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Enzymatic Hydrolysis of Organophosphorus Compounds

IV. Specificity Studies

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The enzymatic hydrolysis of a series of organophosphorus compounds was studied with rabbit plasma and a purified preparation (Fraction IV—1) from human serum as enzyme sources. Most of the compounds studied are split by these enzymes.

Amongst the homologues of tabun, the methoxy compound is split at the highest rate. Amongst the analogues of this compound, the chloride is hydrolysed at a higher rate than the fluoride and the cyanide.

By summation experiments it was shown that the phosphorylphosphatase ("tabunase") of both rabbit plasma and Fraction IV—1 splits tabun, DFP, and mintacol; the latter compound is probably also split by other enzymes. TEPP is hydrolysed by a separate enzyme or enzymes present in these enzyme preparations.

In a previous paper [1] the existence of an enzyme (phosphorylphosphatase, "tabunase") catalysing the hydrolytic breakdown of dimethylamido-ethoxy-phosphoryl cyanide (tabun) was reported. Some properties of the enzyme were also described [2]. A third paper [3] discussed the effect of reversible cholinesterase inhibitors on phosphorylphosphatase and the significance of considering the presence of this enzyme in studies on tabun as a cholinesterase inhibitor. In the present paper the specificity of phosphorylphosphatase is demonstrated.

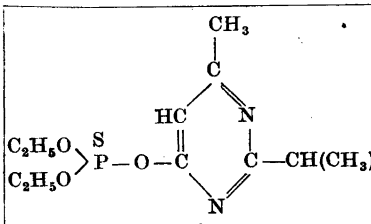
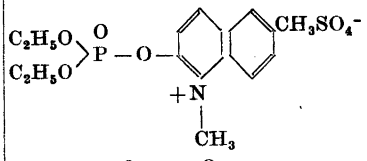
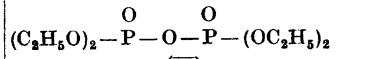
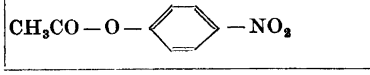
METHODS AND MATERIAL

The Warburg manometric technique was used to follow the enzymatic hydrolysis of various organophosphorus compounds [1]. Rabbit plasma and Fraction IV—1 prepared from human postpartum serum were used as enzyme sources, except as otherwise stated.

The organophosphorus compounds used as substrates are tabulated in Table 1. They were all of high purity, except as otherwise stated. The synthesis and properties of the compounds containing nitrogen were described by Holmstedt [4]. They were, in addition to DFP, TEPP and mintacol, synthesized at the Research Institute of National Defence of Sweden. Chlorothion [5], systox and isosystox [6] were synthesized by Schrader. Malathion and diazinon were commercial products (Geigy, A. G., Basel). Ro3—0422 was prepared

Table 1. Enzymatic hydrolysis of various organophosphorus compounds by rabbit plasma (0.1 ml) and Fraction IV—1 (10 mg) from human serum. Substrate concentration, 5.32 × 10^{-3} M, except for malathion and diazinon (0.2 vol. %), and for chlorothion, systox and isosystox (suspensions). Activity expressed in b_{30} values.

Formula	Name or code	Mol. wt.	Density g/ml 20° C	Spont. hydrol.	Rabbit plasma	Fraction IV—1
$(CH_3)_2N$, C_2H_5O > P(O)—CN	tabun	162.13	1.077	10	315	170
$(CH_3)_2N$, CH_3O > P(O)—CN	dimethylamido-methoxy-phosphoryl cyanide	148.11	1.12	12	≈400	≈450
$(CH_3)_2N$, $(CH_3)_2CHO$ > P(O)—CN	dimethylamido-*iso*propoxy-phosphoryl cyanide	176.16	1.054	11	62	78.5
$(CH_3)_2N$, C_2H_5O > P(O)—Cl	dimethylamido-ethoxy-phosphoryl-chloride	171.57	1.188	very rapid	?	?
$(CH_3)_2N$, CH_3O > P(O)—F	dimethylamido-methoxy-phosphoryl fluoride	141.09	1.155	2	60	25.5
$(CH_3)_2N$, $(CH_3)_2N$ > P(O)—Cl	bis(dimethylamido)-phosphoryl chloride	170.59	1.198	0	—	0
$(CH_3)_2N$, $(CH_3)_2N$ > P(O)—F	bis(dimethylamido)-phosphoryl fluoride	154.10	1.115	0	—	0
$(CH_3)_2CHO$, $(CH_3)_2CHO$ > P(O)—F	DFP	184.15	1.070	1	179	132
C_2H_5O, C_2H_5O > P(O)—O—C$_6$H$_4$—NO_2	mintacol, paraoxon	275.20	1.265	1	208	39
CH_3O, CH_3O > P(S)—O—C$_6$H$_3$Cl—NO_2	chlorothion	297.66	1.433	2	0	0
C_2H_5O, C_2H_5O > P(S)—O—CH_2—CH_2—S—C_2H_5	systox	258.34	1.119	5	0	0
C_2H_5O, C_2H_5O > P(O)—S—CH_2—CH_2—S—C_2H_5	isosystox	258.34	1.132	3	0	0
CH_3O, CH_3O > P(S)—S—CH(CO—O—C_2H_5)—CH_2—CO—O—C_2H_5	malathion	330.36	technical product	3	0	0

	diazinon	304.35	technical product	3	0	0
	Ro 3—0422	406.37		6	75	138
	TEPP	290.20	21.185	12	331	43
	*p*-nitrophenyl acetate (PNPA)	181.14		3	≈370	≈650

by Roche Products, Ltd., England [7]. The synthesis of *p*-nitrophenyl acetate was carried out according to the method described by Huggins and Lapides [8]. The substrates were dissolved in water and their concentrations were in most experiments 5.32×10^{-3} *M* (total volume of the reaction mixture, 2.00 ml).

RESULTS

The enzymatic hydrolysis of various organophosphorus compounds. A series of organophosphorus compounds was tested for their enzymatic hydrolysis by the rabbit plasma and by partly purified phosphorylphosphatase (Fraction IV—1) from human serum. The results are recorded as b_{30} values (initial enzymatic hydrolysis rates) in Table 1 and Fig. 1. Amongst the homologues of tabun, the methoxy compound is split at a higher rate, the *iso*-propoxy compound at a lower rate than tabun. The chloride analogue (dimethylamido-ethoxy-phosphoryl chloride) of tabun is split at a very high, enzymatically not measurable, rate; the spontaneous hydrolysis of this compound in water is likewise very rapid. The dimethylamido-methoxy-phosphoryl fluoride is enzymatically hydrolysed by both enzymes studied at a lower rate than the corresponding cyanide and chloride. From these preliminary studies with amido-substituted alkoxy-phosphoryl compounds, we conclude that the chlorides are enzymatically (and also spontaneously) hydrolysed at a higher rate than the cyanides and these latter derivatives at a higher rate than the fluorides.

The enzymes studied do not split the substituted diamidophosphoryl halogenides.

The three well-known compounds, DFP, TEPP and mintacol were all found to be split by the rabbit plasma and Fraction IV—1. DFP behaves to these two enzyme sources in roughly the same way as do tabun and its analogues. TEPP and mintacol, on the other hand, are hydrolysed at much higher rates by the rabbit plasma compared with the rates found for the Fraction IV—1.

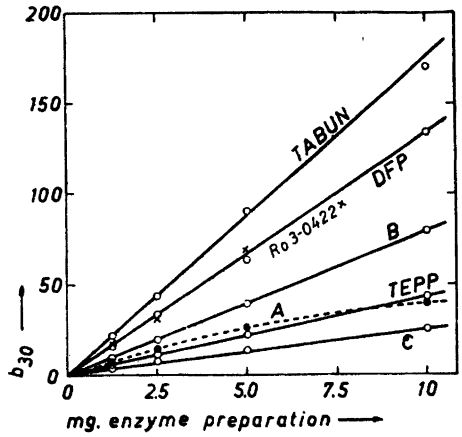


Fig. 1. Enzymatic hydrolysis of various organophosphorus compounds by a purified preparation (Fraction IV—1) of phosphorylphosphatase from human serum. Enzymatic hydrolysis (b_{30}) plotted against enzyme concentration (mg of Fraction IV—1 in the reaction mixture, total volume 2.00 ml). Substrate concentration, 5.32 × 10^{-3} M, except in the case of mintacol which was used in saturated solution. A: dimethylamido-methoxy-phosphoryl cyanide; B: dimethylamido-iso-propoxy-phosphoryl cyanide; C: dimethylamido-methoxy-phosphoryl fluoride.

Ro 3-0422 (diethyl-3-quinolylmethyl-phosphate methylsulphate) is split at a comparatively high rate by Fraction IV—1 and relative to that, at a lower rate by rabbit plasma. Its spontaneous hydrolysis is appreciable in aqueous solution; a 2.66 × 10^{-2} *M* solution is broken down 38 % after 40 minutes at room temperature.

None of a series of compounds recently introduced as insecticides, *viz.* chlorothion, systox, isosystox, malathion, diazinon, were found to be attacked enzymatically. They are all sparingly soluble in water and were therefore used as aqueous suspensions.

Table 2. Enzymatic hydrolysis of tabun, TEPP, and mintacol by various fractions of human postpartum serum. Fractionation carried out by a partly modified (Kabi) method No. 6 of Cohn et al. *Fraction IV-1 was obtained from various fractionation procedures.*

Fraction	b_{30}/10 mg protein		
	tabun	TEPP	mintacol
Original serum	40	13	11
I	8	5	1
II (γ-globulin)	2	9	0
III (β-globulin)	1	4	0
IV-1	135—188	27—43	31—43
IV-4	3	7	0
IV—5 + 6	2	3	0
IV—6 + 9	0	0	0
V (albumin)	2	9	0

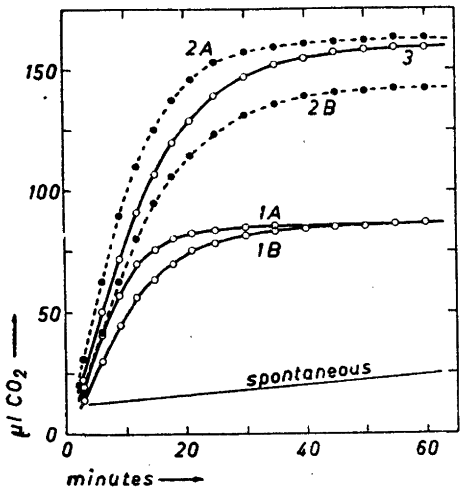


Fig. 2. Enzymatic hydrolysis of tabun and mintacol and a mixture of these substrates by rabbit plasma (0.1 ml). The spontaneous hydrolysis curve refers to a mixture of tabun and mintacol, the concentrations of which are those of the curves 1A and 1B.

Curve	Substrate	$M \times 10^{-3}$	b_{30}
1A	tabun	2.66	175
1B	mintacol	2.66	150
2A	tabun	5.32	290
2B	mintacol	5.32	210
3	tabun + mintacol	2.66 of each	230

In confirmation with the results obtained by other authors (*cf.* below), *p*-nitrophenyl acetate (PNPA) was split by the rabbit plasma. This ester is hydrolysed at a comparatively high rate by Fraction IV—1.

In Fig. 1 the enzymatic hydrolysis rates of some organophosphorus compounds studied are plotted against the concentration of a purified preparation (Fraction IV—1) of phosphorylphosphatase from human serum. In most cases, where the enzyme concentration is not too high, hydrolysis rate is proportional to enzyme concentration.

The phosphorylphosphatase activities of the various fractions of human serum were tested with tabun, TEPP, and mintacol as substrates. As was described in the first paper of this series [1], tabun is almost exclusively split by the Fraction IV—1. These results are compared with those obtained with TEPP and mintacol (Table 2). Mintacol is hydrolysed only by the Fraction IV—1 which splits tabun. TEPP also is destroyed at the highest rate by this fraction, but almost all fractions studied attack this compound at low rates; especially noticeable are the hydrolysis rates for the Fractions II and V. From these results we conclude that the hydrolysis of TEPP by human blood serum is catalysed by at least three factors; one of these is present in the same Fraction IV—1 as the enzyme(s) which hydrolyse(s) tabun and mintacol.

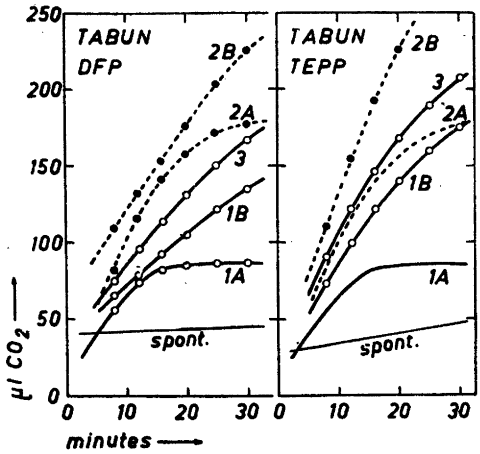


Fig. 3. Enzymatic hydrolysis of tabun mixed with DFP and TEPP. Enzyme: rabbit plasma (0.1 ml). The spontaneous hydrolysis curves refer to the mixtures of substrates.

Curve	Substrate	$M \times 10^{-3}$	b_{30}	μl CO_2 found at total hydrolysis
1A	tabun	2.66	172	86
1B	DFP	2.66	100	195
	TEPP		200	230
2A	tabun	5.32	270	190
2B	DFP	5.32	180	380
	TEPP		330	440
3	tabun + DFP	2.66 of each	120	235
	tabun + TEPP		280	300

Summation experiments. A comparison between the hydrolysis rate, when an enzyme preparation is acting upon a mixture of substrates, with the rates when the substrates are hydrolysed separately, has been frequently used in enzyme specificity studies. Although the results might be misleading in some special cases, as has been pointed out recently by Goldstein [9], they can generally be used as criteria of specificity. Such summation experiments were carried out in the present study as preliminary tests for the presence of phosphorylphosphatases in the rabbit plasma and a purified preparation of human serum (Fraction IV—1).

Figs. 2 and 3 record the results obtained with the rabbit plasma. The experiments performed with tabun and mintacol (Fig. 2) show that there are probably two enzymes present, each splitting each of the substrates. Therefore, the enzyme hydrolysing mintacol, called A-esterase by Aldridge [10], is not identical with the "tabunase" (phosphorylphosphatase) reported recently by the present authors [1]. The possibility remains that the A-esterase of the rabbit plasma also splits tabun or that "tabunase" alsc splits mintacol but no further studies have as yet been performed to elucidate this question (*cf.* below).

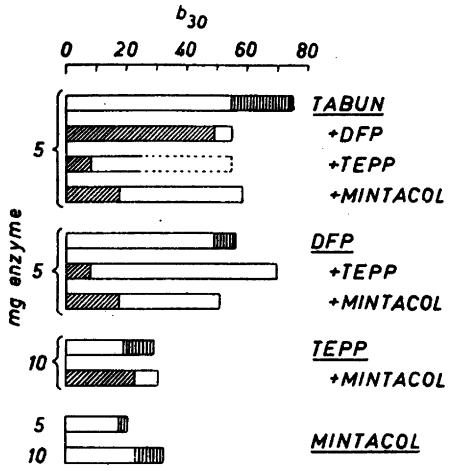


Fig. 4. Summation experiments with Fraction IV—1. Substrate concentrations, 2.66×10^{-3} M; hydrolysis rates, when the concentration of the substrate is twice that value, are marked by vertical shading. Oblique shading marks the enzymatic hydrolysis of the second substrate when present alone.

The results obtained with DFP and TEPP when these substrates are mixed with tabun, are illustrated in Fig. 3. They show that DFP is probably split by the same enzyme as is tabun. There is no additive effect in this case. TEPP, on the other hand, is probably not split by the "tabunase". The effect is not absolutely additive, but the higher hydrolysis rate for the substrate mixture compared with those obtained with separate substrates (tabun and TEPP) is significant. There may be a mutual inhibition by the substrates or their reaction products.

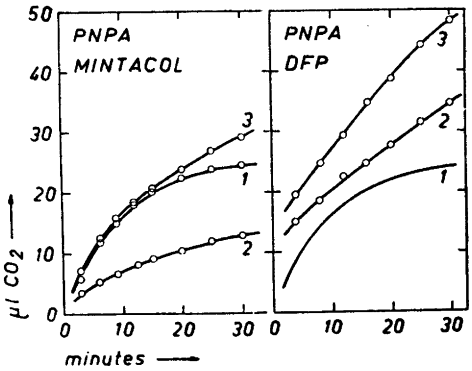


Fig. 5. Enzymatic hydrolysis of p*-nitrophenyl acetate (PNPA) mixed with mintacol and DFP; Fraction IV—1 (2.5 mg per reaction mixture).*
1: PNPA; 2: mintacol or DFP; 3: (PNPA + mintacol) or (PNPA + DFP).

The results in summation experiments with the Fraction IV—1 are illustrated in Fig. 4. They show that the same enzyme in this preparation splits tabun, DFP and mintacol. TEPP, on the other hand, is most probably hydrolysed by a second enzyme. This is demonstrated with all certainty by the DFP-TEPP mixture. The experiments performed with tabun and TEPP are not as conclusive, due to the rapid decline in reaction velocity during the hydrolysis of a mixture of these substrates. There is probably an interaction between the substrates and the two separate enzymes supposed to be present. A similar situation exists for the TEPP-mintacol mixture.

It was demonstrated by Aldridge [10] that the A-esterase of the rabbit plasma splits both mintacol and *p*-nitrophenyl acetate. In summation experiments performed with Fraction IV—1 (Fig. 5), the initial hydrolysis rates obtained show that DFP is probably split by an other enzyme than is *p*-nitrophenyl acetate (PNPA). In the case of mintacol, it is surmised that this substrate is hydrolysed both by an enzyme, which corresponds to the A-esterase and by a phosphorylphosphatase ("tabunase", "DFP-ase").

DISCUSSION

The ability of rabbit plasma and partly purified phosphorylphosphatase from human serum to hydrolyse a series of organophosphorus compounds has been demonstrated. Analogues (halogenides) and homologues of tabun (dimethylamido-ethoxy-phosphoryl cyanide) are all hydrolysed by these enzyme preparations at various rates. The bis(dimethylamido)-phosphoryl derivatives are not attacked, which is of interest, as these compounds are known to undergo another transformation (probably an oxidation) in the organism, to highly active cholinesterase inhibitors. Whether these products are hydrolysed by the phosphorylphosphatase has not been studied. The dialkoxyphosphoryl halogenides are probably split by the same enzyme as tabun, demonstrated in the present paper in experiments with DFP. TEPP, on the other hand, is hydrolysed by an enzyme separate from "tabunase".

It was recently demonstrated by Mounter and Whittaker [11] that human blood plasma contains an "aromatic esterase" hydrolysing phenyl esters. This enzyme was suggested to be probably indentical with the mintacol-insensitive esterase, the so-called A-esterase of Aldridge [10]. The results obtained in the present study allow us to suggest that mintacol is attacked not only by the A-esterase, but also by a phosphorylphosphatase, both these enzymes being present in Fraction IV—1 of human serum. Tabun and DFP are probably not hydrolysed by the A-esterase. Neither of these enzymes appear to hydrolyse TEPP.

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