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THE REACTION OF ORGANOPHOSPHORUS COMPOUNDS WITH HYDROLYTIC ENZYMES.

THE INHIBITION OF HORSE LIVER ALIESTERASE

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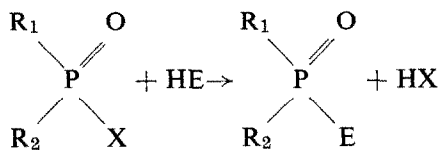
Abstract—Reaction rate constants were determined for the inhibition of horse liver aliesterase by a number of organophosphorus compounds with the general formula $R_1R_2P(O)X$ at pH 7·7 and 25°. The influence of the structure of the groups R_1 and R_2 on the rate of inhibition is generally very small. The influence of the structure of the group X is much greater. There seems to be, however, only a slight correlation between the rate of the enzyme inhibition and the strength of the P—X bond.

In two cases the influence of the temperature on the reaction rate was investigated. In both cases the activation enthalpy was found to be of the same order as in alkaline hydrolysis; the activation entropy was very much higher than in the hydrolytic reactions.

A colorimetric assay method for aliesterase is described.

INTRODUCTION

ORGANOPHOSPHORUS compounds are known to inhibit a number of hydrolytic enzymes, e.g. the cholinesterases, chymotrypsin, trypsin *etc.* (cf. Heath[1]). This paper deals with the inhibition of horse liver aliesterase (carboxylic ester hydrolase, E.C. 3.1.1.1.). From the investigations of Boursnell and Webb[2] it is known that this enzyme is inhibited by DFP (diisopropyl phosphorofluoridate) and related compounds. The inhibition proved to be irreversible and was accompanied by the introduction of the phosphorus atom into the enzyme molecule. Later, Jansz *et al.*[3] found that aliesterase reacts with DFP to form the inactive diisopropyl phosphoryl enzyme, the mechanism of inhibition thus being identical with the mechanism observed with other hydrolytic enzymes, which can be represented in a general scheme.



(1)[1]

In the equation EH stands for the enzyme: R_1 and R_2 represent alkyl, alkoxy, aryl, aryloxy or substituted aminogroups. X is the so called "leaving group" and is acidic in nature, i.e. the conjugated acid HX is more acidic than either R_1H or R_2H.

Concerning the influence of the structure of the groups R_1, R_2 and X on the rate of reaction with liver aliesterase a comparatively small number of investigations have

been carried out. In a comprehensive study Myers[4] measured the I_{50} values of rat brain aliesterase inhibition by a large number of organophosphorus compounds; all three groups mentioned seem to have an influence on the inhibition. However, his enzyme preparation (rat brain homogenate) seemed to dephosphorylate rather rapidly so that no kinetic constants could be determined. Augustinsson[5] also inhibited mammalian aliesterase with a number of organophosphorus compounds but he does not give quantitative data.

As part of a comprehensive study on the relation between chemical structure and rate of inhibition of hydrolytic enzymes we included horse liver aliesterase. In the general formula of an organophosphorus inhibitor we varied R_1, R_2 and X more or less systematically and determined the influence of these variations on the rate of esterase inhibition. As a measure of this rate we have chosen the bimolecular rate constant rather than the I_{50} because the proportionality between the two values (cf. O'Brien[6]) is lost in those cases where enzyme and inhibitor are present in the reaction mixture in comparable concentrations.[7] In two cases the influence of the temperature on the inhibition rate was determined.

EXPERIMENTAL

Enzyme preparation

The aliesterase used in the investigation was obtained from horse liver and purified according to Rozengart *et al.*[8] and Connors *et al.*[9] The procedure consisted of preparing an acetone powder, extraction with 8% ammonia, two ammonium sulphate precipitations, heat denaturation, acetone fractionation, copper acetate precipitation and lyophilization.

The acetone powder was prepared according to Connors, whereas extraction and ammonium sulphate precipitation were carried out following the method described by the Russian authors because of its high yield.

From the heat denaturation step onwards, the directions of Connors were followed. In the purification procedure we did not proceed beyond the lyophilization step; the rate constants found with DFP, sarin and paraoxon obtained with the preparation proved to be identical with the values obtained with a preparation further purified by electrophoresis.*

The preparation used in our investigations had a specific activity of 580 units/mg N^{10} using 0·06 M methyl butyrate as substrate and at pH 7·5.

Nitrogen was determined using the semi micro Kjeldahl method.

Organophosphorus compounds

The following inhibitors were used: 34 *p*-nitrophenyl compounds (Table 4), 2-*p*-nitrothiophenyl compounds (Table 5), 4-*o*-nitrophenyl compounds (Table 5), 4 *m*-nitrophenyl compounds (Table 5), 4 *m*-dimethylaminophenyl compounds (Table 5), 13 fluoridates (Table 6) and 4 miscellaneous compounds (Table 7).

All the compounds were synthesized in this laboratory, the *p*-nitrophenyl compounds according to De Roos and Toet,[11] the fluoridates according to standard methods[12] and the other compounds according to Boter.[13]

* Kindly supplied by dr. H. S. Jansz, Rijswijk.

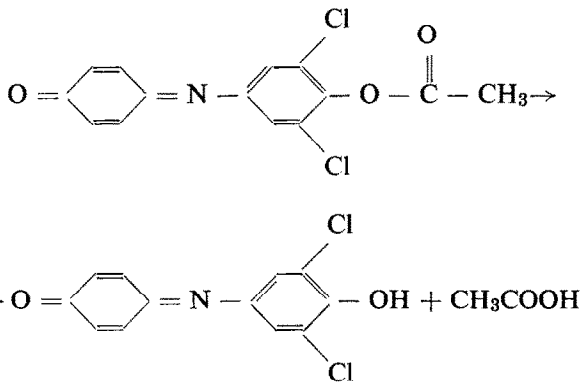
Substrates

Methyl butyrate was obtained commercially (British Drug Houses Ltd.). Benzenone-indo-3′d-5′ichlorophenylacetate was synthesized according to de Borst *et al.*[14]

Enzyme assay method

For standardization purposes we used the titrimetric method with methyl butyrate as described by Jansz[3] 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.

For that reason we developed a colorimetric method using benzenone-indo-3′5′-dichlorophenylacetate (BIDA) as a substrate.



The ester has a faint yellow colour whereas the phenol formed has an intense blue colour with an absorption maximum at 605 mμ.[15] The procedure used 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 benzenone-indo-3′5′-dichlorophenylacetate in alcohol (20 mg/25 ml.) and 1 ml. enzyme solution.

The mixture is kept for 30 min in a constant temperature bath at 25°. The blue colour is then measured at 605 mμ in a colorimeter. Under these conditions 1 unit of enzyme gave an extinction of approximately 0·5 and the extinction was proportional to the amount of enzyme in the 0·05–1 unit range. After we had proved that the same enzyme is responsible for the hydrolysis of methyl butyrate and BIDA (see below) we used the colorimetric method in all our kinetic experiments for reasons described above.

Inhibition rate measurements

The aliesterase preparation is diluted with 0·067 M veronal buffer pH 7·7 so that the activity is approximately 2 units/ml. 3 ml. of this 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 (compare $K_m = 4 \times 10^{-5}$ M for BIDA with $K_m = 2{\cdot}2 \times 10^{-2}$ M for methyl butyrate) 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 6×10^{-8} M. If the concentration of the inhibitor in the mixture is more than 20 times that of the enzyme the following formula is used to calculate the rate constant:

$$k = \frac{2{\cdot}303}{t \times b} \log \frac{a}{a - x}$$

In all other cases the complete formula is used:

$$k = \frac{2{\cdot}303}{t(a - b)} \log \frac{b(a - x)}{a(b - x)}$$

In both formulae a represents the enzyme concentration, b the inhibitor concentration and x the concentration of enzyme (or inhibitor) which has reacted in time t.[7]

In those cases where we investigated the influence of the temperature on the rate of inhibition (see Table 8), only the inhibition was performed at the temperature given; the enzyme assay was always carried out at 25·0°.

RESULTS

The identity of the enzymes hydrolyzing methyl butyrate and BIDA

We determined the activity ratio of aliesterase preparations of different purities, both with methyl butyrate and with BIDA as substrates (Table 1).

Table 1. Ratio of activity of a number of aliesterase preparations with methyl butyrate and with benzenone-indo-3′5′-dichlorophenylacetate as substrate

Preparation	Purification step	U/ml. (methyl butyrate)	U/ml. × 10^2 (BIDA)	U methyl butyrate / U BIDA
A	acetone precip.	4·8	4·1	117
C	copper precip.	2·0	1·6	121
E	lyophilized	14·0	11·0	126

Next to that, an aliesterase preparation was inhibited by sarin, using an excess of enzyme. After termination of the reaction the inhibition percentage was determined, both with methyl butyrate and with BIDA as a substrate. The results are in Table 2.

Table 2. Inhibition of aliesterase by sarin, determined with methyl butyrate and with benzenone-indo-3′5′-dichlorophenyl-acetate as substrate. Inhibition carried out at pH 7·7 and 25·0°

Concentration sarin	Time of inhibition	% inhibition (methyl butyrate)	% inhibition (BIDA)
$1{\cdot}79 \times 10^{-6}$M	20 min	93	95
$5{\cdot}36 \times 10^{-7}$M	8 min	84	84
$1{\cdot}17 \times 10^{-7}$M	65 min	53	55

From the results shown in Tables 1 and 2 we can conclude that methyl butyrate and BIDA are hydrolyzed by the same enzyme.

The enzyme concentration

In order to calculate the bimolecular rate constant of the inhibition reaction in the case of a very rapidly reacting inhibitor, it is necessary to know the concentration of active sites of the enzyme.[7] (In the following the expression "enzyme concentration" will be used for the concentration of active sites.) From the work of Jansz *et al.*[3] one can calculate that the concentration of an enzyme solution containing 1 unit/l is $5{\cdot}3–5{\cdot}5 \times 10^{-11}$M, keeping in mind that Jansz's definition leads to units which are ten times greater than the internationally proposed units.[10]

We have determined independently the concentration by means of a number of inhibition experiments. The enzyme was inhibited by a number of organophosphorus compounds during such a time that the inhibition level had become constant (about one hour). From the percentage of inhibition it was possible to calculate the concentration of the enzyme (see Table 3).

TABLE 3. DETERMINATION OF THE CONCENTRATION OF ALIESTERASE

Inhibitor	Conc. of inhibitor (M)	% age inhibition	Conc. of enzyme (Unit/ml.)	Conc. of enzyme (M)	M/Unit $\times 10^{11}$
sarin	$1{\cdot}07 \times 10^{-7}$	48	3·9	$2{\cdot}19 \times 10^{-7}$	5·6
isopropyl *p*-nitrophenyl isobutylphosphonate	$1{\cdot}97 \times 10^{-8}$	41	0·88	$4{\cdot}85 \times 10^{-8}$	5·5
isopropyl *p*-nitrophenyl isopropylphosphonate	$3{\cdot}27 \times 10^{-8}$	67	0·90	$4{\cdot}90 \times 10^{-8}$	5·5
di-*n* propyl *p*-nitrophenyl-phosphate	$1{\cdot}85 \times 10^{-8}$	37	0·92	$4{\cdot}97 \times 10^{-8}$	5·4
	$2{\cdot}11 \times 10^{-8}$	42	0·99	$5{\cdot}00 \times 10^{-8}$	5·1
	$2{\cdot}58 \times 10^{-8}$	48	1·05	$5{\cdot}32 \times 10^{-8}$	5·1

Mean: $5{\cdot}4 \pm 0{\cdot}2 \times 10^{-11}$M

The result is in good agreement with the value found by Jansz; in our experiments we obtained stoichiometric properties in all cases where this could be expected, i.e. in those experiments where the enzyme was present in excess.

The rate reaction of aliesterase with organophosphorus compounds

The results of the kinetic experiments are listed in the tables 4 through 8. Each rate constant is the mean of at least three values obtained with different inhibitor concentrations, the error being of the order of $\pm$ 5 per cent. The influence of the temperature on the inhibition of aliesterase by two inhibitors is given in Table 8.

TABLE 4. RATE CONSTANTS ($M^{-1}MIN^{-1}$) OF THE REACTION OF ALIESTERASE WITH A NUMBER OF *p*-NITROPHENYL COMPOUNDS

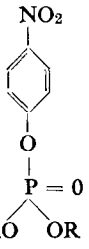
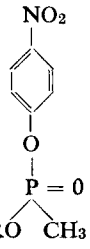
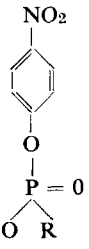
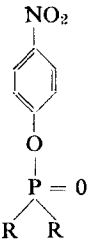
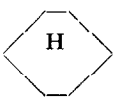
R				
CH_3	$2{\cdot}0 \times 10^5$	$3{\cdot}3 \times 10^6$	$6{\cdot}0 \times 10^6$	—
C_2H_5	$7{\cdot}9 \times 10^6$	$3{\cdot}2 \times 10^6$	$2{\cdot}8 \times 10^6$	$2{\cdot}2 \times 10^6$
nC_3H_7	$1{\cdot}4 \times 10^7$	$7{\cdot}9 \times 10^6$	$8{\cdot}2 \times 10^6$	$1{\cdot}1 \times 10^6$
iC_3H_7	$7{\cdot}6 \times 10^6$	$6{\cdot}0 \times 10^6$	$9{\cdot}7 \times 10^5$	$6{\cdot}1 \times 10^3$
nC_4H_9	$1{\cdot}0 \times 10^7$	$6{\cdot}0 \times 10^6$	$3{\cdot}9 \times 10^6$	$7{\cdot}3 \times 10^5$
iC_4H_9	$3{\cdot}7 \times 10^6$	$3{\cdot}4 \times 10^6$	$2{\cdot}5 \times 10^6$	$4{\cdot}2 \times 10^5$
sec. C_4H_9	$1{\cdot}4 \times 10^7$	$3{\cdot}1 \times 10^6$	$1{\cdot}7 \times 10^6$	$7{\cdot}3 \times 10^4$
tert. C_4H_9	—	—	$1{\cdot}9 \times 10^3$	—
nC_5H_{11}	$3{\cdot}8 \times 10^6$	$6{\cdot}3 \times 10^6$	$3{\cdot}9 \times 10^6$	$1{\cdot}0 \times 10^6$
	—	$1{\cdot}2 \times 10^6$	—	—
$(CH_3)_3C—CH(CH_3)$	—	$7{\cdot}0 \times 10^5$	—	—

TABLE 5. RATE CONSTANTS ($M^{-1}MIN^{-1}$) OF THE REACTION OF ALIESTERASE WITH A NUMBER OF SUBSTITUTED PHENYL COMPOUNDS

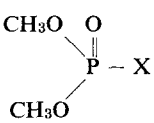
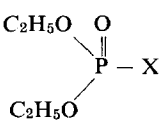
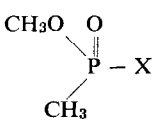
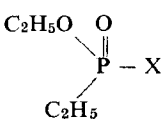
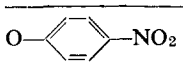
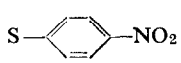
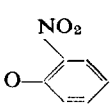
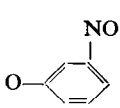
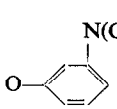
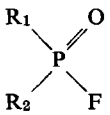
X				
	$2{\cdot}0 \times 10^5$	$7{\cdot}9 \times 10^6$	$3{\cdot}3 \times 10^6$	$2{\cdot}9 \times 10^6$
	—	$3{\cdot}0 \times 10^6$	—	$1{\cdot}5 \times 10^6$
	$1{\cdot}3 \times 10^5$	$4{\cdot}2 \times 10^5$	$3{\cdot}5 \times 10^5$	$4{\cdot}0 \times 10^5$
	$3{\cdot}7 \times 10^4$	$1{\cdot}5 \times 10^6$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

TABLE 6. RATE CONSTANTS ($M^{-1}MIN^{-1}$) OF THE REACTION OF ALIESTERASE WITH A NUMBER OF PHOSPHORO- AND PHOSPHONOFLUORIDATES



R_1	R_2	k	R_1	R_2	k
CH_3O	CH_3O	$2{\cdot}9 \times 10^4$	CH_3	iC_3H_7O	$1{\cdot}7 \times 10^5$
			CH_3	$(CH_3)_2CH—CH(CH_3)O$	$3{\cdot}3 \times 10^5$
C_2H_5O	C_2H_5O	$6{\cdot}1 \times 10^5$	CH_3	$(CH_3)_3C—CH(CH_3)O$	$2{\cdot}3 \times 10^5$
nC_3H_7O	nC_3H_7O	$2{\cdot}4 \times 10^6$	CH_3	$(CH_3)_2CH—CH_2—CH(CH_3)O$	$1{\cdot}3 \times 10^6$
			CH_3		$9{\cdot}8 \times 10^5$
iC_3H_7O	iC_3H_7O	$3{\cdot}2 \times 10^5$	CH_3		$5{\cdot}3 \times 10^5$
			CH_3		$8{\cdot}7 \times 10^5$
			C_2H_5	C_2H_5O	$5{\cdot}5 \times 10^5$
			C_2H_5		$7{\cdot}1 \times 10^5$

TABLE 7. RATE CONSTANTS ($M^{-1}MIN^{-1}$) OF THE REACTION OF ALIESTERASE WITH SOME MISCELLANEOUS COMPOUNDS

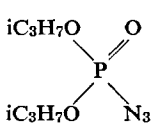
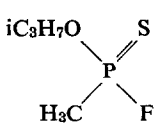
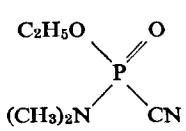
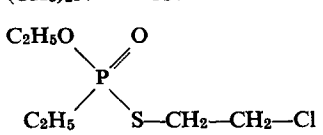
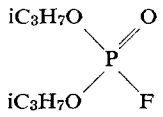
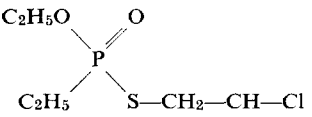
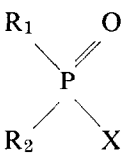
Compound	k
	$1{\cdot}3 \times 10^6$
	$3{\cdot}2 \times 10^4$
	$7{\cdot}0 \times 10^5$
	$1{\cdot}7 \times 10^5$

TABLE 8. THE INFLUENCE OF THE TEMPERATURE ON THE RATE OF REACTION ($M^{-1}MIN^{-1}$) OF ALIESTERASE WITH DFP AND WITH ETHYL S-2-CHLOROETHYL ETHYLPHOSPHONOTHIOLATE

				
	rate constant	E kcal/mol	rate constant	E kcal/mol
5·0°	$7{\cdot}9 \times 10^4$		$1{\cdot}8 \times 10^4$	
20·0°	—	13·0	$1{\cdot}1 \times 10^5$	15·3
25·0°	$3{\cdot}2 \times 10^5$		$1{\cdot}7 \times 10^5$	
35·0°	$8{\cdot}3 \times 10^5$		$4{\cdot}3 \times 10^5$	

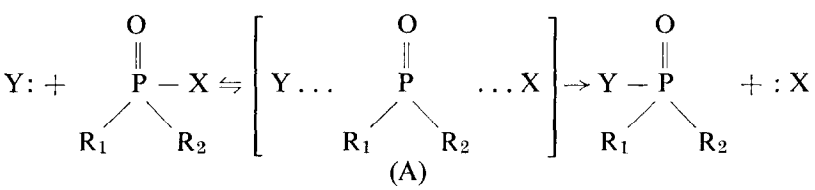
DISCUSSION

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

A bimolecular nucleophilic substitution reaction (Sn-2P) of an organophophorus compound may be represented by the following equation:



(A)

in which (A) represents the transition-state.

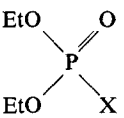
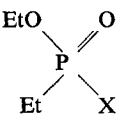
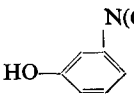
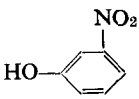
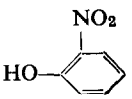
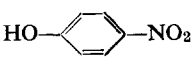
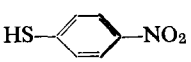
The rate of reaction, groupings R_1 and R_2 being constant, will depend on :—

(1) the strength of the P—X bond
(2) the nucleophilicity of Y:
(3) the charge on the central P-atom, caused by differences in electronegativity of the groups R_1, R_2, X and O
(4) steric factors
(5) "interactions" between X and Y.

The same equation will apply to the enzyme inhibition as has been pointed out by Aldridge.[16] The rate of reaction will then depend on the strength of the P—X bond. A measure for this bond-strength is the pK_a of HX. The smaller this pK_a, the less stable the P—X bond will be and the faster the reaction.

In Table 9 we have listed some results obtained with a series of diethyl phosphoryl- and ethyl ethylphosphonyl compounds taken from Tables 4–6. The rates of hydrolysis were taken from Ginjaar[18] while the pK_a values were taken from Albert and Serjeant.[17]

TABLE 9. THE INFLUENCE OF THE pK_a OF HX ON THE RATE CONSTANTS OF THE HYDROLYSIS AND OF THE ALIESTERASE INHIBITION BY A NUMBER OF PHOSPHORYL AND PHOSPHONYL COMPOUNDS

HX	pK_a of HX				
		log k_{OH^-}	log k_{all}	log k_{OH^-}	log k_{all}
	~11·8	−1·60	2·68	−0·44	1·60
HS—CH_2—CH_2Cl	~10	—	—	< −1	5·23
	8·4	−0·42	6·18	0·77	6·04
	7·2	0·12	5·62	1·35	5·60
	7·1	−0·18	6·90	0·94	6·46
	~4·9	1·18	6·47	1·26	6·18
HF	3·2	2·15	5·78	3·08	5·74

As can be seen from Table 9, there is a definite correlation between the pK_a and the rate of hydrolysis. This correlation seems to be absent in the case of aliesterase inhibition. The reactivity of the organophosphorus molecule is here probably offset by an interaction between the nitrophenyl group and some part of the enzyme molecule.

From results obtained with a series of 2 haloethyl butyrates and related esters, which will be published shortly, we deduced the probability that a cationic site is present in the aliesterase molecule participating in the enzymatic mechanism. The oxygen atoms of the nitrogroup on the phenyl nucleus have a partial negative charge due to resonance. The dimethylamino group on the phenyl nucleus, having a pK_a of about 3·6 will be totally protonated under our conditions and thus have a positive charge. The large difference in reaction rates between the nitro- and dimethylaminophenyl compounds may be explained by assuming electrostatic interaction as one of the possibilities of X—Y interaction.

The high reaction rate of the O-ethyl S-(2-chloroethyl) ethylphosphonothiolate (Table 7), compared with the low rate of alkaline hydrolysis can be explained by an interaction of the partial negative charge on the chlorine atom with the cationic site on the enzyme. Acetylcholinesterase, having an anionic site, indeed shows a lower reactivity with this compound ($k = 2{\cdot}9 \times 10^4$), whereas in general the enzyme is much more reactive towards organophosphorus compounds than aliesterase.

Unexpected results are obtained by comparing the following three compounds: diisopropyl *p*-nitrophenyl phosphate ($\log k_{OH} = -1{\cdot}22$, $\log k_{ali} = 6{\cdot}88$); diisopropyl phosphorofluoridate ($\log k_{OH} = 1{\cdot}70$, $\log k_{ali} = 5{\cdot}10$) and diisopropyl phosphorazidate ($\log k_{OH} = <<-2$, $\log k_{ali} = 6{\cdot}10$) (Tables 4, 6 and 7). In this case the order of the rate constant of the aliesterase inhibition reaction is completely reversed compared to the rate constant of the alkaline hydrolysis. The pK_a of hydrazoic acid being 4·7 it is surprising that the rate of hydrolysis of the azidate is so very low. A possible explanation for this effect is the great number of resonance possibilities of the azido group bound to the P = O group, as a result of which the $P-N_3$ bond is stabilized.

A charged group on the enzyme surface near to the active site may induce an opposite charge in the azido group and as a result, the possibilities for electrostatic interaction are present. This could explain the high rate of enzyme inhibition of the azido compound for enzymes bearing a negative (cholinesterases) or a positive (aliesterase) site compared to the very low reaction rate with enzymes which do not possess a charged group (e.g. chymotrypsin and trypsin).

The influence of the groups R_1 and R_2 on the enzyme inhibition

The influence of the substitution of different groups R_1 and R_2 on the inhibition of aliesterase is very small as can be seen in the Tables 4–6. This is in contrast with the results obtained with the other enzymes investigated. Only the substitution of the diethyl group for the dimethyl group as well as in the *p*-nitrophenyl phosphate as in the phosphorofluoridate series gives rise to a substantial increase of the reaction rate (Tables 4 and 6). Another exception is the low rate of *p*-nitrophenyl diisopropylphosphinate (Table 4).

It is interesting to compare this rather low specificity of aliesterase in regard to organophosphorus inhibitors with the substrate specificity of aliesterase, which is also low, compared with other enzymes investigated by us.

A large number of the organophosphorus compounds investigated have an asymmetric phosphorus atom and one could expect stereospecificity in the inhibition reaction (cf. Ooms and Boter[19]). However, since in virtually 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 order to visualize better the relation between the reaction rate of the enzyme inhibition and of the alkaline hydrolysis we have plotted the data of the *p*-nitrophenyl compounds in Fig. 1. For reasons of convenience we have chosen dimethyl *p*-nitrophenyl phosphate as a reference with reaction rate constants respectively k^o_{enz} and k^o_{OH}.

In the figure ($\log k^i_a - \log k^o_a$) is plotted against ($\log k^i_{OH} - \log k^o_{OH}$), the dimethyl compound thus occupying the origin by definition. The small differences of the enzyme

inhibition rate compared with the rate of hydrolysis are clearly seen. Inhibitors best suitable for practical reasons occupy the upper left hand corner of the graph, thus combining high rate of enzyme inhibition with low rate of hydrolysis.

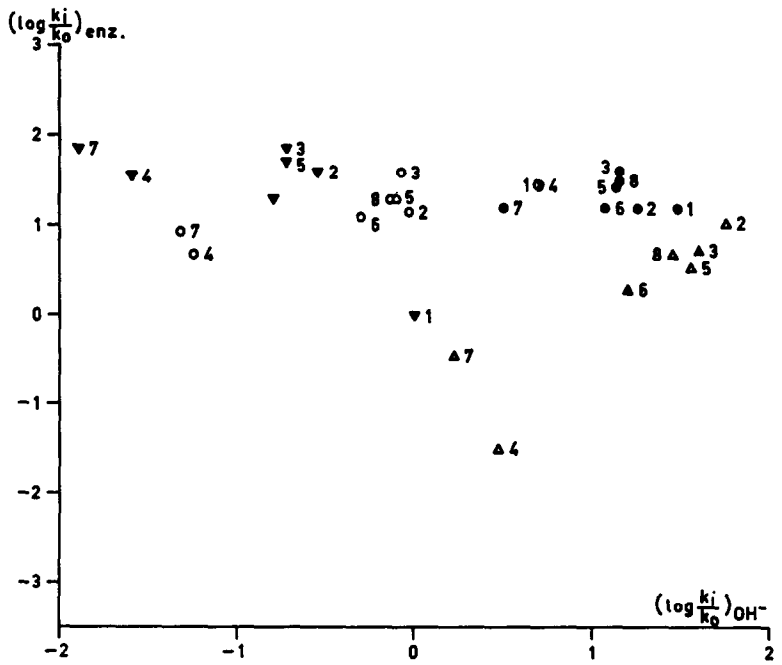


FIG. 1. The relation of the rate of inhibition of aliesterase and the rate of alkaline hydrolysis of a number of *p*-nitrophenyl compounds.

▼ $(RO)_2P(O)OC_6H_4NO_2$	1	R = Me	5	R = n-Bu
○ (i-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

The influence of the temperature on the rate of enzyme inhibition

Of the compounds described in this paper, we only investigated the influence of the temperature on the rate of the reaction of aliesterase with DFP and with O-ethyl S- (2-chloroethyl) ethylphosphonothiolate.

The results (Table 8) obey Arrhenius' law and the following values were calculated:

DFP	$\Delta H^* = 12{\cdot}4$ kcal/mol; $\Delta S^* = 0{\cdot}5$ E.U. and $\Delta G^* = 12{\cdot}3$ kcal/mol
thiolate	$\Delta H^* = 14{\cdot}7$ kcal/mol; $\Delta S^* = 6{\cdot}6$ E.U. and $\Delta G^* = 12{\cdot}8$ kcal/mol

One observes that the activation enthalpy does not differ very much from the values obtained in alkaline hydrolysis,[18] the activation entropy, however, is very much higher (compare the value of −25 E.U. at alkaline hydrolysis[18]).

The rapid reaction of the compounds with the enzyme is obviously not caused by a lowering of the activation enthalpy but rather by a much more favourable activation entropy. Laidler[20] suggests that the high activation entropies can be caused by the

release of water molecules following charge interaction of the enzyme surface. We will come back on this hypothesis in a later paper describing the inhibition of chymotrypsin, an enzyme that does not possess a charged group participating in the substrate or inhibitor interaction.

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