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REFERENCES

1. S. G. B. Amyes and J. T. Smith, *Biochem. biophys. Res. Commun.* **58**, 412 (1974).
2. O. Sköld and A. Widh, *J. biol. Chem.* **249**, 4324 (1974).
3. K. H. Pattishall, J. Acar, J. J. Burchall, F. W. Goldstein and R. J. Harvey, *J. biol. Chem.* **252**, 2319 (1977).
4. S. G. B. Amyes and J. T. Smith, *J. gen. Microbiol.* **107**, 263 (1978).
5. M. E. Fling and L. P. Elwell, *J. Bacteriol.* **141**, 779 (1980).
6. M. E. Fling, L. Walton and L. P. Elwell, *Antimicrob. Agents Chemother.* **22**, 882 (1982).
7. S. S. Joyner, M. E. Fling, D. Stone and D. P. Baccanari, *J. biol. Chem.* **259**, 5851 (1984).
8. P. T. Barth, N. Datta, R. W. Hedges and N. J. Grinter, J. Bacteriol. **125**, 800 (1976).
9. H-K. Young and S. G. B. Amyes, *Proceedings of 13th International Congress of Chemotherapy*, Vienna (1983).
10. R. C. Clowes and D. Rowley, *J. gen. Microbiol.* **11**, 250 (1954).
11. N. Datta, R. W. Hedges, E. J. Shaw, R. B. Sykes and M. H. Richmond, *J. Bacteriol.* **108**, 1244 (1971).
12. M. J. Osborn and F. M. Huennekens, *J. biol. Chem.* **233**, 969 (1958).
13. W. J. Waddell, *J. Lab. clin. Med.* **48**, 311 (1956).
14. B. J. Davis, *Ann. N.Y. Acad. Sci.* **121**, 404 (1964).
15. S. G. B. Amyes and J. T. Smith, *Eur. J. Biochem.* **61**, 597 (1976).
16. M. Dixon and E. C. Webb, in *Enzymes* (1st Edn), p. 8. Longmans, Green and Co., London (1958).

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Carbamylated acetylcholinesterase: Acceleration of decarbamylation by bispyridinium oximes

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Experimental animals can be protected against the lethal effects of organophosphorus anticholinesterase compounds by pretreatment with a carbamate (e.g. pyridostigmine) and therapy with atropine [1, 2]. The theory is that comparatively little phosphorylation of acetylcholinesterase (AChE*; EC 3.1.1.7) occurs, since the enzyme is already partially carbamylated by the carbamate. Rapid elimination of the organophosphate from the body is accompanied by spontaneous decarbamylation of the enzyme, so that sufficient active enzyme to maintain normal function is soon present [3]. In the meantime, atropine blocks the muscarinic receptor from the adverse effects of the elevated concentration of acetylcholine [3]. The level of protection afforded by carbamate prophylaxis can be enhanced by including an oxime in the therapy [1]. Presumably the oxime rapidly reactivates any phosphorylated AChE present to regenerate active AChE. However, it is conceivable that the oxime might also accelerate the spontaneous decarbamylation of carbamylated enzyme, with consequent beneficial effects on the outcome of the intoxication. It is therefore surprising that this question of accelerated decarbamylation has received little attention to date. Wilson *et al.* reported in 1960 [4] that 1 mM pyridine-2-aldoxime methyl iodide (2-PAM) had no effect on the rate of decarbamylation of dimethylcarbamyl-AChE from the electric eel. Since then there have been some suggestions that oximes can affect the rate of decarbamylation, but these are based on indirect evidence [5–7]. We have determined the effects of four oximes on the rate of decarbamylation of AChE from four sources, and report our results below. The four oximes are the monopyridinium oxime 2-PAM, the bispyridinium bis-oxime TMB-4 and two isomeric H-oximes, HS-6 and HI-6 [8]. The sources of AChE were electric eel, bovine erythrocytes (both commercial water-soluble powders), human erythrocytes (commercial membrane-bound enzyme) and rabbit brain (Triton–solubilised enzyme). Most experiments were done with dimethylcarbamyl-AChE, but some studies were made of methylcarbamyl-AChE. Both dimethylcarbamates (e.g. pyridostigmine) and methylcarbamates (e.g. physostigmine) are effective prophylactically *in vivo*. Some experiments were also done with two bispyridinium compounds lacking an oxime group. One of these, 1,1′-(1,3-propanediyl) bispyridinium dibromide, is an analogue of TMB-4 with hydrogen atoms instead of oxime groups. The other, SAD-128, is a compound which can protect experimental animals against some toxic effects of the organophosphate Soman, and which appears to interact allosterically with AChE [9].

Methods

Methods for studying the decarbamylation of AChE inhibited by neostigmine (a dimethylcarbamate) have been reported previously [10]. Similar methods were used for physostigmine. Decarbamylation was found to be relatively rapid in the case of eel AChE, and for this enzyme the reactivation was followed to near completion and the rate constant was determined by non-linear regression analysis of the data [11]. Most experiments were done in 2 mM phosphate–150 mM NaCl, pH 7.0, at 25°; in some cases NaCl was omitted. Corrections were made for non-enzymic hydrolysis of substrate.

Eel AChE was obtained from the Worthington Biochemical Corp. and stabilised in solution with gelatin (0.1%, w/v). Soluble bovine erythrocyte AChE (Type XII) and membrane-bound human erythrocyte AChE (Type XIII) were obtained from the Sigma Chemical Co. Whole rabbit brains were homogenised in 10 vol. 0.32 M sucrose at 4°, and the homogenate was centrifuged at 19,000 *g* for

* Abbreviations: AChE, acetylcholinesterase; 2-PAM, pyridine-2-aldoxime methyl iodide; TMB-4, pyridinium, 1,1′-(1,3-propanediyl)bis[4-(hydroxyimino)methyl]dibromide; HS-6, pyridinium, 1-[[[3-(aminocarbonyl)pyridinio]methoxy]methyl]-2-[(hydroxyimino)methyl]diiodide; HI-6, pyridinium, 1-[[[4-(aminocarbonyl)pyridinio]methoxy]methyl]-2-[(hydroxyimino)methyl]dichloride; SAD-128, pyridinium, 1,1′-[oxybis(methylene)]bis[4-(1,1-dimethylethyl)]dichloride; and DMB, 3,3-dimethyl-1-butanol.

Table 1. Rate constants for decarbamylation

Additive	Dimethylcarbamyl-AChE					Methylcarbamyl-AChE
	Eel	Bovine	Bovine (low ionic strength buffer)	Human	Rabbit	Rabbit
None	1.38 ± 0.07 (7)	0.391 ± 0.012 (7)	0.070 ± 0.002 (6)	0.619 ± 0.019 (4)	0.381 ± 0.021 (5)	0.830 ± 0.031 (5)
2-PAM	1.58 ± 0.23 (4)	0.456 ± 0.016* (7)		0.598 ± 0.064 (6)	0.380 ± 0.027 (4)	0.823 ± 0.062 (3)
TMB-4	2.36 ± 0.18* (9)	0.621 ± 0.014* (8)	0.541 ± 0.034* (6)	0.919 ± 0.086* (6)	0.531 ± 0.041* (4)	1.113 ± 0.065* (3)
HS-6	1.35 ± 0.12 (3)	0.473 ± 0.011* (3)		0.620 ± 0.033 (4)	0.638 ± 0.026* (5)	1.367 ± 0.087* (4)
HI-6	1.34 ± 0.07 (3)	0.779 ± 0.017* (8)	0.847 ± 0.024* (6)	1.139 ± 0.080* (3)	1.050 ± 0.061* (4)	3.250 ± 0.325* (5)
HI-6 (10^{-5} M)		0.545 ± 0.007* (3)			0.506 ± 0.033* (6)	1.421 ± 0.098* (3)
SAD-128		0.634 ± 0.031* (3)	0.720 ± 0.009* (3)		0.451 ± 0.025 (3)	
TMB-4 analogue		0.512 ± 0.008* (3)	0.728 ± 0.026* (5)		0.391 ± 0.009 (3)	
Choline	6.19 ± 0.25 (9)	3.83 ± 0.19 (7)	3.30 ± 0.37 (4)	2.78 ± 0.24 (6)		
DMB	8.88 ± 0.56 (10)	0.96 ± 0.04 (9)	0.85 ± 0.06 (6)	2.25 ± 0.19 (4)		
TMB-4 + choline	5.87 ± 0.44 (6)	2.16 ± 0.01 (3)	1.47 ± 0.08 (3)	2.54 ± 0.13 (3)		
TMB-4 + DMB	12.50 ± 0.45 (6)	1.20 ± 0.04 (5)	2.67 ± 0.41 (4)	2.48 ± 0.25 (4)		
HI-6 + choline	4.45 ± 0.31 (3)	1.83 ± 0.02 (3)	1.42 ± 0.02 (3)	2.10 ± 0.25 (6)		
HI-6 + DMB	6.91 ± 0.33 (5)	2.13 ± 0.12 (5)	2.38 ± 0.06 (4)	3.50 ± 0.69 (3)		

Rate constants are given as mean ± S.E.M., with the number of experiments in parentheses. The values are in units of hr^{-1}. Neostigmine and physostigmine were used to produce dimethylcarbamyl- and methylcarbamyl-AChE respectively. Oximes and their analogues were used at a concentration of 10^{-4} M unless indicated otherwise. Choline was used at a concentration of 0.8 to 2.0 mM [10]. 3,3-Dimethyl-1-butanol (DMB) was used at a concentration of 5 mM for eel AChE, or 10–18 mM for AChE from other sources [10]. The low ionic strength buffer comprised 2 mM phosphate, pH 7.0.

* Significantly different from control (first row of figures), $P < 0.05$. This test was not applied to experiments in which choline or DMB was present.

1 hr at 4°. The pellet was homogenised in 10 vol. 0.32 M sucrose–1% Triton X-100 and centrifuged at 110,000 *g* for 1 hr at 5°, after 30 min in the cold room. The Triton-solubilised AChE in the supernatant fraction was partially purified by affinity chromatography [12]. Triton X-100 (0.01% or 0.05%, w/v) was included in the buffer for all decarbamylation experiments with rabbit brain AChE.

The oximes 2-PAM and TMB-4 (pyridinium, 1,1′-(1,3-propanediyl)bis[4-(hydroxyimino)methyl]dibromide) were obtained from K&K Laboratories. The H-oximes HS-6 (pyridinium, 1-[[[3-(aminocarbonyl)pyridinio]methoxy] methyl]-2-[(hydroximino)methyl]diiodide; lot 1-7362) and HI-6 (4-(aminocarbonyl) isomer of HS-6, dichloride salt; batch DRES-32) were gifts from Drs. P. A. Lockwood and J. G. Clement, Defence Research Establishment Suffield, Alberta, Canada. The two non-oxime bispyridinium compounds were gifts from the Chemical Defence Establishment, Porton Down, U.K.

Statistical tests of significance were performed using Student's *t*-test, with $P < 0.05$ as the criterion of difference.

Results and discussion

The oximes were tested at a concentration of 0.1 mM; higher concentrations caused an unacceptably high non-enzymic hydrolysis of the substrate acetylthiocholine [13,14]. Table 1 lists the rate constants obtained from the decarbamylation experiments. 2-PAM did not affect significantly the rate of decarbamylation of dimethylcarbamyl-AChE from eel, consistent with the results of Wilson *et al.* [4]. It slightly increased the corresponding rate constant for bovine erythrocyte AChE, by 17%, but otherwise was without effect in all systems studied. HS-6 had no effect on the rate constant for eel and human erythrocyte AChE, but increased the rate constant for bovine erythrocyte AChE, by 21%, and rabbit brain AChE (67% increase for dimethylcarbamyl-AChE, 65% for methylcarbamyl-AChE). TMB-4 and HI-6 were the most effective of the four oximes tested. TMB-4 accelerated decarbamylation in all cases, as did HI-6 with one exception, namely eel AChE. However, the magnitude of the acceleration produced by HI-6 (1.8- to 3.9-fold at normal ionic strength) exceeded that produced by TMB-4 (1.3- to 1.6-fold). Because of its effectiveness at a concentration of 0.1 mM, HI-6 was also tested at 0.01 mM for three inhibited enzymes—dimethylcarbamyl-bovine erythrocyte AChE, dimethylcarbamyl-rabbit brain AChE, and methylcarbamyl-rabbit brain AChE. Significant increases (39, 33 and 71% respectively) in the rate constant were observed at this concentration (Table 1). The two non-oxime compounds, SAD-128 and the TMB-4 analogue, had no significant effects on decarbamylation for rabbit brain AChE (dimethylcarbamyl-enzyme) but accelerated decarbamylation for bovine erythrocyte AChE at both low and normal ionic strength.

Further experiments were done in which the buffer also included the alcohols choline or 3,3-dimethyl-1-butanol (DMB), for comparison with earlier experiments in this laboratory in which the influence of allosteric effectors of AChE on the rate of decarbamylation of dimethylcarbamyl-AChE from bovine erythrocytes was studied [10, 15–17]. The present results show (Table 1) that TMB-4 or HI-6 reduced the acceleration of decarbamylation produced by choline in all cases. However, TMB-4 or HI-6 potentiated the acceleration of decarbamylation produced by DMB in all cases, with one exception, namely HI-6 + DMB/eel AChE. It will be recalled that HI-6 alone had no effect on eel AChE. The potentiating effect was additive or synergistic in most cases.

The acceleration of decarbamylation in the presence of an oxime could be due either to nucleophilic attack by the oxime group on the carbamyl-enzyme bond, analogous to oxime-catalysed dephosphorylation, or to the compound binding to a peripheral, allosteric site on the enzyme, thereby bringing about a conformational change at the active site with a consequent increase in the rate of spontaneous hydrolysis of the carbamyl group. It is difficult to distinguish between nucleophilic attack and indirect allosteric effects [15]. Nevertheless, the occurrence of allosteric effects in the present case is suggested by two observations. First, acceleration of decarbamylation can be seen in the presence of compounds having structural similarity to TMB-4 but lacking the nucleophilic oxime group. Second, the influence of ionic strength on the rate of decarbamylation in the presence of an oxime, and the effects of a combination of an oxime with choline or DMB, are typical of allosteric effectors of AChE [10, 15–17].

The results in Table 1 suggest that eel AChE, despite its ready availability as a cheap, commercial preparation, is not a suitable model for mammalian erythrocyte and brain AChE. Not only was the control rate of decarbamylation much higher for the eel enzyme than for the other preparations of AChE (row 1 of Table 1), but the studies on eel AChE did not reveal the substantial accelerating properties of HI-6. A similar conclusion concerning eel AChE was reached by de Jong and Wolring in their study on the reactivation of Soman-inhibited AChE by H-oximes [8].

The phenomenon of successful carbamate prophylaxis has yet to be explained fully. For example, it would be expected that pretreatment with a carbamate anticholinesterase would sensitise animals to poisoning by an organophosphate anticholinesterase, in the absence of atropine and/or oxime. Such is not the case [1]. Evidently many factors operate in addition to the temporary inhibition of AChE and the oxime-catalysed dephosphorylation of phosphorylated enzyme [1]. Acceleration of decarbamylation by oximes may well be one of these factors, since there is evidence that therapeutically-effective levels of H-oximes *in vivo* can be as high as 10^{-5} M to 10^{-4} M, and that such oximes can cross the blood–brain barrier [9]. The results above show that some oximes, particularly HI-6, have an appreciable effect on the rate of decarbamylation in this concentration range in blood and brain.

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REFERENCES

1. J. J. Gordon, L. Leadbeater and M. P. Maidment, *Toxic. appl. Pharmac.* **43**, 207 (1978).
2. P. Dirnhuber, M. C. French, D. M. Green, L. Leadbeater and J. A. Stratton, *J. Pharm. Pharmac.* **31**, 295 (1979).
3. A. L. Green, *Biochem. Pharmac.* **32**, 1717 (1983).
4. I. B. Wilson, M. A. Hatch and S. Ginsburg, *J. biol. Chem.* **235**, 2312 (1960).
5. H. Kuhnen, *Biochem. Pharmac.* **21**, 1187 (1972).
6. I. L. Natoff and B. Reiff, *Toxic. appl. Pharmac.* **25**, 569 (1973).
7. H. E. Whiting and J. W. Byron, *Biochem. Pharmac.* **25**, 353 (1976).
8. L. P. A. de Jong and G. Z. Wolring, *Biochem. Pharmac.* **33**, 1119 (1984).
9. G. Amitai, Y. Kloog, D. Balderman and M. Sokolovsky, *Biochem. Pharmac.* **29**, 483 (1980).

10. R. M. Dawson, *J. Neurochem.* **30**, 865 (1978).
11. R. M. Dawson, *Analyt. Biochem.* **131**, 483 (1983).
12. R. M. Dawson and H. D. Crone, *J. Chromat.* **92**, 349 (1974).
13. J. Patocka, J. Bajgar and J. Bielavsky, *Colln Czech. chem. Commun.* **38**, 3685 (1973).
14. V. Simeon, Z. Radic and E. Reiner, *Croat. chem. Acta* **54**, 473 (1981).
15. R. M. Dawson and M. Poretski, *Neurochem. Int.* **5**, 559 (1983).
16. R. M. Dawson, M. Poretski and C. M. Upsher, *Neurochem. Int.* **3**, 405 (1981).
17. R. M. Dawson, H. D. Crone, M. P. Bladen and M. Poretski, *Neurochem. Int.* **3**, 335 (1981).