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Anticholinesterase Activity of Ethyleneimines and certain other Cytotoxic Agents

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It has previously been shown by Bullock (1955) that cytotoxic activity and anticholinesterase activity do not run parallel. Indeed certain epoxides and methanesulphonyloxy derivatives possessing cytotoxic activity were almost inactive as anticholinesterases. On the other hand, three ethyleneimines inhibited acetylcholinesterase (AChE) of nervous tissue, but more feebly than did *NN*-di-(2-chloroethyl)methylamine hydrochloride (HN2). Unlike HN2 they inhibited pseudocholinesterase of serum more strongly than AChE. In most cases the inhibition was progressive, the progress being in some cases stopped by the addition of the substrate acetylcholine. At the time it was concluded that the most interesting feature of the results might well be that these cytotoxic agents, designed to resemble the nitrogen mustards and their breakdown products, differ from HN2 in being more feeble inhibitors of AChE and in showing their greatest activity against pseudocholinesterase. Four considerations have led to the subject being pursued further: (1) Most of the ethyleneimines and especially 2:4:6-tri(ethyleneimino)-s-triazine (tretamine, TEM), a substance at present undergoing clinical trial in the treatment of malignant disease, are much more active against AChE at pH 6.3 than at 7.4, the pH customarily employed in Warburg determinations. (2) The ethyleneimines are irreversible progressive inhibitors, so that their inhibitory effects may be more important than if the reverse had been true. (3) At pH 6.3 the anti-AChE activity of TEM is so greatly enhanced that the kinetics of the inhibition can reasonably be studied. (4) The study of the anti-AChE activity of the ethyleneimines might throw some light on the

mode of their reaction with proteins. The epoxides and methanesulphonyloxy derivatives were examined for activity at pH 6.3. Diisopropyl phosphorofluoridate (DFP, an irreversible inhibitor, and neostigmine and eserine, two reversible inhibitors of AChE, were included in the tests for comparison.

Among other attempts which have been made to study the actions of cytotoxic agents on proteins may be mentioned those of Alexander, Fox, Stacey & Smith (1952) and Watkins & Wormall (1952). The general chemistry of the cytotoxic alkylating agents has been discussed by Ross (1953) and their behaviour as chemotherapeutic agents in malignant disease has been reviewed by Bergel (1955).

EXPERIMENTAL

Materials. Epichlorohydrin, propylene oxide, DFP, TEM, *NL*- β -acetoxypropyltrimethylammonium chloride (MeCh, methacholine chloride, B.P. 1953) and di-(2-chloroethyl)methylamine (HN2, mustine hydrochloride B.P.C. 1954) were purchased. The other substances examined for anti-AChE activity were very kindly supplied by Dr W. C. J. Ross, Mr J. L. Everratt and Mr G. M. Timmis of the Chester Beatty Cancer Research Institute, London.

pH values. These were determined by a glass electrode and valve potentiometer, except that the value of 7.4 was assumed for the contents of the Warburg flasks containing 0.025 M-NaHCO₃ after equilibration with N₂ + CO₂ (19:1).

Solutions of inhibitors. These were freshly prepared. 4-[*p*-Di-(2-chloroethyl)aminophenyl]butyric acid (CB1348) was dissolved in a slight excess of 0.1 N-NaOH and the pH reduced to 7.4 with 0.1 N-HCl. Sparingly soluble substances (methanesulphonyloxy derivatives) were directly weighed into the Warburg flask or triturated with the required amount of water, or dissolved in a minimum of ethanol.

Erythrocyte envelopes (stromata). As a source of AChE these were prepared by the method of Augustinsson (1948) with slight modifications. Oxalated (0.14% of $K_2C_2O_4 \cdot H_2O$) ox blood was centrifuged, the erythrocytes were washed by centrifuging three times with aqueous 0.94% (w/v) NaCl and then haemolysed by addition of an equal volume of water. This stock solution was preserved with 0.3% (v/v) $CHCl_3$, and stored at 4°. When required, 30 ml. was diluted with water to 110 ml., the pH was reduced to 6.3 by addition of 0.1 N-HCl and the coagulated stromata were deposited by centrifuging. They were washed by centrifuging with water three times and the final deposit was suspended in 25 ml. of water saturated with $CHCl_3$. Such a suspension may be kept for several days at 4° without appreciable loss of activity. This suspension of stromata showed some buffering capacity; 2 ml. required 0.2 ml. of 0.005 N-NaOH to raise the pH to 7.4, and 0.5 ml. of 0.005 N-HCl to lower the pH to 4.0. The enzyme was in a completely insoluble form at pH 6.3. Less than 5% loss in activity resulted from washing four times with water. Preservation of the stromata by drying over P_2O_5 under reduced pressure caused no appreciable loss in activity or alteration in behaviour with inhibitors. Suspensions of the dried stromata in water or in chloroform water lost strength at the same rate on storage at 4° or 37°.

Stromata rather than washed red blood corpuscles were used because: (1) they constitute a purer form of protein and avoid complications resulting from slow diffusion of the inhibitor into and out of the cells; (2) AChE is more strongly inhibited by TEM at pH 6.3, the pH of the stromata suspension, than at pH 7.4, the more natural pH for red blood corpuscles; (3) considerations of isotonicity and haemolysis do not arise; (4) a centrifuged suspension of stromata forms a compact deposit from which the supernatant fluid may easily be drained off.

AChE activity was determined by the usual Warburg technique at 37° in the presence of 0.025 M- $NaHCO_3$, equilibrated for 10 min. with $N_2 + CO_2$ (19:1). The substrate, 0.2 ml. of 0.9 M MeCh in 0.025 M- $NaHCO_3$, was added from the side arm after equilibration for 15 min. The total volume in each flask was 3 ml. As far as possible enzyme quantities causing the evolution of 60–120 μ l. of CO_2 in 30 min. were used. Esterase activity was calculated from the initial, approximately constant, rate of evolution of CO_2 . In all experiments corrections for non-enzymic hydrolysis of the substrate were applied, changes of gas volume not due to hydrolysis of the substrate being taken into account; both corrections were very small. Results quoted are the average of at least two experiments.

Inhibitions. These were carried out in separate glass-stoppered tubes or centrifuge tubes, whose contents were thoroughly mixed at 10 min. intervals. For inhibition in the absence of $NaHCO_3$ (i.e. at pH 6.3) 1 ml. of stromata suspension was added to either 2 ml. of water or 1 ml. of water and 1 ml. of inhibitor solution. For inhibition in the presence of $NaHCO_3$, 1 ml. of stromata suspension and 1 ml. of 0.075 M- $NaHCO_3$ were mixed with 1 ml. of water or 1 ml. of inhibitor solution. At the end of the inhibitory period, unless otherwise stated at 37°, 1.4 ml. of the reaction mixture was mixed in the Warburg flask with 1.4 ml. of $NaHCO_3$ solution so as to give a final concentration of 0.025 M. In the centrifuge tubes the insoluble enzyme could be centrifuged off and repeatedly washed and incubated with water before being made up to the original volume and

tested. Usually inhibition continued, at pH 7.4, after transfer to the Warburg flask, but with some dilution of both enzyme and inhibitor. During the gassing, temperature equilibration and digestion process there was opportunity for a new equilibrium to be set up. Thus the method is not to be recommended for use with reversible inhibitors.

The pH values were checked before the period of inhibition. The value did not usually change by more than 0.2 of a unit during inhibition, except when the pH had been made other than 6.3 or 8.3 (see below). The pH in the Warburg flasks (7.4) was roughly checked at the end of each experiment by addition of a drop of phenol red to each flask.

RESULTS

Effect of pH on inhibition. The anti-AChE activities of the substances examined are shown in Table 1. The eserine results do not conflict with those of Wilson & Bergmann (1950) since, as pointed out above, the conditions in the present investigation were not suitable for judging pH effects on rapidly reversible inhibitors; experiments with *S*-mustard were difficult because of its slight solubility and rapid hydrolysis in water. In experiments in the absence of $NaHCO_3$ the acid produced by hydrolysis soon lowered the pH to such an extent that the enzyme was rapidly destroyed at 37°. Results in the presence of alkali were obtained by dissolving 1 drop of the *S*-mustard in 1 ml. of ethanol and using 0.05 ml. of this solution in 3 ml. of a suspension of stromata in 0.025 M- $NaHCO_3$; allowance was made for the acid generated when transferring to the Warburg flask. According to Bergmann & Shimoni (1953) 1% ethanol itself causes approximately 20% inhibition. The results show that *S*-mustard could not be regarded as a potent inhibitor of AChE.

The effect of pH on the inhibition produced by HN 2 and TEM was further investigated, with the results shown in Fig. 1. It was immaterial whether 0.005 N-NaOH or 0.025 M- $NaHCO_3$ was used as the alkali in adjusting the pH.

Irreversibility of inhibition. The results obtained are shown in Table 2.

Progressive inhibition of AChE by TEM. TEM has a markedly progressive inhibitory action on AChE at pH 6.3, as shown in Fig. 2.

Minimum concentration of TEM causing 50% inhibition (I_{50}) of AChE at pH 6.3. The minimum I_{50} value for TEM obtainable at 37° was 8.2×10^{-4} M and occurred after 5 hr. inhibition. I_{50} values between 3.0×10^{-4} and 4.0×10^{-4} M were obtained at 30° after 3 days' inhibition and at room temperature after 2 weeks' inhibition. These experiments were carried out in the absence and in the presence of chloroform water of various strengths (Gage, 1953), Merthiolate (Davison, 1953), thymol and potassium cyanide (Aldridge & Davison, 1952), but under all conditions the lowest concentration of TEM causing 50% inhibition was 3.0×10^{-4} M.

Small uptake of TEM by stromata at pH 6.3. Identical results were obtained, whether during the period of inhibition at pH 6.3 by TEM the tubes were: (1) not shaken after the first mixing, (2) shaken at 10 min. intervals, (3) mechanically shaken all the time, (4) centrifuged after the first mixing and not again shaken until the end of the period of inhibition, or (5) centrifuged after the first mixing and the supernatant discarded before the period of inhibition (the original volume was restored by addition of water after inhibition). That this was due to a small uptake of TEM was shown by adding an equal volume of double-strength inhibitor to the moist deposit of stromata obtained by centrifuging. The same degree of inhibition was

obtained as by treating the same quantity of stromata with single-strength inhibitor. If, on the other hand, the deposit of stromata was treated with an equal volume of double-strength inhibitor and immediately largely diluted with water, no inhibition resulted on incubation; this showed that inhibitor had not been irreversibly taken up by the stromata.

Action of stromata on TEM. TEM solution was incubated with stromata for 3 hr. at 37° and then centrifuged; the supernatant had not lost any of its inhibitory power against the AChE of a fresh preparation of stromata.

Stromata prepared from whole human blood behaved similarly to ox stromata.

Table 1. *Inhibition of acetylcholinesterase activity of erythrocyte stroma*

Stromata were kept in contact at 37° with the inhibitor at the pH of the stromata preparation (6.3) or in the presence of 0.025 M-NaHCO₃ (pH 8.3). Determination of residual activity by Warburg method (pH 7.4).

Inhibitor	Formula $\left(R = -N \begin{array}{c} \diagup CH_2 \\ \diagdown CH_2 \end{array} \right)$	Molarity	Time of contact (hr.)	Inhibition (%) at pH	
				6.3	8.3
Nitrogen mustards					
HN 2.	CH ₃ .N(CH ₂ .CH ₂ Cl) ₂ .HCl	3.5 × 10 ⁻⁵	2	18	67
CB 1348	(CH ₂ Cl.CH ₂) ₂ N.C ₆ H ₄ .[CH ₂] ₃ .CO ₂ H	7.8 × 10 ⁻⁴	3	63	37
Ethyleneimines					
TEM	$\begin{array}{c} N-C(R)=N \\ R.C \quad N=C.R \end{array}$	3.3 × 10 ⁻³	3	69	5
TEM*	—	3.0 × 10 ⁻³	3	62	0
CB 1263	R.CO.NH.[CH ₂] ₆ .NH.CO.R	6.6 × 10 ⁻⁴	3	80	25
CB 1289	$\begin{array}{c} N-C(R)=N \\ R.C \quad N=C.Cl \end{array}$	5.7 × 10 ⁻⁴	3	71	45
Epoxides					
Epichlorohydrin	$\begin{array}{c} O \\ \diagup \quad \diagdown \\ ClCH_2 \quad CH_2 \end{array}$	1.15 × 10 ⁻¹	3	73	39
		5.8 × 10 ⁻²	3	38	11
Propylene oxide	$\begin{array}{c} O \\ \diagup \quad \diagdown \\ CH_3 \quad CH_2 \end{array}$	1.6 × 10 ⁻¹	3	49	14
		9.5 × 10 ⁻²	3	33	17
CB 1262	$\begin{array}{c} O \quad O \\ \diagup \quad \diagdown \quad \diagup \quad \diagdown \\ CH_2 \quad CH_2 \quad CH_2 \quad CH_2 \end{array}$	1.05 × 10 ⁻¹	2.5	27	13
CB 1161	$\begin{array}{c} O \quad O \quad O \\ \diagup \quad \diagdown \quad \diagup \quad \diagdown \quad \diagup \quad \diagdown \\ CH_2 \quad CH_2 \quad CH_2 \quad O \quad CH_2 \quad CH_2 \end{array}$	—	—	—	—
Methanesulphonyloxy derivatives					
CB 2039	CH ₃ .SO ₂ .O.[CH ₂] ₂ .O.SO ₂ .CH ₃	2.6 × 10 ⁻²	2.5	0	0
CB 2040	CH ₃ .SO ₂ .O.[CH ₂] ₃ .O.SO ₂ .CH ₃	2.1 × 10 ⁻²	2.5	17	13
CB 2041	CH ₃ .SO ₂ .O.[CH ₂] ₄ .O.SO ₂ .CH ₃	6.77 × 10 ⁻³	3	21	0
Miscellaneous					
Eserine sulphate	—	7.35 × 10 ⁻⁷	2	68	75
Neostigmine methylsulphate	—	3.3 × 10 ⁻⁸	3	58	54
DFP	—	6.9 × 10 ⁻⁸	2.5	53	68
		6.0 × 10 ⁻⁸	2	37	42
S-Mustard	CH ₂ Cl.CH ₂ .S.CH ₂ .CH ₂ Cl	1.3 × 10 ⁻³	3	—	51

* 1.35 × 10⁻² M TEM kept at pH 8.3 (0.025 M-NaHCO₃) for 3 hr. and neutralized with 0.1 N-HCl before test.

Fig. 1. Variation with pH of the percentage inhibition of erythrocyte acetylcholinesterase by: x, 6.9×10^{-5} M nitrogen mustard in 2.5 hr., and ●, 3.0×10^{-3} M TEM in 3 hr. Stromata and inhibitor were brought to required pH with 0.005 N-HCl or NaOH and kept at 37° for 2.5 or 3 hr. The solution was then neutralized and the residual AChE estimated by the Warburg method. Controls were similarly treated. The pH values quoted are the average of pH (maximum change 0.35 pH unit) before and after the period of contact with inhibitor.

DISCUSSION

The results in Table 1 confirm the inactivity of the epoxides and methanesulphonyloxy derivatives as anticholinesterases. In contradistinction to HN 2 itself all the ethyleneimines and the nitrogen mustard analogue CB 1348 were considerably more active at pH 6.3 than at pH 7.4 (Bullock, 1955) or pH 8.3. With regard to the use of these substances in the treatment of cancer, Voegtlin, Fitch, Kuhler, Johnson & Thompson (1955) observed that cancerous tissue, unlike normal tissue, produces large quantities of lactic acid from fermentable sugars, and that the acid production can be increased by suitable injection of glucose to the point where the pH of the cancerous tissue falls to 6.3.

Fig. 2. Progressive inhibition of erythrocyte acetylcholinesterase at pH 6.3 and 37° caused by 3.46×10^{-3} M TEM. Residual activity was determined by Warburg method (pH 7.4). Controls were similarly treated.

Table 2. *Reversibility of inhibition of acetylcholinesterase of stromata at pH 6.3*

Inhibition at 37°. Stromata were subsequently centrifuged off, washed three times with water and made up with water to original volume. The washing process was at 37° for the same time as the contact with inhibitor. Controls were similarly treated. AChE activity was determined by Warburg method (pH 7.4).

Inhibitor	Molarity	Time of contact with inhibitor (hr.)	Inhibition (%)	
			Before washing	After washing
HN 2	6.9×10^{-4}	2	60	32
	6.9×10^{-2}	2	100	100
	1.1×10^{-2}	2	82	80
	3.3×10^{-3}	3	64	68
CB 1348	1.3×10^{-3}	2	60	60
CB 1263	1.0×10^{-3}	1.5	86	87
	6.8×10^{-4}	2	80	79
CB 1289	8.5×10^{-4}	2	71	72
Eserine sulphate	1.0×10^{-5}	0.5	96	29
	4.68×10^{-6}	2	86	0
Neostigmine methylsulphate	2.0×10^{-6}	0.5	84	10
DFP	4.16×10^{-7}	2.5	62	58

The lower inhibitory activity of TEM at pH 8.3 was not due to its decomposition in the alkaline solution (Table 1, TEM*). Fig. 1 shows that, although the activity of the HN 2 steadily increased with increasing pH, TEM appeared to exert its optimum inhibitory activity at about pH 6.3. The data in Table 2 show that the ethyleneimines are irreversible inhibitors of AChE. That this was not specifically due to the use of stromata at pH 6.3 is shown by the reversibility of the inhibition induced by eserine and neostigmine at that pH.

Fig. 2 shows that TEM brings about a progressive inhibition of AChE. The inhibition is irreversible and the lowest concentration of TEM which would cause 50% inhibition in an unlimited time was 3.0×10^{-4} M. A limit was placed on the reduction in concentration, since TEM decomposes slowly in aqueous solution and AChE is not so stable at pH 6.3 as at pH 7.4 or 8.3. A very small volume of TEM solution is as effective as a large volume in bringing about inhibition of the AChE of stromata, indicating a small uptake of TEM by the stromata. The rate of decomposition of TEM in solution was not appreciably accelerated in the presence of stromata. No difference was found in the action of TEM on ox or human stromata, and it was immaterial whether the stromata were fresh, preserved with chloroform water or previously freeze-dried.

SUMMARY

1. A convenient method of examining drugs for antiacetylcholinesterase activity at pH 6.3 by the

use of insoluble erythrocyte envelopes has been developed.

2. The inactivity of the epoxides and methane-sulphonyloxy derivatives as anticholinesterases has been confirmed.

3. The ethyleneimines and an HN 2 derivative show greatly increased anticholinesterase activity at pH 6.3 compared with pH 7.4 or 8.3.

4. Anticholinesterase activity of the ethyleneimines is progressive and irreversible.

5. Only very small quantities of TEM are taken up by erythrocyte stromata.

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A Method for the Determination of Oestriol, Oestrone and Oestradiol-17 β in Human Urine by Partition Chromatography and Colorimetric Estimation

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The purpose of this investigation was to devise a method for the determination in human urine of oestriol, oestrone and oestradiol-17 β , suitable for routine use and of sufficient sensitivity for the determination of the oestrogens excreted during the menstrual cycle. This investigation has been conducted independently of that reported by Brown (1955*a, b*), with whom close liaison was maintained at

all stages. The development of the method began with a review of methods of hydrolysis of urinary oestrogens (Marrian & Bauld, 1951). This was followed by investigation of the colorimetric determination, the column partition chromatography, and the sources of error in extraction of urinary oestrogens (Bauld, 1954, 1955*a, b*).

The final method of determination, described in this report, involves acid hydrolysis of the urine, separation of the ether extract into oestriol and

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