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Some adjuncts to oxime–atropine therapy for organophosphate intoxication—Their effects on acetylcholinesterase

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The standard therapy in cases of poisoning by organophosphorus anticholinesterase compounds is administration of atropine, to alleviate the effects of excess acetylcholine, and an oxime, such as pyridine-2-aldoxime methochloride (2-PAM), to reactivate the inhibited acetylcholinesterase (AChE, acetylcholine acetyl-hydrolase; EC 3.1.1.7)[1]. Various other drugs have been tested as adjuncts to this therapy, in order to control the convulsions which are a side-effect of the intoxication, to assist in countering respiratory failure, which is the primary cause of death [1], or because of their antimuscarinic properties. Some of these drugs have been found to enhance protection against the organophosphorus agents, including benactyzine, chlorpromazine, diazepam, mecamylamine and representatives of the Veratrum alkaloids [2–5]. The anticonvulsant, meprobamate [5], and the alkaloid, galanthamine, might well come into this category also. Galanthamine is a potent reversible cholinesterase inhibitor and as such has been recommended as a prophylactic against nerve agents in combination with atropine and an oxime [6]. The proposed mode of action of each of the other supplementary drugs above is unrelated to any effect on AChE. Nevertheless, since the fate of AChE is central to the problem of organophosphorus poisoning, it is surprising that work on the interaction of the above drugs with this enzyme has been limited to semi-quantitative studies of the inhibition of hydrolysis of acetylcholine or its analogues [7–9]. Even recently reported values of the inhibition constant K_i for chlorpromazine [10] and galanthamine [11] neglect the uncompetitive component of inhibition. A more detailed and comprehensive study is reported in this paper.

Materials. Most materials were obtained from commercial sources. Galanthamine was obtained as a 1% aqueous solution from "Medexport", U.S.S.R., for whom the Australian agents are the Malco Agency, Sydney. Cevadine (veratrine), from K & K Laboratories, Plainview, NY, U.S.A., probably includes 25–30% of the related alkaloid, veratridine, as indicated by its n.m.r. spectrum in $CDCl_3$. The spectrum was consistent with that of cevadine [12] but included a sharp peak at $\delta 3.91$, attributable to the aromatic methoxyl groups of veratridine [12]. Diazepam was donated by Roche Products, Dee Why, Australia. Bovine erthrocyte AChE, from the Sigma Chemical Co., St. Louis, MO, U.S.A., was the enzyme used except where mentioned otherwise. *Electrophorus* eel AChE, from the Worthington Biochemical Corp., Freehold, NJ, U.S.A., was used in some experiments. Sarin was synthesized in these laboratories by Mr. D. Amos.

General details. All experiments were done in 45 mM phosphate buffer, pH 7.0, at 25° except where indicated otherwise. Diazepam was introduced into aqueous solution in methanol or ethanol of final concentration 1–2%. Enzyme assays were performed at pH 7.0, 25°, by the method of Ellman *et al.* [13] using a Unicam SP 600 or SP 1750 spectrophotometer.

Hydrolysis of acetylthiocholine (ASCh) by AChE. Six concentrations of ASCh (0.025 to 0.50 mM) were generally employed, and these were run in a convoluted sequence designed to minimize the effect of any enzyme denaturation during the set of assays [14]. When a reversible inhibitor was present, corresponding assays in the presence and absence of inhibitor were run simultaneously. The kinetic parameters K_m (Michaelis constant) and V (maximum velocity) were obtained via the computer program "Median" provided by Dr. A. Cornish-Bowden [15–17].

Inactivation of AChE by physostigmine or phospholine (diethoxyphosphinylthiocholine iodide). Some experiments at 1.6 μM physostigmine were done in the presence of substrate [0.5 mM ASCh, with 0.05 mM 5,5′-dithiobis-(2-nitrobenzoic acid), DTNB] by the method of Hart and O'Brien [18]. Preliminary experiments established that there was no interaction between physostigmine and DTNB at the concentrations used [19]. Other experiments were done in the absence of substrate. At selected times (0.5 to 3.5 min) after mixing solutions of AChE and physostigmine (0.23 μM) or phospholine (0.29 μM), 0.1-ml aliquots were assayed with 1 mM ASCh. No further inhibition occurred during the period of the assay. The apparent first-order rate constant was determined by plotting ln (activity) vs time. The half-life in the absence of a reversile inhibitor was approximately 1 min in all cases.

Reactivation of dimethylcarbamyl-AChE and diethylphosphoryl-AChE. The procedure for reactivation of dimethylcarbamyl-AChE has been described [20]. The same procedure was used for reactivation of diethylphosphoryl-AChE except that the activity of uninhibited enzyme was determined by an appropriate control experiment, rather than by accelerated reactivation. Diethylphosphoryl-AChE was produced by incubation of AChE for 30 min with 0.13 μM phospholine, and its reactivation was followed over 6 hr.

Ageing of isopropylmethylphosphonyl-AChE. AChE in 5 mM Tris, pH 8.7, was incubated with 50 nM Sarin for 30 min at 37°, and for 10 min at room temperature. Approximately 97 per cent inhibition of enzyme activity occurred over this period. Excess Sarin was removed by filtration through a Pharmacia PD 10 column. Studies of the rate of ageing of inactivated AChE were done at 37° in 8.2 ml phosphate buffer. At set times (0–6 hr), 1.0-ml aliquots were treated with 0.2 ml of 3 mM 2-PAM, incubated for 30 min at 25° and freed of 2-PAM by filtration through a PD 10 column. The solution of AChE was then assayed with 0.5 mM ASCh. The first-order rate constant was obtained by plotting ln (activity) vs time.

Hydrolysis of acetylthiocholine (ASCh). All drugs under study inhibited the hydrolysis of ASCh by AChE. The inhibition was of the mixed reversible type, i.e. the drugs increased both K_m/V and $1/V$ where K_m and V are the Michaelis constant and maximum velocity of hydrolysis respectively

Table 1. Inhibition constants for various drugs acting on bovine erythrocyte AChE*

Drug	Acetylthiocholine		Physostigmine		Phospholine
	K_i (mM)†	K_i' (mM)†	Without ASCh K_i (mM)†	With ASCh K_i (mM)†	K_i (mM)†
Benactyzine	0.63 ± 1.01	20.7 (10.2–∞)	2.22 ± 1.25	11.29 ± 6.52	2.11 ± 2.38
Chlorpromazine	0.017 ± 0.020	0.21 (0.09–0.29)	0.079 ± 0.057	> 0.045	0.10 ± 0.17
Diazepam	0.18 ± 0.15	1.1 (0.9–1.3)	0.38 ± 0.30		
Galanthamine	0.095 ± 0.126†	2.0 (1.0–3.8)†	0.29 ± 0.13†	> 0.6†	0.14 ± 0.11†
Mecamylamine	0.84 ± 0.17	47.5‡	1.28 ± 0.97	3.79 ± 2.72	
Meprobamate	5.76 ± 10.45	52.8 (42.2–75.9)	12.7 ± 11.5		
Veratrine	0.24 ± 0.13	0.86 (0.63–3.44)	1.61 ± 1.28		1.13 ± 0.89

* All experiments were done in 45 mM phosphate buffer, pH 7.0, 25°. Values of K_i are shown with 95 per cent confidence limits. For K_i', the range of observed values is given.

† Units are μM for galanthamine.

‡ Mecamylamine reduced V (maximum velocity) only at the highest concentration studied (4.8 mM) and then only by 10 per cent. K_i was calculated from this result using Eqn. 1.

[21]. A plot of K_m/V vs I (concentration of inhibitor) was linear in all cases and K_i, the competitive inhibition constant, could therefore be determined via Equation 1. $(K_m/V)_i$ is the

$$(K_m/V)_i = (K_m/V)_0\ (1 + I/K_i) \qquad (1)$$

value of K_m/V in the presence of inhibitor of concentration I, and $(K_m/V)_0$ is the corresponding value in the absence of inhibitor [21]. Figure 1 (open circles) illustrates Eqn. 1 in the case of galanthamine as inhibitor; in this graph the values on the y-axis are relative units, i.e. $(K_m/V)_i/(K_m/V)_0$. It follows from Eqn. 1 that K_i is the intercept on the x-axis of such a graph. Approximate 95 per cent confidence limits for this intercept can be calculated from the linear regression data [22] but they, and the linear regression itself, require that the dependent variable (K_m/V) follows a Gaussian (normal) distribution. This cannot be assumed [23] and the data, therefore, were analyzed in more detail. The computer program "Median" (see Materials and Methods) prints out the median value of K_m/V and its 95 per cent confidence limits, which are usually asymmetric. Many such print-outs from various experiments were obtained in the course of this study. If K_m/V follows a Gaussian distribution, we would expect that there would be no trend for the value of (upper 95 per cent limit-median) to be greater than the value of (median-lower limit) in all these experiments, or vice versa. Appropriate ratios which would manifest such a trend were calculated, therefore, and a test for a Gaussian distribution (in this case a rankit plot; Ref. 24) applied to a suitable function of them.* It was concluded that the assumption of a normal distribution was not unreasonable. Consequently K_i and its confidence limits were evaluated by linear regression and are listed for each drug in Table 1. The calculated 95 per cent confidence limits approach, and in some cases exceed, the actual value of K_i. Two factors contribute to this: the relatively small number of points in the regression (often n = 5 or 6) and the fact that the intercept on the x-axis necessarily falls outside the range of observations.

A different situation with respect to analysis of data holds in the cast of K_i', , the uncompetitive inhibition constant. A plot of $1/V$ vs I was linear in all cases (but generally with more scatter of points than for the corresponding plot of K_m/V vs I) and Equation 2, therefore, can be applied.

$$(1/V)_i = (1/V)_0\ (1 + I/K_i') \qquad (2)$$

Equation 2 is analogous to Eqn. 1 with 1/V instead of K_m/V and K_i' instead of K_i. However, the appropriate rankit plot (as above) of results from many experiments suggested that the assumption of a Gaussian distribution for $1/V$ is doubtful. Therefore, individual values of K_i' were calculated via Eqn. 2 and the median value was taken as the best estimate of K_i'. This value and the range of values observed is given in Table 1 for each drug under study.

An examination of Table 1 reveals that K_i' is substantially higher than K_i, the competitive inhibition constant, in every case. K_i itself ranged from 5.8 mM (meprobamate) to 0.1 μM (galanthamine). Essentially the same value of K_i was observed for each drug when eel AChE, rather than bovine erythrocyte AChE, was investigated, with the exception of the K_i for the heterogeneous alkaloid veratrine, as the K_i values were 0.24 mM for bovine erythrocyte AChE (Table 1) and 0.022 mM for eel AChE.

The most effective inhibitor, galanthamine, was also tested on bovine erythrocyte AChE at low ionic strength (2 mM phosphate). Should galanthamine possess allosteric properties, an increase in V (i.e. decrease in $1/V$) might be evident [25]. However, an increase in $1/V$ was observed, as at the higher ionic strength, with K_i' being 1.33 μM. K_i was found to be 0.045 ± 0.062 μM.

Inactivation by physostigmine. Physostigmine is a substrate of AChE, as is ASCh, but hydrolysis of the intermediate acyl-enzyme is slow [26]. The acylation (carbamylation or inactivation) and de-acylation (reactivation) steps, therefore, can be studied separately. The effect of the various drugs on reactivation is considered later. In this section we are dealing with the rate of inactivation of AChE by physostigmine, i.e. the rate of formation of methylcarbamyl-AChE. Physostigmine was used at a concentration sufficiently low for the concentration of reversible enzyme–physostigmine complex to be negligible [27]. The appropriate rate equation in the presence of a reversible inhibitor of concentration I is then

$$k_0/k_i = 1 + I/K_i \qquad (3)$$

where k_0 and k_i are first-order rate constants in the absence and presence of reversible inhibitor respectively [28]. Equation 3 is analogous to Eqn. 1 with $1/k_i$ and $1/k_0$ replacing $(K_m/V)_i$ and $(K_m/V)_0$ respectively. K_i has the same meaning as in Eqn. 1, i.e. it is the dissociation constant of the complex of reversible inhibitor with free enzyme. The rate of carbamylation was measured either with or without the additional substrate ASCh. Equation 3 holds regardless of the presence or absence of ASCh [29]. A graph of k_0/k_i vs I appeared to be linear in all cases, and K_i and its 95 per cent confidence limits were determined as the intercept on the x-axis, as described earlier. Values of K_i for the various drugs determined in this

* Full details and a reproduction of the rankit plot have been omitted for the sake of brevity but can be provided by the authors on request.

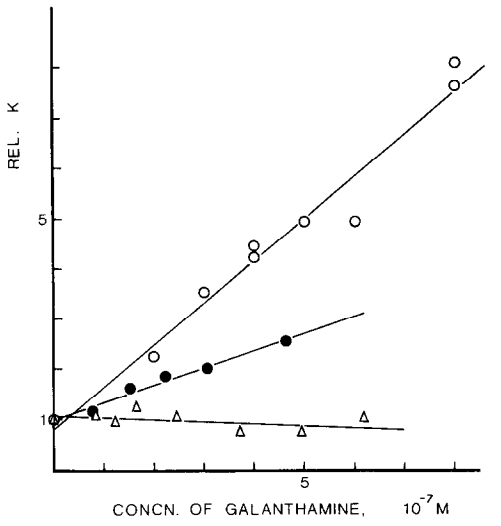

Fig. 1. Inhibition of bovine erythrocyte AChE by galanthamine. Experiments were done in 45 mM phosphate buffer, pH 7.0, 25°. Key: (○) substrate = ASCh. Rel K (y-axis) = relative $K = (K_m/V)_{obs}/(K_m/V)_0$; (●) substrate = physostigmine. Rel. $K = k_0/k_i$; and (△) substrate = physostigmine with ASCh. Rel. $K = k_0/k_i$.

way are listed in Table 1. If the two substrates acetylthiocholine and physostigmine bind to the same active site on the enzyme, then K_i (drug/ASCh) should equal K_i (drug/physostigmine). In fact, when carbamylation by physostigmine was measured in the absence of additional substrate, it was found that K_i (drug/physostigmine) > K_i (drug/ASCh) in all cases. The difference was significant in all cases ($P \leqslant 0.05$), as determined by a statistical comparison of the two regression lines by means of a t-test [22]. When carbamylation by physostigmine was measured in the presence of ASCh with benactyzine or mecamylamine as reversible inhibitor, K_i was found to be larger still (see Table 1); again the difference between K_i (benactyzine or mecamylamine/physostigmine + ASCh) and K_i (drug/physostigmine alone) was significant. Galanthamine and chlorpromazine caused no decrease at all in the rate of carbamylation of AChE by (physostigmine + ASCh). Figure 1 illustrates the varying effectiveness of galanthamine as an inhibitor of ASCh hydrolysis, as an inhibitor of the rate of inactivation by physostigmine alone, and as an inhibitor of the rate of inactivation by physostigmine in the presence of ASCh.

Inactivation by phospholine. Formation of diethylphosphoryl-AChE from phospholine is analogous to formation of methylcarbamyl-AChE from physostigmine. The results were analyzed, therefore, as for inactivation by physostigmine (above). The rate of inactivation was measured in the absence of ASCh. The various values of K_i which were thereby obtained are given in Table 1. For benactyzine, chlorpromazine and veratrine, K_i (drug/phospholine) was significantly different from K_i (drug/ASCh) but not from K_i (drug/physostigmine alone). The reverse situation was observed for galanthamine, i.e. K_i (galanthamine/phospholine) $\simeq K_i$ (galanthamine/ASCh) but K_i (galanthamine/phospholine) $\neq K_i$ (galanthamine/physostigmine).

Reactivation of acyl-enzyme. The half-life for spontaneous reactivation of diethylphosphoryl-AChE was found to be 133 hr. this is longer than that reported for eel AChE (50 hr) under similar experimental conditions [30]. Galanthamine at concentrations up to 0.5 μM, which is five times the value of K_i (galanthamine/ASCh), had no effect on this half-life, nor on the rate of reactivation of dimethylcarbamyl-AChE at normal or low ionic strength (2.5 mM phosphate). Similarly, 1 mM mecamylamine, 1 mM meprobamate, 0.1 mM diazepam and 0.1 mM veratrine had little or no effect on the rate of reactivation of diethylphosphoryl- or dimethylcarbamyl-AChE. Benactyzine at 1 mM also had little effect on the rate of reactivation of diethylphosphoryl-AChE, but it inhibited reactivation of dimethylcarbamyl-AChE. A plot of (first-order rate constant)$^{-1}$ vs concentration of benactyzine, which is analogous to the other plots described above, was linear with dissociation constant $K_i'' =$ intercept on x-axis $= 2.9 \pm 4.2$ mM.

Chlorpromazine showed the most interesting behavior; it inhibited the rate of reactivation of dimethylcarbamyl-AChE with $K_i'' = 5.5 \pm 4.7 \times 10^{-5}$ M, but accelerated the rate of reactivation of diethylphosphoryl-AChE. The net rate constant for reactivation appeared to vary hyperbolically with the concentration of chlorpromazine (0.02 to 0.17 mM) and the results, therefore, were analyzed by the computer program "Median" (see also ref. 20). This analysis gave a dissociation constant $K_a = 0.15$ mM and an acceleration of 5.3-fold. The acceleration was reproducible and was confirmed by additional experiments at 37° over 24 hr.

Ageing of isopropylmethylphosphonyl-AChE. Ageing is the process by which the phosphonylated enzyme is converted to a non-reactivable form by hydrolysis of the P–O–alkyl bond [31]. Ageing was very slow at 25° and experiments were done, therefore, at 37°, pH 7.0. Under these conditions, $t_{\frac{1}{2}} = 2.5$ hr. None of the compounds tested had a significant effect on this half-life at concentrations at which they interact with the free enzyme with respect to substrate hydrolysis. The following concentrations were investigated: benactyzine, 1 mM; chlorpromazine, 0.1 mM; diazepam, 0.1 mM; galanthamine, 1 μM; mecamylamine, 1 mM; meprobamate, 1 mM; and veratrine, 0.1 mM.

With all compounds studied, the observed value of K_i, the competitive inhibition constant, varied significantly according to the substrate or combination of substrates. It is possible that this is due merely to the fact that different techniques were necessarily used in deriving the various values of K_i, and there are precedents for such occurrences. For example, Rosenberry and Bernhard [32] found K_i, for flaxedil with 1-naphthyl acetate as substrate, to vary with the technique of measurement; K_i was found to be 344 ± 64 μM if substrate hydrolysis was measured on a spectrophotometer, but 98 ± 32 μM if substrate hydrolysis was measured on a pH stat apparatus. The errors quoted above are standard deviation. Nevertheless, it is more likely that the affinity of a ligand for the enzyme active site (the logical prelude to competitive inhibition) is not independent of substrate. Iverson [33] made the same observation in reporting that K_i for the tetraethylammonium ion was 32 μM with diisopropylphosphorylthiocholine as substrate, and 400 μM with neostigmine as substrate. It is also relevant in this context that leaving groups of acetic acid ester substrates and organophosphorus esters bind to different loci at the active site of AChE [34].

The present results also indicate that the presence of two substrates, e.g. acetylthiocholine and physostigmine, modify the binding of a reversible inhibitor with respect to its behavior in the presence of only one substrate. A similar observation, but for different substrates and inhibitor, was made by Rosenberry and Bernard [32].

Chlorpromazine accelerates reactivation of diethylphosphoryl-AChE 5-fold. This could be due to allosteric effects, i.e. chlorpromazine may bind to a peripheral site on the enzyme so as to alter its conformation and thereby increase the rate of aqueous hydrolysis [20, 25]. However, such effects usually, but not always [35], occur only at low ionic strength [25]. Alternatively, chlorpromazine may act by nucleophilic attack by its tertiary amine group on the diethylphosphoryl group. The pK_a of chlorpromazine (9.0; Ref. 36) is favorable for such a nucleophilic attack [37], but we are unaware of any other cases of a tertiary amine increasing the rate of reactivation of phosphorylated AChE (see also Ref. 38). Further, most of the other compounds in the present study also contain tertiary amine groups. Therefore, it is not possible to favor either explanation for the effect of chlorpromazine.

Certainly galanthamine did not display any allosteric properties, since it failed to increase the rate of deacetylation or decarbamylation of AChE at low ionic strength [25].

The beneficial effects of the compound under study in the therapy of organophosphate poisoning would appear to be unrelated to their effects on acetylcholinesterase. Most compounds have little or no effect on reactivation or ageing at concentrations at which they inhibit substrate hydrolysis, and they inhibit the hydrolysis of acetylthiocholine (and presumably also the natural substrate acetylcholine; ref. 13) more than they inhibit the reaction of the enzyme with a carbamate and an organophosphorus compound (where investigated). These effects are likely to be detrimental rather than beneficial *in vivo*. The favorable effect of chlorpromazine on reactivation of diethylphosphoryl-AChE is not a dramatic one, and requires a relatively high concentration of chlorpromazine. It is possible that chlorpromazine and 2-PAM could act synergistically, as has been observed for other combinations of nucleophiles and AChE modifiers [20, 39], and this will be the subject of a separate study.

In summary, benactyzine, chlorpromazine, diazepam, galanthamine, mecamylamine, meprobamate and veratrine have been shown or postulated to increase the effectiveness of oxime–atropine therapy in cases of poisoning by anticholinesterase compounds. All are mixed reversible inhibitors of the system acetylcholinesterase/acetylthiocholine with values of K_i, the competitive inhibition constant, ranging from 5.8 mM (meprobamate) to 0.1 μM (galanthamine). The drugs were less effective as inhibitors of acetylcholinesterase/phospholine or acetylcholinesterase/physostigmine, and less effective again as inhibitors of acetylcholinesterase/physostigmine in the presence of acetylthiocholine. Most drugs had no effect on the rate of reactivation or ageing of carbamylated and phosphorylated enzyme. The exceptions were benactyzine and chlorpromazine, which decreased the rate of reactivation of dimethylcarbamyl-acetylcholinesterase. Chlorpromazine increased the rate of reactivation of diethylphosphoryl-acetylcholinesterase. The most effective inhibitor, galanthamine, showed no signs of possessing allosteric properties at low ionic strength. The beneficial effects of the various drugs *in vivo* are probably unrelated to their effects on acetylcholinesterase.

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Materials Research Laboratories, Defence Science and Technology Organization, Ascot Vale, Victoria 3032, Australia

RAYMOND M. DAWSON
MICHAEL P. BLADEN

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