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REACTIVATION OF ORGANOPHOSPHATE-INHIBITED TRYPSIN BY HYDROXYLAMINE

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SUMMARY

The reactivation of trypsin (EC 3.4.4.4), inhibited by reaction with organophosphorus compounds, by hydroxylamine has been studied. There is a slow reactivation of all the inhibited samples with a pH optimum for the reaction in the range of pH 7.0. Di-*n*-butylphosphoryltrypsin is reactivated more readily than the corresponding diethyl derivative. Di-isopropylphosphoryltrypsin shows a low, but significant, reactivation. Di-*n*-butylphosphoryltrypsin prepared from different organophosphorus compounds behaves similarly. The results are discussed.

INTRODUCTION

The reaction of many hydrolytic enzymes with organophosphorus compounds, such as DFP, takes place as follows:

$$\text{Enzyme} + \text{O}=\overset{\text{R'O}}{\underset{\text{R''}}{\text{P}}}-\text{X} \rightarrow \text{Enzyme}-\overset{\text{OR'}}{\underset{\text{R''}}{\text{P}}}=\text{O} + \text{HX} \qquad (1)$$

where R′ is usually an alkyl group, R″ may be alkyl, alkoxy or dialkylamino and X can be fluoride, cyanide, dichlorovinyl or aromatic. The essential structural factors seem to be that R′ and R″ should possess sufficient stability and that the P–X bond possess some acid anhydride-like character[1]. Enzyme inhibition involves the hydrolytic cleavage of the P–X bond to liberate an anion while the remainder of the organophosphorus compound is attached to the active site of the enzyme preventing further interaction of enzyme and substrate[2]. Although the reaction represented above is essentially irreversible under normal physiological conditions, a number of studies have been made of possible mechanisms of reactivation. WILSON[3] showed that hydroxylamine would produce some reactivation of cholinesterase (EC 3.1.1.8) inhibited with tetramethyl pyrophosphate and CUNNINGHAM[4] obtained partial reactivation of chymotrypsin (EC 3.4.4.5) inhibited with diethyl-*p*-nitrophenyl phosphate. Subsequently, many hydroxylamine and nitroso derivatives have been studied for their reactivating effects on inhibited cholinesterases and

considerable success has been achieved[5]. Several investigations of the reactivation of trypsin (EC 3.4.4.4) and chymotrypsin inhibited with diethylphosphoryl compounds have been made; the diethylphosphoryl group can be removed by a number of nucleophilic reagents[6–9].

Organophosphorus compounds will inhibit a number of esterases and proteases[10,11] and in some instances, it has been possible to correlate the rate of reaction with the alkyl group size of the inhibitor. The present report describes experiments on the reactivation of trypsin inhibited with several different organophosphorus inhibitors.

MATERIALS AND METHODS

Trypsin

The enzyme used was two times crystallized (Worthington Biochemical Corporation). In order to check inactivation by inhibitors, preliminary assays were made by the method of KUNITZ[12] using 1% casein in 0.1 M phosphate buffer (pH 7.8) as substrate. Quantitative determinations were made by potentiometric titration using a recording potentiometric titrator[13]. The substrate benzoyl-L-arginine methylester (0.02 M) was obtained from Mann Research Laboratories. It was dissolved in 0.005 M Tris buffer (pH 7.6). The low buffer concentration stabilized the pH without affecting the sensitivity of determination. Titration was carried out at 25°, at pH 7.6, using 0.05 M KOH to neutralize the acid liberated.

The concentrations of enzyme were estimated by absorbance measurements at 280 mμ in the Beckman DU spectrophotometer using an optical factor of 0.067 (see ref. 14).

Organophosphorus compounds

DFP was donated by the U.S. Army Chemical Center. Diethyl- and di-*n*-butylphosphorofluoridate were prepared by an exchange reaction between the corresponding chlorophosphates (Victor Chemical Co.) and sodium fluoride[15]. *O,O*-Di-*n*-butyl-*O*-*p*-nitrophenyl phosphate was prepared by the method of HARTLEY AND KILBY[16] and *O,O*-di-*n*-butyl-*O*-(1-ethoxy-2,2-dichlorovinyl) phosphate was given by Dr. J. WILLARD of the Niagara Chemical Co. Other reagents were obtained commercially and were of analytical grade.

EXPERIMENTAL AND RESULTS

A sample of 150 mg of trypsin was dissolved in 30 ml of 0.001 M HCl containing 0.02 M $CaCl_2$. Aliquots of 0.1 ml of diethyl-, diisopropyl- and di-*n*-butylphosphorofluoridate were dissolved in three separate 40-ml volumes of 0.05 M Tris buffer (pH 8.0) and 10 ml of the enzyme solution added to each of these flasks. These solutions were stored in the cold overnight and then tested for proteolytic activity. The enzyme was completely inactivated.

Aliquots of 1 ml of the inhibited enzyme were added to 2 ml of 0.05 M Tris buffer (pH 8.0) and 3 ml of 2 M hydroxylamine hydrochloride, brought to pH 8.0 by the addition of sodium hydroxide. These solutions were allowed to stand at

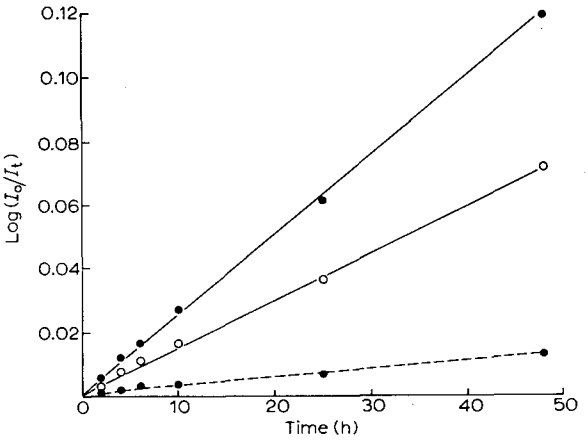

Fig. 1. Time course of reactivation of trypsin inhibited with diethyl- (○—○), diisopropyl- (●— — —●) and di-*n*-butyl- (●—●) phosphorofluoridate. Abscissa, time in hours; ordinate, log I_0/I_t, where I_t/I_0 is the fraction of inhibited enzyme remaining after time t. Reaction carried out at pH 8.0 and in presence of 1 M hydroxylamine.

room temperature; samples of 0.1 ml were drawn off at intervals and their activity against benzoyl-L-arginine methylester determined. A progressive reappearance of enzymic activity was observed (Fig. 1). The control to which reactivation measurements were compared consisted of a sample of uninhibited trypsin (1 mg/6 ml) containing 1 M hydroxylamine and maintained under the same conditions as the samples whose reactivation was being followed.

GREEN AND NICHOLLS[17] showed that the reactivation of isopropylmethylphosphorofluoridate (Sarin)-inhibited chymotrypsin by hydroxamic acids was a bimolecular reaction and that rate constants could be derived from the formula

$$k = \frac{2.3}{At} \log (I_0/I_t) \qquad (2)$$

where A is the concentration of reactivator and I_t/I_0 is the fraction of the inhibited enzyme remaining after time t. The results obtained in the present experiments have been fitted to this equation and presented graphically in Fig. 1. The apparent rate constants are given in Table I.

A further series of samples was set up under similar conditions but using buffers of varying pH. The reactivation reaction was allowed to continue for 48 h and the

TABLE I

APPARENT RATE CONSTANTS FOR REACTIVATION OF ORGANOPHOSPHATE-INHIBITED TRYPSIN BY 1 M HYDROXYLAMINE AT pH 8.0

Organophosphate group	k *(l/mole/h)*
Di-*n*-butylphosphoryltrypsin	0.0057
Di-ethylphosphoryltrypsin	0.0033
Diisopropylphosphoryltrypsin	0.0006

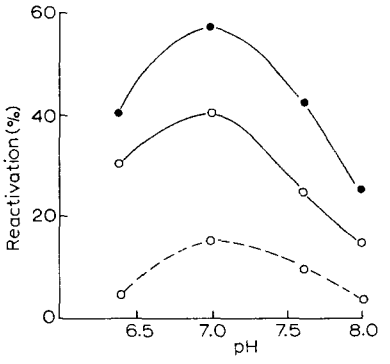

Fig. 2. Effect of pH on reactivation of organophosphate-inhibited trypsin by 1 M hydroxylamine. ○—○, diethylphosphoryltrypsin; ○- - -○, diisopropylphosphoryltrypsin; ●—●, di-*n*-butylphosphoryltrypsin.

activity determined. The results (Fig. 2) show that there is an optimum pH in the region of 7.0, which is the same range found by CUNNINGHAM[4] for the reactivation of diethylphosphorylchymotrypsin.

According to the reactions postulated for the inhibition of trypsin by organophosphorus compounds (Eqn. 1), the product obtained should be dependent only on the nature of the groups R″, R′OP = O and not on the other component of the reaction which is liberated as an anion during the inhibitory reaction. Three samples of trypsin were inhibited by reaction with di-*n*-butyl derivatives and the solutions dialyzed against running tap water for 24 h, against two changes of distilled water and finally with 0.05 M Tris buffer (pH 7.0). The 3 samples were adjusted to equal concentrations with buffer after dialysis and brought to a concentration of 1 M

TABLE II

REACTIVATION BY 1 M HYDROXYLAMINE AT pH 7.0 OF DI-*n*-BUTYLPHOSPHORYLTRYPSINS DERIVED FROM DIFFERENT DI-*n*-BUTYLPHOSPHATE INHIBITORS

Inhibitors	*Per cent reactivation*	
	24 h	*48 h*
Di-*n*-butylphosphorofluoridate	21	52
O,O-Di-*n*-butyl-*O*-(4-nitro)phenyl phosphate	17	47
O,O-Di-*n*-butyl-*O*-1-ethoxy-2,2-dichlorovinyl) phosphate	23	56

hydroxylamine (pH 7.0). These samples were allowed to stand at room temperature and the activity determined after 24 h and 48 h. The results (Table II show approximately the same degree of reactivation, which agrees with the theory that the 3 products of the initial reactions should be identical.

Incubation of di-*n*-butylphosphorofluoridate-inhibited trypsin with saturated

solutions of α-oximino propionic acid, α-oximino butyric acid, β-phenyl-α-oximino propionic acid, and β-(*p*-chlorophenyl)-α-oximino propionic acid produced no significant reactivation.

CONCLUSIONS

The results obtained in this investigation show that treatment of trypsin, that has been inhibited by organophosphorus compounds, with hydroxylamine can produce a considerable degree of reactivation of the enzyme. Complete reactivation is not attained, which is similar to the observations made by CUNNINGHAM[4] with diethyl-*p*-nitrophenylphosphate-inhibited chymotrypsin. Greater reactivation can be obtained by other hydroxamic acids[9] but in the present study the primary objective was to make a comparison of the relative rates of reactivation of trypsin inhibited with different organophosphate esters.

It has been observed previously that it is far more difficult to reactivate hydrolytic enzymes that have been inhibited with diisopropyl-phosphate derivatives than those treated with the corresponding diethyl compounds and it has been suggested that this difference might be due to steric factors[4,18] involving the configuration of the diisopropyl groups. These bulky substituents might limit the attack of the nucleophilic reactivating reagent to the dialkylphosphorylserine group that is believed to be the final locus of the inhibiting reaction of many hydrolytic enzymes[19]. The present results show that this hypothesis may well be correct for there is excellent reactivation of di-*n*-butylphosphoryltrypsin: this takes place even more readily than with the diethylphosphoryl derivative; by contrast, the diisopropylphosphoryltrypsin is only reactivated slowly and to a limited, although significant extent. The rate constants for the inhibitory reactions between homologous series of dialkyl organophosphate compounds and trypsin are in the order: butyl > isopropyl > ethyl[11]; reactivation with hydroxylamine is in the order butyl > ethyl > isopropyl and steric interferences are a logical explanation of this difference.

The proposal that the mechanism of inhibition of hydrolytic enzymes by toxic phosphorus compounds leads to phosphorylated protein derivatives is now generally accepted, although there are still many questions to be answered with respect to details of the process and the effect of the chemical reactions on the final structure and properties of the protein macromolecule. The results presented in Table II support the theory that the end product of the inhibitory reaction is determined by the nature of the dialkylphosphoryl group attached to the enzyme molecule and not by the group liberated in the reaction. It has been shown with chymotrypsin that fluoride is released in the reaction with DFP[2], *p*-nitrophenol by the reaction of diethyl-*p*-nitrophenyl phosphate[16] and dichloroacetaldehyde is derived from *O,O*-di-*n*-propyl-*O*-2,2-dichlorovinyl phosphate[20]. The di-*n*-butylphosphoryltrypsin obtained in the present studies from corresponding compounds appears to be reactivated similarly when treated with hydroxylamine.

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