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Inhibition of acetylcholinesterase by dibenamine and dibenzyline

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Dibenamine and dibenzyline are irreversible inhibitors of acetylcholinesterase (AChE). Kinetic studies show that at pH 9·5 a fast reaction occurs between a group on the enzyme with pK_a 9·1 and the ethyleniminium ion derived from the inhibitor. Either the ϵ-amino-group of a lysine residue is alkylated or else a lysine residue catalyses the alkylation of a non-ionisable group (e.g. hydroxyl). At pH 6·5 there is a slow reaction between a carboxyl anion on the enzyme and the ethyleniminium ion. Studies of the alkylation reactions in the presence of the reversible competitive inhibitor of the enzyme, tetramethylammonium ion, show that alkylation occurs at some distance from the anionic site and probably on the borders of the active site.

The hypothesis concerning the identity of the enzyme acetylcholinesterase (AChE) and the cholinergic (acetylcholine) receptor of mammalian tissues was first proposed by Roepke (1937). Since then an accumulation of conflicting data has cast considerable doubt on its validity (Koelle, 1963; Webb, 1965; Podleski, 1967; Karlin, 1967). We have examined this problem by a comparative study of the reaction between certain alkylating agents and both the enzyme and receptor. This communication is concerned with our findings on the nature of the reactions of dibenamine and dibenzyline with the enzyme.

MATERIALS, METHODS AND RESULTS

Materials

Dibenamine hydrochloride and dibenzyline hydrochloride were recrystallized from isobutanol and had m.p. 187–8° and 136·5–137° respectively. The AChE used was a lyophilized preparation from bovine erythrocytes (Koch—Light Laboratories, 20 000 Schachter units per vial) and solutions were prepared in partially hydrolysed gelatin (2%) containing sodium chloride (0·2M) and stored at 4°. The concentrations of enzyme expressed here as mg refer to the lyophilized preparation. Acetylcholine bromide (BDH, biochemical grade) solutions (4×10^{-2}M) were prepared in sodium chloride (0·2M) and stored at 4°. Fresh solutions of enzyme and substrate were prepared every 3–4 days.

Dissociation constants of dibenamine and dibenzyline

The pK_a values of dibenamine and dibenzyline were determined by Shapiro's method (Shapiro, Isaacs & others, 1962) on solutions of the hydrochloride salts (1×10^{-4}M) in methanol-water mixture (1 : 1) at 25° by titration with alkali using a pH-stat (Radiometer, Copenhagen). The apparent pK_a values were 5·5 and 5·1 respectively.

Inhibition of AChE by dibenamine and dibenzyline

Standard procedure. The enzyme solution (1 ml, 3·6 mg) was added to a solution of the inhibitor (19 ml) in sodium chloride (0·2M). The mixture was mechanically stirred and adjusted to the required pH by the automatic addition of sodium hydroxide solution (20 mM) from a pH-Stat (Radiometer, Copenhagen) or by the dropwise addition of dilute hydrochloric acid to the mixture. The reaction mixture was then incubated in a thermostat bath at $25 \pm 0{\cdot}01°$. It proved extremely difficult even under an atmosphere of nitrogen to maintain the reaction mixture at pH values $> 7{\cdot}5$ due to absorption of atmospheric carbon dioxide each time a sample was withdrawn. A satisfactory method of sampling was devised which used the pressure of nitrogen gas to blow a sample of the mixture from the vessel when required. By this means the pH of the mixture during each experiment was maintained $\not> 0{\cdot}3$ pH unit below the commencing value. The pH values quoted are the means of the initial and final values observed.

Acetylcholine solution (1 ml) was added to a solution of sodium chloride (17 ml, 0·2M) in a jacketed vessel through which water from the thermostat bath circulated. The vessel was connected to the pH-stat and supplied with a continuous flow of dry, carbon dioxide-free nitrogen, the end of the nitrogen tube being placed just above the surface of the solution. Samples (2 ml) of the inhibition reaction mixture were withdrawn at suitable intervals of time and added to the assay vessel. The rate of substrate hydrolysis at pH 7·4 was given by the slope of the trace on the recorder over a period of 5 min. The rate of acid production in the absence of enzyme was negligible.

Irreversible inhibition

A solution of dibenamine hydrochloride ($7{\cdot}5 \times 10^{-5}$M) was incubated with the enzyme at pH 9·5 following the standard procedure. The enzyme activity remaining decreased with time and was eventually zero. The reaction with dibenzyline hydrochloride (1×10^{-4}M) followed a similar course.

Attempted regeneration of inhibited enzyme

Solutions of inhibited enzyme (20 ml) prepared by the standard procedure from either dibenamine or dibenzyline at both pH 5·0 and pH 9·5 were mixed with sodium thiosulphate (1 ml, 1·334M) to remove the alkylating species (*vide infra*). After readjustment of the pH to the initial values the solutions were stored at 25° for 18 h. Control solutions of the enzyme were subjected to the same treatment. There was no change in the enzyme activity of either the control solutions or inhibited enzyme solutions over the 18 h period. Consequently, the inhibition of AChE by dibenamine and dibenzyline is regarded as irreversible for this period of time.

Kinetics of the inhibition reaction

In the general case of a bimolecular reaction between two compounds (A) and (B) the rate of reaction is given by

$$\frac{dx}{dt} = k_2 (a - x)(b - x) \quad \ldots \quad (1)$$

where k_2 is the second-order rate constant, (a) and (b) are the initial concentrations of

(A) and (B) respectively and the concentration of products is (x) at time (t). Integration and rearrangement of equation (1) gives,

$$k_2 = \frac{2{\cdot}303}{t(a-b)} \log \frac{b(a-x)}{a(b-x)}.$$

In the situation where $a \gg b$, this simplifies to,

$$k_2 = \frac{2{\cdot}303}{ta} \log \frac{b}{(b-x)} \qquad \text{(2)}$$

and gives,

$$t = \frac{2{\cdot}303}{k_2 a} \log b - \frac{2{\cdot}303}{k_2 a} \log (b-x),$$

so that a plot of t *vs* log (b − x) for the reaction is linear and the second-order rate constant can be calculated from the slope of the line. However, it is more usual to determine the second-order rate constant from the half life, $t_{0{\cdot}5}$, for the reaction when plotted as a first-order reaction. The first order rate equation on integration gives

$$k_1 = \frac{2{\cdot}303}{t} \log \frac{b}{(b-x)} \qquad \text{(3)}$$

so that for a plot of t *vs* log (b − x),

$$t_{0{\cdot}5} = \frac{2{\cdot}303}{k_1} \log 2,$$

where k_1 is the first order rate constant. In the case of a pseudo unimolecular reaction, k_1 is an apparent first order rate constant and from equations (2) and (3), $k_1 = k_2 a$. The second-order rate constant is then given by the slope of the line for a plot of k_1 *vs* concentration of (a) in a series of experiments where the concentrations of (b) are constant.

Dibenamine hydrochloride over the concentration range $0{\cdot}05 - 2{\cdot}5 \times 10^{-5}$M was incubated with AChE at pH 9·5 following the standard procedure. A similar study was made at pH 6·5 over the concentration range $3{\cdot}75–6{\cdot}25 \times 10^{-5}$M.

At each incubation pH, duplicate control experiments were conducted in which the inhibitor was replaced by sodium chloride (0·2M). There was no change in the enzyme activity of any of the control solutions which showed that AChE is stable at each pH studied during the time of the inhibition studies.

A graph of log (rate of acetylcholine hydrolysis) *vs t* for each inhibition reaction was linear showing that the reaction followed first order kinetics.

The lines of best fit were determined by regression analysis and the apparent first-order rate constants, k_1, calculated. The mean values for k_1 were a linear function of the inhibitor concentration at each pH studied (Fig. 1A) as expected for a pseudo-unimolecular reaction and the lines passed through the origin indicating that the inhibitor does not form a complex with the enzyme before formation of a covalent bond.* The second-order rate constants, k_2, were calculated from the slopes of the graphs and had the values 37·2 and 1093 M^{-1} min^{-1} at pH 6·5 and 9·5 respectively.

Similar results were obtained at pH 6·5 and 9·5 with dibenzyline hydrochloride over the concentration ranges $6{\cdot}0–10{\cdot}0 \times 10^{-5}$M and $2{\cdot}0–6{\cdot}0 \times 10^{-5}$M respectively (Fig. 1B).

* In a preliminary communication (Beddoe & Smith, 1967), complexing was erroneously reported for the dibenamine reaction, attributable to working in excess of the solubility of the base.

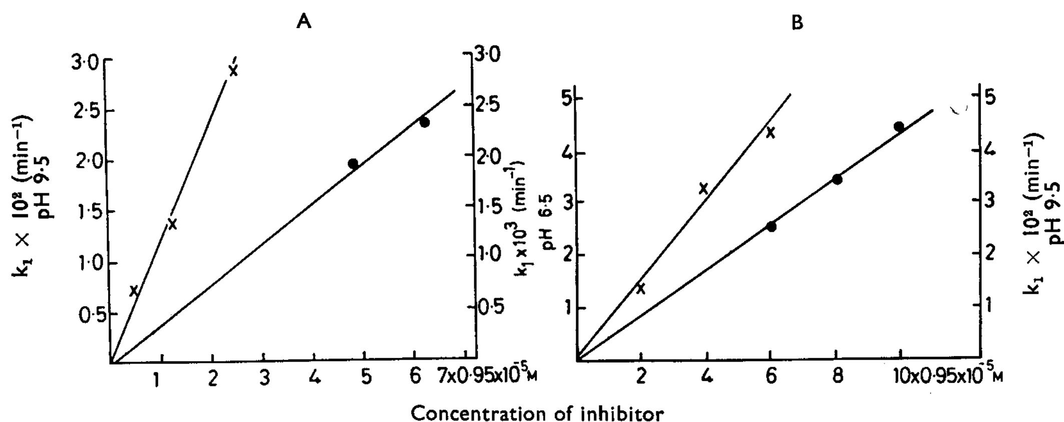


FIG. 1. Inhibition of AChE by dibenamine (A) and dibenzyline (B) at pH 9·5 (x) and pH 6·5 (●) as a function of inhibitor concentration.

The values obtained for the second order rate constants, k_2, at pH 6·5 and 9·5 were 43·5 and 735·4 M^{-1} min^{-1} respectively.

In kinetic studies of the reactions of inhibitors with enzymes the rate constants can vary as much as ± 10% between duplicates (Kézdy, Thomson & Bender, 1967). In this work the coefficient of variance (%) for six determinations at pH 6·5 was 6·1 and for five determinations at pH 9·5 was 8·9%. The coefficient of variance (%) for ten determinations in the enzyme assay alone was 1·18%.

Variation of the concentration of enzyme with constant inhibitor concentration showed that the second-order rate constants for the inhibition reaction were independent of enzyme concentration as expected for a pseudo-unimolecular reaction (Table 1).

Influence of thiosulphate ion on the inhibition reaction

AChE was added to a solution of dibenamine hydrochloride (7·5 × 10^{-5}M) or dibenzyline hydrochloride (1 × 10^{-4}M) in a medium containing sodium thiosulphate [6·67 × 10^{-2}M; ionic strength (I) = 0·2] and the pH was adjusted to 10. The mixture was left at room temperature for 10 min and then the standard inhibition procedure followed. Control experiments were conducted in a similar manner in a medium containing sodium chloride (0·2M). Other experiments were made in a

Table 1. *Influence of AChE concentration on the second-order rate constant for the inhibition reaction*

	Enzyme concentration in inhibition mixture (mg/ml)	Incubation pH	Mean k_2 ($M^{-1}min^{-1}$)
Dibenzyline (1 × 10^{-4}M)	0·18	6·5	48·9
		9·5	435·8
	0·36	6·5	46·9
		9·5	391·4
Dibenamine (7·5 × 10^{-5}M)	0·18	6·5	24·4
		9·5	532·1
	0·36	6·5	22·1
		9·5	534·4

medium containing sodium thiosulphate ($9 \cdot 4 \times 10^{-5}$M) in addition to sodium chloride. The results show that thiosulphate ion prevents the reaction of dibenamine and dibenzyline with AChE.

Influence of ionic strength on the rate of the inhibition reaction

Dibenamine hydrochloride ($7 \cdot 5 \times 10^{-5}$M) and dibenzyline hydrochloride (1×10^{-4}M) were separately incubated with AChE at pH 6·5 and pH 9·5 following the standard procedure but in media of different ionic strengths over the range, $I = 0\text{–}20 \times 10^{-2}$.

A three to four fold increase in the value of the observed second-order rate constant, k, was noted for both inhibitors at pH 6·5 when the ionic strength was decreased from 0·2 to zero (Fig. 2). There was a slight increase (about 8%) in the rate constant when chloride ions were replaced by sulphate ions but this was not regarded as having a special significance. The second-order rate constant at pH 9·5 was independent of

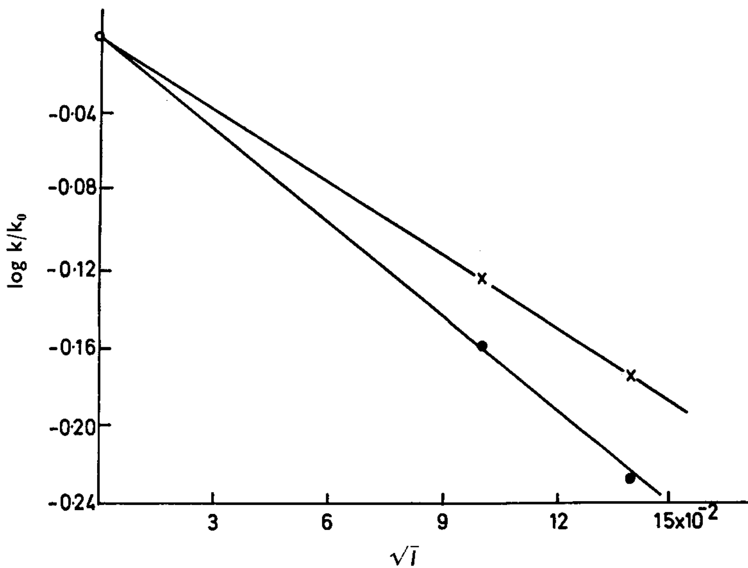


FIG. 2. Influence of ionic strength on the rate of inhibition of AChE by dibenamine (o) and dibenzyline (x) at pH 6·5.

the ionic strength of the medium over the range $I = 0 \cdot 05 - 19 \cdot 05 \times 10^{-2}$ for dibenamine but for dibenzyline the reaction was slowed to half rate at the high ionic strength.

It can be shown (Scatchard, 1932) that for two interacting species,

$$\log k/k_0 = 1 \cdot 018\, Z_A\, Z_B \sqrt{I} \quad \ldots \quad \ldots \quad \ldots \quad (4)$$

where k_0 is the second-order rate constant when $I = 0$, and Z_A and Z_B are the charges on the two interacting species. A plot of $\log k/k_0$ *vs* $\sqrt{I}$ for the results at low pH gave a linear curve with slope $- 1 \cdot 63$ for dibenamine and $- 1 \cdot 24$ for dibenzyline over the low ionic strength range, $I = 0 - 1 \cdot 95 \times 10^{-2}$.

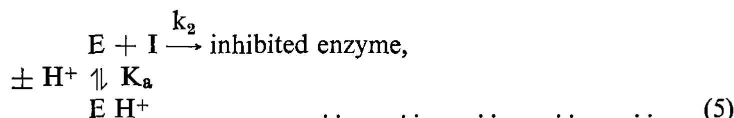
Effect of pH on the inhibition reaction

Solutions of dibenamine hydrochloride ($7\cdot5 \times 10^{-5}$M) and dibenzyline hydrochloride (1×10^{-4}M) were separately incubated with the enzyme over the pH range 4·7–10·6 following the standard procedure.

Duplicate control experiments in which the inhibitor was replaced by sodium chloride (0·2M) were made at pH 4·7 and 10·6 for the same intervals of time as the corresponding inhibition experiments and showed no loss of enzyme activity at the end of these periods.

The observed second-order rate constants, k, for the reactions were pH-dependent and plots of k *vs* pH were sigmoid-shaped.

The relation between the hydrogen ion concentration and the observed second-order rate constant k, for the system

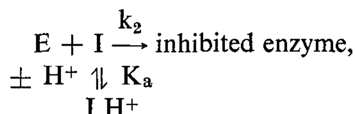


$$\begin{array}{c} E + I \xrightarrow{k_2} \text{inhibited enzyme,} \\ \pm H^+ \upharpoonleft\downharpoonright K_a \\ E\,H^+ \end{array} \quad \ldots \quad (5)$$

where a single ionizable group is on the enzyme, is given (Dixon & Webb, 1964) by

$$\frac{1}{k} = \frac{1}{k_2} + \frac{1}{k_2 K_a}[H^+] \quad \ldots \quad (6)$$

where k_2 is the second-order rate constant for the reaction with the active form of the enzyme. Equation (6) must also apply in the system,



$$\begin{array}{c} E + I \xrightarrow{k_2} \text{inhibited enzyme,} \\ \pm H^+ \upharpoonleft\downharpoonright K_a \\ I\,H^+ \end{array}$$

where the ionizable group is on the inhibitor.

Graphs of $1/k$ *vs* $[H^+]$ for the inhibition reactions with dibenamine and dibenzyline over the pH range 6–10 gave non-linear curves (Fig. 3A and B).

Ionization of a single group would have shown a linear relation so that two ionizable groups must be involved in the inhibition reaction over this pH range. Each graph was divisible into two separate linear sections over the pH ranges 7·8–9·5 ad 6·0–7·3 for dibenamine and 8·5–9·9 and 5·5–6·5 for dibenzyline. The lines of best fit were calculated for each linear section and the pK_a value of the group ionizing over each pH range was calculated from the gradient ($1/k_2K_a$) and intercept ($1/k_2$). The pK_a values for the ionizable groups involved in the inhibition reaction between dibenamine and AChE were 5·8 and 9·05 and the corresponding values for dibenzyline were 5·4 and 9·22. Using these observed values, theoretical ionization curves were constructed and fitted to the experimental points for the reactions (Fig. 4A and B). The theoretical curves fitted the data well except for the points at very high pH. These deviations are readily explicable since small differences in the $t_{0\cdot5}$ values are much more significant when the reaction is fast as at high pH. Accordingly, these points were not used in calculating the pK_a values.

Influence of tetramethylammonium ion (TMA) on the inhibition reaction

Dibenamine hydrochloride ($0\cdot75 \times 10^{-5}$M) and dibenzyline hydrochloride (1×10^{-4}M) were separately incubated with the enzyme at pH 9·5 in media containing

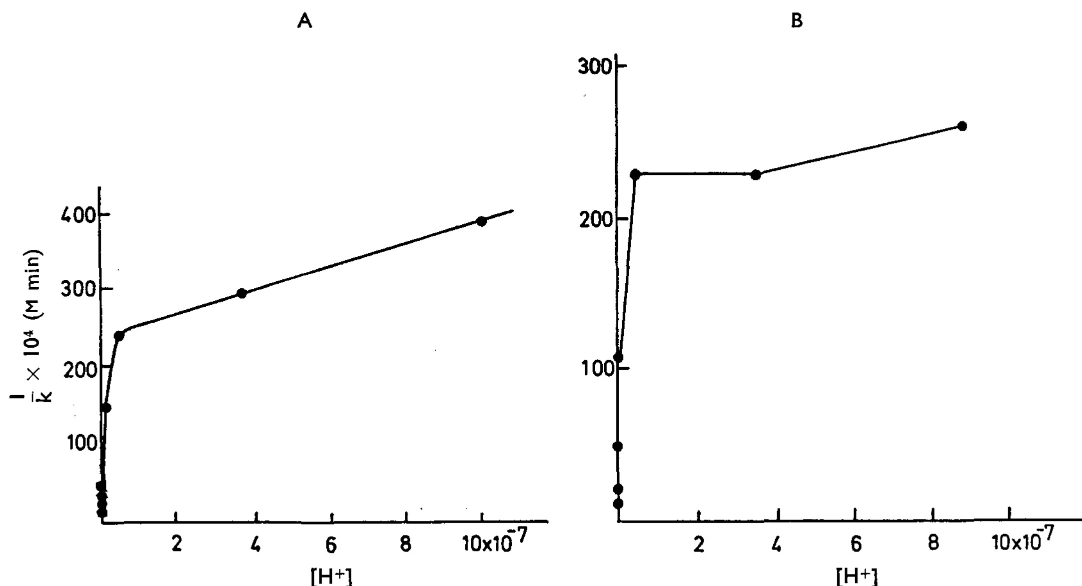
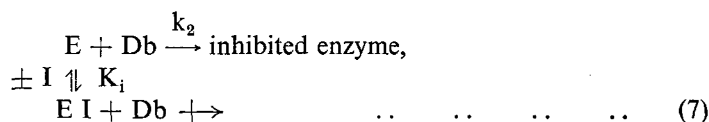


FIG. 3. Inhibition of AChE by dibenamine (A) and dibenzyline (B) as a function of hydrogen ion concentration.

tetramethylammonium ion (0–0·2M) and sodium chloride such that $I_{total} = 0{\cdot}2$ and the standard procedure followed.

In the system



$$\begin{array}{l} E + Db \xrightarrow{k_2} \text{inhibited enzyme,} \\ \pm I \upharpoonleft\downharpoonright K_i \\ E\,I + Db \not\rightarrow \end{array} \qquad \ldots \quad \ldots \quad \ldots \quad \ldots \quad (7)$$

where Db = dibenamine or dibenzyline and I = TMA, then, provided the (EI) complex is unreactive it can be shown that

$$\frac{1}{k} = \frac{1}{k_2} + \frac{1}{k_2 K_i} \cdot [I], \qquad \ldots \quad \ldots \quad \ldots \quad \ldots \quad (8)$$

where k_2 is the second-order rate constant for the reaction with the active form of the enzyme and k is the observed second-order rate constant in the presence of inhibitor.

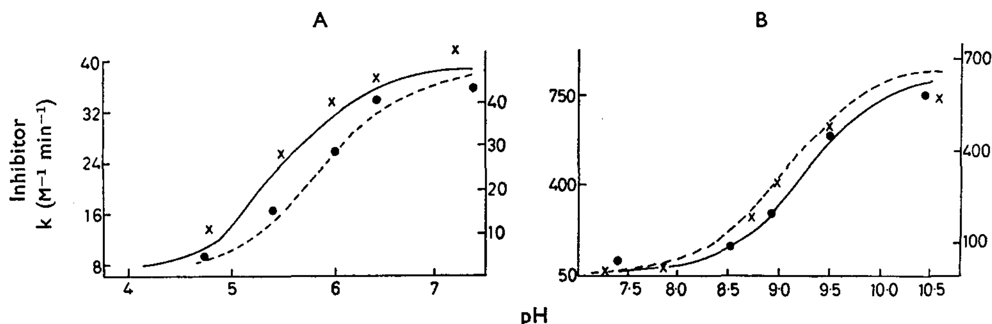
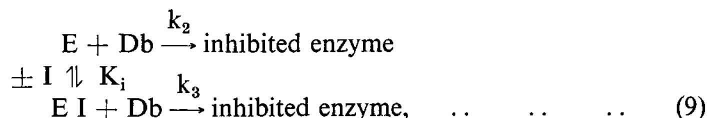


FIG. 4. Inhibition of AChE by dibenamine (left hand ordinate) (● in A, × in B) and dibenzyline (right hand ordinate) as a function of pH. The curve is that calculated for the dissociation of a group with A pK_a 5·8 (- - - -) and 5·4 (———) respectively; B pK_a 9·05 (- - - -) and 9·22 (———) respectively.

This equation resembles equation (6) for the effect of hydrogen ions on the second-order rate constant.

The relation between $\frac{1}{k}$ and TMA concentration was non-linear (Fig. 5A and B) and consequently it was inferred that TMA did not completely prevent access of the irreversible inhibitor to a reactive group at the active site of the enzyme. An acceptable explanation is that in the system



$$\begin{array}{l} E + Db \xrightarrow{k_2} \text{inhibited enzyme} \\ \pm I \rightleftharpoons K_i \\ E\,I + Db \xrightarrow{k_3} \text{inhibited enzyme,} \end{array} \quad \ldots \quad (9)$$

the (EI) complex is capable of reacting with dibenamine or dibenzyline at a reduced rate (i.e. $k_2 > k_3$). The relation between the rate constants k_2 and k_3 and the observed second-order rate constant, k, is then given by the expression

$$\frac{1}{k} = \frac{1}{\left(\frac{k_2}{(1 + \frac{[I]}{K_i})}\right) + \left(\frac{k_3}{(1 + \frac{K_i}{[I]})}\right)} \quad \ldots \quad (10)$$

The theoretical curve calculated using equation (10) approximately fitted the experimental values for dibenamine inhibition, where $K_i = 5 \times 10^{-3}$M, $\frac{1}{k_3} = 90 \times 10^{-4}$M min and $\frac{1}{k_2} = 16{\cdot}4 \times 10^{-4}$M min, and for dibenzyline inhibition, where $K_i = 10 \times 10^{-3}$M, $\frac{1}{k_3} = 85 \times 10^{-4}$M min and $\frac{1}{k_2} = 27{\cdot}8 \times 10^{-4}$M min.

DISCUSSION

The reaction between dibenamine and dibenzyline with AChE leads to progressive loss of enzyme activity until eventually the enzyme is completely inactivated. After

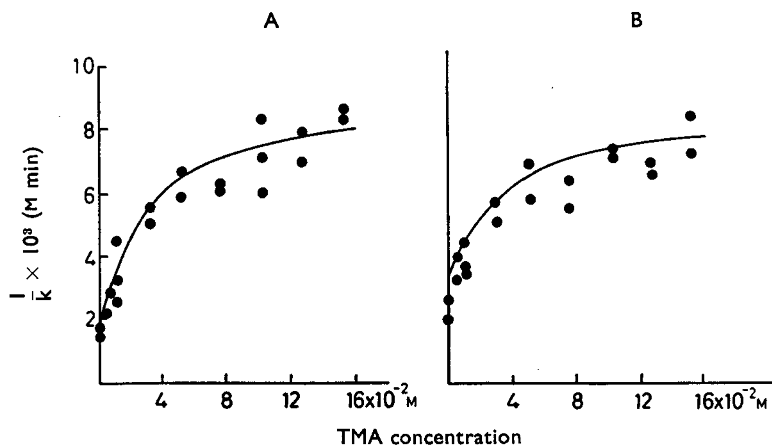
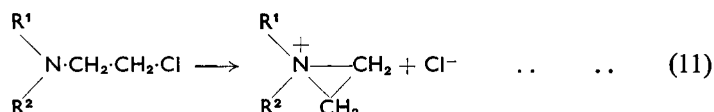


FIG. 5. Inhibition of AChE by dibenamine (A) and dibenzyline (B) at pH 9·5 in the presence of TMA. k is the observed second-order rate constant for the inhibition.

removal of the inhibiting species, enzyme activity cannot be regenerated by prolonged exposure of the inhibited enzyme to either acidic or alkaline conditions. This establishes that these compounds are irreversible inhibitors of the enzyme, and that the inhibitor residue is firmly attached to the enzyme by a very stable covalent bond.

The kinetics for the inhibiton reaction are first-order and are in accord with the occurrence of a biomolecular reaction between the enzyme and the inhibitor where the concentration of inhibitor is in excess. Work with other irreversible inhibitors of AChE such as carbamates and organophosphates (Reiner & Aldridge, 1967) has shown that in a few cases the second-order rate constant for the reaction is dependent on the inhibitor concentration. This has been interpreted in terms of initial complex formation between the inhibitor and enzyme preceding the formation of a covalent bond between the enzyme and inhibitor (Smith & Williams, 1965; Baker, 1964; Singer, 1967). In this work, complex formation between the inhibitor and the enzyme was not observed. However, this may be a result of the low inhibitor concentration used (due to the low aqueous solubility of the bases) which would tend to prevent such an observation (see Ryan, Ginsburg & Kitz, 1969).

Nitrogen mustards (Barlett, Ross & Swain, 1947; Hanby, Hartley & others, 1947) and certain monofunctional β-halogenoethylamines (1) (Graham 1962) are known to cyclize to the corresponding ethyleniminium ions (2) in neutral aqueous solutions, i.e.



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and several of these ions have been shown to be the pharmacologically active species present in solutions of the parent compounds (Chapman & James, 1954; Graham, 1957; Allen & Chapman, 1960). Dibenamine is considered to exert its adrenergic blocking action through its ethyleniminium ion (Nickerson & Gump, 1949) since this action is prevented by the presence of thiosulphate (Nickerson & Goodman, 1948) which is known to react rapidly with ethyleniminium ions but only slowly with alkyl halides (Golumbic, Fruton & Bergmann, 1946).

The reactions of both dibenamine and dibenzyline with the enzyme are prevented by high thiosulphate concentration in the pH region where both inhibitors exist in the base form. The action of the thiosulphate may be attributable to removal of the active intermediate (presumably ethyleniminium ion) formed from the base.

The reaction between the inhibitors and the enzyme is pseudounimolecular and follows first order kinetics at pH 6·5 and 9·5 where the inhibitors are present mainly in the base form. The observation that the active alkylating species is the intermediary ethyleniminium ion requires accommodating within this kinetic scheme. This can be achieved if the concentration of the ion is rapidly built up to a constant level which is maintained thoughout the life of the experiments. That this situation exists is supported by the linear nature of the first order plots for the reactions over the concentration ranges studied together with evidence from previous studies where it was found that the level of ethyleniminium ion in aqueous-organic solvent solutions of dibenamine and dibenzyline is low (Chapman & Tomsett, 1961) and reasonably constant over a period of several hours (Harvey & Nickerson, 1953).

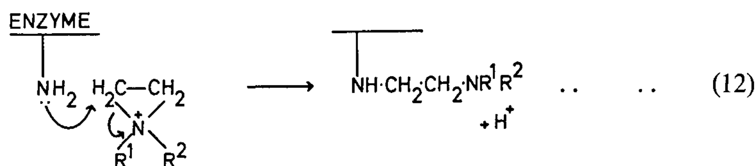
The pH-inhibition rate constant (k_2) profiles for the reactions between both dibenamine and dibenzyline with the enzyme over the pH range 4–10 were sigmoid. Close

examination showed that two ionization steps were involved over this range, one attributable to a group with a low pK_a value and the other to a group having a high value.

The group with a high pK_a has a value of 9·05 and 9·22 for the inhibition reactions using dibenamine and dibenzyline respectively. We have attributed the similarity in both systems to the ionization of an identical group. This group must be on the enzyme surface since dibenamine (pK_a 5·5) and dibenzyline (pK_a 5·1) exist in the base form above pH 7·5 and remain unchanged as the pH is increased.

The effect of ionic strength on reaction rates has been well documented for organic reactions and successfully applied to enzyme-substrate interactions although misleading results have sometimes been obtained (Kézdy, Clement & Bender, 1964; Lumry, Smith & Glantz, 1951). The absence of a significant ionic strength effect on the second-order rate constants, k_2, for the inhibition reactions with both inhibitors at pH 9·5 suggests that an uncharged group on the enzyme is alkylated by the ethyleniminium ion since interaction between two charged ions would have exhibited a considerable effect.

We consider that the neutral group on the enzyme involved in the reaction is the ϵ-amino group of lysine (free base form) which exists in proteins with a pK_a value within the range 9·4–10·6 (Edsall, 1965). This group can either be directly alkylated by the ethyleniminium ion (eqn 12) or else act as a general base and catalyse the



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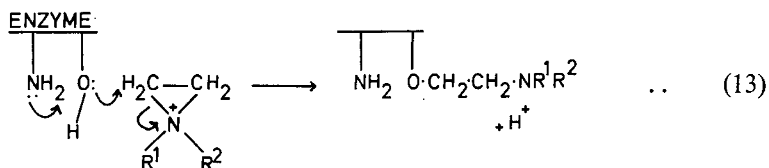
alkylation of an adjacent unionized group such as a hydroxyl group (eqn 13). There is no evidence to suggest that the conformation of the enzyme changes over the pH range 7–10 [cf. α-chymotrypsin (Bender, Gibian & Whelan, 1966)]. This eliminates the possibility that a conformational change, dependent on a group with pK_a 9·1, leads to the unmasking of a neutral group which enters into the alkylation reaction.



.. (13)

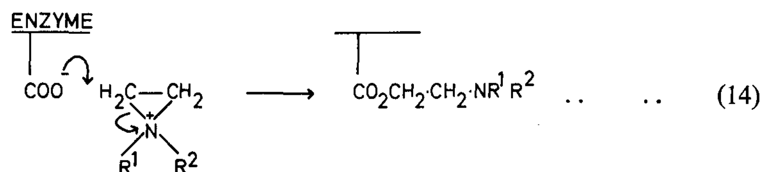
The group with a low pK_a value involved in the inhibition reaction has values of 5·8 and 5·4 for the reaction with dibenamine ($pK_{a_{app.}}$5·5) and dibenzyline ($pK_{a_{app.}}$ 5·1) respectively. These values compare favourably with those for the inhibitors. It is to be expected that the second-order rate constants for the reaction would increase in the observed manner with increase in pH since the concentration of the alkylating species is dependent on the proportion of the inhibitor present in the base form.

The effect of ionic strength on the second order rate constant for the reactions at

pH 6·5 was considerable and contrasted with the absence of such an effect at high pH. The noted values for the slopes of the graphs of log k/k_0 *vs* $\sqrt{I}$ for the reactions with dibenamine (— 1·63) and dibenzyline (— 1·24) are considered high values for a reaction with a slope of — 1. This is the expected value for a reaction occurring between oppositely charged monovalent ions.

We consider that all these data are in accord with alkylation by the ethyleniminium ion of a negatively charged group on the enzyme. This group does not change its state of ionization throughout the low pH range studied and consequently does not influence the pH-rate profile for the reaction. This group is most likely a carboxyl group which in proteins is almost completely ionized over the range considered (α-carboxyl, pK_a 3·0–3·2; aspartyl, 3·0–4·7; glutamyl ca 4·4; Edsall, 1965) and, furthermore, is known to react with dibenamine and dibenzyline (Harvey & Nickerson, 1954).

The reaction between the carboxylate ion on the enzyme and the ethyleniminium ion leads to the formation of an ester (eqn 14) which is stable under acidic and basic conditions for prolonged periods. This observation is in accord with the known resistance to hydrolysis of esters derived from these inhibitors (Graham & Al Katib, 1966).



The pH-activity profile for the reaction between AChE and its substrates is bell-shaped and is attributable to general base catalysis and general acid catalysis by imidazole (histidine) and phenolic hydroxyl (tyrosine) respectively in both the acylation and de-acylation steps (Wilson & Bergmann, 1950a; Bergmann, Segal & others, 1956). The reactions between the enzyme and organophosphorus compounds, e.g. TEPP (Wilson & Bergmann, 1950b) and carbamates, e.g. neostigmine (Reiner & Aldridge, 1967) also have bell-shaped pH-activity profiles. Consequently, it is generally accepted that the reactions of AChE with its substrates (i.e. acylation), organophosphorus compounds and carbamates are analogous processes (Wilson, Harrison & Ginsburg, 1961; Reiner & Simeon-Rudolf, 1966; Winteringham & Fowler, 1966). By contrast, the reactions of both dibenamine and dibenzyline with the enzyme have pH-activity profiles which are sigmoid-shaped and have a different pH-dependence.

Reaction between the alkylating agents and AChE resulted in total loss of activity. This observation is partial evidence that alkylation occurs at, or very near, the active site.

The question arises as to whether the point of attachment of the alkylating agents to the enzyme is to serine at the active site, (Schaffer, May & Summerson, 1953; Jansz, Berends & Oosterbaan, 1959), as for the acylation, phosphorylation and carbamoylation reactions, or elsewhere. The alkylation reaction occurring at low pH is with a carboxylate ion which rules out this possibility and the proposal that alkylation at high pH occurs on lysine similarly eliminates the involvement of serine.

However, the alternative interpretation of the data for the reaction at high pH, where lysine acts as a general base catalyst for the alkylation of an unionized group, could involve the hydroxyl group of serine. The sigmoid-shaped pH-activity profile for the alkylation reaction does not preclude this possibility since the charged nitrogen atom of the alkylating ethyleniminium ion, by acting as a "built-in" electron deficient centre, removes the necessity of a general acid catalyst for the reaction. It seems unlikely for the catalysis, however, that the ϵ-amino group of a lysine residue at the active site is preferred to the adjacent and correctly aligned imidazole nucleus of the histidine residue.

Studies were conducted with a reversible competitive inhibitor, the tetramethylammonium ion (TMA) (Wilson, 1952), to obtain further information on the point of attachment of the inhibitors to the enzyme. TMA is known to bind to AChE at the anionic site and alkylation of a group at the active site should be impeded by this inhibitor in a predictable manner.

TMA slowed the rate of inhibition of the enzyme by both dibenamine and dibenzyline, as would be expected if the reaction were to occur with an amino acid in the vicinity of the active site. However, the quantitative effect of TMA on the reaction differed from that expected for an inhibitor which binds to the active site and completely prevents the alkylation reaction from occurring by either steric or electrostatic repulsive interactions. The results obtained were in agreement with the view that alkylation occurs at some distance from the anionic site and on the borders of the active site so that the bound TMA ion can retard, but not prevent, the alkylation reaction. The equations developed for this situation approximately fit the observed experimental data.

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REFERENCES

Allen, J. F. & Chapman, N. B. (1960). *J. chem. Soc.*, 1482–1487.

Baker, B. R. (1964). *J. pharm. Sci.*, **53**, 347–364.

Bartlett, P. D., Ross, S. D. & Swain, C. G. (1947). *J. Am. chem. Soc.*, **69**, 2971–2977; 2977–2982.

Beddoe, F. & Smith, H. J. (1967). *Nature, Lond.*, **216**, 706–707.

Bergmann, F., Segal, R., Shimoni, A. & Wurzel, M. (1956). *Biochem. J.*, **63**, 684–690.

Bender, M. L., Gibian, M. J. & Whelan, D. J. (1966). *Proc. natn. Acad. Sci.*, **56**, 833–839.

Chapman, N. B. & James, J. W. (1954). *J. chem. Soc.*, 2103–2108.

Chapman, N. B. & Tomsett, A. J. (1961). *Ibid.*, 1291–1297.

Dixon, M. & Webb, E. C. (1964). *Enzymes*, 2nd Edn, p. 133, London: Longmans.

Edsall, J. T. (1965). In *Proteins, Amino Acids and Peptides*, p. 445. Editors: Cohn, E. J. & Edsall, J. T. New York and London: Hafner.

Golumbic, C., Fruton, J. S. & Bergmann, M. (1946). *J. org. Chem.*, **11**, 518–535.

Graham, J. D. P. (1957). *Br. J. Pharmac. Chemother.* **12**, 489–497.

Graham, J. D. P. (1962). In *Progress in Medicinal Chemistry*, Vol. 2, p. 132. Editors: Ellis, G. P. and West, G. B. London: Butterworths.

Graham, J. D. P. & Al-Katib, H. (1966). *Br J. Pharmac. Chemother.*, **28**, 1–14.

Hanby, W. E., Hartley, G. S., Powell, E. O. & Rydon, H. N. (1947), *J. chem. Soc.*, 519–527.

Harvey, S. C. & Nickerson, M. (1953). *J. Pharmac. exp. Ther.*, **109**, 328–339.

Harvey, S. C. & Nickerson, M. (1954). *Ibid.*, **112**, 274–290.

JANSZ, H. S., BERENDS, F. & OOSTERBAAN, R. A. (1959). *Recl Trav. chim. Pays-Bas Belg.*, **78**, 876–878.

KARLIN, A. (1967). *Biochim. biophys. Acta*, **139**, 358–362.

KÉZDY, F. J., CLEMENT, G. E. & BENDER, M. L. (1964). *J. Am. chem. Soc.* **86**, 3690–3696.

KÉZDY, F. J., THOMSON, A. & BENDER, M. L. (1967). *Ibid.*, **89**, 1004–1009.

KOELLE, G. B. (1963). In *Handbuch der Experimentellen Pharmacologie*, Chaps. 9 and 13, Berlin: Springer.

LUMRY, R., SMITH, E. L. & GLANTZ, R. R. (1951). *J. Am. chem. Soc.*, **73**, 4330–4340.

NICKERSON, M. & GOODMAN, L. S. (1948). *Fedn Proc. Fedn Am. Socs exp. Biol.*, **7**, 397–409.

NICKERSON, M. & GUMP, W. S. (1949). *J. Pharmac. exp. Ther.*, **97**, 25–47.

PODLESKI, T. R. (1967). *Proc. natn. Acad. Sci. U.S.A.*, **58**, 268–273.

RYAN, J. F., GINSBURG, S. & KITZ, R. J. (1969). *Biochem. Pharmac.*, 18, 269–278.

REINER, E. & ALDRIDGE, W. N. (1967). *Biochem. J.*, **105**, 171–179.

REINER, E. & SIMEON-RUDOLF, V. (1966). *Ibid., J.*, **98**, 501–505.

ROEPKE, M. H. (1937). *J. Pharmac. exp. Ther.*, **59**, 264–276.

SCATCHARD, G. (1932). *Chem. Rev.*, **10**, 229–240.

SCHAFFER, N. K., MAY, S. C. & SUMMERSON, W. H. (1953). *J. biol. Chem.*, **202**, 67–76.

SHAPIRO, S. L., ISAACS, E. S., BANDURCO, V. & FREEDMAN, L. (1962). *J. Mednl pharm. Chem.*, **2**, 793–799.

SMITH, H. J. & WILLIAMS, H. (1965). *J. Pharm. Pharmac.*, **17**, 529–557; 601–618.

SINGER, S. J. (1967). In *Advances in Protein Chemistry*, Vol. 22, p. 1–54, London and New York: Academic Press.

WEBB, G. D. (1965). *Biochim. biophys. Acta*, **102**, 172–184.

WILSON, I. B. (1952). *J. biol. Chem.*, **197**, 215–225.

WILSON, I. B. & BERGMANN, F. (1950a). *Ibid.*, **186**, 683–692.

WILSON, I. B. & BERGMANN, F. (1950b). *Ibid.*, **185**, 479–489.

WILSON, I. B., HARRISON, M. A. & GINSBURG, S. (1961), *Ibid.*, **236**, 1498–1500.

WINTERINGHAM, F. P. W. & FOWLER, K. S. (1966). *Biochem. J.*, **99**, 6P.