The *in vitro* Protection of Cholinesterases Against some Organophosphorus Inhibitors

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This work was carried out in 1948–9 and formed the subject of confidential reports to the Ministry of Supply. It was part of a search for simple compounds which would protect cholinesterases against inhibition by such compounds as Tabun (ethyl *NN*-dimethylphosphoramidocyanidate), Sarin (*iso*-propyl *P*-methylphosphonofluoridate) and ethyl-Sarin (*iso*propyl *P*-ethylphosphonofluoridate). As with DFP (*diisopropyl* phosphorofluoridate: Mackworth & Webb, 1948) inhibition by Tabun (Augustinsson, 1951, 1953) and Sarin (unpublished work of A. Todrick & L. T. D. Williams) is irreversible, and it is reasonable to suppose that inhibition by ethyl-Sarin is also irreversible. The starting point of our experiments was the supposition that since nothing definite was known about the active centre of cholinesterases, the catalytic activity was probably associated with the amino acid side chains. A number of amino acids were examined for their ability to protect cholinesterase *in vitro*; only dopa (3:4-dihydroxyphenylalanine) was effective, but in high concentration, and it was found that protective ability was a property of catechol derivatives. It appears that Augustinsson (1952), independently and about the same time, discovered the reaction of catechols with Tabun, and published his results almost simultaneously with those of Jandorf, Wagner-Jauregg, O'Neill & Stolberg (1952) on the reaction of phenols with DFP. The present paper deals mainly with the properties of Sarin, but sufficient data are also given to make it clear that the three groups of workers have studied different facets of the same phenomenon.

A brief account of this work has been published (Berry, 1953).

THEORETICAL

One of us (A.T.) in earlier unpublished work on Sarin in collaboration with L. T. D. Williams considered the case of irreversible inhibition by a labile inhibitor. The gist of their treatment is as follows:

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Let *I* be an inhibitor combining irreversibly with an enzyme *E* by means of a bimolecular reaction of rate constant *k*₄. Then

$$-\frac{d(E)}{dt} = k_4(E-x)(I-y). \quad (1)$$

If the inhibitor is simultaneously destroyed by unspecified side reactions of apparent unimolecular rate *k*₀, then

$$-\frac{d(I)}{dt} = k_4(E-x)(I-y) + k_0(I-y). \quad (2)$$

It is supposed that only the changes in enzyme activity can be measured. From the above equations

$$\frac{d(I)}{d(E)} = 1 + \frac{k_0}{k_4(E-x)}, \quad (3)$$

and if the enzyme activity (*E*_∞) is measured at a time when no further inhibition can be detected, and the inhibitor is presumed to be all destroyed,

$$(I) = x_\infty + \frac{k_0}{k_4} \ln \frac{(E_0)}{(E_\infty)}, \quad (4)$$

where *x*_∞ is the amount of enzyme inhibited and (*E*₀) the initial activity. It can be calculated from Berry's (1951) data that the concentration of serum enzyme used in the present studies did not exceed 0.5 μmm, whilst the concentrations of Sarin used were upwards of 10 μmm. Therefore the term *x*_∞ can be neglected, as Todrick & Williams (unpublished) had assumed.

If to such a system is added a substance *P* which reacts bimolecularly with *I*, rate constant *k*_p, and if *P* is in excess of *I*, equation 2 now becomes

$$-\frac{d(I)}{dt} = k_4(E-x)(I-y) + (k_0 + k_p(P))(I-y), \quad (2a)$$

and equation 4 becomes

$$(I) = \frac{k_0 + k_p(P)}{k_4} \ln \frac{(E_0)}{(E_p)}, \quad (4a)$$

where (*E*_p) is the activity in presence of *P* corresponding to (*E*_∞) in its absence. Combining equations (4) and (4a) to eliminate (*I*).

$$\frac{\log(E_p)/(E_\infty)}{(P) \log(E_0)/(E_p)} = \frac{k_p}{k_0} = \text{constant}. \quad (5)$$

The above treatment deals with enzyme activities. Since the only measure of this is the velocity of breakdown of substrate, the corresponding velocities may be used provided that the substrate concentration is the same in all assays.

EXPERIMENTAL

The enzyme preparations were horse serum or rat-brain dispersions diluted with bicarbonate-Ringer to give an output of about 100 μ l. CO₂/30 min. in the Warburg apparatus at 38°. The gas phase was 5% CO₂ in N₂, and with 25 mm-NaHCO₃ the pH was 7.4.

The substrates were benzoylcholine (6 mm final concentration) and acetylcholine (18 mm) respectively. They were kept as stock solutions in distilled water.

For most experiments Sarin was used as a stock solution in dry *n*-propanol, the alcohol being added to the controls, but some experiments were carried out with freshly prepared solutions of this and the other inhibitors in distilled water.

The 'protection test' which, with appropriate modifications, formed the basis of the experiments, consisted in adding inhibitor solution to the mixture of enzyme and substance being tested. The pH was brought to 7.4 if necessary. This mixture, of total volume 2.5 ml., was put into the main compartment of the Warburg vessels, and 0.5 ml. substrate solution in the side bulb. At this stage the mixture contained 0.125 ml. *n*-propanol, 0.67 M. The respirometers were gassed, and incubated in the thermostat at 38° for 3 hr. to allow of complete destruction of the inhibitor. The substrate was then tipped, and the residual enzyme activity determined.

The extent to which enzyme activity in the presence of the substance being tested approached that of the control was a measure of the protection conferred. Since some of the substances examined were themselves slightly inhibitory, 'percentage protection' is defined as

$$100 \times \frac{(\text{percentage inhibition by Sarin}) - (\text{percentage inhibition by Sarin and compound})}{(\text{percentage inhibition by Sarin}) - (\text{percentage inhibition by compound})}$$

Solutions of the oxidation products of dopa were prepared using Ag₂O, by a method similar to that of Mason

(1948). The substances were not isolated, nor were their spectra checked. They were assumed to have the structure assigned by Mason, viz. a red solution, 1:2-dihydroindole-2-carboxylic acid-5:6-quinone; a colourless solution, 5:6-dihydroxyindole-2-carboxylic acid; and a purple solution, indole-2-carboxylic acid-5:6-quinone.

RESULTS

The effect of various compounds on the inhibition of horse-serum cholinesterase by Sarin

Of sixteen representative amino acids, only dopa was found to be able to protect cholinesterase and it did not inhibit of itself. Trials of several phenols and of two aliphatic polyhydroxy compounds, propan-1:2-diol and D-glucose, indicated that protection could be conferred by any catechol derivative, and that blocking of the hydroxy groups diminished the protective potency. Table 1 shows the results obtained with 10 mm concentrations of the compounds tested.

In these tests the initial Sarin concentration was about 20 μ M, so that there was an enormous excess of the test substance in the mixtures. The effect of diluting some of the more active substances is shown in Table 2, which shows that the protective action is mostly absent at a concentration of only 0.1 mm. Almost identical results were obtained using rat-brain dispersions.

No evidence could be obtained to suggest that dopa could reverse the inhibition caused by Sarin.

The action of dopa on other inhibitors

Our initial failure to repeat our work with Sarin was traced to the fact that we used water, not propanol, as solvent. It will be seen from Table 3 that for the most part propanol appears to confer protection against the inhibitors, since in its presence the *I*₅₀ (concentration of inhibitor giving 50% inhibition) is increased. This is probably

Table 1. *Effect of compounds related to 3:4-dihydroxyphenylalanine on the inhibition of horse serum cholinesterase by isopropyl methylphosphonofluoridate (Sarin)*

Percentage protection is defined in the text. Compounds tested at 10 mm.

Group 1. Compounds which inhibit so strongly as to mask any protective action:

1:2-Dihydroxynaphthalene, 2:3-dihydroxynaphthalene, *p*-benzoquinone.

Group 2. Compounds failing to protect:

Glycine, alanine, serine, threonine, lysine, arginine, histidine, tryptophan, hydroxyproline, glutamic acid, aspartic acid, cysteine, methionine, phenylalanine, tyrosine, propan-1:2-diol, D-glucose, *m*-aminophenol, 3:4-dimethoxyphenylalanine, adrenochrome.

Group 3. Protective action 15-30%:

Phenol, *m*-dihydroxybenzene, *o*-aminophenol, *p*-aminophenol, *o*-dihydroxybenzene monomethyl ether, *o*-dihydroxybenzene dimethyl ether, 3-methoxy-4-hydroxyphenylalanine.

Group 4. More than 70% protection:

p-Dihydroxybenzene, *o*-dihydroxybenzene, L- and DL-3:4-dihydroxyphenylalanine, β -(3:4-dihydroxyphenyl)- α -(tetrahydro-1':4'-thiazin-4'-yl)propionic acid 1':1'-dioxide, 2(3':4'-dihydroxyphenyl)-2-hydroxyethylmethylamine (adrenaline), 1:2:4-trihydroxybenzene.

associated with the finding that alcohols are competitive reversible inhibitors (Todrick, Fellowes & Rutland, 1951), and are able to block the approach of the organophosphorus compounds to the enzyme.

Table 4 shows that on increasing the concentration of Sarin, the protective effect of 10 mM dopa diminishes, and that the protection is more in the

Table 2. *Effect of various concentrations of some phenols on the inhibition of horse-serum cholinesterase by Sarin*

	Percentage protection at concentration of phenol of		
	10 mM	1 mM	0.1 mM
DL-dopa	100	60	0
L-dopa	100	69	0
L-Adrenaline	81	52	0
Catechol	84	63	0
Pyrogallol	100	66	18
Hydroquinone	106	36	0

Table 3. *Effect of inhibitor diluent on inhibition of serum and brain cholinesterase*

The I_{50} is the concentration of inhibitor giving 50% inhibition.

Inhibitor	$I_{50} \times 10^9$ M			
	Serum cholinesterase		Brain cholinesterase	
	Water	<i>n</i> -Propanol (0.67 M)	Water	<i>n</i> -Propanol (0.67 M)
Tabun	11	6.5	—	—
Sarin	4	21	4	6.3
Ethyl-Sarin	2	4	—	—
DFP	3	8.2	80	240

Table 5. *Effect of dopa and of inhibitor diluent on inhibition of serum cholinesterase by organophosphorus compounds*

Inhibitor	Diluent	$I_{50} \times 10^9$ M	Percentage inhibition in absence of dopa	Percentage protection by 10 mM dopa
Tabun	Water	12	42	33
		18	63	22
		13	67	81
Sarin	<i>n</i> -Propanol (0.67 M)	5	56	52
		23	56	75
		2.5	59	42
Ethyl-Sarin	<i>n</i> -Propanol (0.67 M)	5	59	83
		6.7	84	15
		11	59	37

Table 6. *Effect of dopa and of inhibitor diluent on inhibition of brain cholinesterase by organophosphorus compounds*

Inhibitor	Diluent	$I_{50} \times 10^9$ M	Percentage inhibition in absence of dopa	Percentage protection by 10 mM dopa
Tabun	Water	3.9	75	68
	<i>n</i> -Propanol (0.67 M)	9.7	72	89
Sarin	Water	3.2	37	72
	<i>n</i> -Propanol (0.67 M)	7.9	56	86
DFP	Water	160	86	34
	<i>n</i> -Propanol (0.67 M)	330	75	43

Table 4. *Effect of inhibitor diluent on the protection by dopa against inhibition of serum cholinesterase by Sarin*

Diluent	Sarin ($M \times 10^9$)	Percentage inhibition in absence of dopa	Percentage protection by 10 mM dopa
Water	5.0	56	52
	8.6	89	52
	54	95	41
	58	96	31
<i>n</i> -Propanol (0.67 M)	23	56	74
	36	88	98
	50	90	81
	54	86	86
	86	99	76
	180	99	50

presence of propanol, in spite of the greater concentration of Sarin used.

Tables 5 and 6 indicate that the protection against other inhibitors is quantitatively similar, and that propanol tends to improve the protection.

Studies on the mechanism of protection

Among the possibilities examined were, that a direct dopa-Sarin (or other inhibitor) reaction occurs, and that dopa was the precursor of a much more active molecule. Evidence in favour of the first possibility is given here.

Quantitative studies on the dopa-Sarin interaction. The protection test was modified so that the time allowed for a possible dopa-Sarin reaction could be varied from 0 to 40 min. at room temperature, before adding cholinesterase. The results are

Table 7. *Effect on protection of cholinesterase of varying the time of contact between dopa and Sarin before adding cholinesterase*

Cholinesterase activities are given as $v = \mu\text{l. CO}_2/30 \text{ min.}$ The regression coefficients in column 4 are the rates of increase of $v/\text{min.}$ of contact. Dopa concentration 0.7 mM. Initial Sarin concentration about 15 μM .

Conditions	No. of determinations	Mean activity at zero time	Regression coefficient and s.d.	P that regression coefficients are zero or identical
dopa + Sarin + cholinesterase	25	69.3	0.470 ± 0.105	0.001
Sarin + cholinesterase	15	39.3	0.098 ± 0.05	0.05-0.1
dopa + <i>n</i> -propanol (0.67 M) + cholinesterase	10	100.2 ± 4.91	$(100.1 \pm 6.51 \text{ at } 40 \text{ min.})$	—
Cholinesterase + <i>n</i> -propanol (0.67 M)	8	104.2 ± 1.5	—	—

Fig. 1. Test of the existence of a direct reaction between dopa and Sarin. For explanation see text p. 4.

expressed as the increase of observed enzyme activity with time of contact of Sarin with the reaction mixture before adding cholinesterase. As Table 7 shows, the activity of the controls was unaltered. The activity in the presence of Sarin only slowly increases, since the inhibitor is labile; but in the presence of dopa the increase is significantly greater, suggesting a progressive reaction between Sarin and dopa.

The results of experiments using Sarin concentrations between 10.5 and 35 μM and dopa between 0.1 and 5 mM, were applied to equation 5, above, as shown in Fig. 1. The experimental points were grouped around a straight line, indicating agreement with the hypothesis.

Protective effect of dopa oxidation products. Among the work carried out in an attempt to explain why protection could be observed only in the presence of relatively enormous concentrations of catechols, were some experiments designed to find out if traces of oxidation products, which might conceivably have arisen during the preliminary aerobic manipulations at slightly alkaline pH, were more active than dopa. Since none of them, whether

with the coloured solutions described above, or involving the use of a polyphenol oxidase preparation, yielded evidence suggesting the existence of a more active compound, these experiments will not be described in detail.

DISCUSSION

Our work was exploratory in character; and points about which our evidence was scanty or largely speculative have since been covered more fully by the work of others, notably Jandorf *et al.* (1952), who have provided the best possible existence of a reaction between catechol and diisopropyl phosphorochloridate, which is more reactive than DFP, by isolating the product. Their manometric studies of the reaction rates between DFP and various phenols have been paralleled by studies on Tabun by Augustinsson (1952). Our evidence of the relative protective activities of phenolic compounds against Sarin conforms broadly to the pattern shown by these studies, and our demonstration that dopa can protect against two other related inhibitors, suggests that this reaction between phenols and organophosphorus compounds may be a general one, although there is insufficient evidence as to the nature of the reactive group in organophosphorus compounds.

At one time we thought, on evidence which was largely speculative, that a partial explanation of the requirements for a very large excess of catechol was that an ion was involved. Attempts to produce a more active compound which had a larger ionic dissociation constant, e.g. nitrophenols, were unsuccessful because of the much greater inhibition of cholinesterase by the catechol which then resulted. Jandorf *et al.* (1952) have produced satisfactory evidence that it is the catecholate ion which reacts with DFP; it is reasonable, therefore, in view of the similarity of the *in vitro* enzyme studies, to assume that catecholate reacts with the other compounds studied.

Augustinsson (1952) seems to place some importance on the observation that adrenaline and noradrenaline can protect cholinesterase. However, our quantitative data show (Table 2) that adrenaline

cannot protect at or below 0.1 mM, and a single observation on adrenochrome, kindly given us by Dr Harley-Mason, shows that it is without protective effect. It is well known to pharmacologists that the concentration of adrenaline in tissues is seldom above 1 $\mu\text{g./g.}$ (about 5 μM) and is often nearer 1 $\mu\text{g./kg.}$ (5 μmM). Thus it seems extremely unlikely that mobilization of adrenaline could influence the inhibition of cholinesterase *in vivo*.

SUMMARY

1. The effect of sixteen amino acids on the inhibition of horse-serum cholinesterase by Sarin (*isopropyl P*-methylphosphonofluoridate) was examined. Only dopa (3:4-dihydroxyphenylalanine) was able, in high concentration, to abolish the inhibition *in vitro*.

2. It was found that catechol derivatives generally are able to protect cholinesterases *in vitro*. Both horse serum and rat brain cholinesterases are protected against Sarin and also against DFP (*diisopropyl* phosphorofluoridate), Tabun (*ethyl-NN*-dimethylphosphoramidocyanide)

date) and *P*-ethyl-Sarin (*isopropyl P*-ethylphosphonofluoridate).

3. Evidence is adduced to suggest that the basis of the protection mechanism is a reaction between catechol and inhibitor.

4. None of the oxidation products of dopa were found to have a greater activity than dopa itself.

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The Role of Glycerylphosphorylcholine and Glycerylphosphoryl-ethanolamine in Liver Phospholipid Metabolism

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The recent demonstration of the presence of free glycerylphosphorylcholine (Schmidt, Hecht, Fallot, Greenbaum & Thannhauser, 1952) and glycerylphosphorylethanolamine (Campbell & Work, 1952; Walker, 1952; Ansell & Norman, 1953) in aqueous extracts of animal tissues has suggested that these compounds may be intermediates in the metabolism of phosphatidylcholine and phosphatidylethanolamine. The immediate phosphorus-containing precursors of these two phospholipids during their synthesis in tissues are still unknown and it is important to know whether glycerylphosphorylcholine (GPC) or glycerylphosphorylethanolamine (GPE) could fulfil this role by reacting with activated fatty acids (Schmidt, Hershman & Thannhauser, 1945). Kornberg & Pricer (1953) have recently shown that an enzyme obtained from liver can cause two coenzyme A-activated fatty acids to combine with glycerophosphoric acid, producing a phosphatidic acid.

Experiments have therefore been made to measure the specific radioactivities of GPC and GPE in rat liver at various times after the injection of labelled phosphate. At the same time the specific radioactivities of liver phosphatidylethanolamine and phosphatidylcholine were measured using a recently devised method of investigating phospholipid labelling (Dawson, 1954*a*). A comparison of these isotopic results suggests that in liver neither GPC nor GPE can act as major phosphorus-containing precursors of the phospholipids. On the other hand, the specific radioactivity of phosphatidylcholine is consistent with the assumption that it is the precursor of GPC, and similarly phosphatidylethanolamine fulfils the criteria for being the precursor of GPE. This would suggest that in liver *in vivo* these water-soluble phosphate esters are present solely as intermediates in the catabolism of the liver phospholipids.

A preliminary report of these results has already been presented to the Biochemical Society (Dawson, 1954*b*).

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