The Inhibition of Serum Cholinesterase by Alkyl Fluorophosphonates*

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During the war, a team of workers under Drs Saunders and McCombie prepared a series of fluorophosphonate esters and related compounds (McCombie & Saunders, 1946). Some of them were found to be highly toxic to animals by inhalation and very rapid in action, and to have a powerful myotic action when present in sub-lethal concentrations. In man, exposure to amounts of vapour insufficient to give any sensory effect produced a pupillary contraction and spasm of accommodation lasting several days. It was suggested that the parasympatheticomimetic activity of the fluorophosphonates, like that of eserine, might be due to an inhibition of cholinesterase. The results reported here have made it clear that these esters do in fact act like eserine in poisoning the enzyme, and that this reaction *in vitro* can be used as a test for the pharmacological potency of the esters.

EXPERIMENTAL

Materials and methods

Enzyme. Cholinesterase was prepared from horse serum by the method of Stedman & Stedman (1935), carrying the preparation as far as the dialysis against chloroform water. The method of estimation was essentially that of Ammon (1933). The enzyme solution was placed in the right-hand flask of a Barcroft manometer, in a total volume of 3 ml. 0·2% $NaHCO_3$; the gas phase was 5% CO_2 in N_2. The reaction, carried out at 20°, was started by tipping in a solution containing 2 mg. acetylcholine chloride. The CO_2 output was usually linear until about 100 μl. had been produced. The activity remained remarkably constant for many weeks when the stock enzyme was stored at 0° in presence of a little chloroform. For the inhibition experiments, sufficient enzyme was taken to produce 100–120 μl. CO_2 in 10 min. at 20°.

Poisons. The fluorophosphonates and related compounds were obtained from Dr B. C. Saunders; eserine sulphate was a commercial product (British Drug Houses Ltd.). Since fluorophosphonates slowly hydrolyze in water, stock solutions of these and of eserine were prepared in ethylene glycol monoethyl ether, and diluted with the same solvent so that an addition of 0·01–0·03 ml. to the 3 ml. of enzyme-buffer solution in the manometer vessel gave the required final concentration of poison. The enzyme, buffer and poison were incubated in the manometer vessel for the required time (15 min. in the standard test mentioned below) at 20° before tipping in the substrate from dangling tubes. For the experiments with a short duration of incubation, the poison solution was tipped from a dangling tube and the acetylcholine from a side tube.

The activity of the treated enzyme was compared against that of a control incubated with the solvent alone. Since it was found that the enzyme was unaffected by the solvent, or by incubation at 20° without substrate for a period of some hours, it was not necessary to repeat the control with every set of estimations.

RESULTS

Progress curve of inhibition. In preliminary experiments with di*iso*propyl fluorophosphonate, it was found that concentrations above 10^{-7} M completely inhibited cholinesterase almost instantaneously. With lower concentrations, the inhibition produced varied with the time of incubation. Fig. 1

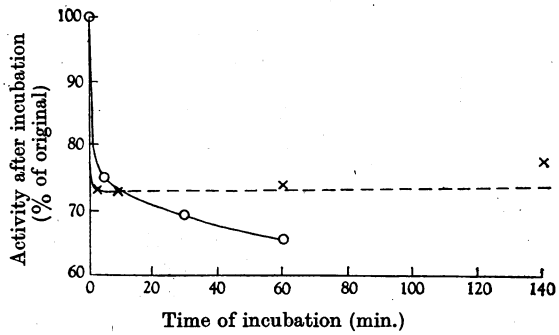


Fig. 1. Progress curve of inhibition of horse serum cholinesterase by eserine and by di*iso*propyl fluorophosphonate, in absence of substrate, at pH 7·4 and 20°. ×—×, 5×10^{-8} M-eserine; ○—○ *c.* 3×10^{-10} M-di*iso*propyl fluorophosphonate.

shows the inhibition of cholinesterase by di*iso*propyl fluorophosphonate, and by a comparable amount of eserine, after varying times. The action of eserine reaches a maximum within 5 min., while the inhibition by fluorophosphonate is initially less rapid, but is progressive and ultimately more complete. The latter effect suggests an irreversible inactivation of the enzyme rather than an equilibrium.

* This work formed part of the research programme carried out for the Ministry of Supply by an Extra-Mural Research Team under the direction of Dr M. Dixon, and was reported to the Ministry of Supply (Mackworth, 1942; Webb, 1944).

Reversibility. It was shown by Matthes (1930) that the effect of eserine on cholinesterase could be reversed completely by prolonged dialysis against water. These results have been confirmed, but on the other hand it proved impossible to obtain any reversal of the poisoning by the fluorophosphonate esters (Table 1). The enzyme solution (5 ml.) was treated with the inhibitor for 15 min. at 38°; 1 ml. was used at once for activity estimation, and the remainder dialyzed against running tap water for 24 hr. in the case of the eserine experiment, 36 hr. in the others. A control was treated in the same manner, in the absence of inhibitor, and corrections were applied for the volume change on dialysis. It is clear that the combination between the fluorophosphonate esters and the enzyme is of a much firmer nature than that between eserine and the enzyme.

Table 1. *Effect of prolonged dialysis against water on activity of cholinesterase poisoned with eserine and fluorophosphonates*

(Enzyme incubated with poison for 15 min. at 38° before dialysis.)

Inhibitor	Conc. (M)	Inhibition (%) Before dialysis	Inhibition (%) After dialysis for 24–36 hr.
Eserine	10^{-6}	100	35
Diethyl fluorophosphonate	4×10^{-9}	70	76
Diethyl fluorophosphonate	10^{-8}	90	85
Di*iso*propyl fluorophosphonate	10^{-9}	50	50

Effect of substrate concentration. In the following experiments the cholinesterase activities were measured by a continuous titration method. The digest of acetylcholine and horse serum cholinesterase (total vol. 10 ml.), containing bromothymol blue and 0·0002 M-phosphate, was titrated with 0·01 N-NaOH to maintain the pH at 7·4. The titrations, which were carried out in a water bath at 20°, were linear over a period of 10–15 min. The velocity was expressed as ml. 0·01 N-NaOH/5 min.; under the conditions used, it was proportional to the enzyme concentration. When an inhibitor was added, this was equilibrated with the enzyme etc., for 5 min. at 20° before adding the substrate contained in a volume of 1 ml.

Under the conditions used the enzyme was markedly less sensitive to fluorophosphonate than in the manometric experiments, due probably to the higher enzyme concentration and the shorter incubation period, so that only the initial rapid inhibition took place. With an acetylcholine concentration of 0·0045 M, 50 % inhibition was produced by approximately 2×10^{-7} M-eserine or $3{\cdot}5 \times 10^{-8}$ M-di*iso*propyl fluorophosphonate. When the substrate concentration was varied over the range 0·0004–0·06 M, the percentage inhibition by the fluorophosphonate (compared with a standard having a similar substrate concentration but no inhibitor) remained more or less constant. On the other hand, the inhibition due to eserine decreased when the acetylcholine concentration was raised. The difference in the behaviour of the two inhibitors is clearly shown when the results are examined by the method of Lineweaver & Burk (1934), which has already been applied to serum cholinesterase and eserine by Eadie (1942). The relationship between enzyme reaction velocity v and substrate concentration S is given by

$$\frac{1}{v} = \frac{1}{V} + \frac{K_p}{V}\frac{1}{S},$$

where V is the limiting velocity and K_p is the effective Michaelis constant. In the presence of a 'competitive' reversible inhibitor, V is unchanged but K_p is increased, i.e. the apparent affinity of the enzyme for its substrate is lowered. If there is no competition between inhibitor and substrate for the enzyme centres, $K_p = K_M$ but V is lowered.

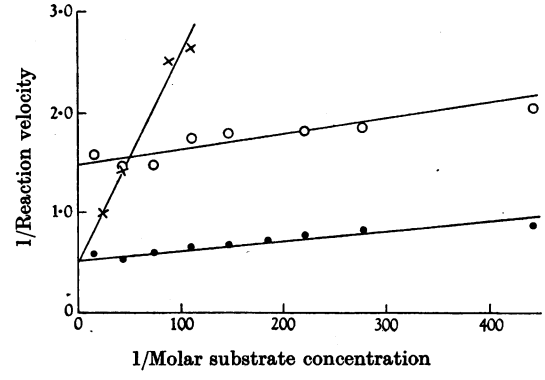


Fig. 2. Effect of substrate concentration on inhibition of horse serum cholinesterase. Enzyme activity estimated by titration with 0·01 N-NaOH at pH 7·4 and 20°. ●—●, control, no inhibitor; ×—×, 2×10^{-7} M-eserine; ○—○, 5×10^{-8} M-di*iso*propyl fluorophosphonate.

Fig. 2 shows the results obtained with 2×10^{-7} M-eserine and 5×10^{-8} M-di*iso*propyl fluorophosphonate. Eserine behaves like a typical competitive

inhibitor, with V unchanged, but the fluorophosphonate gives no indication of competition with the substrate.

True and pseudo-cholinesterase. The crude serum preparation used contained both the 'true' and 'pseudo' cholinesterases of Mendel & Rudney (1943). The effect of di*iso*propyl fluorophosphonate on these components was examined separately by means of the specific substrates described by Mendel, Mundell & Rudney (1943), using the titration method described above. Fluorophosphonate (5×10^{-8} M) gave an inhibition of 57 % of the activity towards 0·0045 M-acetylcholine, 30 % of the activity towards 0·0005 M-acetyl-β-methylcholine, and 40 % of that towards 0·005 M-benzoylcholine, after incubating the enzyme with the poison for 5 min. Thus there is no appreciable difference in sensitivity of the true and pseudo-cholinesterases of horse serum to fluorophosphonates.

Assessment of inhibitory power. In order to compare the inhibitory power of the different compounds, conditions for a standard manometric test were defined. A fixed amount of enzyme was incubated at 20° with varying concentrations of the inhibitor for 15 min. before tipping in the acetylcholine. The CO_2 production in the first 10 min. was used for estimating the activity, and by comparison with a control, the percentage inhibition. Curves showing the percentage inhibition produced under the standard conditions plotted against the logarithm of the inhibitor concentration for eserine and a number of alkyl fluorophosphonates are shown in Fig. 3. They are in general sigmoid in shape, but are not all similar; in particular, those for *iso*propyl and *sec.*-butyl fluorophosphonate are considerably flatter than the others, so that in the range of inhibitions below 50 % these esters are relatively more effective than would be indicated by the pI_{50} values (see below). With different preparations of cholinesterase the absolute positions of the curves tended to vary, but they occupied the same position relative to one another. The graph for eserine agrees with that given by Ellis, Krayer & Plachte (1943). In an extended series of studies, the inhibition-concentration curves for new esters were always compared against that for di*iso*propyl fluorophosphonate, which was redetermined at the same time.

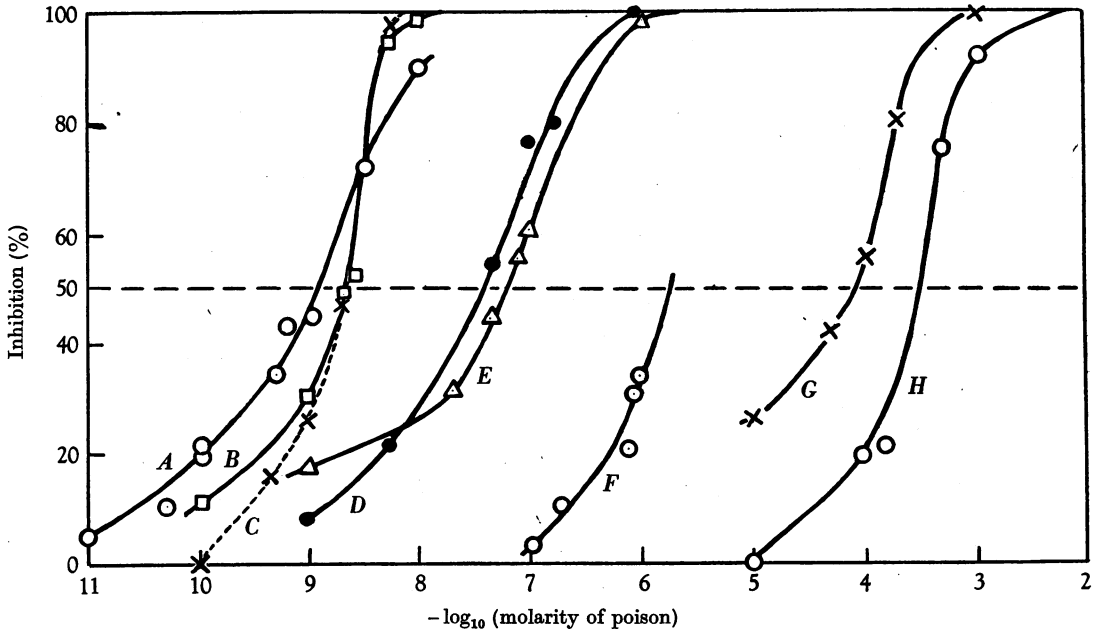


Fig. 3. Inhibition of horse serum cholinesterase by various compounds. Incubated for 15 min. at 20° before addition of 2 mg. acetylcholine chloride. A, di*iso*propyl fluorophosphonate; B, di*sec.*-butyl fluorophosphonate; C, di*iso*amyl fluorophosphonate; D, eserine; E, diphenyl fluorophosphonate; F, dithioethyl fluorophosphonate; G, dimethylaminophosphoryl fluoride; H, diethyl methylaminophosphate.

To obtain a numerical value for inhibitory power, the concentration of poison producing 50 % inhibition in the standard test was read off from the inhibition-log (concentration) curve. This value is conveniently expressed as the pI_{50}, which is the negative logarithm of the concentration producing 50 % inhibition (Mazur & Bodansky, 1944). Values for the poisons examined are given in Table 2. Di*iso*propyl fluorophosphonate was the most active

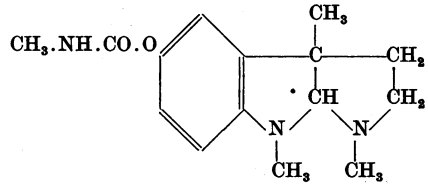
of these compounds, being thirty times more potent than eserine. The other branched-chain esters, namely *sec.*-butyl and *iso*amyl, although less active than the *iso*propyl derivative, are more active than the straight-chain *n*-propyl, ethyl and methyl esters. This suggests a special affinity of the enzyme for methyl groups, which are also present in the substrate and in eserine. Replacement of oxygen in diethyl fluorophosphonate by sulphur reduces the activity to 1/400 of the original. A slight activity is shown by methylaminophosphonate ethyl ester; unesterified fluorophosphonate is virtually inactive.

Table 2. *Inhibitory power of eserine, fluorophosphonates and related compounds on horse serum cholinesterase*

(Standard test conditions; 15 min. incubation at 20° in bicarbonate buffer, pH 7·4, in absence of substrate.)

Inhibitor	Formula	Molarity producing 50% inhibition	pI_{50} ($-\log_{10}$M)
Di*iso*propyl fluorophosphonate	$\left(\begin{matrix}CH_3\\CH_3\end{matrix}\!\!>\!CHO.\right)_2 PO.F$	$1{\cdot}3\times10^{-9}$	8·9
Di*sec.*-butyl fluorophosphonate	$\left(\begin{matrix}C_2H_5\\CH_3\end{matrix}\!\!>\!CHO.\right)_2 PO.F$	$2{\cdot}0\times10^{-9}$	8·7
Di*iso*amyl fluorophosphonate	$\left(\begin{matrix}C_2H_5\\CH_3\end{matrix}\!\!>\!CH.CHO.\right)_2 PO.F$	$2{\cdot}0\times10^{-9}$	8·7
Di-*n*-propyl fluorophosphonate	$(CH_3.CH_2.CH_2O.)_2PO.F$	$5{\cdot}5\times10^{-9}$	8·25
Diethyl fluorophosphonate	$(C_2H_5O.)_2PO.F$	$8{\cdot}0\times10^{-9}$	8·1
Eserine	*	$4{\cdot}0\times10^{-8}$	7·4
Diphenyl fluorophosphonate	$(C_6H_5O.)_2PO.F$	$6{\cdot}3\times10^{-8}$	7·2
Dimethyl fluorophosphonate	$(CH_3O.)_2PO.F$	$1{\cdot}0\times10^{-7}$	7·0
Dithioethyl fluorophosphonate	$(C_2H_5S.)_2PO.F$	$2{\cdot}0\times10^{-6}$	5·7
Dimethylaminophosphoryl fluoride	$(CH_3)_2N.PO(OH).F$	$8{\cdot}0\times10^{-5}$	4·1
Diethyl methylaminophosphonate	$(C_2H_5O.)_2PO.NHCH_3$	$3{\cdot}0\times10^{-4}$	3·5
Trimethyl phosphate	$(CH_3)_3PO_4$	$1{\cdot}0\times10^{-3}$	3·0
Vitamin B_1	—	$1{\cdot}7\times10^{-3}$	2·8
Ammonium fluorophosphonate	$(NH_4O.)_2PO.F$	$1{\cdot}0\times10^{-2}$	2·0
Sodium fluoride	NaF	$1{\cdot}0\times10^{-2}$	2·0

* Eserine has the following structure (Polonovski & Polonovski, 1925):



DISCUSSION

The extremely high affinity for cholinesterase of the fluorophosphonates, especially those with branched-chain alkyl groups, suggests that the toxic and myotic power of these compounds is due to inhibition of cholinesterase *in vivo*. Bloch (1943) showed that the parasympatheticomimetic symptoms of poisoning by cresyl phosphate, which is also an inhibitor of cholinesterase *in vitro*, were paralleled by a fall of serum cholinesterase and serum lipase activities *in vivo*. Similarly, Mazur & Bodansky (1946) have observed a correlation between serum cholinesterase and poisoning by fluorophosphonates, although Hawkins & Gunter (1946) have shown that complete inhibition of the serum (pseudo) cholinesterase *in vivo* is not necessarily accompanied by symptoms of acetylcholine accumulation. The values obtained in this work for inhibitory power towards serum cholinesterase fall into the same series as those from the physiological assay on the rabbit eye, although there is not a strict quantitative agreement. Possibly better agreement would have been obtained if the compounds had been assessed on a tissue true cholinesterase.

The inhibition of cholinesterase by eserine is readily understood by comparing the structure of the alkaloid with that of acetylcholine. Eadie (1942) has shown that eserine is a competitive inhibitor of cholinesterase, and that an equilibrium is reached within a few minutes of mixing, in which two molecules of eserine combine with one molecule of enzyme. The action of other inhibitors which possess

a strongly dissociated quaternary ammonium group, such as methylene blue, miotine (Massart & Dufait, 1941), phenylpyrazolones (Zeller, 1942), and vitamin B_1, which is a quaternary thiazolinium salt (Glick & Antopol, 1939), is probably similar. The action of the alkyl fluorophosphonates, on the other hand, is probably of a different nature; it is not reversible and there is no evidence of substrate competition or of an equilibrium, the destruction being progressive for at least an hour. Nevertheless the sigmoid curves obtained when inhibition is plotted against the logarithm of inhibitor concentration simulate equilibrium curves, and the more flattened, asymmetric curves obtained for the most potent esters resemble those which Straus & Goldstein (1943) have shown are to be expected for enzyme-inhibitor equilibrium when the enzyme concentration is high relative to the enzyme-inhibitor dissociation constant (so that the degree of inhibition is dependent to some extent on the concentration of the enzyme, as well as that of the inhibitor). Probably similar curves are to be expected if the degree of inhibition is determined by the velocity of a reaction between enzyme and inhibitor, dependent on their respective concentrations. It is possible that the initial rapid inhibition is an equilibrium, but that this is followed by an irreversible destruction of the enzyme; this would be consistent with the two-phase nature of the progress curve of inhibition. In any case, the effective irreversibility of the destruction would explain the prolonged action of the fluorophosphonates *in vivo*, in contrast to the transitory effect of eserine.

Easson & Stedman (1936) and Straus & Goldstein (1943) give figures of 1490 and 450 molecules of acetylcholine/sec./enzyme centre respectively for the absolute activity of horse serum cholinesterase at 30°, based on the inhibition by eserine at varying concentrations. (These figures depend on the assumption that there is an equilibrium between one enzyme centre and one molecule of eserine. They presumably need recalculation in the light of the work of Eadie mentioned above, but are probably of the right order of magnitude.) In the manometric experiment with an amount of serum cholinesterase which produced 100 μl. CO_2/10 min. at 20°, complete inhibition was produced by about 10^{-8} M-di*iso*propyl fluorophosphonate. Calculation shows that under these conditions complete inactivation was produced in 15 min. by the presence of 1–3 molecules of fluorophosphonate/enzyme active centre, which is particularly remarkable in view of the low purity of the enzyme preparation used. Clearly the fluorophosphonate has a very specific affinity for the active centres attacked.

SUMMARY

1. The alkyl fluorophosphonates examined were found to be highly potent inhibitors of horse serum cholinesterase. The most active ester, di*iso*propyl fluorophosphonate, was 30 times as active as eserine.

2. The inhibition of cholinesterase by alkyl fluorophosphonates, unlike the action of eserine, is progressive with time. It is not affected by the substrate concentration and cannot be reversed by prolonged dialysis.

3. The most potent esters were those with short branched-chain alkyl groups. The order of inhibitory power of the various esters was roughly the same as that for toxicity and for myotic power.

4. The true and pseudo-cholinesterase components of horse serum were equally sensitive to di*iso*propyl fluorophosphonate.

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* Information available on application to Ministry of Supply, London.