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THE REACTION OF ORGANOPHOSPHORUS COMPOUNDS WITH HYDROLYTIC ENZYMES—III
THE INHIBITION OF CHYMOTRYPSIN AND TRYPSIN

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Abstract—Reaction rate constants were determined for the inhibition reaction of chymotrypsin and trypsin by a number of organophosphorus compounds of the general formula $R_1R_2P(O)X$ at pH 7·7 and 25°. In all cases the rate constants for the inhibition of chymotrypsin were greater than the corresponding rate constants for the inhibition of trypsin. In the two series investigated 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_1 and R_2 on the rate of enzyme inhibition does not parallel the reactivity measured as alkaline hydrolysis. Cycloalkyl methylphosphonofluoridates proved to be the most active inhibitors investigated for both enzymes. The influence of the temperature on the reaction rate has been investigated in five cases. It seems that the rapid reaction of the compounds with the enzymes, compared with alkaline hydrolysis, is caused by a lowering of the activation enthalpy rather than by an increase of the activation entropy. Chymotrypsin shows marked stereospecificity in a number of cases. The enzyme reacted more than a hundred times faster with one of the stereoisomers of pinacolyl methylphosphonofluoridate than with its optical antipode. A calculation method for the determination of the rate constants of the stereoisomers from the data obtained with the racemic compound is given in an appendix.

INTRODUCTION

From the investigations of Jansen *et al.* (c.f. Balls and Jansen[1]), it is known that both chymotrypsin (E.C. 3.4.4.5) and trypsin (E.C. 3.4.4.4) are irreversibly inhibited by a number of organophosphorus compounds. Both the proteolytic and the esterolytic activities of the enzymes were inhibited to the same extent. At complete inhibition one atom of phosphorus was introduced in one molecule of the enzyme. Later Hartley and Kilby[2] for chymotrypsin and Kilby and Youatt[3] for trypsin showed that in the inhibition reaction with diethyl *p*-nitrophenyl phosphate (paraoxon) one molecule of *p*-nitrophenol was formed for each molecule of enzyme that was inhibited. Moreover the authors showed that the rate of *p*-nitrophenol liberation was equal to the rate of the inhibition of the proteolytic, the esterolytic and the amidolytic activities of the enzymes.

Concerning the relation between structure of the organophophorus inhibitors and the rate of reaction with chymotrypsin and trypsin relatively few investigations have been performed up to now.

Mounter *et al.*[4, 5] have measured the pI_{50} of some 20 organophosphorus compounds

with miscellaneous structure for both enzymes whereas Becker and co-workers[6, 7] measured the rate constants for the reaction of chymotrypsin and trypsin with a series of *n*-alkyl-, *ω*-chloroalkyl- and phenylalkyl phosphonates. The first mentioned authors found that the inhibitory power of dialkyl phosphorofluoridates was greater for the larger alkylgroups up to a certain maximum.

The results of Becker *et al.* also indicate that there is a general increase in reaction rates where the alkyl chains are lengthened with a maximum for *n*-heptyl in the case of chymotrypsin and for *n*-hexyl in the case of trypsin. With the *ω*-chloroalkyl compounds the same effects were observed with chymotrypsin, but with trypsin the rate constants were now more or less constant. In the phenylalkyl series both enzymes showed the highest rate constants for the phenylpropyl compound.

In previous publications we described a study on structure-activity relations in the case of the inhibition of horse liver aliesterase (carboxylic ester hydrolase E.C. 3.1.1.1)[8] and of citrus acetylesterase (acetic ester acetyl-hydrolase E.C. 3.1.1.6)[9] by a variety of organophosphorus compounds. The same type of investigation using chymotrypsin and trypsin is described in this paper. 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.[8]

EXPERIMENTAL

Enzyme preparation

The chymotrypsin used was obtained from Worthington Biochemical Corporation as a crystallized salt-free material. The enzyme had a specific activity of $1{\cdot}4 \times 10^3$ units/mg N* using acetyl tyrosine ethyl ester as a substrate according to Balls and Jansen.[1]

The trypsin was a commercial preparation obtained from the British Drug Houses Ltd. and afterwards purified according to Anson.[11] The final product has a specific activity of $8{\cdot}6 \times 10^2$ units/mg N using *p*-toluene sulfonyl arginine methyl ester as substrate according to Balls and Jansen.[1]

Organophosphorus compounds

The same compounds as described earlier[8] were used for this investigation; all compounds have been synthesized in this laboratory.

Substrates

Acetyl tyrosine ethylester was obtained from the British Drug Houses Ltd. and *p*-toluene sulfonyl argininemethylester was obtained from Serva Entwicklungs Labor (Heidelberg, Germany), both as chromatographically homogeneous.

Enzyme assay methods

The titrimetric assay methods, indicated above, are unsuitable for an extended series of successive determinations to be carried out during kinetic measurements. As has been shown,[1, 2, 3] the esterolytic and proteolytic activities of both enzymes are inhibited at the same rate.

* In accordance to the proposal of the Commission on Enzymes[10] units are defined as μ moles substrates hydrolysed per min.

We therefore decided to assay the enzymes using the proteolytic hemoglobin digestion method of Anson,[12] measuring absorption at 280 mμ rather than the colorimetric method for phenols. This method proved to be rapid, reliable and lends itself for a series of consecutive assays.

Inhibition rate measurements

Chymotrypsin and trypsin were dissolved in 0·067 M veronal buffer. pH 7·7, the former enzyme to a concentration of 0·06 mg N/ml, the latter to a concentration of 0·025 mg N/ml.5 ml of the enzyme solution were mixed in a test tube with 5 ml of the dilution of the organophosphorus inhibitor, both solutions being prewarmed to 25·0°. From this mixture 1 ml is pipetted into 5 ml of the hemoglobin substrate solution after definite times. The remaining enzyme was then assayed and the percentage remaining enzyme activity calculated.

The dilution together with the presence of substrate stopped the inhibition reaction completely as has been shown in a number of cases.

Under the described conditions the concentration of chymotrypsin was $6{\cdot}6 \times 10^{-6}$ M and of trypsin 10^{-6} M. If the concentration of the inhibitor in the mixture is more than 20 times that of the enzyme, the rate constant was calculated with the pseudo first-order formula[8] (this was always the case with trypsin). In all other cases the complete second-order formula was used. In the case of stereospecific inhibition the calculation method mentioned in the Appendix was used.

RESULTS

The enzyme concentration

Assuming molecular weights of 25·000 for chymotrypsin and of 24·000 for trypsin, a rough calculation of the molar concentration of the enzymes could be made. This resulted in values of 7×10^{-6} M for chymotrypsin and 1×10^{-6} M for trypsin under the conditions of the inhibition experiments.

The concentration of trypsin was in all cases at least 100 times lower than the concentration of the inhibitors so that this concentration was not used for the calculation of the rate constants. This was not the case with chymotrypsin however. To determine the concentration of the enzyme more exactly we inhibited the enzyme totally with diethyl *p*-nitrophenyl phosphate (10^{-3} M) and with isopropyl *p*-nitrophenyl methylphosphonate (10^{-4} M). The amount of *p*-nitrophenol was measured making corrections for spontaneous hydrolysis. Both experiments gave as a result for the concentration of the enzyme under the conditions of the inhibition experiments $(6{\cdot}7 \pm 0{\cdot}1) \times 10^{-6}$ M, a value corresponding well with the calculated value.

Assuming the concentration of $6{\cdot}7 \times 10^{-6}$ M we obtained good stoichiometry in all cases where this could be expected, i.e. in those experiments, where the enzyme was present in excess.

The rate of reaction of chymotrypsin and trypsin with organophosphorus compounds

The results of the kinetic experiments are listed in the Tables 1–4. Each value is the mean of at least three values obtained with different inhibitor concentrations, the error being of the order of ±5% The influence of the temperature on the inhibition is given in Table 5

TABLE 1. RATE CONSTANTS (1 mol^{-1}/min^{-1}) OF THERE ACTION OF CHYMOTRYPSIN (CT) AND TRYPSIN (T) WITH A NUMBER OF *p*-NITROPHENYL COMPOUNDS

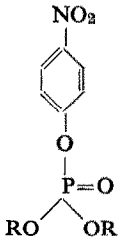
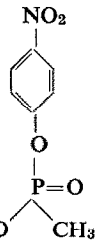
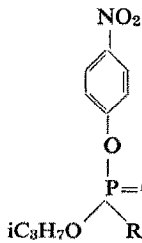
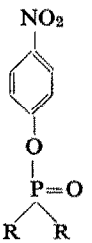
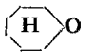
R								
	CT	T	CT	T	CT	T	CT	T
CH_3	$2{\cdot}2 \times 10^{0}$	$2{\cdot}5 \times 10^{-1}$	$5{\cdot}1 \times 10^{2}$	$6{\cdot}6 \times 10^{0}$	$5{\cdot}4 \times 10^{2}$	$6{\cdot}2 \times 10^{0}$	—	—
C_2H_5	$9{\cdot}0 \times 10^{0}$	$1{\cdot}6 \times 10^{0}$	$4{\cdot}8 \times 10^{2}$	$7{\cdot}3 \times 10^{0}$	$5{\cdot}3 \times 10^{0}$	$1{\cdot}7 \times 10^{0}$	$2{\cdot}4 \times 10^{1}$	$3{\cdot}8 \times 10^{0}$
nC_3H_7	$5{\cdot}5 \times 10^{2}$	$2{\cdot}2 \times 10^{1}$	$1{\cdot}2 \times 10^{3}$	$3{\cdot}3 \times 10^{1}$	$7{\cdot}5 \times 10^{0}$	$3{\cdot}1 \times 10^{0}$	$1{\cdot}5 \times 10^{2}$	$4{\cdot}3 \times 10^{1}$
iC_3H_7	$9{\cdot}5 \times 10^{-1}$	$2{\cdot}9 \times 10^{0}$	$5{\cdot}4 \times 10^{2}$	$6{\cdot}2 \times 10^{0}$	$5{\cdot}4 \times 10^{-1}$	$4{\cdot}6 \times 10^{-1}$	$<10^{-1}$	$<10^{-1}$
nC_4H_9	$2{\cdot}6 \times 10^{3}$	$1{\cdot}2 \times 10^{1}$	$4{\cdot}5 \times 10^{3}$	$3{\cdot}5 \times 10^{1}$	$2{\cdot}7 \times 10^{1}$	$4{\cdot}7 \times 10^{0}$	$5{\cdot}1 \times 10^{3}$	$1{\cdot}9 \times 10^{2}$
iC_4H_9	$7{\cdot}1 \times 10^{3}$	$1{\cdot}8 \times 10^{1}$	$2{\cdot}3 \times 10^{3}$	$1{\cdot}9 \times 10^{1}$	$2{\cdot}7 \times 10^{1}$	$3{\cdot}4 \times 10^{0}$	$2{\cdot}6 \times 10^{1}$	$2{\cdot}8 \times 10^{0}$
sec C_4H_9	$2{\cdot}2 \times 10^{0}$	$1{\cdot}4 \times 10^{0}$	$6{\cdot}7 \times 10^{2}$	$3{\cdot}3 \times 10^{0}$	$1{\cdot}7 \times 10^{0}$	$1{\cdot}5 \times 10^{0}$	$<10^{-1}$	$<10^{-1}$
nC_5H_{11}	$6{\cdot}3 \times 10^{3}$	$1{\cdot}5 \times 10^{1}$	$1{\cdot}1 \times 10^{4}$	$4{\cdot}0 \times 10^{1}$	$2{\cdot}0 \times 10^{2}$	$7{\cdot}7 \times 10^{1}$	$2{\cdot}3 \times 10^{4}$	$6{\cdot}0 \times 10^{2}$
iC_5H_{11}	—	—	—	—	—	—	$8{\cdot}1 \times 10^{3}$	—
$3C_5H_{11}$	—	—	$4{\cdot}4 \times 10^{2}$	—	—	—	—	—
	—	—	$3{\cdot}6 \times 10^{3}$	$4{\cdot}0 \times 10^{1}$	—	—	—	—
	—	—	$1{\cdot}5 \times 10^{4}$	$1{\cdot}5 \times 10^{1}$	—	—	—	—
	—	—	$1{\cdot}4 \times 10^{4}$	$1{\cdot}6 \times 10^{1}$	—	—	—	—
$(CH_3)_3C.CH(CH_3)$	—	—	$4{\cdot}0 \times 10^{2}$	$9{\cdot}4 \times 10^{-1}$	—	—	—	—

TABLE 2. RATE CONSTANTS (1 mol^{-1}/min^{-1}) OF THE REACTION OF CHYMOTRYPSIN (CT) AND TRYPSIN (T) WITH A NUMBER OF SUBSTITUTED PHENYL COMPOUNDS

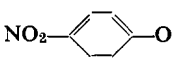
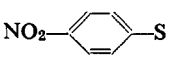
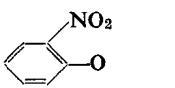
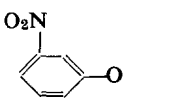
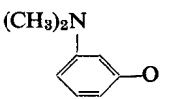
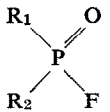
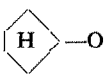
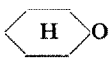
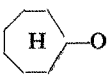
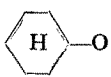
X	$(CH_3O)_2P(O)X$		$(C_2H_5O)_2P(O)X$		$(CH_3O)(CH_3)P(O)X$		$(C_2H_5O)(C_2H_5)P(O)X$	
	CT	T	CT	T	CT	T	CT	T
	$2{\cdot}2 \times 10^{0}$	$2{\cdot}5 \times 10^{-1}$	$9{\cdot}0 \times 10^{0}$	$1{\cdot}6 \times 10^{0}$	$5{\cdot}1 \times 10^{2}$	$6{\cdot}6 \times 10^{0}$	$8{\cdot}2 \times 10^{0}$	$1{\cdot}9 \times 10^{0}$
	—	—	$2{\cdot}5 \times 10^{0}$	$1{\cdot}6 \times 10^{0}$	—	—	$1{\cdot}3 \times 10^{1}$	$4{\cdot}8 \times 10^{0}$
	$1{\cdot}3 \times 10^{1}$	$7{\cdot}5 \times 10^{-1}$	$1{\cdot}0 \times 10^{1}$	$8{\cdot}5 \times 10^{-1}$	$1{\cdot}5 \times 10^{2}$	$6{\cdot}4 \times 10^{0}$	$2{\cdot}0 \times 10^{1}$	$2{\cdot}3 \times 10^{0}$
	$8{\cdot}9 \times 10^{-1}$	$8{\cdot}6 \times 10^{-1}$	$4{\cdot}1 \times 10^{0}$	$<10^{-1}$	$1{\cdot}2 \times 10^{1}$	$1{\cdot}4 \times 10^{0}$	$5{\cdot}0 \times 10^{0}$	$<10^{-1}$
	$<10^{-1}$	$<10^{-1}$	$<10^{-1}$	$<10^{-1}$	$3{\cdot}1 \times 10^{-1}$	$4{\cdot}6 \times 10^{-1}$	$<10^{-1}$	$<10^{-1}$

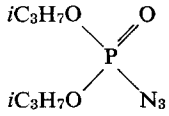
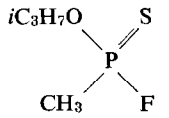
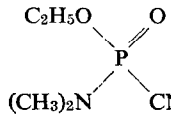
TABLE 3. RATE CONSTANTS (1 mol^{-1}/min^{-1}) OF THE REACTION OF CHYMOTRYPSIN (CT) AND TRYPSIN (T) WITH A NUMBER OF PHOSPHORO- AND PHOSPHONOFLUORIDATES



R_1	R_2	k		R_1	R_2	k	
		CT	T			CT	T
CH_3O	CH_3O	$1{\cdot}7 \times 10^3$	$4{\cdot}2 \times 10^2$	CH_3	$(CH_3)_2CH{\cdot}CH(CH_3)O$	$2{\cdot}0 \times 10^5$*	$6{\cdot}8 \times 10^2$
C_2H_5O	C_2H_5O	$1{\cdot}6 \times 10^4$	$2{\cdot}5 \times 10^3$	CH_3		$\geqslant 2 \times 10^6$*	$3{\cdot}7 \times 10^3$
nC_3H_7O	nC_3H_7O	$2{\cdot}0 \times 10^6$	$1{\cdot}8 \times 10^4$	CH_3		$\geqslant 2 \times 10^6$*	$2{\cdot}4 \times 10^3$
iC_3H_7O	iC_3H_7O	$1{\cdot}5 \times 10^4$	$9{\cdot}7 \times 10^2$	CH_3		$\geqslant 2 \times 10^6$*	$3{\cdot}6 \times 10^3$
CH_3	iC_3H_7O	$2{\cdot}3 \times 10^4$*	$1{\cdot}8 \times 10^3$	C_2H_5	C_2H_5O	$3{\cdot}4 \times 10^4$*	$2{\cdot}1 \times 10^3$
CH_3	$(CH_3)_2CH{\cdot}CH_2{\cdot}CH(CH_3)O$	$8{\cdot}0 \times 10^5$*	$1{\cdot}3 \times 10^3$	C_2H_5		$\geqslant 2 \times 10^6$*	$1{\cdot}9 \times 10^3$
CH_3	$(CH_3)_3C{\cdot}CH(CH_3)O$	$2{\cdot}0 \times 10^5$*	$2{\cdot}7 \times 10^2$	C_2H_5O	$N(CH_3)_2$	—	$2{\cdot}4 \times 10^2$

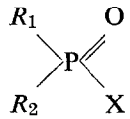
* Rate constant for the fast reacting stereoisomer.

TABLE 4. RATE CONSTANTS (1 mol^{-1}/min^{-1}) OF THE REACTION OF CHYMOTRYPS IN (CT) AND TRYPSIN (T) WITH SOME MISCELLANEOUS COMPOUNDS

Compound	k	
	CT	T
	$8{\cdot}0 \times 10^{1}$	$<10^{-1}$
	$5{\cdot}0 \times 10^{2}$	$1{\cdot}8 \times 10^{2}$
	$1{\cdot}6 \times 10^{3}$	$2{\cdot}6 \times 10^{2}$

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 papers on the inhibition of aliesterase[8] and of acetylesterase[9] we have compared the rate constants of the inhibition reactions 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 6 we have listed the results obtained with a series of diethylphosphoryl- and ethyl ethylphosphonyl compounds (from Tables 1–3). The rates of hydrolysis were taken from Ginjaar[13] whereas the pK_a values were taken from Albert and Serjeant.[14]

From the results of Table 6 we observe a definite correlation between the pK_a and the rates of enzyme inhibition as in the case of the inhibition of acetylesterase[9] and contrary to the case of aliesterase.[8] With chymotrypsin and trypsin the correlation is even better than with acetylesterase where diisopropyl phosphorazidate showed a very high reaction rate compared with the rate of alkaline hydrolysis. Here, the compound (Table 4) reacts only slowly with the enzymes in agreement with its slow hydrolysis. The only exception seems to be the slow rate of the diethyl S-*p*-nitrophenyl phosphorothiolate both with chymotrypsin and trypsin compared with the rate of alkaline hydrolysis and the pK_a of the *p*-nitrothiophenol. This exception was not found with the other enzymes investigated (aliesterase, acetylesterase, acetylcholinesterase and butyrylcholinesterase).

TABLE 5. THE INFLUENCE OF THE TEMPERATURE ON THE RATE OF REACTION (1 mol^{-1}/ min^{-1}) OF CHYMOTRYPSIN AND TRYPSIN WITH A NUMBER OF COMPOUNDS

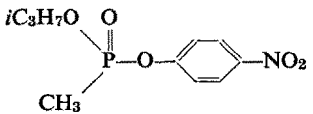
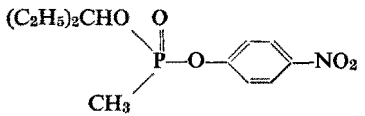
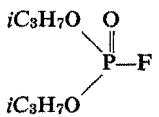
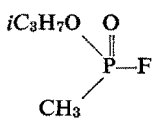
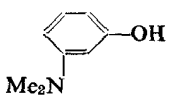
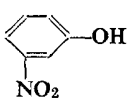
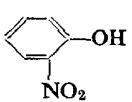
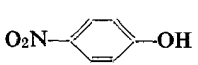
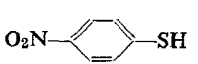
Compound	Temperature	Chymotrypsin		Trypsin	
		k	E kcal/mol	k	E kcal/mol
	18·0°	$3{\cdot}8 \times 10^{2}$			
	25·0°	$5{\cdot}4 \times 10^{2}$	9·6		
	30·0°	$7{\cdot}4 \times 10^{-2}$			
	10·0°	$2{\cdot}0 \times 10^{2}$			
	25·0°	$4{\cdot}4 \times 10^{2}$	9·0		
	37·0°	$8{\cdot}3 \times 10^{2}$			
	5·0°	$5{\cdot}3 \times 10^{3}$		$2{\cdot}9 \times 10^{2}$	
	20·0°	—		$7{\cdot}4 \times 10^{2}$	
	25·0°	$1{\cdot}4 \times 10^{4}$	9·3	$9{\cdot}7 \times 10^{2}$	9·3
	35·0°	$2{\cdot}8 \times 10^{4}$		$1{\cdot}4 \times 10^{3}$	
	10·8°			$6{\cdot}5 \times 10^{2}$	
	20·0°			$1{\cdot}2 \times 10^{3}$	
	25·0°			$1{\cdot}8 \times 10^{3}$	10·8
	35·0°			$3{\cdot}0 \times 10^{3}$	

TABLE 6. THE INFLUENCE OF THE pK_a OF HX ON THE RATE CONSTANTS OF THE HYDROLYSIS AND OF THE INHIBITION OF CHYMOTRYPSIN (CT) AND TRYPSIN (T) BY A NUMBER OF PHOSPHORYL AND PHOSPHONYL COMPOUNDS

HX	pK_a of HX	$(EtO)_2P(=O)X$			$EtO(EtO)P(=O)X$		
		log k_{OH^-}	log k_{CT}	log k_T	log k_{OH^-}	log k_{CT}	log k_T
	approx 11·8	−1·60	<0	<0	−0·44	<0	<0
	8·4	−0·42	0·61	<0	0·77	0·70	<0
	7·2	0·12	1·01	−0·07	1·35	1·31	0·35
	7·1	−0·18	0·95	0·20	0·94	0·91	0·28
	approx 4·9	1·18	0·38	<0	1·26	1·01	0·68
HF	3·2	2·15	4·20	3·39	3·08	4·53	3·33

On the whole, rate constants for chymotrypsin are definitely higher than for trypsin, a fact that corresponds with the results of Mounter *et al.*[5]

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

Generally speaking, the influences of the structure of the groups R_1 and R_2 on the rate of inhibition are qualitatively the same for both chymotrypsin and trypsin. We will discuss in a more or less systematic way the structure-activity relations for both enzymes. The results are presented in Tables 1 and 3.

Dialkyl phosphates. There is a definite rise in the rate constants for both chymotrypsin and trypsin going from methyl to *n*-propyl as well with the *p*-nitrophenyl compounds as with the fluoridates. In the case of chymotrypsin the rate constants continue to increase up to *n*-pentyl whereas in the case of trypsin the rate constants remain constant.

These results are in marked contrast with the alkaline hydrolysis where the rate constants decrease when the alkylchains are lengthened.[15, 16] Our results are in agreement with those of Mounter *et al.*[5]

Alkyl methylphosphonates. The same tendency is observed as with the phosphates but on a smaller scale. Again this result is in contrast with the results obtained with the alkaline hydrolysis. It seems that the influence of changes in the alkyl group on inhibition rates in the series of the fluoridates are greater than in the series of the *p*-nitrophenyl compounds (c.f. the difference in rates of the isopropyl compound with the pinacolyl compound). A very high reactivity was found with the cycloalkyl compounds, these compounds are the most active chymotrypsin inhibitors investigated. With our technique a precise determination of the rate constants was not even possible. In the series of the fluoridates chymotrypsin showed marked stereospecificity (see below).

Isopropyl alkyl phosphonates. For both enzymes a minimum is observed: with the *n*-propyl compound for chymotrypsin and with the ethyl compound for trypsin. The differences are much greater for chymotrypsin than for trypsin.

Alkaline hydrolysis shows a lowering with the lengthening of the alkyl chain. The minimum could be explained as the result of the lowering of the reactivity (as expressed in the hydrolysis) with the lengthening of the alkyl chain and an increase in interaction.

The rate constant of the inhibition of chymotrypsin with the isopropyl compound is remarkably low.

Dialkylphosphinates. For both enzymes the effects are completely the reverse compared with the alkaline hydrolysis and the inhibition of acetylesterase: there is a marked rise in the rate constants as the alkyl groups are lengthened. The rate constants of the compounds with secondary alkyl groups are very low compared with the *n*-alkyl compounds.

Isosteric substitution. If we compare isosteric phosphates with phosphinates in the *p*-nitrophenyl series (e.g. diethyl *p*-nitrophenyl phosphate with *p*-nitrophenyl di-*n*-propyl-phosphinate) we observe that the phosphinates have rate constants about a factor 10 higher than the corresponding phosphates (except the diisobutyl-phosphinate). This is in general agreement with the alkaline hydrolysis and in contrast with the results obtained with acetylesterase.

General remarks on the influence of the structure of the groups R_1 *and* R_2 *on the reactivity.* In general the effects observed do not parallel those found with the alkaline hydrolysis,[11, 13] this in contrast to the inhibition of acetylesterase.[9] The results lead to the following requirements for a compound with a high reaction rate.

Chymotrypsin

(a) dialkoxy- and dialkyl-groups, if present, have to be primary and have to contain 4 C-atoms;

(b) alkylgroups in the presence of an alkoxy group, have to be small.

Trypsin

(a) dialkoxy- and dialkylgroups, if present, have to be primary and have to contain 3 C-atoms for alkoxy- and 4–5 C-atoms for alkylgroups;

(b) alkylgroups in the presence of an alkoxygroup, have to be small. In Fig. 1 the rate constant of the inhibition of chymotrypsin and trypsin 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 reference compound with rate constants k°_{enz} and k°_{OH} respectively.

The graphs show ($\log k^{i}_{enz} - \log k^{\circ}_{enz}$) plotted against ($\log k^{i}_{OH} - \log k^{\circ}_{OH}$). Thus the dimethyl compound gives by definition the origin. Compounds which combine high reaction rate with the enzyme and low rate of hydrolysis and which may be of practical significance are situated in the upper left hand corner. They are virtually absent in the case of trypsin.

Stereospecificity of the inhibition of chymotrypsin

The course of the reaction of chymotrypsin with sarin (isopropyl methylphosphonofluoridate) did not follow second order kinetics. The first part of the reaction was too fast, the latter part too slow. This effect was explained by Michel[17] by assuming that the two possible stereoisomers of sarin reacted with different rates with the enzyme (for a further introduction on the stereospecificity of enzymes for inhibitors see the paper of Ooms and Boter[18]).

Using the formulae in the Appendix we were able to calculate both rate constants. This was also the case in the reaction of chymotrypsin with 3-methylbutyl-2 methylphosphonofluoridate and with ethyl ethylphosphonofluoridate. In the other cases the rate constant for the slowest component could be determined after the reaction with the fastest component was terminated. The results found are given in Table 7.

From the results of Table 7 it is evident that chymotrypsin does show stereospecificity for organophosphorus inhibitors, in some cases very markedly. Three compounds have, besides an asymmetric phosphorus atom, an asymmetric carbon atom and thus contain four diastereoisomers. However the reaction course with chymotrypsin could be explained completely by assuming the existence of only two components reacting with different rates with the enzyme. It seems that two pairs of diastereoisomers are present showing different rate constants with the enzyme but that there is no difference in rate within the pairs. From experiments with resolved compounds which will be presented in a later publication, we could show that chymotrypsin is very sensitive to asymmetry around the central phosphorus atom but much less sensitive to asymmetry

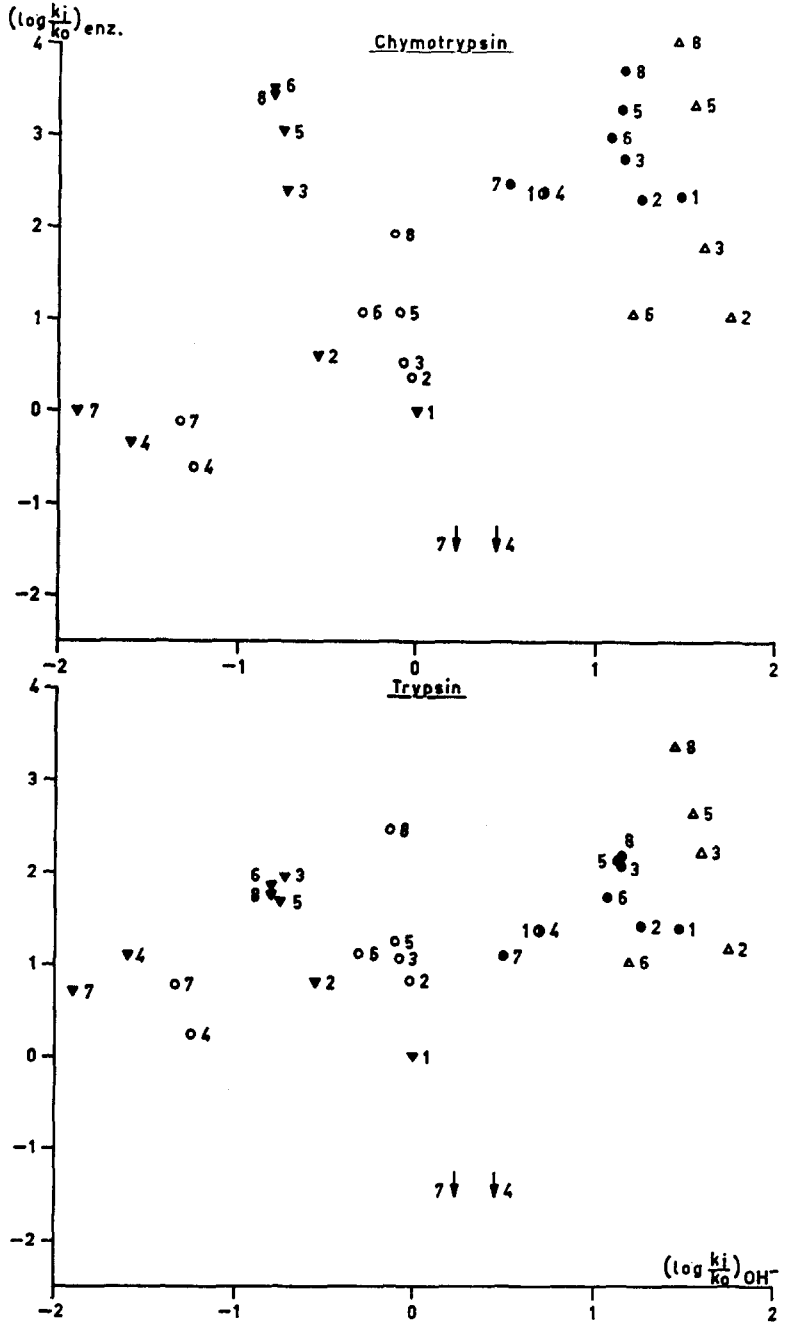
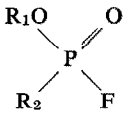


FIG. 1. Relation between the rate constants of the inhibition of chymotrypsin and of trypsin and the alkaline hydrolysis of a number of *p*-nitrophenyl compounds.

▼—$(RO)_2$ P(O) $OC_6H_4NO_2$; ○—(*i*PrO) RP(O) $OC_6H_4NO_2$; ●—(RO)Me P(O) $OC_6H_4NO_2$; △—$R_2P(O)$ $OC_6H_4NO_2$.

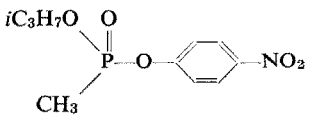
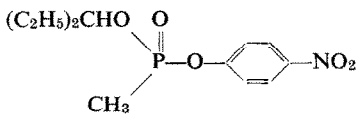
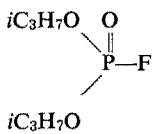
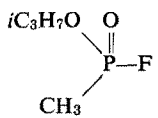
1 R = Me	5 R = *n* Bu
2 R = Et	6 R = *i* Bu
3 R = *n*Pr	7 R = *sec* Bu
4 R = *i*Pr	8 R = *n*Pe.

TABLE 7. RATE CONSTANTS (1 mol^{-1}/min^{-1}) OF THE FAST AND THE SLOWLY REACTING COMPONENT (k_1 AND k_2 resp.) OF THE REACTION OF ALKYLPHOSPHONOFLUORIDATES WITH CHYMOTRYPSIN AND THEIR RATIOS OF ACTIVITY



R_1	R_2	k_1	k_2	k_1/k_2	R_1	R_2	k_1	k_2	k_1/k_2
iC_3H_7	CH_3	$2{\cdot}2 \times 10^4$	3×10^3	7·6		CH_3	$\geqslant 2 \times 10^6$	$1{\cdot}3 \times 10^5$	$\geqslant 15$
$(CH_3)_2CH.CH(CH_3)$	CH_3	$2{\cdot}0 \times 10^5$	$3{\cdot}2 \times 10^4$	6·3		CH_3	$\geqslant 2 \times 10^6$	$6{\cdot}6 \times 10^4$	$\geqslant 30$
$(CH_3)_2CH.CH_2.CH(CH_3)$	CH_3	$8{\cdot}0 \times 10^5$	$1{\cdot}0 \times 10^4$	80		CH_3	$\geqslant 2 \times 10^6$	$7{\cdot}5 \times 10^4$	$\geqslant 27$
$(CH_3)_3C.CH(CH_3)$	CH_3	$2{\cdot}0 \times 10^5$	$<10^3$	>100					
C_2H_5	C_2H_5	$3{\cdot}4 \times 10^4$	$4{\cdot}9 \times 10^3$	6·9		C_2H_5	$\geqslant 2 \times 10^6$	$1{\cdot}6 \times 10^5$	$\geqslant 12{\cdot}5$

TABLE 8. ACTIVATION ENTHALPY, ACTIVATION ENTROPY AND FREE ENTHALPY OF ACTIVATION OF THE REACTION OF SOME ORGANOPHOSPHORUS COMPOUNDS

	OH^-			Chymotrypsin			Trypsin		
	ΔH^* *k*cal/mol	ΔS^* e.u.	ΔG^* *k*cal/mol	ΔH^* *k*cal/mol	ΔS^* e.u.	ΔG^* *k*cal/mol	ΔH^* kcal/mol	ΔS^* e.u.	ΔG^* *k*cal/mol
	11·4	−23·6		9·0	−24·0	16·2			
				8·4	−26	16·2			
	21·2	−12·7	25·0	8·7	−18·3	14·0	8·7	−24·0	15·6
							10·2	−16·5	15·1

around carbon in the alkoxy group. With these results it is probable that the stereospecificity of chymotrypsin for all compounds of Table 7 are all caused by phosphorus asymmetry.

The influence of the temperature on inhibition

In Table 5 the results are shown obtained with four compounds. The results obey Arrhenius' law and the following values were calculated.

In all cases a large negative value for the activation entropy is observed, together with rather low values for the activation enthalpy. This is in agreement with the result obtained with acetylesterase,[9] but in contrast with the results obtained with aliesterase[8] and with the cholinesterases[19] where positive values are found for the activation entropy and large values for the activation enthalpy (>10 kcal/mol). Laidler[20] explains the negative activation entropy in the case of the reaction of an uncharged substrate with an uncharged enzymatic active site as a result for certain electron shifts in the activated complex. The activated complex will then be more polar than the reactants; an increase in electrostriction and a corresponding negative activation entropy will result. On the other hand, when charge neutralization occurs, the release of water molecules can give rise to an increase of activation entropy to positive values.

If we assume that charged groups on the active site of the cholinesterases ("anionic site") and of aliesterase ("cationic site") participate in the phosphorylation reaction and that such groups do not participate in the cases of chymotrypsin, trypsin and acetylesterase, the difference in thermodynamic constants can be explained.

APPENDIX

The calculation of rate constants of the reaction of a racemic inhibitor with an enzyme

The following reactions occur:

$$I_1 + E \xrightarrow{k_1} EI_1$$

$$I_2 + E \xrightarrow{k_2} EI_2$$

The only variable we can measure is the rate of disappearance of E. The initial concentrations of I_1 and I_2 are equal in the case of a racemic inhibitor. We introduce the following symbols:

$$[I_1]_0 = [I_2]_0 = a; \qquad [E]_0 = C$$

The concentration of I_1 that has reacted in time t we call x_1, the concentration of I_2 reacted in the same time x_2 and the concentration of the enzyme x.

The following equation can be derived:

$$x = x_1 + x_2 \tag{1}$$

$$\frac{dx_1}{dt} = k_1(a - x_1)(c - x) \tag{2}$$

$$\frac{dx_2}{dt} = k_2(a - x_2)(c - x) \tag{3}$$

If we define the "ratio of activity" r_a as (k_2/k_1) (see [18]), the division and integration result in

$$\frac{a - x_2}{a} = \left\{\frac{a - x_1}{a}\right\}^{r_a} \tag{4}$$

Substituting (1) in (4) gives:

$$\frac{a - x + x_1}{a} = \left\{\frac{a - x_1}{a}\right\}^{r_a}$$

or

$$x = a + x_1 - a\left\{\frac{a - x_1}{a}\right\}^{r_a} \tag{5}$$

Addition of (2) and (3) gives:

$$\frac{dx}{dt} = (c - x)\{k_1(a - x_1) + k_2(a - x_2)\} = k_1(c - x)\{(a - x_1) + r_a(a - x_2)\} \tag{6}$$

We also have the "normal" second order reaction:

$$\frac{dx}{dt} = k_x(c - x)(2a - x) \tag{7}$$

where k_x is the experimentally determined rate "constant" for every time t. Combination of (6) and (7) results in

$$k_x = k_1 \cdot r_a + k_1(1 - r_a)\frac{a - x_1}{2a - x} \tag{8}$$

With equations (5), (7) and (8) the problem can be solved.

From the experimental data a graph of x against k_x is drawn. With an estimated value of r_a, obtained from k_x values in the beginning and the end of the reaction, a number of values for x are calculated with equation (5) for a number of assumed x_1 values. For the above mentioned graph the corresponding values for k_x can be found. These values, substituted in equation (8) give a series of values for k_1. The iterative method of Lansner[21] is then used to find the value for r_a which gives a constant value for k_1.

With this method a number of rate constants and r_a values from Table 7 were calculated.

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