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SELECTIVE INHIBITION OF PSEUDO-CHOLINESTERASE BY DI*ISO*PROPYL FLUOROPHOSPHONATE

BY

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The inhibition of cholinesterase by fluorophosphonates was discovered in 1941 by Adrian, Feldberg, and Kilby (1947), when they examined dimethyl fluorophosphonate. In 1941 McCombie and Saunders prepared di*iso*propyl fluorophosphonate and Adrian *et al.* (1942), as well as Mackworth (1942) found that it had an even stronger inhibitory action on cholinesterase than the dimethyl ester. At that time it was not known that there were two enzymes, true cholinesterase and pseudo-cholinesterase (Mendel and Rudney, 1943a), which are not necessarily affected similarly by inhibitors (Mendel and Rudney, 1944; Hawkins and Gunter, 1946). In the experiments to be reported in this paper it will be shown that di*iso*propyl fluorophosphonate, unlike eserine or prostigmine (Hawkins and Mendel, 1946, and unpublished experiments), exhibits a much stronger inhibitory action on pseudo-cholinesterase than on true cholinesterase. With low concentrations of di*iso*propyl fluorophosphonate it is therefore possible to inhibit pseudo-cholinesterase selectively without affecting true cholinesterase.

The possibility of such selective inhibition of pseudo-cholinesterase by di*iso*propyl fluorophosphonate was suggested by the following two observations:

(1) Bodansky (1945) as well as Mazur and Bodansky (1946) found that on exposure of human beings to low concentrations of the vapour of di*iso*propyl fluorophosphonate almost complete inhibition of cholinesterase activity in the plasma could be obtained without causing serious distress. It should be borne in mind, however, that human plasma contains predominantly pseudo-cholinesterase (Mendel, Mundell, and Rudney, 1943) and that the inhibition of this enzyme, as shown by Hawkins and Gunter (1946), will not interfere with the destruction of acetylcholine released by nervous activity. These workers found that certain concentrations of a prostigmine analogue, the dimethylcarbamate of 2-hydroxy-5-phenyl-benzyltrimethylammonium bromide (Hoffman-LaRoche Nu-683), are capable of inhibiting completely the activity of pseudo-cholinesterase *in vitro* without affecting significantly that of true cholinesterase. This

compound, when injected into dogs in amounts sufficient to inhibit pseudo-cholinesterase almost completely, elicits no symptoms indicative of the accumulation of acetylcholine; such symptoms appear only if the dose injected is sufficiently large to depress the activity of the true cholinesterase as well. Pseudo-cholinesterase, therefore, plays no essential role in the hydrolysis of acetylcholine *in vivo*.

(2) Bodansky and Mazur (1946) and Mazur and Bodansky (1946) found that the concentration of di*iso*propyl fluorophosphonate necessary for the inhibition of cholinesterase varied according to the enzyme preparations used; the negative logarithm of the molar concentration of di*iso*propyl fluorophosphonate necessary to produce a 50 per cent inhibition of the activity towards acetylcholine ($1.5 \times 10^{-2}M$) was 7.7 and 8.1 for human and horse serum respectively, the corresponding value for rabbit serum was 4.1, and the values for red blood cells and brain varied between 5.2 and 6.0. Since the sera of man and the horse contain predominantly pseudo-cholinesterase (Mendel, Mundell, and Rudney, 1943), rabbit serum mainly true cholinesterase (Mendel and Rudney, 1945), and brain and red blood cells throughout the animal kingdom true cholinesterase only (Mendel and Rudney, 1943a, 1943b), the results obtained by Mazur and Bodansky can be interpreted as indicating that pseudo-cholinesterase is approximately 100 times more sensitive to the inhibitory action of di*iso*propyl fluorophosphonate than is true cholinesterase.

METHODS

Cholinesterase activity was measured manometrically by Warburg's method at 37° C. in $2.5 \times 10^{-2}M$ $NaHCO_3$, saturated with 5 per cent CO_2 in N_2. The di*iso*propyl fluorophosphonate was added to the bicarbonate medium containing the enzyme preparation in the main compartment of the Warburg flask, the substrate being placed in the side arm. After the enzyme preparation had been shaken for 15 min. to attain temperature equilibrium the substrate was tipped into the main compartment. From a stock solution, freshly prepared every third day, of di*iso*propyl fluorophosphonate ($10^{-2}M$) in propylene glycol, greater dilutions were made with distilled water as required. The final concentration of propylene glycol present in the experimental vessels caused by itself no inhibition of the cholinesterases.

The activities of the true and pseudo-cholinesterases were measured, as described by Mendel, Mundell, and Rudney (1943), by the rates of hydrolysis of acetyl-β-methylcholine and benzoylcholine respectively.

RESULTS

In vitro.—Preliminary experiments were carried out to determine the inhibitory action of di*iso*propyl fluorophosphonate on enzyme preparations containing either only true cholinesterase or only pseudo-cholinesterase. This was done in order to ascertain whether the substrate acetylcholine could be replaced by acetyl-β-methylcholine in measuring the activity of true cholinesterase, or by benzoylcholine in measuring the activity of pseudo-cholinesterase, without affecting the percentage inhibition by di*iso*propyl fluorophosphonate. Haemolysed dog erythrocytes were used as a source of true cholinesterase, and an extract of dog pancreas as a source of pseudo-cholinesterase. As shown in Table I, the degree of

inhibition of true cholinesterase and pseudo-cholinesterase by di*iso*propyl fluorophosphonate is not altered when acetyl-β-methylcholine or benzoylcholine respectively are substituted for acetylcholine.

TABLE I

INHIBITION OF PSEUDO-CHOLINESTERASE AND TRUE CHOLINESTERASE BY DI*iso*PROPYL FLUOROPHOSPHONATE, USING VARIOUS SUBSTRATES

Enzyme preparation	Substrate*	Molar concentration of di*iso*propyl fluorophosphonate	Percentage inhibition of enzymatic activity
True cholinesterase (haemolysed dog erythrocytes)	Ach. $1.2 \times 10^{-3}M$	1×10^{-7}	4
	Mch. $3 \times 10^{-2}M$	1×10^{-7}	3
	Ach. $1.2 \times 10^{-3}M$	5×10^{-7}	25
	Mch. $3 \times 10^{-2}M$	5×10^{-7}	26
Pseudo-cholinesterase (suspension of dog pancreas)	Ach. $6 \times 10^{-2}M$	1×10^{-8}	87
	Bch. $6 \times 10^{-3}M$	1×10^{-8}	86
	Ach. $6 \times 10^{-2}M$	5×10^{-8}	100
	Bch. $6 \times 10^{-3}M$	5×10^{-8}	100

* Ach. = acetylcholine; Mch. = acetyl-β-methylcholine; Bch. = benzoylcholine.

Moreover, the presence of pseudo-cholinesterase does not interfere with the inhibition of the true cholinesterase. Table II shows that the hydrolysis of acetyl-β-methylcholine by the true cholinesterase of haemolysed human erythrocytes is inhibited 26 per cent by $5 \times 10^{-8}M$ di*iso*propyl fluorophosphonate. When highly purified pseudo-cholinesterase prepared from horse serum is mixed with the haemolysed erythrocytes in an amount possessing an activity approximating that of the pseudo-cholinesterase of human plasma, no diminution of the inhibitory action of di*iso*propyl fluorophosphonate on true cholinesterase is observed.

In subsequent experiments, therefore, acetyl-β-methylcholine and benzoylcholine could be used to estimate separately the activities of the two cholinesterases in enzyme preparations which in most cases contained a mixture of both.

TABLE II

INHIBITORY ACTION OF DI*iso*PROPYL FLUOROPHOSPHONATE ON TRUE CHOLINESTERASE IN THE PRESENCE OF PSEUDO-CHOLINESTERASE

Type of cholinesterase	Molar concentration of di*iso*propyl fluorophosphonate	Activity* (μl. CO_2/15 min.)	Percentage Inhibition
True cholinesterase (haemolysed human erythrocytes)	—	100.0	—
True cholinesterase (haemolysed human erythrocytes)	5×10^{-8}	74.0	26.0
True cholinesterase (haemolysed human erythrocytes) in the presence of pseudo-cholinesterase† (purified horse serum)	5×10^{-8}	74.5	25.5

* Substrate in all cases: acetyl-β-methylcholine ($3 \times 10^{-2}M$).

† The pseudo-cholinesterase from horse serum was kindly supplied by Miss F. Strelitz, who purified it according to her method (Strelitz, 1944). This preparation exhibited no activity towards acetyl-β-methylcholine.

The enzyme preparations tested were the plasma of man, dog, cat, rat, rabbit, and sheep. Sheep plasma contains no pseudo-cholinesterase, while the plasma of the other species contains both cholinesterases, although in different proportions. For each enzyme preparation, with the exception of sheep plasma, the minimal concentration of di*iso*propyl fluorophosphonate required to cause complete inhibition of pseudo-cholinesterase activity was determined, using benzoylcholine as substrate. The inhibitory action of this concentration of di*iso*propyl fluorophosphonate on the true cholinesterase in the plasma was then examined, using acetyl-β-methylcholine as substrate.

The results of these experiments are shown in Table III. Although pseudo-cholinesterase is inhibited completely in all instances, the true cholinesterase is inhibited only partially: 35, 34, and 33 per cent in human, rabbit, and rat plasma respectively, and only 7 per cent in dog plasma.

TABLE III

SELECTIVE INHIBITION OF PSEUDO-CHOLINESTERASE BY DI*ISO*PROPYL FLUOROPHOSPHONATE

Source of enzyme	Substrate*	Molar concentration of di*iso*propyl fluorophosphonate	Activity (expressed as μl. CO_2 evolved by 1 ml. plasma in 15 min.) Without inhibitor	With inhibitor	Percentage inhibition
Human plasma	Ach. $6 \times 10^{-2}M$	1×10^{-8}	1280	13	99
	Mch. $3 \times 10^{-2}M$	,,	26	17	35
	Bch. $6 \times 10^{-3}M$	,,	570	0	100
Dog plasma	Ach. $6 \times 10^{-2}M$	5×10^{-8}	590	60	90
	Mch. $3 \times 10^{-2}M$	,,	82	76	7
	Bch. $6 \times 10^{-3}M$	,,	294	0	100
Cat plasma	Ach. $6 \times 10^{-2}M$	5×10^{-8}	426	14	96
	Mch. $3 \times 10^{-2}M$	,,	30	25	16
	Bch. $6 \times 10^{-3}M$	,,	109	0	100
Rat plasma	Ach. $6 \times 10^{-2}M$	1×10^{-6}	123	16	87
	Mch. $3 \times 10^{-2}M$	,,	54	36	33
	Bch. $6 \times 10^{-3}M$	,,	20	0	100
Rabbit plasma	Ach. $6 \times 10^{-2}M$	5×10^{-7}	45	20	56
	Mch. $3 \times 10^{-2}M$	,,	47	31	34
	Bch. $6 \times 10^{-3}M$	,,	4	0	100
Sheep plasma	Ach. $6 \times 10^{-2}M$	5×10^{-7}	13	10	23
	Mch. $3 \times 10^{-2}M$	,,	14	11	22
	Bch. $6 \times 10^{-3}M$	,,	0	0	—

* Ach. = acetylcholine
Mch. = acetyl-β-methylcholine } all in the form of the chloride.
Bch. = benzoylcholine

Besides disclosing the difference between the sensitivities of pseudo-cholinesterase and true cholinesterase towards di*iso*propyl fluorophosphonate, these experiments show that when acetylcholine serves as substrate, the inhibition brought about by di*iso*propyl fluorophosphonate depends on the proportion of true cholinesterase and pseudo-cholinesterase present in the plasma; the greater the content of pseudo-cholinesterase, the greater the discrepancy between the inhibition of true cholinesterase and the inhibition observed when acetylcholine

is the substrate; conversely, the lower the pseudo-cholinesterase activity, the closer the parallelism between the inhibition of true cholinesterase and the inhibition of the acetylcholine hydrolysis.

In vivo.—Mazur and Bodansky found that in human beings exposed to di*iso*propyl fluorophosphonate, an inhibition of 98–99 per cent of the activity of the plasma towards acetylcholine did not result in symptoms of acetylcholine accumulation. In the present series, animals were injected intramuscularly with di*iso*propyl fluorophosphonate in order to ascertain whether in species, the plasma of which contains true cholinesterase and pseudo-cholinesterase in a proportion different from that in human plasma, there is also no correlation between the inhibition of the activity of the plasma towards acetylcholine and the symptoms to be expected from this inhibition.

Rabbits were chosen as experimental animals because the plasma of this species contains a smaller proportion of pseudo-cholinesterase to true cholinesterase, and therefore (see Table III) the discrepancy between the degree of inhibition of the activity towards acetylcholine, on the one hand, and towards acetyl-β-methylcholine, on the other, is not so pronounced as with human plasma, in which pseudo-cholinesterase predominates.

It will be seen from the typical experiment outlined in Table IV that rabbits receiving intramuscular injections of di*iso*propyl fluorophosphonate display their first symptoms of acetylcholine poisoning (i.e., masticatory movements of the jaws and slight generalized fibrillation) at a time when an appreciable activity (18 per cent) of the plasma towards acetylcholine is still present. These results confirm Mazur and Bodansky's findings in their experiments with rabbits. However, these authors did not attempt to explain why in rabbits symptoms of acetylcholine poisoning appear when the cholinesterase of their serum still displays a considerable activity towards acetylcholine, whereas in man an almost complete inhibition of the activity of the plasma towards acetylcholine causes no symptoms of serious distress.

TABLE IV

RELATIONSHIP BETWEEN THE INHIBITION OF CHOLINESTERASES BY DI*iso*PROPYL FLUOROPHOSPHONATE AND THE ONSET OF SYMPTOMS OF ACETYLCHOLINE POISONING

Rabbit II—2.5 kg.
11.26 Activity of plasma tested.
11.27 0.65 mg. di*iso*propyl fluorophosphonate* in saline injected intramuscularly.
11.29 localized twitching of hind leg at site of injection.
11.40 masticatory movements, which continued until
11.49 0.13 mg. di*iso*propyl fluorophosphonate in saline intramuscularly.
11.56 generalized fibrillation.
11.57 chewing, swallowing and fibrillation; activity of plasma tested.

Time	Activity (expressed as μl. CO_2 evolved by 1 ml. plasma in 15 min.) towards:					
	Bch.† ($6 \times 10^{-3}M$)	Inhibition %	Mch.† ($3 \times 10^{-2}M$)	Inhibition %	Ach.† ($6 \times 10^{-2}M$)	Inhibition %
11.26	6.1	—	62.8	—	69.5	—
11.57	0	100	17.3	73	13.3	82

* An initial dilution (1 in 500) was made with propylene glycol.
† Bch. = benzoylcholine; Mch. = acetyl-β-methylcholine; Ach. = acetylcholine.

Discussion

The plasma of most species contains, in varying proportions, a mixture of two enzymes: pseudo-cholinesterase, which plays no essential role in the hydrolysis of acetylcholine *in vivo*, and true cholinesterase, the inhibition of which results in symptoms of acetylcholine poisoning. The experiments reported here have shown that appropriate concentrations of di*iso*propyl fluorophosphonate completely inhibit pseudo-cholinesterase without affecting the true cholinesterase significantly (see Table III).

Since acetylcholine is hydrolysed by both cholinesterases, measurements with acetylcholine as substrate can yield no information about the contribution made by each of these enzymes to the total activity, and the extent of inhibition of the activity towards acetylcholine in the presence of a selective inhibitor of pseudo-cholinesterase will depend on the relative proportions of pseudo- and true cholinesterases in the mixture which is being tested. Therefore, the degree of inhibition of acetylcholine hydrolysis by di*iso*propyl fluorophosphonate is no index of the inhibition of the true cholinesterase.

In the light of the above facts it is not surprising that human beings exposed to low concentrations of di*iso*propyl fluorophosphonate exhibit no symptoms indicative of acetylcholine accumulation when their plasma has lost 98–99 per cent of its original activity towards acetylcholine; 99 per cent of the activity of human plasma towards acetylcholine ($6 \times 10^{-2}M$) is due to pseudo-cholinesterase, true cholinesterase accounting only for about 1 per cent of the total activity (Mendel, Mundell, and Rudney, 1943). Consequently, when di*iso*propyl fluorophosphonate causes a 98–99 per cent inhibition of the activity of human plasma towards acetylcholine, the inhibition of the pseudo-cholinesterase activity should be complete, while the activity of the true cholinesterase may be depressed less than 35 per cent (see Table III).

The results are entirely different with animals whose plasma contains predominantly true cholinesterase (e.g., rabbits). The hydrolysis of acetylcholine by the plasma of such animals is due mainly to the true cholinesterase; therefore, when a 98 per cent inhibition of the activity of their plasma towards acetylcholine is achieved, it must be the true cholinesterase which is inhibited to a great extent. Consequently, symptoms of acetylcholine poisoning should set in at a much lower level of inhibition of acetylcholine hydrolysis than would be the case in species, such as man, where the hydrolysis of acetylcholine by the plasma is due mainly to pseudo-cholinesterase. Indeed, our experiments with rabbits have shown that the injection of di*iso*propyl fluorophosphonate leads to parasympathomimetic symptoms and fibrillation at a time when the activity of the plasma towards acetylcholine is inhibited not more than 80–82 per cent. In sheep, whose plasma contains true cholinesterase only, these symptoms would probably appear at a still lower level of inhibition of the acetylcholine hydrolysis. It would seem, therefore, that the higher the ratio of true cholinesterase to

pseudo-cholinesterase, the lower the degree of inhibition of acetylcholine hydrolysis prevailing at the time of onset of symptoms.

As mentioned previously, it is the inhibition of true cholinesterase which results in the appearance of symptoms of acetylcholine poisoning. On the basis of experiments in which the level of true cholinesterase activity was correlated with the appearance of symptoms after the injection of eserine, Gunter and Mendel (1945) concluded that the body possesses a surplus of this enzyme ; they observed no ill-effects until the activity of the true cholinesterase was inhibited 70–80 per cent. Similarly, Hawkins and Gunter (1946) found that symptoms of acetylcholine accumulation made their first appearance in dogs when the true cholinesterase activity of their plasma had been depressed to 23 per cent of its original level. Koelle and Gilman (1946) reported only slight parasympathomimetic symptoms in rats when the activity of the true cholinesterase in the brain had been depressed to 21–28 per cent of the normal by intramuscular injection of di*iso*propyl fluorophosphonate, and in the present study symptoms of acetylcholine accumulation appeared in rabbits when the activity of the true cholinesterase in the plasma had been depressed to 27 per cent of its original level (see Table IV).

Therefore, di*iso*propyl fluorophosphonate, in order to produce symptoms of acetylcholine poisoning, must be present in a concentration which is sufficient to remove the true cholinesterase in excess of that required for normal function. To estimate to what extent this objective has been achieved by injection of, or exposure to, di*iso*propyl fluorophosphonate it is necessary to determine the degree of inhibition of the activity towards acetyl-β-methylcholine. The use of acetylcholine as substrate would yield no such information except in the rare cases in which pseudo-cholinesterase is absent or is present in negligible amounts only.

SUMMARY

1. Although di*iso*propyl fluorophosphonate inhibits both true and pseudo-cholinesterases, higher concentrations are required for the inhibition of true cholinesterase than of pseudo-cholinesterase. With appropriate concentrations of di*iso*propyl fluorophosphonate it is therefore possible, in a mixture of both enzymes, to inhibit selectively the activity of pseudo-cholinesterase without affecting that of true cholinesterase.

2. Acetylcholine is hydrolysed *in vitro* not only by true cholinesterase, but also by pseudo-cholinesterase ; therefore, measurements of cholinesterase activity in which acetylcholine is used as substrate cannot be used to correlate the degree of inhibition of true cholinesterase by di*iso*propyl fluorophosphonate and the effects resulting from this inhibition *in vivo*. Since true cholinesterase is the enzyme responsible for the hydrolysis of acetylcholine released at nerve endings, it is the degree of inhibition of true cholinesterase which must be determined

when a correlation between anti-cholinesterase action and pharmacological effects is sought.

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