The Mode of Recovery of Cholinesterase Activity in vivo after Organophosphorus Poisoning

1. ERYTHROCYTE CHOLINESTERASE

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It is now generally accepted that the inhibition of cholinesterase by organophosphorus compounds consists of a direct phosphorylation of the active centre with the formation of a disubstituted phosphonyl cholinesterase (Aldridge, 1953). If the inhibited enzyme is kept in vitro in the absence of inhibitor, a certain amount of spontaneous reactivation occurs by hydrolysis of the phosphorylated enzyme, the exact amount being dependent on the groups attached to the phosphorus atom (Aldridge, 1953; Burgen & Hobbiger, 1951; Aldridge & Davison, 1953).

Reactivation of the inhibited cholinesterase can be markedly accelerated by treating it with certain nucleophilic reagents such as choline (Wilson, 1951), hydroxamic acids (Wilson, 1955) and oximes (Childs, Davies, Green & Rutland, 1955; Wilson & Ginsberg, 1955; Davies & Willey, 1957), the rate and extent of reactivation again being dependent on the groups attached to the phosphorus atom. The oximes are by far the best reactivators and under suitable conditions, with freshly inhibited enzyme, oxime treatment will restore all the enzyme activity (Davies & Green, 1956).

If the freshly inhibited and completely reactivatable cholinesterase is stored it gradually changes into a form which can no longer be reactivated by oximes (Davies & Green, 1956; Wilson, Ginsberg & Meislich, 1955; Hobbiger, 1958; Hobbiger, 1956; Jandorf, Michel, Schaffer, Egan & Summerson, 1955). This may be due either to the migration of the phosphoryl group to a more stable position in the inhibited enzyme (Wilson, Ginsberg & Meislich, 1955; Hobbiger, 1956; Jandorf et al. 1955), or to loss of one of the substituents on the phosphorus atom which would then acquire a negative charge and consequently become resistant to nucleophilic attack (Oosterbaan, Warringa, Janasz, Berends & Cohen, 1958). The change which occurs during storage will be referred to here as 'aging' although some authors have in fact referred to it as a transphosphorylation process (Hobbiger, 1956; Jandorf et al. 1955).

Little is known about the extent to which these reactions influence the in vivo recovery of inhibited cholinesterase. Davison (1955) suggested that the return to normal levels of activity was probably the combined result of spontaneous hydrolysis of the inhibited enzyme and of new enzyme synthesis. He found that after inhibition in vivo by diethyl p-nitrophenyl phosphate (E 600) the activities of rat-brain cholinesterase and of a variety of erythrocyte cholinesterases returned to normal in two
stages, a rapid partial recovery followed by a slower completion of the recovery process. Davison (1955) attributed this to the existence of two types of cholinesterase which formed inhibited enzymes of different stabilities.

The object of the present work was to ascertain whether the recovery of erythrocyte cholinesterase activity in vivo after inhibition by various organophosphorus compounds could be accounted for in terms of spontaneous reactivation, aging and the synthesis of new enzyme. The inhibitors used were chosen so that inhibited cholinesterases of widely differing stabilities would be produced.

**MATERIALS AND METHODS**

**Inhibitors.** These were synthesized in the Chemistry Section, Chemical Defence Experimental Establishment (C.D.E.E.), by the following methods: diisopropyl phosphoryl fluoridate (DFP), Ford-Moore, Lernit & Stratford (1953); tetramethyl pyrophosphate (TEPP), Arbos & Arbusew (1932); isopropyl methylphosphonofluoridate (Sarin), Bryant, Ford-Moore, Perry, Wardrop & Watkins (1960); dimethyl p-nitrophenyl phosphate (DMNP) was presented by Dr W. N. Aldridge, Toxicology Research Unit, Carshalton, Surrey.

**Reactivators.** These were also synthesized in the Chemistry Section, C.D.E.E.: monoisonitrosoacetone (hydroxymimoacetone; MINA) by the method of Preon (1939) and 2-(hydroxymimonoethyl)-N-methylpyridinium methanesulphonate (P2S) by the method of Creasey & Green (1959).

**Special chemicals.** Acetylcholine chloride was supplied by Roche Products Ltd.

**Animals.** Sheep were adult females or castrated males 40-60 kg.; rabbits were albino males of approx. 2 kg.

**Preparation of packed erythrocytes.** The plasma was removed from a sample of heparinized whole blood by centrifuging. The erythrocytes were then washed twice with 10 times their own volume of 0-9% NaCl soln. and packed by centrifuging at 1500 g for 10 min. The NaCl soln. was removed by aspiration and the cells were thoroughly stirred before sampling.

**Estimation of cholinesterase activity.** (a) Cholinesterase was estimated manometrically at 37° with the following reaction mixture: 0-1 ml. of packed erythrocytes' or 0-2 ml. of 50% erythrocyte suspension, 0-5 ml. of 0-12 M NaHCO₃, 0-5 ml. of 0-056 M-acetylcholine chloride, water to 3-0 ml.; gas phase N₂ + CO₂ (95:5). Activity was expressed as µl. of CO₂/30 min./0-1 ml. of packed erythrocytes', allowance being made for non-enzyme hydrolysis.

(b) In certain experiments cholinesterase activity was estimated by continuous titration; 0-2 ml. of packed erythrocytes' haemolsed in 2-0 ml. of water and 5-0 ml. of 0-007 M-acetylcholine chloride-0-42 M-KCl added. After equilibration at 25° the pH was adjusted to 7-5 and the mixture titrated continuously by the addition of portions (2 µl.) of 0-1 N-NaOH over a period of 6 min. The time was noted at which each addition of NaOH was neutralized and a titration curve was plotted. The slope was measured and activity expressed as µl. of 0.1-n NaOH used/0-1 ml. of packed erythrocytes'/min.

**Rate of reactivation of inhibited erythrocyte cholinesterase** by 2-(hydroxymimonoethyl)-N-methylpyridinium methanesulphonate and monoisonitrosoacetone. "Packed erythrocytes," diluted with an equal volume of 0-9% NaCl, were mixed with equal volumes of 2 µM-DMNP, 2 µM-TEPP, 2 µM-Sarin or 20 µM-DFP, all solutions were in 0-9% NaCl, and the mixtures were kept at 37° for 30 min. Excess of inhibitor was then removed by washing the erythrocytes at least three times with 10 times their volume of 0-9% NaCl soln. Samples of the inhibited erythrocytes were then mixed with an equal volume of 5 mM-2-(hydroxymimonoethyl)-N-methylpyridinium methanesulphonate in 0-9% NaCl or with an equal volume of 20 mM-monoisonitrosoacetone in 0-9% NaCl and kept at room temperature. Samples of the mixtures were removed at intervals, the erythrocytes were washed twice with 10 times their volume of 0-9% NaCl to remove excess of oxime and finally packed by centrifuging at 1500 g for 10 min. After removing the supernatant NaCl soln., the erythrocyte cholinesterase activity was measured manometrically.

**Measurement of aging.** With cholinesterase inhibited by DMNP, TEPP or DFP equal volumes of 'packed erythrocytes' and 2 mM-2-(hydroxymimonoethyl)-N-methylpyridinium methane sulphonate were mixed and kept at room temperature for 1 hr. at pH 7-5. With Sarin-inhibited cholinesterase the erythrocytes were mixed with an equal volume of 20 mM-MINA and kept for 1 hr. The erythrocytes were then washed twice with 10 times their volume of 0-9% NaCl soln. to remove excess of oxime and packed by centrifuging at 1500 g for 10 min. After removing the supernatant the cholinesterase activity of the erythrocytes was measured manometrically.

The percentage of aged enzyme was that percentage of the initial activity which could not be recovered by treatment with oximes.

**Inhibition in vivo.** Sheep were given 0-045 mg. of atropine sulphate/kg. subcutaneously; 10 min. later, solutions of the inhibitors in 0-9% NaCl soln. were injected as follows: TEPP (0-1 mg./kg.) and Sarin (0-016 mg./kg.) were given subcutaneously; DFP (0-5 mg./kg.) or DMNP (0-2 mg./kg.) was given intravascularly, DFP because subcutaneous administration produced only a slow inhibition, and DMNP because the inhibited cholinesterase has a half-life of only 90 min. (Aldridge & Davison, 1953) and it was therefore necessary to bring the enzyme and inhibitor into contact as quickly as possible. Subcutaneous injections were given in the axilla and intravenous injections into the jugular vein.

**Storage and recovery of inhibited erythrocyte cholinesterase** in vivo and in vitro. At suitable intervals after injecting the inhibitors (5 min. after DMNP, 30 min. after TEPP and Sarin, and 4 hr. after DFP) 10-20 ml. of blood was withdrawn, 'packed erythrocytes' were prepared and the cholinesterase activity was determined manometrically before and after oxime treatment as described under 'measurement of aging'.

The packed erythrocytes remaining after the cholinesterase determination were mixed with an equal volume of the following preservative solution: potassium oxalate 1-8%, 8-hydroxyquinoline 0-1%, NaCl 0-9%, glucose 6-48% and stored at pH 7-4 and 37°. When stored in this manner normal erythrocytes retained their cholinesterase activity for 7 days, very little haemolysis occurred and bacterial growth was prevented. Samples were taken at the same time from both the stored erythrocytes and from the
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Table 1. Reactivation of inhibited sheep-erythrocyte cholinesterase by oximes

The inhibited erythrocyte cholinesterase was incubated at 37°C with the stated concentration of oxime. At the times stated samples were taken, the erythrocytes washed with 0-9% NaCl and the cholinesterase activity was measured. The initial inhibition of the cholinesterase in vitro was produced as described under Methods.

Percentage of initial cholinesterase activity

<table>
<thead>
<tr>
<th>Time of contact with oxime (min.)</th>
<th>DMNP</th>
<th>TEPP</th>
<th>DFP</th>
<th>Sarin</th>
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<tr>
<td></td>
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<td>45</td>
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sheep, and the erythrocyte cholinesterase activity was estimated before and after treatment with oxime. All the erythrocyte cholinesterase activities were expressed as a per cent of a normal value determined immediately before the sheep was poisoned.

RESULTS

Measurement of the 'aging process'

In order to follow the aging process one must be able to reactivate completely the non-aged inhibited cholinesterase present at any particular time. Ideally, therefore, the rate of reactivation by oxime should be very much greater than the rate of the aging process. With TEPP- and Sarin-inhibited cholinesterase, treatment with the appropriate oxime for 1 hr. resulted in complete reactivation of the inhibited enzyme, consequently the aging process could easily be followed. Table 1 shows that for TEPP-inhibited cholinesterase 2-(hydroxymimonomethyl)-N-methylpyridinium methanesulphonate was the faster reactivator and for Sarin-inhibited cholinesterase MINA was the faster reactivator; these combinations of inhibited cholinesterase and oxime were therefore used in the routine aging measurements. With DFP- and DMNP-inhibited cholinesterase, however, complete reactivation of the inhibited enzyme could not be observed. Reactivation of the DFP-inhibited cholinesterase continued for 4–5 hr. and became very slow when only about 50% of the inhibited enzyme initially formed was reactivated. Reactivation of the DMNP-inhibited cholinesterase ceased after contact for 45 min. with the oxime with about 76% of the inhibited enzyme initially formed being reactivated. It is clear that the reactivation and aging of DFP- and DMNP-inhibited cholinesterase proceeded at comparable rates, and although it was possible to tell whether the aging process was still proceeding, it was not possible to observe it as accurately as with Sarin- and TEPP-inhibited cholinesterase.

Recovery of erythrocyte cholinesterase activity in poisoned sheep

Preliminary experiments with rabbits showed that small animals could not be used in this work because the removal of the necessary quantities of blood, in all a considerable proportion of the total blood volume, caused an increase in the proportion of circulating reticulocytes, blood constituents which contain three times the cholinesterase activity associated with a normal red-cell population (Pritchard, 1949; Allison & Burn, 1955). The erythrocyte cholinesterase activity of the poisoned rabbits consequently returned much faster than could be accounted for either by published rates of erythrocyte turnover (Neuberger & Niven, 1951), or by the rate of spontaneous reactivation of the inhibited cholinesterase as observed in vitro. No such difficulty was experienced with sheep.

To see whether spontaneous reactivation, aging and new enzyme synthesis could satisfactorily explain the changes in the erythrocyte cholinesterase activity during the recovery from poisoning, a sample of blood was taken from a sheep soon after it had received a non-lethal dose of the cholinesterase inhibitor. The erythrocytes were separated, stored at 37°C with a preservative and the rates of spontaneous reactivation and aging in the stored erythrocytes compared with their rates in the circulating erythrocytes of the intact sheep. Each inhibitor was given to three sheep and typical results are shown in Figs. 1–4.

The return of inhibited erythrocyte cholinesterase to the normal level of activity in vivo occurred
in one or two stages depending on which inhibitor was used.

With TEPP-, Sarin- or DMNP-poisoned sheep the recovery of inhibited erythrocyte cholinesterase in vivo occurred in two stages, a rapid partial recovery being followed by a slower linear return to the normal level of activity. In vitro only the first stage was observed.

Figs. 1–3 show that the rates of the separate reactions involved in the first stage, namely spontaneous reactivation and aging were the same in vivo as they were in vitro. Both reactions occurred simultaneously and when all the residual inhibited enzyme was converted into the aged form no further spontaneous reactivation appeared

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**Fig. 1.** Behaviour in vitro (a) and in vivo (b) of erythrocyte cholinesterase of sheep poisoned with 0-1 mg. of TEPP/kg subcutaneously. Broken lines show the decrease in cholinesterase reactivatable by P2S and represent the aging of the inhibited enzyme. Full lines show the cholinesterase activity before oxime treatment and represent spontaneous recovery (in vitro) or spontaneous recovery plus new enzyme synthesis (in vivo).

**Fig. 2.** Behaviour in vitro (a) and in vivo (b) of erythrocyte cholinesterase of sheep poisoned with 0-015 mg. of Sarin/kg. subcutaneously. Broken lines show the decrease in cholinesterase reactivatable by monoisonitrosacetone and represent the aging of the inhibited enzyme; full lines show the cholinesterase activity before oxime treatment and represent spontaneous recovery (in vitro) or spontaneous recovery plus new enzyme synthesis (in vivo).

**Fig. 3.** Behaviour in vivo of erythrocyte cholinesterase of sheep poisoned with 0-2 mg. of DMNP/kg. intravenously; the broken line shows the decrease in cholinesterase reactivatable by P2S and represents the aging of the inhibited enzyme; the full line shows the cholinesterase activity before oxime treatment and represents spontaneous recovery plus new enzyme synthesis.

**Fig. 4.** Behaviour of erythrocyte cholinesterase of sheep poisoned with 0-5 mg. of DFP/kg. intravenously. Spontaneous reactivation did not occur in vitro (a) and the increase in erythrocyte cholinesterase activity in vivo (b) was due solely to new enzyme synthesis.
to be possible. The relative rates of the two processes determined the extent to which the inhibited enzyme recovered spontaneously. For example, in the case of the TEPP-inhibited enzyme spontaneous reactivation proceeded at the same rate as aging and at the end of the first stage 49% of the inhibited activity had recovered in vitro; with the Sarin-inhibited enzyme, on the other hand, the aging process was much faster than spontaneous reactivation and at the end of the first stage only 2% of the inhibited activity had returned in vitro. The absolute rates of the two reactions determined how long the first stage of the recovery lasted; thus with Sarin the first stage was completed in 24 hr., whereas with TEPP the first stage lasted 3 days.

Spontaneous reactivation and aging were observed in vivo with DMNP-inhibited cholinesterase (Fig. 3), although the rates were much faster than with either Sarin- or TEPP-inhibited cholinesterase, the first stage being completed in 1 hr. Because of the difficulty of completing all the analyses on the same animal during the first hour, only the behaviour of DMNP-inhibited cholinesterase in vivo was studied. However, the rate of spontaneous reactivation in vitro has been studied by Aldridge & Davison (1953), who found that with rabbit erythrocytes the inhibited enzyme had a half-life of only 90 min.

With DFP-poisoned sheep (Fig. 4) no spontaneous reactivation was detected in vitro, and in vivo the return of the erythrocyte cholinesterase activity proceeded throughout the entire recovery period at the slower linear rate typical of the second stage. Aging of the DFP-inhibited enzyme appeared to be complete in about 6 hr. This could only be roughly assessed, however, because of the slow rate at which the inhibited enzyme was reactivated by 2-(hydroxyiminomethyl)-N-methylpyridinium methanesulphonate.

The rate of the later linear stage was similar for all the inhibitors used; thus with TEPP, Sarin, DFP and DMNP the daily rates at which the inhibited erythrocyte cholinesterase was replaced were 0.6, 0.7, 0.5 and 0.7% respectively, suggesting that the mechanism involved in this stage of the recovery was the same for each. As this rate of replacement is similar to that measured by different methods (Neuberger & Niven, 1951) for the normal turnover of erythrocytes in other mammals the second or linear stage in the recovery of inhibited erythrocyte cholinesterase activity was assumed to be due to new red-cell formation.

DISCUSSION

In the present experiments erythrocyte cholinesterase, inhibited with organophosphorus compounds, recovered in one or two stages depending on which inhibitor was used. During the first stage, which occurred both in vivo and in vitro, the inhibited enzyme underwent two simultaneous reactions: spontaneous reactivation by hydrolysis, and aging in which the inhibited enzyme was converted into a form which could no longer reactivate spontaneously or be reactivated by oxime treatment.

When all the inhibited enzyme was present in the aged form no further recovery of the erythrocyte cholinesterase activity occurred in vitro, and in vivo the second stage of recovery was reached in which the rate of erythrocyte cholinesterase recovery became that at which the inhibited erythrocytes were replaced by new ones. Thus the characteristics of the first stage were dependent on the inhibitor, whereas those of the second stage were not, and were the same for all the inhibitors used.

When the cholinesterase was inhibited with TEPP, Sarin or DMNP the two stages in the recovery could clearly be seen; with DFP-inhibited erythrocyte cholinesterase, however, no spontaneous recovery was detected in vitro, and in vivo only one stage of recovery was observed, the rate being that of new erythrocyte formation.

If failure to detect spontaneous reactivation was due to aging of the DFP-inhibited enzyme within the first hour or so, then it would not be possible to reactivate it by prolonged oxime treatment as has been described. The absence of spontaneous reactivation immediately after inhibition was therefore attributed to the freshly inhibited enzyme's being highly resistant to hydrolysis. It will be observed that the rate of spontaneous reactivation of the inhibited erythrocyte cholinesterase decreased markedly in the order dimethyl-, diethyl-, diisopropyl-phosphoryl cholinesterase.

The inhibition of cholinesterase and the first stage in its recovery may be summarized as shown in Fig. 5.

Since the nature of the inhibited enzyme produced by an organophosphorus compound depends on the dialkylphosphoryl residue, provided that this group is kept constant the same inhibited enzyme can be obtained from a variety of inhibitors differing from one another only in the 'X' group (Aldridge & Davison, 1953). This group may, however, modify the behaviour of the inhibitor in vivo. For example Vandekar & Heath (1957) found that, in contrast with DMNP, the dimethyl phosphate esters (MeO)2PO·S·C2H5R, where R is -SEt, -SOEt or -SO2Et, persisted in the tissues in vivo; and although spontaneous reactivation and aging of the inhibited cholinesterase proceeded normally, any free cholinesterase so produced was immediately reinhibited, so that eventually all the inhibited cholinesterase was present in the aged
form and no spontaneous recovery could be detected. Recovery of erythrocyte cholinesterase activity in vivo therefore occurred only at the slow linear rate associated with new erythrocyte formation.

Thus it is possible to explain the behaviour of erythrocyte cholinesterase inhibited in vivo by organophosphorus compounds in terms of the spontaneous hydrolysis and aging of the inhibited enzyme and the formation of new red cells.

The two forms of inhibited cholinesterase with different stabilities originally postulated by Davison (1955) are probably the freshly inhibited enzyme, which can undergo spontaneous reactivation, and the aged enzyme, which cannot. As the first form may undergo spontaneous and complete conversion into the second form there is no reason to believe that there are initially two types of uninhibited cholinesterase.

SUMMARY

1. Recovery of sheep-erythrocyte cholinesterase inhibited in vivo by dimethyl p-nitrophenyl phosphate, tetraethyl pyrophosphate or isopropyl methylphosphonofluoridate occurred in two stages, a rapid partial recovery being followed by a slower linear return to normal activity. With diisopropyl phosphorofluoridate-inhibited erythrocyte cholinesterase, recovery occurred throughout at the slower linear rate.

2. During the first stage in the recovery spontaneous hydrolysis and aging of the inhibited cholinesterase occurred simultaneously; the absolute rates of these two processes determined the speed of recovery and their relative rates determined the extent of recovery. The characteristics of this stage varied with the inhibitor used.

3. The second stage in the recovery was correlated with new red-cell formation and the rate of recovery in this stage, 0.5-0.7% per day, was independent of the inhibitor used.

REFERENCES


