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STUDIES ON REACTIVATION AND AGEING OF BLOOD CHOLINESTERASES OF TABUN INTOXICATED DOGS

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Abstract—The effects of pralidoxime (N-methylpyridinium-2-aldoxime) and TMB-4 (N,N′-trimethylene bis(pyridinium-4-aldoxime)) on blood cholinesterases (ChE) in Tabun poisoned dogs have been studied.

When given prophylactically TMB-4 dibromide (7 mg/kg intravenously) partly prevented the decrease of the ChE activity caused by 1 LD_{50} of Tabun. When given after the organophosphorus compound an initial enzyme inhibition was first observed followed by a rapid reactivation. Pralidoxime methane sulphonate (20 mg/kg intravenously) had no significant reactivating effect, and it made no difference whether the oxime was given before or after Tabun. The lowest plasma concentration of TMB-4 dibromide that produced within 2 hr a significant reactivation of the erythrocyte ChE was $4–7 \times 10^{-6}$ M. This concentration produced a 60 per cent reactivation within 48 hr (dose of Tabun 10 LD_{50}).

Ageing of phosphorylated dog erythrocyte ChE was found to be slower *in vivo* than *in vitro* ($t_{1/2}$ about 48–72 and 30 hr respectively).

IN RECENT years it has been shown that certain oximes are valuable adjuncts to atropine in the treatment of organophosphorus anticholinesterase poisoning.[1, 2] These oximes are believed to act principally by reactivating the phosphorylated enzyme and thus provide a causal therapy. The most thoroughly studied is pralidoxime (N-methylpyridinium-2-aldoxime, 2-PAM)[3, 4] which has been successfully used in human intoxications.[5–8] However, it has been shown that another oxime, TMB-4, N,N′-trimethylene bis(pyridinium-4-aldoxime) is a more potent antidote,[9, 10] especially in Tabun poisoning where pralidoxime is almost without effect.[11–14].

In vitro reactivation of Tabun inhibited cholinesterase has been shown to proceed very slowly with low concentrations of pralidoxime.[13, 15] At an oxime concentration of 5×10^{-5} M the rate constant was estimated to 13×10^{-4} min^{-1}.[15] In spite of this slow reactivation a certain antidote effect of pralidoxime has been demonstrated in Tabun poisoned animals.[11–14, 21] This has been attributed to inactivation of Tabun due to the rapid reaction between the organophosphorus compound and the oxime.[11] This possibility was studied further in the present investigation. In contrast to pralidoxime TMB-4 has been shown to be a potent reactivator *in vitro* after Tabun inhibition. No data are available on the effect of TMB-4 on cholinesterases in Tabun poisoned animals. In this paper the effect of TMB-4 on blood cholinesterase activity and on the toxic symptoms has been studied in severe Tabun poisoning. Plasma concentrations of the injected oximes were determined. The transformation of phosphorylated cholinesterase to a non-reactivatable form (ageing), being a limiting factor for the

efficacy of oxime therapy, has been followed *in vivo* and compared with the rate of ageing *in vitro*.

MATERIALS AND METHODS

Dimethylamido ethoxyphosphorylcyanide (Tabun) was synthesized according to Holmstedt.[16] N-methylpyridinium-2-aldoxime methane sulphonate (P2S) and N,N'-trimethylenebis(pyridinium-4-aldoxime) dibromide (TMB-4) were synthesized according to Creasy and Green[17] and Poziomek *et al.*[9] respectively.*

Seventeen unanaesthetized dogs were used in this study. In all experiments atropine sulfate (2 mg/kg) was given intravenously 10 to 15 min before the intravenous injection of Tabun. The oximes were given either intravenously or intramuscularly either before or after Tabun. The clinical condition of the dogs was carefully observed during the first 3–5 hr and then intermittently for 10–30 days.

Venous blood samples were collected in heparinized test tubes at different time intervals. The samples were immediately cooled in ice water and all further treatment before determination of enzyme activities was done at 0°. After centrifugation (10 min at 10,000 rev/min) of the blood the cholinesterase activity of the red blood cells (ChE) was determined as well as their percentage of aged inhibited enzyme (see below). Some of the blood samples were also analysed for the presence of 'free' cholinesterase inhibitor. A standard cholinesterase preparation was incubated with the samples and the degree of inhibition measured. The amount of inhibitor was then calculated from a standard graph. The plasma was used for analysis of the oxime concentration. It was found that the ultraviolet method previously used[17, 21] for the determination of pralidoxime in plasma could be adapted for the determination of TMB-4. Following deproteinization the samples were made alkaline and the absorption at 345 mμ was determined (P2S in the original method was determined at 330 mμ).

Determination of cholinesterase activity was performed after washing the erythrocytes with 2 × 10 volumes of cold isotonic saline to remove any traces of free inhibitor.[15] Before enzyme activity determinations the erythrocytes were diluted 1:1 with veronal buffer pH 7·4 and the measurements were performed with the electrometric method.[18] The final concentration of acetylcholine iodide was $7{\cdot}9 \times 10^{-3}$ M.[19] The enzyme activity (E) is given in per cent of the erythrocyte cholinesterase activity of a blood sample taken immediately before the administration of Tabun.

Ageing of the Tabun inhibited dog erythrocytes *in vivo* was measured after incubation of the samples with $8{\cdot}5 \times 10^{-3}$ M TMB-4 *in vitro* at pH 7·4 and 37° for 70 min and subsequent determination of enzyme activity. The values given in the figures represent the total amount of enzyme activity after *in vitro* reactivation (Er) expressed in per cent of normal dog erythrocyte samples containing the same amount of TMB-4. The percentage of aged enzyme at each point of determination therefore is given by

$$\frac{100\ (100 - \mathrm{Er})}{100\ (\mathrm{E})}$$

It was considered necessary to determine the variability of the enzyme activity determinations. The following method was used: ten consecutive blood samples were taken from a dog during about 6 hr and treated separately as described above. The coefficient of variation between the samples was found to be 4·9 per cent of the mean.

* The compounds were synthesized in the department of organic chemistry of this institute.

RESULTS

The effect of Tabun on cholinesterase activity and ageing in vivo *and* in vitro

In two experiments 84 μg/kg of Tabun (approximately 1 LD_{50})[20] were injected intravenously into atropinized dogs. Following this dose the animals showed slight dyspnoea and slight weakness of the muscles of the hind legs for a few minutes. After 5 min the cholinesterase activity of plasma and erythrocytes was inhibited about 80 per cent. Maximal inhibition of erythrocyte ChE was obtained after 60 min. A recovery of about 10 per cent of the enzyme activity was noticed during the following 2 hr (Fig. 1). Very little further spontaneous return of erythrocyte cholinesterase

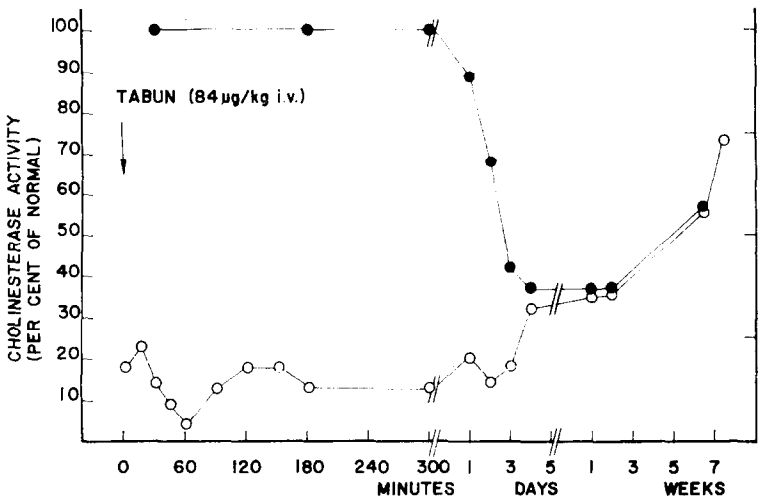

FIG. 1. Erythrocyte ChE activity (○) and total amount of enzyme activity after *in vitro* reactivation (●) following 1 LD_{50} intravenously of Tabun in an atropinized unanaesthetized dog.

could be detected during the next three days while 80 per cent of the activity of plasma ChE returned during the same time. A continous return of plasma ChE activity occurred and only a small portion of the originally inhibited enzyme was transformed to a nonreactivatable form. After 24 hr about 10 per cent of the original plasma enzyme activity could not be reactivated by oximes.

After about $2\frac{1}{2}$ days 50 per cent of the inhibited erythrocyte enzyme was transformed to a form which could not be reactivated by TMB-4. After 4–6 days all of the still inhibited enzyme seemed to be aged. The rate constant for ageing of Tabun inhibited dog erythrocytes *in vitro* (k_1) was determined at pH 7·4 and 37° and found to be $3{\cdot}8 \times 10^{-4}$ min^{-1} ($t_{1/2} \approx 30$ hr). This means that ageing *in vivo* is slower than *in vitro*.

Effect of P2S and TMB-4 intravenously

P2S (20 mg/kg) was given intravenously 10 min before the intravenous injection of 84 μg/kg of Tabun. The symptoms that developed following the injection of Tabun were essentially the same as described in the preceding paragraph. A very slow increase from 15 to 28 per cent of the original erythrocyte ChE activity occurred during the time 1–6 hr after the injection of Tabun (Fig. 2). No further increase

was noticed during the following week. Ageing proceeded approximately as in the absence of oxime.

P2S (20 mg/kg) was then given intravenously 17 min after an intravenous injection of 84 μg/kg of Tabun. No immediate effect upon enzyme activity was observed. During the period 1–3 hr a slight reactivation was seen (about 5 per cent) but the enzyme activity then declined to that before administration of oxime (Fig. 3). The plasma concentration of oxime was $3{\cdot}9 \times 10^{-5}$ M 20 min after the Tabun injection and declined exponentially, $t_{1/2}$ was 40 min.

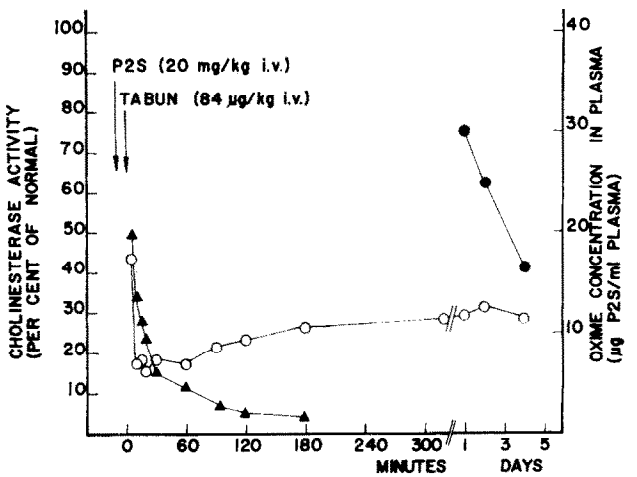

FIG. 2. Erythrocyte ChE activity (○) and total amount of enzyme activity after *in vitro* reactivation (●) following 20 mg/kg intravenously of P2S and 1 LD_{50} intravenously of Tabun given 10 min later. ▲ represents the plasma concentration of the oxime.

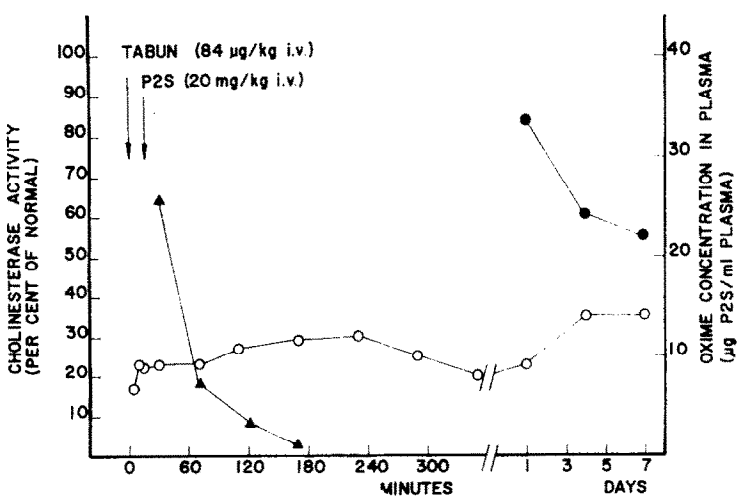

FIG. 3. Erythrocyte ChE activity (○) and total amount of enzyme activity after *in vitro* reactivation (●) following 1 LD_{50} intravenously of Tabun and 20 mg/kg intravenously of P2S given 17 min later. ▲ represents the plasma concentration of the oxime.

Three dogs were pretreated with TMB-4 (7 mg/kg intravenously) 10 min before the intravenous injection of 84 μg/kg of Tabun. In none of the experiments the enzyme activity of the erythrocytes decreased to less than 35 per cent of original value. A small reactivation to 40, 45 and 55 per cent of the original activity was obtained within 4 hr. The results from one of the experiments are shown in Fig. 4. The plasma con-

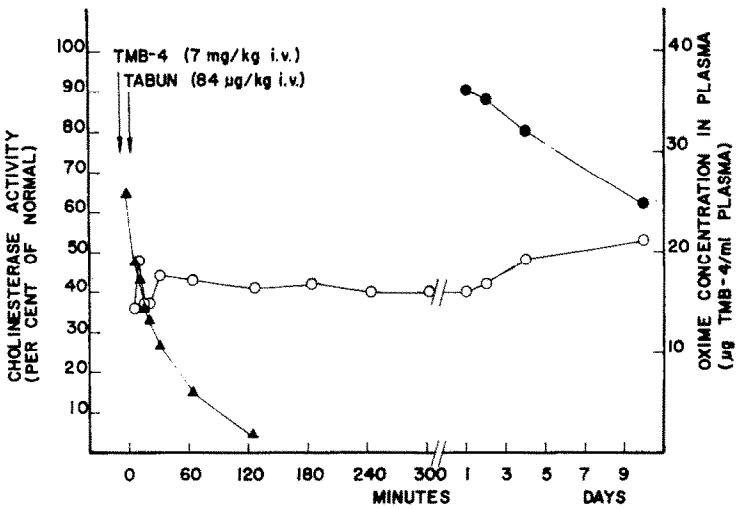

FIG. 4. Erythrocyte ChE activity (○) and total amount of enzyme activity after *in vitro* reactivation (●) following 7 mg/kg intravenously of TMB-4 and 1 LD_{50} intravenously of Tabun given 10 min later. ▲ represents the plasma concentration of the oxime.

centration of TMB-4 declined exponentially, and had a calculated half life of 37 min. 20 min after Tabun the mean plasma concentration of the dogs was $3{\cdot}6 \times 10^{-5}$ M. TMB-4 (7 mg/kg intravenously) was then given to two dogs which had received one LD_{50} of Tabun 23 min before. An immediate reactivation effect was observed and within 5–10 min the enzyme activity had increased from 20 to 50 per cent of the original value (Fig. 5). After 4 hr 60–70 per cent of the original enzyme activity was recovered and no further reactivation was observed.

The dose of Tabun was then increased to 420 μg/kg (about 5 LD_{50}) intravenously. After about 30 sec this animal showed severe symptoms consisting of severe dyspnoe followed by respiratory failure and pronounced cyanosis, weakness and tremor of the legs. After 3 min TMB-4 (7 mg/kg) was given intravenously. The condition of the animal improved gradually and after 3 hr the animal had recovered completely. The Tabun injection produced a 98 per cent inhibition of the erythrocyte ChE activity.

After TMB-4 an increase to 10 per cent of the original value was obtained after 3 min and to 30 per cent after 25 min. A further increase was noticed during the next few hours.

The occurrence of free inhibitor in the blood could be demonstrated during the first 6 min after the injection of 5 LD_{50} of Tabun. The concentration of free inhibitor in the blood was $1{\cdot}9 \times 10^{-8}$ M after 2 min and about $1{\cdot}2 \times 10^{-8}$ M after 6 min.

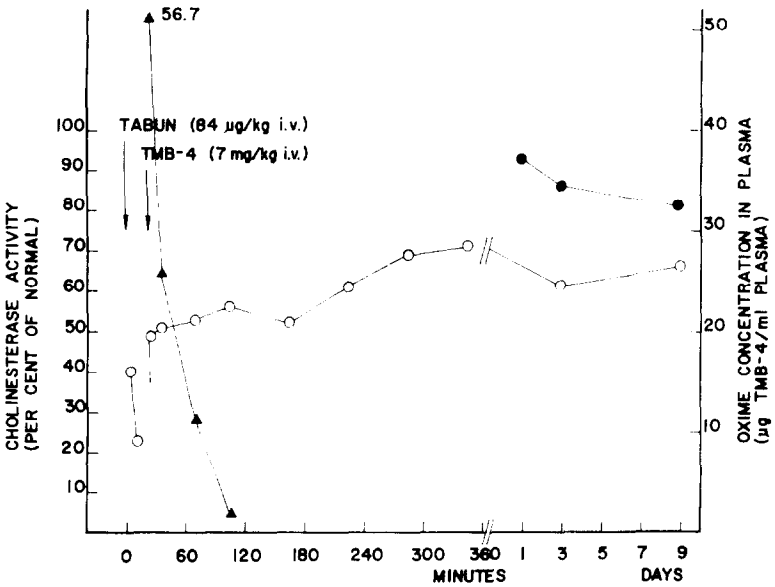

FIG. 5. Erythrocyte ChE activity (○) and total amount of enzyme activity after *in vitro* reactivation (●) following 1 LD_{50} intravenously of Tabun and 7 mg/kg intravenously of TMB-4 given 23 min later. ▲ represents the plasma concentration of the oxime.

The effect of intramuscular injection of TMB-4 after large doses of Tabun

The dose of Tabun was increased to 840 µg/kg intravenously (approx 10 LD_{50}). Following this dose severe dyspnoe and cyanosis, prostration and extended fasciculations occurred within 1 min. TMB-4 (1·75 mg/kg intramuscularly) was given within 1 minute. Despite this treatment respiratory failure developed after 4 min. Another injection of 1·75 mg TMB-4/kg was then given. 6 min counted from the first doses of TMB-4 respiratory movements reappeared in the diaphragm and fasciculations diminished. After 8 min cyanosis had disappeared and intercostal breathing had reappeared. After 25 min the animal could stand up and only slight fasciculations on the skull were seen. The experiment is illustrated in Fig. 6. No reactivation of the erythrocyte ChE activity was seen 30 min after the injection of TMB-4. The oxime concentration in the plasma during that time was about 1×10^{-5} M. After 60 min 5 per cent of the ChE activity had returned.

In another experiment only one dose of TMB-4 (1·75 mg/kg intramuscularly) was given initially within 1 min after 840 µg Tabun/kg. Artificial respiration had to be given from 4–11 min after the Tabun injection. After 30 min the dog was able to stand on its feet and fasciculations had markedly diminished. After 60 min muscle weakness and fasciculations reappeared. Another dose of TMB-4 (1·75 mg/kg) was given after 90 min and then repeated approximately every third hour except during early morning hours. The experiment is illustrated in Fig. 7. After the second injection of TMB-4 the enzyme activity increased (18 per cent) and continued gradually to increase during the following days. When the oxime injections were stopped (4th day), 67 per cent of

original enzyme activity had reappeared. The rest of the original enzyme activity (33 per cent) had been transformed to the nonreactivatable form. The oxime concentration in plasma during the whole experiment varied between $3{\cdot}4 \times 10^{-6}$ M and $6{\cdot}8 \times 10^{-6}$ M.

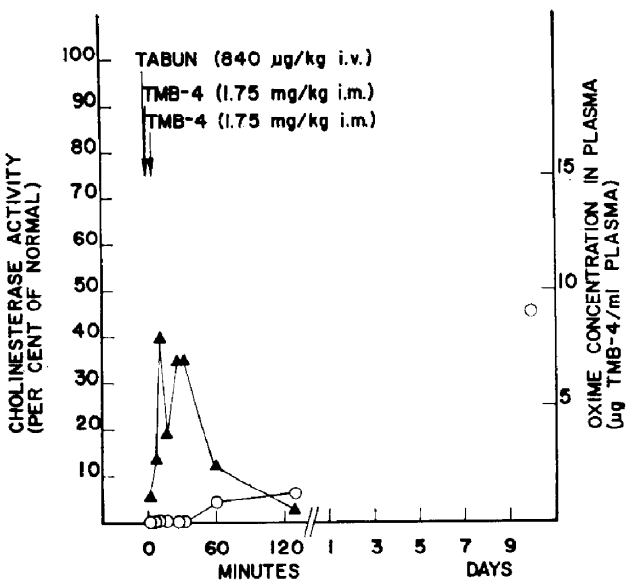

FIG. 6. Erythrocyte ChE activity (○) following 10 LD_{50} intravenously of Tabun and $2 \times 1{\cdot}75$ mg/kg intramuscularly of TMB-4 given 1 and 4 min later, respectively. ▲ represents the plasma concentration of the oxime.

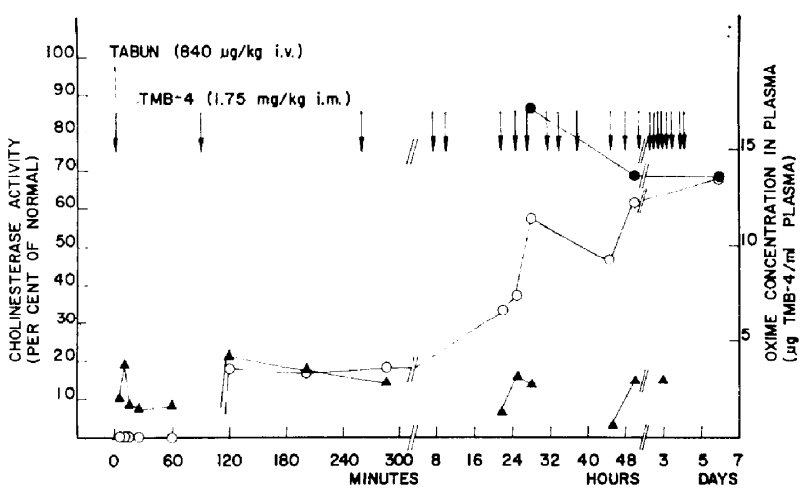

FIG. 7. Erythrocyte ChE activity (○) and total amount of enzyme activity after *in vitro* reactivation (●) following 10 LD_{50} intravenously of Tabun and repeated doses of 1·75 mg/kg of TMB-4 during 4 days. ▲ represents the plasma concentration of the oxime.

DISCUSSION

The experiments described in this paper show that there is no significant increase in erythrocyte cholinesterase activity following inhibition by Tabun and subsequent administration of P2S.

If the antidote effect of P2S is due to a rapid *in vivo* reaction between P2S and Tabun or an enzyme protecting effect of P2S, the enzyme should be less inhibited[22] in the presence of P2S. However, the experiments show that 1 LD_{50} of Tabun inhibits the enzyme to the same degree in the presence and in the absence of P2S. Possibly inhibition of erythrocyte ChE by Tabun was quicker than any inactivation of Tabun by P2S. The possibility also exists that an anticholinesterase is formed by a reaction between P2S and Tabun and that the latter is inhibiting the enzyme. As a matter of fact it is known that 4-PAM and Sarin form an anticholinesterase as potent as Sarin itself.[23]

Intravenous injection of TMB-4 before Tabun gives an initial higher enzyme activity than that which is found after the same amount of Tabun alone. A rapid and marked reactivation is obtained when TMB-4 is given after Tabun. This is in agreement with *in vitro* reactivation experiments and the good antidote effect of the oxime. The minimum concentration of TMB-4 giving a significant reactivation in the blood within 2 hr is of the order $4–7 \times 10^{-6}$ M. This concentration reactivates about 60 per cent of the original enzyme activity within 48 hr (Fig. 7). However, following 10 LD_{50} of Tabun, this concentration is not enough to prevent a respiratory paralysis. Concentrations of $1–1{\cdot}5 \times 10^{-5}$ M on the other hand overcome this effect of Tabun and also produce a slight reactivation in the blood within 60 min (Fig. 6). In Tabun poisoning death is caused by respiratory failure resulting from progressive paralysis of the respiratory muscles and depression of the respiratory centre. In the atropinized dogs used in these experiments the respiratory paralysis is caused by a periferal neuromuscular block[16] since atropine counteracts the central inhibition of respiration. The fact that the respiratory failure could be overcome by TMB-4 without any measurable reactivation in the blood during the first 30 min may be explained by the observation that TMB-4 also possesses some curare-like properties, which promote the restoration of neuromuscular function.[25, 26] Another possibility is that the oxime concentration in skeletal muscle is higher than in blood.

From the results presented here and in a previous paper[15] it is obvious that Tabun inhibited ChE's from different animals have different rates of ageing. *In vitro* the rate constants for ageing of Tabun inhibited ChE from human and dog erythrocytes were found to be $8{\cdot}7 \times 10^{-5}$ and $3{\cdot}8 \times 10^{-4}$ min^{-1} at 37° and pH 7·4. The experiments with Tabun in dogs show that ageing proceeds slower *in vivo* and is not complete until 4–6 days. Both results show that the therapeutic effect of TMB-4 would not be seriously impaired by ageing of Tabun inhibited cholinesterase during the first day.

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