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# *IN VITRO* REACTIVATION AND "AGEING" OF TABUN-INHIBITED BLOOD CHOLINESTERASES

## STUDIES WITH N-METHYLPYRIDINIUM-2-ALDOXIME METHANE SULPHONATE AND N,N'-TRIMETHYLENE BIS (PYRIDINIUM-4-ALDOXIME) DIBROMIDE

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**Abstract**—Tabun-inhibited acetyl- and butyrylcholinesterases from human and horse blood were stored at varying conditions and the degree of reactivation after incubation with the oximes TMB-4 and P2S was measured. The influence of the concentration of the oxime upon reactivation was studied. When possible, rate constants for the reactivation were calculated and compared with rate constants for the transformation of the phosphorylated enzyme to a non-reactivable form. Reactivation of human erythrocyte and plasma cholinesterase with P2S was shown to be very slow. Reactivation with TMB-4 was more rapid, but decreasing the concentration of TMB-4 below $10^{-3}$ M resulted in incomplete reactivation. At therapeutical concentrations of the reactivator ($\sim 2{\cdot}5 \times 10^{-5}$ M) at least 75 per cent of the enzyme activity was restored.

Phosphorylated plasma cholinesterase from human blood was shown to "age" much more rapidly than phosphorylated plasma cholinesterase from horse blood and to have a promoting effect upon the "ageing" of phosphorylated, purified horse serum cholinesterases. The "ageing" of the purified serum cholinesterase preparations was found to be slower than that of the corresponding untreated plasma enzyme.

IT IS generally accepted that organophosphorus anticholinesterases inhibit cholinesterases through phosphorylation. The phosphorylated enzymes can often be reactivated by nucleophilic compounds such as certain oximes provided conversion of the inhibited enzyme to a non-reactivable form ("ageing") has not occurred.[3] There are, however, inhibitors which do not allow reactivation of the inhibited enzyme. Methylfluoro-phosphorylcholines obstruct reactivation by 2-PAM, the explanation being a shielding of the anionic site from the reactivator through an ionic bond between enzyme and inhibitor.[1] The *in vivo* reactivation[2] by 2-PAM of Tabun-inhibited cholinesterases is supposed to be difficult. An attempt to analyse the possibilities of reactivation after inhibition with Tabun of human blood, horse plasma and purified cholinesterase preparations from human serum and horse serum is described here. Reactivators were TMB-4[3] and P2S[3].

### MATERIALS AND METHODS

*Enzymes*

*Acetylcholinesterases.* Human erythrocytes were obtained after centrifugation of heparinized blood (blood bank), and washed three times with 0·9% sodium chloride solution.

*Butyrylcholinesterases.* Human plasma or horse plasma was obtained after centrifugation of heparinized blood. Purified horse serum cholinesterase[4] was dissolved in 0·9% sodium chloride solution, 2 mg/ml solution. Serum fraction IV-6-3 (AB Kabi, Stockholm) obtained from human postpartum blood, was dissolved in veronal buffer pH 8·0, 0·5 mg/ml solution.

*Substrates*

Acetylcholine iodide, synthesized in this laboratory, was used. The final concentration in all experiments was $7{\cdot}3 \times 10^{-3}$ M.

*Inhibitors*

Dimethylamido ethoxyphosphoryl cyanide (Tabun) and *iso*propoxy methylphosphoryl fluoride (Sarin) were used. The inhibitors were synthesized in this laboratory by L. Fagerlind. Their concentration during the inhibition procedure was $10^{-5}$ M. The inhibitors were either dissolved in 0·9% sodium chloride solution or in veronal al buffer pH 8·0.

*Reactivators*

N-methylpyridinium-2-aldoxime methane sulphonate (P2S) and N,N′-trimethylene bis(pyridinium-4-aldoxime) dibromide (TMB-4) were used. The reactivators were synthesized in this laboratory by I. Enander. When kept in solution at pH 7·4 and 37 °C, 31 per cent of P2S or 9 per cent of TMB-4 were broken down after 14 days.[5]

*Buffer solutions*

Veronal buffer pH 8·0 (Michels buffer) was prepared as described earlier.[7] Veronal buffer pH 7·4 was prepared as described in *Methods in Enzymology* (Vol. 1).

*Inhibition of the enzymes*

Human erythrocytes, diluted 1:1 with saline solution were incubated with the inhibitor for 1 hr at 0 °C. The incubation mixture was centrifuged for 15 min at 4000 × *g*, 0 °C and the erythrocytes were washed three times with a fivefold excess of cold (0°) saline. Immediately before use the erythrocytes were diluted with twice their volume of buffer. In experiments with the automatic recording titrator[6] the erythrocytes were haemolysed by addition of twice their volume of cold distilled water. After incubation with the inhibitor, all other enzyme preparations were dialysed continuously for 2 days against saline or buffer at +4 °C. A shorter dialysis time resulted in a presence of traces of free inhibitor in the enzyme preparation. Enzyme solutions without inhibitor were treated in the same way and used as controls. No loss of enzyme activity due to the dialysis was noticed.

*Test for free inhibitor*

After washing or dialysis of the inhibited enzyme solutions these solutions were used as "inhibitors" on their controls. The controls were incubated with the inhibited enzymes for 30 min at 25 °C and pH 8·0, after which the cholinesterase activity of the solutions was measured and compared with the activity of the controls. The same activity in all samples was taken as an indication of the absence of free inhibitor.

*Measurement of enzyme activity*

Enzyme activity was either determined with the electrometric method[7] or with the automatic recording constant pH-titrator.[6] When the electrometric method was used, the vessels contained 3 ml of veronal buffer pH 8·0, $(3 - a)$ ml of distilled water, $a$ ml of enzyme solution and 0·6 ml of substrate solution. When the titrator was used the vessel contained 2 ml of enzyme solution, 37 ml of 0·1 M potassium chloride and 1 ml of substrate solution. All enzyme activities were determined at 25 °C and pH 8·0. All values used are mean values of two determinations.

*Determination of reactivation and "ageing"*

Control and inhibited enzyme solution were diluted in the same manner, usually 1:1 with veronal buffer pH 8·0 or pH 7·4 and placed in a thermostat either at 25 °C (pH 8·0) or 37 °C (pH 8·0 or pH 7·4). In all but one series of experiments (those of short time reactivation; the pH was kept at 7·4 by the use of the constant pH-titrator) the pH was kept constant by means of buffer solutions. The pH was measured at the beginning and at the end of each experiment and was found to be constant. When larger amounts of reactivator were used, the pH was lower (7·8, resp. 7·2) during the whole experiment.

Samples of controls and of inhibited enzyme solution were withdrawn after varying incubation times and incubated with reactivators without further dilution. From these solutions samples were again withdrawn after certain time intervals and their enzyme activities were determined. In experiments with a long duration the first enzyme activity determination was made 70 min after addition of the reactivator. Further measurements were made at intervals of about 24 hr.

For calculation of rates of reactivation some of the symbols introduced by Davies and Green[8] were used. $E_\infty$ thus is the total inhibited enzyme concentration available for reactivation and $E_t$ is the concentration of reactivated enzyme after time $t$. Rate constants were calculated only from freshly inhibited enzyme preparations. The following equations were used:

$$k_{obs} = \frac{2{\cdot}301}{t} \log \frac{E_\infty}{E_\infty - E_t}$$

and

$$k' = \frac{k_{obs}}{Ox}$$

were $Ox$ is the concentration of the reactivator. With the reactivator P2S at concentrations above $1 \times 10^{-3}$ M, $E_\infty$ was identical with the activity of the control containing the same amount of reactivator. This was also true within 5 per cent with TMB-4 at high concentrations. At lower concentrations reactivation with TMB-4 reached a plateau at a value below the value of the control.

Activities of untreated enzyme solutions with or without reactivator and of inhibited enzyme solutions were always measured in order to control spontaneous reactivation or denaturation of the preparations.

When "ageing" was observed for several days, corrections for changes in the activity of the control and the inhibited enzyme preparation were made. For calculations of rate constants ($k_1$) for the "ageing" of the phosphorylated enzymes $\log [(E - E_i)100/(E_o - E_i)]$ was plotted against time, where $E$ is the activity of the reactivated enzyme,

$E_i$ the activity of the inhibited enzyme (including spontaneous hydrolysis of the substrate) and $E_0$ the activity of the control incubated with the same amount reactivator. $E$ was obtained after 1 day's incubation with TMB-4 or 2 day's incubation with P2S.

## RESULTS

*Human plasma cholinesterase*

After inhibition with Tabun enzyme activity could be restored at pH 7·2, 37 °C and in the presence of veronal buffer much quicker with $8{\cdot}5 \times 10^{-3}$ M TMB-4 than with $10^{-2}$ M P2S. The effect of the two reactivators on Sarin inhibited human plasma was more equal (Table 1). The Sarin-inhibited enzyme showed, in contrast to the

TABLE 1. REACTIVATION OF TABUN- OR SARIN-INHIBITED HUMAN PLASMA AT 37 °C AND PH 7·2

(Enzyme inhibited at 0 °C for 1 hr and dialysed at 4 °C for 2 days. Controls incubated with the same amount of reactivator as inhibited enzymes.)

| Time after addition of reactivator | | Enzyme activities in % of controls | | | |
|---|---|---|---|---|---|
| | | TMB-4 $8{\cdot}5 \times 10^{-3}$ M | | P2S $10^{-2}$ M | |
| TMB-4 | P2S | Tabun-inhib. enzyme | Sarin-inhib. enzyme | Tabun-inhib. enzyme | Sarin-inhib. enzyme |
| 70 min | 70 min | 65 | 91 | 17 | 88 |
| 22 hr | 23 hr | 84 | 100 | 46 | 84 |
| 68 hr | 67 hr | 83 | 91 | 49 | 88 |

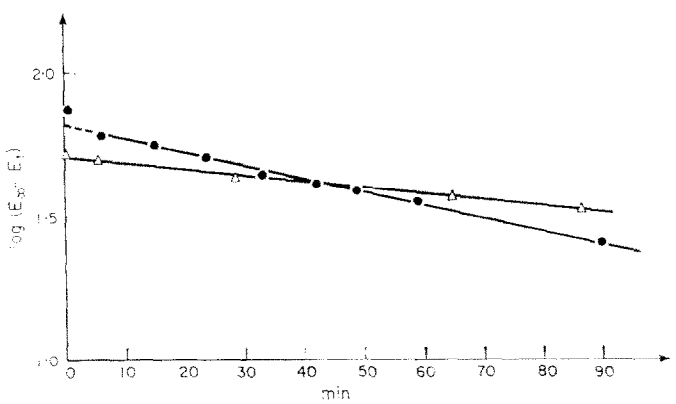


FIG. 1. Reactivation at pH 7·2, 37 °C of dimethylamido-ethoxyphosphorylated cholinesterase in human plasma. △ = $10^{-2}$ M P2S, ● = $8{\cdot}5 \times 10^{-3}$ M TMB-4.

Tabun-inhibited enzyme, a certain amount of spontaneous reactivation, which followed first-order kinetics. At constant reactivator concentration reactivation was also found to follow first-order kinetics (Fig. 1). Rate constants for the reactivation of the dimethylamido-ethoxyphosphorylated enzyme were calculated from experiments with the automatic recording pH-titrator and are reported in Table 2.

When the Tabun- or Sarin-inhibited plasma was stored at 37 °C in veronal buffer pH 7·4, conversion to a stable phosphorylated form occurred. This "ageing" followed first-order kinetics with respect to the amount of reactivable enzyme and was stopped by the addition of reactivator. Rate constants are given in Table 3.

TABLE 2. RATE CONSTANTS FOR THE REACTIVATION OF TABUN-INHIBITED HUMAN ERYTHROCYTES (*E*) AND TABUN-INHIBITED HUMAN PLASMA (*P*) WITH $8{\cdot}5 \times 10^{-3}$ M TMB-4 OR $10^{-2}$ M P2S AT 37 °C AND PH 7·4

| | TMB-4 | | P2S | |
|---|---|---|---|---|
| | *E* | *P* | *E* | *P* |
| $k_{obs}$ ($min^{-1}$) | $1{\cdot}62 \times 10^{-1}$ | $1{\cdot}32 \times 10^{-2}$ | $1{\cdot}05 \times 10^{-2}$ | $6{\cdot}04 \times 10^{-3}$ |
| $k$ ($l.\ mol^{-1}\ min^{-1}$) | $1{\cdot}91 \times 10$ | 1·55 | 1·05 | $6{\cdot}04 \times 10^{-1}$ |

TABLE 3. RATE CONSTANTS FOR THE "AGEING" AT PH 7·4 (VERONAL BUFFER) AND 37 °C OF SOME PHOSPHORYLATED CHOLINESTERASES

| Source of enzyme | Inhibitor | $k_1$ ($min^{-1} \times 10^4$) |
|---|---|---|
| Human plasma* | Tabun | 18 |
| Human plasma† | Tabun | 19 |
| Human plasma* | Sarin | 20 |
| Human plasma† | Sarin | 18 |
| Purified from human serum* | Tabun | 1·4 |
| Purified from human serum† | Tabun | 1·2 |
| Horse plasma* | Tabun | 0·9 (slowed down after 5 days) |
| Purified from horse serum* | Tabun | 0·4 |
| Purified from horse serum* | Sarin | 0·7 |
| Human erythrocytes* | Tabun | 8·7 |

Reactivator * TMB-4 or † P2S.

*Purified human serum cholinesterase fraction* IV-6-3

The experiments were run at 25 °C and in veronal buffer pH 8·0. Again, reactivation with TMB-4 was quicker, maximal restoration of enzyme activity was reached within 1 day with TMB-4, while only 47 per cent of the enzyme activity was restored with P2S during the same time. "Ageing" of the phosphorylated enzyme followed first-order kinetics and could be prevented by the addition of reactivator. Rate constants are given in Table 3 and show that "ageing" of the purified enzyme preparation was more than ten times slower than of the untreated human plasma enzyme.

The preparation was tested for Tabun-splitting enzymes (phosphorylphosphatases)[3] with $10^{-3}$ M Tabun and found to contain no such enzymes.

*Purified horse serum cholinesterase*

At 25 °C, pH 7·8 (veronal buffer) and constant concentration of reactivator, reactivation of the Tabun-inhibited enzyme preparation was found to be a first-order reaction with respect to the amount of inhibited enzyme available for reactivation. With $10^{-2}$ M P2S the rate of reactivation was $6 \times 10^{-5}$ $min^{-1}$. Reactivation with TMB-4 was much quicker (Fig. 2). No spontaneous reactivation was noticed during

the time of the experiment (7 days). A small decrease of enzyme activity with time noticed in the uninhibited preparation was somewhat higher in the presence of TMB-4 (Fig. 2).

At 25 °C and pH 8·0 (veronal buffer) "ageing" of the Tabun-inhibited enzyme was hardly noticeable, the percentage of restored enzyme activity after treatment with TMB-4 for 1 day being approximately constant during 6 days. At 37 °C and in veronal

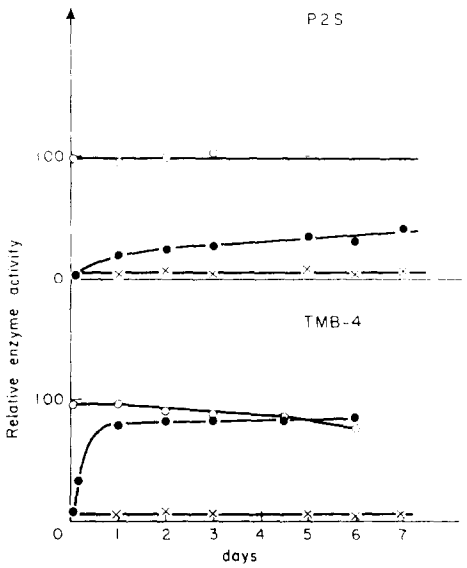


FIG. 2. Reactivation at pH 7·8, 25 °C of dimethylamido-ethoxyphosphorylated cholinesterase in a purified horse serum fraction. $10^{-2}$ M P2S and 8·5 × $10^{-3}$ M TMB-4. × inhibited enzyme, ● reactivated enzyme, ○ control.

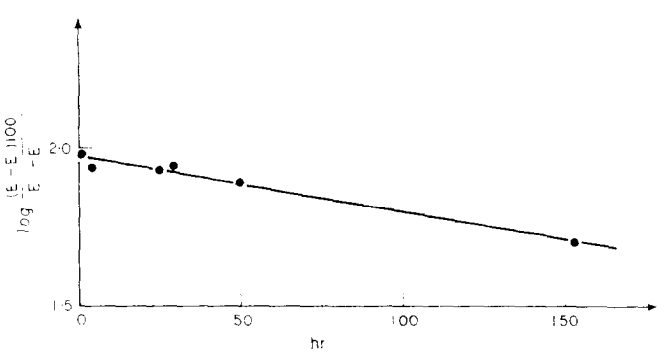


FIG. 3. "Ageing" at pH 7·4, 37 °C of methyl-*iso*propoxyphosphorylated cholinesterase in a purified horse serum fraction. Reactivation with 8·5 × $10^{-3}$ M TMB-4, 24 hr.

buffer pH 7·4 a very slow "ageing" was observed (Table 3). Jansz *et al.*[9] have described rapid "ageing" of purified horse serum cholinesterase after inhibition with DFP and slower "ageing" after inhibition with Sarin. To compare the enzyme preparation used in the Tabun experiments with the one used by Jansz, "ageing" was studied at 37 °C in veronal buffer pH 7·4 after inhibition with Sarin and found to be rather slow (Fig. 3).

The rate constant is seen in Table 3. Corrections for spontaneous reactivation had to be made. From the first part of the curve obtained from values given by Jansz *et al.* ("restored enzymic activity (%) Table 4") a rate constant of approximately $9 \times 10^{-5}$ $min^{-1}$ at pH 7·5 (buffer unknown) and 25 °C can be calculated. Considering the difference in temperature, this value is somewhat higher than the value seen in Table 3.

An attempt was made to determine if the difference in the rate of "ageing" of phosphorylated human plasma cholinesterase and phosphorylated purified horse serum cholinesterase depends upon the presence in human plasma of some enzymic or non-enzymic factor that promotes "ageing". Dimethylamido-ethoxyphosphorylated cholinesterase from horse serum was incubated at pH 7·4 and 37 °C either with buffer alone or with buffer plus human plasma or with buffer plus boiled and centrifuged human plasma. The rate of "ageing" was then observed. In Fig. 4 the curves are plotted. They suggest the existence of an "ageing-promoting" factor in human plasma.

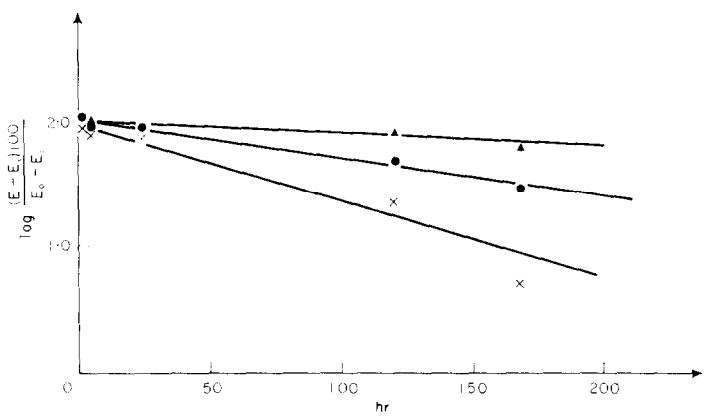


FIG. 4. "Ageing" at pH 7·4, 37 °C of dimethylamido-ethoxyphosphorylated cholinesterase in a purified horse serum fraction. ▲ = incubated alone, ● = incubated with boiled and centrifuged human plasma, × = incubated with human plasma. Reactivation with $8·5 \times 10^{-3}$ M TMB-4, 24 hr.

*Horse plasma cholinesterase*

To determine whether the very slow "ageing" of purified horse serum was caused by a change of enzyme properties during purification the experiments were repeated with Tabun-inhibited horse plasma at 37 °C and pH 7·4 (veronal buffer). "Ageing" was still very slow although somewhat faster than with the purified preparation (Table 3). Reactivation of freshly inhibited plasma with $8·5 \times 10^{-3}$ M TMB-4 was complete within 1 day.

*Human erythrocyte cholinesterase*

Reactivation and "ageing" were studied at 25 °C and pH 7·8 (8·0) (veronal buffer) and at 37 °C and pH 7·2 (7·4) (veronal buffer). Reactivation with $8·5 \times 10^{-3}$ M TMB-4 was quicker than with $10^{-2}$ M P2S. Both substances reactivate this enzyme better than human plasma cholinesterase (Table 2). The total enzyme activity could be recovered when freshly inhibited enzyme was used.

"Ageing" occurred according to first-order kinetics (Fig. 5) in veronal buffer. $k_1$ calculated after reactivation with P2S or TMB-4 was $2{\cdot}4 \times 10^{-4}$ min$^{-1}$ at 25 °C. pH 8·0 and $8{\cdot}7 \times 10^{-4}$ min$^{-1}$ at 37 °C pH 7·4. "Ageing" was prevented by the addition of high concentrations of reactivator (Table 4).

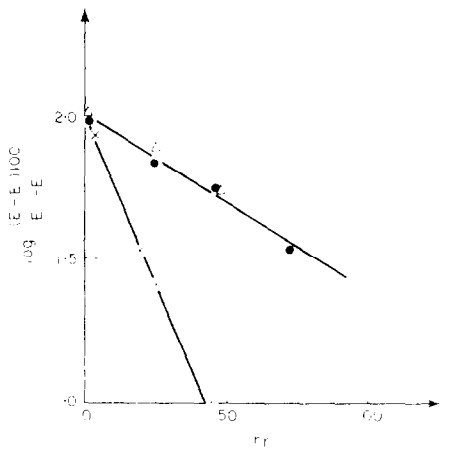


FIG. 5. "Ageing" at pH 7·4, 37 °C (○) and pH 8·0, 25 °C (●, △) of dimethylamido-ethoxy-phosphorylated cholinesterase in human erythrocytes. Reactivation with $8{\cdot}5 \times 10^{-3}$ M TMB-4, 24 hr (○, ●) and $10^{-2}$ M P2S, 48 hr (△).

TABLE 4. PREVENTION OF "AGEING" OF TABUN-INHIBITED ACETYLCHOLINESTERASE BY ADDITION OF REACTIVATOR

(Activity of inhibited enzyme before addition of reactivators 2 per cent of control.)

| Time after addition of reactivator | Activity in % of control | |
|---|---|---|
| | TMB-4 | P2S |
| 70 min | 94 | 50 |
| 1 day | 92 | 101 |
| 2 days | 91 | 105 |
| 3 days | 93 | 104 |
| 4 days | 92 | 108 |
| 6 days | 91 | 103 |

*Influence of oxime concentration on the reactivation of inhibited human erythrocyte cholinesterase*

Reactivation with 2-PAM at varying concentrations of reactivator has been studied by Green *et al.*[3, 10, 11] They proposed the following mechanism for the reactivation, assuming the formation of a complex

$$EI + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EIA \xrightarrow{k_3} E + IA \qquad (1)$$

where $A$ is the reactivator, and $EIA$ the complex. With a large excess of reactivator, the observed velocity of the reaction is given by

$$v = k_{obs}\,[EI_{total}] \qquad (2)$$

where

$$k_{obs} = k_3 \frac{[A]}{K + [A]} \tag{3}$$

and

$$K = \frac{k_2}{k_1} \tag{4}$$

Green *et al.* tested the validity of this equation over a range of reactivator concentrations and for that purpose they rearranged equation (3) to

$$\frac{1}{k'} = \frac{K}{k_3} + \frac{[A]}{k_3} \tag{5}$$

where

$$k' = \frac{k_{obs}}{[A]} \tag{6}$$

Their results have now been confirmed with Tabun-inhibited erythrocytes and concentrations of reactivator between $10^{-2} - 10^{-3}$ M, as is seen in Fig. 6, where $1/k'$ is plotted against the concentration of the reactivator. When the concentration of the reactivator was kept at $5 \times 10^{-5}$ M, which is what can be expected to be the *in vivo*

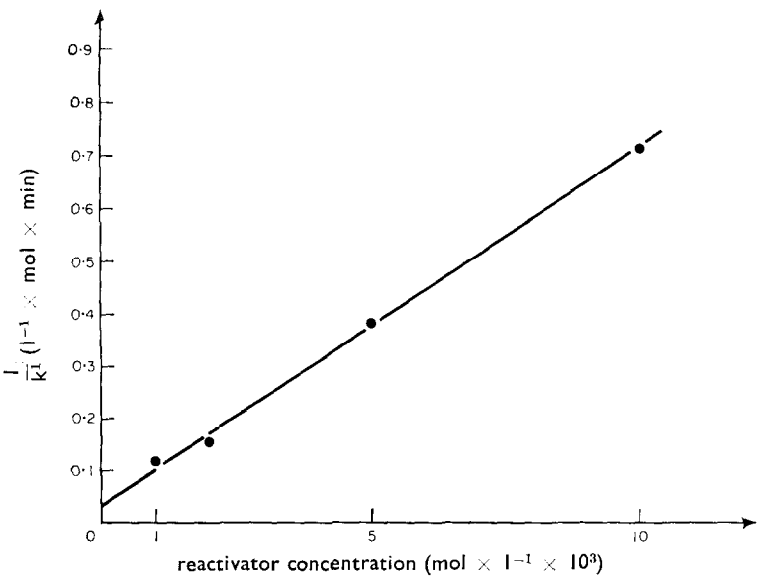


FIG. 6. Reactivation at pH 7·4, 37 °C of dimethylamido-ethoxyphosphorylated cholinesterase in human erythrocytes by P2S. $k'$ is $\frac{k_{obs}}{[\text{reactivator}]}$.

concentration during therapy,[12] only 67 per cent of enzyme activity was restored. An increase in the added amount of P2S did not restore more of the enzyme activity.

While reactivation with $8{\cdot}5 \times 10^{-3}$ M and $4{\cdot}25 \times 10^{-3}$ M TMB-4 was found to follow first-order kinetics, it was observed that at lower concentrations of the reactivator, reactivation reached a plateau. The degree of reactivation decreased with

decreasing concentration of reactivator, but in the examined range of oxime concentrations 75 per cent of enzyme activity was always restored (Fig. 7). The time for reaching 75 per cent of reactivation as a function of TMB-4 concentration is reported in Table 5. Increase of the added amount of TMB-4 to a concentration of $8{\cdot}5 \times 10^{-3}$ M after the plateau was reached could not restore the total enzyme activity when the

TABLE 5. TIME AT pH 7·4, 37 °C FOR A 75% RETURN OF ERYTHROCYTE CHOLINESTERASE ACTIVITY AFTER INCUBATION WITH VARYING AMOUNTS OF TMB-4

| M TMB-4 | Time (min) |
|---|---|
| $8{\cdot}50 \times 10^{-3}$ | 8 |
| $4{\cdot}25 \times 10^{-3}$ | 8·5 |
| $8{\cdot}50 \times 10^{-4}$ | 11 |
| $5{\cdot}00 \times 10^{-4}$ | 12 |
| $1{\cdot}00 \times 10^{-4}$ | 22 |
| $5{\cdot}00 \times 10^{-5}$ | 44 |
| $2{\cdot}50 \times 10^{-5}$ | 77 |

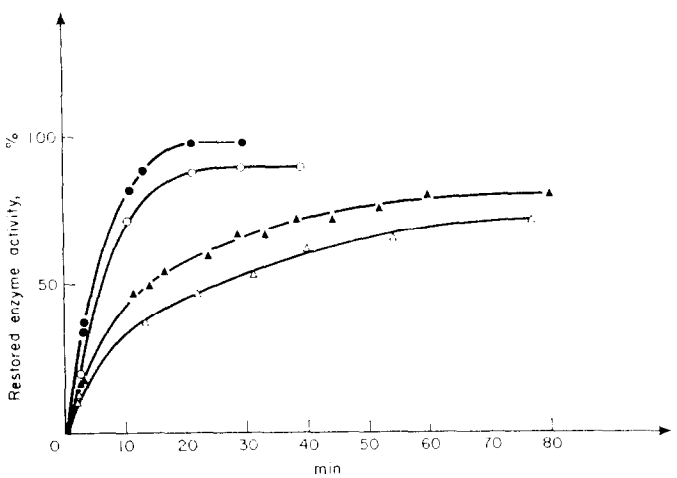


FIG. 7. Reactivation at pH 7·4, 37 °C of dimethylamido-ethoxyphosphorylated cholinesterase in human erythrocytes at different concentrations of TMB-4. ● = $8{\cdot}5 \times 10^{-3}$ M, ○ = $8{\cdot}5 \times 10^{-4}$ M, ▲ = $5 \times 10^{-5}$ M, △ = $2{\cdot}5 \times 10^{-5}$ M.

reaction was studied in potassium chloride solution (titrator). When the reaction was studied in veronal buffer an additional small portion of the enzyme activity was restored.

## DISCUSSION

It has been suggested that the low antidotal effect of 2-PAM in Tabun intoxication is due to inability to reactivate the formed phosphorylated cholinesterases.[13] From the results presented here it is seen that *in vitro* Tabun-inhibited cholinesterases are reactivated by P2S, but that the rate of reactivation is slow, much

slower than, for example, after Sarin inhibition. Experiments with Tabun intoxicated dogs[14] have shown that *in vitro* reactivation of the inhibited blood cholinesterases from these animals can be obtained. Judging from the results seen in Fig. 7 and Table 3, reactivation at therapeutical concentrations of P2S (30 mg/kg, resulting in plasma concentrations of $10^{-4}$ to $5 \times 10^{-5}$ M during 1–2 hr after intramuscular injection)[12] after Tabun intoxication can not be expected within a reasonable length of time. *In vivo* practically no reactivation can be expected after a single dose of P2S. Provided equation (3) holds at therapeutical concentrations of P2S, $k_{obs}$ would be $13 \times 10^{-4}$ $min^{-1}$ which means a slow reactivation. Low concentrations of reactivator do not seem to be able to protect the inhibited enzyme from "ageing." Therefore complete reactivation cannot be expected, since the rates of reactivation and "ageing" are of competitive orders of magnitude. This has been indicated by the experiments described above. Even repeated injections of P2S would therefore not be able to restore total enzyme activity.

Reactivation with TMB-4 after Tabun inhibition proceeds more rapidly. This reactivator has been shown to be slightly more toxic than P2S. The $LD_{50}$ as calculated after intraperitoneal injection into white mice is 3·5 times that of P2S.[15] One would therefore perhaps consider a therapeutical concentration of $3 \times 10^{-5}$ to $10^{-5}$ M. At these concentrations the onset of reactivation is rapid *in vitro*, 50 per cent of enzyme activity being restored within 30 min. However, a total of only 75–85 per cent is restored. Whether this effect of TMB-4 is enough for treatment of Tabun intoxications has to be determined by *in vivo* experiment.

So far it has not been possible to give a satisfactory explanation to the plateau effect. When working in veronal buffer, addition of a high amount of reactivator after the plateau was reached resulted in a small additional reactivation. At the same time enzyme activity of both control and reactivated sample decreased owing to inhibition by the reactivator. When working in potassium chloride (titrator) addition of more reactivator could not restore total enzyme activity. The difference between total enzyme activity and the reactivated amount was approximately twice that which could be expected if "ageing" had occurred as calculated from the rate constant for "ageing" obtained from experiments in veronal buffer. It is possible that the exclusion of buffer results in a more rapid "ageing". If the following two reactions are supposed to occur simultaneously

$$EI + Ox \xrightarrow{k_{obs}} E + P$$

$$EI \xrightarrow{k_1} EI_{aged}$$

then plots of the logarithm for the disappearance of *EI* against time, result in straight lines. The value of $k_1$ can be obtained from these curves and was found to vary between 0·008 $min^{-1}$ and 0·015 $min^{-1}$. These figures are from ten to twenty times higher than the one found for the "ageing" of dimethylamido-ethoxy-phosphorylated erythrocyte cholinesterase in veronal buffer (Table 3). This would be an astonishingly high buffer effect. The found values of $k_{obs}$ vary with the concentration of the oxime as has been proposed by Green *et al.*[16] Further experiments are needed to clarify the problem.

Originally reactivation and "ageing" of butyrylcholinesterase after Tabun inhibition was studied with purified horse serum cholinesterase since this enzyme had been extensively purified.[4] When it was noticed that this phosphorylated enzyme "aged" very slowly, untreated horse plasma enzyme and human plasma enzyme were included in the study. Calculation of the rate constants for the "ageing" of dimethylamido-ethoxyphosphorylated plasma cholinesterases revealed a species difference as well as a difference between the untreated and the purified enzymes. If one assumes the splitting off of an alkoxy group from the phosphorylated enzymes[3, 9] to be responsible for the "ageing" of the Tabun-inhibited cholinesterases it would seem that human plasma contains an "ageing-promoting" factor, which is partly separated or destroyed during purification of the enzyme and which seems to be able to promote "ageing" of another phosphorylated cholinesterase (Fig. 6). The possibility that phosphorylphosphatases are able to split off an alkoxy group from the phosphorylated enzyme is small. Augustinsson[17] has demonstrated the occurrence of a Tabun-splitting enzyme present in horse plasma which hydrolyses the P—CN bond of Tabun at a rate similar to that of human plasma phosphorylphosphatase. To the author's knowledge no splitting of P—O bonds has been demonstrated to occur with these enzymes. Still the possibility exists, that the phosphorylphosphatase in human plasma is able to split a P—O bond while the one in horse plasma is not. Other enzymic or non-enzymic factors can equally well be involved. However, the explanation may also be found in the structure of the cholinesterase molecule itself. The three-dimensional structure of the enzyme might well be disturbed during purification which could result in a decrease in the rate of "ageing". A difference in structure between the human and the horse plasma enzyme may also explain the observed differences between the two enzymes. This argument can also be applied if instead of the alkoxy group the dimethylamido group is split off as has been shown, for example, by Larsson[18] to occur in acid hydrolysis of Tabun.

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## REFERENCES

1. I. ENANDER, *Acta Chem. Scand.* **12**, 780 (1958).
2. I. W. COLEMAN, P. LITTLE and G. A. GRANT, *Canad. J. Biochem. Physiol.* **38**, 667 (1960).
3. R. D. O'BRIEN, *Toxic Phosphorus Esters*. Academic Press, New York and London (1960).
4. E. HEILBRONN, *Biochim. Biophys. Acta* **58**, 222 (1962).
5. R. BARKMAN. Personal communication.
6. L. LARSSON and B. HANSEN, *Svensk kem. Tidskr.* **68**, 521 (1956).
7. L.-E. TAMMELIN, *Scand. J. clin. Lab. Invest.* **5**, 267 (1955).
8. D. R. DAVIES and A. L. GREEN, *Biochem. J.* **63**, 529 (1956).
9. H. S. JANSZ, R. A. OOSTERBAAN, F. BERENDS and J. A. COHEN, *Fifth International Congress of Biochemistry, Moscow* 1961. Preprint no. 130, symposium no. IV.
10. W. K. BERRY, D. R. DAVIES and A. L. GREEN, *Brit. J. Pharmacol.* **14**, 186 (1959).
11. A. L. GREEN and H. I. SMITH, *Biochem. J.* **68**, 28 (1958).
12. A. SUNDWALL, *Biochem. Pharmacol.* **5**, 225 (1960).
13. I. B. WILSON and F. SONDHEIMER, *Arch. Biochem. Biophys.* **69**, 468 (1957).
14. E. HEILBRONN and A. SUNDWALL. To be published.
15. F. HOBBIGER and P. W. SADLER, *Brit. J. Pharmacol.* **14**, 192 (1959).
16. A. L. GREEN and H. I. SMITH, *Biochem. J.* **68**, 32 (1958).
17. K.-B. AUGUSTINSSON and G. HEIMBÜRGER, *Acta Chem. Scand.* **8**, 753 (1954).
18. L. LARSSON, *Acta Chem. Scand.* **7**, 306 (1953).