

Effect of pH on Inhibition and Spontaneous Reactivation of Acetylcholinesterase Treated with Esters of Phosphorus Acids and of Carbamic Acids

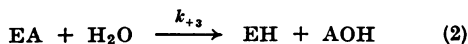
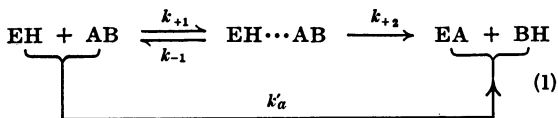
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1. The second-order rate constants of inhibition, k_a , of acetylcholinesterase were measured at pH values between 5.5 and 10.5 for two esters of phosphorus acids and five esters of carbamic acids. Two of the carbamates and one of the phosphates contained a quaternary nitrogen group. 2. For the three positively charged compounds the k_a -pH plots are bell-shaped, with a pH optimum between 7.5 and 9.0. The changes in k_a above and below the optimum pH fit theoretical curves for the dissociation of groups on the protein of pK 6.2 and 10.25. 3. For the uncharged compounds, the k_a -pH plot on the alkaline side is identical with the one obtained for charged inhibitors. On the acid side they do not fit such a curve and the k_a for two of the carbamates is independent of pH changes between 5.5 and 8.0. 4. The first-order rate constants, k_{+3} , for spontaneous reactivation were measured at pH values between 5.0 and 11.0 for *N*-methylcarbamoylated, *NN*-dimethylcarbamoylated and di-(2-chloroeth)phosphorylated cholinesterase. For all three derivatives the k_{+3} -pH plots are bell-shaped, with a pH optimum between 8.0 and 8.5. The changes in k_{+3} above and below the optimum fit theoretical curves for the dissociation of groups of pK 6.9 and 9.8. 5. The relevance of these results to binding, acylation and deacylation of both inhibitors and substrates is discussed.

It is now accepted that the reactions of cholinesterase with substrates, organophosphates and carbamates are analogous processes (Aldridge, 1953; Becker, Fukuto, Boone, Canham & Boger, 1963; Wilson, Hatch & Ginsburg, 1960; Reiner & Simeon-Rudolf, 1966; Winteringham & Fowler, 1966). All three types of reactions seem to proceed via an acylated enzyme (EA), the formation of which is preceded by a complex (EH...AB) and followed by hydrolysis of EA:



AB stands for an ester, which can be either substrate or inhibitor; k_a is the second-order rate constant of acylation independent of pH and k_{+3} is the first-order rate constant of deacylation.

Acylation and deacylation are fast reactions when substrates are involved, and it is therefore difficult

to distinguish experimentally between the separate steps in eqns. (1) and (2). For phosphates and carbamates deacylation is relatively slow and k_a (the second-order rate constant for acylation) and k_{+3} can be measured separately.

We have studied the influence of pH on k_a and k_{+3} for some esters of phosphorus acids and carbamic acids. This is a simpler system to study than the enzyme-substrate system; if similarities are found between the pH-dependence of acylation or deacylation after treatment with different inhibitors it may indicate that the results are applicable to substrate hydrolysis.

MATERIALS AND METHODS

Enzyme preparations. The enzyme preparation was purified bovine erythrocyte cholinesterase (Winthrop Laboratories Inc., New York, N.Y., U.S.A.). When inhibition was studied stock solutions were prepared in water (16.8 mg./ml.) and kept at 4° no longer than 1-2 days; further dilutions were prepared in 0.02M-phosphate-pyrophosphate buffer immediately before use. When reactivation was studied stock solutions (35.0 mg./ml.) were prepared in 0.01M-phosphate buffer immediately before use.

Buffers. The phosphate-pyrophosphate buffer was prepared by titrating 0.02 M-Na₂H₂P₂O₇ with 0.02 M-Na₃PO₄ to the required pH. This buffer has proved very useful for it can be applied over a pH range 5.5-11.

Universal buffer was prepared by titrating 0.02 N-NaOH with a solution containing KH₂PO₄ (0.02 M), citric acid (0.02 M), diethylbarbituric acid (0.02 M) and boric acid (0.02 M).

Phosphate buffer was used at three different concentrations: 0.5 M-, 0.05 M- and 0.01 M-Na₂HPO₄, titrated with 0.5 M-, 0.05 M- and 0.01 M-KH₂PO₄ respectively.

Inhibitors. The following inhibitors were used: neostigmine (Koch-Light Laboratories Ltd., Colnbrook, Bucks.); monomethylnestigmine (given by Mr D. R. Davies); phosphostigmine (given by Professor F. Hobbiger); dimethyl 4-nitrophenyl phosphate (prepared by Dr D. F. Heath); haloxon (Cooper's Technical Bureau, Berkhamsted, Herts.); 3-isopropylphenyl *N*-methylcarbamate, 2-isopropoxyphenyl *N*-methylcarbamate and 3-isopropylphenyl *NN*-dimethylcarbamate (provided by the World Health Organisation, Geneva, Switzerland). (The full chemical names of compounds with trivial names are given in Table 1.) Stock solutions (0.01 M) were prepared as follows: neostigmine, monomethylnestigmine and phosphostigmine in water; haloxon and 3-isopropylphenyl *N*-methylcarbamate in ethanol; dimethyl 4-nitrophenyl phosphate, 2-isopropoxyphenyl *N*-methylcarbamate and 3-isopropylphenyl *NN*-dimethylcarbamate in *NN*-dimethylformamide. All solutions were stored at 4°, except monomethylnestigmine, which is unstable and was prepared immediately before use. Further dilutions from these stock solutions were made in water or buffer immediately before use.

Substrate and thiol reagent. Stock solutions of acetylthiocholine iodide (British Drug Houses Ltd., Poole, Dorset) in water were prepared daily.

The reagent for thiols, 5,5'-dithiobis-(2-nitrobenzoic acid), was obtained from Aldrich Chemical Co., Milwaukee, Wis., U.S.A. Stock solution (16.6 mM) was prepared in 0.05 M-phosphate buffer, pH 8.0, containing NaHCO₃ (1.5 mg./ml.). The solution was stored at 4° in the dark for no longer than 4-5 days.

Procedure for measuring enzyme inhibition. Enzyme and inhibitor were incubated in phosphate-pyrophosphate buffer at a given pH. Then phosphate buffer, containing substrate and the thiol reagent, was added to bring the pH to 7.3, at which enzyme activity was always measured spectrophotometrically, by the method of Ellman, Courtney, Andres & Featherstone (1961). All experiments were carried out at 25°.

The detailed procedure was as follows: into the photocell 1.7 ml. of 0.02 M-phosphate-pyrophosphate buffer was added, followed by 0.30 ml. of solution of inhibitor in water and 1.0 ml. of enzyme in 0.02 M-phosphate-pyrophosphate buffer. The concentration of enzyme preparation during inhibition was 0.056 mg./ml. At suitable time-intervals (0.25-4.0 min.) 7.0 ml. of solution, containing 5.8 ml. of 0.5 M-phosphate buffer, pH 7.3, 0.2 ml. of thiol reagent and 1.0 ml. of substrate in water, was added. The substrate concentration during enzyme determination was 1.0 mM. After rapid mixing the extinction was read on a modified Unicam SP. 1400 spectrophotometer at 15 sec. intervals for 1 min., the first reading being taken 15 sec. after the 7.0 ml. of solution had been added. The increase in extinction was linear with time for both inhibited and uninhibited enzyme

samples. No correction for spontaneous hydrolysis of the substrate was necessary.

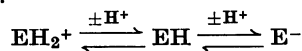
Procedure for measuring spontaneous reactivation. Cholinesterase (0.4 ml. of solution containing 35.0 mg./ml. in 0.01 M-phosphate buffer, pH 7.3) and inhibitor (0.1 ml. of various concentrations in 0.01 M-phosphate buffer, pH 7.3) were incubated for about 20 min. to obtain about 90% inhibition. The concentrations of inhibitor were as follows: 2-isopropoxyphenyl *N*-methylcarbamate, 5 μM; haloxon, 2.5 μM; neostigmine, 0.5 μM. The enzyme-inhibitor solution was then diluted 1:500 with 0.02 M-phosphate-pyrophosphate buffer or universal buffer of a given pH. At suitable time-intervals 3.0 ml. samples were withdrawn and the activity of the enzyme was determined as described in the procedure for enzyme inhibition. All experiments were carried out at 25°.

RESULTS

Theoretical treatment

In almost all previous reports of the effect of pH on the inhibition of cholinesterase by esters of phosphorus acids and of carbamic acids, pH has been related to the wrong parameters (cf. the Discussion section). The following is the theoretical derivation showing that the pH should be related to the second-order rate constant for inhibition or to the first-order constant for deacylation.

The usual (Michaelis, 1922) concept for ionization states of an enzyme with only one active form (EH) is given by:



The concentration of EH at a given pH is given by:

$$K_1 = \frac{[\text{EH}][\text{H}^+]}{[\text{EH}_2^+]} \quad (3)$$

$$\text{and} \quad K_2 = \frac{[\text{E}^-][\text{H}^+]}{[\text{EH}]} \quad (4)$$

where K_1 and K_2 are equilibrium constants. If only EH can react with an inhibitor AB as shown in eqn. (1) and if the concentration of the Michaelis complex between EH and EA is neglected, then:

$$\frac{d[\text{EA}]}{dt} = k'_a[\text{EH}][\text{AB}] \quad (5)$$

Where k'_a is the second-order rate constant independent of pH, the total concentration of enzyme, $[\text{E}_0]$, in the presence of inhibitor AB is:

$$[\text{E}_0] = [\text{E}^-] + [\text{EH}] + [\text{EH}_2^+] + [\text{EA}] \quad (6)$$

Inserting eqns. (3) and (4) into eqn. (6) to eliminate $[\text{EH}_2^+]$ and $[\text{E}^-]$ and then inserting the resultant expression for $[\text{EH}]$ into eqn. (5):

$$\frac{d[\text{EA}]}{dt} = \frac{k'_a[\text{AB}](\text{E}_0 - [\text{EA}])}{1 + \frac{K_2}{[\text{H}^+]} + \frac{[\text{H}^+]}{K_1}} \quad (7)$$

Rearrangement and integration of eqn. (7) yields:

$$\ln\left(\frac{[E_0]}{[E_0]-[EA]}\right) = \frac{k'_a[AB]t}{1 + \frac{K_2}{[H^+]} + \frac{[H^+]}{K_1}} \quad (8)$$

This equation relates the ratio of the activities of enzyme before inhibition and the enzyme in the presence of inhibitor to the concentration of inhibitor and k'_a , the maximum second-order rate constant of inhibition independent of pH. None of the inhibitors used changes its concentration (due to dissociation of groups in the compound) in the pH range studied. The ratio:

$$\frac{k'_a}{1 + \frac{K_2}{[H^+]} + \frac{[H^+]}{K_1}} \quad (9)$$

yields k_a , the second-order constant at a given pH. The same form of equation applies to the pH-dependence of the rate of deacylation expressed as the first-order constant k_{+3} (cf. eqn. 2).

When $[H^+] \gg K_2$ or $[H^+] \ll K_1$, eqn. (9) becomes respectively:

$$k_a = k'_a - \frac{1}{K_1} \cdot k_a[H^+] \quad (10)$$

and

$$k_a = k'_a - K_2 \cdot \frac{k_a}{[H^+]} \quad (11)$$

Eqns. (10) and (11) were used to evaluate K_1 and K_2 as illustrated in Figs. 5(a) and 5(b).

Inhibition of acetylcholinesterase

Seven compounds were used to study the inhibition of cholinesterase (Table 1): two esters of phosphoric acid (compounds 1 and 7) and five esters

of carbamic acid (compounds 2-6), one of the phosphates (compound 1) and two of the carbamates (compounds 2 and 3) containing a quaternary nitrogen group. Of the five carbamates two are *NN*-dimethyl- and three are *N*-monomethyl-substituted esters.

All inhibition studies were performed in 0.02M-phosphate-pyrophosphate buffer at pH values in the range 5.5-10.5. To obtain k_a , the second-order rate constant of inhibition, at least two different inhibitor concentrations were used, and for each concentration the degree of inhibition was determined for at least three different times of incubation. Short incubation times (0.25-4.0 min.) and short times of assay (1.0 min.) were chosen to eliminate errors due to spontaneous reactivation of the inhibited enzyme during inhibition and assay.

Inhibition by compounds containing a quaternary nitrogen group. An example of the results obtained from a typical experiment with an inhibitor (neostigmine) that produces an unstable inhibited enzyme and the experimental procedure described above is given in Fig. 1. The rates of inhibition by neostigmine are first-order and the lines pass through the origin ($\log 100\% = 2$, in Fig. 1a). The first-order rate constants are a linear function of the inhibitor concentration (Fig. 1b), the reaction thus showing the characteristics of a bimolecular reaction with one component, the inhibitor, in excess. The second-order rate constants, k_a , could thus be calculated. Two carbamates have been examined, neostigmine and monomethylneostigmine; the values of k_a at optimum pH are given in Table 1. Because of its instability, monomethylneostigmine was not studied at pH values above 7.7.

When phosphostigmine was examined by the same procedure, although the rate of inhibition was first-order at any given concentration of inhibitor

Table 1. *Second-order rate constants for inhibition of acetylcholinesterase by esters of phosphorus acids and of carbamic acids*

Structure of inhibitor (abbreviation in square brackets)	Second-order constant k'_a (l. mole ⁻¹ min. ⁻¹)
Positively charged compounds	
Methyl sulphate of diethyl 3-trimethylaminophenyl phosphate [phosphostigmine]	4.2 × 10 ⁴
Methyl sulphate of 3-trimethylaminophenyl <i>NN</i> -dimethylcarbamate [neostigmine]	2.6 × 10 ⁶
Bromide of 3-trimethylaminophenyl <i>N</i> -methylcarbamate [monomethylneostigmine]	2.8 × 10 ⁷
Uncharged compounds	
3-Isopropylphenyl <i>NN</i> -dimethylcarbamate	2.9 × 10 ⁸
3-Isopropylphenyl <i>N</i> -methylcarbamate	5.4 × 10 ⁵
2-Isopropoxyphenyl <i>N</i> -methylcarbamate	4.9 × 10 ⁴
Dimethyl 4-nitrophenyl phosphate	2.0 × 10 ⁵
Di-(2-chloroethyl) 3-chloro-4-methylcoumarin-7-yl phosphate [haloxon]	—

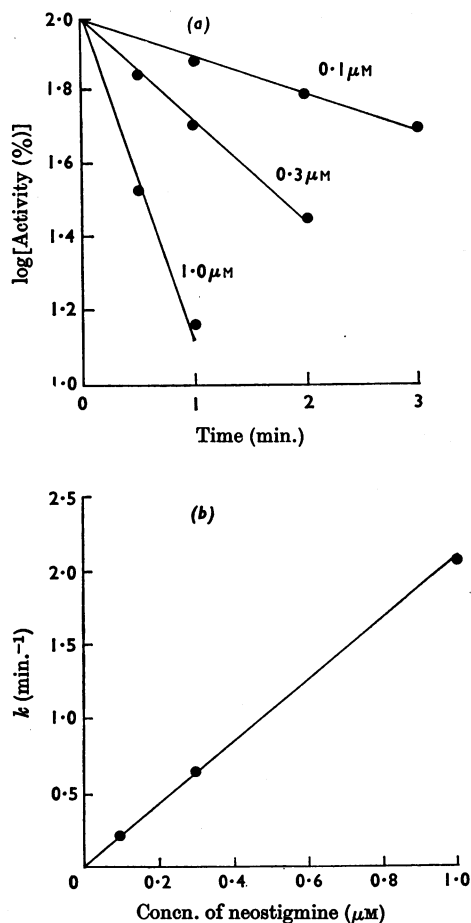


Fig. 1. Inhibition of acetylcholinesterase by neostigmine at pH 9.6. (a) Plot of log [activity (%)] against time; the concentration of neostigmine before the addition of substrate is shown against each curve. (b) Plot of the first-order rate constant, k , against neostigmine concentration; the second-order rate constant, k_a , derived from these results is $2.1 \times 10^6 \text{ l. mole}^{-1} \text{ min.}^{-1}$.

the first-order constants were not a linear function of the inhibitor concentration (Fig. 2). A reversible intermediate before formation of the phosphorylated enzyme is probably present. With such an intermediate (probably analogous to the Michaelis complex) present, the observed first-order rate constant for inhibition is (Main, 1964):

$$k = \frac{k_{+2}[\text{AB}]}{K_m + [\text{AB}]} \quad (12)$$

where k_{+2} is the first-order constant of acylation (eqn. 1) and K_m is the Michaelis constant for the reversible intermediate between cholinesterase and

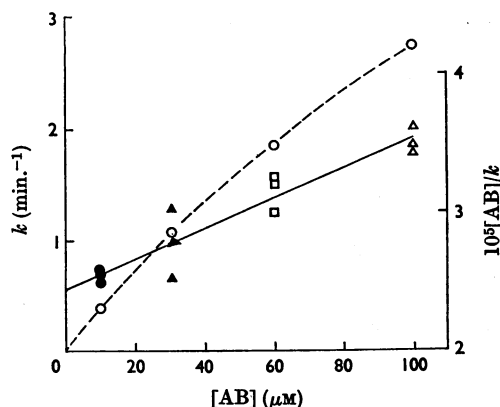


Fig. 2. Inhibition of acetylcholinesterase by phosphostigmine pH 8.6. The points marked \circ and the broken line through them show the non-linearity of the relation between the first-order rate constant, k , and the concentration of phosphostigmine. The results of these experiments are also shown plotted (\bullet , \blacktriangle , \square and \triangle , solid line) by the method described in the text. The values of K_m and k_{+2} derived from this and similar experiments are given in Table 2. $[\text{AB}]$ is the concentration of inhibitor. The symbols \bullet , \blacktriangle , \square and \triangle indicate separate experiments, each with different concentrations of inhibitor and a range of times of incubation.

phosphostigmine. Rearranged according to Wilkinson (1961) eqn. (12) becomes:

$$\frac{[\text{AB}]}{k} = \frac{K_m}{k_{+2}} + \frac{[\text{AB}]}{k_{+2}} \quad (13)$$

In Fig. 2 $[\text{AB}]/k$ is plotted against $[\text{AB}]$. Values of k were calculated for each inhibitor concentration and each time of incubation. At each pH, k_{+2} and K_m was derived from the slope and the intercept of the regression line respectively (eqn. 13, Table 2). The results are rather scattered, but this is probably a reflection of the limitations of inhibitor concentration that could be used. With our minimum time of incubation of 0.25 min., maximum concentration of inhibitor $100 \mu\text{M}$ and K_m $180 \mu\text{M}$, only 36% of the enzyme would be present as a reversible intermediate. An accurate determination of K_m would require shorter incubation times and higher concentrations of inhibitor. k_{+2} did not vary in any consistent way with pH, but there is evidence that K_m is higher at the lowest pH, namely 5.6 (Table 2).

When $[\text{AB}] < K_m$ rearrangement of eqn. (13) yields:

$$\frac{k}{[\text{AB}]} = \frac{k_{+2}}{K_m} \quad (14)$$

$k/[\text{AB}]$ is the usual bimolecular rate constant, k_a .

All the results with positively charged inhibitors are given in Fig. 3, where k_a is plotted against pH,

Table 2. Kinetic data for the inhibition of cholinesterase by phosphostigmine

k_{+2} (\pm s.e.m.) and K_m were calculated from the slope and intercept of the regression lines according to eqn. (13) (cf. Fig. 2); k_a was obtained according to eqn. (14).

pH	k_{+2} (min. ⁻¹)	$10^4 K_m$ (M)	$10^{-4} k_a$ (l. mole ⁻¹ min. ⁻¹)
5.6	7.86 \pm 0.60	6.14	1.28
6.5	5.12 \pm 0.42	1.99	2.57
7.1	6.53 \pm 0.59	1.85	3.53
7.6	5.20 \pm 0.66	1.23	4.23
7.95	7.36 \pm 0.86	1.86	3.96
8.6	9.1 \pm 1.10	2.23	4.07
9.6	6.08 \pm 0.05	1.61	3.79
10.2	—	—	2.0

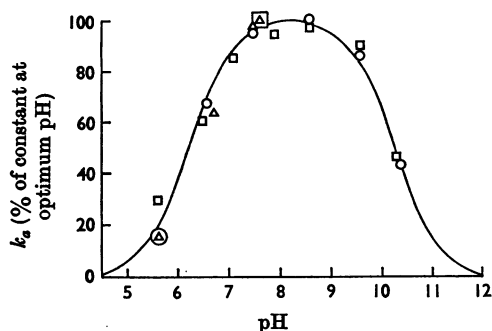


Fig. 3. Inhibition of acetylcholinesterase by neostigmine (○), monomethylneostigmine (△) and phosphostigmine (□) as a function of pH. k_a is the second-order rate constant for inhibition at a particular pH and the curve is that calculated for the dissociation of two groups of pK 6.2 and 10.25.

k_a being given as a percentage of the value obtained at the optimum pH. The curve relating k_a to pH is bell-shaped for all these compounds. The optimum pH is between 7.5 and 9.0. To evaluate pK on each side of the optimum pH eqns. (10) and (11) were used. The results are given in Fig. 4, pK₁ and pK₂ being 6.2 and 10.25 respectively. The respective values of K_1 and K_2 were used to construct the theoretical curve in Fig. 3. The experimental points fit well to this curve.

Inhibition by compounds without a quaternary nitrogen group. Three carbamates and one phosphate were examined (Table 1). In every case and at all pH values the rate of inhibition was first-order and the rate constant was a linear function of inhibitor concentration. The values of k_a at optimum pH are given in Table 1 and the variation of k_a with pH is shown in Fig. 5. The line in Fig. 5 is the theoretical curve (cf. Fig. 3) calculated from

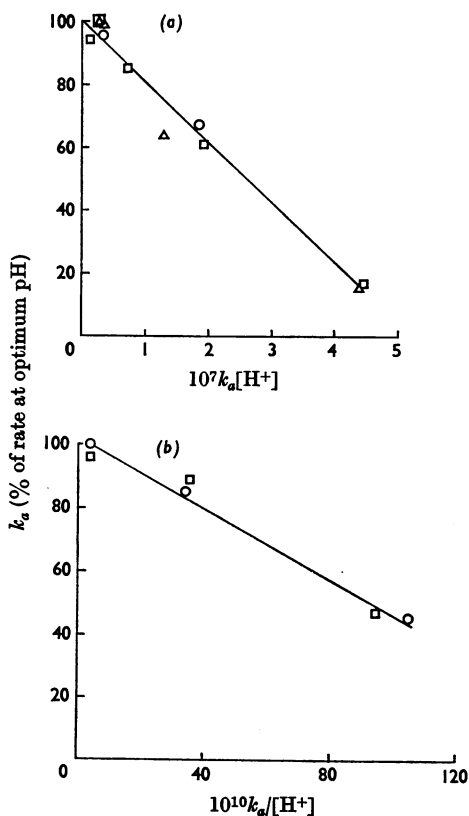


Fig. 4. Inhibition of acetylcholinesterase by neostigmine (○), monomethylneostigmine (△) and phosphostigmine (□). (a) At pH values below 8.5. Under these conditions $[H^+] > K_2$ and the results are plotted according to eqn. (10) (see the text). The pK₁ derived from the slope of this graph is 6.2. (b) At pH values above 8.5. Under these conditions $[H^+] < K_1$ and the results are plotted according to eqn. (11) (see the text). The pK₂ derived from the slope of this line is 10.25. The theoretical dissociation curve for these two groups is given in Fig. 3.

the results obtained with inhibitors with a positive charge. On the alkaline side the results for all the inhibitors studied fit this curve with pK 10.25. However, there are considerable differences on the acid side, no uncharged compound falling near the curve. The inhibitory power of 2-isopropoxyphenyl *N*-methylcarbamate and of 3-isopropylphenyl *NN*-dimethylcarbamate is unaffected by a decrease in pH from 8 to 5.5. Some slight decrease in activity occurs with 3-isopropylphenyl *N*-methylcarbamate and dimethyl 4-nitrophenyl phosphate, but the decrease does not fit a curve for the dissociation of a single group and no pK can be calculated.

Some difficulties were experienced with 3-isopropylphenyl *NN*-dimethylcarbamate. It is a poor

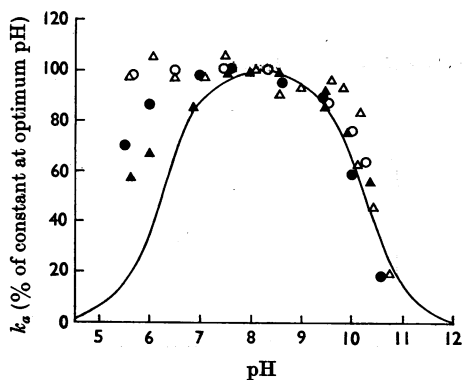


Fig. 5. Inhibition of acetylcholinesterase by compounds not containing a quaternary nitrogen group. k_a is the second-order rate constant for inhibition at a particular pH. The curve is that for the dissociation of two groups of pK 6.2 and 10.25 shown in Fig. 3. The compounds used are 2-isopropoxyphenyl *N*-methylcarbamate (○), 3-isopropylphenyl *NN*-dimethylcarbamate (△), 3-isopropylphenyl *N*-methylcarbamate (●) and dimethyl 4-nitrophenyl phosphate (▲).

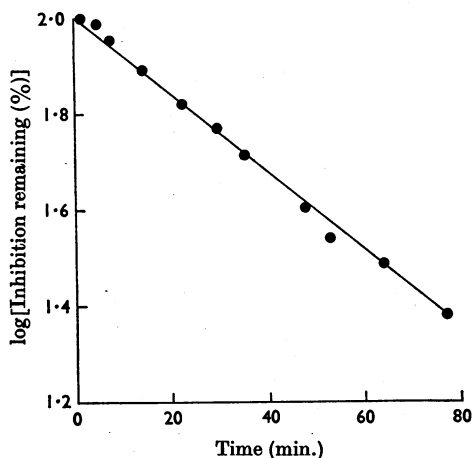


Fig. 6. Spontaneous reactivation of *N*-methylcarbamoylated acetylcholinesterase. The reactivation took place in phosphate-pyrophosphate buffer, pH 7.45. The first-order rate constant derived from these results is $1.88 \times 10^{-2} \text{ min.}^{-1}$.

inhibitor and not very soluble in water. Repeated determination of k_a gave results varying more than those for other compounds. This may be associated with the low solubility. The values given in Fig. 5 are mean values for four or five individual experiments and the k_a reported in Table 1 is the mean for all values obtained between pH 5.5 and 8.0.

Table 3. First-order rate constants for the spontaneous reactivation of inhibited cholinesterase at optimum pH

Acyl group	$10^2 k_{+3}$ (min. ⁻¹)	Half-life (min.)
-CO·NH·CH ₃	2.34	29.5
-CO·N(CH ₃) ₂	1.23	56.5
-PO(O·C ₂ H ₄ Cl) ₂	1.68	41.0

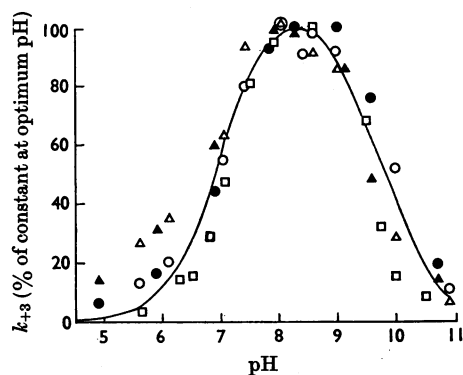


Fig. 7. Spontaneous reactivation of inhibited cholinesterases as a function of pH. The inhibited enzymes were *N*-methylcarbamoylated (○ and ●), *NN*-dimethylcarbamoylated (□) and di-(2-chloroethyl)phosphorylated (△ and ▲), in phosphate-pyrophosphate buffer (○, △ and □) or universal buffer (● and ▲). k_{+3} is the first-order rate constant for reactivation at a particular pH. The curve is that for the dissociation of two groups of pK 6.9 and 9.8. The calculated peak of the two dissociation curves at pH 8.3 is 96% dissociation of the group of pK 6.9. The whole curve has been corrected (by multiplying by 100/96) so that the peak coincides with 100%, the optimum rate constant for reactivation.

Spontaneous reactivation of inhibited cholinesterase.

The three types of inhibited cholinesterase studied were monomethylcarbamoylated (obtained by incubating cholinesterase with 2-isopropoxyphenyl *N*-methylcarbamate), dimethylcarbamoylated (incubation with neostigmine) and di-(2-chloroethyl)phosphorylated (incubated with haloxon). The enzyme was incubated with sufficient inhibitor to produce an inhibition greater than 90%. For the first and third of the above inhibitors, the concentration calculated from known k_a values (the enzyme concentration being ignored) was adequate. For neostigmine such a calculation gave a concentration of inhibitor that was less than the enzyme concentration used, and a concentration of inhibitor tenfold higher was used. After inhibition the solution of enzyme and inhibitor was diluted 500-fold with buffer of various pH values, the decrease in inhibitor concentration being such that less than

1% further inhibition could occur during the 1–2 hr. period of reactivation.

In all cases and at all pH values the rate of reactivation was first-order. Experiments were continued until more than 50% of the activity had returned or for not more than 2 hr. The activity of controls (cholinesterase treated in an identical fashion without the addition of inhibitor) remained unaltered at all pH values studied. The values of k_{+3} (cf. eqn. 2) were derived graphically as shown in Fig. 6, where a typical experiment is illustrated, and those at optimum pH are listed in Table 3. The results were identical in phosphate–pyrophosphate or universal buffer.

The changes in k_{+3} with pH give a bell-shaped curve (Fig. 7) with optimum pH 8.0–8.5. A theoretical curve for the dissociation of two groups of pK_1 and pK_2 6.9 and 9.8 has been calculated and this fits all of the results with the exception of those for di-(2-chloroethyl)phosphorylated cholinesterase at pH below 6.0. An optimum of about pH 8 was found by Davison (1955), but the assay procedure used does not allow precise values for k_{+3} to be calculated.

DISCUSSION

The evidence that the reactions of cholinesterase with esters of phosphorus acids, of sulphuric acids and of carbamic acids are analogous to its reaction with substrates (Aldridge & Davison, 1952; Aldridge, 1953) has recently been extended by the demonstration of kinetics indicating a reversible

intermediate analogous to the Michaelis complex (Kitz & Wilson, 1962; Main, 1964; Main & Iverson, 1966). In our experiments there was no indication of a reversible complex at any pH between 5.5 and 10.5 with six compounds out of seven studied. However, with phosphostigmine there is kinetic evidence for the presence of a Michaelis-type reversible intermediate. The K_m values for phosphostigmine ($2.1 \times 10^{-4} M$) and acetylcholine ($2 \times 10^{-4} M$; Ellman *et al.* 1961) are similar. Phosphostigmine and acetylcholine differ markedly in their rates of acylation, which are about 8 min.^{-1} for phosphostigmine and at least $295\,000 \text{ min.}^{-1}$ for acetylcholine (V_{max} at 37° according to Cohen & Warringa, 1953). The second-order rate constant for acylation by phosphostigmine is $4.2 \times 10^4 \text{ l. mole}^{-1} \text{ min.}^{-1}$, whereas for acetylcholine it must be at least:

$$k_a = \frac{V_{\text{max}}}{K_m} = \frac{295\,000}{2 \times 10^{-4}} = 1.5 \times 10^9 \text{ l. mole}^{-1} \text{ min.}^{-1}$$

The k_a found for the most effective inhibitors of cholinesterase approaches this value (Hobbiger, 1954; Heath & Vandekar, 1957; Tammelin, 1958).

With the exception of the 'aging' process (Berends, Posthumus, Sluys & Deierkauf, 1959; Janz, Brons & Warringa, 1959; Heilbronn, 1963*a,b*; Lamb & Steinberg, 1964; Coult, Marsh & Read, 1966; Berry & Davies, 1966) the reactions of inhibitor and substrate with enzyme seem to be formally identical, the major difference being the rates of the partial reactions (Table 4). This is the basis for the experiments described in the present

Table 4. Catalytic-centre activity for inhibitors or substrates of acetylcholinesterase

Acyl-enzyme or substrate	Enzyme (temp., pH)	Catalytic-centre activity	Reference
Diethylphosphoryl-enzyme	Rabbit erythrocyte (37°, pH 7.8)	~0.0005	Aldridge (1954)
Dimethylphosphoryl-enzyme	Rabbit erythrocyte (37°, pH 7.8)	0.00855	Aldridge (1953)
Di-(2-chloroethyl)phosphoryl-enzyme	Sheep erythrocyte (27°, pH 7.2)	0.017	Lee (1964)
Di-(2-chloroethyl)phosphoryl-enzyme	Bovine erythrocyte (25°, pH 8.0–8.5)	0.0168	This paper
Monomethylcarbamoyl-enzyme	Electric eel (25°, pH 7.0)	0.018	Wilson, Harrison & Ginsburg (1961)
Monomethylcarbamoyl-enzyme	Bovine erythrocyte (37°, pH 7.4)	0.0284	Winteringham & Fowler (1966)
Monomethylcarbamoyl-enzyme	Bovine erythrocyte (25°, pH 7.4)	0.0113	Reiner & Simeon-Rudolf (1966)
Monomethylcarbamoyl-enzyme	Bovine erythrocyte (25°, pH 8.0–8.5)	0.0234	This paper
Monomethylcarbamoyl-enzyme	Bee heads (30°, pH 8.0)	0.027	Kunkee & Zweig (1965)
Monomethylcarbamoyl-enzyme	Fly heads (30°, pH 8.0)	0.029	Kunkee & Zweig (1965)
Dimethylcarbamoyl-enzyme	Electric eel (25°, pH 7.0)	0.025	Wilson <i>et al.</i> (1961)
Dimethylcarbamoyl-enzyme	Bovine erythrocyte (25°, pH 8.0–8.5)	0.0123	This paper
Dimethylcarbamoyl-enzyme	Rat brain (<i>in vivo</i>)	0.013	Myers (1956)
Carbamoyl-enzyme	Electric eel (25°, pH 7.0)	~0.35	Wilson <i>et al.</i> (1961)
Acetylcholine	Bovine erythrocyte (37°, pH 7.4)	295 000	Cohen & Warringa (1953)
Acetylcholine	Electric eel (30°, pH 7.0)	530 000–2 200 000	Lawler (1961)
Acetylcholine	Electric eel (25°, pH 7.4)	610 000	Kremzner & Wilson (1964)

paper, for the rates of acylation and deacylation for inhibitors may be directly determined; this is not possible with substrates.

The influence of pH on the rate of return of enzyme activity after formation of dimethyl-carbamoyl-, monomethylcarbamoyl- and di-(2-chloroethoxy)phosphoryl-cholinesterase is identical over the range pH 6.5–11.0 (pK_1 6.9 and pK_2 9.8). This fact, in view of the widely different acyl groups involved, suggests that a similar pH-dependence for acetylated cholinesterase is to be expected. This is contrary to the conclusion of Krupka (1966*a,b*) that pK_1 for deacetylation is 6.2; Krupka's (1966*a,b*) conclusion, however, depends on evidence taken to indicate that deacetylation is rate-limiting in the reaction of cholinesterase with acetylcholine (Krupka, 1964). On the basis of observations on other enzymes, conformation change in proteins is being considered generally as a part of the catalytic mechanism. If this is accepted then it is extremely difficult to be sure, in experiments with substrates, which is the rate-limiting step.

Lowering of the pH below 8 decreases the rate of carbamylation and phosphorylation by compounds containing a positive charge, and the extent of the decrease fits very well the dissociation curve for a group of pK 6.2. Carbamates and phosphates without a positive charge do not fit such a curve; for this reason we deduce that a group of pK 6.2 is concerned in the approach of compounds with a positively charged group to the enzyme. The increase of the Michaelis constant for phosphostigmine at pH 5.6 is consistent with this conclusion. Krupka's (1966*a,b*) results on cationic substrates agree with this conclusion; Bergmann, Segal, Shimoni & Wurzel (1956) obtained the same pK for inhibition of cholinesterase with tetraethylammonium. Theoretically this group could attract or repel a positively charged group. For cholinesterase, attraction to a negatively charged group seems much less likely than repulsion by a positively charged group (cf. Krupka, 1966*a,b*).

Two non-cationic carbamates were equally effective as inhibitors from pH 8.0 down to 5.5, and two other compounds, one carbamate and one phosphate, have slightly decreased inhibitory power at low pH. We have no explanation for these findings, but it seems safe to conclude that acylation can take place without the aid of any groups ionizing between pH 5.5 and 8.0.

Previous work by other authors on the influence of pH on inhibitory power of carbamates or phosphates cannot be directly compared with our results. Comparisons have been made between pH and either percentage inhibition by a given concentration of inhibitor or the concentration of inhibitor to produce 50% inhibition (Mounter, Alexander, Tuck & Dien, 1957; Wilson & Bergmann, 1950).

Experimental procedures and lack of information prevent recalculation of these results in order that they may be compared with the present ones. In one paper (Wilson, 1951) the effect of pH on inhibition of electric-eel cholinesterase by neostigmine has been studied and a suitable rapid determination of enzyme activity was made. By making certain assumptions, recalculation from these results of the bimolecular rate constants shows excellent agreement with our results between pH 5.2 and 8.0.

What can be derived from comparisons of pH effects studied in the present paper? It is not possible to attribute the pK to individual amino acids in the protein structure. Substitution of an amino acid into a protein may considerably alter its pK (Tanford, 1962; Edsall & Wyman, 1958). Suggestions about the importance of histidine must be derived primarily from other information. Also, if conformation or other changes are accepted, the possibility cannot be excluded that the values pK_2 9.8 for deacylation and pK_2 10.25 for acylation are reflections of the same group. Only differences in the form of the curve with different compounds allow an attempt at interpretation. The fact that increasing the pH above 8.5 decreases the inhibitory power of all compounds studied to an identical extent may mean that the ionization of one group influences the general properties of the protein, but this group may not necessarily be involved in the chemical mechanism of acylation.

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