The Inhibition of Hydrolytic Enzymes by Organophosphorus Compounds*

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It has been shown that several hydrolytic enzymes with different specificities undergo similar reactions with diisopropyl phosphofluoridate (1) and other related toxic organophosphorus compounds (2). These conclusions were based on kinetic studies, particularly the influence of pH on the rates of reaction, and on inhibition characteristics. Reactions of this type have provided a unique method of attacking the problem of the nature of the active site of enzymes, supplementing the approaches via substrate specificity studies and the structural analysis of the enzyme protein itself (3-5). Although the physiological response of animals intoxicated with organophosphorus esters has led to the recognition of the anticholinergic action of these substances, there is evidence that reaction with other enzymes may influence toxicity and a more precise understanding of the specificity of action of various organophosphorus compounds is desirable (6). The present report describes the effect of a number of organophosphorus compounds on seven hydrolytic enzymes, comprising three esterases (acetylcholinesterase, plasma cholinesterase, and phosphorus compounds) and four proteolytic enzymes (chymotrypsin, trypsin, plasmin, and thrombin).

EXPERIMENTAL PROCEDURE

Enzymes—The following enzymes were used.
(a) α-Chymotrypsin, obtained from Worthington Biochemical Corporation as a twice crystallized material, was assayed by casein hydrolysis by the method of Anson (7). Solutions of chymotrypsin (1 mg per ml in 0.001 N HCl) were diluted immediately before use to a concentration of 10 µg per ml with 0.5 M Tris buffer, pH 7.8. Serial dilutions of the inhibitors were prepared and added to samples of the enzyme, and the mixtures were incubated for 30 minutes at 37°C. Aliquots (0.5 ml) were added to 2 ml of 1% casein in Tris buffer (pH 7.8, 0.05 M) and incubated at 37°C. The addition of 5% trichloroacetic acid stopped the reaction and precipitated undigested casein. One sample was precipitated immediately after mixing with the substrate; other samples were precipitated at intervals during a period of 1 hour. The precipitates were removed by filtration, and 5 ml of the filtrate were made alkaline with 10 ml of 0.5 N NaOH prior to the addition of 3 ml of diluted Folin-Ciocalteau reagent. The intensity of the resulting blue color was measured at 650 mµ in a Bausch and Lomb Spectronic 20 colorimeter.

(b) Trypsin, obtained from Worthington Biochemical Corporation as a twice crystallized material, was assayed as for chymotrypsin (7). The trypsin solutions (1 mg per ml in 0.001 N HCl) were diluted immediately before use to 10 µg per ml with Tris buffer at pH 7.8, 0.05 M, containing 0.03 M Ca++. The Ca++ stabilized the enzyme in this high pH range (8). Serial dilutions of inhibitor were incubated with trypsin for 30 minutes at 37°C before determining enzyme activity.

(c) Plasmin, derived from plasminogen, was assayed by casein hydrolysis (9), which is essentially the same method as that described for chymotrypsin. The plasminogen was prepared from fraction III of human blood by the method of Kline (10), activated by the addition of 1000 units of streptokinase (provided as a lyophilized powder (Varidase) by Lederle Laboratories Division, American Cyanamid Company) and incubated for 5 minutes at 37°C prior to the addition of any inhibitor.

(d) Thrombin, obtained from Parke, Davis and Company, was assayed by Laki's (11) procedure for the clotting time of fibrinogen. To 0.1 ml of thrombin solution (0.5 mg per ml) was added 0.2 ml of Tris buffer (pH 7.8, 0.05 M) and 0.2 ml of inhibitor solution, and the mixture was incubated for 30 minutes at 37°C. At the end of this time, 4 ml of 1% fibrinogen, prepared by the method of Ferguson (12), in 0.05 M phosphate buffer (pH 7.5) were added, and the tube was transferred immediately to the Bausch and Lomb Spectronic 20 colorimeter. The transmittance, initially set at 100% at 640 mµ, was read at 15-second intervals. When these readings were plotted against time, a sigmoid curve was obtained, and the point of inflection was taken as a measure of clotting time. Enzymic activity was expressed as the reciprocal of clotting time.

(e) Acetylcholinesterase, derived from bovine erythrocytes, was obtained from Nutritional Biochemicals Corporation. The activity was determined manometrically by the method of Ammon (13). The enzyme solution (2 mg per ml) was made up in 0.025 M NaHCO₃ immediately before use. Inhibitor (0.1 ml) and enzyme (0.2 ml) were incubated together for 30 minutes at 37°C in the side arm of the flasks before tipping into the main compartment that contained 3 ml of the substrate acetyl β-methylcholine chloride (0.030 M dissolved in 0.025 M NaHCO₃) obtained from Distillation Products Industries. Equilibration with a gas phase of 85% N₂-5% CO₂ gave a pH of 7.4. Under these conditions, the uninhibited enzyme hydrolyzed 0.030 M acetyl β-methylcholine chloride at a rate of 55 µmoles per mg per hour.

(f) Cholinesterase, donated by A. Kabi, Stockholm, Sweden, was a partially purified product from human placental blood. The activity was determined manometrically as for acetyl-
Inhibition of hydrolytic enzymes by organophosphorus compounds

Organophosphorus compounds

<table>
<thead>
<tr>
<th>Inhibition</th>
<th>Chymotrypsin</th>
<th>Trypsin</th>
<th>Plasmin</th>
<th>Thrombin</th>
<th>Wheat germ esterase</th>
<th>Cholinesterase</th>
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<td>4.0</td>
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*The abbreviation used is: DFP, diisopropyl phosphorofluoridate.*

cholinesterase but with 0.015 M acetylcholine bromide (Distillation Products Industries) as substrate. The enzyme concentration was 0.4 mg per ml and hydrolyzed 0.015 M acetylcholine bromide at the rate of 120 umoles per mg per hour.

(g) Wheat germ esterase was obtained from Worthington Biochemical Corporation. The activity was determined manometrically at pH 7.4 with 0.1 M triacetin as substrate (14). The enzyme concentration was 25 mg per ml and 0.2 ml of this solution was incubated in the side arm of the flask with 0.1 ml of inhibitor for 30 minutes at 37° before tipping. The control enzyme hydrolyzed 0.1 M triacetin at a rate of 7 umoles per mg per hour.

Inhibitors—DFP; isopropylmethyl phosphonofluoridate (Sarin) and ethyl N-dimethylphosphoramidocyanate (Tabun) were donated by the United States Army Chemical Corps. Diethyl phosphorofluoridate (b.p. 68° at 17 mm) and di-1-butyl phosphorofluoridate (b.p. 127° at 30 mm) were prepared as described previously (2); they were purified by fractional distillation, and the middle third of the distillate was retained. O,O-Diethyl-O-(4-nitrophenyl) and O,O-di-1-butyl-O-(4-nitrophenyl)-phosphates were prepared as described by Hartley and Kilby (15). Other organophosphorus compounds were provided by Dr. J. R. Willard of the Niagara Chemical Company, and were representative compounds of analytical purity from a large number of potential pesticides.

**Determination of Inhibition**—The reaction of organophosphorus compounds with esterases and proteases to give a phosphorylated enzyme derivative (16, 17) may be represented as follows:

Enzyme + (RO)zPO-X → Enzyme-OP(OR)z + HX

To make a true comparison of the relative effects of different inhibitors, the bimolecular rate constants for reactions of the type indicated in the equation above should be determined (18).

In surveys of the nature described in this report, these experiments are very time consuming. If the concentration of enzyme, pH, and time of reaction are kept constant, then the extent of inhibition in a particular system is proportional to the concentration of inhibitor (2). In most cases, there is a direct relationship between the negative logarithm of the concentration of inhibitor required to produce 50% inhibition (pI50) under standard conditions and the value of the bimolecular rate constant (k). It must be emphasized that this form of comparison is valid only if studies are made on a single enzyme system with a series of inhibitors and it is only an approximation in extremely fast or slow reactions (6). In the present investigation, the enzyme assays were performed after the inhibitor and enzyme had been allowed to react together for 30 minutes at 37° and pH 7.8 before adding the substrate. The percentage inhibition...
observed in each determination was plotted against the negative logarithm of the inhibitor concentration. The intercepts of these curves with the lines representing 50% and 30% inhibition gave the values of $p_{150}$ and $p_{130}$, respectively (Fig. 1). The $p_{130}$ value indicates the extent of inhibition for slow reactions where high degrees of inactivation may not be observed due to limitations of inhibitor concentration or time of reaction.

**RESULTS**

Data for the inhibiting effects of the series of organophosphorus compounds used in this study on seven enzymes are summarized in Table I. Representative data for the inhibition of the enzymes by $O,O$-di-(1-butyl)-$O$-(1-ethoxy-2,2-dichlorovinyl) phosphate (I)

$$
\begin{align*}
\text{C}_4\text{H}_8\text{O} & \text{OC}_4\text{H}_4 \quad \text{O-P-C=CCl}_2 \\
\text{C}_4\text{H}_8\text{O} & 
\end{align*}
$$

are presented in Fig. 1 where the logarithmic scale of inhibitor concentration shows the wide differences in the rates of reaction of the inhibitor with the various enzymes. More information can be obtained from this figure when it is considered in relation to the experimental conditions. The curve for chymotrypsin is very steep, there being a sharp fall in the inhibition between $10^{-4}$ and $10^{-7}$ M; above this concentration, there is complete inhibition of the enzyme. When the concentration of chymotrypsin reacting with the inhibitor was calculated (assuming a molecular weight of 25,000 (19)), it was found to be approximately $4 \times 10^{-5}$ M. If the reaction between enzyme and inhibitor is bimolecular, where one molecule of inhibitor combines with one molecule of enzyme, lower inhibitor concentrations could only give partial inhibition. The observed results suggest that there is a rapid reaction between this inhibitor and chymotrypsin. A further experiment was made to study the kinetics of the reaction between $O,O$-di-(1-butyl)-$O$-(1-ethoxy-2,2-dichlorovinyl) phosphate and chymotrypsin. A solution of the enzyme (5 X $10^{-4}$ M) was mixed with a solution of the inhibitor (4 X $10^{-6}$ M) and samples were assayed for activity at intervals. Immediately after mixing, there was an appreciable loss of activity when the enzymic hydrolysis was compared to that of a control enzyme sample without added inhibitor: at the end of 15 minutes, only 20% of the enzymic activity remained, indicating that all of the inhibitor had reacted in this time. These data, together with additional results for the reaction of the inhibitor (4 X $10^{-5}$ M) with trypsin and for the reaction of the two enzymes with DFP, are presented in Fig. 2. It is impossible to calculate a rate constant for this fast reaction, since the method is valid only for relatively slow reactions in which there is an excess of inhibitor present.

Many of the toxic organophosphorus esters are hydrolyzed spontaneously in dilute solutions and especially at alkaline pH (20). An experiment was performed to determine if the in-
Inhibitors were stable under the experimental conditions corresponding to those under which the results presented in Fig. 1 were obtained. Solutions of two of the inhibitors (DFP and O,0-di(1-butyl)-O-(1-ethoxy-2,2-dichlorovinyl) phosphate) in Tris buffer (pH 8, 0.05 M) were prepared, and the pI_{1/2} for chymotrypsin was determined immediately after preparation of the inhibitor solutions and again after they had stood for 2 hours. It can be seen (Table II) that the chlorovinyl phosphate has considerably greater stability than the phosphorofluoridate and that at concentrations sufficient to produce appreciable inhibition there was relatively little spontaneous hydrolysis during the experiment. Jandorf (17) has also pointed out the relative stability of chlorovinyl phosphates under similar conditions.

**Discussion**

Examination of the results illustrates the importance of alky group size of the dialkoxy substituent of organophosphorus compounds in determining their relative rates of reaction with different enzymes. The proteolytic enzymes, chymotrypsin and trypsin, react more rapidly with those compounds containing the larger alkyl group of 1-butyl, but when the size is increased to that of 2-ethylhexyl, the inhibition of these enzymes decreases. Wheat germ esterase reacts faster with compounds containing small groups. None of the present data conflict with the generalization first established with a more limited series of compounds (2). If a wider range of compounds was investigated, more specific differential rates of reaction might be found with different enzymes.

Determination of the p{I}_{1/2} and p{I}_{10} values for the inhibition of the enzymes has been used in this survey, yet it is apparent that this is invalid for studying the effects of rapidly reacting compounds and does not give a true comparison between different enzymes of unknown molecular weight.

Cholinesterases have a very high turnover number and in partially purified preparations, the molar concentration of active sites is much smaller than that for the proteolytic enzymes used in these studies. This accounts in part for the very high dilutions at which the majority of the organophosphorus compounds will inhibit cholinesterases. Turnover numbers of $5 \times 10^4$ and $5 \times 10^6$ molecules per active center per minute have been reported for bovine erythrocyte acetylcholinesterase and horse plasma cholinesterase, respectively (21, 22). These figures were obtained from data for binding of DFP and rates of hydrolysis of 0.008 mM acetylcholine chloride. In the present investigation, acetyl β-methylcholine chloride (0.03 M) was hydrolyzed by bovine erythrocyte acetylcholinesterase at the rate of 55 pmol/mg per hour, and acetylcholine bromide (0.025 M) was hydrolyzed by the human plasma preparation at 120 pmol/mg per hour. If one assumes that acetyl β-methylcholine is hydrolyzed at approximately half the rate of acetylcholine (23), then the concentration of active centers in the erythrocyte acetylcholinesterase studies is of the order of $10^{-8}$ M; the concentration of human plasma cholinesterase is also in the region of $10^{-8}$ M, if the values for the turnover number are similar to that of the horse plasma enzyme. It is probable, therefore, that inhibition curves that give p{I}_{10} values greater than 7.5 do not represent relative rates of reaction but are titrations of the number of active centers of the cholinesterase.

Care is necessary also in the interpretation of inhibition data where the p{I}_{10} value is low, for chemical contaminants may be responsible for the observed inhibition. This point has been well illustrated in studies of cholinesterase inhibition by parathion preparations; the pure compound (O-O-diethyl-O-4-nitrophenyl thiophosphate) is almost without effect on cholinesterase, but very divergent results have been obtained with impure samples (24). Aldridge and Davison (25) have shown that inhibition due to contaminants can be distinguished by kinetic studies. In the present investigation, no significant effects were found which could be interpreted as being due to impurities in the organophosphorus compounds tested. Reproducible results were obtained when the experimental conditions were maintained constant. Individual determinations of the degree of inhibition at a particular inhibitor concentration might differ by as much as ±10%, but p{I}_{10} values obtained from separate series of experiments with the same enzyme and inhibitor did not differ by more than 0.2 unit.

The large differential effects of some compounds studied is of importance from two points of view. Toxicity may be divorced to an appreciable extent from the ability of a material to react in vitro; the most potent inhibitors of the proteolytic enzymes while highly toxic are not outstanding inhibitors of esterases. The di-1-butyl phosphate derivatives are probably the most effective proteolytic enzyme inhibitors yet recorded; O,O-di(1-butyl)-O-(1-ethoxy-2,2-dichlorovinyl) phosphate appears to react very rapidly with chymotrypsin at a concentration that is almost equal to the molecular weight is of two orders of magnitude less than that of naturally occurring proteolytic inhibitors (26). Compounds of this type might be useful to control the reactions of proteolytic enzymes in biochemical studies, including degradation reactions.

**Summary**

The reactions of a series of organophosphorus compounds with the enzymes acetylcholinesterase, plasma cholinesterase, wheat germ esterase, chymotrypsin, trypsin, plasmin, and thrombin have been studied. Di-1-butyl phosphate derivatives react rapidly with chymotrypsin and trypsin.

**References**

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