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THE REACTION OF ORGANOPHOSPHORUS COMPOUNDS WITH HYDROLYTIC ENZYMES—II.

THE INHIBITION OF CITRUS ACETYLESTERASE

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Abstract—A purification method, using acetone precipitation, and a simple colorimetric assay method for citrus acetylesterase, using 2,6-dichlorobenzenone indophenyl-acetate as a substrate are described.

Reaction rate constants have been determined of the inhibition of citrus acetylesterase at pH 7·5 and 25° by a number of organophosphorus compounds of the general formula R′R″. P(O)X. There is a correlation between the rate of enzyme inhibition and the strength of the P—X bond as expressed by alkaline hydrolysis and pK_a values of HX.

The influence of the structure of the groups R′ and R″ on the rate of inhibition is parallel to that of the reactivity measured as the rate of alkaline hydrolysis but a number of exceptions occur. In a series of alkyl *p*-nitrophenyl methylphosphonates the rate of enzyine inhibition increases when the alkyl chain is lengthened whereas the rate of the alkaline hydrolysis decreases. In one case the influence of the temperature on the reaction rate has been investigated. It seems that the rapid reaction of the compound with the enzyme, compared with alkaline hydrolysis is caused by a lowering of the activation enthalpy rather than by an increase of the activation entropy.

In a number of cases the influence of the pH on the inhibition rate was studied. This influence was found to be rather small.

INTRODUCTION

FROM the investigations of Jansen *et al.*[1, 2] it is known that citrus acetylesterase (acetic ester acetyl-hydrolase EC 3.1.1.6) is inhibited by di-isopropyl phosphorofluoridate (DFP) and by tetra-ethyl pyrophosphate (TEPP). The inhibition proved to be "irreversible" and the inhibition reaction was bimolecular.

Substrates could protect the enzyme against inhibition by the organophosphorus compounds; hence the point of attack of the inhibitor must involve the site of attachment of the enzyme to the substrate. In a previous publication we described a study on structure-activity relations in the case of the inhibition of horse liver aliesterase (carboxylic ester hydrolase EC 3.1.1.1) by a variety of organophosphorus compounds.[3] As virtually nothing is known about the influence of the structure of the inhibitor molecules on the rate of inactivation of acetylesterase we decided to carry out a similar investigation as described for the aliesterase with the citrus acetylesterase.

As a measure of the inhibition rate we have again chosen the bimolecular rate constant rather than the I_{50} for reasons stated in our previous publication.[3]

EXPERIMENTAL

Enzyme preparation

The acetylesterase used was obtained from the outer coloured layer (flavedo) of orange rinds. At first we followed the purification method described by Jansen *et al.*[1] based on extraction by pressing, precipitation with ammonium sulphate and dialysis. In our hands however, the product obtained had a much lower specific activity (2·7 units/mg N as against the published value of 18 units/mg N)*. It also had a very deep brown colour, which interferes in the colorimetric assay method (see below). We tried therefore to improve the purification method in order to obtain a colourless product and to raise the specific activity. The rinds of citrus fruits are minced in a meat grinder. Four hundred grams of the pulp are then mixed with 350 g of acid washed sand.† The mixture is pressed through cheese cloth with a Carver Laboratory Press with a pressure of 12,000 lb/in^2. To the press-juice is added 6·8 g/l. sodium oxalate (0·2 S). The solution is then filtered to eliminate insoluble matter and etherial oils. To the filtrate is added 243 g/l. ammonium sulphate (0·4 S). After 30–60 min the liquid is filtered through a Büchner funnel.

The precipitate is taken up in distilled water ($\sim$ 1/10th of the original volume). The solution is brought to pH 7·0 and dialyzed in the cold until negative reaction to sulphate. To the dialyzed solution is added ammonium sulphate (114g/l., 0·2 S). The (inactive) precipitate was discarded after 10 min centrifugation (0°, 16000 *g*). To the clear supernatant, brought to $-5°$ is added an equal volume of cold acetone with vigorous stirring. After the addition of the acetone, the mixture is left during 1 hr at $-5°$ without stirring.

The solution separates into two layers, a light brown coloured upper layer and a colourless lower layer. The precipitate is found at the interphase. The mixture is filtered through a Büchner funnel with a thin (1–2 mm) layer of Hyflo. The precipitate is dissolved in 60 ml of water for each litre of starting product for step 2. The solution is brought to pH 7·0 and the Hyflo filtered off.

The solution is then dialyzed in the cold against distilled water until negative reaction to sulphate. The final product is kept frozen at $-15°$ and is thus stable for at least one year. The slightly yellow final preparation had a specific activity of 14 units/mg N using 0·23 M($\pm$ 5%) triacetin as a substrate and at pH 6·5.

Nitrogen was determined using the semi-micro Kjeldahl method.

Organophosphorus compounds. The same compounds as used in our previous investigations were used[3] (see Tables 5–8).

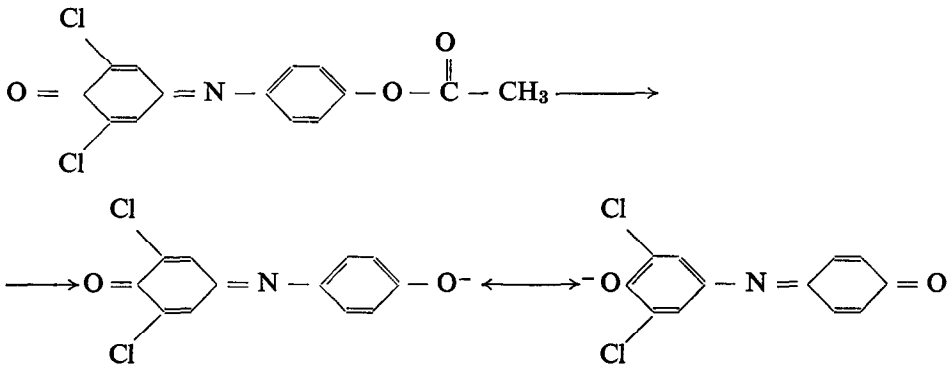
Substrates. Glycerol triacetate (triacetin) was obtained from British Drug Houses Ltd.; 2,6-dichlorobenzenone indophenylacetate (DIBIA) was synthesized according to de Borst *et al.*[4]

Enzyme assay method. For standardisation purposes we used the titrimetric method with triacetin as a substrate and in the presence of 0·15 M sodium chloride as described by Jansen *et al*,[1] making use of a Radiometer Titrigraph SBR_2/SBU_1. This method, however, is unsuitable for a series of successive determinations to be carried out during kinetic measurements. Jansen *et al.*[2] introduced a colorimetric assay method for acetylesterase using *o*-nitrophenyl acetate as a substrate. This substrate has a

* In accordance to the proposal of the International Union of Biochemistry all enzyme activity units are expressed as μ moles substrate hydrolyzed per minute.

† British Drug Houses Ltd.

much higher affinity for the enzyme than triacetin (K_m resp. $1{\cdot}2 \times 10^{-4}$ M and 3×10^{-2} M)[1, 5] and is therefore in a better position to compete with the inhibitors for the active site of the enzyme, a necessary condition for our measurements. However *o*-nitrophenylacetate showed a poor stability in aqueous solutions. During an investigation of the properties of the indophenyl acetates we observed that 2,6-dichloro benzenone indophenyl acetate (DIBIA) was a good substrate for acetylesterase, whereas the isomeric benzenone-indo 3'5'-dichloro phenylacetate was hydrolyzed much slower. This is in contrast with the results obtained with aliesterase.[3] Moreover the affinity of DIBIA for the enzyme proved to be even higher than that of *o*-nitrophenyl acetate ($5{\cdot}4 \times 10^{-5}$ M).[5]



The ester has a red colour whereas the phenolate has an intense blue colour with an absorption maximum at 605 mμ.[6]

The procedure is as follows. In a test tube are added 10 ml 0·05 M phosphate buffer pH 7·5; 0·2 ml of a solution of 2,6-dichlorobenzenone indophenylacetate in ethanol (20 mg/25 ml) and 1 ml enzyme solution. The mixture is kept for 30 min in a constant temperature bath at 25·0°. The blue colour is measured at 605mμ in a colorimeter. Under these conditions 0·01 (triacetin) unit of enzyme gave an extinction of approximately 0·50 and the extinction was proportional to the amount of enzyme in the 0·001–0·02 unit range. After we had proved that the same enzyme is responsible for the hydrolysis of triacetin and DIBIA (see below) we used the colorimetric method in all our kinetic experiments.

Inhibition rate measurements

The acetylesterase preparation is diluted with 0·067 M veronal buffer pH 7·5 so that the activity is approximately 0·02 units/ml. Three millilitres of the enzyme solution are mixed in a test tube with 3 ml of the dilution of the organophosphorus compound, both solutions being prewarmed to 25·0°. From this mixture 1 ml is pipetted into the reagent medium (see above) after 1, 2, 3, 4 and 5 min reaction time. The remaining enzyme is then assayed with the colorimetric method and the percentage remaining enzyme activity is calculated. The dilution, together with the presence of a substrate with a high affinity for the enzyme stopped the inhibition reaction as has been shown in a number of cases.

Under the described conditions the concentration of the enzyme in the enzyme–inhibitor mixture is $5{\cdot}6 \times 10^{-10}$ M. As in all cases investigated the concentration of

the inhibitor was at least 1000 times higher than the enzyme concentration; the rate constants could be calculated using the first order kinetics.[3]

In those cases where we investigated the influence of the temperature and of the pH on the rate of inhibition (see Tables 9 and 10), only the inhibition was performed at different temperatures and at different pH values; the enzyme assay was always carried out at 25° and pH 7·5.

RESULTS

The identity of the enzyme hydrolyzing triacetin and DIBIA

We determined the activity ratio of acetylesterase preparations of different purities, both with triacetin and with DIBIA as substrates (Table 1).

Table 1. Ratio of activities of a number of acetylesterase preparations with triacetin and with 2,6-dichlorobenzenone-indophenylacetate as substrate

Preparation	Purification Step	U/ml (triacetin)	U/ml (DIBIA)	$\frac{U_{triacetin}}{U_{DIBIA}}$
XXV	press juice	0·11	0·07	1·69
IV_2	precip. 0·3 S ammonium sulphate	0·47	0·26	1·80
A_2	precip. 0·5 S ammonium sulphate	1·17	0·69	1·71
C	acetone precipitate	0·87	0·52	1·69

Next to that, an acetylesterase preparation was inhibited by sarin. The inhibition percentage was determined both with triacetin and with DIBIA as a substrate (see Table 2).

Table 2. Inhibition of acetylesterase by sarin, determined with triacetin and with 2,6-dichlorobenzenone indophenylacetate as substrate. Inhibition carried out at pH 7·5 and 25°

Concentration sarin	Time of inhibition	% inhibition (triacetin)	% inhibition (DIBIA)
$8{\cdot}93 \times 10^{-8}$ M	20 min	16	13
$2{\cdot}23 \times 10^{-7}$ M	20 min	28	30
$4{\cdot}41 \times 10^{-7}$ M	27 min	38	40
$6{\cdot}70 \times 10^{-7}$ M	20 min	40	36
$8{\cdot}93 \times 10^{-7}$ M	20 min	45	45

From the results shown in Tables 1 and 2 we can conclude that triacetin and DIBIA are hydrolyzed by the same enzyme.

The enzyme concentration

In the literature no value of the "turn over number" of acetylesterase is given from which the enzyme concentration can be calculated. (As in the case of aliesterase[3] we use the expression "enzyme concentration" for concentration of active sites.) We therefore determined the concentration by measuring the amount of phosphorus that was bound to the enzyme after inhibition by sarin. For this purpose acetylesterase

preparations were incubated with ^{32}P labelled sarin in a concentration of 4×10^{-2} M at pH 7·5 and 20° during 14 hr. Total inhibition was observed in all cases. The inhibited preparations were dialyzed against water until the amount of ^{32}P within the dialysis bags was constant. The results of this experiment are collected in Table 3.

TABLE 3. THE BINDING OF PHOSPHORUS ON THE INHIBITION OF ACETYLESTERASE WITH SARIN ^{32}P

Preparation	Nitrogen (mg/ml)	Units of activity (triacetin)	Amount of protein nitrogen per bound g atom P (g atoms)	Amount of protein per bound g atom P, (g)
1	0·020	2·6	$1·8 \times 10^5$	$1·7 \times 10^7$
2	0·015	2·0	$2·0 \times 10^5$	$1·9 \times 10^7$
3	0·010	1·3	$2·1 \times 10^5$	$2·0 \times 10^7$

The small amount of phosphorus which is bound to the protein suggests that the reaction of sarin with acetylesterase is very specific. If we assume that inhibition is caused by the binding of one atom of phosphorus we can calculate the following concentration identity

$$1 \text{ U/litre} \equiv 5·6 \times 10^{-11} \text{ M}.$$

From this result we can calculate the enzyme concentration under the conditions of the kinetic experiments: $5·6 \times 10^{-10}$ M. As has been stated before, the concentration of inhibitors we had to add to the enzyme in order to obtain measurable inhibition within reasonable times was at least 1000 times the enzyme concentration and we do not need the value of the enzyme concentration for the determination of the rate constants.

Reactivation of inhibited enzyme

It is known from the literature that enzymes, inactivated by organophosphorus compounds can be reactivated by nucleophilic reagents.[7] Although Jansen *et al.*[2] could demonstrate a slight spontaneous reactivation after inhibition with TEPP, it was not known if nucleophilic reagents could reactivate the inhibited enzyme. We therefore studied the reactivation of sarin inhibited acetylesterase by monoisonitrosoacetone (MINA). 1·7 Units of enzyme were incubated during 30 min with $2·14 \times 10^{-4}$ M sarin. The excess of inhibitor was removed by four washings with peroxide free ether.

TABLE 4. REACTIVATION OF ACETYLESTERASE, INHIBITED BY SARIN, BY MONO-ISONITROSOACETONE

Incubation time with 0·1 M MINA (days)	Reactivation in percentages		
	MINA immediately	MINA after 24 hr	No MINA
1	52	52	0
2	51	56	0
3	55	58	0
4	58	58	0
6	65	64	0

TABLE 5. RATE CONSTANTS (1 $mol^{-1}min^{-1}$) OF THE REACTION OF ACETYLESTERASE WITH A NUMBER OF *p*-NITROPHENYL COMPOUNDS

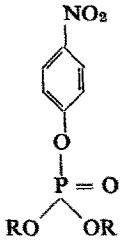
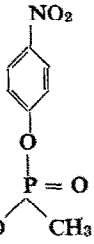
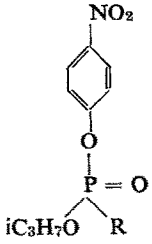
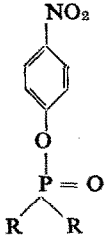
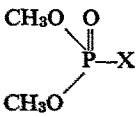
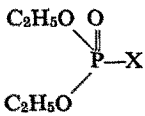
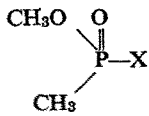
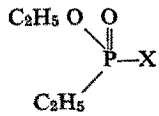
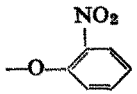
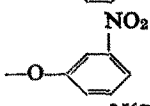
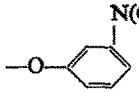
R				
CH_3	$7{\cdot}1 \times 10^{2}$	$6{\cdot}0 \times 10^{3}$	$1{\cdot}6 \times 10^{3}$	—
C_2H_5	$5{\cdot}3 \times 10^{2}$	$7{\cdot}9 \times 10^{3}$	$8{\cdot}5 \times 10^{2}$	$2{\cdot}8 \times 10^{3}$
nC_3H_7	$8{\cdot}5 \times 10^{1}$	$6{\cdot}4 \times 10^{3}$	$1{\cdot}9 \times 10^{2}$	$6{\cdot}5 \times 10^{2}$
iC_3H_7	$1{\cdot}6 \times 10^{0}$	$1{\cdot}6 \times 10^{3}$	$6{\cdot}2 \times 10^{0}$	$1{\cdot}1 \times 10^{0}$
nC_4H_9	$5{\cdot}4 \times 10^{1}$	$1{\cdot}5 \times 10^{4}$	$2{\cdot}7 \times 10^{1}$	$9{\cdot}2 \times 10^{0}$
iC_4H_9	$2{\cdot}7 \times 10^{0}$	$2{\cdot}4 \times 10^{3}$	$4{\cdot}3 \times 10^{0}$	$2{\cdot}1 \times 10^{0}$
sec C_4H_9	$1{\cdot}2 \times 10^{0}$	$2{\cdot}3 \times 10^{2}$	$3{\cdot}2 \times 10^{0}$	$5{\cdot}9 \times 10^{-1}$
tert C_4H_9	—	—	$<<10^{0}$	—
nC_5H_{11}	$1{\cdot}4 \times 10^{1}$	$1{\cdot}8 \times 10^{5}$	$8{\cdot}3 \times 10^{1}$	$3{\cdot}1 \times 10$
iC_5H_{11}	—	—	—	$<10^{-1}$
nC_6H_{13}	—	—	—	$<10^{-1}$
	—	$5{\cdot}7 \times 10^{2}$	—	—
$(CH_3)_3$ C—CH(CH_3)	—	$1{\cdot}1 \times 10^{1}$	—	—

TABLE 6. RATE CONSTANTS (1 $mol^{-1}min^{-1}$) OF THE REACTION OF ACETYLESTERASE WITH A NUMBER OF SUBSTITUTED PHENYL COMPOUNDS

				
	$7{\cdot}1 \times 10^{2}$	$5{\cdot}3 \times 10^{2}$	$6{\cdot}0 \times 10^{3}$	$1{\cdot}6 \times 10^{4}$
	—	$6{\cdot}4 \times 10^{3}$	—	$9{\cdot}4 \times 10^{4}$
	$7{\cdot}4 \times 10^{2}$	$2{\cdot}6 \times 10^{2}$	$6{\cdot}5 \times 10^{3}$	$1{\cdot}7 \times 10^{4}$
	$1{\cdot}5 \times 10^{1}$	$4{\cdot}7 \times 10^{1}$	$1{\cdot}8 \times 10^{2}$	$1{\cdot}7 \times 10^{2}$
	$<10^{0}$	$1{\cdot}6 \times 10^{1}$	$6{\cdot}9 \times 10^{1}$	$6{\cdot}0 \times 10^{-1}$

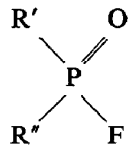
The solution obtained was divided into three parts. To the first part MINA was added to a final concentration of 0·1 M. To the second part MINA was added also, but only after 24 hr at room temperature. To the third part no MINA was added. We also added MINA to an untreated acetylesterase sample to correct for a slight inhibition of the enzyme. The pH of incubation with MINA was 6·5. The enzymatic activities were determined from time to time. The results are shown in Table 4.

The results from Table 4 indicate that the enzyme is reactivated to about 50 per cent within the first 24 hr and that thereafter only a slight increase in reactivation is observed. There is virtually no spontaneous reactivation and the phenomenon of "ageing" which is observed with the cholinesterases[8] is absent in the case of acetylesterase.

The rate of reaction of acetylesterase with organophosphorus compounds

The results of the kinetic experiments are listed in the Tables 5–8. Each value is the mean of at least three values obtained with different inhibitor concentrations, the error being of the order of ± 5 per cent. The influence of the temperature on the inhibition rate of acetylesterase by DFP is given in table 9 and the influence of the pH on the inhibition rate in Table 10.

TABLE 7. RATE CONSTANTS (1 $mol^{-1}min^{-1}$) OF THE REACTION OF ACETYLESTERASE WITH A NUMBER OF PHOSPHORO- AND PHOSPHONOFLUORIDATES



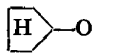
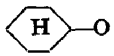
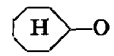
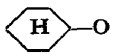
R'	R"	k	R'	R"	k
CH_3O	CH_3O	$4·0 \times 10^4$	CH_3	$(CH_3)_2CH—$ $CH_2—CH(CH_3)O$	$4·0 \times 10^4$
C_2H_5O	C_2H_5O	$1·9 \times 10^4$	CH_3		$3·3 \times 10^4$
nC_3H_7O	nC_3H_7O	$1·3 \times 10^3$	CH_3		$1·8 \times 10^5$
iC_3H_7O	iC_3H_7O	$2·6 \times 10^1$	CH_3		$3·5 \times 10^5$
CH_3	iC_3H_7O	$4·1 \times 10^3$	C_2H_5	C_2H_5O	$7·0 \times 10^3$
CH_3	$(CH_3)_2CH—$ $—CH—(CH_3)O$	$1·2 \times 10^4$	C_2H_5		$3·9 \times 10^5$
CH_3	$(CH_3)_3C—$ $—CH(CH_3)O$	$5·7 \times 10^2$	C_2H_5O	$N(CH_3)_2$	$3·7 \times 10^2$

TABLE 8. RATE CONSTANTS (1 $mol^{-1}min^{-1}$) OF THE REACTION OF ACETYLESTERASE WITH SOME MISCELLANEOUS COMPOUNDS

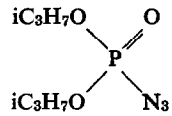
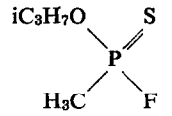
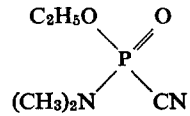
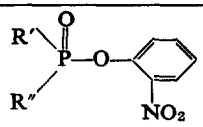
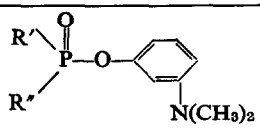
Compound	k
	$2{\cdot}0 \times 10^3$
	$6{\cdot}6 \times 10^2$
	$6{\cdot}9 \times 10^5$

TABLE 9. THE INFLUENCE OF THE TEMPERATURE ON THE REACTION RATE (1 $mol^{-1}min^{-1}$) OF ACETYLESTERASE WITH DFP

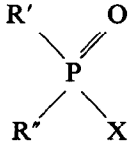
Temperature	rate constant	Activation energy kcal/mol
5°	$1{\cdot}2 \times 10^1$	
20°	$5{\cdot}5 \times 10^1$	
25°	$8{\cdot}0 \times 10^1$	9·75
35°	$2{\cdot}0 \times 10^2$	

TABLE 10. THE INFLUENCE OF THE pH ON THE REACTION RATE (1 $mol^{-1}min^{-1}$) OF ACETYLESTERASE WITH A NUMBER OF *o*-NITRO- AND *m*-DIMETHYLAMINO PHENYL COMPOUNDS

							
R′	R″	pH = 6·0	7·5	8·5	6·0	7·5	8·5
CH_3O	CH_3O	$4{\cdot}7 \times 10^2$	$7{\cdot}4 \times 10^2$	$7{\cdot}3 \times 10^2$	$< 10^0$	$< 10^0$	$< 10^0$
CH_3	CH_3O	$1{\cdot}9 \times 10^3$	$6{\cdot}5 \times 10^3$	$6{\cdot}2 \times 10^2$	$3{\cdot}4 \times 10^1$	$6{\cdot}9 \times 10^1$	$4{\cdot}5 \times 10^1$
C_2H_5O	C_2H_5O	$1{\cdot}4 \times 10^2$	$2{\cdot}6 \times 10^2$	$3{\cdot}4 \times 10^2$	$1{\cdot}6 \times 10^1$	$1{\cdot}6 \times 10^1$	$1{\cdot}5 \times 10^1$
C_2H_5	C_2H_5O	$6{\cdot}3 \times 10^3$	$1{\cdot}7 \times 10^4$	$1{\cdot}8 \times 10^4$	$1{\cdot}1 \times 10^0$	$6{\cdot}0 \times 10^{-1}$	$1{\cdot}1 \times 10^0$

DISCUSSION

The discussion will be based on the general formula of an organophosphorus inhibitor



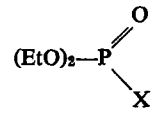
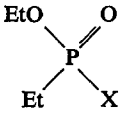
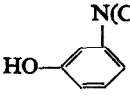
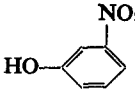
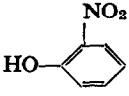
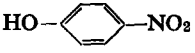
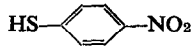
The influence of the structure of group X on the enzyme inhibition

In the paper on the inhibition of aliesterase[3] we have compared the rate constants of the inhibition reaction and of the alkaline hydrolysis with the pK_a of HX, the latter being a measure for the strength of the P-X bond.

In Table 11 we have listed some results obtained with a series of diethylphosphoryl- and ethyl ethylphosphonyl compounds (from Tables 5–7). The rates of hydrolysis were again taken from Ginjaar[9] whereas the pK_a values were taken from Albert and Serjeant.[10]

From Table 11 we observe a definite correlation between the pK_a and the rates of hydrolysis and enzyme inhibition. The latter correlation was found to be absent in the

TABLE 11. THE INFLUENCE OF THE pK_a OF HX ON THE RATE CONSTANTS OF THE HYDROLYSIS AND OF THE ACETYLESTERASE INHIBITION BY A NUMBER OF PHOSPHORYL- AND PHOSPHONYL COMPOUNDS

HX	pK_a of HX				
		log k_{OH^-}	log k_{enz}	log k_{OH^-}	log k_{enz}
	~11·8	−1·60	1·28	−0·44	0·07
	8·4	−0·42	1·68	0·77	2·22
	7·2	0·12	2·28	1·35	3·67
	7·1	−0·18	2·72	0·94	4·21
	~4·9	1·18	3·80	1·26	4·97
HF	3·2	2·15	4·27	3·08	3·84

case of aliesterase; the absence we explained tentatively by assuming an interaction of X with some groups of the enzyme molecule. It seems that such interactions are much less important in the case of acetylesterase; at any rate these interactions do not offset the sequence of the reactivity of the inhibitors as they do in the cases of the cholinesterases and aliesterase.[3] That interaction of X with acetylesterase cannot be completely absent follows from the comparison of the reactivity of the following three compounds (taken from Tables 5, 7 and 8).

di-isopropyl *p*-nitrophenylphosphate	$\log k_{OH} = -1{\cdot}22$,	$\log k_{enz} = 0{\cdot}20$
di-isopropyl phosphorofluoridate	$\log k_{OH} = 1{\cdot}70$,	$\log k_{enz} = 1{\cdot}90$
di-isopropyl phosphorazidate	$\log k_{OH} = << -2$,	$\log k_{enz} = 3{\cdot}30$

The very small rate of hydrolysis of the latter compound can be explained by resonance stabilization. The high rate of the enzyme inhibition reaction may then be explained by the elimination of certain resonance possibilities by interaction of the azido group with some group in the enzyme molecule.

There is a difference of a factor 2000 between the rate constants of ethyl N-dimethylphosphoramidofluoridate and ethyl N-dimethylphosphoramidocyanidate (tabun) (Tables 7 and 8).

If we compare the pK_a values of hydrofluoric acid (3·2) and of hydrocyanic acid (9·1) one would expect the reverse.

Perhaps the polarizability of the cyanide group causes the electronic charge to become localized in the leaving group during the approach to the active enzyme centre as has been suggested by Heath.[14]

The influence of the groups R′ and R″ on the enzyme inhibition

In the case of aliesterase we found that the influence of the structure of groups R′ and R″ on the enzyme inhibition was very small.[3]

This is definitely not the case with acetylesterase. We will in a more systematic way discuss the structure-activity relations.

1. *Dialkyl phosphates.* In the series of the dialkyl *p*-nitrophenylphosphates (Table 5) we observe the following sequence of rate constants.

$$\text{Me} \geqslant \text{Et} > \text{nPr} > \text{nBu} > \text{nPe};\ \text{iPr} = \text{sec Bu}$$

The reactivity of the inhibitor decreases with increasing chain length, in the same manner as has been observed in the case of alkaline hydrolysis.[11, 12]
Both α-(Et > iPr and nPr > sec Bu) and β − (nPr > iBu) substitution give a lowering of the reaction rate.

Similar effects are found with the dialkyl phosphorofluoridates. Our results correspond with those obtained by Mounter *et al.*[13] using wheat germ esterase, an enzyme probably closely related to our acetylesterase.[1]

2. *Alkyl methylphosphonates.* In the series of the alkyl *p*-nitrophenyl methylphosphonates (Table 5) we observe the following sequences in the inhibition rates

$$\text{Me} = \text{Et} = \text{nPr} < \text{nBu} < \text{nPe}$$
$$\text{iPr} > \text{sec Bu} \leqslant \text{cyclohexyl} < \text{pinacolyl}$$

From the *n*-propyl compound onwards the rate constants increase, this in contrast

to the alkaline hydrolysis which decreases with increasing chain length. As in the previous case both α- and β- substitutions tend to result in lower rate constants.

In the series of the phosphonofluoridates (Table 7) one of the most striking results is the low value of the inhibition rate for the pinacolyl compound whereas the isomeric methyl pentyl-2 compound is very active. Also the pinacolyl *p*-nitrophenyl methylphosphonate has a low rate constant. The cycloalkyl methylphosphonofluoridates are very reactive; this in contrast with the low reactivity of the cyclohexyl *p*-nitrophenyl compound. In general the series of the alkyl methylphosphonates includes the best acetylesterase inhibitors among those investigated.

3. *Isopropyl alkylphosphonates.* In the series of the isopropyl *p*-nitrophenyl alkylphosphonates (Table 5) we observe the following sequences

$$\text{Me} > \text{Et} > \text{n–Pr} > \text{n–Bu} < \text{n–Pe}, \quad \text{iPr} > \text{sec Bu}$$

There is a gradual decrease in reactivity as the alkyl chain is lengthened as is the case in alkaline hydrolysis.[12]

In the case of the acetylesterase inhibition however there seems to be a minimum for the *n*-butyl compound.

As in the other series, α- and β- substitution tend to result in lower rate constants.

4. *Dialkylphosphinates.* In the series of the *p*-nitrophenyl dialkylphosphinates (Table 5) the sequence is

$$\text{Et} > \text{n–Pr} >> \text{nBu} > \text{nPe} > \text{nHex}$$
$$\text{iPr} > \text{sec Bu and iBu} > \text{iPe}$$

Here we observe a sharp fall in reactivity on lengthening the alkyl chains. The falling off is much more dramatic than in the case of the alkaline hydrolysis[2] where qualitatively the same trend is observed. Both α- and β-substitutions are highly unfavourable for high reaction rates.

5. *Isosteric substitution.* If we compare isosteric phosphates and phosphinates in the *p*-nitrophenyl series (e.g. diethyl *p*-nitrophenyl phosphate and *p*-nitrophenyl di-*n*-propylphosphinate) we observe that the diethylphosphinate is more reactive than its isosteric phosphate, the di-*n*-propylphosphinate has about the same reactivity as the corresponding phosphate but that with the other members of the series the phosphates have higher rate constants than the corresponding isosteric phosphinates. This is in contrast to the alkaline hydrolysis where the phosphinates always hydrolyze considerably faster than the phosphates. The same effect is found with aliesterase.

Probably the P-O-C oxygen interacts with the enzyme surface in the formation of the transition state.

6. *General remarks on the influence of the groups* R′ *and* R″ *on the reactivity towards acetylesterase.* In general the effects observed parallel those found in the alkaline hydrolysis, the main exceptions being that in the series of the alkyl *p*-nitrophenyl methylphosphinates the reactivity towards acetylesterase increases whereas the rate of hydrolysis increases with the lengthening of the alkyl chain and that the majority of the phosphates react faster with acetylesterase than the corresponding phosphinates.

The results lead to the following requirements for a compound with a high reaction rate with acetylesterase:

(a) dialkoxy- and dialkylgroups, if present, have to be small

(b) an alkylgroup in the presence of an alkoxygroup, has to be small
(c) an alkoxygroup in the presence of an alkylgroup, has to be primary and has to contain (at least) 5 C-atoms.

In Fig. 1 the rate constant of acetylesterase inhibition is plotted against the rate constant of alkaline hydrolysis for the series of the *p*-nitrophenyl compounds. In order to compare this graph with similar ones obtained with other enzymes dimethyl *p*-nitrophenylphosphate was chosen as a reference compound with rate constants $k°_{enz}$ and $k°_{OH}$ respectively. The graph shows the (log k^{i}_{enz} — log $k°_{enz}$) plotted against (log k^{i}_{OH} — log $k°_{OH}$). Thus the dimethylcompound is by definition situated in the origin. Compounds which combine high reaction rate with the enzyme and low rate of hydrolysis and which should be situated in the left upper corner are virtually absent.

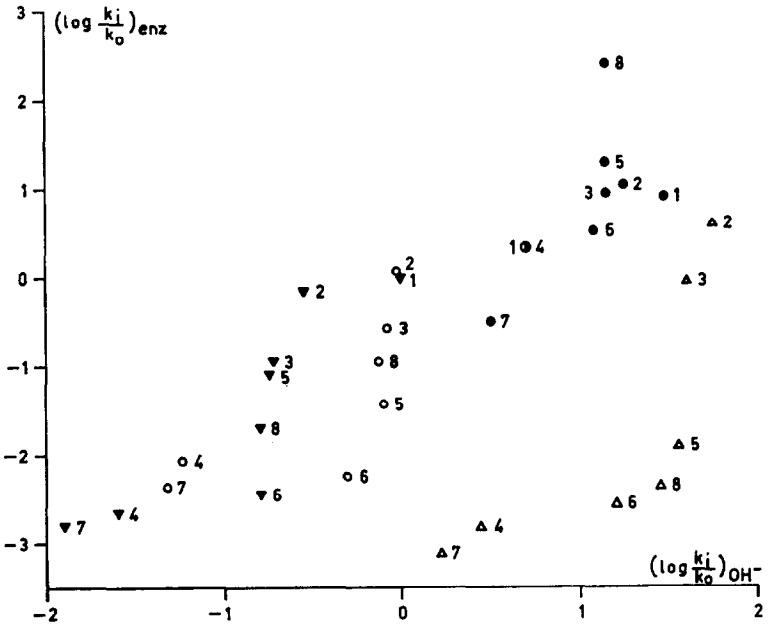


Fig 1. The relation of the rate of inhibition of acetylesterase and the rate of alkaline hydrolysis of a number of p-nitrophenyl compounds.

▼	$(RO)_2P(O)OC_6H_4NO_2$	1	R = Me	5	R = n–Bu
○	$(i\text{–}PrO)RP(O)OC_6H_4NO_2$	2	Et	6	i–Bu
●	$(RO)MeP(O)OC_6H_4NO_2$	3	n–Pr	7	sec–Bu
△	$R_2P(O)OC_6H_4NO_2$	4	i–Pr	8	n–Pe

A large number of the organophosphorus compounds investigated have an asymmetric phosphorus atom and one could expect stereospecificity in the inhibition reaction (*c.f.* Ooms and Boter[14]). However, since in all cases a large excess of inhibitor over enzyme was necessary to obtain a fair amount of inhibition within a reasonable time, it was impossible to determine whether or not the optical isomers of inhibitors

containing an asymmetric phosphorus atom show different rates of reaction with the enzyme. In a subsequent article we will come back on the stereospecificity.

The influence of the temperature on acetylesterase inhibition

In Table 9 the results obtained with DFP are shown. They obey Arrhenius' law and the following values were calculated (Table 12).

TABLE 12. KINETIC CONSTANTS FOR THE ALKALINE HYDROLYSIS, THE REACTION WITH HORSE LIVER ALIESTERASE AND WITH ACETYLESTERASE, OBTAINED WITH DFP

	OH^-	aliesterase	acetylesterase
$\triangle H^*$	21·2 kcal/mol	12·4 kcal/mol	9·1 kcal/mol
$\triangle S^*$	−12·7 E.U.	0·5 E.U.	−27 E.U.
$\triangle G^*$	25·0 kcal/mol	12·3 kcal/mol	17·1 kcal/mol

If we compare the rate of reaction of DFP with aliesterase with that of acetylesterase we find that the former is higher. However the activation enthalpy of the acetylesterase inhibition is lower, but this is more than offset by the much lower activation entropy, a value more in agreement with the activation entropy of the alkaline hydrolysis. The rapid reaction with acetylesterase is caused here by a lowering of the activation enthalpy, in contrast with the reaction with aliesterase, where the high activation entropy is the cause of the rapid reaction.[3]

The influence of the pH on acetylesterase inhibition

The results of Table 10 indicate that the influence of the pH on the inhibition is rather small. The optimum pH will be somewhere between 7·5 and 8·5. Mounter *et al.*[15] observed an optimum pH of 7·8 for the wheat germ esterase, a value not in disagreement with our results. However Jansen *et al.*[1] observed an optimal pH for the hydrolysis of triacetin at pH 6·5, a value that was confirmed by our experiments.

It seems that the optimum pH for substrate hydrolysis and for inhibition are not identical in this case. Our results differ from those of Wilson and Bergmann[16] (with acetylcholinesterase) and of Mounter *et al.*[15] (for acetylcholinesterase, butyrylcholinesterase, chymotrypsin, trypsin and wheat germ esterase) where the optimal pH values of substrate hydrolysis and inhibition were the same.

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