behaviour by means other than the inhibition of acetylcholinesterase. It is possible, however, that significant depletion in acetylcholinesterase levels in specific regions of the brain could occur but not be revealed by the relatively crude procedure of homogenising the whole brain and determining the enzyme activity in the homogenate.

An alternative possibility is that the behavioural effects observed are due to inhibition of other brain esterases or indeed that they are manifestations of peripheral rather than central actions of the drugs. The precise significance of the effects on open-field behaviour is not clear. This test, although useful as a screening procedure, seems to be relatively non-specific and of limited value in predicting what precise effects on human behaviour a compound will have. It has been possible[9] with certain classes of psychotropic drugs to extrapolate to man from results obtained using the open-field, but in this case the compounds did not produce a common effect and extrapolation is impossible. It seems clear that, in comparison with other reported results with cholinesterase inhibitors, these halogen-containing phosphorus esters can produce certain behavioural effects at doses which have little or no effect on brain and blood acetylcholinesterase levels. The mechanism whereby these effects are produced is unknown and the significance of the effects obscure. Further experiments, particularly behavioural studies using other test methods and other species, are required.

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