The Effect of some Amine Oxides and Disulphide Compounds on the Activity of Acetylcholinesterase

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Three amine oxides, two aliphatic and one aromatic, and 2,2'-dithiodipyridine were found to be weak reversible inhibitors of acetylcholinesterase. 5,5'-Dithiobis-(2-nitrobenzoic acid) had no effect on the enzyme under the conditions of the Ellman spectrophotometric assay.

Many studies have been made of the effect of ionic charge on some aspects of the biochemistry of acetylcholinesterase (EC 3.1.1.7; O'Brien, 1971). Amine oxides are an interesting class of compounds in this context, because the N→O bond is highly polarized, but the NO group does not possess a net electrical charge. Many amine oxides are also of significant biological interest (Bickel, 1969), but few quantitative results have been published on the effect of amine oxides on cholinesterases. This paper reports on the nature of the inhibition of bovine erythrocyte acetylcholinesterase by an aliphatic amine oxide (tri-*n*-butylamine oxide), a phenyl-substituted aliphatic amine oxide (*NN*-dimethylaniline *N*-oxide) and an aromatic amine oxide (pyridine *N*-oxide).

Enzymic activity was measured by the spectrophotometric method of Ellman *et al.* (1961), in which the hydrolysis of acetylthiocholine in the presence of 5,5'-dithiobis-(2-nitrobenzoic acid) yields the coloured 5-thio-2-nitrobenzoate ion. Augustinsson & Eriksson (1974) showed that 5,5'-dithiobis-(2-nitrobenzoic acid) does not inhibit acetylcholinesterase under the assay conditions, contrary to an earlier report by Brownson & Watts (1973). In both of these reports, modifications of the Ellman *et al.* (1961) spectrophotometric assay were used, and in addition the later study used a manometric assay to check the results. In the present work assays of acetylcholinesterase in the presence of a disulphide were performed by the pH-stat method, and the results were found to confirm those of Augustinsson & Eriksson (1974). The nature of the inhibition of acetylcholinesterase by 2,2'-dithiodipyridine (aldrithiol-2), suggested by Brownson & Watts (1973) as an alternative chromogenic reagent, was also investigated by the pH-stat assay.

Experimental

Pyridine *N*-oxide (Fluka A.G., Buchs, Switzerland), 5,5'-dithiobis-(2-nitrobenzoic acid) (Sigma Chemical

Co., St. Louis, Mo., U.S.A.) and aldrithiol-2 (Aldrich Chemical Co., Milwaukee, Wis., U.S.A.) were obtained commercially. Bovine erythrocyte and electric eel acetylcholinesterases were obtained from Sigma and Worthington Biochemical Corp., Freehold, N.J., U.S.A., respectively. Tri-*n*-butylamine oxide and *NN*-dimethylaniline *N*-oxide were prepared from the parent amines by published procedures (Ferris *et al.*, 1968; Craig & Purushothaman, 1970). The purity of each amine oxide was checked by proton- and ¹³C-magnetic-resonance spectroscopy and by t.l.c.

pH-stat assay. The hydrolysis of acetylcholine or acetylthiocholine (0.05–0.80 mM) was studied with a Radiometer apparatus at pH 7.4, 30°C, in an N₂ atmosphere and in a medium of ionic strength 0.13 M (NaCl; total volume, 25.0 ml). These conditions correspond to those of Brownson & Watts (1973) and Augustinsson & Eriksson (1974). Aldrithiol-2 was added to the assay medium as a solution (87.5 mM) in 0.2 M-HCl, and 5,5'-dithiobis-(2-nitrobenzoic acid) was added as a solution (8.75 mM) in 2.5 M-sodium acetate, pH 6. The latter solution was prepared by careful addition of dilute NaOH to a stirred suspension of 5,5'-dithiobis-(2-nitrobenzoic acid) in 5 M-sodium acetate, to avoid base-induced decomposition of the disulphide (Danehy *et al.*, 1971). Reactions were initiated by addition of acetylcholinesterase (100 μl) and the titrant was 2 mM-NaOH. Assays were corrected for non-enzymic acid production.

The results of assays in the presence of 5,5'-dithiobis-(2-nitrobenzoic acid), with acetylthiocholine as substrate, were corrected for the acidity of a product of the reaction, 2-nitro-5-mercaptopbenzoate ion (p*K*_a, thiol group, 4.4–4.8; Danehy *et al.*, 1971; Little & Brocklehurst, 1972). Results of assays in the absence of a disulphide, with acetylthiocholine as substrate, were corrected for the partial dissociation of the thiol group of thiocholine at pH 7.4 (Krupka, 1964). No corrections to the experimental results were required for the hydrolysis of acetylthiocholine in the presence of aldrithiol-2 (Albert & Barlin, 1959; Brocklehurst & Little, 1972).

Spectrophotometric assays. Assays were performed with a Unicam SP.600 spectrophotometer on solutions in 0.1M-sodium phosphate, pH8.0 (total volume, 3.15 ml) at 20°C (Ellman *et al.*, 1961).

Calculations. In most cases, values of $V_{\max}$ (maximum velocity) and K_m (apparent Michaelis constant) were determined for a set of assays ($n = 5-6$) by a direct linear plot (Eisenthal & Cornish-Bowden, 1974). Values of K_i (competitive inhibition constant) and K'_i (uncompetitive inhibition constant) were then calculated as the ratio of the intercept to the slope of plots $K_m/V_{\max}$ and $1/V_{\max}$, respectively against inhibitor concentration (Rosenberry & Bernhard, 1972). The intercept and slope were determined in each case by simple linear regression. The maximum concentration of inhibitor studied varied from $1.7 K_i$ to $3 K_i$.

Results and discussion

Inhibition of enzyme activity was observed to be independent of time in all cases, indicating that the inhibition was reversible.

Effect of amine oxides on acetylcholinesterase. The amine oxides were found to be very weak inhibitors of acetylcholinesterase. The inhibition was predominantly competitive in nature, and the uncompetitive component was either comparatively small or undetectable. The inhibition constants at pH8.0 and 20°C are given for each compound in Table 1. At pH8.0 the amine oxides are essentially unprotonated (Ochiai, 1967). The same competitive inhibition constant (K_i) was found, within experimental error, for each amine oxide, despite their structural dissimilarity. Evidently the presence of an N→O group substantially diminishes the affinity of a compound for the enzyme. Thus the value of K_i for tri-*n*-butylamine oxide is approx. 100 times that for the protonated parent amine (tri-*n*-butylamine hydrochloride; see Table 1). The difference in the value of the uncompetitive inhibition constant (K'_i) is even greater. The large difference in the values of K_i for

2,2'-dithiodipyridine (0.5 mM; see below) and pyridine *N*-oxide (24 mM) also illustrates the effect of the N→O group, notwithstanding the difference in assay conditions and the fact that the structures of the two compounds are not strictly analogous.

The results above are consistent with those of Cannon *et al.* (1971), who found that *N*-oxidation of acetylcholine and related compounds greatly decreased their cholinergic activity. A possible explanation of the results is that the attractive forces between the electrophilic nitrogen atom of an amine oxide and the anionic site(s) of acetylcholinesterase are countered by the presence of the adjacent negatively charged oxygen atom. Amine oxide-type compounds do not appear to have the potential to be efficient regulators of acetylcholinesterase activity.

Effect of disulphides on acetylcholinesterase. To evaluate statistically the effect of 5,5'-dithiobis-(2-nitrobenzoic acid) on acetylcholinesterase, the relevant values of K_m and $V_{\max}$ and their standard errors were determined by the method of Wilkinson (1961) (see Table 2). When comparing two sets of results, steps were taken to minimize systematic errors by alternating assays under the two conditions.

The values of K_m and $V_{\max}$ for acetylthiocholine in the presence of 0.35 mM-5,5'-dithiobis-(2-nitrobenzoic acid) were indistinguishable from the corresponding values in its absence. This concentration of 5,5'-dithiobis-(2-nitrobenzoic acid) corresponds to that present in the spectrophotometric assay medium (Ellman *et al.*, 1961) and is the same as that studied by Brownson & Watts (1973) and Augustinsson & Eriksson (1974). With acetylcholine as substrate the results showed $V_{\max}$ (but not K_m) in the presence of 0.35 mM-5,5'-dithiobis-(2-nitrobenzoic acid) to be statistically lower ($P = 0.01-0.05$) than in its absence. However, the values in Table 2 illustrate that the difference is minimal. Certainly no activation of the enzyme by 5,5'-dithiobis-(2-nitrobenzoic acid) was observed (Brownson & Watts, 1973). The hydrolysis of acetylcholine in the presence and absence of 0.35 mM-5,5'-dithiobis-(2-nitrobenzoic

Table 1. Inhibition of bovine erythrocyte acetylcholinesterase by various compounds

Each value is accompanied by 95% confidence limits ($n = 5-6$) (Lark *et al.*, 1968). K_i = competitive inhibition constant; K'_i = uncompetitive inhibition constant (Rosenberry & Bernhard, 1972). N.A., Not available (slope of graph of $1/V_{\max}$ versus inhibitor concentration was statistically indistinguishable from zero). The inhibitors were as follows: aldrithiol-2, 2,2'-dithiodipyridine; Bu_3NO , tri-*n*-butylamine oxide; $(\text{Bu}_3\text{NH})^+$, tri-*n*-butylamine hydrochloride; PhNMe_2O , *NN*-dimethylaniline *N*-oxide.

Inhibitor	Substrate	Assay method	K_i (mM)	K'_i (mM)
Aldrithiol-2	Acetylcholine	pH-stat	0.49 ± 0.10	4.7 ± 3.2
Aldrithiol-2	Acetylcholine	pH-stat	0.47 ± 0.21	N.A.
Bu_3NO	Acetylthiocholine	Spectrophotometric	31.3 ± 19.4	85.1 ± 21.6
$(\text{Bu}_3\text{NH})^+$	Acetylthiocholine	Spectrophotometric	0.36 ± 0.26	0.34 ± 0.09
PhNMe_2O	Acetylthiocholine	Spectrophotometric	26.6 ± 9.0	N.A.
Pyridine <i>N</i> -oxide	Acetylthiocholine	Spectrophotometric	24.2 ± 5.2	261 ± 175

Table 2. *Effect of 5,5'-dithiobis-(2-nitrobenzoic acid) (0.35 mM) on the kinetic constants of bovine erythrocyte acetylcholinesterase at pH 7.4, 20°C, 1 0.13 mol/litre*Each value is accompanied by 95% confidence limits ($n = 5-7$) (Wilkinson, 1961).

Substrate	Additive	$V_{\max}$. ($\mu\text{mol/min per mg}$)	K_m (μM)
Acetylthiocholine	5,5'-Dithiobis-(2-nitrobenzoic acid)	2.33 ± 0.14	75 ± 16
Acetylthiocholine	None	2.32 ± 0.24	82 ± 27
Acetylcholine	5,5'-Dithiobis-(2-nitrobenzoic acid)	2.43 ± 0.09	122 ± 13
Acetylcholine	None	2.54 ± 0.07	132 ± 9

acid) was also studied with electric-eel acetylcholinesterase and crude Triton-solubilized ox brain acetylcholinesterase (Dawson & Crone, 1974). The disulphide had no significant effect on K_m or $V_{\max}$. in either case.

Constants for the inhibition of acetylcholinesterase by aldrithiol-2 (Augustinsson & Eriksson, 1974) are given in Table 1. As expected, the same competitive inhibition constant was obtained whether the substrate was acetylthiocholine or acetylcholine.

The results above, obtained by the pH-stat assay of acetylcholinesterase, are consistent with the results of Augustinsson & Eriksson (1974) and are in direct contrast with the conclusions of Brownson & Watts (1973). In view of the widespread use of the Ellman *et al.* (1961) spectrophotometric assay, the above confirmation of the results of Augustinsson & Eriksson (1974) by a different experimental method is considered to be important.

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