Biochem. J. (1966) **100**, 572

Factors Influencing the Rate of 'Aging' of a Series of Alkyl Methylphosphonyl-acetylcholinesterases

By W. K. BERRY and D. R. DAVIES
Army Department, Chemical Defence Experimental Establishment, Porton Down, Salisbury, Wilts.

(*Received* 6 *January* 1966)

1. The progressive development of resistance to reactivation by an oxime ('aging') shown by a series of alkyl methylphosphonyl-acetylcholinesterases is slow when the alkyl group is a primary alcohol, whether or not the carbon chain is branched, but is much more rapid if the alkyl group is a secondary or cyclic alcohol. 2. Aging is accelerated by increase of temperature or decrease of pH. 3. Aging is inhibited by the quaternary amine *N*-methylpyridinium iodide. 4. The results are discussed in relation to the role played by aging in the therapy of poisoning by organophosphorus compounds.

Organophosphorus compounds inactivate acetylcholinesterase (EC 3.1.1.7) by phosphorylating the enzyme. The inactive enzyme can usually be dephosphorylated by reaction with quaternary pyridine aldoximes, which thus restore the activity of the enzyme. If the inactivated enzyme is kept at room temperature for some time, the proportion of reactivatable enzyme becomes progressively less (Davies & Green, 1956). This spontaneous transformation of inactivated but reactivatable enzyme into a form that is resistant to the action of nucleophilic reagents, particularly oximes, has been termed 'aging' (Jandorf, Michel, Schaffer, Egan & Summerson, 1955).

The rate of aging appears to differ with the nature of the alkylphosphoryl group; but only a limited number of studies have been reported, from which it appears that the decreasing order of rates of aging is dimethylphosphoryl, di-isopropylphosphoryl, isopropyl methylphosphonyl and diethylphosphoryl, these being the groups attached to the enzyme (Davies & Green, 1956; Hobbiger, 1956; Vandekar & Heath, 1957).

In the present work, some of the factors influencing the rate of aging have been more extensively examined, in particular the effect of the structure of the alkyl group in a series of alkyl methylphosphonyl-acetylcholinesterases. This is important, since branching on the α-carbon atom, especially when accompanied by branching elsewhere in the chain, increases the aging rate greatly, compared with the corresponding straight-chain derivatives. The effects of temperature and pH have also been examined.

MATERIALS AND METHODS

Organophosphorus compounds. A series of freshly prepared alkyl methylphosphonofluoridates were used. All were more than 98% pure according to the Schoenemann method (Marsh & Neale, 1956). For use, stock solutions of about 5mM in propan-2-ol were prepared (about 1μl./ml.) from which further dilution in 0·9% NaCl was made as required.

Oxime. TMB-4* was obtained from the Aldrich Chemical Co. Inc. (Milwaukee, Wis., U.S.A.) and used without further purification. Each week a stock solution of 12·5mM (117·9mg./25ml.) in water was prepared, and from this dilution at pH 8 was made daily; to 4ml. was added 0·6ml. of 0·1N-NaOH (or such other volume as may have been determined by previous trial) to bring the pH to 8, and water to 5ml.

Modified Michel buffer. This was made by dissolving 2·5g. of sodium barbitone B.P., 0·33g. of KH_2PO_4 (anhydrous) and 26·89g. of KCl in about 800ml. of water, adding sufficient N-HCl to bring the pH to 8·1 and making up the volume to 1l.

Acetylcholine. A stock solution of 2% (w/v) of the chloride (Roche Products Ltd., Welwyn Garden City, Herts.) in water was used.

Acetylcholinesterase. Heparin-treated human blood, freshly collected each week, was centrifuged, and the cells were washed twice with 0·9% NaCl and stored at 0–4° as a 1:1 suspension in 0·9% NaCl.

Inactivation and reactivation of acetylcholinesterase. The principle of the method of measuring the rate of aging was to inactivate with organophosphorus compound, wash off the surplus inhibitor and haemolyse, all at 0°, warm to

* Abbreviations: TMB-4, 1,3-di-(4-hydroxyiminomethylpyridinium)propane dichloride; Soman, 1,2,2-trimethylpropyl methylphosphonofluoridate.

the required temperature and remove portions of the preparation at intervals for reactivation by TMB–4.

The exact procedure was as follows. One vol. of erythrocyte suspension was mixed at 0° with 1 vol. of a solution of the organophosphorus compound prepared by diluting the stock solution $1:10^5$ with 0·9% NaCl. After this mixture had stood at 0° for 30 min., 3 vol. of ice-cold 0·9% NaCl was added, and after mixing the cells were separated by centrifuging at 2500 rev./min. in an M S E Major centrifuge for 10 min. The supernatant was rejected and the cells were washed twice more with 5 vol. of ice-cold 0·9% NaCl. Finally the packed cells were haemolysed with water to a total of 5 vol. The pH of the haemolysate was 7·1–7·3.

In some experiments the pH of the haemolysate was adjusted to different required values by adding a few microlitres of N-NaOH with magnetic stirring while observing the pH with the glass electrode. The effect of *N*-methylpyridinium iodide on aging was measured by adding 0·01 vol. of concentrated solution to both the control and inhibited haemolysates.

The haemolysate was placed in a bath at 25° or 35°. After allowing exactly 10 min. for temperature equilibration a 6 ml. sample was transferred to a stoppered flask containing 0·67 ml. of alkaline TMB–4 solution. Further samples were transferred at intervals. If the haemolysate had been made alkaline, each sample was restored to pH 7·1–7·3 by adding a suitable volume of N-HCl, and this was conveniently done by adding the HCl to the alkaline TMB–4 solution. At 30 min. after transfer, 2 ml. portions of the mixture were placed in Michel pots containing 9 ml. of buffer and 1 ml. of acetylcholine solution. The cholinesterase activity was then determined in triplicate by the electrometric method (Michel, 1949).

To allow for spontaneous loss of activity of the enzyme and for spontaneous reactivation, control samples were run in which 0·9% NaCl replaced the organophosphorus compound or the TMB–4 solutions respectively. The percentage reactivation was:

$$100 \times \frac{E_r - E_i}{E - E_i}$$

where E was the activity of the enzyme in the absence of organophosphorus compound, E_i was the activity of the enzyme after inactivation and E_r was the activity of the inactivated enzyme after treatment by TMB–4. The inhibitory effect of 1 mM-TMB–4 did not exceed 5% and was neglected. The greater inhibitory effect of *N*-methylpyridinium iodide was allowed for by including it in the controls.

Preliminary experiments in which the activity of the enzyme was measured by continuous titration at constant pH showed that the reactivation of the inactivated acetylcholinesterase by TMB–4 took place in two stages. The first was completed in about 10 min. and the second was much slower. For example, after inhibition by ethyl methylphosphonofluoridate 0·1 mM-TMB–4 gave 75% reactivation in 10 min., which increased to 82% by 60 min.; after inhibition by the isopropyl (1-methylethyl) homologue, 0·1 mM-TMB–4 gave 62% reactivation in 10 min., which increased to 73% by 90 min.; and after inhibition by the 1,2-dimethylpropyl homologue, 1 mM-TMB–4 gave 33% reactivation in 10 min. with no further increase by 120 min. A standard time of reactivation of 30 min. was therefore chosen to ensure completion of the first stage but negligible progress of the second. By the present methods a concentration of 1 mM-TMB–4 produced 65–100% reactivation of the freshly inhibited enzyme, depending on the organophosphorus compound used, but less than 5% inhibition of the normal enzyme, and was thus adequate to ensure good reactivation without necessitating correction for inhibition of the reactivated enzyme.

The increase in activity caused by TMB–4 after aging for a given time was assumed to be a constant proportion of the reactivatable inhibited enzyme and was taken as the measure of reactivatable enzyme. When the logarithm of the increase was plotted against time of aging a straight line was obtained, from which the time to age 50%, i.e. the half-life of the inhibited enzyme, was calculated.

RESULTS

Influence of the alkyl group on the rate of aging. The half-lives of a series of alkyl methylphosphonylacetylcholinesterases during storage at 25° are shown in Table 1.

Table 1. *Effect of the structure of the alkyl ester group on the rate of aging of alkyl methylphosphonylacetylcholinesterases*

The structure of the inhibited acetylcholinesterase is $RO \cdot PO(CH_3) \cdot$enzyme. Measurements of half-life were made at 25° and pH 7·1–7·3.

Structure of the alkoxy group (RO–)	Half-life of the phosphonylated enzyme (hr.)	Spontaneous reactivation in 24 hr. (%)
A. Straight-chain primary		
$CH_3 \cdot O-$	60	41
$CH_3 \cdot CH_2 \cdot O-$	60	10
$CH_3 \cdot [CH_2]_2 \cdot O-$	60	22
$CH_3 \cdot [CH_2]_3 \cdot O-$	60	46
$CH_3 \cdot [CH_2]_4 \cdot O-$	60	64
$CH_3 \cdot [CH_2]_5 \cdot O-$	60	65
B. Branched-chain primary		
$(CH_3)_2CH \cdot CH_2 \cdot O-$	60	35
$(CH_3)_3C \cdot CH_2 \cdot O-$	60	25
$(CH_3)_3C \cdot CH_2 \cdot CH_2 \cdot O-$	>100	34
C. Straight-chain secondary		
$(CH_3)_2CH \cdot O-$	12	4
$CH_3 \cdot CH_2 \cdot CH(CH_3) \cdot O-$	0·47	0
$CH_3 \cdot [CH_2]_2 \cdot CH(CH_3) \cdot O-$	0·60	0
$CH_3 \cdot [CH_2]_3 \cdot CH(CH_3) \cdot O-$	0·80	0
D. Branched-chain secondary		
$(CH_3)_2CH \cdot CH(CH_3) \cdot O-$	0·2	0
$(CH_3)_3C \cdot CH(CH_3) \cdot O-$	$<0·04$*	0
E. Cyclic		
$CH_2 \cdot CH_2 \cdot CH_2\ CH_2 \cdot CH_2 \cdot CH \cdot O-$ (cyclohexyl, ring closure as drawn)	4·6	0

* Reactivation could not be achieved under the standard conditions used for the rest of the compounds in this Table.

When the alkyl ester radical consisted of a primary alkoxy group, the half-life was fairly long (about 60hr.) and independent of chain length (group A). Branching had no measurable effect, provided that the primary structure was maintained (group B). However, branching on the α-carbon atom to produce secondary alkoxy derivatives markedly increased the rate of aging (group C), and in such cases further branching, on the β-carbon atom, caused further acceleration (group D); for example, the half-life of the 1-methylbutoxy derivative is 36min., whereas that of the more highly branched homologue, the 1,2-dimethylpropoxy derivative, is only 12min. No reactivation of the 1,2,2-trimethylpropoxy derivative could be demonstrated under these conditions, and reasons are presented below for supposing that this was because aging was virtually instantaneous. The one example of a cyclic alkyl derivative (group E) aged moderately rapidly.

The primary alkoxy derivatives showed considerable spontaneous reactivation, whereas the others showed little or none (the method of calculating oxime-induced reactivation allowed for spontaneous reactivation). The alkylphosphonylated enzyme has two routes of breakdown, aging and spontaneous reactivation, and these results show that the nature of the alkyl ester group determines which one predominates.

Effect of temperature on aging. Increase in temperature accelerated aging. The temperature coefficients, defined as the ratio of the rate at 35° to the rate at 25°, for some alkyl methylphosphonyl-acetylcholinesterases were: isopropyl, 4·9; *n*-propyl, 3·1; 2-methylpropyl, 3·2; 1,2-dimethylpropyl, 2·8. These values suggest that the coefficient varies with the alkyl group.

Effect of pH *on aging.* The effect of varying the pH on the aging of 1,2-dimethylpropyl methylphosphonyl-acetylcholinesterase is shown in Fig. 1. The linear relationship between pH and the logarithm of the first-order rate constant shows that the process is acid-catalysed.

Reactivation of acetylcholinesterase inhibited by Soman. In the experiments just reported it was not possible to measure the aging of Soman-inhibited acetylcholinesterase because it could not be reactivated. The results of the study on the effect of pH suggested that it might be possible to demonstrate aging by increasing the pH; and, since aging at physiological pH during the preliminary manipulation might still be very rapid at 0°, it was thought necessary to carry out both inhibition and washing at a high pH. Accordingly a suspension of erythrocytes was brought to pH 9, cooled and treated with a concentration of Soman ten times that previously used. After washing and haemolysis the pH had fallen to 8·5, and to minimize aging reactivation was carried out without further adjustment of pH towards 7·2. When reactivation was carried out as soon as the temperature reached 25° a small but significant increase in activity from 2% to 15% was observed, but after keeping for 30min. at 25° no reactivation could be demonstrated. The half-life would thus be 10min. or less at pH 8·5–9, and if the effect of pH were as in Fig. 1 the half-life at pH 7·2 would be 2min. or less.

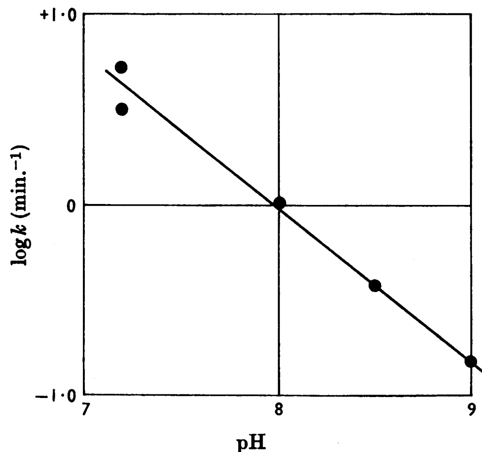


Fig. 1. Effect of pH on the aging of 1,2-dimethylpropyl methylphosphonyl-acetylcholinesterase at 25°.

In later experiments with the Warburg method it was possible to produce measurable reactivation at 38° by adding a mixture of acetylcholine (7·3mM) and 2-hydroxyiminomethyl-*N*-methylpyridinium methanesulphonate (10mM) 1min. after a concentration of Soman just sufficient to produce 95% inhibition in this time. The Soman was not removed, and under these conditions the half-life of the inactivated reactivatable enzyme was 1·7min.

Effect of N-*methylpyridinium iodide on the rate of aging of some alkyl methylphosphonyl-acetylcholinesterases.* It has been shown that substitution on the α-carbon atom of the alkyl group results in considerable acceleration of aging, and additional substitution on the β-carbon atom, so that the structure spatially resembles choline, gives further acceleration. This led to the idea that the anionic site of the enzyme is in some way involved, and preliminary experiments with *N*-methylpyridinium iodide, which inhibits the hydrolysis of acetylcholine by the normal enzyme, appear to confirm this. Table 2 shows that various concentrations of *N*-methylpyridinium iodide inhibit the aging of the inactivated enzyme formed from 1,2-dimethyl-

Table 2. *Effect of* N-*methylpyridinium iodide on the rate of aging of some alkyl methylphosphonyl-acetylcholinesterases, and on the activity of the native enzyme, at* 25° *and* pH 7·2

The activity of the native enzyme was measured by the Warburg method with 7·3 mM-acetylcholine as substrate. —, Not measured.

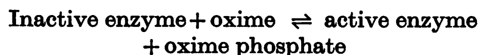
Concn. of *N*-methyl-pyridinium iodide (mM)	Rate of aging with alkyl group indicated (% of rate with *N*-methylpyridinium iodide absent)			Activity of native enzyme (% of activity with *N*-methyl-pyridinium iodide absent)
	1,2-Dimethyl-propyl	1-Methyl-pentyl	Cyclohexyl	
40	31	4	0	—
10	38	28	19	27
2	67	42	48	65

propyl, 1-methylpentyl and cyclohexyl methylphosphonofluoridate. For comparison results are also given for the inhibition of the normal enzyme, as determined by the Warburg method, with 7·3 mM-acetylcholine.

DISCUSSION

The purpose of these experiments was to confirm and extend earlier observations that failure to reactivate acetylcholinesterase that had been inactivated by organophosphorus compounds was a time-dependent process, and to obtain information about the rates at which it proceeded.

The preliminary observations on the rates of reactivation after inactivation by some organophosphorus compounds were consistent with the suggestion by Scaife (1959) that reactivation by TMB-4 involved the rapid formation of a fairly stable equilibrium:



Inactive enzyme + oxime $\rightleftharpoons$ active enzyme + oxime phosphate

Once this equilibrium had been formed, the effect of aging *in vitro* would be to shift it to the left, and if this shift occurred during measurement of the final activity of the enzyme it would be shown as a perceptible diminution in the rate of hydrolysis of substrate, whether this was measured as a fall in pH (Michel method) or as output of carbon dioxide (Warburg method). The fact that such diminution was not observed is taken as evidence that the addition of TMB-4 and substrate had effectively stopped aging.

Aging *in vitro* at pH 7·1–7·3 proceeds at a finite rate, which is sometimes very rapid; for example, the half-life of the 1,2-dimethylpropyl methylphosphonyl-enzyme at 35° is only 3–4 min., and of the Soman-inhibited enzyme probably less than 2 min. as suggested by two independent approaches. The first involved direct measurement but without removal of excess of Soman. The second involved inference from observations made at an unphysiologically high pH. The relevance of these observations might be questioned on the grounds that at this high pH both the attachment of the inhibitor to the enzyme and the reactivation process are qualitatively changed; however, in previous studies of this kind (Davies & Green, 1956, 1958; Hobbiger, 1956) it has been assumed that essentially the same processes occur, and that the effect of pH is merely to influence the degree of ionization of the reacting molecules.

Whether aging is fast enough in any given instance to influence the treatment by oximes of animals poisoned by organophosphorus compounds can be assessed only indirectly. The key question is: what is the minimum amount of acetylcholinesterase in vital organs required to maintain life? Barstad (1960) has suggested 10–15% in rat diaphragm. The problem in therapy is whether this amount can be regenerated by the concentration of oxime reaching an organ from a therapeutically tolerable dose in competition with aging. After intramuscular injection of some of these organophosphorus compounds the first signs of poisoning are observed in 5–30 min., depending on the dose. Slow aging that occurs in poisoning by an organophosphorus compound derived from a primary alcohol is not important. Aging after poisoning by isopropyl methylphosphonofluoridate (Sarin) is also unimportant, since the half-life of the inactivated enzyme is about 2·5 hr. at body temperature. It is probably critical in poisoning by some of the other *sec.*-alkyl derivatives because it is so rapid in comparison with the rate of onset of lethal processes, and may be the primary factor in poisoning by Soman.

The possibility of exploiting for therapeutic purposes the inhibition of aging by *N*-methylpyridinium iodide has been considered. Table 2 shows that, to do this, enough *N*-methylpyridinium iodide would have to be used to inhibit a large

proportion of the available acetylcholinesterase; and preliminary trials have shown that the maximum dose of oxime that does not produce signs of poisoning is diminished in proportion to the dose of *N*-methylpyridinium iodide given with it. Since it is a reasonable presumption that quaternary oximes will also inhibit aging, it seems preferable to give the tolerable dose of quaternary compound as oxime.

The authors thank Miss E. M. Grant (Mrs M. Puckey) for her technical assistance.

REFERENCES

Barstad, J. A. B. (1960). *Arch. int. Pharmacodyn.* **128**, 143.
Davies, D. R. & Green, A. L. (1956). *Biochem. J.* **63**, 529.
Davies, D. R. & Green, A. L. (1958). *Advanc. Enzymol.* **20**, 283.
Hobbiger, F. (1956). *Brit. J. Pharmacol.* **11**, 295.
Jandorf, B. J., Michel, H. O., Schaffer, N. K., Egan, R. & Summerson, W. H. (1955). *Disc. Faraday Soc.* **20**, 134.
Marsh, D. J. & Neale, E. (1956). *Chem. & Ind.* p. 494.
Michel, H. O. (1949). *J. Lab. clin. Med.* **34**, 1564.
Scaife, J. F. (1959). *Canad. J. Biochem.* **37**, 1301.
Vandekar, M. & Heath, D. F. (1957). *Biochem. J.* **67**, 202.