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Effect of imidazoles and pH on aging of phosphylated acetylcholinesterase

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Cholinesterase inhibited by organophosphorus compounds can be reactivated by treatment with nucleophilic agents such as pyridine oximes [1]. However, the enzyme fraction susceptible to reactivation decreases exponentially with the time after inhibition [2, 3]. This is due to a process called aging, which involves the release of an oxygen-bounded alkyl group from the enzyme phosphorus moiety [4–6]. Since the enzyme can no longer be dephosphylated by nucleophilic attack, aging is an important problem in therapy of organophosphate poisoning. The molecular mechanisms of aging is far from understood. The rate of aging depends on the structure of the organophosphorus compound [7]. It is pH dependent [8–11], and is retarded by quarternary ammonium compounds [5, 12, 13]. Present evidence suggests that the mechanism involves a unimolecular fission of the C—O bond of the alkoxy group [14, 15], which may be catalyzed by a protonated group on the enzyme itself [8, 11, 15].

Previously only the effect of quarternary ammonium compounds on aging has been studied. In this communication the effect of imidazole and its derivatives on aging of acetylcholinesterase (AChE, acetylcholine acylhydrolase, ED 3.1.1.7)phosphylated by Sarin (isopropylmethylphosphonofluoridate) and DFP (diisopropylphosphorofluoridate) has been investigated. The effect of different imidazoles, *N*-methylpyridinium iodide, and morpholin on aging is compared with their effect as reversible inhibitors of AChE. The influence of steric factors, the degree of basicity and hydrophobic binding ability, and the effect of pH on aging of isopropylmethylphosphonylated AChE itself both in the presence and absence of effector compounds are discussed.

The effector compounds were of highest purity available from Fluka AG (imidazole, morpholin), Koch–Light (2-methylimidazole), and K & K (2-ethylimidazole, 2,4-dimethylimidazole), or was synthetized in this laboratory (*N*-methylpyridinium iodide) [16].

1.26 mg/ml AChE from bovine erythrocytes (Type 1, Sigma) in 5 mM NaK-phosphate buffer pH 6.0, 7.0, 7.4 and 7.9, and Na-pyrophosphate buffer pH 8.9, was completely inhibited by incubation at 37° for 10 min with 3.15 μM Sarin or 30 min with 3.15 μM DFP. The solution was then mixed with either an equal vol. of the incubation buffer containing the effector compound (Table 1), or with buffer containing NaCl to achieve corresponding ionic strength, although the aging was almost independent of the ionic strength within the concentration range used. After incubation for 0.5 to 4.5 hr at 37°, 0.4 ml was mixed with an equal volume of 20 mM pralidoxime mesylas (P2S, 2-hydroxyiminomethyl-*N*-methylpyridinium methanesulphonate) (Norsk Medisinaldepot) at pH 7.0–7.9. The P2S will reactivate the enzyme, and the proportion of nonreactivated enzyme corresponds to the proportion of aged enzyme. The enzyme was allowed to reactivate for 1 hr at 37°, although separate experiments showed that more than 95 per cent was reactivated after 15 min at pH 7.9. Then 0.4 ml of the reactivation mixture was added to a 29.6 ml solution containing 3.04 mM acetylcholine iodide (Fluka AG) and 137.4 mM NaCl. The activity was determined by continuous titration at pH 7.4 and 25° [17]. P2S itself is a reversible inhibitor of AChE, and the results were corrected for the inhibition caused by P2S itself (25%). After dilution in 30 ml the effector compounds did not affect AChE activity. At the same time as samples of incubate were taken for reactivation, samples (0.2 ml) were also taken to control complete inhibition during the aging experiment. Only in the case of imidazole itself was spontaneous reactivation observed (maximal reactivation was 19 per cent after 3.5 hr at pH 7.9), and this was corrected for. Plots of the logarithm of the percentage of reactivated AChE vs time of aging were linear, and therefore consistent with first-order kinetics. The slope of the lines was found by the method of least squares. The results are expressed as first-order rate constants (Table 1). A linear relationship was found (Fig. 1) between the concentration of 2,4-dimethylimidazole and the ratio of rate constants in absence (k_0) and presence (k_e) of 2,4-dimethylimidazole:

$$k_0/k_e = 1 + (\text{2,4-dimethylimidazole})/\text{constant}$$

The relative potency of the other effectors to 2,4-dimethylimidazole was determined from this relationship. In the relative values presented in Table 2, the different degree of protonation at pH 7.9 was also taken into account.

AChE is reversibly inhibited by quarternary ammonium salts and several protonated compounds by their interaction with the so-called anionic site [18–20] in the active centre of the enzyme. The activity of AChE (6.2 μg AChE/ml 0.1 M phosphate buffer pH 7.9) in the presence of the effector compound and 465 μM (final concentration) acetylthiocholine iodide (Sigma) was determined by the method of Ellman *et al.* [21]. Five concentrations of each effector compound within the range 0.09–1.9 mM (2,4-dimethylimidazole, *N*-methylpyridinium iodide), 0.9–9 mM (2-ethylimidazole), 0.9–47 mM (imidazole, 2-methylimidzole), or 1.9–93 mM (morpholin) were used. The results were corrected for non-enzymatic substrate hydrolysis caused by imidazole, morpholin, or 2-methylimidazole at high concentrations. The ratio of activities in absence and presence of effector compounds was a linear function of the concentration of the compounds. The results are expressed as inhibition constants for competitive inhibition (Table 2). The inhibition constants were used to calculate the relative affinity of AChE for the compounds (Table 2) after correctiong for the different degree of protonation at pH 7.9.

The results (Table 1) show that imidazole itself and particularly its more basic derivatives retard the rate of aging of AChE phosphylated by Sarin or DFP. The mechanism by which aging is retarded was investigated using 2,4-dimethylimidazole at various concentrations. As mentioned above, the effect on aging was found to be a linear function of the concentration of the compound (Fig. 1). The result indicates [22] that phosphylated enzyme can no longer undergo aging following binding of the effector compound. The effect of pH on the rate of aging in the presence and absence of effector compounds is very complex. Firstly, the strong effect by 2,4-dimethylimidazole at pH 6.0, when it is almost completely protonated, indicates that the imidazoles are active in their protonated form. Secondly, the effect of *N*-methylpyridinium iodide, a quarternary ammonium salt, and that of all of the imidazoles were more pronounced at pH 7.9 than at pH 6.0. Since

Table 1. Rate constants of aging of phosphylated AChE incubated with different effector compounds at 37°

pH	Effector compound	conc (mM)	pK_a*	% protonated	$10^3 \times k$ (hr^{-1}) Sarin	DFP
	None				439 ± 17	
	Imidazole	100	7.1	92.6	396 ± 44	
6.0	2,4-dimethylimidazole	5	8.36	99.6	145 ± 3	
	N-methylpyridinium iodide	2			283 ± 5	
	None				247 ± 17	333 ± 10
	Imidazole	100	7.1	13.7	61 ± 10	
	2-methylimidazole	2	7.85	47.1	134 ± 3	196 ± 4
7.9	2-ethylimidazole	2	8.0	55.7	135 ± 7	
	2,4-dimethylimidazole	1	8.36	74.3	73 ± 5	167 ± 3
	N-methylpyridinium iodide	1			60 ± 3	
	Morpholin	30	8.33	72.9	160 ± 5	187 ± 11

The results are expressed as mean ± S.D. from 5–25 experimental points.
* Values from Perrin, D.D. [27].

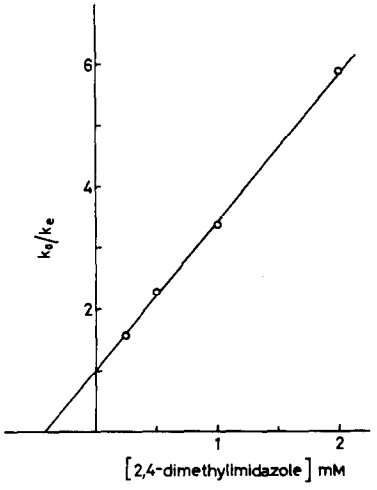

Fig. 1. The effect on aging of isopropylmethylphosphonylated AChE at pH 7.9 by various concentrations of 2,4-dimethylimidazole. The effect is expressed as ratio of rate constants in absence (k_0) and presence (k_e) of 2,4-dimethylimidazole.

N-methylpyridinium is a cation at any pH, this pH effect must be ascribed to a group on the enzyme. Thus the increase in retardation with an increase in pH obtained with the imidazoles may be accounted for by a pH effect on the enzyme rather than a pH effect on the imidazoles. Thirdly, the rate of aging of phosphylated AChE are increased with a lowering of pH (Fig. 2).

A good correlation was found between the relative effect on aging by imidazoles, *N*-methylpyridinium iodide, and morpholin, and their ability to inhibit AChE by binding to its anionic site (Table 2). The increasing effectiveness of the more basic imidazoles could be due to an increasing hydrophobic binding ability. Morpholin which is as basic as 2,4-dimethylimidazole showed only a weak effect, showing that basicity is not the very important factor. The weak effect on aging by morpholin may be due to a poor ability to bind by noncoulombic forces. This is in agreement with previous results suggesting that van der Waal's dispersion forces are important for binding of ammonium ions to the anionic site of AChE [20, 23–25]. In addition, steric hindrance may influence the binding of the effector compounds. The imidazoles and *N*-methylpyridinium are coplanar molecules, whereas morpholin is not. The good correlation found between the relative effect on aging by the compounds and their ability to inhibit AChE through interaction with its anionic site show that the binding sites involved in producing these two effects may be similar.

Table 2. Inhibition constants, relative affinity to AChE, and relative effect on aging of isopropylmethylphosphonylated AChE at pH 7.9 by different effector compounds

Effector compound	K_i (mM)	Relative affinity to AChE	Relative effect on aging
Morpholin	9.9	0.09	0.1
Imidazole	4.9	1	1
2-methylimidazole	1.3	1.1	3.7
2-ethylimidazole	0.63	1.9	3.2
2,4-dimethylimidazole	0.1	8.9	14.3
N-methylpyridinium iodide	0.076	8.8	14.2

The relative values apply to the catonic form of the compounds.

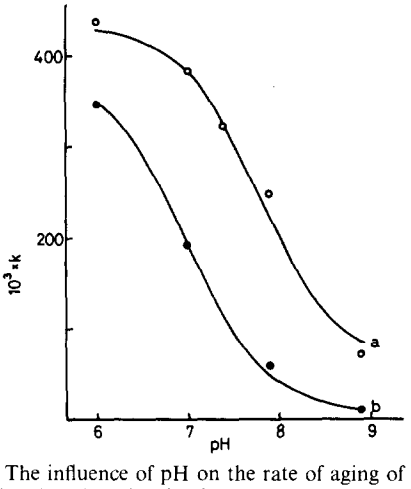

Fig. 2. The influence of pH on the rate of aging of isopropylmethylphosphonylated AChE at 37° in (a) absence and (b) presence of 1 mM *N*-methylpyridinium iodide. Experimental points represent $10^3 \times k$ values, the lines represent dissociation curves of acids with (a) $pK_a = 7.8$ and (b) $pK_a = 7.0$.

In an attempt to characterize the residue on the enzyme taking part or influencing the aging, the logarithm of the rate constants of aging of isopropylmethylphosphonylated AChE in absence of effector compounds was plotted against pH. The points fitted the dissociation curve ($\%_u$ undissociated form vs pH) of an acid with $pK_a = 7.8$ (Fig. 2), indicating that an undissociated enzyme group with $pK_a = 7.8$ participates in the aging of the phosphonylated enzyme [11]. This pK_a is higher than the pK values previously reported [11, 26]. However, the pK_a values found by Keijer [26] differed with different organophosphates, the highest pK_a was obtained with the organophosphate most similar to Sarin. The variation in the pK_a may be due to varying degrees of stabilization of the undissociated enzyme group by formation of a hydrogen bond and/or nonpolar environment, which may considerably enhance the pK_a of the group [11]. When the logarithm of the rate constants of aging in the presence of *N*-methylpyridinium was plotted against pH, the points fitted the dissociation curve of an acid with $pK_a = 7.0$ (Fig. 2). The results show that the affinity of the enzyme for *N*-methylpyridinium increases when pH is increased.

In conclusion, we have found that imidazoles in the protonated form retards the rate of aging of phosphylated AChE, possibly by binding to a group on the enzyme similar to the anionic site. The results are consistent with a retarding mechanism by which aging is completely blocked following binding of effector compound. The results also show that the pH dependence of aging in absence and presence of cationic effector compounds differs, probably because the affinity of the enzyme for the effector compounds increases with increasing pH.

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