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EFFECT OF KINETIC PARAMETERS AND INHIBITORS ON INTESTINAL ESTERASE

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SUMMARY

1. Effect of temperature on the activity (K_m and V) of intestinal esterase has been investigated with a good (*p*-nitrophenyl-*n*-butyrate, NPB) and a poor substrate (*p*-nitrophenyllaurate). The energy of activation values, 8.75 and 10.4 kcal/mole, respectively, for the two substrates are very close. In each case K_m is practically unaffected by temperature showing only a slight decreasing trend as the temperature is raised from 20 to 40 °C suggesting that the enzyme–substrate binding is primarily of entropic origin.

2. The effect of pH on K_m and V of two substrates has been investigated. In each case the K_m changes only slightly with a change in pH. The variation of V of each substrate is consistent with the participation of two groups of enzyme molecule with pK values 5.8 and 8.1.

3. Intestinal esterase is inhibited by low concentrations of metal ions like Ag^+, Hg^{2+} *etc.* The inhibition by Ag^+ is non-competitive with K_i equal to $9.1 \cdot 10^{-7}$ M.

4. Organic phospho compounds like diisopropylfluorophosphonate, diethyl-*p*-nitrophenylphosphate (Paraoxon) and bis-(monoisopropylamino)-fluorophosphine oxide inactivate the enzyme. About one mole of Paraoxon inactivates 1 mole (mol. wt 135 000) of the enzyme.

INTRODUCTION

Enzyme-catalysed ester hydrolysis has been investigated mostly with proteolytic enzymes. Carboxyl ester hydrolases (other than cholinesterases) with little or no proteolytic activity have received very little attention. Specificity studies with an intestinal esterase using aryl esters of aliphatic acids showed that substitution in aryl moiety had very little effect on the rate of enzymic hydrolysis[1]. Slope of the Hammett plot (log V *versus* substituent constant) was found to be −0.16 only. In

Abbreviations: DFP, diisopropylfluorophosphonate; Mipafox, bis(monoisopropylamino)-fluorophosphine oxide; NPA, *p*-nitrophenylacetate; NPB, *p*-nitrophenyl-*n*-butyrate; NPL, *p*-nitrophenyllaurate; Paraoxon, diethyl-*p*-nitrophenyl phosphate.

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this respect the enzymic reaction is similar to the acid-catalysed ester hydrolysis[2] and is in contrast to the esterolytic action of proteolytic enzymes like chymotrypsin. The latter are known to exhibit characteristics of general base catalysis[3]. This marked difference between the esterolytic reactions catalysed by intestinal esterase and proteolytic enzymes prompted us to investigate further the properties of the former enzyme. The present paper reports studies on the effect of various kinetic parameters and inhibitors on the intestinal esterase.

In the case of this enzyme the substrate-binding has been shown to involve non-polar interactions[1]. Such forces are primarily of entropic origin and exhibit zero or very slightly positive enthalpy values for the complex formation. Moreover, they are independent of any acid–base dissociation. Therefore, the formation of enzyme–substrate complex in the present case should be practically unaffected by changes in pH or temperature. It has been shown that both these parameters affect K_m to a minor extent only. Moreover, the effect of pH on V has been shown to be consistent with the participation of two dissociable groups (pK 5.8 and 8.1) in the catalysed reaction. The enzyme is inhibited by metal ions and organic phospho compounds.

EXPERIMENTAL

Reagents

Enzyme[4] and substrates[1] were prepared as described earlier. $AgNO_3$, $CuSO_4$, $ZnSO_4$, lead acetate, $CdSO_4$, $Na_2HPO_4 \cdot 12\ H_2O$ and $NaH_2PO_4 \cdot H_2O$ were analytical grade reagents. Tris was from L. Light and maleic acid from Riedel-de-Haen. $HgCl_2$ was recrystallized in the laboratory. Diisopropylfluorophosphonate (DFP), diethyl-*p*-nitrophenylphosphate (Paraoxon, E600) and bis(monoisopropylamino)fluorophosphine oxide (Mipafox) were kind gifts from Dr W. N. Aldridge. Only double-distilled water from an all-glass assembly was used.

Estimations

Hydrolysis of *p*-nitrophenyl esters (acetate, *n*-butyrate and laurate) was monitored by estimating the liberated *p*-nitrophenol spectrophotometrically at 405 nm[4]. The K_m and V values were obtained from Lineweaver and Burk plots[5].

For inhibition experiments the enzyme and inhibitor were incubated in 5.0 ml 0.05 M Tris–acetic acid buffer, pH 7.3, at 30 °C till the activity became constant. The reaction was started by blowing a small volume (0.2 ml) of the methanolic solution of *p*-nitrophenyl-*n*-butyrate (NPB) into the test solution. Final NPB concentration was 1 mM. Inhibitor and enzyme concentrations are shown in respective figures. Relative activity represents the percentage residual activity. For inhibition by organic phospho compounds the incubation with inhibitor was carried out at high enzyme concentration (0.74 mg/ml) and an aliquot of the incubation mixture diluted before addition of the substrate (NPB).

RESULTS AND DISCUSSION

Effect of temperature

Earlier work showed that the intestinal esterase was relatively specific for short-chain fatty acid esters. The long-chain fatty acid esters are hydrolysed at the

same site but at a very much smaller rate. Thus when a poor substrate (small V) is used as a competitive inhibitor in the hydrolysis of a good substrate (large V), the K_i value so obtained is equal to the K_m value of the poor substrate[1]. A comparison of the energy of activation for the hydrolysis of the two types of substrates has been carried out in order to determine whether the mechanism of the reaction in the two cases are energetically similar or different. Effect of temperature on K_m and V of a

TABLE I

ENZYMIC HYDROLYSIS OF NPB AND NPL AT DIFFERENT TEMPERATURES IN 0.05 M PHOSPHATE BUFFER, pH 7.0

V is expressed as μmoles substrate hydrolysed per min per mg enzyme protein.

Temperature (°C)	*NPB*		*NPL*	
	$K_m \times 10^5$ *(moles/l)*	V	$K_m \times 10^6$ *(moles/l)*	V
20	4.5	151.5	3.3	1.24
25	4.3	210.0	2.6	1.58
30	4.0	280.0	2.1	2.37
35	3.7	336.0	1.8	3.17
40	3.4	420.0	1.6	3.87

short (NPB) and a long chain fatty acid ester (*p*-nitrophenyllaurate, NPL) is shown in Table I. Energy of activation for NPB and NPL is found to be 8.75 and 10.4 kcal/mole, respectively. When one considers the close values of energy of activation together with the fact that both the substrates are hydrolysed at the same site, it may be concluded that the reaction in the two cases must proceed by the same or energetically very similar mechanisms. This is in agreement with the fact that the length of alkyl chains in such substrates does not significantly alter the electronic environments of the sensitive ester bond. Thus, the low rates of enzymatic hydrolysis of long-chain fatty acid esters must be attributed to some factor other than any change in the overall mechanism. We have earlier pointed out that this difference in the rates could be readily explained by a steric model. According to this model there is a greater probability of "incorrect" (unproductive) binding of the long alkyl chain on the non-polar binding site of the enzyme[1].

In both cases K_m is practically unaffected by temperature, although a slight decreasing trend is observed as the temperature is raised. This suggests that the enthalpy of enzyme–substrate complex formation is practically zero. The binding of substrate to the enzyme must, therefore, be essentially of entropic origin.

Effect of pH

K_m and V values of *p*-nitrophenylacetate (NPA) and NPB at different pH values (4.5–9.0) are shown in Table II. Tris–maleic acid buffers were used for most cases. Citrate–phosphate and Tris–HCl were used, respectively, for the lower and upper extremes of the pH range. Control experiments showed that at a given pH the nature and concentration of buffers had no effect on the K_m and V values. Therefore, data obtained in different buffers can be compared directly.

For each substrate K_m changes to a small extent only as the pH is varied from

TABLE II

EFFECT OF pH ON THE ACTIVITY OF GOAT INTESTINAL ESTERASE AT 30 °C IN CITRATE–PHOSPHATE (pH 4.5), 0.05 M TRIS–MALEIC ACID (pH 5.2–8.0) AND 0.05 M TRIS–HCl (pH 8.5 and 9.0) BUFFERS K_m values are expressed as moles/l and V as μmoles substrate hydrolysed·min^{-1}·mg^{-1} enzyme protein.

pH	*p-Nitrophenylacetate*		*p-Nitrophenyl-n-butyrate*	
	K_m ($\times 10^4$)	V	K_m ($\times 10^5$)	V
4.5	10.0	5.3	6.25	24.2
5.2	4.34	38.5	9.9	73.3
5.5	7.14	58.5	6.25	126.5
6.0	4.54	85.6	3.7	166.2
6.5	3.33	113.5	4.5	235.6
7.0	4.87	143.3	5.0	281.9
7.3	3.84	133.3	4.5	257.2
7.5	2.38	103.6	3.9	230.1
8.0	1.96	75.5	2.8	160.6
8.5	—	—	1.7	61.3
9.0	—	—	1.0	21.6

4.5 to 9.0. A slight decreasing trend is noticed as pH is increased. However, the changes are too small to suggest involvement of any group dissociating in the pH range 4.5–9.0 in the binding of substrate to the enzyme[6].

The variation of V of NPA and NPB show a typical bell-shaped curve with a

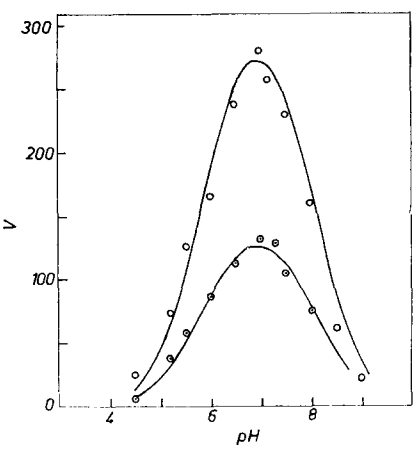

Fig. 1. Influence of pH on intestinal esterase activity with NPA (○) and NPB (○) as substrates. V values were calculated as usual. Citrate–phosphate (pH 4.5), 0.05 M Tris–maleic acid (pH 5.2–8.0) and 0.05 M Tris–HCl (pH 8.5, 9.0) buffers, 30 °C. The curves were calculated as described in the text.

pH optimum at 6.9–7.0 in each case (Fig. 1). Considering the nature of the substrates (neutral esters), these changes must be due to the effect of pH on the enzyme or enzyme–substrate complex only. In such a case the rate equation for the effect of

pH on enzyme activity under conditions of substrate saturation may be expressed as follows:

$$V_{obsd} = \frac{k_{cat}\,[E]_0}{1 + \frac{[H^+]}{K_A} + \frac{K_B}{[H^+]}}$$

where K_A and K_B are dissociation constants of groups dissociating below and above pH optimum, respectively, V_{obsd} is the observed V at any given pH, $[E]_0$ the total enzyme concentration and k_{cat} the overall pH-independent unimolecular rate constant for the conversion of enzyme–substrate complex into products. $k_{cat}[E]_0$ would then stand for the hypothetical V when both the groups (defined by K_A and K_B) were in the correct state of dissociation. It can be shown that V at pH optimum would approach $k_{cat}[E]_0$ only if $K_A \gg K_B$, *i.e.* when the two pK values are very far removed from each other. In all other cases V at pH optimum would be smaller than

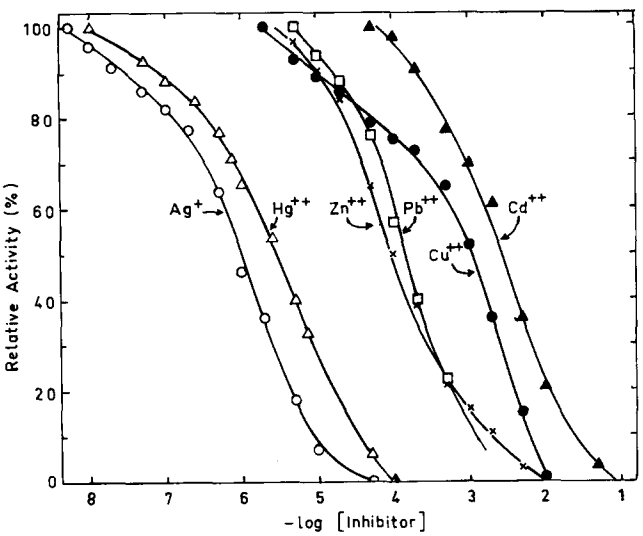

Fig. 2. Inhibition of intestinal esterase with metal ions. Enzyme and inhibitor dissolved in buffer (pH 7.3) were preincubated for 10 min at 30 °C. Volume 5.2 ml. The reaction was started by blowing 0.2 ml methanolic solution of NBP into the enzyme–inhibitor solution and followed at 405 nm. Final concentrations 0.042 μg protein·ml^{-1} and 10^{-3} M NPB.

this value. In Fig. 1 the curves have been calculated on the basis of the above equation taking pK_A and pK_B as 5.8 and 8.1 and $k_{cat}[E]_0$ as 143 and 308 μmole·min^{-1}·mg^{-1} enzyme for NPA and NPB, respectively. The experimental points for the two substrates lie very close to the respective curves. Thus, the V–pH variations are consistent with the participation of two groups with pK 5.8 and 8.1 in the catalysed reaction. An identification of these groups is not possible at this stage. As is known the pK values of groups in proteins are often perturbed by their microenvironments.

Inhibition by metal ions

The intestinal esterase is inhibited by metal ions, like Hg^{2+}, Ag^{+} *etc.* Their effect is shown in Fig. 2. It is noteworthy that their inhibitory action, $Ag^{+} > Hg^{2+} > Zn^{2+} > Pb^{2+} > Cu^{2+} > Cd^{2+}$, is more or less in the same order as their affinity towards SH groups. The inhibition appears to be non-competitive in character (Fig. 3).

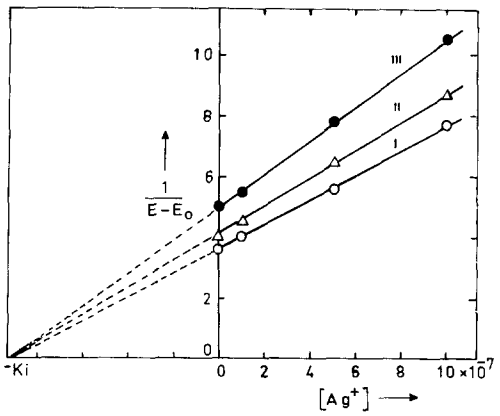

Fig. 3. Non-copetitive inhibition of intestinal esterase by Ag^{+}. Conditions and procedure as given in Fig. 2. NPB concentrations were I, 10^{-3} M; II, $2.5 \cdot 10^{-4}$ M and III, 10^{-4} M. K_i value was found to be $9.1 \cdot 10^{-7}$ M.

Effect of organic phospho compounds

A number of esterolytic enzymes react stoichiometrically with organic phospho compounds like DFP, Paraoxon and Mipafox with a resultant loss of enzyme activity. The same is true of intestinal esterase. All the three compounds tested were equally

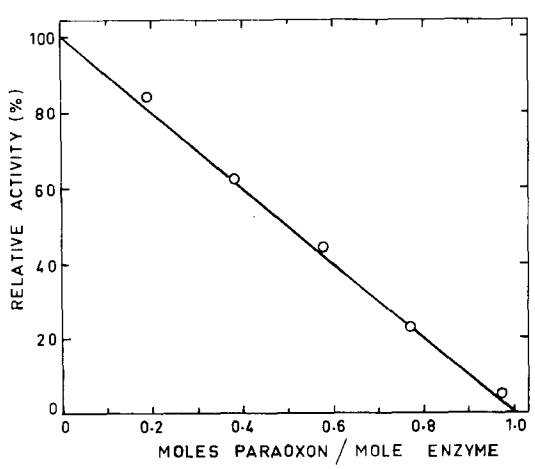

Fig. 4. Effect of Paraoxon on intestinal esterase. The enzyme solution (3.7 mg per 5 ml) in 0.05 M phosphate buffer, pH 7.3, was treated with 0.05 ml portions of 10^{-4} M Paraoxon at 30 °C at about 30-min intervals. Before each addition, a 0.05-ml aliquot was withdrawn, diluted 500 times and tested as usual with 10^{-3} M NPB for enzyme activity. ○, experimental points. The line has been drawn for a stoichiometric reaction for a mol. wt of 135 000 for the enzyme.

effective. Typical results with Paraoxon are shown in Fig. 4. One mole of the reagent is required for complete inactivation of one mole (mol. wt 135 000)[4] of the enzyme. The reaction appears to be strictly stoichiometric. The inactivation is not reversed by simple dilution.

Besides the "serine active site" enzymes, several "SH-proteases" are known to react with DFP[7–10]. With bromelain[9] and papain[10], it has been shown that the reaction involves one or more tyrosyl residues and does not lead to any loss of enzyme activity. In the case of chymopapain, a large excess of DFP is required for complete inactivation[7]. Gould and Leiner[8] showed that the observed inactivation of ficin with excess DFP was due to the presence of an impurity in the commercial preparations of this reagent. If any such impurity were responsible for inactivation of intestinal esterase, different effects should have been observed with the three reagents tested, namely DFP, Mipafox and Paraoxon. No such differences have actually been observed. Moreover, the reaction is strictly stoichiometric (Fig. 4). In these respects, the present observations are similar to the effect of these reagents on "serine active site" enzymes. Evidently, further experiments are required to locate the site of attack of DFP and its analogues in the case of intestinal esterase.

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