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SPONTANEOUS REACTIVATION OF ACETYLCHOLINESTERASE FOLLOWING ORGANOPHOSPHATE INHIBITION

I. AN ANALYSIS OF ANOMALOUS REACTIVATION KINETICS

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Summary

The first kinetic studies on the spontaneous reactivation of Sarin-inhibited acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) are reported. With increasing pH the extent of reactivation increases while the observed rate constant decreases. An analysis of the change in aging rate constant as a function of pH suggests that the aging of alkyl-alkoxy phosphonylated acetylcholinesterases is not solely acid catalyzed.

Introduction

While the induced reactivation of phosphorylated ** acetylcholinesterase by oximes and hydroxamic acids has been extensively investigated, spontaneous return of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) activity following inhibition represents a somewhat neglected area of research. For example, for every 50 papers on induced reactivation there has been approximately one paper on spontaneous reactivation. This relative neglect is surprising since an understanding of the basic parameters and mechanisms underlying spontaneous reactivation is mandatory if one hopes to advantageously perturb this system as part of a therapeutic approach in organophosphate poisoning. Specifically, enhancement of spontaneous reactivation would be a

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** The term phosphorylation will be used to include both phosphonylation and phosphorylation [1]. Abbreviations: Sarin, isopropyl methylphosphonofluoridate; TMB-4, 1,1'-trimethylene-bis(4-formylpyridinium bromide)dioxime; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Bicine, *N,N*-bis(2-hydroxyethyl)glycine.

useful adjunct to the current regimen of oxime therapy that is now prescribed [2]. It would be of even greater significance in those situations that are unresponsive to current oxime therapy [3,4].

Our first studies in this area were carried out using a series of para-substituted phenyl methylphosphonochloridates [5]. These spontaneous reactivation studies produced the first evidence for hydrophobic interactions participating in the spontaneous dephosphonylation of inhibited acetylcholinesterase.

We have subsequently investigated the spontaneous reactivation of acetylcholinesterase inhibited with Sarin. Although Hobbiger [6] stated in 1963 that Sarin-inhibited acetylcholinesterase does not spontaneously reactivate, limited qualitative observations to the contrary had been published in 1960 by Fredriksson and Tibbling [7]. Their qualitative observations have been subsequently reinforced by at least two "physiological studies" [8,9]. The results reported herein are the first kinetic studies of this phenomenon.

Materials and Methods

The zwitterionic MOPS and Bicine buffers used were described by Good et al. [10], and are available commercially from several sources. MOPS was used in the pH 7.60 studies and Bicine in the pH 8.44 and pH 9.16 studies. Unless otherwise noted, all were 0.10 M and contained 0.01 M Mg^{2+}, 0.0002% NaN_3, and 0.01% bovine serum albumin.

Acetylcholinesterase from *Electrophorus electricus* was obtained from Worthington Biochemical Corp. and in-house preparations. The latter were solubilized by the procedure of Rothenberg and Nachmansohn [11] and purified by affinity chromatography. Generally 10 mg of the commercial material, a salt-free powder with an activity of 1000 μmol of acetylcholine hydrolysed/min per mg (25.0°C, pH 7.0), was dissolved in 1.5 ml of a previously boiled aqueous solution (pH approx. 7.4) containing 0.225 M KCl, 0.10% gelatin, and 0.02% NaN_3 to give the enzyme concentrate. The addition of 5.0 μl of a 50-fold dilution of the concentrate to 2.0 ml of 0.10 M MOPS buffer, pH 7.60, containing 5.0 μl of 1.47 M phenyl acetate in acetonitrile, gives an absorbance change at 272.5 nm of approx. 0.60 absorbance unit/min (25.0°C). Assay concentration of the substrate equals $4 \cdot 10^{-3}$ M. It is important that the concentration of acetonitrile in the cuvette be kept equal to or less than 0.25%. If acetonitrile is increased to 1.20%, enzymatic activity is reduced 5.5%. At 3.12% acetonitrile, enzymatic activity is reduced 30.1%.

Inhibition and spontaneous reactivation of acetylcholinesterase. A detailed description of the tecnique involved in a double inhibition-spontaneous reactivation of acetylcholinesterase at pH 8.44 is given below. All kinetic investigations were handled in an analogous manner.

An enzyme stock solution was prepared by adding 200 μl of enzyme concentrate to 300 μl of 0.10 M Bicine buffer, pH 8.44. For a control, 0.50 μl of this stock solution was added to 25.0 ml of the buffer. Phenyl acetate assays of this 1 to 50000 dilution showed an activity of 0.134 absorbance unit/min, allowance being made for the non-enzymatic hydrolysis of phenyl acetate in the buffer medium (0.0072 absorbance unit/min).

The enzyme stock solution was inhibited with 0.50 μl of Sarin. In approx.

2 min 250 μl of inhibited enzyme solution was added to a 0.5 × 4.5 inch column bed of Sephadex G-25 Fine and eluted with the pH 8.44 buffer (flow rate 0.5 ml/min). The zero time for reactivation was taken as the time when the 250 μl sample of inhibited acetylcholinesterase had passed into the gel bed of the column. The fraction of 0—10.0 ml was collected without dilution. Previous tests established that all the acetylcholinesterase and no inhibitor were present in the fraction.

The 10.0 ml fraction was incubated at 25.0°C. At appropriate intervals 10.0 μl of this solution were added to 1.0 ml of the buffer. Activity was determined from phenyl acetate assays, the reaction being monitored in this manner for 80 h. Using Eqn. 2, k_{obsd} = 0.028 h^{-1} with an 11.5% return of activity (see Fig. 1).

After 13 days, a little more than 9 ml of remaining enzyme solution was reduced in volume to approx. 0.5 ml using an Amicon 8MC ultrafiltration cell with an XM-50 filter at 34 lb/inch^2. A new control was prepared by diluting 5.0 μl of the concentrate to 10.0 ml with the buffer. Phenyl acetate assays showed an activity of 0.157 absorbance unit/min. The remaining concentrate was inhibited with 0.50 μl Sarin. The separation of the phosphonylated acetylcholinesterase from excess inhibitor was carried out as described above for the first inhibition. Assays and calculations were also carried out as previously described. The results of this second inhibition-spontaneous reactivation gave k_{obsd} = 0.0272 h^{-1} with an 11.6% return of activity.

Calculations. Two methods were used to calculate the observed rate of spontaneous reactivation (k_{obsd}). In all experiments corrections were made for the loss of activity in the control during the time period. As it was practical in the pH 7.60 investigations to measure the end point (final activity value), the rate was determined from the usual first-order kinetic equation, i.e. Eqn. 1. Here V_∞

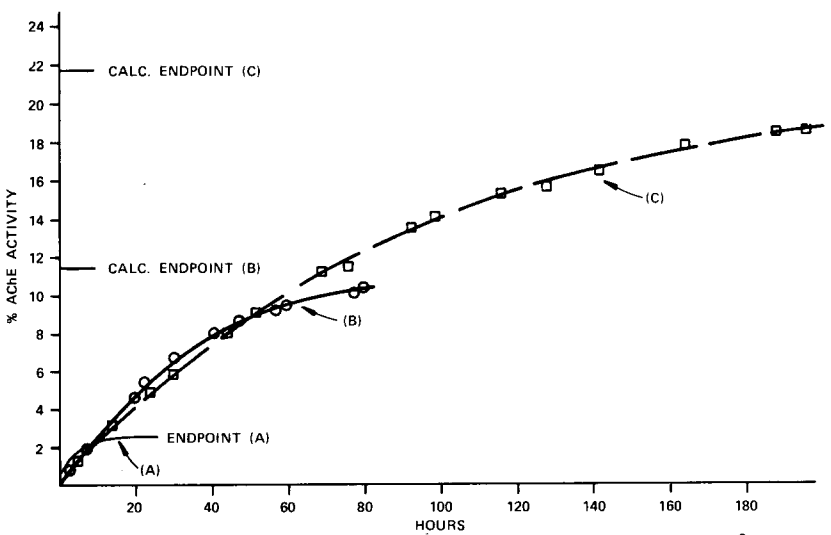


Fig. 1. Spontaneous reactivation of Sarin-inhibited acetylcholinesterase at 25.0°C at pH 7.60 (A), pH 8.44 (B), and pH 9.16 (C). The solid line corresponding to results at pH 7.60 (A) is based on 28 points, with $t_{1/2}$ = 154.3 min.

and V_t represent enzyme activities at times ∞ and t, respectively.

$$\ln\left(\frac{V_\infty}{V_\infty - V_t}\right) = k_{obsd}t \qquad (1)$$

In the pH 8.44 and pH 9.16 experiments, where the time frame made it impossible to measure a valid experimental end point, a computer program developed by Hayo and Wilcoxson [12] was used. The program fits the data (V_t, t) to Eqn. 2. The best value for the end point (V_∞) and the first-order rate constant (k_{obsd}) for return of enzymatic activity are calculated.

$$V_t = V_\infty[1 - e^{-k_{obsd}(t - t_0)}] \qquad (2)$$

We have reported previously on the validity of Eqn. 2 [5]. In the calculation of first-order kinetic constants from data in which no experimental end point is available, the Hayo and Wilcoxson program offers a distinct advantage over the more common method of Guggenheim [13] in that there is no need to space the kinetic parameter at a constant Δt.

Oxime reactivation of isopropyl methylphosphonylated acetylcholinesterase. An enzyme stock solution was prepared by adding 300 μl enzyme concentrate to 1200 μl of 0.10 M MOPS buffer, pH 7.60. A control was prepared by diluting 50 μl of this stock solution to 50.0 ml with the buffer. Phenyl acetate assays of this 1 to 10000 dilution showed an activity of 0.372 absorbance unit/min (272.5 nm) after correcting for the non-enzymatic hydrolysis of phenyl acetate in the buffer (0.002 absorbance unit/min).

After the control was prepared, the remaining enzyme stock solution was inhibited with 1.0 μl of $5 \cdot 10^{-4}$ M Sarin. In approx. 2 min, 250 μl of the inhibited enzyme and unreacted Sarin solution was added to a 0.5 × 4.5 inch column bed of Sephadex G-25 Fine and eluted with the pH 7.60 buffer (flow rate, 0.5 ml/min). Zero time was designated as the time when the 250 μl sample

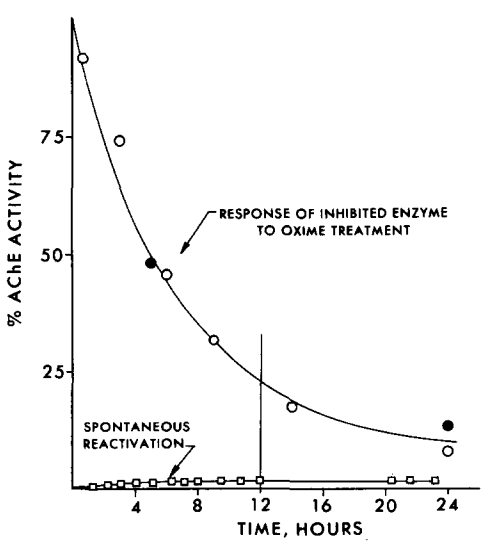


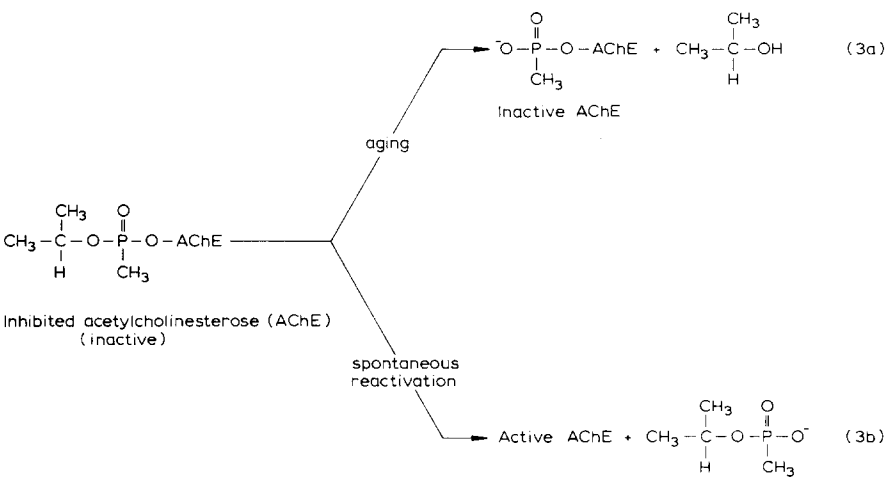
Fig. 2. Reactions of acetylcholinesterase inhibited by Sarin: 1, (o) response to oxime treatment, Harris et al. [16] (in vivo, rat brain acetylcholinesterase; 2, (•) response to oxime treatment, this work (pH 7.60, 25.0°C); and 3, spontaneous reactivation observed under the same conditions as 2.

of inhibited acetylcholinesterase had passed into the bed of the column. The fraction of 0—10.0 ml was collected without dilution. Previous tests established that all the acetylcholinesterase and no inhibitor were present in this fraction.

At the end of 5 h an aliquot of the inhibited acetylcholinesterase solution was added to TMB-4 in a conical-bottom vial. The concentration of TMB-4 was 1.0 M. After 30 min the TMB-4 was separated from the acetylcholinesterase on the Sephadex G-25 Fine column. Activity of the resulting acetylcholinesterase solution was 48% of that of the control. At the end of 24 h analogous TMB-4 reactivation resulted in an activity corresponding to 14% of the control (see Fig. 2).

Results and Discussion

Inhibition of acetylcholinesterase by Sarin is accompanied by the formation of a covalent phosphorus-enzyme linkage at the active site serine. This inhibition is an extremely facile reaction as evidenced by its rate constant of $1 \cdot 10^7$ $M^{-1} \cdot min^{-1}$ [14]. Spontaneous reactivation involves displacement of the phosphonyl group with concomitant return of enzymatic activity (Eqn. 3b).



Its counterpart has been termed aging and has been shown to involve loss of the alkoxy group from the phosphonate moiety [15—17] (Eqn. 3a). This pathway results in an enzymatic species which is refractory to reactivation by hydroxamic acids and oximes.

Since spontaneous reactivation and aging of phosphonylated acetylcholinesterase are paralled first-order reactions (Eqn. 3), the observed rate constant of reactivation (Eqns. 1 and 2) is actually the sum of the true reactivation rate constant and the true aging rate constant (Eqn. 4).

$$k_{obsd} = k_{react} + k_{aging} \tag{4}$$

Furthermore, the ratio of reactivated to aged acetylcholinesterase is the same as the ratio of their first-order rate constants of formation (Eqn. 5) [18].

$$\frac{k_{react}}{k_{aging}} = \frac{\%\ \text{React}}{\%\ \text{Aged}} \tag{5}$$

At the end of the reaction, where % React + % Aged = 100%, k_{react} can be calculated from Eqn. 6.

$$k_{react} = \frac{(\% \text{ React})\ (k_{obsd})}{100} \tag{6}$$

Thus, from the observed rate of reactivation (k_{obsd}) and the percent reactivation, the true rate constants of reactivation (k_{react}) and aging (k_{aging}) may be evaluated.

In our work we monitored the return of acetylcholinesterase activity following inhibition by Sarin. The phosphonylated enzyme was separated from excess inhibitor by gel filtration prior to assaying for the spontaneous return of activity. Examples of the quality of the rate data collected in these investigations are shown in Fig. 1. A summary of our studies at pH 7.60, 8.44, and 9.16 are shown in Table I. The percent of final spontaneous reactivation is noted as "Δ" to exclude the small residual activity (0.5% or less) observed at "zero time" in several experimental runs. The results indicate (1) an increase in the percentage of inhibited enzyme that recoveres with increasing pH (1—2% at pH 7.60, 13% at pH 8.44, and 23% at pH 9.16, and (2) a decrease in the observed rate with increasing pH ($t_{1/2}$ = 154 min at pH 7.60, $t_{1/2}$ = 28 h at pH 8.44, and $t_{1/2}$ = 70 h at pH 9.16).

As noted in Table I, at pH 8.44 we observed a 13.0 ± 1.7% return of activity with k_{obsd} = 0.0245 ± 0.0035 h^{-1}. Subjecting one of these reactivated samples to a second Sarin inhibition and spontaneous reactivation resulted in an 11.73% (that is, 11.73% of 13.0%) return of activity with k_{obsd} = 0.0272 h^{-1}. These results are, within experimental error, exactly what one would expect to see if (1) they reflect the behavior of the most abundant enzyme species, and (2) if this species of the Sarin-inhibited enzyme is partitioning via the two parallel reactions of spontaneous reactivation and aging (Eqn. 3).

Additional support for (1) and (2) above is afforded by further analysis of our pH 8.44 results coupled with available literature data. As has been pointed out, according to Eqn. 4 the k_{obsd} is equal to the sum of the true spontaneous reactivation rate constant and the true aging rate constant. Thus, breakdown of the pH 8.44 data in Table I using Eqns. 6 and 7 yields k_{react} = 0.0032 h^{-1} ($t_{1/2}$ = 216.6 h) and k_{aging} = 0.0213 h^{-1} ($t_{1/2}$ = 32.5 h).

These results are basically consistent with the work of Davies and Green [19] in spite of differences in experimental design. Their studies on the aging of Sarin-inhibited enzyme (human erythrocyte acetylcholinesterase, 37°C, veronal buffer) show k_{aging} = 0.072 h^{-1} ($t_{1/2}$ = 9.62 h) at pH 8.5. Since it is

TABLE I

SPONTANEOUS REACTIVATION OF ISOPROPYL METHYLPHOSPHONYLATED ACETYLCHOLINESTERASE

pH	Buffer	No. of runs	Δ (%)	k_{obsd}	$t_{1/2}$
7.60	MOPS	6	2.1 ± 0.7	0.0045 ± 0.0006 min^{-1}	154 ± 20 min
8.44	Bicine	3	13.0 ± 1.7	0.0245 ± 0.0035 h^{-1}	28.3 ± 4.0 h
9.16	Bicine	4	23.4 ± 2.1	0.0099 ± 0.0006 h^{-1}	70.0 ± 4.2 h

known that an increase in temperature accelerates aging, to a first approximation we can adjust our 25.0°C result at pH 8.44 to 37°C by multiplying it by 5.88, i.e. 0.49 per degree [20]. This brings our result to 0.125 h^{-1}, which is quite close to their value of 0.072 h^{-1} observed with human erythrocyte acetylcholinesterase.

If one couples our pH 8.44 aging value with the result of an analogous breakdown of the pH 9.16 data in Table I (via Eqns. 6 and 7: k_{react} = 0.0023 h^{-1} with $t_{1/2}$ = 301.3 h and k_{aging} = 0.0076 h^{-1} with $t_{1/2}$ = 91.2 h), calculation of $\Delta \log k_{aging}/\Delta pH$ gives a slope of —0.62. Similar treatment of Davis and Green's results at pH 8.0 and pH 8.5 gives a slope of —0.8. These negative slopes of less than 1 mean that the aging rate is retarded less as the pH is raised than would be expected from operation of only an acid-catalyzed aging mechanism. These results are important as a number of previous studies [21—24] indicate that aging of alkoxy phosphonylated acetylcholinesterase is solely acid catalyzed.

We have previously documented the importance of base-catalyzed aging in acetylcholinesterase inhibited with phenyl methylphosphonochloridates [5]. In that case, involvement of an acid-catalyzed, carbonium ion mechanism was obviated by the presence of aryl leaving groups. However, the data reported here implicate base-catalyzed aging in the conversion of Sarin-inhibited acetylcholinesterase to the oxime resistant form, particularly at pH values above the physiological range. Thus, base-catalyzed aging may be more general than previously thought, and should be considered when investigating the development of oxime resistance in all phosphorylated cholinesterases.

As is evident from Fig. 2, the spontaneous reactivation we observe at pH 7.60 is not consistent with the concept that spontaneous reactivation and aging of Sarin-inhibited acetylcholinesterase are parallel first-order reactions (Eqn. 3). The upper curve, showing the decreasing susceptibility of the inhibited enzyme to oxime reactivation, i.e. aging, agrees with the results of investigations by Harris and co-workers [16]. The lower curve reflects the observed spontaneous reactivation under the same conditions. It should be noted that the lower curve is a kinetically determined result (Table I). The vertical line on the figure emphasizes that the observed spontaneous reactivation (lower curve) is essentially finished (>95%) when the aging reaction (upper curve) still has approx. 25% of its course to complete.

The 1—2% spontaneous reactivation observed at pH 7.60 appears to be independent of the source of the enzyme. To date we have used four different lots from Worthington and two different in-house preparations. In addition, to eliminate the possibility that the results obtained at pH 7.60 are peculiar to Sarin or a trace impurity associated with it, several studies were carried out using isopropyl *p*-nitrophenyl methylphosphonate [25] and isoproyl *m*-nitrophenyl methylphosphonate [26] to effect inhibition. These phosphonates would be expected to give the same active site-inhibited derivative of the enzyme, and to spontaneously reactivate in a manner analogous to the lower curve in Fig. 2. Our results substantiated this point. Spontaneous reactivation following inhibition by isopropyl *p*-nitrophenyl methylphosphonate amounted to a 0.89% return of activity, and a 1.96% return when isopropyl *m*-nitrophenyl methylphosphonate was used as the inhibitor.

In the early stages of this work the authors considered the possibility that

the spontaneous reactivation observed at pH 7.60 might have its origin in a "contaminating esterase". However, extensive studies clearly identified the active material as acetylcholinesterase [27]. Our present effort is directed at elucidating the challenging considerations presented in this paper.

Acknowledgements

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