

## Enzymatic Hydrolysis of Organophosphorus Compounds

### I. Occurrence of Enzymes Hydrolysing Dimethyl-amido-ethoxy-phosphoryl Cyanide (Tabun)

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Compared with the biochemical effects of the organophosphorus compounds as cholinesterase inhibitors, little is known about the degradation and detoxication mechanism of these agents in the living organism. The presence of an enzyme ("phosphofluorase" or "dialkylfluorophosphatase") capable of catalysing the hydrolysis of diisopropyl fluorophosphate (DFP) in the plasma and tissues of rabbit and man was first reported by Mazur<sup>1</sup>. The purification and properties of such an enzyme (DFP-ase) from kidney was recently described by Mounter *et al.*<sup>2</sup>, who in a second paper<sup>3</sup> reported studies on the activation and inhibition by metals of this enzyme. In these three reports DFP was used as substrate and no serious attempts have as yet been made to elucidate the specificity of the enzyme or enzymes.

Aldridge<sup>4,5</sup> has demonstrated that an esterase, widely distributed in the tissues of rabbit and rat and present in the sera from many different species, hydrolyses diethyl *p*-nitrophenyl phosphate (mintacol, E 600). This esterase is identical with the so-called A-esterase which hydrolyses *p*-nitrophenylacetate. The enzymatic hydrolysis of mintacol by human plasma was confirmed in this laboratory<sup>6</sup>. The *in vivo* degradation of parathion (E 605), the sulphur analogue of mintacol, was studied by Pankaskie *et al.*<sup>7</sup> and by Lieben *et al.*<sup>8</sup>, who showed that no parathion was excreted from the body.

It has been shown previously in this laboratory<sup>9</sup> that the organophosphorus compounds react spontaneously with dihydroxyphenyl derivatives, the reaction resulting in phosphorylation of one of the phenolic groups. Such a reaction could be of importance in the mechanism of cholinesterase inactivation or in the mechanism of an enzymatically controlled detoxication process. A series of experiments were carried out *in vitro* on the enzymatic degradation of organophosphorus compounds; a short communication of the results obtained was recently published<sup>10</sup>. The present report deals with some preliminary studies on the enzymatic hydrolysis of tabun or dimethylamido-ethoxy-phosphoryl cyanide.

## METHODS AND MATERIAL

Enzyme activity was determined by the Warburg technique. Generally, 1.6 ml of the enzyme preparation was placed in the main compartment of a Warburg flask and 0.4 ml of the substrate solution in one of the side bulbs. All enzyme preparations were made up in a solution containing 0.034 *M* sodium bicarbonate and 0.12 *M* chloride. In the first of these experiments 0.0012 *M* magnesium chloride was also present, but was later omitted since it was found that magnesium ions do not influence the enzymatic hydrolysis of the compounds studied. The solutions were equilibrated with a mixture of 95 % N<sub>2</sub> + 5 % CO<sub>2</sub>. The pH of the reaction mixture after mixing substrate and enzyme was 7.6. The liberation of CO<sub>2</sub> from the bicarbonate buffer was taken as evidence of the production of acid. All experiments were made at 25° C. Readings were taken at various intervals over 40 minutes or longer. Activities were calculated from the initial slope of the reaction curve (in a CO<sub>2</sub> (in  $\mu$ l) — time diagram). The extrapolated 30 minute value ( $a_{30}$ ), minus the amount of CO<sub>2</sub> evolved during the same period of time by non-enzymatic hydrolysis, is generally taken as unit of enzyme activity, symbolised by  $b_{30}$ .

*Substrates.* A great variety of organophosphorus compounds have been tested as substrates. In the present paper, the experiments carried out mainly with tabun will be described; in some cases the results were compared with those obtained with DFP (diisopropyl fluorophosphate). In general, 0.4 % (by volume) solution of pure tabun in distilled water was prepared immediately before use; the concentration of tabun (mol. wt. = 162.13,  $d_{20} = 1.077$ ) in the reaction mixture (2.00 ml) during activity determinations was  $5.3 \times 10^{-3}$  *M*. The time between the dilution procedure and mixing the substrate and enzyme in the Warburg flask was in most instances 60 minutes (for technical reasons). During this period the substrate solutions became somewhat acid due to spontaneous hydrolysis.

In the preliminary studies various solvents were tried for the dilution of the organophosphorus compounds. Highly purified propylene glycol (1,2-propanediol) is not suitable as it was found that CO<sub>2</sub> is evolved spontaneously when such a solution is mixed with the bicarbonate solution or with plasma diluted with that buffer. In addition, the initial rate of enzymatic hydrolysis of tabun is unfavorably influenced when the enzyme preparation is mixed with propylene glycol prior to the addition of substrate. The propylene glycol probably interferes with the enzyme in some way which has not yet been investigated in detail.

Peanut oil is not suitable when the enzymatic hydrolysis of these compounds are studied. When it is used as a solvent for the substrates, a lower initial enzyme activity is observed compared with controls. The actual substrate concentration in the aqueous phase is reduced for such an oil-water system. Due to the spontaneous hydrolysis of organophosphorus compounds in alkaline solutions, a bicarbonate solution is not suitable for the preparation of substrate solutions. It was decided therefore to use distilled water as a solvent and this was done throughout the investigations described.

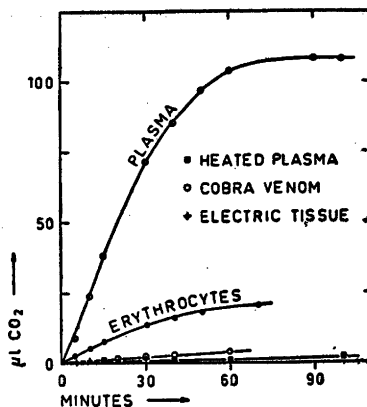
*Enzyme preparations.* The catalytic effect of blood plasma from various animals on the hydrolysis of tabun has been investigated. Blood was taken with heparin and centrifuged free of blood cells. The enzyme activity of rabbit tissues was also studied. The organs were homogenised and extracts made up in bicarbonate solution. The hydrolysis capacity of kidney and adrenal glands from rabbit was compared with that of the same organs from pig and cow.

The various fractions of human serum (postpartum) studied were those obtained by a partly modified method No. 6 of Cohn *et al.*<sup>11</sup>. These fractions were prepared by A/B Kabi (Stockholm).

## RESULTS

*Introduction.* The hydrolysis of tabun catalysed by blood plasma (*e. g.*, human plasma) is of an enzymatic nature. The rate of this reaction is much higher than the hydrolysis in aqueous solution without the plasma. The catalytic reaction is continuous and the rate of hydrolysis proportional to the amount of plasma added (*cf.* below and Fig. 3). The catalysis is completely

Fig. 1. Enzymatic hydrolysis of tabun (correction made for spontaneous hydrolysis) by various preparations known to contain cholinesterases. Plasma and erythrocytes from human blood. Electric tissue from *Torpedo marmorata*. Plasma heated for 15 minutes at 70° C.



prevented by heating for 10 minutes at 70° C (Fig. 1). After dialysis for 24 hours against running water, the catalytic activity of the plasma is reduced only a few per cent. It was concluded therefore that this hydrolysis of tabun by blood plasma is an enzymatic reaction. For the sake of convenience this enzyme will provisionally be referred to as "tabunase". It should be noted, however, that the enzyme activity demonstrated in various animal tissues is not specific for tabun, other organophosphorus compounds being hydrolysed as well; at least some of the enzymes studied split DFP and sarin. "Phosphorylphosphatase" is suggested by the authors as a more general name for enzymes hydrolysing organophosphorus compounds, characterised by the phosphoryl (PO) group and a variety of organic substituents. It should be noted also that these enzymes, as far as can be discussed, are not related to the ordinary phosphatases, *e. g.*, the kidney and plasma phosphatases.

The phosphorylphosphatases have no relation to the cholinesterases. However, the importance of the discovery of such enzymes is obvious when studying the organophosphorus compounds as anticholinesterase drugs. Preparations known to contain acetylcholinesterase as the only esterase, *e. g.*, those of electric tissue and cobra venom, do not hydrolyse tabun (Fig. 1) or other organophosphorus compounds studied. Haemolysate of human erythrocytes splits tabun at a comparatively low but detectable rate. It is known, however, that acid is produced when the compounds react with cholinesterases<sup>9</sup>, but the concentrations necessary to inactivate cholinesterase completely are too low to give sufficiently large amounts to be detected by the technique used in the present study. Systems containing cholinesterases and phosphorylphosphatases as enzyme mixture, and acetylcholine and an organophosphorus compound as substrate-inhibitor mixture will be discussed in more detail in a subsequent paper.

*Blood plasma as enzyme source.* Typical hydrolysis curves for the enzymatic destruction of tabun are illustrated in Fig. 2 where horse plasma was used as the enzyme source. As will be described in a subsequent paper the production of CO<sub>2</sub> from a bicarbonate buffer when tabun is used as substrate, is due to the liberation of dimethyl-amido-ethoxy-hydroxy-phosphine oxide which

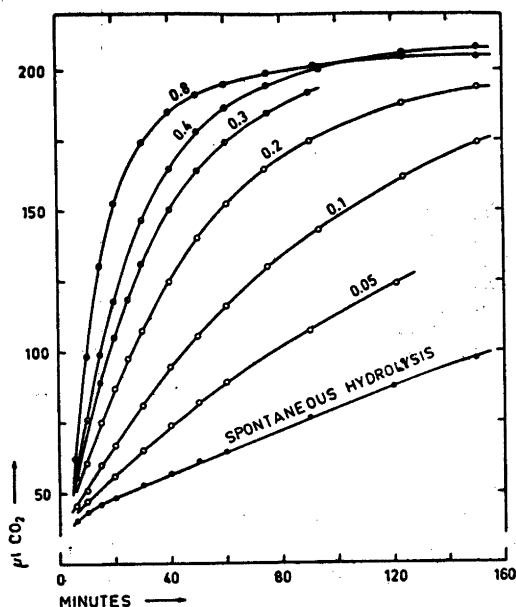


Fig. 2. Hydrolysis of tabun by the action of horse plasma. Numbers refer to ml plasma per reaction mixture (2.00 ml). Substrate,  $5.3 \times 10^{-3}$  M tabun.

is a strong acid<sup>12</sup>. Hydrocyanic acid is too weak an acid to be the cause of  $\text{CO}_2$  production and therefore no more than one mole  $\text{CO}_2$  per mole tabun is liberated. Generally we have found that 80 to 90 % of the theoretical amount of  $\text{CO}_2$  (238  $\mu\text{l}$  in Fig. 2) was evolved when crude enzyme preparations were used. In Fig. 3 the enzyme activity is recorded as a function of plasma concentration.

Blood plasma from other animal species, including man, have been tested. The results are demonstrated in Fig. 3 where enzyme activity is plotted against the amount of plasma present. For most preparations direct proportionality was found between hydrolysis rate and plasma concentration. Rabbit plasma has the highest activity and is about 10 times more active than human plasma. Amongst the eight plasma examined guinea-pig plasma is least active. Bovine plasma, which is known to have no cholinesterase activity, has a moderate tabunase activity. The results obtained with a purified preparation (Fraction IV—1) from human serum are inserted in Fig. 3.

*Separation of tabunase from cholinesterase.* In order to purify tabunase of blood plasma and to separate it from the plasma cholinesterase, ammonium sulphate fractionation was tried without much success. Table I shows that the enzyme hydrolysing tabun cannot be separated by precipitation with this salt; even in 70 % saturated solutions appreciable amounts of active material were still detected in solution. It is known from earlier studies<sup>13</sup> that cholinesterase is completely precipitated at this concentration of ammonium sulphate. With

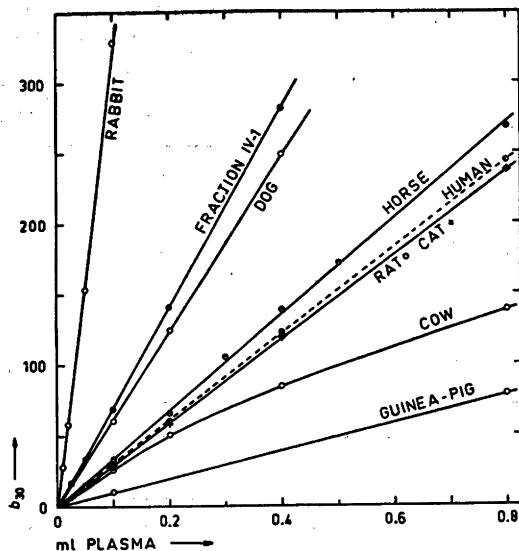


Fig. 3. Enzymatic hydrolysis of tabun by the plasma of various animals as a function of plasma concentration. Substrate,  $5.3 \times 10^{-3}$  M tabun. Fraction IV—1 was used as a 2.5 % solution.

a similar fractionation procedure, Aldridge<sup>5</sup> could not precipitate his A-esterase, which hydrolyses mintacol, successfully from rabbit serum.

Using the fractionation procedure of Cohn *et al.*<sup>11</sup> with human postpartum serum the tabunase came down almost completely in the globulin Fraction IV—1. Using this method the cholinesterase is found in Fraction IV—6 and no active material comes down in Fraction IV—1. The results obtained are recorded in Table 2. The direct proportionality between tabunase activity and concentration of Fraction IV—1 is shown in Fig. 3.

*Tissues as enzyme source.* The ability of various tissues of rabbit to hydrolyse tabun was then examined. The results are tabulated in Fig. 4 together

Table 1. Fractionation of human plasma with ammonium sulphate to separate the enzymes hydrolysing tabun and acetylcholine.

| Fraction   | $b_{30}$ |               |
|--|----------|---------------|
|  | Tabun    | Acetylcholine |
| Original plasma                                  | 58.5     | 213           |
| Precipitation, 50 % saturation with sulphate     | 47       | 35.5          |
| Precipitation, 70 % saturation with sulphate     | 49       | 88.5          |
| Centrifugate after 70 % saturation with sulphate | 53       | 0             |

Table 2. Fractionation of human postpartum serum according to a partly modified (Kabi) method No. 6 of Cohn *et al.*<sup>11</sup> to separate the enzymes hydrolysing tabun and acetylcholine.

| Fraction                           | $b_{30}/10$ mg proteine |               |
|------------------------------------|-------------------------|---------------|
|                                    | Tabun                   | Acetylcholine |
| Original serum                     | 40                      | 111           |
| I                                  | 8                       | 14            |
| II ( $\gamma$ -globulin)           | 2                       | 0             |
| III ( $\beta$ -globulin)           | 1                       | 11            |
| IV-1                               | 135                     | 14            |
| IV-4                               | 3                       | 510           |
| IV-5 + 6                           | 2                       | 475           |
| IV-6 + 9                           | 0                       | 880           |
| IV-6                               | —                       | 4 500         |
| V (albumin)                        | 2                       | 0             |
| Electric tissue ( <i>Torpedo</i> ) | 0                       | 5 000         |

with those obtained with acetylcholine as substrate. Adrenal glands are the most active; kidney and liver come next in activity amongst the tissues tested. Those tissues known to contain acetylcholinesterase (*e. g.*, brain, spinal cord) are weakly active. It will be shown in a subsequent paper that tabunase is not identical with the phosphatases known to be present in plasma, adrenal glands, kidney, and liver.

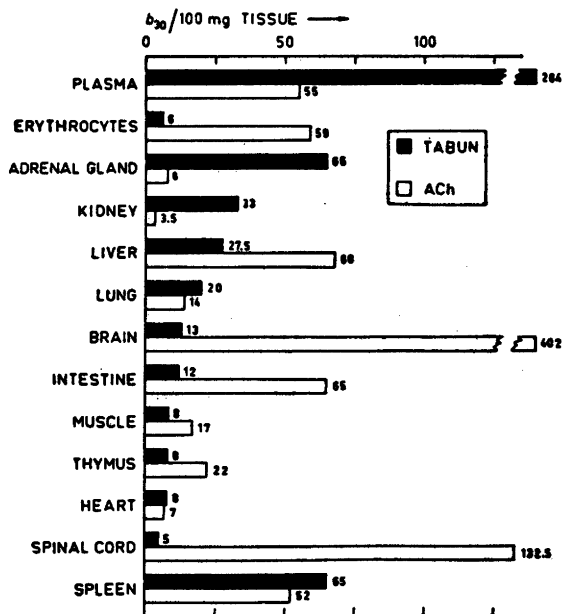


Fig. 4. The activity of various rabbit tissues in the hydrolysis of tabun and acetylcholine (ACh).

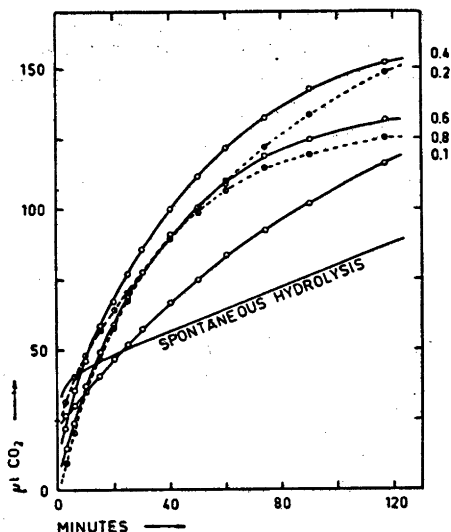


Fig. 5. Hydrolysis of tabun by the action of rabbit liver (not perfused). Numbers refer to grams of liver per reaction mixture (2.00 ml). Substrate,  $5.3 \times 10^{-3}$  M tabun.

An example of the hydrolysis of tabun by a tissue phosphorylphosphatase (rabbit liver) is shown in Fig. 5. Due to the relatively high spontaneous hydrolysis of tabun there is an immediate liberation of  $\text{CO}_2$  when the enzyme (in bicarbonate buffer) and the substrate (in aqueous solution) are mixed. The

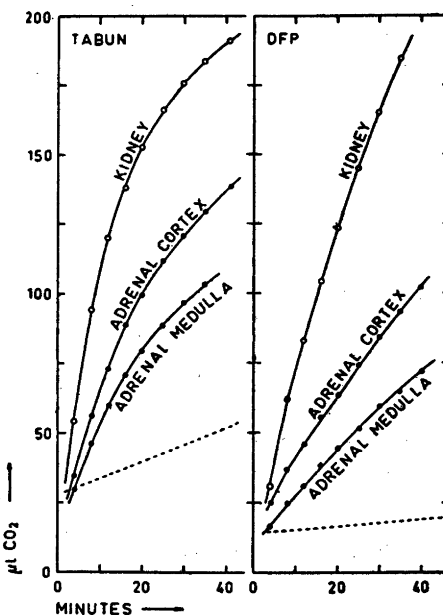


Fig. 6. Hydrolysis of tabun and DFP by kidney cortex and by the cortex and medulla of adrenal glands (pig). Substrate,  $5.3 \times 10^{-3}$  M; tissue, 200 mg per reaction mixture (2.00 ml). Dotted lines refer to spontaneous hydrolysis of substrates.

Table 3. The hydrolysis of tabun and DFP by kidney and adrenal glands of cow and pig. Enzyme activities are expressed in  $b_{50}$  per 100 mg tissue. Cf. Fig. 6.

| Animal | Kidney cortex |     | Adrenal gland |     |         |      |
|--------|---------------|-----|---------------|-----|---------|------|
|        |               |     | Cortex        |     | Medulla |      |
|        | Tabun         | DFP | Tabun         | DFP | Tabun   | DFP  |
| Cow    | 101.5         | 40  | 41            | 28  | 27      | 17   |
| Pig    | 140           | 158 | 51            | 31  | 34.5    | 22.5 |

recorded amount of this spontaneously produced  $\text{CO}_2$  is the less, the larger the amount of crude extract used.

Mazur<sup>1</sup> and Mounter *et al.*<sup>2</sup> have shown that kidney hydrolyses DFP. The hydrolysis of this substrate and that of tabun were compared using kidney and adrenal glands of pig and cow. The results obtained are shown in Fig. 6 and Table 3. In both animals the kidney is more active against the two substrates than are the adrenal glands. The adrenal cortex is more active than the adrenal medulla. The various tissue preparations show proportionate activities for each substrate and most probably the same enzyme in these tissues is responsible for the hydrolysis of tabun and DFP. Evidence will be presented later that the phosphorylphosphatase of plasma is not identical with that of kidney.

#### DISCUSSION

The presence of enzymes which hydrolyse the highly toxic organophosphorus compounds, is of great importance for our understanding of the pharmacological and biochemical effects of the latter on living organisms. Such enzymes in all probability interfere with the inactivation process of cholinesterases by such compounds. They will also be actively engaged in the detoxication mechanism of these anticholinesterase drugs, of which mechanism we know comparatively little.

From biochemical point of view a study of the enzymatic hydrolysis of organophosphorus compounds is likely to be of value for understanding of the mechanism of the inactivation of cholinesterases, which process underlies the toxicity of such compounds. In the opinion of the authors the hydrolysis of the compounds and cholinesterase inactivation by them involve principally the same reaction mechanism. In both these reactions the same linkage of the active molecule is broken, *i. e.*, the bond between the phosphorus atom and the acid group (halogen, cyanide, or an organic radical). In the hydrolysis process the phosphorus compound formed by the splitting of the toxic molecule is in itself not toxic. In the cholinesterase inactivation process that very part of the original molecule is more or less irreversibly bound to the esteratic site of a cholinesterase molecule. Further evidence for this working hypothesis is being presented later together with a more complete discussion of the results obtained.



## SUMMARY

The presence of an enzyme, provisionally called tabunase (general name suggested, phosphorylphosphatase) and having the property of hydrolysing dimethylamido-ethoxy-phosphoryl cyanide (tabun), has been demonstrated in the blood plasma of eight various animals including man; the rabbit plasma is the most active. Erythrocytes are almost inactive. Amongst the tissues of rabbit the adrenal glands, kidney, and liver are particularly active. It appears probable from experiments with pig and bovine tissues that tabun and DFP are split by the same enzyme of kidney and adrenal glands.

The phosphorylphosphatase and cholinesterase of human serum were separated from each other by the method of Cohn.

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 the various serum fractions.

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