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Enzymatic Hydrolysis of Organophosphorus Compounds

V. Effect of Phosphorylphosphatase on the Inactivation of Cholinesterases by Organophosphorus Compounds *in vitro*

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The effect of phosphorylphosphatase of human serum on the inactivation of cholinesterases by organophosphorus compounds *in vitro* was studied.

The esterase inactivation is a progressive reaction. Phosphorylphosphatase cannot reactivate inactivated cholinesterases when the former is added after maximum esterase inactivation is reached. On the other hand, phosphorylphosphatase stops the inactivation at a certain level when added before maximum inactivation is reached.

The results are discussed from a kinetic point of view.

The existence of enzymes, phosphorylphosphatases, catalysing the hydrolytic breakdown of organophosphorus compounds has been reported recently [1,2]. A series of previous papers discussed some properties of the phosphorylphosphatase ("tabunase") of human blood serum [3], the effect of reversible cholinesterase inhibitors on that enzyme [4], and the enzyme specificity [5]. In the present paper the effects were studied when a phosphorylphosphatase preparation was added to cholinesterases, inactivated by various organophosphorus compounds.

METHODS AND MATERIAL

Phosphorylphosphatase and cholinesterase activities were measured by the Warburg technique [1,6]. Fraction IV-1 of human postpartum serum was used as enzyme source for phosphorylphosphatase. Acetylcholinesterase (AChE) was a partly purified preparation of the electric tissue of *Torpedo marmorata*, and Fraction IV-6 of human serum was used as a (butyryl-) cholinesterase (ChE) preparation.

The following organophosphorus compounds were used: tabun (dimethylamidoethoxy-phosphoryl cyanide), DFP (di*iso*propoxyphosphoryl fluoride), TEPP (tetraethylpyrophosphate), and mintacol (E 600, diethyl-*p*-nitrophenyl-phosphate). Acetylcholine chloride was employed as substrate for cholinesterases; its concentration during the esterase determination was usually 1.10×10^{-2} *M* (total volume of the reaction mixture, 2.00 ml).

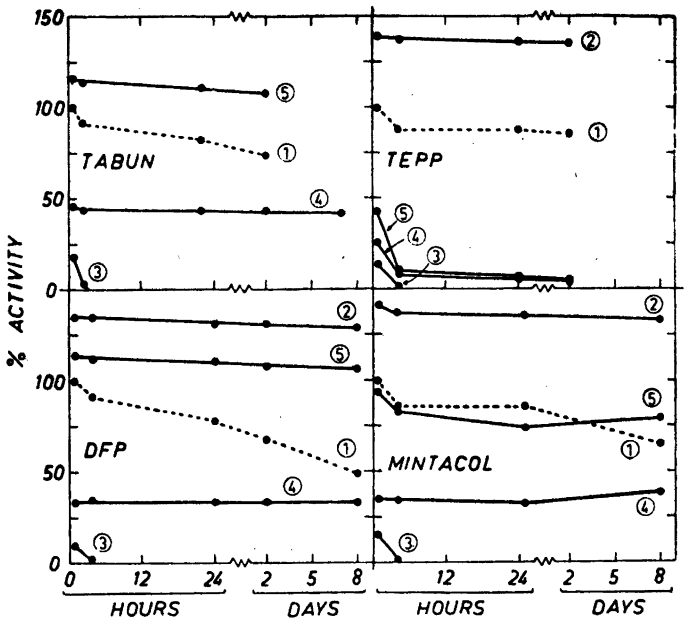

Fig. 1. Effect of phosphorylphosphatase (Fraction IV-1) on acetylcholinesterase (AChE, electric tissue) and on the inhibition of this esterase by various organophosphorus compounds (P) tested after various periods of time. Esterase activity tested against acetylcholine (1.10 × 10^{-2} M). Total volume of the incubation mixture, 1.6 ml, kept at 4° C. Esterase and phosphatase respectively were incubated 15 minutes with the organoposhphorus compound prior to further treatment. Concentrations of the organophosphorus compounds during incubation: tabun, 4.65 × 10^{-6} M; DFP, 1.17 × 10^{-3} M; TEPP, 1.58 × 10^{-5} M; mintacol, 7.42 × 10^{-5} M. 1) AChE; N.B. the phosphatase preparation has an esterase activity which is appr. 9 % of that of AChE; 2) AChE + phosphatase; 3) AChE + P; 4) (AChE + P) + phosphatase; 5) (Phosphatase + P) + AChE.

RESULTS

The effect of phosphorylphosphatase on inactivated cholinesterases. Acetylcholinesterase (AChE) and cholinesterase (ChE) were incubated for 15 minutes with an organophosphorus compound (P). Phosphorylphosphatase was then added and the first measurement of the esterase activity was performed after a further period of 45 minutes (Figs. 1 and 2, No. 4). In another series the phosphorus compound was incubated for 15 minutes with phosphorylphosphatase; then the cholinesterase was added and the activity of the latter determined (No. 5). The concentration of the phosphorus compound in each case was chosen to give 95 % or more inactivation of cholinesterases when the esterase was incubated for 45 or 60 minutes prior to the addition of substrate (acetylcholine) (No. 3). In a control series, the effect of phosphorylphosphatase on the cholinesterase activity was determined (Nos. 1 and 2). All five reaction mixtures were kept at 4° C and the cholinesterase activities tested after various periods of time. The results thus obtained with tabun, DFP, TEPP and mintacol are recorded in Fig. 1 (AChE) and Fig. 2 (ChE).

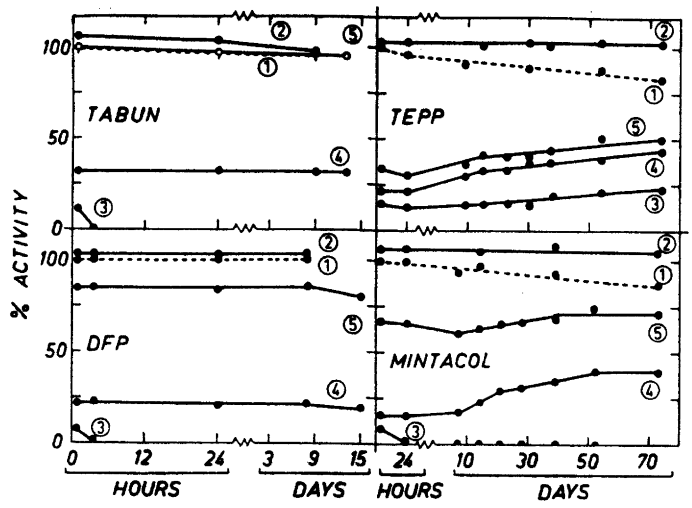

Fig. 2. Effect of phosphorylphosphatase (Fraction IV-1) on serum cholinesterase (Fraction IV-6) and on the inhibition of this esterase by various organophosphorus compounds (P) tested after various periods of time. Concentrations of the organophosphorus compounds during incubation: tabun, 2.33 × 10^{-6} M; DFP, 9.36 × 10^{-7} M; TEPP, 8.44 × 10^{-7} M; mintacol, 4.45 × 10^{-6} M. Numbers and further explanations as in Fig. 1.

It will be noted that the phosphatase preparation (Fraction IV—1) has a weak cholinesterase activity not exceeding 10 % of the activity of the cholinesterase preparation used.

Acetylcholinesterase (Fig. 1). A characteristic property of the phosphatase preparation is that it activates the acetylcholinesterase activity but not the (butyryl)cholinesterase activity (*cf.* Fig. 2). This activation (curve No. 2) will be discussed in a forthcoming publication. As was expected from the results described earlier —, the esterase inhibiting effect of tabun is lost by incubation with phosphatase (No. 5). This is also true for DFP. In both these cases the activating effect of the phosphatase preparation is partly retained even after incubation with the phosphorus compound. The latter effect, however, is lost when phosphatase hydrolysed mintacol. The situation is completely different for TEPP. We know that TEPP is enzymatically hydrolysed by the phosphatase preparation, but contrary to the other compounds tested the degradation products of TEPP seem to have a strong esterase inhibiting effect (No. 5).

The effect of phosphatase on inactivated acetylcholinesterase is seen in curve No. 4. After 15 minutes incubation with tabun, DFP and mintacol, the addition of phosphatase stops the esterase inactivation at a certain level, but there is no reactivation of the esterase activity even after 15 days (not indicated in Fig. 1) of incubation with phosphatase. TEPP seems to inactivate acetylcholinesterase to a maximum more rapidly (in 15 minutes or less) than do the other compounds tested. We conclude from these results that phosphatase stops the progressive irreversible inactivation of acetylcholinesterase

when added before the maximum effect of the inactivator is reached. Phosphatase, however, is not capable of reactivating the inactivated acetylcholinesterase under the experimental conditions used.

The effect of phosphatase on the process of acetylcholinesterase inactivation has been studied further and is discussed below.

Cholinesterase (Fig. 2). Phosphatase has no activating effect on human serum cholinesterase: there is a small increase of cholinesterase activity in the presence of phosphatase, but this effect is due to the weak esterase activity of the phosphatase preparation itself. A great part of the cholinesterase inactivating effect of phosphorus compounds is lost after incubation with phosphatase. The reaction products from this hydrolytic procedure, however, are still effective as inactivators of cholinesterase; this is contrary to the results obtained with acetylcholinesterase.

Phosphatase has no reactivating effect on cholinesterase inactivated by tabun or DFP (No. 4). For mintacol a slow but significant reactivation was observed. This reactivation must be catalysed by the phosphatase present, since a control experiment in the absence of phosphatase did not show this phenomenon. For TEPP, however, a spontaneous reactivation by hydrolysis is known to occur from previous observations [7,8]; phosphatase seems to catalyse this reactivation procedure (No. 4) to a small extent during the first days. From these experiments we conclude that phosphatase has no (as far as tabun and DFP are concerned) or a very weak reactivating effect on inactivated cholinesterase. However, as in the case of acetylcholinesterase, phosphatase stops the inactivation of cholinesterase when it is added before maximum inactivation is reached.

The progressive nature of the cholinesterase inactivation by tabun. The reaction rate at which cholinesterases are inactivated by an organophosphorus compound is different for various compounds and is also dependent on the nature of the enzyme preparation and on the concentration of the phosphorus compound used. This progressive nature of cholinesterase inactivation by tabun is illustrated in Fig. 3. Acetylcholinesterase was incubated with tabun and the esterase activity was then measured after 0, 10 and 20 minutes by adding acetylcholine to the incubation mixture (Fig. 3, A). The concentration of tabun used was such that after 60 minutes incubation of the esterase, the activity of the latter was reduced 95 %. It is seen in Fig. 3 that after 10 minutes incubation the esterase activity is reduced to 58.5 % and after 20 minutes to 39 %.

In a previous paper [9] it was demonstrated that acetylcholinesterase is protected by acetylcholine against the inactivation by tabun if the acetylcholinesterase is added before or at the same time as the inactivator. In Fig. 3 (B) the results are shown when acetylcholine is present from zero time and tabun is added to the enzyme-substrate mixture 10 and 20 minutes afterwards. The protective effect of the substrate against esterase inactivation by tabun is significantly demonstrated in this experiment.

The effect of phosphorylphosphatase on the process of cholinesterase inactivation. In a preliminary experiment, performed with acetylcholinesterase, and somewhat different from those described above, the effect of phosphorylphosphatase on the tabun-inactivated esterase was studied (Table 1) by simul-

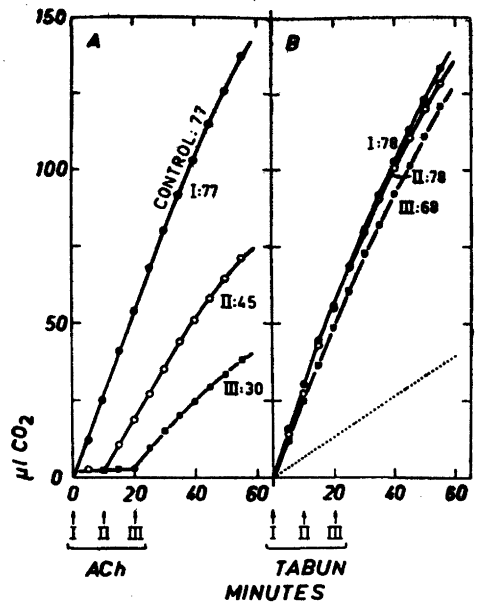

Fig. 3. The progressive nature of acetylcholinesterase inactivation by tabun. A) Esterase incubated with tabun from zero time; acetylcholine (ACh) added simultaneously with tabun (I), and after 10 (II) and 20 (III) minutes incubation. Arabic numbers refer to esterase activity (in b_{30} values). B) Acetylcholine present from zero time and tabun added simultaneously with ACh (I) and after 10 (II) and 20 (III) minutes.

taneous addition to the esterase of the phosphatase preparation and the acetylcholine (substrate). It was found that the activating effect of the phosphatase preparation on the esterase activity is not influenced by tabun. Furthermore, the low cholinesterase activity of the phosphatase preparation is reduced by

Table 1. Effect of phosphorylphosphatase (Fraction IV-1) on the inactivation of acetylcholinesterase (AChE, electric tissue) by tabun. AChE was incubated 45 minutes with tabun before the substrate (acetylcholine) or substrate + phosphatase was added. Phosphatase was added to AChE at the same time as the substrate, except in the last experiment where tabun and phosphatase were together 45 minutes before acetylcholine was added. Reaction mixture, 2.00 ml. In the two experiments (I and II) the concentration of tabun was different (appr. 2×10^{-8} M).

Enzyme system	Exp. I	Exp. II
AChE	91.5	154
AChE + Phosphatase	108.5	167
AChE + Tabun	20	76.5
(AChE + Tabun) + Phosphatase	31	89.5
Tabun + Phosphatase	8	10.5
(Tabun + Phosphatase)	1.5	6

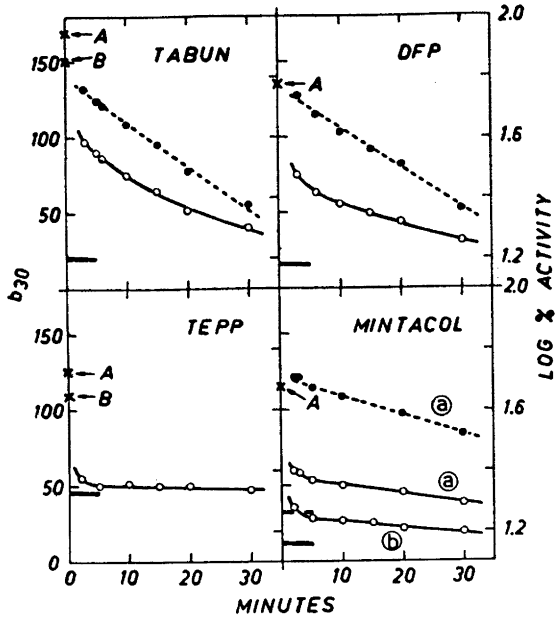

Fig. 4. Inactivation of acetylcholinesterase (AChE, electric tissue) by organophosphorus compounds. The reaction was stopped by adding phosphorylphosphatase (Fraction IV-1) after various periods of time. Esterase activity expressed in b_{30} values (left ordinate and full drawn lines). The logarithm of per cent activity (right ordinate and dotted lines) was calculated with the activity of the mixture AChE + phosphatase as control. Short and wide lines (on the ordinates) give the activity at maximum inactivation. A: activity of the AChE-phosphatase mixture; B: activity of the AChE preparation alone. Concentrations of the organopshophorus compounds: tabun, 4.16 × 10^{-8} M; DFP, 3.63 × 10^{-6} M; TEPP, 2.56 × 10^{-8} M; mintacol, a) 2.16 × 10^{-7} M; b) 4.32 × 10^{-7} M.

tabun. In the complex system, consisting of acetylcholinesterase + phosphatase + tabun + acetylcholine, we have to consider these two facts, *i. e.*, the activating effect on the esterase by phosphatase not influenced by tabun, and the inhibiting effect of tabun on the esterase activity of both acetylcholinesterase and the low one of the phosphatase preparation.

We have learned from the experiments described above that the inactivation of cholinesterases in most cases is progressive and it is surmised that it proceeds as a stepwise reaction (see further "Discussion" below). The effect of a phosphorylphosphatase preparation on the process of inactivation was therefore studied in more detail, especially the effects obtained by a certain concentration of the phosphorus compound before maximum inactivation was reached. The results of these experiments are shown in Figs. 4 and 5 and were obtained by employing the following experimental conditions. The phosphorus compound was added to the esterase and after various short periods of time (2 to 30 minutes) a phosphorylphosphatase preparation (Fraction IV—1) was added. The cholinesterase activity of that mixture was then determined with

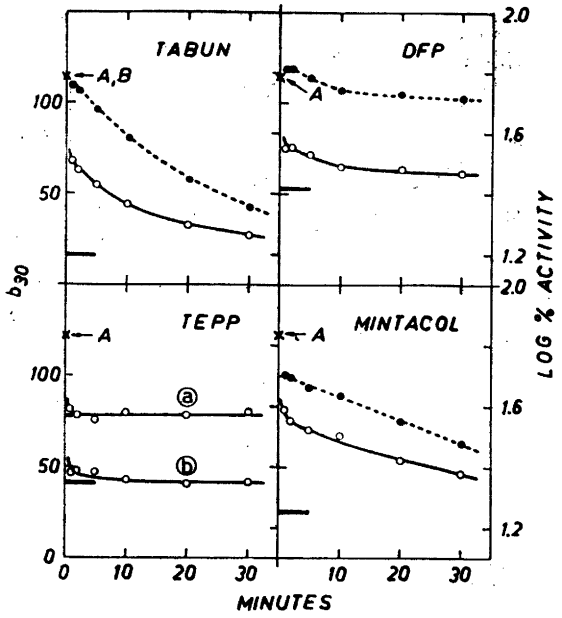

Fig. 5. Inactivation of cholinesterase (Fraction IV-6 of human serum) by organophosphorus compounds. See Fig. 4 for further explanations. Concentrations of the organophosphorus compounds: tabun, 1.66 × 10^{-5} M; DFP, 2.90 × 10^{-5} M; TEPP, a) 1.53 × 10^{-5} M; b) 3.07 × 10^{-5} M; mintacol, 1.15 × 10^{-5} M.

acetylcholine as substrate. It will be remembered (see Figs. 1 and 2) that the phosphatase has no effect on the esterase which beforehand was inactivated maximally by a specific concentration of a phosphorus compound.

For both acetylcholinesterase (Fig. 4) and serum cholinesterase (Fig. 5) the progressive enzyme inactivation by tabun, DFP and mintacol can be stopped by adding phosphorylphosphatase. Once the phosphatase is added there is no further decrease of esterase activity. It has been shown that all of the inactivator added reacts immediately with the esterase and this reaction can be reversed by phosphatase. In a second irreversible reaction the esterase is inactivated and this reaction is not influenced by the phosphatase. TEPP differs from the other three compounds studied in the very rapid reaching of maximum inactivation of the esterases. Even after one minute incubation with TEPP maximum inactivation is reached and therefore the addition of phosphatase after that period has no influence on esterase activity.

DISCUSSION

The mechanism of enzyme inhibition by organophosphorus compounds has been discussed in a variety of publications during the last few years. These compounds seem to be general inhibitors for enzymes having carboxylic esterase activity, *e. g.*, chymotrypsin, trypsin, liver esterase, acetylesterase, B-

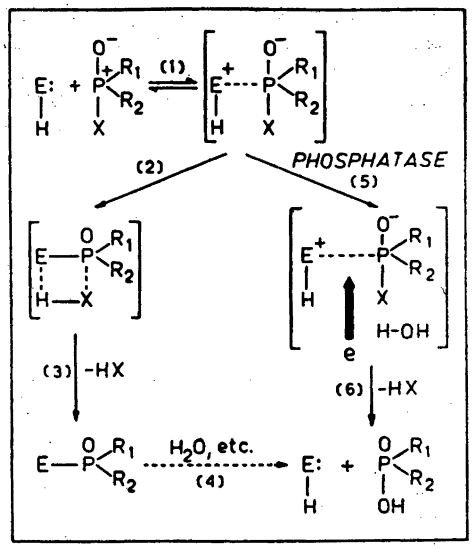

Fig. 6. Tentative scheme of the reaction mechanism for the inactivation of cholinesterases (E) by organophosphorus compounds, and the effect of phosphorylphosphatase (e) on that reaction.

esterase [10]. The inhibitory process for all these enzymes is a first order reaction and bimolecular. It has been demonstrated for some of these enzymes that the inhibitor phosphorylates the enzyme [11]. As far as cholinesterases are concerned most studies are consistent with the view that a similar reaction mechanism is valid for the inactivation of these enzymes by organophosphorus compounds. The analogy between this phosphorylating process and the reaction mechanism for the hydrolysis of acetylcholine by cholinesterases has been pointed out by Wilson *et al.*[12]

The reaction mechanism of the spontaneous hydrolysis of organophosphorus compounds was discussed by Larsson [13] and the similarity between the base-catalysed hydrolysis and the enzymic hydrolysis of these compounds was postulated by the present authors [3]. In some preliminary experiments carried out to study the effect of phosphorylphosphatase on phosphorylated cholinesterases the results were leading to the idea that the phosphatase is able to reactivate inactivated cholinesterases [14]. A more detailed study of this phenomenon, as made in the present paper, showed that there is no true enzymic reactivating effect, but that the phosphatase can stop the progressive inactivation when added before maximum inactivation is reached.

On the basis of investigations by Jansen, Aldridge, Wilson, Wagner-Jauregg, Myers, and others, and those from this laboratory, the following reaction mechanism is suggested (Fig. 6). The enzyme (E) combines reversibly (1) with the phosphorus compound by a one-point contact at the ester-bonding site (*i. e.*, the "esteric" site [7] or active group II [8] of the acetylcholinesterase molecule); then the acid group (X) of the phosphorus containing part (CN in

tabun) of the complex reacts (2, 3) with a hydrogen atom of the enzyme molecule releasing the corresponding acid. The dephosphorylating reaction (4) of the inhibited enzyme is, for most phosphorus compounds, very slow compared with reactions (1), (2) and (3), and therefore the enzyme remains inhibited (inactivated). Catalysis of reaction (4) by replacing water with hydroxylamine or derivatives of hydroxamic acid has recently been demonstrated by Wilson and Meislich [15].

On the basis of the results described in the present paper it is now suggested that phosphorylphosphatase (e) prevents the reactions (2) and (3) from taking place (see reaction 5). The phosphorus containing moiety of the loosely bound complex (reaction 1) is thereby hydrolytically split, acid (HCN in the case of tabun) and a phosphoric acid are released, and the cholinesterase becomes active (6). The degree of the effect of the phosphatase depends both on the time elapsing after cholinesterase and phosphorus compound have come in contact with each other, and on the various groups (alkyl, substituted amido) of the phosphate inhibitor.

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