SHORT COMMUNICATIONS

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Inhibition and reactivation of acetylcholinesterase modified by reaction with 1,1-dimethyl-2-phenylaziridinium ion*

In a previous note[1], we described the modification of the esteratic activity of acetylcholinesterase by reaction with 1,1-dimethyl-2-phenylaziridinium ion (DPA) An increased hydrolysis of indophenyl acetate by this modified cholinesterase (DPA-enzyme) made it possible to examine its reaction with organophosphorus compounds and preliminary results were described This work was continued, and the reactivation of phosphorylated DPA-enzyme was investigated

The acetylcholinesterase used was a purified bovine erythrocyte acetylcholinesterase obtained commercially from Winthrop Laboratories The contents of a vial (nominally 600–800 I U) were dissolved in physiological saline (5 ml) containing bovine serum albumin (40 mg/ml) To prepare DPA-enzyme, an aliquot (1 ml) of the above solution was mixed with a solution of DPA (1 mg) in 0 1 M phosphate buffer (pH 8, 1 ml) and diluted to 10 ml with buffer The solution was allowed to stand 18 h before use Organophosphate solutions were made up at the required concentrations in dimethylformamide These solutions are stable Dimethylformamide in equivalent amounts was added to the blank

Inhibition was studied at 25° by diluting DPA-enzyme solution (0 1 ml) with distilled water (4 8 ml) and adding inhibitor solution (0 1 ml) In most experiments, a solution of 10 μM Tetram (0 1 ml) was added 15 min before the inhibitor to suppress any residual acetylcholinesterase activity After the inhibition, a further dilution followed with the pH 8 phosphate buffer (4 9 ml) and indophenyl acetate (0 1 ml, 0 04 M in alcohol) was added The solution was assayed at 25° against an appropriate blank in a Cary Model 14 spectrophotometer using the method of KRAMER AND GAMSON[2]

Reactivation was studied by treating 1–3 ml of a concentrated solution of DPA-enzyme of known activity with enough concentrated inhibitor solution to destroy all activity in 15–30 min An aliquot (0 1 ml) was diluted with a 0 1 M NaCl–0 04 M $MgCl_2$ solution (8 9 ml) and treated with an aqueous oxime solution (1 ml) to give a total oxime concentration between 1 10^{-3} and 1 10^{-4} M Except where otherwise implied, reactivation was allowed to proceed for 30 min at constant pH, in a Metrohm 30 combititrator, and the solution was assayed with isoamyl acetate (0 1 ml, 10% (w/v) in ethanol) The corrected rate was compared to that previously determined for the same batch of enzyme before inhibition

Quantitative values of the rate constants of inhibition could not be obtained, apparently because of the presence of two or more forms of DPA-enzyme Graphical analysis of the biphasic reaction curves (based on 30–80 points each) gave an order of magnitude for the slower rate constant with tetraethyl pyrophosphate of 1 10^{-4}M^{-1} min^{-1} and 1 10^{-5} M^{-1} min^{-1} with tabun, sarin, and soman The faster reacting component usually represented about one quarter of the enzymatic activity, and its rate constants were an order of magnitude greater, *i e* of the same order as for unmodified

Abbreviations DPA, 1,1-dimethyl-2-phenylaziridinium ion, TMB-4, 1 1-trimethylene-bis-pyridinium-4-aldoxime dibromide

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acetylcholinesterase Neither component is inhibited by $1\ 10^{-5}$ M Tetram at an appreciable rate, excluding the possibility that unmodified enzyme is involved

Many efforts to affect the proportion of the two forms present were unfruitful These included the use of resolved DPA, examining DPA-enzyme produced by partial inhibition and suppression of residual acetylcholinesterase with Tetram, maintaining the DPA concentration roughly constant during inhibition, and treatment to remove one of the two DPA groups shown to be present by B BELLEAU AND V DITULLIO (private communication) Recently, MAIN[3] has shown that such multiphasic reaction curves can be obtained with inhibitors and acetylcholinesterase itself

Other evidence has also been obtained for inhomogeneity of the DPA preparation, including differential reaction rates with neostigmine, and degree of residual activity of DPA-enzyme toward acetylcholine (2-5% of initial acetylcholinesterase activity remains in DPA-enzyme It is refractory to Tetram)

Neostigmine bromide reacted very slowly, if at all, with DPA-enzyme at pH 7 4, but at pH 8 and with $1\ 10^{-5}$ M neostigmine about 50% of the activity was inhibited within one hour after which there was little further change In another experiment, $1\ 10^{-3}$ M neostigmine solution (0 3 ml) was added each hour to a solution of DPA-enzyme (10 ml) The final constant activity after 8 h, corrected for dilution, was 19% of the original activity Solutions of DPA-enzyme inhibited by neostigmine and dialysed did not regain any activity over a period of 8 days On the contrary acetylcholinesterase, inhibited by neostigmine and subsequently treated with DPA, acquired activity slowly over a period of several days, until it reached approximately the full activity (to indophenyl acetate) expected of DPA-enzyme It is to be noted that the final activity is greater than that which would have been expected of acetylcholinesterase itself and that only about 15% of the recovered activity can be inhibited by Tetram This indicates that the major part of the carbamylated acetylcholinesterase has reacted with DPA

Reactivation must be assessed with the very poor substrate isoamyl acetate, because of vigorous reaction of oximes with indophenyl acetate (Removal of the oxime by dialysis or gel filtration was unreliable) It was shown that the uncharged oximes monoisonitrosoacetone and diacetyl monoxime did not reactivate DPA-enzyme, nor did the two common acetylcholinesterase reactivators, 1-methyl pyridinium-2-aldoxime methanesulfonate and 1-methylpyridinium-4-aldoxime iodide However 1,1-trimethylene-bis-pyridinium-4-aldoxime dibromide (TMB-4) consistently reactivated DPA-enzyme inhibited with sarin, soman or tabun to an extent ranging from 25 to 35% of the original activity The corresponding dimethylene analogue of TMB-4 also reactivated, but more slowly than TMB-4, while 1-(3-trimethylammoniopropyl)pyridinium-4-aldoxime dibromide, and 1-benzylpyridinium-2-aldoxime chloride gave small but measurable reactivations under similar conditions About 18-20% of the material is reactivated in 10 min, after which there is a further gain in activity to about 40% of the original value, with incubation for 1 5-2 h, after which little change is noted

The recovered activity could not be removed by incubation with Tetram indicating that the restored activity has the characteristics of DPA-enzyme Also, again in sharp contrast to the behaviour of acetylcholinesterase, there is no loss of reactivatability with time for either sarin- or soman-treated DPA-enzyme

Tetraethyl pyrophosphate-inhibited DPA-enzyme gave only about 4% re-

activation under the same conditions This might be due to reactivation of small amounts of unreacted acetylcholinesterase

Reactivation was studied over the pH range 7–8 in steps of 0 2 pH units The results were rather erratic but in the range of 23–28% reactivation and indicate that pH has no marked effect on reactivation in this range

There is abundant evidence for the inhomogeneity of DPA-enzyme preparations which precludes obtaining quantitative measurements of its characteristics The components could consist of forms with different numbers of DPA molecules, in either of the diastereomeric forms or could be due to inhomogeneity of the initial acetylcholinesterase as postulated by MAIN[3]

The failure of phosphonylated DPA-enzyme to show the "aging" phenomenon (development of resistance to reactivation by oximes) is very interesting BERENDS *et al*[4] have shown that "aging" results from dealkylation of the organophosphorus residue, a reaction which occurs very rapidly with soman, at a moderate rate with sarin, and hardly at all with tetraethyl pyrophosphate It is known to be acid catalysed[5,6] The reaction with DPA has probably added a dimethylamino group near the active centre of the enzymes, which would be protonated at physiological pH Our results could be explained by assuming that a very rapid dealkylation has occurred for one of the sarin- and soman-inhibited species, catalysed by the protonated amine group, and that it is the dealkylated form which is reactivated by TMB-4, assisted by the effective neutralization of the charged oxygen by hydrogen bonding with the same group The undealkylated species are hindered from reactivation by steric shielding of the phosphorus by the alkyl group The pH independence would be ascribed to the approximate equality of the pK's of the oxime, which must be in the anionic form, and the dimethylamino group which must be in the cationic form

An alternative explanation is that protonation of the anionic site of the active centre is responsible for the dealkylation phenomenon Since this site is thought to have been blocked by DPA, aging would not be expected to occur, but one might also expect a difference in reactivatability between soman and sarin for steric reasons This is not observed

Although these speculations could be readily verified by tracer experiments, changing programs make it impossible to continue with these studies in our laboratories

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