Dealkylation Studies on Inhibited Acetylcholinesterase

By D. B. COULT AND D. J. MARSH

Chemical Defence Experimental Establishment, Porton Down, Salisbury, Wilts.

AND G. READ

Department of Chemistry, University of Exeter

(Received 17 August 1965)

1. A rapid method for measuring the rate of dealkylation of organophosphorus-inhibited enzymes is described. 2. The dealkylation rates have been measured for acetylcholinesterase inhibited by isopropyl, *sec.*-butyl, 1,2-dimethylpropyl, 1,2,2-trimethylpropyl and cyclohexyl methylphosphonofluoridate, and by di-isopropyl phosphorofluoridate; they are first-order under the conditions used.

Cholinesterase inhibited by organophosphates can be reactivated by powerful nucleophiles such as hydroxamic acids and those based on pyridinium aldoximes, e.g. pyridine-2-aldoxime methiodide (Childs, Davies, Green & Rutland, 1955). Hobbiger (1955) observed that the extent of reactivation with nicotinoxyhydroxamic acid as reactivator depended on the length of time the inhibitor had been in contact with the enzyme. The change in the inhibited enzyme to a form that cannot be reactivated by oximes has become known as 'aging', and has been observed with several enzymes (Berends, 1964). It has also been shown that the speed of 'aging' depends to a marked degree on the structure of the organophosphate used for inhibition, di-isopropyl phosphoryl-acetylcholinesterase 'aging' more rapidly than diethyl phosphonyl-acetylcholinesterase (Hobbiger, 1957).

Loomis & Salafsky (1963) showed that oximes were virtually ineffective in reactivating acetylcholinesterase inhibited by Soman (1,2,2-trimethylpropyl methylphosphonofluoridate) and that there was a 100% mortality in mice poisoned by Soman despite the administration of atropine and oxime. They suggested that rapid 'aging' could account for these observations.

Jansz, Brons & Warringa (1959) observed that the degradation of di-isopropyl phosphorofluoridate (DFP)-inhibited chymotrypsin, trypsin and liver *ali*-esterase gave serine residues that contained the di-isopropyl phosphoryl group, whereas pseudocholinesterase under the same conditions gave a monoisopropyl phosphorylserine derivative. It is to be noted that, for chymotrypsin, trypsin and liver *ali*-esterase, when this 'aging' phenomenon occurs at all it is considerably slower than for pseudocholinesterase. On this basis Jansz *et al.* (1959) suggested that 'aging' was caused by dealkylation of the dialkyl phosphoryl-enzyme.

This hypothesis has been established for pseudocholinesterase by Berends, Posthumus, Sluys & Dieirkauf (1959), who have shown that the rate of 'aging' is the same as the rate of dealkylation. Usdin, Mitz & Killos (1964) report that other inhibitors behave similarly but no details have been given.

It was considered desirable to measure the rates of dealkylation of *sec.*-alkyl methylphosphonyl-acetylcholinesterases to assess the effect of the *sec.*-alkyl group on the rate of dealkylation, as certain *sec.*-alkyl groups increase the rate of 'aging'. The reactions involved in these measurements of dealkylation are given in Scheme 1.

MATERIALS AND METHODS

Enzyme. Aqueous dilutions of bovine erythrocyte acetylcholinesterase (Winthrop Laboratories Inc., New York, N.Y., U.S.A.) was used at concentrations of approx. 100 units/ml. The enzyme was found to contain no measurable amounts of *ali*-esterase or *arylesterase*.

Inhibitors. The ³²P-labelled inhibitors were prepared at Suffield Experimental Station, Ralston, Alberta, Canada (for their preparation see Reesor, Perry & Sherlock, 1960) and were shown to be at least 95% pure by the Schönemann method (Marsh & Neale, 1956) with the exception of the di-isopropyl phosphorofluoridate which was 93% pure. The initial specific activities used were approx. 120 mc/g. The nature of the impurities is not known.

Radioactive counting. All the counts were made with a Geiger-Müller counter for liquids of the type CV2145 housed in a lead 'castle' and connected to an Ekco automatic scaler, type N530B. A 5 ml. volume of solution was used in each determination, and the count was over a period of 1000 sec. Correction for the background count was applied in each case.

Column chromatography. The following column-chromatographic procedures were used.

(1) Sephadex (Pharmacia, Uppsala, Sweden) was used to separate the protein containing the inhibited acetylcholinesterase from the excess of inhibitor and hydrolysis

Scheme 1. Reaction products of *sec.*-alkyl methylphosphonofluoridates and cholinesterases.

products of the inhibitor. Sephadex G-50 (medium grade) in water was used to make up columns (40 cm. $\times$ 3 cm.). The protein was eluted with water, 5 ml. samples of the effluent were collected and the radioactivity of each was determined. Each column was only used once.

(2) Amberlite (British Drug Houses Ltd., Poole, Dorset) was used to separate the *sec.*-alkyl hydrogen methylphosphonate (Scheme 1, compound II) from the methylphosphonic acid (Scheme 1, compound III) when these acids had been produced as the alkaline-hydrolysis products of partially 'aged' inhibited acetylcholinesterase. Amberlite CG-120 type II resin, which had been washed by the continuous-flow technique to remove fine particles, was filtered, stirred with 2M-LiCl-N-HCl and used to prepare the column. Sufficient resin to give a column 10 cm. high was poured in the form of a slurry into a 30 cm. $\times$ 2 cm. column surrounded by a water jacket maintained at 50° by a continuous-flow system. The base of the column was supported by a filter-paper disk bedded on acid-washed sand. Eluent solution (700 ml.) was passed down the column before use (flow rate approx. 0.4 cm./min.), and when the column had settled a disk of filter paper covered by a little acid-washed sand was used to preserve an even surface. Between runs, 300 ml. of eluent solution was passed down the column.

For isopropyl methylphosphonofluoridate (Scheme 1, compound I, R = isopropyl, R' = methyl) and di-isopropyl phosphorofluoridate (Scheme 1, compound I, R = isopropyl, R' = isopropoxy) a column of resin 20 cm. long was used and elution was effected with 4M-LiCl-2N-HCl.

Phosphorus analysis. An aqueous solution of the alkyl hydrogen methylphosphonate or methylphosphonic acid (4 ml.), containing an equivalent of up to 60 μ g. of phos-

phorus, was decomposed by adding N-NaOH (1 ml.) and 10% (w/v) $(\text{NH}_4)_2\text{S}_2\text{O}_8$ (1 ml.) and heating at 100° for 30 min. After cooling, 5N- H_2SO_4 (2 ml.) was added and the solution was diluted to 8 ml. and mixed. Then 1 ml. of 10% (w/v) sodium molybdate solution was added with stirring, followed by 1 ml. of 0.2% SnCl_2 solution and further mixing. After 5 min. the intensity of the blue colour formed was measured spectrophotometrically and the concentration of phosphorus read off from a calibration curve prepared from KH_2PO_4 solutions of suitable concentrations (Fiske & Subbarow, 1925).

Measurement of dealkylation. Each estimation required a 10 ml. sample of enzyme in 0.01 M-phosphate buffer, pH 7.4, and eight to ten samples were used for each determination of dealkylation rate. These were maintained at 25° with an additional sample that was used as a control (see below). Inhibition was carried out with a concentration of inhibitor that gave 100% inhibition in 1 min. This was 0.5 μ M for the *sec.*-alkyl methylphosphonofluoridates and 1 μ M for di-isopropyl phosphorofluoridate. Each solution was allowed to 'age' for an appropriate time-interval before the reaction was stopped by the addition of 6 g. of urea to the solution. The urea solution obtained was heated at 100° for 5 min. Acetylcholinesterase has been reported to be irreversibly denatured in 8M-urea (Coleman, 1957), and since Berends (1964) has shown that the dealkylation process does not occur with denatured inhibited butyrylcholinesterase no further dealkylation was expected. However, preliminary studies showed that dealkylation was resumed on removal of urea from cyclohexyl methylphosphonyl-acetylcholinesterase by Sephadex separation. It appears that at least some of the functional groups at the active centre of the enzyme resume their original positions after the removal of

urea. The procedure described above was used since it was thought that dealkylation might be initially accelerated by heat treatment alone.

The denatured inhibited enzyme was placed on a Sephadex column and eluted with water. The radioactive protein fraction was found to separate completely from the excess of inhibitor and its hydrolysis products. The radioactive protein fraction was treated with *N*-NaOH to bring the pH of the solution to 12.0 and then heated at 100° for 1 hr. to liberate the *sec*.-alkyl hydrogen methylphosphonate and methylphosphonic acid. Chromatography on an Amberlite column established that there was no dealkylation of *sec*.-alkyl hydrogen methylphosphonates under these conditions.

The two expected phosphorus-containing acids were added to the hydrolysate to act as carriers (1 ml. of a 0.1 *N* solution of each acid). The solution was then reduced in volume to approx. 1 ml. and quantitatively transferred to the Amberlite column by using 6 ml. of the LiCl-HCl eluting solution. The column was developed and the effluent collected in 5 ml. fractions. Each fraction was examined for total radioactivity and total phosphorus content. In this manner it was established that the retention volumes for the radioactive phosphorus-containing acids corresponded to those of the carrier acids used. The order in which the acids were eluted from the column was determined beforehand and in every case the dealkylated phosphonic acid preceded the alkylated acid. This separation procedure was based on the work of Jakob, Park, Ciric & Rieman (1961), who have shown that under carefully controlled conditions a quantitative separation of phosphorus-containing esters and acids can be achieved with a Dowex cation-exchange resin of low capacity. The recovery of the phosphorus-containing acids from the Amberlite resin was 95–100%.

The possibility that non-specifically bound phosphorus was present in the deactivated enzyme was examined, since Cohen & Warringa (1953) have shown that non-specific phosphorylation can occur in some preparations of acetylcholinesterase. It was assumed that the binding of the phosphorus residue to sites other than the active site of the enzyme would be unaffected by denaturation. A control sample of the enzyme, which was taken for each inhibitor, was denatured with urea before treatment with the inhibitor. The sample was then subjected to the procedure outlined above and the amount of bound ³²P determined. In no case did the activity of the control sample exceed 5% of that of the other samples and no dealkylation of the non-specifically bound phosphorus was found to occur. Corrections for non-specifically bound phosphorus were made, based on the values determined in this way.

A further possible source of error lay in spontaneous dephosphorylation of the inhibited enzyme, equivalent to the spontaneous reactivation that has been observed in some organophosphorus-inhibited enzymes (Aldridge & Davison, 1953). The influence of this on the rate measurements would depend on whether the dephosphorylation of the dealkylated enzyme occurred more rapidly than the dephosphorylation of the alkylated enzyme. This possibility was studied by determining the radioactivity of the enzyme after its separation on the Sephadex column. Equivalent samples were examined after they had been standing for up to 72 hr. The radioactivity in the samples showed that the extent of dephosphorylation was no more than 5% after

72 hr. and was probably much less. It was not possible to correct for this in the rate values.

From the amount of the radioactive alkyl hydrogen methylphosphonate and the sum of the esterified and unesterified radioactive methylphosphonic acids from each sample the percentages of alkylated and dealkylated methylphosphonyl-acetylcholinesterase were obtained.

RESULTS AND DISCUSSION

When the logarithm of the percentage of alkylated methylphosphonyl-acetylcholinesterase present in each sample was plotted against the time of standing, a straight line was obtained, suggesting first-order kinetics. Fig. 1 shows such a plot for 1,2,2-trimethylpropyl methylphosphonyl-acetylcholinesterase, the most rapid of the dealkylations. In each case agreement with first-order kinetics was found when the results were subjected to a modification of the least-squares treatment described by Bond (1935), which takes the logarithmic nature of the concentration term into account. The inhibitors studied and the rates of dealkylation obtained are given in Table 1.

Di-isopropyl phosphorofluoridate has been included to compare the behaviour of acetylcholinesterase with that of pseudocholinesterase studied by Berends *et al.* (1959). Di-isopropyl phosphorofluoridate-inhibited acetylcholinesterase was found to dealkylate more slowly than the corresponding inhibited pseudocholinesterase. These

Fig. 1. Time-course of the spontaneous dealkylation of ³²P-labelled Soman-inhibited acetylcholinesterase at pH 7.4 and 25°. The dealkylation process in the samples was stopped by the addition of urea and heat treatment; after removal of excess of inhibitor by Sephadex separation, the inhibited enzyme was hydrolysed and the dealkylated and alkylated methylphosphonic acids formed were estimated by column chromatography and radioactive counts on the effluent.

Table 1. *First-order rate coefficients for the dealkylation of organophosphorus-inhibited acetylcholinesterase at pH 7.4 and 25°*

The rate coefficients were obtained from first-order kinetics applied to the percentage of alkyl methylphosphonyl-acetylcholinesterase present after standing for a known time at pH 7.4 and 25°. The method of estimating the percentage of alkyl methylphosphonyl-acetylcholinesterase is given in the text.

Trivial name	Inhibitor		$10^3 k$ (min. ⁻¹)	Half-life (min.)
	R	R'		
Sarin	Isopropyl	Methyl	0.37	1870
—	<i>sec.</i> -Butyl	Methyl	8.22	85
—	1,2-Dimethylpropyl	Methyl	12.0	58
Soman	1,2,2-Trimethylpropyl	Methyl	115	6
—	Cyclohexyl	Methyl	0.17	4000
DFP	Isopropyl	Isopropoxy	0.32	2140

workers used two methods to determine the rate of dealkylation of the inhibited enzyme. In the first, excess of ^{32}P -labelled di-isopropyl phosphorofluoridate was removed from the inhibited enzyme preparation by dialysis at low temperature before the enzyme was allowed to age at 24°. Samples were taken at intervals and subjected to alkaline hydrolysis. Radioautography of chromatograms of the hydrolysed samples showed the presence of radioactive di- and mono-isopropyl phosphates, which were estimated quantitatively. In the second method, di-isopropyl phosphorofluoridate labelled with ^{14}C in the isopropyl groups and with ^{32}P was used. It was shown that the ^{14}C content of the inhibited enzyme decreased at the same rate as the inhibited enzyme 'aged', whereas the ^{32}P content remained unchanged. The dealkylation step was found to have a half-life of about 5 hr. at 24°.

For our studies with *sec.*-alkyl methylphosphonofluoridates it was necessary to modify Berend's (1964) first method so that faster dealkylation rates could be measured. The method used is more rapid than that used by Berends *et al.* (1959) as Sephadex separation replaces dialysis and column chromatography, and liquid counting on the effluent replaces paper chromatography and radioautography of the chromatograms. The concentration values obtained by extrapolating the plots to zero time correspond to $100 \pm 4\%$ of fully alkylated phosphorus residue on the enzyme for all the inhibitors examined, and this is a measure of the accuracy of the method. However, the rates would be expected to vary with the environment of the enzyme and it would be dangerous to extend these results to other situations *in vitro* and the situation *in vivo* without experimental support. Extrapolation to 100% alkyl methylphosphonyl-acetylcholinesterase supports

the suggestion that the urea and heat treatment of the inhibited enzyme stops the dealkylation process by denaturing the enzyme; no other means of denaturing the enzyme was tried in these studies.

It is clear that a wide variation in the dealkylation rate exists for the different phosphorylating groups under the conditions used. These differences in rate parallel the ease of formation of carbonium ion from the secondary alkyl group (Hughes, 1951). 'Aging' studies (D. B. Coult, D. J. Marsh & G. Read, unpublished work) carried out in connexion with this work show that 'aging' and dealkylation can only be considered synonymous under special circumstances.

Since this work was completed a study of the 'aging' and dealkylation of Soman-inhibited acetylcholinesterase has been published by Fleischer & Harris (1965). These workers used a different technique to determine the rate of dealkylation, for which they found a rate coefficient of 0.29 min.^{-1} at 37° and pH 7.4, which is in reasonable agreement with the value of 0.115 min.^{-1} at 25° and pH 7.4 found in the present study when the temperature difference is taken into account. They also found that the rate of 'aging' was not significantly different to that of dealkylation, which indicates that 'aging' and dealkylation are very closely connected.

These studies indicate that from a practical point of view any alkyl methylphosphonofluoridate in which the alkyl group easily forms a carbonium ion would form a rapidly dealkylating inhibited acetylcholinesterase, and poisoning by this compound would be resistant to therapeutic treatment with oximes and atropine.

The authors are grateful for the supply of ^{32}P -labelled *sec.*-alkyl methylphosphonofluoridates from the Suffield Experimental Station, Ralston, Alberta, Canada.

REFERENCES

- Aldridge, W. N. & Davison, A. N. (1953). *Biochem. J.* **55**, 763.
- Berends, F. (1964). Ph.D. Thesis: University of Leiden.
- Berends, F., Posthumus, C. H., Sluys, I. v.d. & Dieirkauf, F. A. (1959). *Biochim. biophys. Acta*, **34**, 576.
- Bond, W. N. (1935). *Probability and Random Error*, p. 37. London: E. Arnold and Co.
- Childs, A. F., Davies, D. R., Green, A. L. & Rutland, P. (1955). *Brit. J. Pharmacol.* **10**, 462.
- Cohen, J. A. & Warringa, M. G. P. (1953). *Biochim. biophys. Acta*, **11**, 32.
- Coleman, M. H. (1957). Ph.D. Thesis: University of Bristol.
- Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
- Fleischer, J. H. & Harris, L. W. (1965). *Biochem. Pharmacol.* **14**, 641.
- Hobbiger, F. (1955). *Brit. J. Pharmacol.* **10**, 356.
- Hobbiger, F. (1957). *Brit. J. Pharmacol.* **12**, 438.
- Hughes, E. D. (1951). *Quart. Rev. chem. Soc., Lond.*, **5**, 243.
- Jakob, F., Park, K. C., Ciric, J. & Rieman, W., III (1961). *Talanta*, **8**, 431.
- Jansz, H. S., Brons, D. & Warringa, M. G. P. (1959). *Biochim. biophys. Acta*, **34**, 573.
- Loomis, A. T. & Salafsky, B. (1963). *J. Tox. appl. Pharmacol.* **5**, 685.
- Marsh, D. J. & Neale, E. (1956). *Chem. & Ind.* p. 494.
- Reesor, J. B., Perry, B. J. & Sherlock, E. (1960). *Canad. J. Chem.* **38**, 1416.
- Usdin, E., Mitz, M. A. & Killos, P. J. (1964). *Abstr. 6th int. Congr. Biochem., New York*, IV-184.