The Influence of pH on the Enzymic Hydrolysis of Acetylcholine

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Since Sørensen¹ in 1909 pointed out the influence that pH has on the activity of enzymes a great many systems have been investigated in this respect. As far as acetylcholinesterase is concerned, the estimation of the activity of the enzyme is usually based upon a determination of the acetic acid liberated by the hydrolysis of acetylcholine. The acid can be determined either by titration or by measuring the amount of CO₂ which is evolved from a bicarbonate buffer. The Warburg manometric method is most frequently used, but is not suitable for studies on the influence of pH because the pH of the reaction mixture can only be varied within narrow limits. Recently two new procedures for the estimation of the activity have been reported, one colourimetric (Hestrin²) and one electrometric (Michel³). In the present investigation, the colourimetric method was employed.

One of the main purposes of this investigation was to determine the influence of pH on the activity-pS curve of the system acetylcholine-acetylcholinesterase, *i.e.*, the plot of enzyme activity versus pS (= -log molar concentration of the substrate). In addition the stability of enzyme solutions at various values of pH was studied. The stability of the enzyme in various buffers at 4° and 25° C was also measured, and the rate of enzymic hydrolysis of some esters other than acetylcholine has been determined at different values of pH and pS.

METHODS

The enzymic hydrolysis was studied by determining the amount of unchanged substrate, by conversion of the ester to a hydroxamic acid by treatment with alkaline hydroxylamine. Treatment of the hydroxamic acid with ferric chloride in acid solution gives a red complex, and the extinction coefficient of the solution at 5400 Å was measured

Fig. 1. Relative extinction of a coloured solution as function of the time of standing.

promptly with an EEL* colorimeter. As pointed out by Hestrin and clearly demonstrated by Fig. 1 the colour fades rather rapidly.

Crystallised cobra venom was used as the enzyme preparation as it contains no other esterases⁴. The substrates used were acetylcholine iodide (ACh), acetylsalicylcholine chloride (ASaCh), acetylsalicylic acid (ASa) and salicylcholine chloride (SaCh)⁵. Substrates and enzyme were dissolved in buffers, and in order to get optimal activity, all the solutions contained magnesium ions in the form of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ at a concentration of 1.73×10^{-3} molar. All reactions were carried out in a thermostat at 25° C.

RESULTS

The reaction of hydroxylamine with esters. Various esters were converted to the corresponding hydroxamic acids and treated with ferric chloride, and the extinctions of the coloured solutions were determined. A 2.00×10^{-3} molar solution of ACh was taken as standard, and the extinction of the solution of the derived complex was assigned the value 100. The extinctions of other compounds investigated, at the same molarity, are collected in Table 1. Substances insoluble in water were dissolved in 95 % ethanol (0.1 ml) which does not affect the colour reaction.

The relative extinction values for substances with only one ester linkage are very scattered, varying from 210 (ASa) to 0 (prostigmine). On the other hand, compounds containing two or more ester linkages seems to have extinctions predominantly around 100.

One pair of isomers, 9- and 3-acetoxyretenes, have extinctions of 85 and 87, but the difference can hardly be regarded as significant.

Hestrin has determined the extinction of about 40 compounds, and some of his values are collected in Table 1. With the exception of the values for ethyl acetate and methyl butyrate the agreement is good.

Influence of pH on enzyme stability. In order to determine the influence of pH on the stability of enzyme solutions, the activities of solutions of various pH, all with ACh as substrate, were determined every month for six months.

* Evans Electroselenium LTD, Harlow, Essex.

Table 1. Relative extinction of the ferric complex of hydroxamic acids of some esters.

Compound	Extinction	Extinction per ester- linkage	Values obtained by Hestrin
Acetylcholine iodide	100	100	100
Acetyl- β -methylcholine chloride (methyl)	106	106	107
Salicylcholine chloride	160	160	—
Benzoylcholine chloride	139	139	—
N-acetyl- <i>p</i> -aminobenzoylcholine chloride	146	146	—
Ethyl acetate	50	50	97
Iso-propyl acetate	33	33	—
Methyl butyrate	50	50	87
Ethyl butyrate	33	33	—
Methyl benzoate	142	142	—
Ethyl benzoate	117	117	—
Acetylsalicylic acid	210	210	—
Prostigmine	0	0	0
3-Acetoxyphenyltrimethyl- ammonium iodide	103	103	—
9-Acetoxyretene	87	87	—
3-Acetoxyretene	83	83	—
Succinylcholine iodide	150	75	—
<i>o</i> -Acetylsalicylcholine chloride	250	125	—
Diacetylmorphine chloride	206	103	—
Triacetin	294	98	98
Tripropionin	314	105	—
Tributylin	285	95	95
Glucose pentacetate	540	108	110
Maltose octacetate	840	105	—

The molar concentration of the substrate was 7.32×10^{-3} , and the enzyme solutions were kept in the refrigerator at 4° C. The results are shown in Fig. 2.

The activity seems to decrease during the first three months, but then appears to reach a constant value. The final activity depends on the pH but is 53–64 per cent of the original value. A measure of the stability may be obtained by determining the time required for the activity to be reduced to a certain degree, *e.g.* 75 per cent. On this basis the stability is greater at pH 6.7 than at pH 5.8 or 8.1.

Enzyme stability in various buffer solutions, and the influence of these solutions on the colour reaction and enzyme activity. It is well known that the buffer

Fig. 2. Enzyme activity in buffers of various pH as function of time.

1. pH 5.8 2. pH 6.7 3. pH 8.1

solutions may influence the activity of enzymes. The activity of acetylcholinesterase has been determined in three different buffer solutions (bicarbonate, veronal and phosphate), all having a pH of 7.2. None of these buffers was found to disturb the colour reaction. 1.46×10^{-3} molar ACh was used as substrate.

In connection with these determinations the stability of the enzyme in different buffers was studied. The stability was measured both at 4° and 25° C. The activity was determined every week for three weeks with the results shown in Table 2.

It is clear from this table, that the rate of hydrolysis of ACh is the same in all three buffer solutions. At 4° C the solutions buffered with phosphate or bicarbonate have the greatest stability, the stability of the enzyme in a veronal buffer is much lower. Naturally the stability at 25° C is lower, but when a phosphate buffer is employed the change is not great. The enzyme dissolved in bicarbonate buffer shows a remarkably low stability at 25° C.

The hydrolysis of acetylsalicylcholine. Augustinsson⁴ has shown that only one of the two ester linkages of ASaCh, the acetate link, is split with cobra venom when the concentration of the substrate is between 6.62×10^{-3} and

Table 2. Relative enzyme activity in various buffers as function of time and temperature.

Buffer		$t = 0$	$t = 1$ week	$t = 2$ weeks	$t = 3$ weeks
Phosphate	4° C	100	96	91	87
	25° C	100	83	74	70
Veronal	4° C	100	70	57	48
	25° C	100	52	39	32
Bicarbonate	4° C	100	100	87	83
	25° C	100	56	17	0

Fig. 3. Calibration curves of ACh(1), ASaCh(2), ASa(3) and SaCh(4).

1.99×10^{-4} molar, although both ester linkages are split when the molarity of the substrate was 6.62×10^{-2} – 1.99×10^{-2} . These measurements were made at pH 7.4.

To investigate the influence of pH on these reactions ASaCh was enzymically hydrolysed at pH values in the range 5.0 to 8.1, and the extinction was determined when the hydrolysis had ceased. The molar concentration of the substrate used were 8.00×10^{-3} and 1.46×10^{-3} . From the calibration curves of ACh, ASaCh, ASa and SaCh (Fig. 3) the extinctions of the possible hydrolysis products have been calculated (Table 3).

Table 3. Calculated extinction when the hydrolysis of ASaCh has ceased as function of pS and the number and type of broken ester linkage.

	pS	
	2.1	2.8
Two ester linkages split	0	0
One » linkage » : SaCh left	0.22 *	0.40
» » » » : ASa »	0.30 *	0.52

* dilution 1:10

Because the three curves are quite distinct the difference in the final extinction is significant in the two cases where only one ester linkage is split. The results (Table 4) show that with ASaCh only one ester linkage is split irrespective of the pH of the reaction mixture, and that the ester linkage split is that of the acetyl group. The same results were obtained at both substrate concentrations.

Table 4. The extinction when the hydrolysis of ASaCh has ceased as function of pS and pH.

pH		5.0	5.8	6.7	8.1
pS	2.1	0.22 *	0.22 *	0.23 *	0.22 *
	2.8	0.41	0.42	0.40	0.38

* dilution 1:10

The quantitative determination of ASaCh is complicated by the fact that when the acetyl group is split off, the compound formed, SaCh, also gives a red colour with the hydroxylamine reagent. A theoretical calibration curve may be calculated as follows. The extinction of ASaCh can be written $E_{\text{ASaCh}} = k_{\text{ASaCh}} \cdot c_{\text{ASaCh}}$ where k_{ASaCh} is a constant and c_{ASaCh} is the molar concentration of ASaCh. Likewise we obtain $E_{\text{SaCh}} = k_{\text{SaCh}} \cdot c_{\text{SaCh}}$. If the concentration of ASaCh is s initially and x after a certain time then the concentration of SaCh at the same time will be $s-x$, and the extinction is given by:

$$E = E_{\text{ASaCh}} + E_{\text{SaCh}} = k_{\text{ASaCh}} \cdot x + k_{\text{SaCh}}(s-x) \quad \text{or}$$

$$E = E_0 + k \cdot x$$

where $E_0 = s \cdot k_{\text{SaCh}}$ and $k = k_{\text{ASaCh}} - k_{\text{SaCh}}$. k_{ASaCh} and k_{SaCh} may be found from the calibration curves for ASaCh and SaCh, and thus x may be calculated from E . This method has been used in the following.

Influence of pH on the activity-pS curve of ACh. ACh solutions of various concentrations were enzymically hydrolysed at pH values varying from 5.8 to 9.8 (Fig. 4). All values have been corrected for non-enzymic hydrolysis which was noticeable only at pH values greater than 8.1.

Fig. 4 shows that with increasing pH pS_{opt} and the activity at pS_{opt} increase to a maximum. After that pS_{opt} and the activity at pS_{opt} decrease. These results are in agreement with the general tendency predicted by Wilson and Bergmann⁶, and will be discussed below.

It is known that acetylcholinesterase of cobra venom gives a bell-shaped activity-pS curve both with ACh and ASaCh^{4,7}. Two cases may be visualized. Either the two curves intersect, or one of them will lie inside the other. In the first case the enzyme will split one substrate at a higher rate than the other at a certain concentration and at a lower rate at another concentration, in the second case one substrate will be split at a higher rate at all substrate concentrations.

Fig. 4. Influence of pH on the activity- pS curve of ACh.

1. pH 5.8 2. pH 6.7 4. pH 9.8.
The uppermost curve pH 8.1.

Fig. 5. Activity- pS curves of ACh(1) and ASaCh(2) at pH 7.4.

A preliminary experiment using the Warburg manometric method at pH 7.4 gave the result shown in Fig. 5, and clearly demonstrated that at this pH the system ACh—ASaCh is of the former type.

Enzymic hydrolysis were carried out with ACh and ASaCh at acidities varying between 5.8 and 8.1, and the substrate concentration given in Table 4. For comparison SaCh and ASa were also hydrolysed enzymically, but only at the higher concentration (Table 5). All the values are corrected for non-enzymic hydrolysis which, however, was significant only at pH 8.1.

When the molarity of the substrates is 8.00×10^{-3} ($pS = 2.1$) ASaCh is split at a higher rate than ACh, SaCh or ASa, the last two substances being almost unaffected. The difference between the rates of hydrolysis of ASaCh

Table 5. Relative enzyme activity towards ACh, ASaCh, ASa and SaCh as function of pH and pS .

Substrate \ pH	5.8		6.7		8.1	
	pS 2.1	pS 2.8	pS 2.1	pS 2.8	pS 2.1	pS 2.8
ACh	25	11	42	25	61	77
ASaCh	31	12	56	20	100	52
ASa	1	—	1	—	1	—
SaCh	—	—	—	—	—	—

and ACh decreases with decreasing pH. When the substrate concentration is 1.46×10^{-3} ($pS = 2.8$) and the pH 6.7 or 8.1 ACh on the other hand is split more rapidly than is ASaCh. The difference at pH 5.8 is insignificant.

DISCUSSION

Deductions on the influence of pH on the enzymic hydrolysis with acetylcholinesterase have been made by Wilson and Bergmann⁶. Between pH 7 and 9, $[S]_{opt}$ is expected to have a minimum, 3.2×10^{-3} molar, when the activity at $[S]_{opt}$ is a maximum. Outside this pH range $[S]_{opt}$ is higher and the activity at $[S]_{opt}$ lower.

As shown in Table 5, it is necessary to use the same substrate concentrations when comparing the activity of the enzyme towards various esters. If ACh is taken as the standard ester, it is convenient to make the comparison at the optimum concentration of ACh.

At pH 6.7 where pS_{opt} is 2.1 (Fig. 4) the rate of enzymic hydrolysis of ASaCh is higher than that of ACh (Table 5). On the other hand at pH 7.4 where pS_{opt} is 2.5 the rate of enzymic hydrolysis of ACh is higher than that of ASaCh (Fig. 5).

The results clearly show the importance of pH in studies on specificity.

SUMMARY

The determination of acetylcholinesterase (cobra venom) was carried out using the colourimetric method recently described by Hestrin². The relative extinctions of the coloured complexis from various esters were determined.

The stability of enzyme solutions of different pH was examined and the stability of the enzyme in various buffers was studied at 4° and 25° C.

The influence of pH on the activity-pS curve of ACh was investigated.

The rate of enzymic hydrolysis of ACh, ASaCh, ASa and SaCh has been investigated at different pH and substrate concentrations.

The importance of pH in specificity studies has been discussed.

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REFERENCES

1. Sørensen, S. P. L. *Biochem. Z.* **21** (1909) 131.
2. Hestrin, S. *J. Biol. Chem.* **180** (1949) 249.
3. Michel, H. *J. Lab. Clin. Med.* **34** (1949) 1546.
4. Augustinsson, K.-B. *Acta Chem. Scand.* **5** (1951) 712.
5. Synthetised according to Euler, H. v., Euler, U. S. v., and Hasselquist, H. *Arkiv Kemi, Mineral. Geol.* **20A** (1945) No. 16.
6. Wilson, I. B., and Bergmann, F. *J. Biol. Chem.* **186** (1950) 689.
7. Augustinsson, K.-B. *Acta Chem. Scand.* **5** (1951) 699.

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