Formation of Nonreactivatible Isopropylmethylphosphonofluoridate-Inhibited Acetylcholinesterase*

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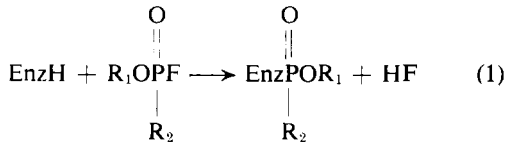
ABSTRACT: Isopropylmethylphosphonofluoridate (Sarin) inhibits acetylcholinesterase by interacting with the active site. Sarin can be displaced from the active site by exposing the complex to strong nucleophilic agents, such as oximes.

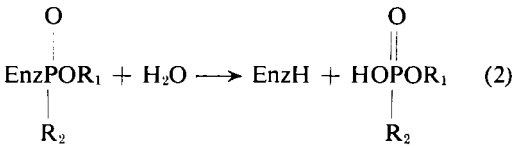
The Sarin–cholinesterase complex, however, undergoes an apparent intramolecular reaction in which an alkyl or alkoxy group is eliminated. This group has been identified by reverse isotope dilution and by countercurrent distribution as the isopropyl group. It was observed that the elimination of the isopropyl group from the Sarin–cholinesterase complex is accompanied by a loss in the ability of oximes to displace the inhibitor to give a reactivated enzyme.

The inhibition of cholinesterases (acetylcholine acetyl-hydrolase, EC 3.1.1.7, and acylcholine acyl-hydrolase, EC 3.1.1.8) by organophosphate compounds such as diisopropylphosphonofluoridate (DFP),[1] isopropylmethylphosphonofluoridate (Sarin), and pinacolylmethylphosphonofluoridate (Soman) involves a phosphorylation of the enzyme at the active site (Aldridge, 1950; Cohen *et al.*, 1955; Jandorf *et al.*, 1955; Schaffer *et al.*, 1954). This may be represented schematically as shown in eq 1.



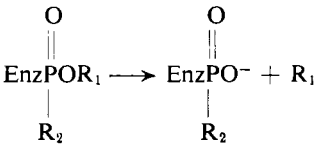
$$\mathrm{EnzH} + \mathrm{R_1OPF} \longrightarrow \mathrm{EnzPOR_1} + \mathrm{HF} \qquad (1)$$

Hydrolytic cleavage of the organophosphate from the phosphorylated enzyme may occur physiologically at an extremely slow rate by a base-catalyzed reaction as shown in eq 2.



$$\mathrm{EnzPOR_1} + \mathrm{H_2O} \longrightarrow \mathrm{EnzH} + \mathrm{HOPOR_1} \qquad (2)$$

Because this reaction is so slow, for all practical purposes the phosphorylated complex may be considered stable. Displacement of the organophosphate group from the enzyme–inhibitor complex can be made to occur, however, by reaction of the complex with strong nucleophilic agents such as quaternary oximes (Wilson, 1951). The ability of oximes to displace the organophosphate from the enzyme decreases with time, presumably because the phosphorylated complex undergoes an additional reaction in which one of the alkyl groups of the organosphosphate moiety is eliminated (eq 3).



$$\mathrm{EnzPOR_1} \longrightarrow \mathrm{EnzPO^-} + \mathrm{R_1} \qquad (3)$$

Berends *et al.* (1959) showed that the percent of nonreactivatible horse serum pseudo-cholinesterase after inhibition by DFP is directly proportional to the amount of monoisopropyl phosphate bound to the enzyme and inversely proportional to the amount of diisopropyl phosphate bound to the enzyme. Harris *et al.* (1965), using [^{32}P]Sarin, and Fleisher and Harris (1965), using [^{32}P]Soman, have shown that the formation of nonreactivatible acetylcholinesterase is related to the formation of a dealkylated methylphosphorylated enzyme. These data indicate that the loss of reactivatibility of DFP-, Sarin-, and Soman-inhibited pseudocholinesterase or acetylcholinesterase is due to the cleavage of an alkoxy group from the organophosphate moiety of the cholinesterase–inhibitor complex. The availability of Sarin labeled with tritium specifically in the isopropyl group has allowed an identification of the R group eliminated in eq 3. Moreover, the data show a 1:1 stoichiometric relationship between the loss of the isopropyl group from the Sarin–cholinesterase

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[1] Abbreviations used: DFP, diisopropylphosphonofluoridate; Sarin, isopropylmethylphosphonofluoridate; [^{3}H]Sarin for [2-^{3}H-propyl]isopropylmethylphosphonofluoridate; Soman, pinacolymethylphosphonofluoridate; TMB-4, 1,1′-trimethylenebis-4-(hydroxyiminomethyl)pyridinium bromide.

complex and the inability of oximes to reactivate the cholinesterase of the complex.

Materials and Methods

Materials. Bovine erythrocyte and electric eel cholinesterases were obtained from the Sigma Chemical Co.[2] Sarin containing tritium solely in the 2 position of the isopropyl alcohol moiety was prepared by the New England Nuclear Corp. and had a specific activity of 96 mc/mmole. [^{14}C]Isopropyl alcohol was also obtained from the New England Nuclear Corp. TMB-4 was obtained from Aldrich Chemicals.

Enzymatic Assay and Reactivation by TMB-4. In a pH-Stat, 0.1 ml of an appropriately diluted enzyme was added to 3.9 ml of a reaction mixture containing 25 mM acetylcholine bromide, 4.8 mM $MgCl_2$, and 20 mM barbital buffer at pH 7.4. Enzymatic activity was followed by recording the rate and amount of 20 mM NaOH added to maintain a constant pH of 7.4. A unit of activity is defined as the amount of enzyme necessary to require the addition of 1 μmole of base/min to maintain a constant pH of 7.4.

The amount of reactivatible enzyme was determined after incubating aliquots of 10–50 μl of the inhibited enzyme solution for 24 hr in the presence of 0.1–0.2 ml of TMB-4 at a final concentration of 0.1 M. Better than 90% of the initial enzymatic activity could be accounted for when this TMB-4 treatment was started immediately after Sephadex filtration. Assays for reactivatible enzyme were carried out in the presence of the TMB-4. Kinetic experiments, in which the reciprocals of the reaction rates were plotted against the reciprocals of substrate concentrations in the presence and absence of TMB-4, showed that the rates of reactions in the presence of 25 mM acetylcholine and 2.5 mM TMB-4 were 51 and 46% of optimum rates for eel and erythrocyte cholinesterases, respectively. Optimum rates for both cholinesterase preparations were obtained at approximately 1 mM. The shape of the double-reciprocal plots, however, indicated that no substrate inhibition occurred in the presence of 25 mM acetylcholine when 2.5 mM TMB-4 was present. All data in which moles of enzymes were calculated have been corrected to the optimum rates that were used to determine turnover numbers.

Estimation of Isopropyl Alcohol by Reverse Isotope Dilution. Aliquots (0.5–1 ml) of the inhibited enzyme were taken at zero time (immediately after Sephadex filtration) and after a 24-hr incubation period. Carrier isopropyl alcohol (1 ml) was added immediately to each of the aliquots, the solutions were saturated with NaCl, and the isopropyl alcohol was extracted two times with 2 ml of carbon tetrachloride. Most of the water was removed by filtration of the combined carbon tetrachloride extracts through Whatman No. 1 filter paper and by shaking the extract with anhydrous sodium sulfate. Isopropylphenylurethan was prepared by standard techniques (Shriner and Fuson, 1948), but increasing the quantity of phenyl isocyanate threefold (1.5 instead of 0.5 ml). Because some water remained in the extract after sodium sulfate treatment, the initial crystals contained an appreciable amount of diphenylurea. The diphenylurea was removed by recrystallization of the isopropylphenylurethan twice from carbon tetrachloride and twice from petroleum ether (bp 35–80). A considerable reduction in the yield of isopropylphenylurethan was obtained by this procedure, but the final crystals appeared to be pure (see Table I) with a melting point of 88 ± 0.5° (Shriner and Fuson, 1948). Unlike the 3,5-dinitrobenzoate of isopropyl alcohol, the isopropylphenylurethan does not quench scintillation counting even at the 100-mg level. Moles of isopropyl alcohol formed were calculated from the label in the isopropylphenylurethan by the following relationship [(theoretical yield in mg/mg counted) × cpm]/(cpm/mole of starting [^{3}H]Sarin) = moles of isopropyl alcohol.

TABLE I: Isopropyl Alcohol Formation and Nonreactivatibility of [^{3}H]Sarin-Inhibited Erythrocyte Cholinesterase.[a]

Assay Method	Moles of Nonreactivatible Enzyme
Enzyme activity	1.13×10^{-10}
Isopropylphenylurethan	
4th recrystallization	1.16×10^{-10}
8th recrystallization	1.02×10^{-10}

[a] A 100-mg/ml solution of erythrocyte cholinesterase was inhibited with [^{3}H]Sarin as described. After two passes through a Sephadex G-25 column (0.9 × 16 cm), samples were removed and assayed for spontaneously regenerated activity (2) and TMB-4 reactivatible activity. Isopropyl alcohol (1 ml) was added to two-fifths of the remaining volume. The alcohol was extracted into carbon tetrachloride and the isopropylphenylurethan was prepared as outlined. The mole equivalents of enzyme not susceptible to TMB-4 reactivation were calculated using the turnover number of 3.0×10^5 (Cohen and Warringa, 1953). Corrections were made as outlined in a previous section for TMB-4 inhibition. Moles of isopropyl alcohol were calculated from the formula outlined in Methods.

Preparation of Inhibited Enzyme. Electric eel cholinesterase (1.67 mg) or 100 mg of bovine erythrocyte cholinesterase in 1 ml of pH 7.4 Tris–chloride buffer that was 0.05 M with respect to chloride was treated

[2] The bovine erythrocyte cholinesterase as obtained from the Sigma Chemical Co. contained about 97 mg of gelatin, NaCl, and buffer salts/100 mg of dry weight; it had 1000 units/mg of protein. The electric eel cholinesterase was in aqueous solution and contained 15,000 units/mg of protein.

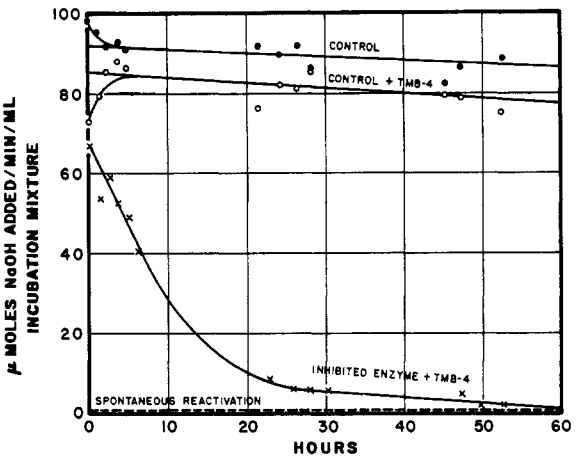

FIGURE 1: Loss of reactivatibility of acetylcholinesterase of the Sarin–cholinesterase complex. Electric eel cholinesterase (20 μl, about 200 units, diluted to 1 ml with a 1% gelatin–10% albumin solution) was divided into two aliquots. One aliquot was put through a precalibrated column of Sephadex G-25 (0.9 × 16 cm) equilibrated with 0.02 M sodium barbital buffer at pH 7.4. The protein was collected in approximately 1 ml and used as the control enzyme. The other aliquot was incubated for 5 min with 10^{-5} M Sarin and put through a similar column of Sephadex. Samples were removed from each one of these at predetermined time intervals and assayed for enzymatic activity before and after TMB-4 treatment. Thus, control plus TMB-4 and inhibited enzyme plus TMB-4 indicate samples to which TMB-4 was added at the times indicated on the abscissa. TMB-4 was not removed before the assay for enzymatic activity. All incubations were performed in the presence of 25 mM acetylcholine. All rates shown are based on an equivalent 280-mμ absorbance.

with a final concentration of 2×10^{-4} M [^{3}H]Sarin. An assay for enzymatic activity after 5 min showed that inhibition was complete. Excess [^{3}H]Sarin was removed from the inhibited enzyme complex by filtration through a 0.9 × 20 cm Sephadex G-25 column that had been equilibrated with the same Tris-chloride buffer. A sample of this mixture (arbitrarily taken as zero time but actually at approximately 30 min after the initial contact between enzyme and inhibitor) was assayed for spontaneously regenerated enzymatic activity, TMB-4 reactivatible activity, and free isopropyl alcohol. The remainder was incubated at 25° without additional treatment or alterations. At predetermined time intervals, the assays for free tritiated isopropyl alcohol, TMB-4 reactivatible, and spontaneously regenerated enzymatic activity were repeated on appropriate samples.

Countercurrent Distribution. An incubation mixture corresponding to approximately 1.67 mg of the originally inhibited eel enzyme was subjected to countercurrent distribution in a 60-tube apparatus using benzene and 2 M lithium sulfate as solvents. The incubation mixture, usually 1 ml, was added to the first tube along with 2 mmoles of solid lithium sulfate. Benzene-saturated 2 M lithium sulfate was then added to the appropriate volume (10 ml) of the aqueous phase of the countercurrent train. After the lithium sulfate had dissolved, the distribution was begun and run at room temperature.

Radioactivity Measurements. All determinations of radioactivity were made in a Packard Tri-Carb automatic liquid scintillation counter using the dioxane-counting solution described by Bray (1960).

Results and Discussion

Acetylcholinesterase completely inhibited by Sarin can be reactivated completely by TMB-4 treatment provided that the TMB-4 is added soon enough. With time, however, less and less of the original cholinesterase activity can be recovered. This decrease in reactivatible enzyme activity is a measure of the extent of the aging reaction shown in eq 3. Thus, by adding TMB-4 to an organophosphate-inhibited cholinesterase complex at various times, the rate of this reaction can be measured. In a similar manner, spontaneous reactivation (2) can be measured if the TMB-4 treatment is omitted.

In order to get the best possible conditions to identify the R group eliminated in the aging reaction, several criteria must be met. The most important of these, however, is to get an enzyme–inhibitor complex in which this reaction is rapid and spontaneous reactivation is negligible. Initial experiments indicated that the [^{3}H]Sarin–pseudocholinesterase complex lost protein-bound radioactivity at a rate identical with the rate by which it lost its susceptibility to reactivation by TMB-4. The over-all rate for this reaction was quite slow, having a half-time of the order of 114 hr. On the other hand, eel and erythrocyte acetylcholinesterase at catalytic concentrations undergo aging at a much faster rate, with a half-time of 9–10 hr. A typical experiment for eel cholinesterase–Sarin complex is shown in Figure 1. It may be seen here that spontaneous reactivation (2) is negligible. Essentially the same results were obtained for the erythrocyte cholinesterase except that spontaneous reactivation occurred to about 7% in 20 hr. With stoichiometric concentrations of enzyme, the kinetics appeared to be altered in that the half-time for aging was slightly longer. However, the data of Table II show that the per cent of radioactivity loss from the complex is the same as the per cent of enzymatic activity that is not reactivatible by TMB-4.

Identification and Quantification of the Radioactive Material Released from Sarin–Cholinesterase Complex. Using the techniques described above, the alkyl or alkoxy group released from Sarin-inhibited acetylcholinesterase by aging has been identified as isopropyl alcohol. Data for the erythrocyte cholinesterase are shown in Table I. During a 24-hr incubation, the number of mole equivalents of the erythrocyte cholinesterase–Sarin complex that had undergone reaction 3

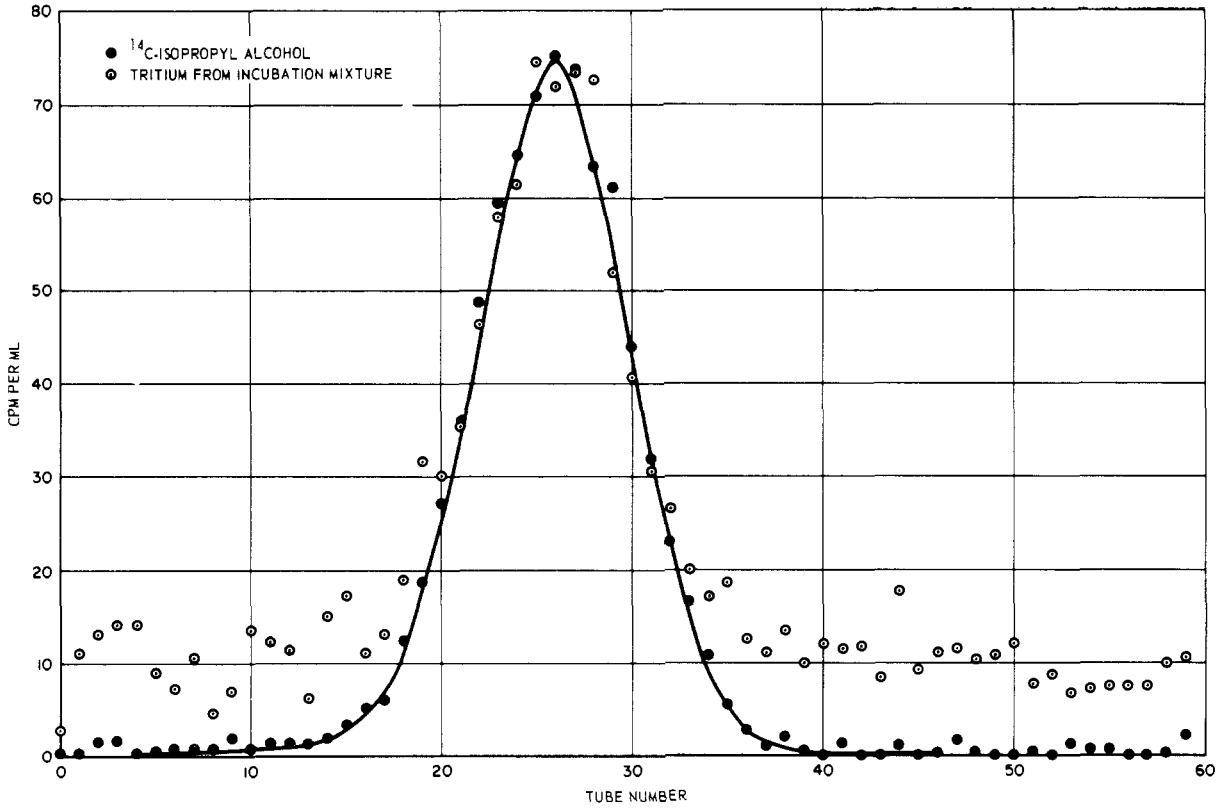

FIGURE 2: Countercurrent distribution analysis of an incubation mixture. Electric eel cholinesterase was inhibited with [^{3}H]Sarin and subjected to countercurrent distribution after a 24-hr incubation period as described in the section entitled Methods.

TABLE II: Loss of Protein-Bound Radioactivity from [^{3}H]Sarin-Inhibited Erythrocyte Cholinesterase.[a]

Hr	Reactivatible Act. (units)	% Lost	Radioactivity/Absorbance Unit (after gel filtration) (cpm)	% Lost
0	30.1	0	1250	0
24	17.2	43	635	49

[a] Erythrocyte cholinesterase (1 ml, 100 mg/ml) inhibited with [^{3}H]Sarin as described was passed in succession through two Sephadex G-25 columns (0.9 × 16 cm). Immediately after the second column passage, a sample (0.1 ml) was removed and treated with TMB-4 to determine reactivatible activity. Another sample was removed for scintillation counting. After a 24-hr incubation at 25°, the TMB-4 reactivation step was repeated. Another sample was subjected to gel filtration on Sephadex G-25 and the radioactivity coming through the column with the protein was determined.

was the same as the number of moles of isopropyl alcohol formed. The former was calculated from the relative changes in reactivatible enzyme activity and the latter was determined by the appearance of tritium in the isopropylphenylurethan prepared by the reverse isotope dilution technique. This is true also for the electric eel cholinesterase (Table III). After a 24-hr incubation of the Sarin–cholinesterase complex, 24.2 × 10^{-5} μmole of the inhibited enzyme complex could not be reactivated by TMB-4 treatment and 27 × 10^{-5} μmole of isopropyl alcohol had been formed. Thus it appears from these data that 1 mole of isopropyl alcohol was formed for each mole of Sarin–cholinesterase complex that was no longer reactivatible by TMB-4.

Additional evidence showing that isopropyl alcohol was the labeled material released upon incubation of the Sarin–cholinesterase complex was obtained by countercurrent analysis of an incubation mixture. It may be seen in Figure 2 that the codistribution of an incubation mixture containing [^{3}H]Sarin cholinesterase (eel) with authentic [^{14}C]isopropyl alcohol gave a single symmetrically labeled peak containing both of the isotopes. Separate distribution analysis in the same solvent system showed that methyphosphonate appeared predominantly in the aqueous phase

TABLE III: Isopropyl Alcohol Formation and Nonreactivatibility of [^{3}H]Sarin-Inhibited Electric Eel Cholinesterase.[a]

Aging Time or Rate (hr)	Enzyme Act./ml (units)	Isopropyl Alcohol/ml (cpm)
0	165.7	880
24	59.7	7540
Nonreactivatible enzyme/24 hr	106.0	6660
Calculated μmoles/ml	24.2×10^{-5}	27×10^{-5}

[a] The same experimental conditions were used here as for Table I except that 1.67 mg of electric eel cholinesterase was substituted for the erythrocyte enzyme. Mole equivalents of enzyme were calculated from the turnover number of 4.38×10^5 derived from the data of Michel and Krop (1951).

with a peak at tube 4 while Sarin appeared predominantly in the organic phase with a peak at tube 57. No peak was seen for the [^{3}H]Sarin–cholinesterase complex that had not undergone aging presumably because this protein-bound tritium precipitated on the walls of the first few tubes of the distribution train. In fact, about 50% of the activity expected in this fraction could be recovered in a 0.1 M NH_4OH wash of the first two or three tubes of the countercurrent distribution train after the separation was completed. These data confirm the reverse isotope dilution studies in showing that the R group in aging is isopropyl alcohol when cholinesterase is inhibited by Sarin.

The mechanism of aging, alkyl or alkoxy elimination, has not been determined. This reaction could occur by either PO or CO scission resulting in an alcohol which does or does not retain the original oxygen of the organophosphate compound. It is known that aging is favored by low pH (Davies and Green, 1956; Hobbiger, 1956; Michel, 1958) showing a general acid catalysis and is mediated by some group on the protein. Protonation of the oxygen in the POC linkage may occur with subsequent elimination of the alkyl group as a carbonium ion. Should this be the case, the alcohol formed would contain oxygen from the incubation medium. The resulting altered enzyme complex would be more nucleophilic in the vicinity of the active binding site and less susceptible to reactivation by the nucleophilic oximes. The net results, therefore, are the formation of a 1:1 stoichiometric amount of isopropyl alcohol and an altered nonreactivatible organophosphate–cholinesterase complex.

Acknowledgments

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References

Aldridge, W. N. (1950), *Biochem. J. 46*, 451.

Berends, F., Posthumus, C. H., Sluys, I. v. d., and Dierkauf, F. A. (1959), *Biochim. Biophys. Acta 34*, 576.

Bray, G. A. (1960), *Anal. Biochem. 1*, 279.

Cohen, J. A., Oosterbaan, R. A., Warringa, M. G. P. J., and Jansz, H. S. (1955), *Discussions Faraday Soc. 20*, 114.

Cohen, J. A., and Warringa, M. G. P. J. (1953), *Biochim. Biophys. Acta 11*, 52.

Davies, D. R., and Green, A. L. (1956), *Biochem. J. 63*, 529.

Fleisher, J. H., and Harris, L. W. (1965), *Biochem. Pharmacol. 14*, 641.

Harris, L. W., Fleischer, J. H., Braswell, L. M., and Michel, H. D. (1965), *Federation Proc. 24*, 361.

Hobbiger, F. (1956), *Brit. J. Pharmacol. 11*, 295.

Jandorf, B. J., Michel, H. O., Schaffer, N. K., Egan, R., and Summerson, W. H. (1955), *Discussions Faraday Soc. 20*, 134.

Michel, H. O. (1958), *Federation Proc. 17*, 275.

Michel, H. O., and Krop, S. (1951), *J. Biol. Chem. 190*, 119.

Schaffer, N. K., May, C. S., and Summerson, W. H. (1954), *J. Biol. Chem. 206*, 201.

Shriner, R. L., and Fuson, R. C. (1948), The Systematic Identification of Organic Compounds, New York, N. Y., Wiley.

Wilson, I. B. (1951), *J. Biol. Chem. 190*, 110.