# The pH-Dependence of Enzymic Ester Hydrolysis

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The esterases of animal tissues have generally been characterized by their substrate specificity and by their behaviour towards various inhibitors. The irreversible inhibition by organic phosphates, which is due to phosphorylation of the active group (Burgen, 1949; Wilson & Bergmann, 1950*a*; Aldridge, 1953*d*), is a common property of most esterases, an interesting exception being the A-esterase of Aldridge (1953*a*, *b*). This general behaviour suggests a similar structure of most of the esteratic groups responsible for the hydrolytic reaction.

True cholinesterase has been characterized, in addition to the properties mentioned, by the dissociation constants of its esteratic site (Wilson & Bergmann, 1950*b*). However, the negative charges present in the active centre of this enzyme are also pH-dependent and thus interpretation of the experimental data obtained with acetylcholine (ACh) as substrate is difficult (Bergmann & Shimoni, 1952*a*). This difficulty can be met by studying the influence of pH changes on the enzymic hydrolysis of uncharged esters, since in such a system all variations of activity can be ascribed solely to structural changes of the esteratic site.

In the present investigation, such procedures have been applied to true and pseudocholinesterase and to an unspecific liver esterase. From these experiments certain conclusions about the chemical structure of the esteratic sites can be drawn, and a rational interpretation can be derived for the bell-shaped curves relating hydrolytic rates to substrate concentration which have been found not only in the system true cholinesterase–acetylcholine, but with other esterases and a variety of uncharged substrates (Bergmann & Shimoni, 1953). In addition, the dissociation constants of the anionic sites in both types of cholinesterases have been determined and a tentative explanation of the curious values obtained has been advanced.

Part of the results described in this paper have been communicated at the Meeting of the Faraday Society held at Oxford in August 1955 (Bergmann, 1955).

## MATERIALS AND METHODS

*Substrates.* The esters used were commercial samples, purified by distillation. The preparation and properties of the fluoroacetates have been described previously (E. D. Bergmann & Blank, 1953; Bergmann & Shimoni, 1953). For the pH range 5–8·5 the buffer used was 0·1 M phosphate, for higher pH values 0·1 M borate. Sodium was the only cation present.

*Enzyme preparations.* Two samples of true cholinesterase were used. (*a*) A preparation from the electric organ of *Torpedo marmorata* was obtained through the courtesy of Professor D. Bovet, Istituto Superiore de Sanita, Rome. The purified solution of the enzyme, when diluted 1:30 000, hydrolysed 9·6 $\mu$moles/ml./hr., with $4 \times 10^{-3}$ M ACh as substrate. (*b*) The enzyme from *Electrophorus electricus* was a gift of Professor C. Chagaz, Department of Physiology, University of Brazil, Rio de Janeiro. The eel enzyme, diluted 1:1000, hydrolysed 6 $\mu$moles of ACh/ml./hr., with the same substrate concentration as before.

The pseudocholinesterase used was the crystalline fraction IV-6-III from human serum, prepared in the laboratory of the late Professor E. Cohn, Harvard Medical School, Boston. This preparation (0·1 mg./ml.) hydrolysed 8 $\mu$moles/ml./hr., with $6 \times 10^{-2}$ M ACh as substrate.

*Dog-liver esterase.* This was prepared as described previously (Bergmann & Shimoni, 1952*b*). However, the colour of the preparation interfered with the analytical procedure and its high protein content caused turbidity and precipitation at acid pH. Therefore, the protein fraction, obtained from dog liver between 17 and 25% (w/v) $(NH_4)_2SO_4$, was dissolved in distilled water, brought to 15% (w/v) $(NH_4)_2SO_4$ and the precipitate discarded. The next fraction, precipitating between 15 and 25% (w/v) $(NH_4)_2SO_4$, represented approximately two-thirds of the total enzymic activity. It was further purified by repetition of the above procedure, but fractionating within narrower limits, i.e. (*a*) 0–15%, (*b*) 15–20%, (*c*) 20–23% and (*d*) 23–25% (w/v) $(NH_4)_2SO_4$. The last precipitate (III*d*), with a protein content of 16·6 mg./ml., was practically colourless and served for the preparation of the stock solution. When diluted 1:100, it produced 6·6 $\mu$moles of $CO_2$/ml./hr., with 0·43 M diacetin as substrate.

It is noteworthy that the ratio of activity for diacetin and *iso*propyl fluoroacetate remained approximately constant during the various purification steps, whereas the enzymic hydrolysis of acetyl- and propionyl-choline decreased steadily. The liver enzyme III*d*, which was used in the colorimetric experiments, showed such a low activity towards choline esters that measurements with these substrates had to be extended over long periods. Therefore, extrapolation of the enzymic activity to zero time, a necessary procedure in view of the competition between $H^+$ ion and cationic substrate (Wilson & Bergmann, 1950*b*), became unreliable.

*Hydrolysis.* This was measured by the method of Hestrin (1949), which was found applicable to alkyl halogenoacetates with the modifications described in Table 1.

*Ester concentrations.* These were selected according to the following considerations: (1) Initial solutions had to be made without ethanol, which inhibits the enzymes studied to various degrees (Bergmann & Shimoni, 1953). (2) Addition of the ester to the incubated mixture of enzyme plus buffer at zero time should not dilute this mixture appreciably, in order not to disturb the equilibrium established between enzyme and hydrogen ions. (3) The final concentrations of substrate should be near that yielding maximal rates as determined previously (Bergmann & Shimoni, 1953). (4) The samples taken out for colorimetric determination should be large enough for accurate analysis, the upper limit being set by a colorimetric reading of 300 on a Klett–Summerson photoelectric colorimeter. (5) The equilibrium between $H^+$ ions and enzyme is disturbed by the substrate, which competes for the active surface. Therefore, the rate of hydrolysis has to be measured under non-competitive conditions, i.e. by extrapolation to zero time. This requires sampling at short intervals. Thus, at pH 6·5–8 samples were usually taken out at 1, 2, 3 and 4 min. In the acid range, increasingly longer reaction times had to be used, in view of the rapidly decreasing activity of the enzymes. In the alkaline range, such an adjustment was not feasible because of the spontaneous hydrolysis of the esters. The conditions used in these experiments are shown in Table 2.

The rates were calculated by plotting ester hydrolysis as a function of time and extrapolating to zero time. The spontaneous hydrolysis was measured at the same time and subtracted from the enzymic rates. The correction was negligible for *n*-propyl acetate at any pH. In the alkaline range (above pH 9) all halogenoacetates underwent non-

Table 1. *Colorimetric determination of esters as hydroxamic acids*

The sample taken was made up to 1 ml., then 2 ml. of a mixture of equal parts of 2 M hydroxylamine sulphate and 3·5 N-NaOH was added and the solution incubated, as indicated. Then 1 ml. each of 3·5 N-HCl and of 0·74 M-$FeCl_3$, dissolved in 0·1 N-HCl, were added. Readings were taken immediately with a Klett–Summerson photoelectric colorimeter. Results are given per μmole of ester/ml. of the final mixture.

| | Reaction with $NH_2OH$ | | | | |
|---|---|---|---|---|---|
| Ester | Temp. (°) | Time (min.) | Klett units/ μmole/ml. | Klett filter no. | Fading rate of ferric complex, Klett units/min. |
| *n*-Propyl fluoroacetate | 30 | 10 | 59 | 50 | 2 |
| *iso*Propyl fluoroacetate | 35 | 40 | 58 | 50 | 2 |
| *n*-Butyl fluoroacetate | 35 | 30 | 58 | 50 | 3 |
| Ethyl chloroacetate | 27 | 4 | 60 | 50 | 10 |
| *n*-Propyl chloroacetate | 27 | 4 | 60 | 50 | 6 |
| Ethyl bromoacetate | 27 | 4 | 58 | 50 | 5 |
| *n*-Propyl acetate | 27 | 2 | 82 | 54 | 0 |
| Acetylcholine | 27 | 1 | 98 | 54 | 1 |

Table 2. *Conditions of enzymic hydrolysis of various esters*

Initial substrate concentration defines the standard solution prepared, portions of which were added to the previously incubated mixture of enzyme and buffer, so as to obtain the final substrate concentration. Enzyme dilution refers to the stock solutions, described in Methods.

| | | | Substrate concn. (M) | | | |
|---|---|---|---|---|---|---|
| Ester | Temp. (°) | Enzyme dilution | Initial | Final | Enzymic hydrolysis at pH 7 (μmoles/ ml./min.) | Size of sample (ml.) |
| (1) Liver esterase | | | | | | |
| *n*-Propyl acetate | 24 | 1:30 | $3 \times 10^{-2}$ | $1 \times 10^{-2}$ | 1·0 | 0·3 |
| *iso*Propyl fluoroacetate | 25 | 1:27 | $1{\cdot}5 \times 10^{-1}$ | $2{\cdot}5 \times 10^{-2}$ | 1·4 | 0·2 |
| Ethyl chloroacetate | 24 | 1:50 | $7{\cdot}5 \times 10^{-2}$ | $2{\cdot}5 \times 10^{-2}$ | 1·2 | 0·2 |
| (2) Serum cholinesterase | | | | | | |
| Acetylcholine | 36·5 | 1:3000 | $10^{-1}$ | $10^{-2}$ | 0·5 | 0·3 |
| *n*-Propyl fluoroacetate | 36·5 | 1:1000 | $9 \times 10^{-2}$ | $3{\cdot}6 \times 10^{-2}$ | 0·75 | 0·15 |
| *n*-Propyl chloroacetate | 37 | 1:1000 | $2 \times 10^{-2}$ | $10^{-2}$ | 0·6 | 0·5 |
| (3) Torpedo esterase | | | | | | |
| Acetylcholine | 23 | 1:10 000 | $6{\cdot}6 \times 10^{-2}$ | $3{\cdot}3 \times 10^{-3}$ | 0·5 | 0·8 |
| *n*-Propyl fluoroacetate | 23 | 1:1000 | $9 \times 10^{-2}$ | $3{\cdot}6 \times 10^{-2}$ | 0·4 | 0·15 |
| *n*-Propyl chloroacetate | 23 | 1:500 | $2{\cdot}4 \times 10^{-2}$ | $8 \times 10^{-3}$ | 0·6 | 0·6 |
| (4) Eel esterase | | | | | | |
| Acetylcholine | 24 | 1:125 | $5 \times 10^{-2}$ | $5 \times 10^{-3}$ | 0·8 | 0·6 |
| *n*-Butyl fluoroacetate | 23 | 1:300 | $3 \times 10^{-2}$ | $10^{-2}$ | 0·6 | 0·5 |
| Ethyl chloroacetate | 23 | 1:30 | $7{\cdot}5 \times 10^{-2}$ | $2{\cdot}5 \times 10^{-2}$ | 2·0 | 0·2 |
| Ethyl bromoacetate | 23 | 1:50 | $9 \times 10^{-2}$ | $2{\cdot}4 \times 10^{-2}$ | 2·2 | 0·2 |

enzymic hydrolysis, the descending order being bromo-, chloro-, fluoro-acetate. All figures were expressed as percentage of the maximum rate of the enzyme–substrate system under consideration.

## RESULTS

### *Dissociation constants of the esteratic sites*

In Fig. 1 the results with dog-liver esterase have been summarized. All curves are similar and demonstrate that a polar substituent in the acyl portion of the ester has little influence on the position of the pH optimum, which for this enzyme is rather sharply defined. Acidic and basic dissociation constants of the esteratic site are thus practically independent of the type of substrate used. Both *iso*propyl fluoroacetate and ethyl chloroacetate have been found previously to possess a p$S$ ($-\log S$, where $S$ is the substrate concentration) maximum (Bergmann & Shimoni, 1953), showing that in the system liver esterase–halogenoacetate a non-hydrolysable $ES_2$ complex is formed by excess of substrate.

We can thus describe the system by the same four equilibria, applied previously to the combination true cholinesterase–acetylcholine (Wilson & Bergmann, 1950*b*), and use the equations derived there for the determination of p$K_a$ and p$K_b$, the dissociation constants of the nucleophilic and the electrophilic groups of the esteratic site. The figures obtained in this way are represented in Table 3.

When applying the same procedure to pseudocholinesterase, we have to take into account the presence of an anionic site (Bergmann & Wurzel, 1954; Bergmann & Segal, 1954), which becomes progressively inactivated below pH 7 by recombination with $H^+$ ions. However, with uncharged substrates we may neglect as a first approximation the influence of the anionic site on ester hydrolysis at the esteratic site or the changing polar forces

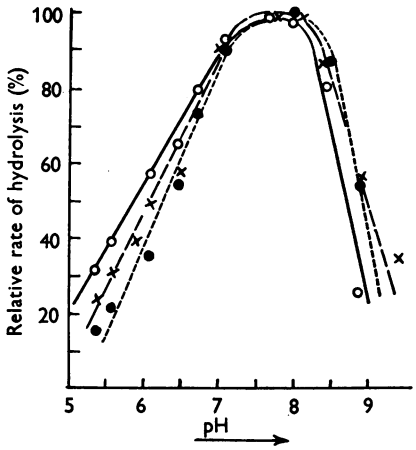


Fig. 1. pH-dependence of enzymic hydrolysis by dog-liver esterase. ○, Ethyl chloroacetate; ×, *iso*Propyl fluoroacetate; ●, *n*-Propyl acetate. Enzyme and substrate concentrations are given in Table 2. Buffer used for pH <8·5 is 0·1 M phosphate, for pH >8·5, 0·1 M borate, as their sodium salts.

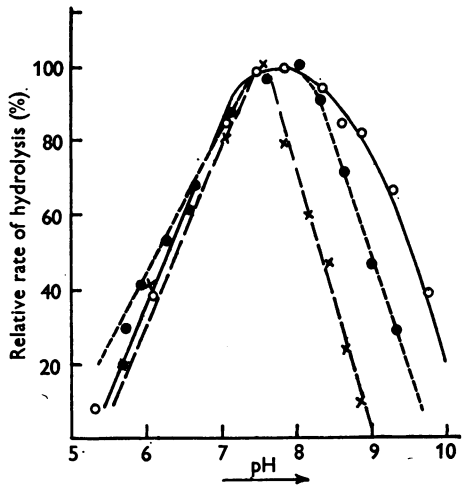


Fig. 2. pH-dependence of enzymic hydrolysis by human-serum cholinesterase. ○, Acetylcholine; ●, *n*-propyl chloroacetate; ×, *n*-propyl fluoroacetate. For details of experimental conditions see Table 2.

Table 3. *Dissociation constants of the components of the active centre of various esterases*

| Enzyme | Substrate or inhibitor | p$K_a$ of esteratic site | p$K_b$ of esteratic site | p$K$ of anionic site |
|---|---|---|---|---|
| Dog-liver esterase | Ethyl chloroacetate | 6·1 | 9·4 | — |
| | *iso*Propyl fluoroacetate | 6·8 | 9·2 | — |
| Human-serum cholinesterase | *n*-Propyl chloroacetate | 6·2 | 9·0 | — |
| | Tetraethylammonium bromide | — | — | 6·5 |
| True cholinesterase from *Torpedo marmorata* | *n*-Propyl fluoroacetate | 6·2 | 9·0 | — |
| | Tetraethylammonium bromide | — | — | 6·3 |
| True cholinesterase from *Electrophorus electricus* | Ethyl chloroacetate | 6·7 | 9·6 | — |
| | Ethyl bromoacetate | 6·5 | 9·0 | — |
| | Tetraethylammonium bromide | — | — | 6·2 |

between the anionic site and the halogen atom. We apply therefore the same equations as above. Indeed, justification for this procedure can be found in the similarity of the pH activity curves in Fig. 2 with those in Fig. 1. The dissociation constants, derived for human-serum cholinesterase, are in the same range as those for liver esterase (see Table 3). However, the curve for ACh deviates from those for all other substrates, mainly in the alkaline range. This phenomenon, which is probably related to the interaction of the cationic group in ACh with the anionic site, is discussed below.

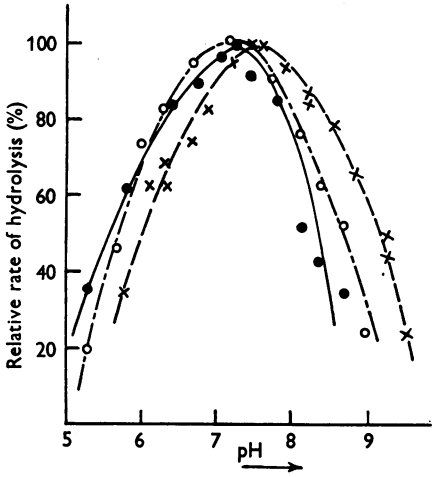


Fig. 3. pH-dependence of enzymic hydrolysis by *Torpedo* esterase. ×, Acetylcholine; ●, *n*-propyl chloroacetate; ○, *n*-propyl fluoroacetate. For details of experimental conditions see Table 2.

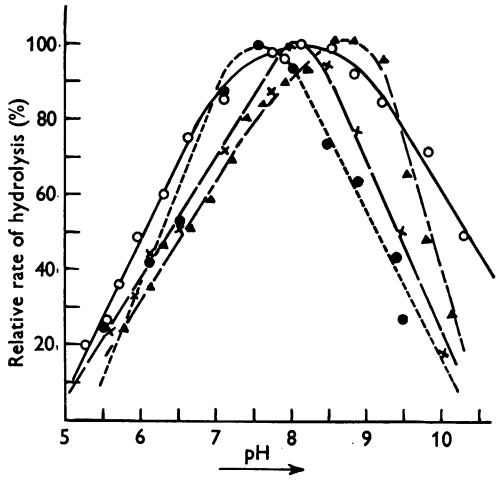


Fig. 4. pH-dependence of enzymic hydrolysis by eel esterase. ○, Acetylcholine; ×, ethyl chloroacetate; ●, ethyl bromoacetate; ▲, *n*-butyl fluoroacetate. For details of experimental conditions see Table 2.

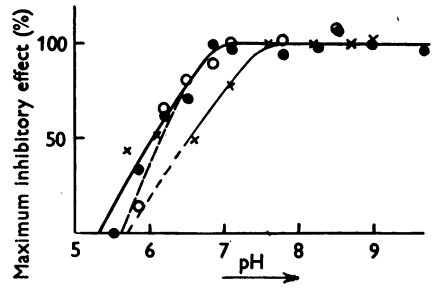


Fig. 5. pH-dependence of the inhibitory effect of tetraethylammonium bromide on cholinesterases. ●, Eel esterase 1:125, ACh $4 \times 10^{-3}$ M, $Et_4N^+$ $5 \times 10^{-3}$ M; ○, *Torpedo* esterase 1:5000, ACh $4 \times 10^{-3}$ M, $Et_4N^+$ $2{\cdot}5 \times 10^{-2}$ M; ×, serum cholinesterase (quoted from Bergmann & Wurzel, 1954).

The two representatives of true cholinesterase, used in our experiments, show a different pH dependence (Figs. 3, 4). The *Torpedo* enzyme is similar to serum cholinesterase, but for the system eel esterase–*n*-butyl fluoroacetate a shift of the pH optimum to about 9 has been observed. This behaviour is shared by the ethyl and *n*-propyl esters and may be ascribed to an interaction of the fluorine atom with a group at the enzyme surface.

*Dissociation constants of the anionic sites*

For the evaluation of these constants the pH-dependence of inhibition by tetraethylammonium bromide has been used. The enzyme and a constant concentration of inhibitor were incubated with buffers of various pH for 1 hr., then ACh was added and hydrolysis determined after 1, 2, 3 and 4 min. The rates obtained by extrapolation to zero time were compared with the inhibitor-free controls for the calculation of the percentage inhibition. The maximum inhibitory effect (between pH 7 and 9) was then put equal to 100 and the results in the acid pH range were expressed as a percentage of this maximum, to compare different sets of experiments with different enzymes (see Fig. 5). The descending branches of the curves in Fig. 5 have been treated as titration curves to evaluate the apparent dissociation constants of the anionic sites. The figures derived in this way are also included in Table 3.

## DISCUSSION

The results in Table 3 demonstrate that for all esterases used in the present study the dissociation constants of the esteratic site, $pK_a$ and $pK_b$, fall within a rather narrow range. This supports the assumption that the active group, responsible for hydrolysis, is essentially the same in all of them.

Since no prosthetic group has been found in esterases, we may assume that the components of

the active centre are amino acids. On this basis, the value of $pK_a \approx 6{\cdot}5$ suggests the presence of histidine, the iminazole group of which is in equilibrium with its cation and possesses a p$K$ of 6–7 in various peptide combinations (Cohn & Edsall, 1943). It should be noted that our value for $pK_a$ is lower than the corresponding value of $pK_{EH_3}^+ = 7{\cdot}2$, derived previously from the system eel esterase–ACh (Wilson & Bergmann, 1950*b*).

The second dissociation constant, $pK_b \approx 9{\cdot}3$, has been interpreted previously to indicate an $\alpha$-amino group (Bergmann, 1955). However, in peptides the p$K$ of this group acquires a value of about 7·1–8·2 (Ellenbogen, 1952). Our previous assumption thus needs correction. It now appears likely that the group responsible for the second dissociation constant is represented either by the *p*-hydroxyl group of tyrosine (p$K$ in various peptides, 9·3–9·8) or, less probably, by the $\epsilon$-amino group of lysine (p$K$, 9·6–10·7). In the former, we would have to assume that the un-ionized phenolic group is the active form and that dissociation into the phenoxide ion destroys the enzyme activity. For the $\epsilon$-amino group of lysine, on the other hand, we would have to conclude that only the ammonium form is active and that its conversion into the free base abolishes enzymic activity.

If, as is usually assumed, the active portion of the enzyme molecule is composed of amino acids only, the anionic sites are most probably represented by the free carboxyl group of aspartic or glutamic acid (Bergmann, 1955). However, the $\beta$-carboxyl of the former in various peptides has a p$K$ of 3·0–4·5, and the $\gamma$-carboxyl of the latter shows a p$K$ of 4·2–4·5. Thus, the experimental values found (see Table 3) are 2–3 units too high, but are surprisingly near the p$K$ value of the iminazole group. This suggests that we are measuring, in our experiments concerned with the pH variation of the anionic site (Fig. 5), not its inactivation by recombination with $H^+$ ions, but its association with the iminazolinium ion of the esteratic site. The anionic site is, so to speak, titrated by iminazolinium ion. In the true enzyme, we would have to assume that both carboxylate groups combine electrostatically with the same cation. The probability of the formation of such ion complexes is enormously increased on the surface of a protein molecule, because both anion and cation have only restricted freedom of motion, and the water, bound to the surface, behaves like a medium of low dielectric constant. In this connexion it is of interest that neutralization of the two anionic sites in the true enzyme occurs at a somewhat lower pH than that of the single negative charge in the pseudo enzyme. In the former, the iminazolinium ion has to compete with two positive ions in the surrounding solution.

To summarize, we conclude that the active site of esterases comprises at least two amino acid residues, namely histidine and tyrosine. For pseudocholinesterase one residue of glutamic (or aspartic) acid has to be included, for the true enzyme two. In the latter, the two glutamate fragments may either be separated by one or more of the other amino acids required or they may be adjacent to each other. A model based on the latter alternative has been constructed and shows that in this case the distance between the two negative charges is of the appropriate size to accommodate such a bis-quaternary ion as decamethonium.

The structure of the active surface, suggested by the above considerations, sheds light on several experimental facts, which have so far eluded a rational explanation.

(1) A bell-shaped p$S$–activity curve is observed not only for the system true cholinesterase–ACh, but also for the other esterases used in the present experiments, especially when halogenoacetates form the substrates (Bergmann & Shimoni, 1953). It should be noted that in these cases a second polar substituent is usually present in the molecule in addition to the ester grouping. Apparently, this favours the formation of an $ES_2$ complex. If there are two polar groups available, which can combine with corresponding polar portions of the protein molecule in the neighbourhood of the active surface, the affinity of the ester molecule becomes sufficiently large to replace water on the active surface. (Within the active centre, we assume, a small number of water molecules required for hydrolytic reactions are being held tightly.) Accordingly, when the affinity of a given ester type is increased by lengthening the carbon chain of the alcoholic portion, the p$S$ optimum shifts to higher values (Bergmann & Shimoni, 1953). It is of great interest that pseudocholinesterase cannot form an $ES_2$ compound with ACh, but does so with a substrate like ethyl fluoroacetate. This finding again stresses the importance of the second anionic site in the true enzyme, which provides the anchor to which the second molecule of ACh can be attached.

(2) A further problem arises from consideration of the relative rates of hydrolysis within the homologous series of choline esters. With the pseudo enzyme, the rate increases with increasing length of the acyl side chain, the values reported being acetylcholine : propionylcholine : butyrylcholine = 1 : 1·5 : 2 (Augustinsson, 1949). On the other hand, for the true enzyme the relative rates are approximately 1 : 0·9 : 0·01, the absolute values differing somewhat for different preparations (e.g. Aldridge, 1953*c*; Nachmansohn & Rothenberg, 1945). Butyrylcholine (BuCh) is, however, well adsorbed by the active surface of the true enzyme, as indicated by the concentration ($2{\cdot}5 \times 10^{-3}$ M) required to produce

50% inhibition of ACh hydrolysis (Bergmann & Shimoni, 1953). This shows that it is the step involving reaction of BuCh with water that does not proceed at a satisfactory rate. We may express this fact by saying that for true cholinesterase autoinhibition of hydrolysis occurs with ACh, when *two* molecules combine with the active surface, whereas with BuCh *one* molecule produces the same effect. We shall therefore try to explain these two phenomena on a common basis. From a model of the active surface built from the four units mentioned above, it became apparent that the space available around the esteratic site is very restricted, being limited by the presence of the two anionic groups, so that the amount of water that can be adsorbed simultaneously with a substrate molecule may become the critical factor. Therefore, with a short acyl chain as in ACh, the second substrate molecule competes with the water necessary for reaction. With BuCh, the acyl chain of one ester molecule is already long enough to replace practically all the water which can be adsorbed on the active surface and thus prevents its own hydrolysis.

The curves for ACh in Figs. 2–4 show rather broad pH maxima. This is especially pronounced with eel esterase, in accordance with the observations of earlier investigators (Hestrin, 1950; Wilson & Bergmann, 1950*b*). In the pH range of 5–7 the curve for ACh deviates little from that for the halogenoacetates examined, but a pronounced difference appears on the alkaline side, where the decay of enzymic hydrolysis is delayed for ACh. This is another expression of the fact that the anionic sites play a major role in the combination enzyme–ACh. As has been calculated previously (Bergmann & Segal, 1954), the coulombic forces represent about 90% of the total binding force of ACh to true cholinesterase. Therefore the affinity of the cationic substrate for the anionic sites outweighs, to a certain extent, the decrease in affinity produced by inactivation of the electrophilic group in the esteratic site. In the acid pH range such an effect cannot be observed, owing to the phenomenon mentioned above, namely, the iminazole group, becoming inactivated by combination with $H^+$ ion, neutralizes the anionic sites.

These assumptions are supported by the following observations: we have tried to construct the pH–activity curve of ACh in Figs. 2 and 4 by superimposing the dissociation curve of the anionic sites as exemplified in Fig. 5, and the pH–activity curve of an uncharged ester, namely ethyl chloroacetate. Although the selection of just this substrate is arbitrary, similar if somewhat less satisfactory results are obtained with the other halogenoacetates. In Fig. 6 the superimposed curves are compared with the experimental data.

A surprising shift of the pH optimum has been observed for the system eel esterase–alkyl fluoroacetate (see Fig. 4). Since the corresponding phenomenon is absent with the *Torpedo* enzyme (and all other esterases), this may indicate that near the active centre of the eel esterase a group is present with a high affinity for fluorine, probably by hydrogen bonding, which thus outweighs to a certain extent the decrease in affinity occurring in the alkaline pH range.

As a final point, we should mention that in degradation studies of various hydrolytic enzymes, including cholinesterases, after their irreversible inhibition by di*iso*propyl phosphorofluoridate

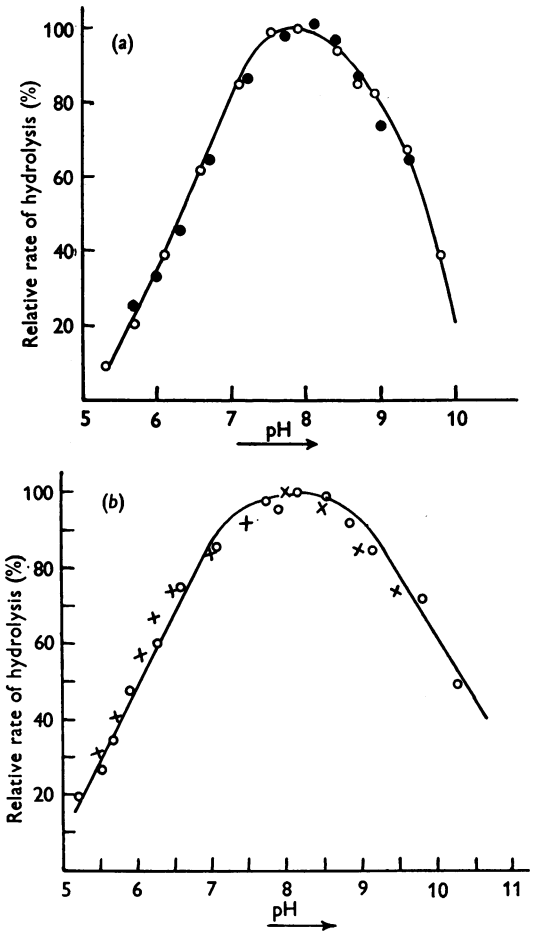


Fig. 6. Comparison of the experimental data for the pH-dependence of the system cholinesterase–ACh with theoretical values. The theoretical points represent the arithmetic mean of the values in Fig. 5 on one hand and the values for chloroacetate in Fig. 2 (serum cholinesterase) or Fig. 4 (eel esterase) on the other. (*a*) Plasma cholinesterase. ○, Experimental; ●, calculated. (*b*) Eel esterase. ○, Experimental; ×, calculated.

(DFP), Schaffer, May & Summerson (1953, 1954) and Oosterbaan, Kunst & Cohen (1955) found the organic phosphate linked to a serine residue. It has been shown by Jandorf, Michel, Schaffer, Egan & Summerson (1955) and by Hobbiger (1955) that the inhibitory reactions with phosphates pass through two reaction steps, so that one may assume that the phosphate–serine linkage represents the final stage of the interaction between enzyme and inhibitor. It is difficult to see how the hydroxyl group of serine participates in ester hydrolysis, since a spontaneously reversible acylation of the esteratic site is required (Bergmann, Wilson & Nachmansohn, 1950). In addition, the dissociation constants of the esteratic site give no information about the possible participation of serine. Nevertheless, one has to admit that the active centre of an enzyme may comprise units which do not undergo any change within the pH range measurable for the enzymic reaction. Therefore the problem of a possible role of serine in enzymic ester fission remains unsolved at present.

## SUMMARY

1. The change of hydrolytic rate with pH has been studied for different types of esterases and with a variety of uncharged (non-cationic) substrates. From these measurements, the dissociation constants of the nucleophilic ($pK_a$) and the electrophilic ($pK_b$) group of the esteratic site have been calculated for each enzyme–substrate system.

2. The esteratic sites of all esterases studied possess approximately the same dissociation constants. The chemical structure of the active surfaces of various esterases is thus closely related.

3. The dissociation constants of the negative sites of true cholinesterase and pseudocholinesterase have been derived from the pH-dependence of the inhibitory effect of tetraethylammonium ion. The curious values obtained, 6·2–6·5, seem to indicate neutralization at acid pH of the anionic sites by the iminazolinium ion of the esteratic group.

4. Possible structures of the esteratic sites have been discussed, and explanations put forward for the inhibition by large substrate concentration of the hydrolysis of cationic and uncharged esters and for the different behaviour of true cholinesterase and pseudocholinesterase towards the homologous series of aliphatic choline esters.

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