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Can allosteric effectors of acetylcholinesterase control the rate of ageing of the phosphonylated enzyme?

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IT IS now reasonably well established that compounds related to (+)-tubocurarine bind to electric eel acetylcholinesterase (EC 3.1.1.7; AChE) in low ionic strength media at a point distinct from the catalytic site, and in so doing alter the hydrolytic activity of the enzyme.[1,2] Similar allosteric effects have been noted for AChE from mammalian sources.[3–5] The inhibition of AChE by organophosphorus compounds can be relieved by the administration of pyridinium aldoximes such as 2-PAM (2-hydroxyiminomethyl-*N*-methylpyridinium iodide), but the reactivation by the oximes becomes diminished with time after poisoning, an effect known as "ageing".[6] This is due to the loss of an O-bonded alkyl group from the

phosphorus atom, which renders the phosphorus to enzyme bond no longer susceptible to attack by the nucleophilic oxime. The rate of this process is so rapid for organic phosphonates bearing a secondary alkyl radical, that it is in practice impossible to reactivate the phosphonylated-AChE at all, and therefore 2-PAM has no antidotal value. Thus poisoning by Soman, (1,2,2-trimethylpropyl methylphosphonofluoridate) cannot be treated by oximes due to the extremely rapid loss of the sec.-alkyl group to form the aged, phosphonylated enzyme.[7] If this process of ageing is influenced by allosteric effectors of AChE, then this would be a matter of considerable practical interest in the treatment of poisoning by organophosphorus compounds. This communication describes a study of the effect of some quaternary nitrogen compounds on the rate of ageing of AChE inhibited by Sarin (isopropyl methylphosphonofluoridate). The latter inhibitor was chosen due to the practical difficulties of measuring ageing of Soman-inhibited AChE.[7]

Alcuronium chloride (diallyl-bis-nortoxiferine dichloride; Alloferin) was donated by Roche Products Pty. Ltd., and 3-chloro-4-methyl-7-hydroxycoumarin[8] by the Cooper Technical Bureau, England. Other drugs were obtained commercially. Two forms of AChE were used; a partially-purified, soluble enzyme from bovine erythrocytes (Sigma Chemical Company, U.S.A.), and intact rabbit erythrocytes stored in acid citrate dextrose and washed in 5 mM sodium phosphate, 150 mM NaCl, pH 7·4.

The enzyme preparation (100 μg pf protein from bovine erythrocytes or erythrocytes from 0·75 ml whole rabbit blood) was incubated at 37° in 10 ml of 5 mM sodium phosphate, pH 7·4. The buffer also contained the effector compound under investigation, and in some experiments 150 mM NaCl was present. Racemic Sarin was added in 0·1 ml of buffer to give a final concentration of $4{\cdot}5 \times 10^{-8}$ M, which resulted in 90 per cent inhibition of the AChE after 30 min. At regular time intervals to 6·5 hr samples of 0·5 ml incubate were removed and mixed with 0·1 ml 3×10^{-3} M 2-PAM in buffer. The mixture was incubated for 20 min at 37°, then 0·1 ml was removed for assay of AChE activity. The radiometric method 3 of Potter[9] as modified by Crone[10] was used. This employed 2·2 mM acetylcholine at pH 7·0, ionic strength greater than 0·14, for 10 min at 37°. Appropriate control measurements were made of the inhibition of AChE by 2-PAM and the effector compounds. The degree of inhibition by Sarin did not change from 30 min to 6·5 hr. The percentage of the Sarin-inhibited AChE that had been reactivated by 2-PAM was calculated at each time point, and the log of this value was plotted against time. The slope of this line was found by the method of least squares, and compared to that of the control experiment by Student's t-test.

All the ageing experiments exhibited first order kinetics over the period of observation, usually one to two half-lives. Table 1 summarizes results from the bovine erythrocyte AChE at low ionic strength, and Table 2 shows the effects of changing salt concentration and enzyme form. Increasing the ionic strength of the controls resulted in a slowing of ageing, as reported by others,[6] whereas the membrane-bound AChE of whole cells showed the same half-life as the soluble enzyme at the same ionic strength (Table 2). At low ionic strength, gallamine triethiodide, tubocurarine and alcuronium all reduced the rate of ageing, alcuronium being the most effective as the change in the rate became noticeable at 1×10^{-5} M (Table 1). When the ionic strength of the medium was increased to 0·163, none of these compounds altered the rate of

TABLE 1. EFFECT OF SEVERAL DRUGS ON THE HALF-LIFE OF SOLUBLE UNAGED, PHOSPHONYLATED AChE AT LOW IONIC STRENGTH (I = 0·013)

Compound	Concn (M)	$t_{\frac{1}{2}}$ (hr)	P
Control		2·94	
(+)-Tubocurarine chloride	1×10^{-5}	3·55	>0·05
	3×10^{-5}	4·9	0·02
	1×10^{-4}	*	0·001
Alcuronium chloride	3×10^{-6}	2·6	>0·05
	1×10^{-5}	4·7	0·01
	3×10^{-5}	8·7	0·001
Gallamine triethiodide	1×10^{-5}	3·2	>0·05
	3×10^{-5}	4·7	0·01
	1×10^{-4}	13·1	0·001
Atropine	3×10^{-5}	2·5	>0·05
N-methylatropinium nitrate	1×10^{-4}	2·7	≫0·05
3-chloro-4-methyl-7-hydroxycoumarin	3×10^{-5}	2·75	≫0·05

The half-life of the AChE activity which can be reactivated by 2-PAM is shown. P is the probability (from Student's t-test) that the result does not differ from the control.

* No ageing observed over the 6 hr of the experiment.

TABLE 2. AGEING OF PHOSPHONYLATED FORMS OF AChE FROM DIFFERENT SOURCES IN THE PRESENCE OF VARIOUS DRUGS AT PHYSIOLOGICAL IONIC STRENGTH (I = 0·163)

Enzyme	Compound	$t_{\frac{1}{2}}$ (hr)	P
Soluble erythrocyte	Control	5·6	
	1×10^{-4} M (+)-tubocurarine	4·7	>0·05
	3×10^{-5} M alcuronium	4·8	>0·05
	1×10^{-4} M gallamine	6·3	>0·05
Whole erythrocytes	Control	5·4	
	1×10^{-4} M (+)-tubocurarine	14·5	0·001
	3×10^{-6} M alcuronium	4·4	0·1
	3×10^{-5} M alcuronium	7·9	0·01
	1×10^{-4} M gallamine	4·85	>0·05
	3×10^{-5} M coumarin derivative	4·9	>0·05
	5×10^{-3} M tetramethylammonium chloride	4·35	>0·05
	5×10^{-3} M *N*-methylpyridinium iodide	42	0·001

ageing (Table 2) a result which could be anticipated from the previous work on these compounds.[1,5] However, the enzyme when present in the cell membrane in its normal position is still influenced by tubocurarine and alcuronium, though not by gallamine at 1×10^{-4} M. The compound 3-chloro-4-methyl-7-hydroxycoumarin was shown by others[8] to be an allosteric effector of AChE, but it had no effect on ageing in the present study (Tables 1 and 2) at 3×10^{-5} M. Atropine has been reported[11] to influence AChE activity, but neither it nor the quaternary derivative *N*-methylatropinium nitrate changed the rate of ageing under our conditions (Table 1). Ageing was inhibited by a high concentration of *N*-methylpyridinium iodide (Table 2) as found by others,[7] but at this concentration of 5×10^{-3} M the compound inhibited AChE enzymic activity to 27 per cent of the control rate.

These results demonstrate that curariform drugs bind to AChE at low ionic strength, and cause a reduction in ageing, that is in the rate of loss of the isopropyl group from the phosphonylated enzyme. When the ionic strength is raised, the effect on ageing disappears, presumably due to decrease in the affinity of the compound for the enzyme. The membrane-bound AChE is still responsive to some of the drugs under these conditions, which could imply that the binding site in the membrane is shielded from the ionic influences of the medium, an idea advanced by the author previously.[5] Although alcuronium and tubocurarine can slow the ageing process under physiological conditions, they have no practical value in the treatment of organophosphorus poisoning as the concentrations necessary are far in excess of what could be tolerated *in vivo*. Indeed, many cholinergic drugs (and the coumarin derivative) may influence the rate of ageing at sufficiently high concentrations, at which they are also potent AChE inhibitors. *N*-methylpyridinium iodide is an example of such a compound. The curariform drugs show effects on ageing at concentrations at which inhibition is less than 10 per cent. The answer to the initial question is that these effectors can control the rate of ageing, but have no practical use unless compounds can be found which are potent effectors of AChE and have no undesirable physiological side-effects.

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Propargyl amine-induced irreversible inhibition of non-flavin-linked amine oxidases

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THE INHERENT specificity of enzymes is primarily based on the k_{cat} term(s). Although many molecules may bind at the active site of a particular enzyme, only a very limited number will actually serve as substrates, and notions such as induced-fit, non-productive binding, and the "rack" mechanism all are intended in one way or another to rationalize this fact.[1] This being the case, irreversible enzyme inhibitors whose

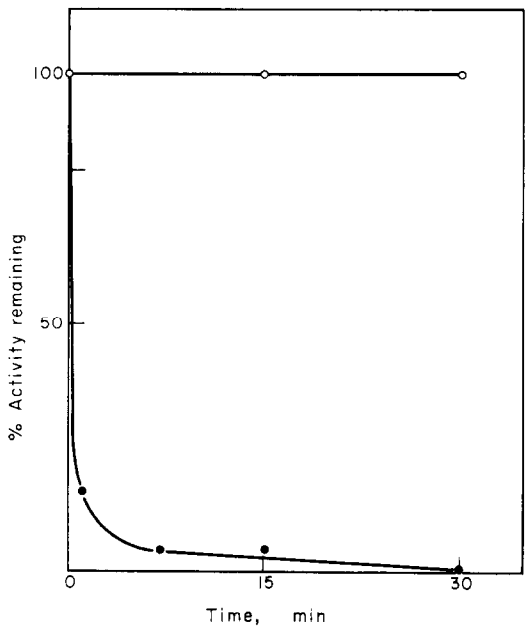

FIG. 1. Irreversible inhibition of plasma monoamine oxidase by propargyl amine. (○) = control, (●) = 3 mM propargyl amine. Ten units of enzyme (sp. act. = 200 units/mg)[4] was incubated in 0·2 M phosphate buffer, pH 7·5, with 3 mM freshly distilled propargyl amine (Aldrich Chemical Co.) along with a control. The remaining activity was measured with time by diluting the enzyme 50-fold in 3·3 mM benzyl amine and measuring the increase in O.D. at 250 nm (benzaldehyde λ_{max} = 250 nm).[4] The activity of the inhibited enzyme could not be restored in the slightest by continued dialysis against the phosphate buffer.