The Action of Alkyl Fluorophosphonates on Esterases and other Enzymes

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(*Received* 25 *October* 1946)

Work carried out during the war showed that alkyl fluorophosphonates, especially those with short branched-chain alkyl groups, have a very powerful inhibitory action on serum cholinesterase *in vitro* (Mackworth & Webb, 1948), and it was at first supposed that this action might be specific for cholinesterase. To test this hypothesis, the action of a potent alkyl fluorophosphonate was studied on a number of enzymes *in vitro*, and the results are reported in this paper. They show that esterases other than serum cholinesterase are sensitive to fluorophosphonate.

EXPERIMENTAL AND RESULTS

Tissue cholinesterase. The cholinesterase activity of a rabbit brain preparation, obtained by grinding the brain in bicarbonate Ringer solution and straining through muslin, was estimated in Barcroft manometers in the presence of 8 mg. acetylcholine. Under the conditions of treatment reported in the previous paper for serum cholinesterase, the brain enzyme was 50% inhibited by $6{\cdot}7 \times 10^{-8}$ M-ethyl fluorophosphonate, or by 5×10^{-7} M-eserine. Thus the inhibitory activity of diethyl fluorophosphonate against rabbit brain cholinesterase ($pI_{50} = 7{\cdot}2$) is about one eighth of its activity against horse serum cholinesterase ($pI_{50} = 8{\cdot}1$) but the relative activities of the two inhibitors on each enzyme remains the same.

Liver esterase. Simple esterase was estimated, with ethyl butyrate as substrate, by the continuous titration method of Harrer & King (1941). The titration mixture (10 ml.) contained M/450 ethyl butyrate, M/5000 phosphate and 0·003% bromothymol blue, and was maintained at pH 7·4 by titration with 0·01 N-NaOH at 20°. The enzyme had been partially purified from an aqueous extract of acetone-dried ox liver by fractional precipitation with ammonium sulphate and fractional adsorption on calcium phosphate gel. It had no detectable action on acetylcholine (cholinesterase activity/simple esterase activity < 1/1500).

Table 1 shows the inhibition of the enzyme by fluorophosphonate, eserine, and fluoride. To prevent reversal of the inhibition on dilution, the enzyme was incubated with the poison in a volume of 2 ml. for 15 min. at 20°, and then the reaction was started by adding 8 ml. of substrate, phosphate, etc., containing the same concentration of poison as the enzyme solution. It will be seen that liver esterase is strongly inhibited by fluorophosphonate but is relatively insensitive to eserine and fluoride.

Table 1. *Inhibition of liver esterase*

(Ethyl butyrate as substrate. Enzyme incubated with poison for 15 min. at 20° before estimation.)

Inhibitor	Conc. (M)	Inhibition (%)
Di*iso*propyl fluorophosphonate	10^{-6}	91
	5×10^{-7}	77
	3×10^{-7}	32
	10^{-7}	<10
Eserine	10^{-2}	65
	10^{-3}	25
NaF	10^{-2}	>85
	2×10^{-3}	73
	10^{-3}	48
	10^{-4}	7

Milk lipase. Human milk contains a powerful lipase, which can easily be estimated by a continuous titration method (Hottinger & Bloch, 1943). The most convenient substrate was a 1·5% emulsion of tributyrin in 1% sodium glycocholate (British Drug Houses Ltd,), neutralized to pH 7·5. A sample of human milk (4 weeks *post partum*) was skimmed by centrifuging and used directly as the enzyme; 0·06 ml. was sufficient for an activity estimation with tributyrin. The enzyme, together with 0·5 ml. 0·02 M-phosphate and 0·3 ml. 0·1% indicator (bromothymol blue or cresol red) was diluted to 8 ml., and the reaction started by addition of 2 ml. of the tributyrin emulsion. The mixture, contained in a boiling tube, was shaken gently and continuously in a water bath at 20°, and 0·01 N-NaOH was run in from a micro-burette to maintain the pH constant. The titration was completely linear over a period of 10–15 min. for a total addition of 1–2 ml. NaOH, the velocity being directly proportional to the amount of enzyme taken. Control titrations, (*a*) without enzyme and (*b*) without substrate, were zero. The optimal pH was found to be 8·0 ($\pm 0{\cdot}2$), and the experiments with poisons were carried out at pH 8 with cresol red as indicator, after a preliminary 15 min. incubation of enzyme and poison

at pH 7·4. A few experiments were carried out with other substrates, and the activities obtained are shown in Table 2.

Table 2. *Hydrolytic activity of skimmed human milk on various substrates*

(At pH 8, 20°, in presence of 0·2% glycocholate. 1 unit = the amount of enzyme which produces free acid equivalent to 1 ml. 0·01 N-NaOH in 5 min.)

Substrate	Activity (units/ml. milk)
Tributyrin	17·5
Olive oil	0·25
Ethyl butyrate	0·28
Acetylcholine	$\leqslant$0·004

The tributyrinase activity was strongly inhibited by fluorophosphonate, considerably less so by eserine, and was insensitive to fluoride. The inhibition by eserine increased continuously, even after addition of substrate, to a maximum at 2 hr. (Table 3). Similar results were obtained with olive oil as substrate.

Kidney phosphatase. The enzyme was prepared from rabbit kidney by the method of Delory & King (1944), and was estimated by the method of Bodansky (1933), using sodium β-glycerophosphate as substrate. The acid phosphatase activity was only moderately sensitive to fluorophosphonate, the alkaline phosphatase still less so (Table 4). According to Belfanti, Contardi & Ercoli (1935) acid phosphatase is sensitive to fluoride, while alkaline phosphatase is not.

Yeast pyrophosphatase. The purified yeast pyrophosphatase of Bailey & Webb (1944) was 48% inhibited by 10^{-3} M-di*iso*propyl fluorophosphonate, and unaffected by eserine. However, it was far more sensitive to free fluoride (50% inhibition by 2×10^{-5} M-NaF), and the result obtained with fluorophosphonate might be due to about 2% of free fluoride present as impurity in the preparation.

Enzymes insensitive to fluorophosphonate. Diethyl and dimethyl fluorophosphonates were found to be without effect on the following enzymes, when tested in concentrations up to 0·002 M: choline dehydrogenase, cytochrome oxidase, catalase, peroxidase, succinic dehydrogenase, lactic dehydrogenase (of muscle), alcohol dehydrogenase (of liver), yeast hexokinase, carboxylase, amylase, diaphorase, carbonic anhydrase, and the glycolytic system of muscle.

Table 3. *Inhibition of human milk lipase*

(At pH 8, 20°; in 0·2% glycocholate, 0·001 M-phosphate. Preliminary incubation at pH 7·4.)

Substrate	Inhibitor	Concentration (M)	Time of incubation without substrate (min.)	Inhibition (%)
Tributyrin	Di*iso*propyl fluorophosphonate	10^{-4}	15	100
		10^{-5}	15	66
		5×10^{-6}	15	50
		2×10^{-6}	15	32
		10^{-6}	15	16
Tributyrin	Eserine	10^{-3}	15	35–60
		10^{-3}	120	>97
		3×10^{-4}	120	86
		10^{-4}	120	50
Tributyrin	NaF	10^{-1}	15	40
		10^{-2}	15	0
Olive oil	Di*iso*propyl fluorophosphonate	10^{-5}	15	77
Olive oil	Eserine	10^{-3}	15	60–95

Table 4. *Inhibition of kidney phosphatases*

(38°, 0·02 M-glycerophosphate. Acid phosphatase estimated in 0·1 M-acetate, pH 5·5. Alkaline phosphatase estimated in 0·08 M-veronal-acetate buffer pH 9, containing 0·005 M-$MgCl_2$. Enzyme and poison incubated for 15 min. before addition of substrate.)

Inhibitor	Concentration (M)	pH	Inhibition (%)
Di*iso*propyl fluorophosphonate	10^{-3}	5·5	60
	3×10^{-4}	5·5	5
	10^{-4}	5·5	0
Eserine	10^{-3}	5·5	18
Di*iso*propyl fluorophosphonate	10^{-3}	9·0	12
Eserine	10^{-3}	9·0	7

Table 5. *Sensitivity of various esterases to fluorophosphonate ester, eserine and fluoride*

($pI_{50} = -\log_{10}$ (molarity producing 50% inhibition).)

Enzyme	pI_{50} Di*iso*propyl fluorophosphonate	Eserine	NaF
Serum cholinesterase	8·9*	7·4*	2·0*
	7·45	6·7	—
Liver esterase	6·4	*c.* 2·5	*c.* 3
Human milk lipase	5·3	*c.* 3 (15 min. incubation)	<1
	—	*c.* 4 (2 hr. incubation)	—
Kidney acid phosphatase	*c.* 3	<3	(+)
Kidney alkaline phosphatase	⩽3	⩽3	(−)
Yeast pyrophosphatase	3 (?)	0	4·7

* These values were calculated from manometric results; all the others were obtained by a titration method.

DISCUSSION

It is clear that the fluorophosphonates are not, as was at first supposed, specific inhibitors of cholinesterase. They poison a range of esterases, some of which have no activity at all towards acetylcholine. In this they resemble tri-*o*-cresyl phosphate and tri-*o*-chlorophenyl phosphate, which have been shown to inhibit serum and tissue cholinesterase, serum lipase, and human milk lipase, but not pancreatic lipase (Bloch, 1943; Hottinger & Bloch, 1943). Bloch has drawn attention to the fact that the enzymes which are inhibited by cresyl phosphate are also sensitive to eserine. However, Table 5 shows that there is no quantitative relationship between the sensitivity of enzymes to fluorophosphonate and to eserine, or between the sensitivity to fluorophosphonate and to free fluoride.

Mackworth & Webb (1948) in the preceding paper gave reasons for believing that the action of fluorophosphonates on serum cholinesterase is of quite a different nature from the action of eserine on the enzyme. The distinction is emphasized by the differing specificity of inhibition reported above; in particular, by the powerful action of fluorophosphonate on simple esterase and milk lipase, which are comparatively insensitive to eserine.

SUMMARY

1. Brain cholinesterase was inhibited by an alkyl fluorophosphonate, but not as strongly as serum cholinesterase.

2. Liver esterase, human milk lipase and kidney phosphatase were also sensitive to di*iso*propyl fluorophosphonate, though not to the same extent as cholinesterase. These enzymes were also inhibited by eserine, but there was no correlation between the sensitivity towards fluorophosphonate and towards eserine or sodium fluoride. Liver esterase in particular is highly sensitive to fluorophosphonate but relatively insensitive to eserine. The specificity of inhibition by fluorophosphonates is similar to that reported for tri-*o*-cresyl phosphate and tri-*o*-chlorophenyl phosphate (Bloch, 1943).

3. Alkyl fluorophosphonates had no effect on a number of other enzymes tested, with the possible exception of yeast pyrophosphatase.

The author is grateful to Dr B. C. Saunders for the methyl, ethyl and *iso*propyl fluorophosphonates used.

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