J. Pharm. Pharmac., 1968, **20**, *Suppl.*, 213S–217S Received May 1, 1968

The anti-acetylcholinesterase activities of the alkaloids of *Physostigma venenosum* seeds*

B. ROBINSON AND J. B. ROBINSON

Anti-acetylcholinesterase activities of physostigmine, physovenine, N_a-norphysostigmine, geneserine and eseramine have been investigated *in vitro* using erythrocyte acetylcholinesterase. The former three bases show comparable activities whereas geneserine and eseramine are inactive under the same experimental conditions. The biologically active form of these bases is discussed in light of these enzyme inhibitory studies.

PHYSOSTIGMINE (eserine) (I, R = Me, X = N-Me), the major alkaloid of *Physostigma venenosum* seeds, and a large number of synthetic analogues have been evaluated for anti-acetylcholinesterase activity and the chemical features essential for such activity have been established (Stempel & Aeschlimann, 1956). The structures of the minor alkaloids physovenine (I, R = Me, X = O), N_a-norphysostigmine (I, R = H, X = N-Me), geneserine [I, R = Me, X = (N→O)Me] and eseramine (I, R = Me, X = N-CONHMe) have been established and they have been shown to have the same absolute configurations as physostigmine about the B/C ring junction, which must be *cis*-fused (Robinson, 1968).

The structure-action relations in this related group of compounds have been examined using erythrocyte acetylcholinesterase.

Experimental

Substrate. Acetylcholine perchlorate (B.D.H.—biochemical grade).

Inhibitors. A stock solution of each alkaloid was prepared by dissolving an accurately-weighed quantity (approximately 10 mg) of the alkaloid in 95% ethanol (100 ml). Test solutions were prepared by diluting (1 in 100) with phosphate buffer (pH 7·4; 1×10^{-3}M) (Documenta Geigy, 1962).

Enzyme solution. Erythrocyte acetylcholinesterase (Sigma Chemical Co., London) (250 μM units) was dissolved in a 1% solution of partially-hydrolysed gelatin containing magnesium chloride (0·04M) and sodium chloride (0·05M) (200 ml); this solution was stored at 0° in the dark until required. The enzyme is stable for 3–4 weeks under these conditions.

HYDROLYSIS RATES

Acetylcholine hydrolysis rates were measured by the pH-stat method (Alles & Hawes, 1940) using a Radiometer Automatic Titrator (Type TTT 1c) equipped with a recorder (SBR 2c) and syringe burette (SBU 1a) (Radiometer, Copenhagen). The reactions were carried out at pH 7·4 in a 50 ml jacketed vessel at 25 ± 0·1°, the reaction mixture being stirred and bubbled with carbon dioxide-free nitrogen. Sodium hydroxide (0·02N) was used as the titrant.

The reaction mixture was made up to 0·04M in magnesium chloride

From the Department of Pharmacy, University of Manchester, Manchester, England.

* For the preceding paper in this series see Longmore & Robinson (1966).

and to 0·05M in sodium chloride and contained enzyme, inhibitor and substrate in a total volume of 25 ml of solution.

Reaction mixtures (less inhibitor and substrate) were pre-incubated for 10 min before adjusting the solution to pH 7·4. Inhibitor was added and allowed to equilibrate with the enzyme for varying times (see Table 1) before initiating the hydrolytic reaction by addition of the substrate.

The velocity of the reaction was calculated from the slope of the recording of volume of sodium hydroxide added against time, obtained during the second and third minute of the incubation. The results were plotted in the manner of Lineweaver & Burk (1934) and the values of K_I calculated from the gradients of the resulting plots using the equation:

$$\text{Gradient} = \frac{K_m (1 + [I]/K_I)}{V_{max}}$$

where K_m is the Michaelis constant, K_I is the enzyme-inhibitor dissociation constant, [I] is the concentration of inhibitor and V_{max} is the maximum velocity of the reaction.

The activity of the enzyme was checked at intervals during the experiments and gave a consistent value for K_m of $4{\cdot}4 \times 10^{-4}$ with a V_{max} of $2{\cdot}1 \times 10^{-6}$ M/min.

The calculated values of K_I are shown in Table 1 and a typical set of experimental results is given in Fig. 1a,b.

Discussion

Table 1 shows that physostigmine, physovenine and N_a-norphysostigmine are highly active inhibitors of the enzyme acetylcholinesterase whereas geneserine and eseramine are inactive under the experimental conditions used. These conclusions are in agreement with the results of previous pharmacological studies on the rat diaphragm-phrenic nerve and frog rectus abdominus muscle. In these preparations the activity of physovenine in potentiating the action of acetylcholine is of the same order as physostigmine, whereas that of eseramine is much lower (Robinson, 1968).

TABLE 1. ENZYME-INHIBITOR DISSOCIATION CONSTANTS OF THE ALKALOIDS OF *Physostigma venenosum* SEEDS

Alkaloid	Molar conc. ($\times 10^7$)	Dissociation constants ($\times 10^7$) after pre-incubation of the inhibitor with acetylcholinesterase for:		
		1 min	3 min	10 min
Physostigmine	1·438	5·5*	2·3†	2·5‡
	2·876	—	2·9†	—
Physovenine	1·534	5·4*	4·2†	2·4‡
	3·068	—	3·3‡	—
N_a-Norphysostigmine ..	1·463	4·1*	3·3†	1·9‡
	2·926	—	2·9‡	—
Eseramine	1·25 and 2·50	Inactive at both these molar concentrations		
Geneserine	1·433 and 2·866	Inactive at both these molar concentrations		

* Competitive inhibition. † Mixed inhibition. ‡ Non-competitive inhibition.

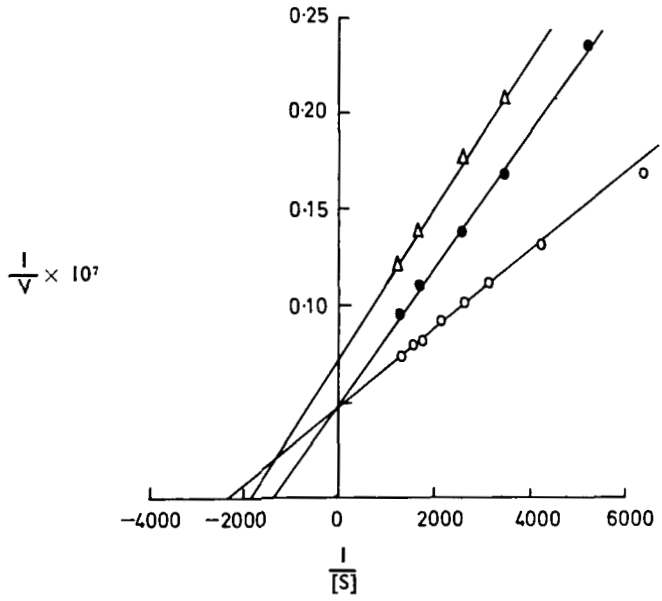


FIG. 1a. Effect of concentration on the kinetics of physostigmine inhibition of acetylcholinesterase, 3 min pre-incubation. —○—○— acetylcholine. —●—●— $1{\cdot}438 \times 10^{-7}$ molar physostigmine. —△—△— $2{\cdot}876 \times 10^{-7}$ molar physostigmine.

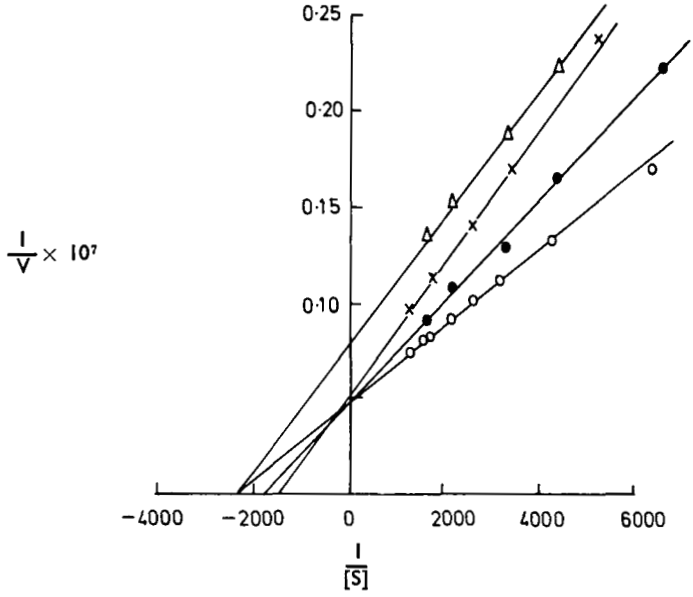


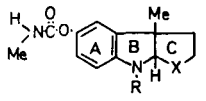
FIG. 1b. Effect of time of pre-incubation on the kinetics of physostigmine inhibition of acetylcholinesterase. Concentration of physostigmine = $1{\cdot}438 \times 10^{-7}$ molar. —●—●— 1 min pre-incubation. —×—×— 3 min pre-incubation. —△—△— 10 min pre-incubation. —○—○— acetylcholine.

Since a decrease in the value of K_I can be taken as an index of increasing inhibitory strength then our results show that the intensity of the inhibition depends on the time of pre-incubation of the inhibitor with the enzyme. Coupled with the increase in inhibitory strength, the kinetics of the inhibition change from competitive to non-competitive, and this may be explained by the formation of a carbamoylated enzyme by transfer of a carbamyl group from the inhibitor as shown below (cf. Main & Hastings, 1966).

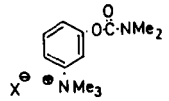
$$E + AcCh \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [E.AcCh] \xrightarrow{k_2} E.Ac + choline \xrightarrow{k_3} E + AcOH \text{ (acetic acid)}$$

$$E + I \underset{k'_{-1}}{\overset{k'_1}{\rightleftharpoons}} [E.I] \xrightarrow{k'_2} E - \textit{N}\text{-Methylcarbamoyl} \xrightarrow{k'_3} E + \textit{N}\text{-Methylcarbamic acid}$$

Thus if k'_3 is much smaller than k_3, we may expect to observe a non-competitive component in the inhibition (Wilson, 1963). Table 1 shows that the three very active alkaloids act initially as competitive inhibitors but during pre-incubation of the inhibitor with the enzyme, some carbamoylation of the enzyme occurs (i.e. these compounds are acting as very poor substrates for the enzyme). It is interesting to note that earlier studies (Nachmansohn, Rothenburg & Feld, 1948; Augustinsson & Nachmansohn, 1949) on the inhibition of acetylcholinesterase by physostigmine and neostigmine (II) report only a competitive inhibition. However, the pre-incubation periods reported by these authors (using enzyme



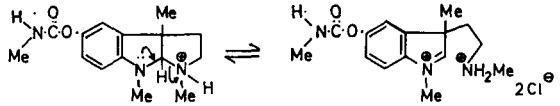
I



II

isolated from electric eel) were up to 150 min, and since later work (Wilson, Harrison & Ginsburg, 1961) showed that the half-life of the methylcarbamoylated acetylcholinesterase is about 38 min, the carbamoylated enzyme would have undergone considerable hydrolytic recovery under these prolonged pre-incubation conditions.

Comparative studies on the effect of the pH on the inhibition of acetylcholinesterase by physostigmine and neostigmine (II) (Wilson & Bergmann, 1950) led to the suggestion that the protonated form (III)



III IV

of physostigmine is the enzyme inhibitor (Nachmansohn & Wilson, 1951). Such a hypothesis will also account for the high anti-acetylcholinesterase activity exhibited by N_a-norphysostigmine and the inactivity (under the

present experimental conditions) of eseramine and geneserine, in which the nitrogen atoms corresponding to N_b (protonated in physostigmine) are non-basic. It does not account, however, for the high inhibitory activity shown by physovenine, in which the physostigmine N_b-Me group is replaced by a relatively non-basic oxygen atom. This may be explained by considering the acid-catalysed opening of ring C which occurs in physostigmine and related compounds. It has been shown on the basis of ultraviolet absorption and proton magnetic resonance studies (Jackson & Smith, 1964) that opening of ring C in physostigmine as shown in (III) takes place in 6N hydrochloric acid to give (IV). We find that both N_a-norphysostigmine and physovenine behave similarly and in 6N-hydrochloric acid undergo opening of ring C to yield the corresponding 3*H*-indolium cations as in (IV), λ_{max} 236, 298, λ_{inf} 241 mμ (ϵ = 6,400, 5,300 and 6,000) and 233, 239 and 284 mμ (ϵ = 6,600, 6,100 and 5,400) respectively. In 6N hydrochloric acid the ultraviolet spectrum of geneserine is indicative of a mixture of 3*H*-indolium cation and indoline chromophores whereas eseramine is fully protonated on N_a under such conditions (indicated by a typical benzenoid absorption). Ring C in both geneserine and eseramine is, however, opened in 11N hydrochloric acid, both alkaloids showing typical 3*H*-indolium cation absorption under these conditions with λ_{max} 238, 242 and 294–5 mμ (ϵ = 5,800, 5,750 and 5,500) and λ_{max} 236, 293–4, λ_{inf} 240 mμ (ϵ = 6,650, 5,500 and 6,300) respectively.

Although the pH values at which such cleavages of ring C in physostigmine, physovenine and N_a-norphysostigmine have been effected are too low to be considered applicable to a biological system, the possibility that analogous enzyme-catalysed reactions occur at the acetylcholinesterase surface is not precluded. It is therefore suggested that the reactive species responsible for the anti-acetylcholinesterase activity of physostigmine is the ring C-opened 3*H*-indolium cation (IV), analogous cations also being the active forms of physovenine and N_a-norphysostigmine.

References

Alles, G. A., & Hawes, R. C. (1940). *J. biol. Chem.*, **133**, 375–390.

Augustinsson, K.-B. & Nachmansohn, D. (1949). *Ibid.*, **179**, 543–559.

Documenta Geigy Scientific Tables (1962). 6th edn, pp. 314–315. Manchester: Geigy Pharmaceuticals Co. Ltd.,

Jackson, A. H. & Smith, A. E. (1964). *J. chem. Soc.*, 5510–5517.

Lineweaver, H. & Burk, D. (1934). *J. Am. chem. Soc.*, **56**, 658–666.

Longmore, R. B. & Robinson, B. (1966). *Chemy Ind.*, 1638–1639.

Main, A. R. & Hastings, F. L. (1966). *Science, N.Y.*, **154**, 400–402.

Nachmansohn, D., Rothenburg, M. A. & Feld, E. A. (1948). *J. biol. Chem.*, **174**, 247–256.

Nachmansohn, D. & Wilson, I. B. (1951). *Advances in Enzymology*, Vol. 12, Editor: Nord, F. F., pp. 259–339. London: Interscience Publishers Ltd.

Robinson, B. (1968). *The Alkaloids*, Vol. 10, Editor: Manske, R. H. F., Ch. 5, pp. 383–400. London & New York: Academic Press, and references therein.

Stempel, A. & Aeschlimann, J. A. (1956). *Medicinal Chemistry*, Editors: Blicke, F. F. & Cox, R. H., Ch. 4, pp. 238–339. London: Chapman & Hall Ltd.

Wilson, I. B. (1963). *Metabolic Inhibitors*, Vol. 2, Editors: Hochster, R. M. & Questel, J. H., pp. 193–203. London and New York: Academic Press.

Wilson, I. B. & Bergmann, F. (1950). *J. biol. Chem.*, **185**, 479–489.

Wilson, I. B., Harrison, M. A. & Ginsburg, S. (1961). *Ibid.*, **236**, 1498–1500.