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IONISATION OF HORSE SERUM BUTYRYLCHOLINESTERASE

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SUMMARY

The effect of pH on horse serum cholinesterase (acylcholine acyl-hydrolase, EC 3.1.1.8) catalyzed *o*-nitrophenyl butyrate hydrolysis shows a non-competitive type of inhibition. The K_m value is independent of the pH of the medium, and may be regarded as the true dissociation constant of the *ES* complex. Its value varies only slightly at 25 and 35°. The group responsible for the catalytic hydrolysis of *o*-nitrophenyl butyrate has a pK value of 5.6. This is indicative of an imidazole group of histidine rather than an amino group as has been suggested. Acylation of the enzyme does not cause a change of pK value. In salt medium both the pK of the enzyme and the *ES*H complex shift to a lower value. This shift is interpreted as evidence of conformational change in the enzyme.

INTRODUCTION

The effect of pH on butyrylcholinesterase-acetylcholine hydrolysis has been the object of a study by a number of workers[1-4]. Since acetylcholine is a charged molecule and the anionic site of the enzyme itself may also be pH dependent, interpretation of the results is rather complicated. For this reason, a neutral ester which attacks primarily the esteratic site of the enzyme has been used. Studies with such a substrate, with special reference to the pH effects of the medium, were reported by BERGMANN *et al.*[5] and more recently by YAKOVLEV AND AGABEKYAN[6].

In the present work, *o*-nitrophenyl butyrate was used as substrate. The investigations were carried out at 25 and 35° in phosphate and acetate buffers containing 15% (v/v) isopropanol, with and without the addition of NaCl. It was found that H^+ acts as a non-competitive inhibitor, and K_m may be regarded as the true dissociation constant of the substrate–enzyme complex. The ionisation constants of the enzyme and the complex are given and an explanation is offered for the shift of pK value caused by medium changes.

MATERIALS AND METHODS

The partially purified horse serum butyrylcholinesterase having an activity of 4–6 units/mg was obtained from Nutritional Biochemicals Corporation. A solution of 2.0 mg/ml was prepared with twice-distilled water. When working in salt solution, the enzyme solution was used without dilution. When working in buffer medium, however, one part of the enzyme solution was diluted with two parts of buffer of appropriate pH.

The *o*-nitrophenyl butyrate substrate[7] of various concentrations was freshly prepared for each set of experiments from a 0.05-M stock solution in isopropanol with 0.05 M phosphate or acetate buffer. The final solution contained 15% (v/v) isopropanol which is the minimum amount required to ensure complete solution of *o*-nitrophenyl butyrate at all concentrations used. The effect of salt was studied in a concentration of 0.47 M NaCl.

The enzyme reaction was started by addition of 10 μl of diluted enzyme solution (or undiluted solution when investigations were carried out in salt medium) to 3.19 ml of substrate in a 1-cm silica cuvette, and the absorbance of *o*-nitrophenol liberated was measured *in situ* at the isosbestic point of 370 mμ (371 mμ in salt solution) against a blank of the same composition, with a recording DK-2 Beckman spectrophotometer equipped with time drive and temperature-regulated cell holder. Temperatures were maintained at 25 $\pm$ 0.1 or 35 $\pm$ 0.1°. Some of the experiments were performed on a 10-inch "slave" recorder (modified Philips PM 8000) whereby a 0.1-section of the absorbance scale of the Beckman DK-2 spectrophotometer is expanded to fill the whole height of the chart. The absorbance scale expansion unit was constructed according to the description of DIXON[8].

The method as described is based on the fact that at 370 mμ the molar absorbance coefficient of *o*-nitrophenyl ($\varepsilon_M = 2240$) is 17 times greater than that of *o*-nitrophenyl butyrate ($\varepsilon_M = 129$). Calculation shows that the change in concentration of *o*-nitrophenyl butyrate due to liberation of *o*-nitrophenol by enzyme action may be ignored if the extent of hydrolysis is restricted to about 3–10%, depending on concentration of the substrate. In other words, the observed increase in absorbance can be entirely attributed to the *o*-nitrophenol liberated; no correction is necessary. The method agrees well with the one based on the measurement of *o*-nitrophenol liberated at 414 mμ at which *o*-nitrophenyl butyrate shows no absorption[7].

pH measurements were carried out with a Metrohm pH-meter E300.

RESULTS

Dependence of the activity of butyrylcholinesterase on pH was measured over the range of concentrations of *o*-nitrophenyl butyrate from 0.2 to 1.6 mM and from pH 5.2 to 7.8 in phosphate or acetate buffer and in salt medium at 25 and 35°. The initial rate was taken within 3–10% hydrolysis of the substrate in which the *o*-nitrophenol liberated is linear with time. The plot of reciprocals of the Lineweaver–Burk equation at different values of pH is shown in Fig. 1 for the system containing no added salt at 25°. The lines were drawn in accordance with the method of least squares. Similar plots were obtained at 35° and with added salt. They reveal that H^+ acts as non-competitive inhibitor of the enzyme–*o*-nitrophenyl butyrate hydrolysis.

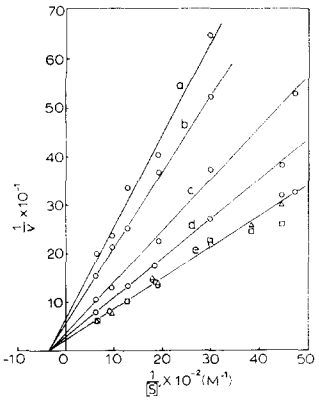

Fig. 1. Lineweaver–Burk plot of horse serum butyrylcholinesterase-*o*-nitrophenyl butyrate interaction at different values of pH at 25°. The assay mixture consisted of 3.19 ml of substrate solution and 10 μl of enzyme solution (2 mg/ml). The activity was arbitrarily expressed in terms of absorbance per min. pH of the substrate: a, 5.25; b, 5.38; c, 5.77; d, 6.32; and e, 6.83 (□); 7.18 (○); and 7.79 (△). All lines were drawn in accordance with the method of least squares.

Values of K_m and V were calculated from the intercepts of the equation of least squares by letting $x=0$ and $y=0$ respectively. The K_m values shown in Table I may be regarded as the dissociation constant of the ES complex since it remains constant throughout the range of pH studied while V varies. The effect of temperature from 25 to 35° on the value of K_m is slight (Table 1). The present results differ from the ones showing competition inhibition of H^+ on the enzyme–*p*-nitrophenyl acetate hydrolysis in which K_m is of pH dependence reported by YAKOVLEV AND AGABEKYAN[6].

Since H^+ acts as a non-competitive inhibitor and K_m does not change with pH of the medium, the constant defining the ionisation of the esteratic site of the enzyme can be regarded as identical with the inhibition constant. Thus $K_i = [E][H^+]/[EH^+]$ where $[E]$ is the concentration of active enzyme and $[EH^+]$ that of inhibited one. From equations for substrate–enzyme hydrolysis and non-competitive inhibition, one obtains:

$$\frac{[E_0]}{[E]} = 1 + \frac{[H^+]}{K_i}$$

TABLE I

K_m VALUES AND IONIZATION CONSTANTS OF THE ENZYME

Values of 95% confidence limits[15] were calculated from K_m values determined at various pH's.

Medium	K_m *(mM)*		*pK*	
	25°	*35°*	*25°*	*35°*
0.05 M phosphate or acetate buffer containing 15% (v/v) isopropanol	2.9 ± 0.6	4.3 ± 0.7	5.6	5.5
0.05 M phosphate or acetate buffer containing 15% (v/v) isopropanol and 0.47 M NaCl	3.1 ± 0.3	3.0 ± 0.5	5.4	5.0

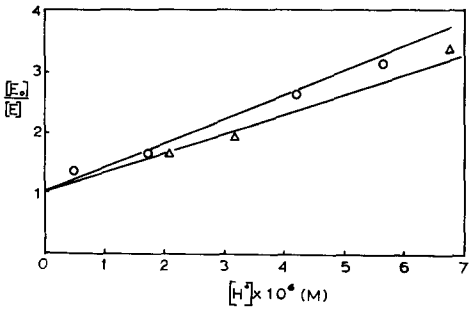

Fig. 2. Effect of H^+ concentration on the maximum velocity of the hydrolysis of *o*-nitrophenyl butyrate by the enzyme in accordance with equation $[E_0]/[E] = 1 + [H^+]/K_i$ at 25° (○) and at 35° (△).

where $[E_0]$ is the total enzyme concentration and $[E]$ is the effective concentration at a given pH.

Fig. 2 shows a plot of $[E_0]/[E]$ against $[H^+]$, taking $V = k_2[E_0]$ near pH 7.8—k_2 being the rate of breakdown of the complex according to the reaction scheme

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\rightarrow} E + P$$

The slope of the lines in Fig. 2 gives $1/K_i$, from which values of pK_i (later designated as pK for simplicity) were calculated (Table I). The pK value of 5.6 is considerably lower than the value of 7.7 given by HASE[1] but close to the values 5.85 by SHUKUYA AND SHINODA[2] and 5.87 and 6.6 by YAKOVLEV AND AGABEKYAN[6], respectively. In buffer medium a change of about 0.1 unit in pK on raising the temperature by 10° was observed. Since the temperature effect is small, it is not possible to estimate the apparent heat of ionisation with certainty under the experimental conditions studied.

DISCUSSION

The results shown in Fig. 1 demonstrated that the enzyme–*o*-nitrophenyl butyrate hydrolysis has the characteristic of a non-competitive inhibition by H^+, being similar to the α-chymotrypsin-catalyzed acetyl-L-phenylalanine ethyl ester reaction[9]. It differs, however, from the enzyme–acetylcholine interaction which is of a competitive nature[1,2]. In the former case, K_m is the dissociation constant of the ES complex, while in the latter case, it is a composite one, involving both the dissociation constant of the complex and the rate constant for breakdown of the complex. This difference is also reflected in the results found in the presence of salt. The value of K_m obtained with *o*-nitrophenyl butyrate as substrate changes very little with change of the salt content up to 0.47 M NaCl. With acetylcholine as substrate, however, a 5-fold increase in K_m was observed in buffer solution containing from zero to 0.264 M NaCl[10]. Since *o*-nitrophenyl butyrate is a neutral molecule, it reacts primarily with the esteratic site; the H^+ does not affect the combination of the substrate with the enzyme but affects only V. On the other hand, the acetylcholine is a charged molecule, its ability to react with enzyme is affected by combination of H^+ to a certain group of the enzyme, and by this combination the formation of the enzyme–substrate

complex or its dissociation is modified. Because of this difference in nature of the substrate a different mechanism of enzyme action occurs.

The pK value of 5.6 for the ionisation constant of the enzyme in the buffer medium suggests an imidazole group rather than an amino group reported previously[1]. This value of pK appears to be somewhat more strongly acidic than would be expected from the model compound imidazole which has a value of about 6.0 (see ref. 11). These abnormalities seem to indicate that the basic group of imidazole (—N=) may have become oriented closer to the neighbouring amino group, or possibly more accessible to hydrogen bonding, as a result of a break in the native helical form. In other words, there is a change in conformation of the enzyme under the experimental conditions studied.

In the salt solution the value of pK is further decreased (Table I). Obviously this decrease is not caused by the effect of the medium but by denaturation, since in imidazole the pK increases with increase of the salt concentration[12]. Thus, a study of enzyme in salt solution would provide further evidence of conformational changes in enzyme.

Since the kinetics of inhibition by H^+ is of non-competitive nature in which ESH is not catalytically active, and the rate is entirely attributed to the breakdown of ES, it requires that the ionization constants of the enzyme and the complex be equal. Thus acylation of the enzyme by a butyryl group does not bring about a change of pK. The change in pK value of the enzyme caused by substitution of an electrophilic group, however, appears to be characteristic. For example, phosphorylation of human serum cholinesterase increases the pK value amounting to 2.5 units, taking the pK of human serum cholinesterase as 5.9 (see ref. 2) and that of the inhibited enzyme as 8.4 (see ref. 13). The exact cause of such a change of pK is not clear but it seems likely to have been induced by a change of enzyme conformation forced upon it by a substituting group, the extent of change depending on the size and nature of the group concerned. Direct evidence of conformation changes due to phosphorylation and acylation by method of optical rotatory dispersion has recently been reported by KITZ AND KREMZNER[14].

In salt medium the pK of the complex shifts to a lower value (Table I). It follows a pattern similar to that found in a study of reactivation of diethylphosphoryl human serum cholinesterase in which the ionisation constant of inhibited enzyme decreased from 8.4 to 7.8 (see ref. 13). This is also interpreted as due to a conformational change of the enzyme induced by the addition of salt, although some contribution by the Brønsted's secondary salt effect may not be ruled out completely.

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