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Enzymatic Hydrolysis of Organophosphorus Compounds

II. Analysis of Reaction Products in Experiments with Tabun and Some Properties of Blood Plasma Tabunase

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In a previous paper [1] evidence has been put forward for the existence of an enzyme (phosphorylphosphatase, "tabunase") which catalyses the hydrolysis of dimethylamido-ethoxy-phosphoryl cyanide or tabun. The enzyme is present in blood plasma and various animal tissues. In the present paper the analysis of the products formed in such a reaction are described together with some properties of tabunase.

METHODS AND MATERIAL

The enzymatic hydrolysis of tabun was followed by the Warburg technique [1]. Enzyme activity is expressed in b_{30} values, *i. e.*, μl CO_2 evolved from a bicarbonate solution in 30 minutes, corrections being made for the spontaneous hydrolysis of the substrate.

Tabun was used as substrate exclusively and the plasma from rabbit and horse were used as enzyme preparations. In studies on the pH stability, fraction IV—1 of human serum (prepared as described by Cohn) [1] was employed.

Dimethylamine was determined by Dowden's [2] method which is based upon the reaction of dimethylamine with carbon disulphide and copper ion, forming cupric dimethyl-dithiocarbamate which is water-insoluble and imparts a deep yellow colour to chloroform, benzene, and other organic solvents in which it is soluble. The method was previously used by Larsson [3] to study the spontaneous hydrolysis of tabun and by Hall, Stohlman and Schechter [4] for the determination of octamethyl pyrophosphoramide (OMPA, Schradan).

The principle of Epstein's method [5] used to analyse the reaction mixture for cyanide involves the conversion of cyanide to cyanogen chloride with chloramine T solution; a blue colour, stable for at least 30 minutes, is obtained by adding pyridine, containing 0.1 % bis-pyrazolone and 1-phenyl-3-methyl-5-pyrazolone. With this method 0.2 to 1.2 μg cyanide can be determined. Recently Holmstedt [6] followed the spontaneous hydrolysis of tabun by this method.

The bicarbonate buffer, pH 7.6, used in the enzyme activity determinations contained 0.034 *M* sodium bicarbonate and 0.12 *M* sodium chloride. In the determination of the pH stability of human serum tabunase, *M*/15 phosphate buffers were used.

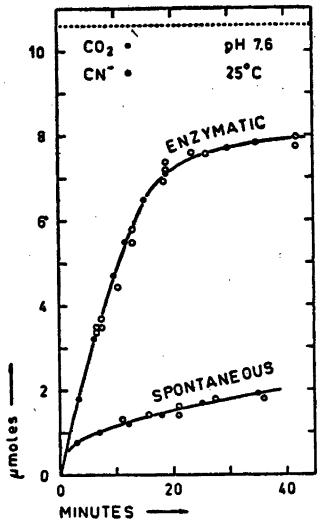


Fig. 1. Enzymatic hydrolysis of tabun by rabbit plasma followed by the CO_2 production in bicarbonate solution and the simultaneous production of cyanide. Original amount of tabun present, 10.6 μmoles.

RESULTS

Enzymatic hydrolysis of tabun by rabbit plasma and analysis of the reaction products at equilibrium and at intervals during the reaction were carried out. There was no release of inorganic phosphate during the reaction, nor was ethanol liberated.

Determination of dimethylamine. Compared with the spontaneous hydrolysis of tabun as control no extra dimethylamine was found to be split off by enzymatic degradation. When hydrolysis had reached equilibrium, the reaction mixture, containing 1.06×10^{-5} moles per ml of tabun originally, contained 11.5 μg or 1.64×10^{-6} moles of dimethylamine per ml. Based upon the amount of free dimethylamine, only 15 % of the tabun was destroyed. The production of dimethylamine was not due to an enzyme as the spontaneous hydrolysis of tabun over the same period (3.5 hours) and under the same experimental conditions (pH 7.6, temp. 25° C) yielded about the same amount, 11.0 μg of dimethylamine per ml.

Determination of cyanide. The liberation of CO_2 from a bicarbonate buffer during the enzymatic hydrolysis of tabun by rabbit plasma parallels the production of cyanide. This result is illustrated in Fig. 1. When equilibrium is reached about 80 % of tabun is hydrolysed, as determined by both the CO_2 and CN methods. After the same period (40 minutes) 16 % of the same initial amount of tabun was hydrolysed spontaneously. These experiments show that the enzymatic hydrolysis of tabun by rabbit plasma involves splitting of the P-CN linkage. The production of CO_2 is not due to hydrocyanic acid ($K_a = 7.2 \times 10^{-10}$), but to the other strong acid formed, dimethylamido-ethoxy-hydroxy-phosphine oxide.

Effect of the reaction products on the tabunase and acetylcholinesterase activities. When equilibrium is reached the reaction mixture has a weak inhibiting effect on the tabunase activity. Cyanide of the same concentration as that at equilib-

Table 1. The effect of cyanide, and the reaction products after "complete" hydrolysis of tabun by tabunase, on the enzymatic hydrolysis of tabun by a purified preparation of tabunase (Fraction IV—1). The heated preparation of the reaction mixture was prepared by incubation for 3 minutes in boiling water. Substrate, 5.3 × 10^{-3} M tabun, which was also the concentration of tabun in the original reaction mixture.

Inhibitor	b_{30}	Percentage inhibition
No inhibitor present (control)	78	—
NaCN, 10^{-3} *M*	67.5	13
Reaction mixture, heated	62.5	20
» » not heated	77.5	0

rium inhibits tabunase to about 10 %. The inhibiting effect of the reaction mixture is somewhat higher than that and it is therefore concluded that the other phosphorus containing part of the original tabun molecule also inhibits the tabunase activity. This inhibiting effect of the reaction products explains the decrease of reaction velocity during the enzymatic hydrolysis of tabun. The results obtained are illustrated in Table 1. In order to test the inhibiting effect of the reaction products the remaining tabunase activity of a mixture containing these products was destroyed by heating in boiling water. The reaction mixture before heating has still sufficient tabunase activity to split additional amounts of tabun and therefore the inhibiting effect of the reaction mixture in this case is masked.

It was suggested that the phosphorus containing part of the tabun molecule liberated during the enzymatic hydrolysis shows no cholinesterase inhibiting effect. This suggestion is a logical conclusion from previous studies on cholinesterase inhibiting activity and chemical structure of the organophosphorus compounds. The compound in question has not been available, but it has been found that the reaction products formed after "complete" enzymatic hydrolysis of tabun, using a purified enzyme preparation (Fraction IV—1), have no inhibiting effect on acetylcholinesterase in the concentrations used. Table 2 demonstrates the results in one of a series of experiments performed. The activating effect of the tabunase preparation on acetylcholinesterase (electric tissue), as seen in Table 2, will be discussed in a future paper.

pH stability of purified tabunase. The stability at various pH values of a purified preparation of tabunase (Fraction IV—1 of human serum) was deter-

Table 2. The effect of the reaction products after "complete" hydrolysis of tabun by tabunase on the acetylcholinesterase (electric tissue of T o r p e d o). Tabunase was a purified preparation (Fraction IV—1). Substrate, 3.3 × 10^{-3} M acetylcholine chloride. Concentration of tabun during esterase determination, 2.6 × 10^{-6} M.

Enzyme-inhibitor-system	b_{30}	
Acetylcholinesterase	168	153
Tabunase	14	14
Acetylcholinesterase + Tabunase	194	185.5
Acetylcholinesterase + Tabun	30.5	6
Acetylcholinesterase + Reaction mixture	195	189.5

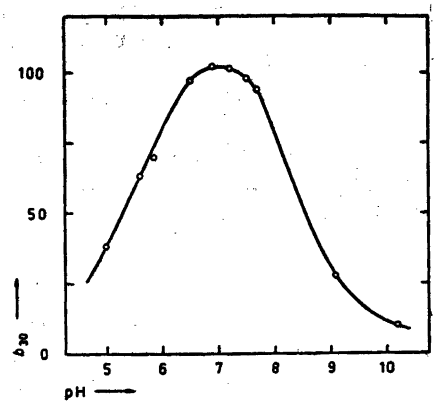


Fig. 2. The pH stability of a purified preparation (Fraction IV—1) of human serum tabunase. Incubation for 24 hours at 4.5° C.

mined in the following way. Solutions of the preparation in phosphate buffers, containing 25 mg tabunase per ml, were incubated at various pH and 4.5° C. After 24 hours 0.3 ml of the test solutions were diluted with 1.3 ml bicarbonate buffer to give pH 7.6. The enzyme activity was then determined using 5.3 $\times$ 10^{-3} *M* tabun. The results obtained are shown in Fig. 2 where enzyme activity is plotted against pH. The tabunase preparation was found to be most stable in neutral solutions.

Determination of the dissociation constant of the tabun-tabunase complex. The most convenient graphical method of evaluating the relationship between enzyme activity and substrate concentration is to plot the initial reaction velocity (v) of the enzyme reaction against v/[S] where [S] is the molar substrate concentration [7]. For simple enzyme-substrate systems this method gives, by extrapolation, the values of maximum velocity (V_{max}) and the dissociation constant (K_S) of the enzyme-substrate complex. This method was applied

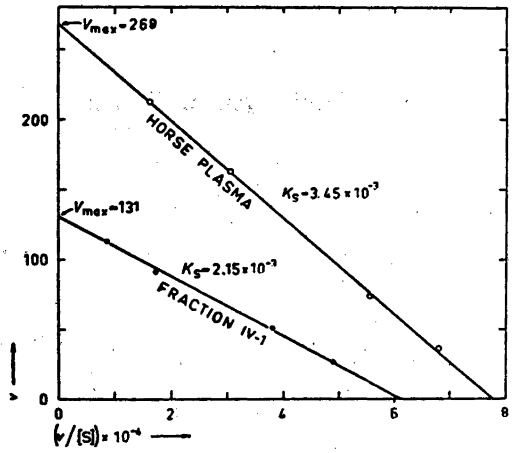
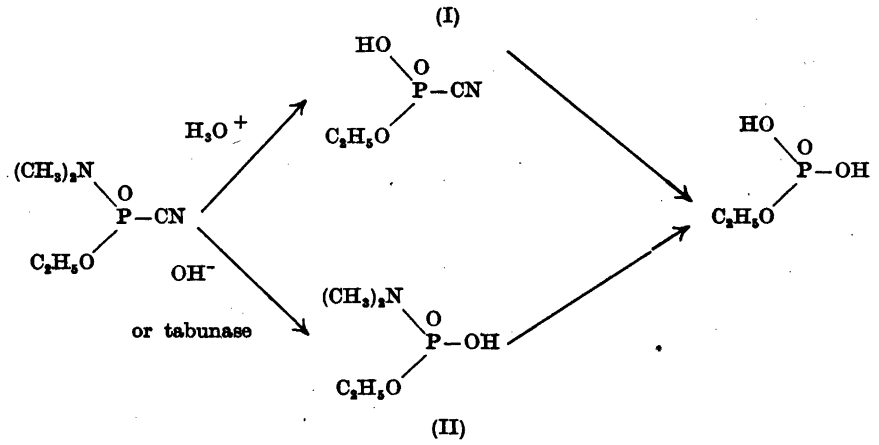


Fig. 3. Evaluation of K_S *for the enzymatic hydrolysis of tabun by horse plasma and Fraction IV—1.* v, *expressed in* b_{30} *values;* [S], *molarity of tabun in the reaction mixture.*

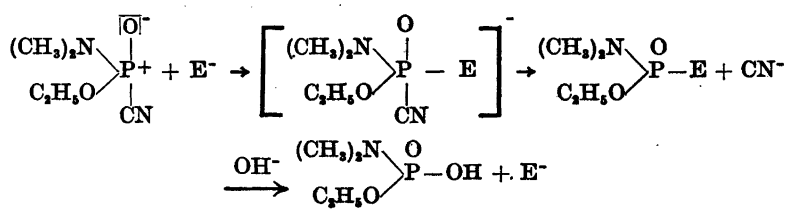
to the enzymatic hydrolysis of tabun by horse plasma and purified tabunase (Fraction IV—1 of human serum). The results are demonstrated in Fig. 3. Horse plasma, containing a variety of esterases including cholinesterase, gave a somewhat higher K_S value (3.45×10^{-3}) than did the preparation free from cholinesterase (2.15×10^{-3}). The difference obtained is significant. It is due to the presence of cholinesterase in horse plasma which in its turn necessitates a higher concentration of substrate (tabun) to saturate tabunase and thus giving maximum reaction velocity for this enzyme.

DISCUSSION

Larsson [3] showed that the decrease in toxicity of tabun during spontaneous hydrolysis parallels the decrease in cholinesterase inhibiting activity. From the fact that neither dimethylamine nor hydrocyanic acid alone is liberated at the same rate as the toxicity decreases, he concluded that the spontaneous hydrolysis must proceed by the two parallel reactions I and II:



The splitting off of the dimethylamido group (I) is catalysed by hydroxonium ions and that of the cyanide group (II) by hydroxyl ions. In the present investigation on the enzymatic hydrolysis of tabun it has been found that the splitting of the P—N linkage (I) is not influenced by enzymes, but that hydrocyanic acid is liberated (II). Hence the mechanism of the enzyme reaction is similar to the base-catalysed hydrolysis. This enzyme reaction probably takes place according to the following scheme:



The reaction is supposed to be controlled by the reaction of a nucleophilic group ("esteric site") of the enzyme molecule with the phosphorus atom, which, dependent on the electrophilic strength of the acid substituent (CN in the case of tabun), is more or less in an anionic state.

The phosphorus containing hydrolysis product does not inhibit cholinesterase in concentrations which are ten fold higher than that of tabun giving almost complete inhibition of that enzyme. Due to the inhibition of tabunase by the reaction products (cyanide and dimethylamido-ethoxy-hydroxy-phosphine oxide) it is concluded that about 15 % of the tabun originally present is not or is very slowly attacked by enzymatic hydrolysis. This amount of unreacted tabun may be responsible for the cholinesterase inhibition of the reaction mixture when used in higher concentrations.

SUMMARY

The enzymatic hydrolysis of tabun by rabbit plasma involves splitting off of the cyanide group. The production of a strong acid, dimethylamido-ethoxy-hydroxy-phosphine oxide, parallels that of hydrocyanic acid. No dimethylamine, ethanol, or inorganic phosphate are liberated by enzymatic degradation of tabun.

Cyanide and the phosphorus containing compound formed in the reaction have a weak inhibiting effect on the tabunase activity, but no such effect on acetylcholinesterase.

The reaction mechanism is discussed.

A purified tabunase preparation from human serum has its optimum stability at pH 7. The Michaelis' constant for the tabunase of horse plasma and that of a purified preparation of human serum were determined and found to differ due to the interaction of cholinesterase in the untreated plasma.

Tabun was prepared at the Research Institute of National Defence, Department 1, Chief Professor Gustaf Ljunggren, to whom we are indebted for his continual interest. The authors wish to express their sincere thanks to Ing. Henrik Björling of A/B Kabi, Stockholm, for the preparation of Fraction IV—1.

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