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THE REACTIVATION BY OXIMES OF PHOSPHONYLATED ACETYLCHOLINESTERASE: THE POSSIBLE ERRONEOUS INTERPRETATION OF REACTIVATING POTENCY

BRIAN HARVEY, DAVID J. SELLERS and PETER WATTS
Chemical Defence Establishment, Porton Down, Salisbury, Wiltshire, U.K.

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Abstract—A comparative study of the reactivation by two oximes of acetylcholinesterase inhibited by several organophosphates has been made, with particular reference to the dependence of the degree of reactivation produced by an oxime (reactivating potency) upon the concentration of inhibited enzyme. In the case of one inhibitor it is demonstrated that the relative reactivating potency of the two oximes can be reversed by a change in experimental conditions. It is concluded that the measurement of the reactivation produced by two or more oximes, particularly when carried out under standardized conditions, is of little value in determining their relative reactivating potencies, and of negligible value in predicting their likely therapeutic effectiveness against organophosphate poisoning.

In the search for more effective oximes for use in therapy against organophosphorus poisoning in animals and man much effort has been devoted to comparisons of the *in vitro* reactivation by different oximes of organophosphorus inhibited acetylcholinesterase (AChE). Rate coefficients of reactivation, or simply the percentage reactivation attained after certain times, have been compared and used to rank oximes in order of reactivating potency [1–6]. However, the complex nature of the reactivation process demonstrated in a recent study* suggests that such an approach is of little value. This view is supported by results of earlier workers who reported that the results obtained from reactivation of inhibited AChE by an oxime vary according to the reaction conditions [7, 8].

It is the purpose of the present study to demonstrate the importance of reaction conditions in the reactivation process. This has been achieved by measuring the reactivation produced by two oximes, 2-hydroxyiminomethyl-1-methyl pyridinium methanesulphonate (P2S) and 1-(2-hydroxyiminomethyl pyridinium-1-3-carboxamide pyridinium) dimethylether dichloride (HS6), of AChE inhibited by VX (*O*-ethyl-*S*-di-isopropylaminoethyl methylphosphonothiolate), sarin (isopropyl methylphosphonofluoridate) and soman (1,2,2,-trimethylpropyl methylphosphonofluoridate) under a range of experimental conditions. Because of the previously mentioned complexity of the system no kinetic analysis of the system has been attempted; only internal comparisons have been made.

MATERIALS AND METHODS

Materials

Sarin and soman (>95% pure), VX (>90% pure) and P2S (>98% pure) and HS6 (>95% pure) were synthesized at CDE Porton. AChE from bovine erythrocytes (EC.3.1.1.7) was obtained from Sigma Chemicals, and acetylcholine iodide (AChI) from BDH.

AChE activities were measured titrimetrically using a Radiometer automatic titrator (pH meter 26, titrator 11, titrigraph SBR2c, syringe burette SBU 1a) fitted with twin syringes containing NaOH (0.01 N) and AChI (0.01 M).

Methods

Preparation of inhibited AChE. To solutions of AChE (10 μM units ml^{-1}) in 0.1 M NaCl at pH 7.4 (5×10^{-3} M sodium phosphate buffer) at 4° was added sarin or VX to give concentrations of 10^{-6} and 10^{-7} M respectively. The solutions were kept for 15–30 min to produce 100% inhibition, after which excess inhibitor was removed by ultrafiltration through an XM50 membrane in an Amicon cell (10 ml) using a pressure of 25–30 psi of N_2. The volume was reduced from 10 to 1 ml three times, and was adjusted to 10 ml each time with 0.1 M NaCl at pH 7.4 (5×10^{-3} M sodium phosphate). Control experiments showed that no loss of AChE activity was caused by this procedure.

AChE inhibited by soman was prepared differently from above because of the rapid ageing of this species at pH 7.4. AChE (100 μM units) was dissolved in 0.1 M NaCl at pH 7.4 (5×10^{-3} M sodium phosphate) (4 ml) and the pH was adjusted to 9.0 with NaOH. This solution (4°) was made 5×10^{-8} M with soman and stood for 20 min to produce 100% inhibition. The pH was then raised to 10.0 and the solution kept at room temperature for 1 hr to hydrolyse excess soman. The volume was adjusted to 5.0 ml and the pH to 9.0.

Complete removal of excess inhibitor was confirmed in all three cases by incubation at 37° of equal volumes of inhibited AChE with free AChE (10 μM units ml^{-1}), no observable inhibition after 2 hr being taken as the criterion for complete

* Work done in this laboratory, to be published.

removal. The solutions were made up freshly each day and stored at 4° throughout the day.

Reactivation experiments. Solutions of VX or sarin inhibited AChE (10 μM units ml^{-1}) were brought to 37°, made 10^{-4} M with P2S or HS6 and aliquots of 0.1 ml were removed at various times, diluted into 0.1 M NaCl (10 ml), and the AChE activities were assayed by making this solution 2.5×10^{-4} M with AChI and pH statting on the radiometer at 7.4.

Solutions of VX or sarin inhibited AChE (0.1 μM units ml^{-1}, made by dilution of the original solution) at 37° were made 10^{-4} M with P2S or HS6, aliquots of 10 ml were removed and the AChE activities were assayed as above. Samples taken shortly after oxime addition did not show a zero order rate of substrate hydrolysis (due to reactivation occurring during the 2 min assay) and in these cases the initial rate of substrate hydrolysis was used as a measure of AChE activity.

Soman inhibited AChE (20 μM units ml^{-1}, 0.5 or 0.1 ml) was added to solutions of P2S or HS6 (10^{-3} or 10^{-4} M, 10 ml) in 0.1 M NaCl and sodium phosphate (5×10^{-3} M). The pH was slighly less than 7.4 but was raised to this value by the addition of inhibited AChE at pH 9.0. AChE activities were assayed by the addition of AChI as above.

In all cases control experiments to determine the rate of AChI hydrolysis at the appropriate concentrations of P2S or HS6 were performed in duplicate.

RESULTS

The results shown are the means of duplicate experiments.

i. *VX inhibited AChE*

Figure 1 compares the reactivation profiles produced by 10^{-4} M HS6 and P2S when the concentration of inhibited AChE ([EP]) is either 10 or 0.1 μM units ml^{-1}. Reducing the initial concentration of EP clearly has a much greater effect upon the P2S results than those of HS6.

ii. *Sarin inhibited AChE*

Figure 2 similarly compares the reactivation profiles produced by 10^{-4} M HS6 and P2S when [EP] is either 10 or 0.1 μM units ml^{-1}. Reducing the initial concentration of EP again affects the P2S results to a much greater extent than those of HS6.

* Work done in this laboratory, to be published.

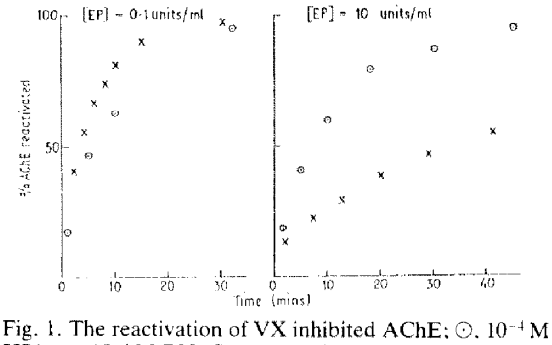

Fig. 1. The reactivation of VX inhibited AChE; ⊙, 10^{-4} M HS6; ×, 10^{-4} M P2S. Each experiment was performed in duplicate. Maximum error is ±4% reactivation.

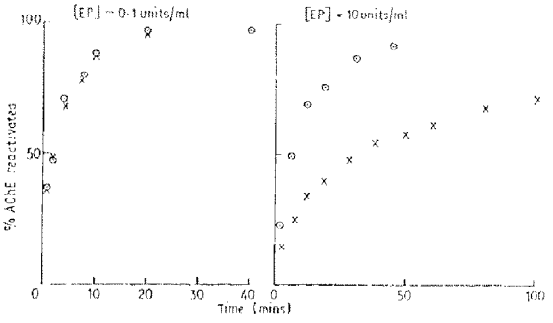

Fig. 2. The reactivation of sarin inhibited AChE. Symbols as in Fig. 1. Each experiment was performed in duplicate. Maximum error is ±3% reactivation.

iii. *Soman inhibited AChE*

Figure 3 shows the reactivation profiles produced by 10^{-3} and 10^{-4} M HS6 and P2S with EP concentrations of 1.0 and 0.2 μM units ml^{-1}. Reduction of the initial concentration of EP does not produce greater reactivation in this case. Experiments with EP concentrations greater than 1.0 μM units ml^{-1} were not performed.

Only preliminary studies were carried out on the reactivation by HS6 of AChE inhibited by tabun because AChE from Sigma Chemicals became unavailable before completion of the work. These studies indicated that no differences in reactivation profiles were apparent upon reduction of the initial concentration of inhibited AChE. Similar findings were observed for the reactivation of tabun inhibited AChE by P2S.*

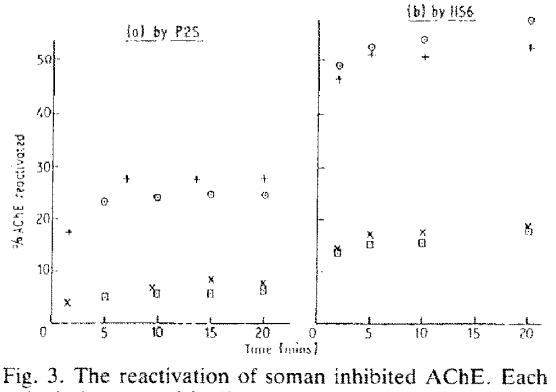

Fig. 3. The reactivation of soman inhibited AChE. Each point is the mean of duplicate experiments. (a): ⊙, inhibited AChE at 0.2 units/ml ([P2S] = 10^{-3} M); +, inhibited AChE at 1 unit/ml ([P2S] = 10^{-3} M); ⊡, inhibited AChE at 0.2 units/ml ([P2S] = 10^{-4} M); ×, inhibited AChE at 1 unit/ml ([P2S] = 10^{-4} M). Maximum error is ±3% reactivation. (b): ⊙, inhibited AChE at 0.2 units/ml ([HS6] = 10^{-3} M); +, inhibited AChE at 1 unit/ml ([HS6] = 10^{-3} M); ⊡, inhibited AChE at 0.2 units/ml ([HS6] = 10^{-4} M); ×, inhibited AChE at 1 unit/ml ([HS6] = 10^{-4} M). Maximum error is ±2.5% reactivation.

DISCUSSION

The results of the reactivation of VX and sarin inhibited AChE by P2S and HS6 (shown in Figs 1 and 2) demonstrate the sensitivity of the time profiles to changes in reaction conditions. Indeed by appropriate selection of the reaction conditions under which comparisons between the two oximes are made quite different results may be obtained. For example, using sarin inhibited AChE at 0.1 μM units ml^{-1} the two oximes are equally effective, with complete reactivation occurring within *ca.* 20 min. Using VX inhibited AChE under the same conditions it is seen that P2S is slightly more effective than HS6, with complete reactivation occurring in *ca.* 30 min. Upon increasing the concentration of both sarin and VX inhibited enzyme to 10 μM units ml^{-1} whilst maintaining the same oxime concentration it is seen that in both cases HS6 is the more effective reactivator, and that P2S produces only partial reactivation even after 1 hr.

The reactivation profiles of soman inhibited AChE, shown in Fig. 3, show that no further reactivation occurs after the first 5 min of reaction. This is due to ageing competing with reactivation. Because of this competition it is impossible to say whether the observed differences in the extent of reactivation are caused by changes in the rate of reactivation or in the rate of ageing. The conditions of reaction under which soman inhibited AChE is reactivated do not appear to have any great effect upon the results obtained. HS6 appears more effective than P2S whether the oximes are at 10^{-3} or 10^{-4} M, or whether the inhibited enzyme is at 1.0 or 0.2 μM units ml^{-1}.

Selection for *in vivo* testing of a few oximes from a group or series of compounds on the basis of reactivating potency measured *in vitro* is likely to be of little predictive value because of the effects of other factors which operate *in vivo* but not *in vitro*. For example the effects of phosphonylated oximes, which are usually potent anticholinesterases, and are necessarily formed during the reactivation reaction, are unknown in *in vivo* situations. It is the re-inhibition of reactivated AChE by this chemical species which is at least partially responsible for the differences in reactivation seen when using different initial concentrations of inhibited AChE *in vitro* (Figs 1 and 2). Some oximes have been shown to have direct pharmacological actions which are beneficial in the treatment of organophosphate poisoning [9], and nothing can be learnt of these actions from reactivation studies. The rate and extent of distribution to sites of importance *in vivo* of different oximes is likely to differ, and since higher oxime concentrations will result in a greater degree of reactivation, knowledge of how well any oxime reactivates inhibited AChE *in vitro* is again of little value unless at least approximate *in vivo* concentrations are also known. The rates of excretion and metabolism of the organophosphates, therapeutic drugs and products of their interaction are also unknown factors which are of importance *in vivo* but not *in vitro*.

The value of *in vitro* reactivation investigations lies then not in their predictive value in determining *in vivo* treatment studies but as an adjunct to such studies.

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