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Enzymatic Hydrolysis of Organophosphorus Compounds

III. Effect of Cholinesterase Inhibitors and Inhibition of Cholinesterase in the Presence of Tabunase

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The existence of an enzyme (phosphorylphosphatase," tabunase") catalysing the hydrolysis of dimethylamido-ethoxy-phosphoryl cyanide (tabun) has been reported recently [1]. The properties of the enzyme and the analysis of the reaction products have also been described [2]. In the present paper the effect of reversible cholinesterase inhibitors (physostigmine, prostigmine) on tabunase is described and the significance of considering the presence of tabunase in studies on tabun as a cholinesterase inhibitor is demonstrated.

METHODS AND MATERIAL

The enzymatic hydrolysis of tabun was measured by the Warburg manometric technique [1]. Tabun and DFP (di*iso*propoxyphosphoryl fluoride) of high purity were used as substrates. The influence of the following compounds on the tabunase activity was studied: acetylcholine chloride (ACh), choline chloride, physostigmine sulphate, and prostigmine bromide.

Human and rabbit plasma were employed as enzyme preparations. In the determination of the influence of tabunase on the inhibiting effect of tabun on human serum cholinesterase, Fraction IV—1 (tabunase) and Fraction IV—6 (cholinesterase) were used, prepared by the method of Cohn [1].

RESULTS

Effect of acetylcholine and choline on the enzymatic hydrolysis of tabun. In these experiments human plasma was used as enzyme source for tabunase. The results from one series of experiments are shown in Fig. 1. Neither choline nor acetylcholine has any influence on the tabunase activity. In the experiment with acetylcholine which, in the absence of tabun, is hydrolysed at a high rate by the plasma used, tabun has two functions, *i.e.*, to inactivate cholinesterase completely and to act as a substrate for tabunase. In the system "tabun + ACh" (Fig.1) therefore the cholinesterase activity is not involved. The concentration of tabun in that system was 2.66×10^{-3} *M*; the concentration

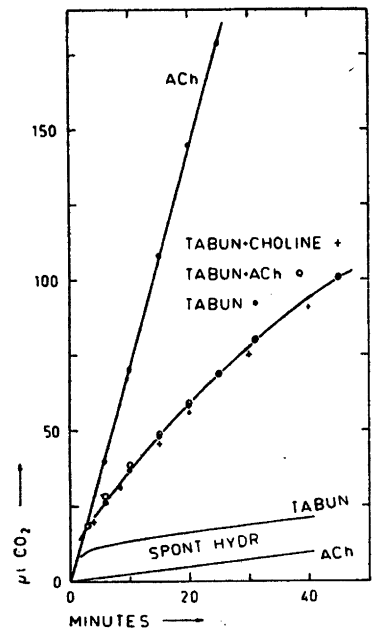

Fig. 1. The effect of acetylcholine (ACh) and choline on the enzymatic hydrolysis of tabun by human serum. Tabun, 2.66 × 10^{-3} M; ACh-chloride, 1.65 × 10^{-3} M; choline chloride, 1.00 × 10^{-3} M.

necessary to give complete inhibition of cholinesterase is about 10^{-8} *M*. From these experiments we conclude firstly that tabunase is a separate enzyme from cholinesterase, and secondly that acetylcholine or its hydrolysis products do not have any influence on the former enzyme.

Effect of physostigmine and prostigmine on the enzymatic hydrolysis of tabun and DFP. The effect of the two best-known reversible inhibitors of cholinesterase, physostigmine and prostigmine, was studied for the ezymatic hydrolysis of tabun by rabbit plasma. This plasma differs from the human plasma in that tabun is hydrolysed at a higher rate than is acetylcholine; the reverse holds for human plasma. Those concentrations of physostigmine as well as of prostigmine, which are sufficiently high to give complete inhibition of the plasma cholinesterase present (acetylcholine as substrate), have no effect on the tabunase activity. These results are demonstrated in Fig. 2. In all these experiments the enzyme preparation was incubated for about 45 minutes with the inhibitor before the substrate (an organophosphorus compound) was added.

Similar experiments were performed with DFP as a substrate and with rabbit plasma as an enzyme source. As seen in Fig. 3 physostigmine has no influence on the enzymatic reaction in this case either. When tabun and DFP are present at the same time the results obtained are in agreement with the suggestion [1] that both these compounds are split by the same enzyme. In a summation experiments, such as that shown in Fig. 3, the initial reaction velocity is somewhat higher than in the system in which the substrates were present alone. This higher reaction velocity, however, is by no means as high as it should be if two enzymatic reactions were going on simultaneously; it is

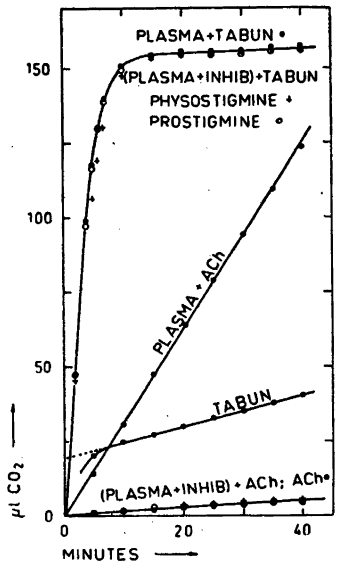

Fig. 2. The effect of physostigmine and prostigmine on the enzymatic hydrolysis of tabun and acetylcholine (ACh) by rabbit plasma. Tabun, 5.3 × 10⁻³ M; ACh-chloride, 1.10 × 10⁻² M; inhibitor (physostigmine sulphate or prostigmine bromide), 1.00 × 10⁻³ M; concentration values refer to the reaction mixture (2.00 ml). Amount of plasma present, 0.4 ml.

due to the higher (two-fold) concentration of the two substrates (taken together as a single entity). The same increase in reaction velocity was obtained when the concentration of each substrate (tabun or DFP) was doubled. Further results of this kind obtained with a variety of organophosphorus compounds will be published later.

The inhibition of cholinesterase by tabun mixtures containing tabunase. One of the present authors has recently [3,4] reported that in experiments with crude enzyme preparations the inhibition of plasma (butyryl-) cholinesterase by tabun is less than that of the acetylcholinesterase (erythrocytes, electric tissue). It is now known that this difference is not due to a true selective difference in inhibition, but is the result of the interference by tabunase present in most animal blood plasma. It is obvious that the simultaneous presence of enzymes which split organophosphorus compounds has to be taken into consideration when these compounds are studied as cholinesterase inhibitors. In Fig. 4 the inhibiting effect of tabun on the cholinesterase of human serum is demonstrated. This effect obtained with the original crude serum was compared with the effects observed with a purified preparation (Fraction IV—6) of cholinesterase and with a mixture containing this preparation and tabunase (Fraction IV —1); both these fractions were prepared from the serum used for comparison. The pI_{50} value (negative log of the molar concentration of tabun which gives 50 % inhibition) obtained for the original serum is much lower than that for a cholinesterase preparation free of tabunase. It is also demonstrated in Fig. 4 that when the tabunase preparation is added to the fraction containing cholinesterase the pI_{50} value is lowered, *i.e.*, a higher concentration of tabun is necessary to give 50 % inhibition. The results show that tabun actually inhibits butyrylcholinesterase and acetylcholinesterase of human blood to

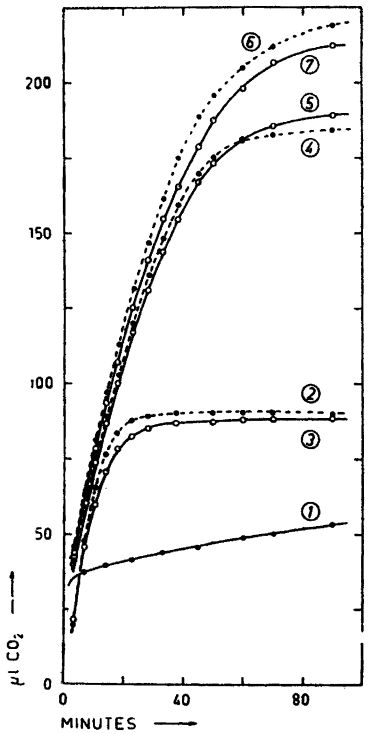

Fig. 3. The effect of physostigmine on the enzymatic hydrolysis of tabun and DFP by rabbit plasma. Tabun and DFP, 2.65 × 10^{-3} M; physostigmine sulphate, 1.00 × 10^{-3} M; concentration values refer to the reaction mixture (2.00 ml). Amount of plasma present, 0.1 ml. 1. Tabun + DFP + physostigmine; 2. Tabun + plasma; 3. Tabun + (plasma + physostigmine); 4. DFP + plasma; 5 DFP + (plasma + physostigmine); 6. (Tabun + DFP) + plasma; 7. (Tabun + DFP) + (plasma + physostigmine).

about the same extent. These results are in agreement with the fact that in crude serum there are at least two factors which combine with tabun: cholinesterase which is inactivated, and tabunase which splits tabun into two molecules each being inactive as a cholinesterase inhibitor. In addition to the catalytic effect on the hydrolysis of tabun, tabunase also reactivates the inhibited cholinesterase, as was recently demonstrated in this laboratory; a special report on this enzymatic reactivation of tabun-inhibited cholinesterase will be published elsewhere.

DISCUSSION

It was demonstrated in previous papers [1,2] and in the present report that tabun is enzymatically hydrolysed by a variety of animal tissues and that the enzyme (tabunase, phosphorylphosphatase) responsible for this reaction is a separate enzyme with no relation to the group of cholinesterases or phosphatases. In any experiment carried out with crude extracts or untreated blood plasma in order to determine the inhibiting effect of tabun or another organophosphorus compound on the cholinesterase activity, the possible presence of a phosphorylphosphatase must be considered. The presence of such an enzyme will always give a distorted picture as regards the cholinesterase inhibiting effect of an organophosphorus compound. The conflicting

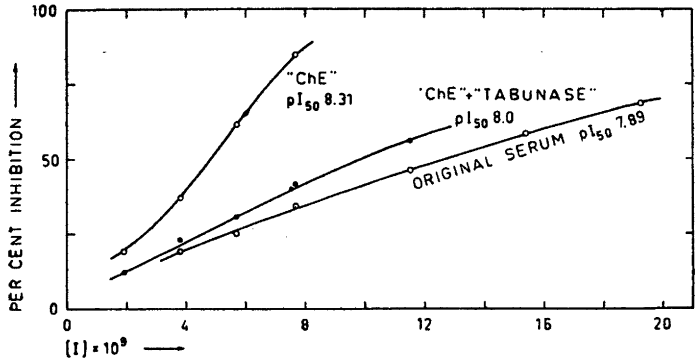

Fig. 4. The inhibiting effect of tabun on the cholinesterase of human serum in the absence and presence of tabunase. [I], molar concentration of tabun during enzyme incubation; pI_{50}, the concentration which gives 50 % inhibition. "ChE": Fraction IV—5+6; "tabunase": Fraction IV—1; the same amount (10 mg) of each fraction was used; the same result was obtained with 1·0 mg of Fraction IV—6. Substrate, 1.10 × 10^{-2} M acetylcholine.

results obtained in this respect by various groups of workers with different methods may be explained by this observation. The suggestion of other authors that the organophosphorus compounds react with proteins other than cholinesterases *in vivo* is confirmed by the demonstration of the presence of the phosphorylphosphatases. These latter enzymes, however, may not be the only representatives of such proteins.

Little is known about the degradation and detoxication mechanism of the organophosphorus compounds. The phosphorylphosphatases may play an important part in that mechanism. The reaction products formed from tabun by enzymatic degradation are biologically inactive in the concentrations to be considered. The toxic effects on the organism may therefore be reduced when these enzymes are present. In addition, it has been demonstrated that these enzymes also reactivate cholinesterases which are completely inactivated by tabun *in vitro*. This latter reaction may be one of the factors which is responsible for the restoration of normal cholinesterase activity in a living organism intoxicated by a phosphorus compound.

SUMMARY

Acetylcholine and choline do not interfere with the tabunase activity of human serum. Reversible cholinesterase inhibitors, such as physostigmine and prostigmine, do not inhibit the enzymatic hydrolysis of tabun and di*iso*-propoxyphosphoryl fluoride (DFP) by rabbit plasma; tabun and DFP are most probably split by the same enzyme in this case.

A much higher concentration of tabun is necessary to inhibit the cholinesterase of untreated human serum and isolated serum cholinesterase when mixed with tabunase, than a cholinesterase preparation free of tabunase.

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