Pig Liver Esterase. Some Kinetic Properties*

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ABSTRACT: Pig liver esterase purified by the method of Adler and Kistiakowsky is subject to activation up to 2.4-fold by organic solutes, such as acetone and dioxane, as well as by a second molecule of substrate. Acetone increases the maximal velocity in both the low and high substrate concentration regions. Enzyme dissociated into half-molecules by treatment at pH 4.5 exhibits activation by substrates and by acetone, showing that interaction between subunits in an intact molecule is not necessary for activation. The dissociated and intact enzymes exhibit the same activity and pH dependence for the hydrolysis of *p*-nitrophenyl acetate; the dissociated enzyme exhibits a higher activity at low concentrations of *m*-(*n*-heptanoyloxy)benzoic acid. The enzyme exhibits an increase in activity toward *m*-(*n*-pentanoyloxy)benzoic acid at low pH values that is attributed to a relatively rapid hydrolysis of the uncharged, protonated species. The hydrolysis of anionic substrates is accelerated and that of neutral substrates is inhibited by salts. It is concluded that each half-molecule of enzyme contains an activator site, which can be occupied by substrate or other activator molecules, as well as a catalytic site.

We describe here some of the kinetic properties of whole and half-molecules of pig liver carboxylesterase (EC 3.1.1.1) purified by the method of Adler and Kistiakowsky (1961). The physical properties of this enzyme preparation have been described in the preceding paper (Barker and Jencks, 1969). Adler and Kistiakowsky (1962) carried out a careful kinetic study of the substrate activation exhibited by this enzyme in the presence of methyl butyrate. Hofstee (1954b) has reported a similar substrate activation with medium chain-length aliphatic esters of *m*-hydroxybenzoate and made the interesting observation that the faster hydrolysis of longer chain esters is a consequence primarily of an increased maximal velocity, rather than of a more favorable Michaelis constant, indicating that the hydrocarbon moiety of the substrate can bring about a decrease in the free energy of activation; this can be interpreted in terms of strain in the enzyme–substrate complex induced by hydrophobic bonding (Lumry, 1959). Several other groups have independently carried out kinetic studies of pig liver esterase (Krisch, 1963; Bernhammer and Krisch, 1965; Krisch, 1966; Ocken, 1967; Levy and Ocken, 1967; Stoops *et al.*, 1969) which will be described further in the Discussion section; in particular, many of the results reported here are in agreement with those of Stoops *et al.* (1969). Some of our results have been described in a preliminary report (Barker and Jencks, 1967).

Experimental Procedure

Most of the materials and methods used in this work were described in the preceding paper (Barker and Jencks, 1969). Reagent grade organic solvents were used without further purification except for dioxane, which was freshly distilled from a purified stock prepared by Dr. Gerald Fasman. *m*-Carboxyl-*p*-nitrophenyl acetate was a gift of Dr. Stephen Marburg. Solutions of *p*-nitrophenyl acetate up to 2.5×10^{-3} M were prepared in water without the addition of organic solvents by prolonged stirring at room temperature. Solutions of *m*-(*n*-heptanoyloxy)benzoic acid (mC_7)[1] and *m*-(*n*-pentanoyloxy)benzoic acid (mC_5) were dissolved at room temperature in 0.01 M Tris buffer (pH 8.0) containing an equimolar amount of sodium bicarbonate to neutralize the carboxyl group, unless noted otherwise; sodium bicarbonate at concentrations up to 0.05 M was shown to have no effect on enzyme activity.

Unless noted otherwise, the enzyme was diluted from a concentrated stock solution into assay buffer to give a concentration of 1.2×10^{-7} M or less 1 day before a kinetic experiment, stored at 2° overnight, and brought to 25° for at least 1 hr before beginning an experiment. The kinetic experiments were carried out at 25° in cuvets contained in the thermostated cell compartment of a Zeiss PMQII or Gilford 2000 spectrophotometer. The observed rates were corrected for nonenzymatic hydrolysis of the substrate under the same experimental conditions if necessary; such corrections were always less than 15% of the observed rates. All kinetic constants are based on enzyme *molarity* and a molecular weight of 168,000.

Results

General Kinetic Properties. A number of unusual kinetic observations and problems were encountered in our attempts to examine the kinetics of this enzyme preparation, some of which remain unexplained. Preliminary experiments with enzymes prepared by different methods and the experience of others suggest that some of these properties may be peculiar to this preparation. A tendency toward drift and irreproducibility during a series of kinetic determinations was reduced to manageable proportions by diluting the enzyme in the

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[1] Abbreviations used are: mC_5, *m*-(*n*-pentanoyloxy)benzoic acid; mC_7, *m*-(*n*-heptanoyloxy)benzoic acid.

buffer to be used for assay 1 day before carrying out a series of experiments. Measurements of viscosity and nonenzymatic hydrolysis with increasing concentration of mC_7 under the conditions of the kinetic experiments provided evidence for micelle formation at concentrations above 0.02 M, so that kinetic experiments were generally carried out at a concentration of less than 0.02 M; a critical micelle concentration of 0.06 M for this substrate under slightly different conditions has been reported by Hofstee (1958). A differing activity of the enzyme toward different solutions of the same preparation of mC_5 substrate was traced to the formation of micelles that were stable for hours after dilution to concentrations less than the critical micelle concentration. It is of interest that enzyme activity toward these aggregates, although irreproducible, was often higher than toward the completely dissolved substrate. Micelle systems frequently require only milliseconds to reach equilibrium (Kresheck *et al.*, 1966), but with longer chain anionic esters (Hofstee, 1958) and ferriprotoporphyrin micelles (Blauer and Zvilichovsky, 1968) equilibration requires minutes or hours.

Rate determinations with *p*-nitrophenyl acetate frequently exhibited a lag phase of up to 2 min, during which the enzyme initially exhibited a rate some 20% less than the steady-state rate. Although this lag was reduced or eliminated by prior incubation of the enzyme in the reaction mixture for a few minutes in the absence of substrate, it cannot be attributed to dissociation of the enzyme because the enzyme does not undergo dissociation in the presence or absence of substrate during the time required for assay (Barker and Jencks, 1969). A similar lag was observed by Craig and Kistiakowsky (1958) in the hydrolysis of methyl propionate catalyzed by horse liver esterase. Furthermore, enzyme activity was found to be nonlinear with respect to enzyme concentration over a wide range of enzyme concentration, from 6×10^{-11} to 6×10^{-8} M, with a 20–40% increase in specific activity over a tenfold increase in enzyme concentration. The same nonlinearity was observed with enzyme which had been dissociated to half-molecules at pH 4.5 and assayed in the presence of 0.5 M sodium chloride at pH 8, under conditions in which reassociation to whole molecules does not take place during the time of the assay. Such nonlinearity has not been reported previously for purified esterase preparations, but it is not apparent from the literature that experiments have been carried out over a wide enough range of enzyme concentrations for its detection. The obvious explanations for such nonlinearity are the presence of activator molecules in the enzyme preparation and inactivation of the enzyme at low concentrations under the conditions of the assay, but a number of experiments designed to evaluate these explanations or avoid their consequences were negative.

Effect of pH. In Figure 1 are shown the effects of pH on the rate of hydrolysis of 0.33 mM *p*-nitrophenyl acetate catalyzed by whole molecules and half-molecules of esterase in the presence (lower line) and absence (upper line) of 0.5 M sodium chloride. The dissociated enzyme was prepared by incubation of 0.04 mg/ml of enzyme in 0.01 M acetate buffer (pH 4.5) for 40 min, followed by reneutralization to pH 8.0 by the sequential addition of 2.0 M sodium chloride, 1.5 M Tris buffer, and water to give a final concentration of 0.05 M Tris buffer and 0.5 M sodium chloride. Under these conditions, the enzyme remained at least 70% in the dissociated form during the course of the experiment as measured by chromatography on Sephadex G-100 gel. There is no significant effect of dissociation to half-molecules on enzyme activity toward *p*-nitrophenyl acetate over this pH range. Sodium chloride inhibits the hydrolysis of this substrate, but does not cause a significant shift in the midpoint of the pH–rate profile. The activity decreases with decreasing pH, with a midpoint near pH 6.0, but does not follow a normal ionization curve (dashed line).

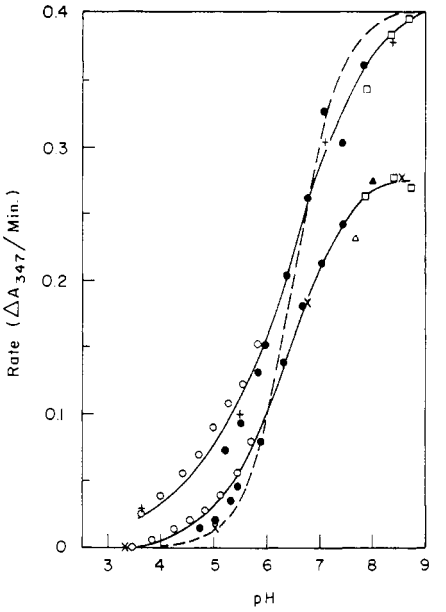


FIGURE 1: The effect of pH on the rate of hydrolysis of 0.33 mM *p*-nitrophenyl acetate catalyzed by 4×10^{-9} M esterase in the presence (lower line) and absence (upper line) of 0.5 M NaCl. The enzyme was preincubated in the reaction mixture for 3 min before the addition of substrate. The upper set of symbols shows rates obtained with native enzyme except for the crosses, which were obtained with dissociated enzyme prepared as described in the text. The lower set of symbols shows rates obtained with dissociated enzyme except for the x's, which were obtained with native enzyme. Rates were determined at 347 nm, the isosbestic point of *p*-nitrophenol. Buffers (0.05 M): squares, Tris-chloride; triangles, sodium borate; solid circles, potassium phosphate; open circles, sodium acetate. The dashed line shows a normal titration curve, $pK = 6.5$.

In contrast, the activity toward the anionic substrate mC_5 falls off as the pH is reduced from pH 8 to 6, but increases strikingly as the pH is lowered further (Figure 2). This indicates that protonation of the carboxylate group of this substrate greatly increases its effectiveness as a substrate. At higher substrate concentrations, there is a further rate decrease between pH 5 and 4, presumably caused by the formation of inhibitory micelles as the charge on the substrate molecules is decreased.

Effect of Substrate Concentration. The esterase exhibits biphasic kinetics, with substrate activation at high concentrations of mC_7 similar to that observed with the horse liver enzyme by Hofstee (1954a,b) for a series of related substrates (Figure 3, lower line). The kinetic constants estimated from these results in 0.05 M Tris buffer (pH 8.0) are given in Table I, in which K_M^H and K_M^L are the Michaelis constants and V^H and V^L are the maximal velocities for the high and low substrate portions of the curve. Similar behavior, but with somewhat different kinetic parameters, was observed in phosphate buffer at pH 7.75 and over a 200-fold range of enzyme concentration (Table I).

Substrate activation is also observed for enzyme which has

TABLE I: Kinetic Parameters for the Esterase-Catalyzed Hydrolysis of mC_7 at 25°.

Conditions	Enzyme Concn[a] (M × 10^9)	K_M^H (M × 10^3)	V^H (min^{-1} × 10^{-4})	K_M^L (M × 10^4)	V^L (min^{-1} × 10^{-4})
0.05 M Tris buffer, pH 8.0	2.0	3.3	3.6	2.2	1.6
0.07 M potassium phosphate buffer,	0.20	2.2	1.8	1.1	1.0
pH 7.75[b]	2.0	4.8	3.1	0.7	1.3
	40	3.5	3.4	0.6	1.4
Dissociated enzyme[c,d]	8.0	4.0	3.6	2.3	2.5
0.5 M LiBr[d]	2.0	1.0	3.5	2.3	2.4

[a] Based on molecular weight 168,000. [b] Each assay contained 0.1 mg of bovine serum albumin in 3.0 ml. [c] The enzyme was dissociated at pH 4.5 and reneutralized in the presence of 0.5 M sodium chloride; see text. [d] In 0.05 M Tris buffer (pH 8.0).

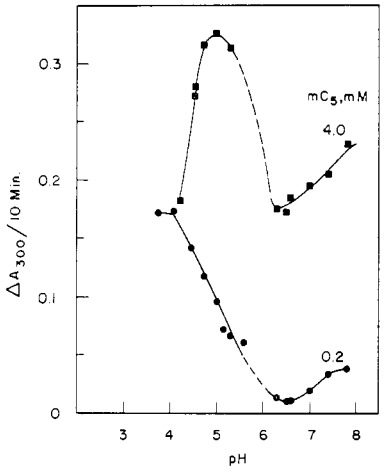


FIGURE 2: The effect of pH on the rate of hydrolysis of mC_5 catalyzed by 2 × 10^{-9} M esterase at 4.0 and 0.2 mM mC_5 in 0.05 M potassium phosphate (pH >6) or sodium acetate (pH <6) buffers.

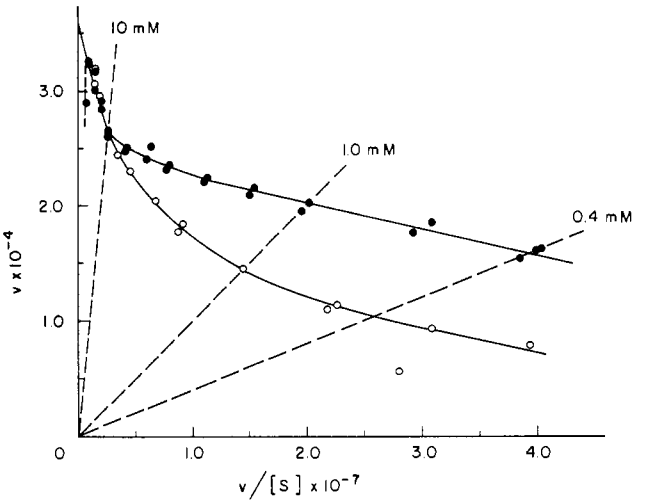


FIGURE 3: Eadie plot of the esterase-catalyzed hydrolysis of mC_7 in 0.05 M Tris buffer (pH 8.0) at 25°. Open circles: 2 × 10^{-9} M native enzyme. Closed circles: 8 × 10^{-9} M dissociated enzyme, prepared by incubating 2.4 × 10^{-7} M (0.04 mg/ml) enzyme in 0.01 M sodium acetate buffer (pH 4.5) at 20° for 40 min. Then appropriate amounts of 2.0 M sodium chloride, 1.5 M Tris, and water were added *in that order* to give a solution containing 0.5 M sodium chloride, 0.05 M Tris (pH 8.0), and 1.2 × 10^{-7} M enzyme.

been dissociated into half-molecules at pH 4.5, as described above (Figure 3, upper curve). The dissociated enzyme has an activity very similar to that of the native enzyme at high substrate concentration, but shows a faster rate at low substrate concentration. The enzyme was shown to remain at least 85% in the dissociated form in the presence of 0.5 M sodium chloride over the 3 hr required for this experiment and gave strictly linear reaction rates upon dilution into the reaction mixtures. In another series of experiments the dissociated enzyme was examined at a smaller number of substrate concentrations over a range of enzyme concentrations from 1.6 × 10^{-8} to 2 × 10^{-9} M. Although an increase in specific activity occurs with increasing enzyme concentration, biphasic behavior was always observed and the break between the high and low substrate portions of the curve occurred at the same substrate concentration, 6 mM, at all enzyme concentrations.

Biphasic kinetics are also exhibited for the hydrolysis of *p*-nitrophenyl acetate catalyzed by the Adler and Kistiakowsky (1961) enzyme (Figure 4, circles). Enzyme prepared by the procedure of Horgan *et al.* (1966)[2] shows similar kinetic behavior at low substrate concentrations, but shows little or no substrate activation (Figure 4, squares). Hydrolysis catalyzed by the Adler and Kistiakowsky enzyme showed a lag phase in the range of 0.083–0.83 mM substrate concentration and the rate constants were calculated from the strictly linear reaction rate after this lag; no such lag was observed with the enzyme prepared by the procedure of the Australian workers.

The anionic substrate *m*-carboxyl-*p*-nitrophenyl acetate is hydrolyzed less than 1% as fast as *p*-nitrophenyl acetate, although the chemical reactivities of the two esters are very similar, as judged from their rates of nonenzymatic hydrolysis. Accurate kinetic data could not be obtained because of the unfavorable ratio of enzymatic to nonenzymatic reaction rates, but the values of the Michaelis constant and maximal velocity were estimated to be on the order of 10^{-3} M and 85 min^{-1}, respectively, in 0.05 M sodium borate buffer, pH 8.1 (this buffer was chosen to minimize nonenzymatic hydrolysis;

[2] P. Greenzaid, unpublished experiments.

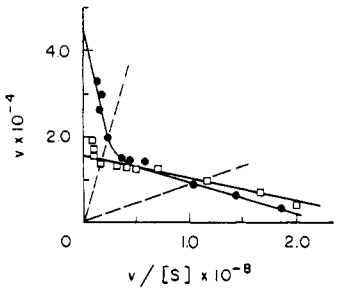


FIGURE 4: Hydrolysis of *p*-nitrophenyl acetate catalyzed by esterase purified by the method of Adler and Kistiakowsky (1961) (circles) and by the method of Horgan *et al.* (1966) (squares). The reactions were initiated by adding 0.02 ml of esterase to give a final concentration of 6.6×10^{-10} M enzyme in 0.033 M potassium phosphate buffer (pH 7.4).

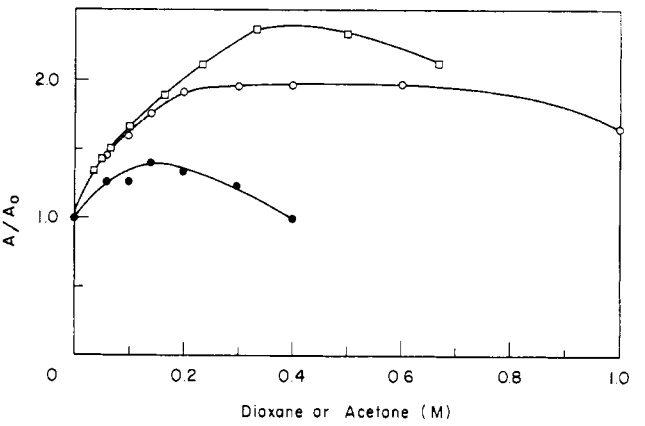


FIGURE 5: Activation by dioxane (circles) and acetone (squares) of the enzymatic hydrolysis at 25°. Conditions: 10 mM (open circles) and 0.4 mM (closed circles) mC_7 in 0.067 M potassium phosphate buffer (pH 7.75); 5 mM mC_7 (squares) in 0.05 M Tris-chloride (pH 7.7) and 2×10^{-9} M esterase. A/A_0 = ratio of initial rates of hydrolysis in the presence and absence of dioxane and acetone. Assays were started by the addition of substrate after the enzyme had been incubated at 25° in buffer and organic reagent for 3–5 min.

similar but even less accurate results were obtained in Tris buffer). These values may be compared with values of $V^L = 1.7 \times 10^4$ min^{-1} and $K_M^L = 8 \times 10^{-5}$ M for *p*-nitrophenyl acetate in 0.05 M Tris buffer (pH 7.8).

Effect of Salts. The presence of 0.44 M salt generally increases the rate of esterase-catalyzed hydrolysis of the anionic substrate mC_7 and inhibits the hydrolysis of *p*-nitrophenyl acetate (Table II). A kinetic analysis of the effect of 0.5 M lithium bromide on the hydrolysis of mC_7 indicates that V^L is increased by about 50% and K_M^H is decreased by a factor of 3.3 compared with the rates under the same conditions in the absence of salt (Table I, first and last lines). The values of V_M^H and K_M^L are unchanged, so that the effect of the salt is in the direction of a linearization of the biphasic kinetic curve with no change in the maximum velocity. The opposite effect was observed with *p*-nitrophenyl acetate hydrolysis, which was most inhibited by 0.5 M lithium bromide at low substrate concentrations to give a decrease in V_M^L and an increase in the biphasic character of the curve, although the value of V_M^H was again found to be unchanged. In no case did the presence of salt result in a time-dependent change in enzyme activity during a kinetic run which might suggest a time-dependent conformation change; in fact the lag phase usually observed in the hydrolysis of *p*-nitrophenyl acetate was not observed in the presence of lithium bromide.

Effect of Organic Reagents. The esterase-catalyzed hydrolysis of *p*-nitrophenyl acetate is increased by up to 75% by a variety of organic reagents at a concentration of 0.1 M (Table III); similar activations were observed with mC_7. At high substrate concentrations the activation reaches a plateau at about 0.5 M acetone or dioxane and then gradually decreases with a further increase in the concentration of organic reagent;

TABLE II: The Effect of 0.44 M Salts on the Esterase-Catalyzed Hydrolysis of mC_7 and *p*-Nitrophenyl Acetate at 25°.

	Relative Activity[a]	
Salt	4.4 mM mC_7	0.3 mM *p*-Nitrophenyl Acetate
(None)	100	100
NaCl	138	86
KCl	147	97
LiCl	136	92
$LiNO_3$		39
LiBr	142	76
$NaNO_3$		52
NaBr	134	68
KBr	145	86
NaSCN	119	81
NaI	62	17
KI	144	67

[a] In 0.05 M Tris buffer (pH 7.8). Assays were initiated with enzyme to give a final concentration of 5.8×10^{-10} M for -*p*nitrophenyl acetate and 2.9×10^{-9} M for mC_7 hydrolysis.

TABLE III: The Effect of Organic Reagents on the Esterase-Catalyzed Hydrolysis of *p*-Nitrophenyl Acetate.

Reagent (0.1 M)	Enzyme Act.[a] (% of Control)
Acetone	175
Dioxane	156
Ethanol	149
Acetonitrile	120
Dimethyl sulfoxide	114
Methanol	114
Tetrahydrofuran	107

[a] The reaction mixtures contained 0.52 mM substrate, 0.8×10^{-9} M esterase, and 0.1 M potassium phosphate (pH 7.62). The enzyme was incubated in buffer and organic solution for 5 min at 25° before substrate was added to start the reaction.

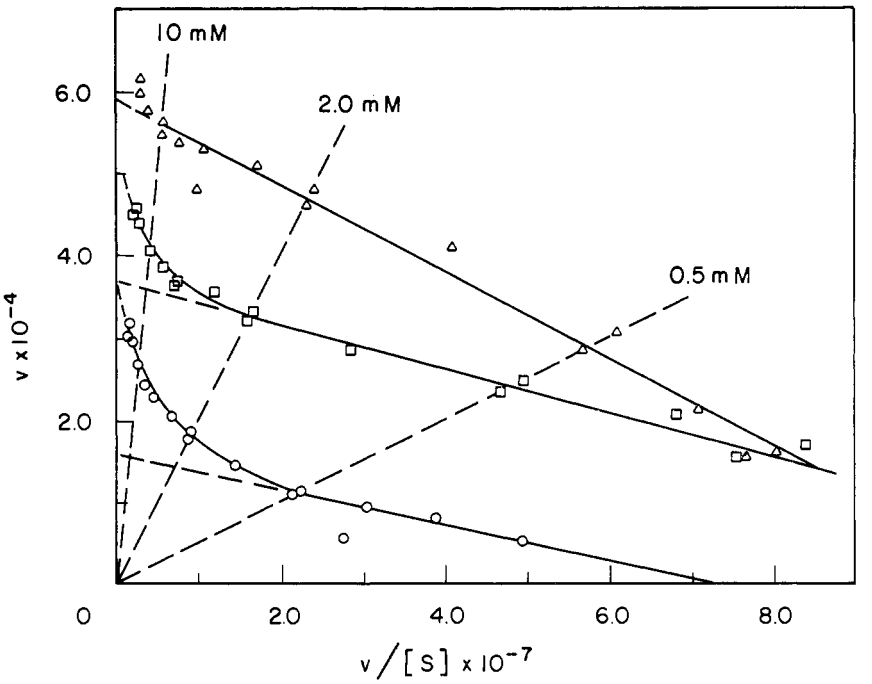


FIGURE 6: Effect of 0.1 M acetone (squares) and 0.25 M acetone (triangles) on the esterase-catalyzed hydrolysis of mC$_7$ in 0.05 M Tris buffer (pH 8.0) at 25°. Assays were started by the addition of substrate after preincubation of the enzyme in the reaction mixture for 3–5 min, except for assays at the two highest substrate concentrations, which were initiated with enzyme.

at low substrate concentrations the inhibition sets in at a lower concentration of organic reagent after an initial smaller activation (Figure 5).

The effects of 0.1 and 0.25 M acetone on the kinetics of hydrolysis of mC$_7$ as a function of substrate concentration are shown in Figure 6. The principal effect is an increase in the value of V_M^L. There is also a definite increase in V_M^H, although the substrate concentration cannot be increased sufficiently to make possible a quantitative estimate of the increase. There is no significant effect of 0.1 M acetone on K_M^L, but this quantity is increased about twofold in the presence of 0.25 M acetone (Table IV).

The activation of p-nitrophenyl acetate hydrolysis by 0.1 M acetone was examined at pH 4.5 with enzyme which had been previously incubated at 0.02 mg/ml at pH 4.5 for varying periods of time, conditions which are known to cause dissociation of the enzyme to half-molecules (Figure 7). The absence of a significant change in the amount of activation by acetone with time indicates that the whole and half-molecules are activated to a comparable extent. The small increase in the activity of both the activated and inactivated enzyme with time is probably not significant; it may be a consequence of the fact that the enzyme was diluted directly into the reaction mixture without preliminary incubation at 2° overnight.

TABLE IV: The Effect of Acetone on the Kinetic Parameters for mC$_7$ Hydrolysis at pH 8.0, 25°.

[Acetone] (M)	K_M^L (M × 10^4)	V^L (min^{-1} × 10^{-4})
0.0	2.2	1.6
0.10	2.7	3.7
0.25	5.4	5.9

Modification of Kinetic Properties. Adler and Kistiakowsky (1961) have shown that substrate activation in the hydrolysis of methyl butyrate is eliminated by heating the enzyme at 67° until 81% of the enzyme activity is lost. We have observed a similar decrease in substrate activation in the hydrolysis of p-nitrophenyl acetate with enzyme which had been exposed to acid pH during chromatography or electrofocusing at pH 4.5–5.5 and, in one experiment, with enzyme which had been allowed to stand for 4 days in 0.02 M potassium phosphate buffer (pH 7.4) at 2°. In some of these experiments recoveries of activity (at low substrate concentration) of over 70% were obtained. The effect of these changes in properties appears to be to make the enzyme resemble more closely the enzyme prepared by the procedure of Horgan *et al.* (1966).

Discussion

The substrate activation that gives rise to the biphasic kinetics exhibited by liver esterase could be caused by (A) the presence of two active sites with differing kinetic characteristics on different enzyme molecules or half-molecules, (B) interaction between the two active sites which is transmitted between the two half-molecules, perhaps in a manner similar to that which occurs between hemoglobin subunits upon oxygenation, and (C) activation by an activator site in each half-molecule which is different from the active site, so that there would be a total of four substrate binding sites in each whole molecule. Adler and Kistiakowsky (1962) concluded that the homogeneity of their enzyme preparation ruled out expla-

nation A involving two enzymes of differing kinetic properties, but they did not rule out the presence of two different active sites on each whole molecule. They chose to discuss their kinetic data in terms of mechanism B. Levy and Ocken (1967) and Ocken (1967) have shown that substrate activation in the hydrolysis of ethyl butyrate can be blocked by *n*-butyl alcohol and have proposed a complex mechanism involving an esteratic site and a second binding site which can accelerate the reaction by "transferring" a substrate molecule to the first site; the second site becomes inactive in the presence of inhibitors. Heymann and Krisch (1967) have found that phosphorylation of the enzyme by bis(*p*-nitrophenyl) phosphate does not follow simple first- or second-order kinetics and have analyzed their data in terms of two active sites which react at tenfold different rates. However, the data do not give a satisfactory fit to the theoretical curve for this mechanism and Horgan *et al.* (1969) have not noted biphasic behavior of this magnitude in the analogous reaction of the enzyme with stoichiometric amounts of *p*-nitrophenyl dimethylcarbamate. Furthermore, the observation that approximately half the original activity remains after reaction with 1 mole of diethyl *p*-nitrophenyl phosphate for each whole molecule of enzyme (Krisch, 1966) suggests that the enzyme does not contain two sites of markedly different activity.

Physical and immunological data from this and other laboratories confirm the conclusion of Adler and Kistiakowsky (1961) that liver esterase can be prepared in an essentially homogeneous state, so that the kinetic behavior does not result from the presence of two different enzymes in the preparation. Physical and immunological studies on the dissociated enzyme (Barker and Jencks, 1969) suggest that the half-molecules are closely similar or identical, so that it is unlikely that the biphasic kinetics can be explained by the presence of two kinetically different active sites in the whole molecule. The fact that the enzyme is activated by small organic molecules, such as acetone and dioxane, as well as by a second molecule of substrate shows that the activity of the catalytic site may be increased by binding of an appropriate molecule to a second site and, therefore, provides more direct evidence that the kinetic behavior cannot be explained by mechanism A. The low concentration of activator required to stimulate the enzyme suggests that the activation is not a nonspecific solvent effect—acetone causes a 25% rate increase at 0.02 M and benzene activates at even lower concentrations (Stoops *et al.*, 1969).

A choice between mechanisms B and C is made possible by the observation that the enzyme can be separated into two closely similar or identical half-molecules at acid pH and maintained in the active, dissociated state in the presence of salt near neutral pH for times which are long compared to the time required for kinetic analysis. The facts that substrate activation is observed under these conditions with the dissociated enzyme and that activation by acetone is observed to approximately the same extent with intact and dissociated enzyme at pH 4.5 show that interaction between the half-molecules is not required for activation. Therefore mechanism C, with two catalytic sites and two activator sites, must be correct. Stoops *et al.* (1969) have independently reached the same conclusion from the observations that the esterase-catalyzed hydrolysis of phenyl butyrate is accelerated by benzene and that substrate activation is shown by enzyme after exposure to pH 3.57 (until 90% of the original activity is lost) followed by dialysis against Tris buffer at pH 7.6 containing 0.5 M sodium chloride, conditions which would be expected to result in dissociation.

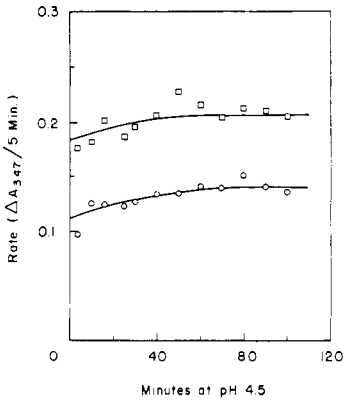
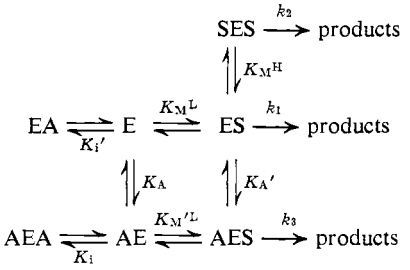


FIGURE 7: Activation by acetone during the dissociation of enzyme to half-molecules at pH 4.5. At zero time, the enzyme was diluted to 1.2×10^{-7} M in 0.01 M sodium acetate, pH 4.5, 20°. At subsequent times aliquots were assayed by diluting further to 2×10^{-9} M into an assay mixture containing 0.67 mM *p*-nitrophenyl acetate and 0.01 M sodium acetate (pH 4.5) in the absence (circles) or presence (squares) of 0.10 M acetone, 25°.

The available data indicate that the change in catalytic properties mediated by this activator site is not a simple consequence of the occupancy or nonoccupancy of the site; *i.e.*, occupancy of the activator site may lead to several different kinetic consequences. All modifier molecules tend to decrease the biphasic character of the dependence of rate upon substrate concentration, but the maximum rate obtained depends upon the nature of the activator, the substrate, and the particular enzyme preparation being examined. Thus, activation of mC_7 hydrolysis by acetone gives a higher maximal velocity than activation by substrate (Figure 6), activation of phenyl butyrate hydrolysis by benzene gives the same maximal velocity as does substrate activation (Stoops *et al.*, 1969), and the addition of *n*-butyl alcohol prevents activation altogether, giving a maximum velocity which is the same as that extrapolated from rates measured at low concentrations of ethyl butyrate (Ocken, 1967).[3] Furthermore, the enzyme prepared by the Adler–Kistiakowsky method shows pronounced substrate activation with *p*-nitrophenyl acetate but the Horgan *et al.* (1966) preparation does not (Figure 4). These results suggest that occupancy of the activator site by an activator molecule may cause a greater, equal, or smaller activation compared to the activation brought about by occupancy by a second substrate molecule and may have no effect at all except to prevent occupancy by an activating substrate molecule. The situation may be described by the scheme of eq 1, in which, E, A, and S refer to enzyme, activator and substrate, respectively, and binding to either the activator or catalytic sites is indicated by placing the symbol for the bound molecule either before or after E. This scheme is similar to that proposed by Stoops *et al.* (1969), but contains some modifications suggested by the

[3] The interpretation of the inhibitory effect of *n*-butyl alcohol is clouded by the possibility that the alcohol acts as an acyl group acceptor. This uncertainty is decreased, but not dispelled, by the fact that 2-butanone exhibits somewhat similar inhibitory behavior.

available data. It is too complex for quantitative evaluation at this time, but inspection of the kinetic plots indicates qualitative agreement with the experimental results. Formation of the activated complexes SES and AES, which decompose to products with the rate constants k_2 and k_3, corresponds to the activation by substrate and activator, respectively. The maximum activation reached with acetone is greater than that



(1)

observed with mC$_7$, corresponding to the case in which $k_3 > k_2$. With benzene and phenyl butyrate $k_3 = k_2$, and with ethyl butyrate and *n*-butyl alcohol only the substrate activates, so that $k_1 = k_3$ and $k_2 > k_1$. Apparently the alcohol occupies the activator site, preventing substrate activation, but does not induce an activity change. Low concentrations of acetone activate the hydrolysis of mC$_7$ without changing the K_M of the substrate appreciably, indicating that the dissociation constant K_A' is smaller than K_i' or K_i for this activator and that the other primed and unprimed constants do not differ greatly from each other. However, at higher acetone concentration, the Michaelis constant for mC$_7$ is increased, suggesting that the activator may bind to the catalytic site and prevent binding of substrate. Similar inhibition is observed with *n*-butyl alcohol and 2-butanone in the hydrolysis of ethyl butyrate (Ocken, 1967; Levy and Ocken, 1967).

We know almost nothing about the mechanism by which activation is transmitted from the activator to the catalytic site or about the function of this activation. Although the metabolic role of the enzyme is not clear, it seems unlikely that the relatively small substrate activation shown by the isolated esterase could be an important regulatory mechanism for the pig. Therefore, it may be that the function of the activation process is more closely related to the catalytic process, *per se*, than to metabolic regulation. A hint may be found in the observations that the enzyme appears to be remarkably sensitive to its history as well as to its environment. Different methods of purification give preparations with different kinetic properties, and these properties are sensitive to the environment in ways that are not all understood. Also, esterase may be isolated in an aggregated form which is active toward long-chain substrates and which may be reversibly converted into the ordinary esterase by the removal or addition of lipid (Schantz and Cortner, 1967; Okuda and Fuji, 1968). These observations suggest that the enzyme is a conformationally labile molecule which can be isolated in several metastable states different from the native state, and that the native state may be one in which binding to a specific lipid structure in the cell makes reaction possible with longer chain substrates than the purified enzyme will attack. Although a high concentration of micelles inhibits the enzyme, the fact that an enhanced activity is observed in the presence of low concentrations of certain substrate micelles is consistent with this interpretation.

The occurrence of activation by substrates and activators has significant implications for the interpretation of the mechanism of action of these enzymes. The observation of substrate activation means that the substrate binds more tightly to the catalytic than to the activator site; if it bound more tightly to the activator site only the high activity kinetic curve would be observed. If a given substrate shows no substrate activation (with an enzyme preparation that exhibits substrate activation with other substrates), there are three possible explanations: (1) the substrate binds more tightly to the activator than to the catalytic site, so that only the high activity kinetic curve will be observed; (2) the substrate does not bind to the activator site; (3) the substrate binds to the activator site but this binding has no effect on the rate; *i.e.*, $k_1 = k_2$. If maximal velocities are being compared for a series of different substrates which do not show substrate activation, it is important to know whether the high or low activity of the enzyme is being measured before mechanistic conclusions can be drawn. This can sometimes be determined by observing whether or not a known activator molecule increases the rate at high substrate concentration. Alternatively, the high and low activities may be distinguished by the different dependencies of V^L and V^H upon pH. Adler and Kistiakowsy found that the pK values for the dependencies of maximal activity upon pH at high and low concentrations of methyl butyrate are 3.4 and 5.7, respectively. These pK values are difficult to reconcile with an involvement of the imidazole group of a histidine residue in the rate-determining step of the enzyme-catalyzed reaction. The pK of 5.7 might be ascribed to a perturbed imidazole ionization (Adler and Kistiakowsky, 1962; Ocken, 1967), but it is difficult to attribute the much lower pK, under conditions in which the activator site is filled, to an imidazole group. The ionization at pH 5.7 is more easily attributed to a group which affects activity indirectly by controlling a conformation change of the enzyme.

Effects of Salts and Charged Substrates. The 100-fold decrease in the activity of esterase toward *m*-carboxyl-*p*-nitrophenyl acetate compared with *p*-nitrophenyl acetate is a reflection of the low activity of this enzyme toward charged substrates, which has been noted by Ocken (1967). The striking increase in the rate of hydrolysis of mC$_5$ with *decreasing* pH as the carboxylate group is protonated provides further evidence for the unfavorable effect of this charged group. Since both the maximal velocity and the Michaelis constant are unfavorable for the charged substrate, the bound substrate is probably oriented in an unfavorable position for reaction relative to the catalytic groups at the active site. The effect of concentrated salt solutions on the catalytic activity of the enzyme can be interpreted in terms of two effects: a conformation change which gives a decrease in activity toward uncharged substrates and a decrease in the unfavorable electrostatic interaction with charged substrates, with a consequent increase in the observed activity toward these substrates. The maximal activity at high substrate concentration is not changed by 0.5 M lithium bromide for either charged or uncharged substrates, which suggests that salts may alter the conformation and charge of the enzyme at low and intermediate substrate concentrations, but do not affect the enzyme when the activator site is occupied. This salt-induced conformation change does not represent dissociation of the enzyme into subunits, since dissociation does not take place during the time required for assay, but the fact that dissociation is favored by salt over

long time periods suggests that it is a preliminary step in the salt-induced dissociation process.

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Titration of Ribonuclease T_1*

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ABSTRACT: Potentiometric and spectrophotometric titrations have been carried out to investigate the structure and properties of ribonuclease T_1. The results of these experiments suggest the following conclusions: nine out of the twelve side-chain carboxyl groups have an abnormally low pK_0 of 3.85, and the other three have a high one of 5.0; the three imidazole groups can be classified into two sets, two groups with pK_0 = 6.6, and one with pK_0 = 7.4; the single lysine has a strikingly low pK_0 of 8.6; the terminal carboxyl and amino appear normal, with pK_0 = 3.85 and 7.4, respectively; only two of the nine phenolic groups titrate normally. The existence of such an unusually large number of abnormal groups presumably reflects the tertiary structure of the ribonuclease T_1 molecule. Titrations in the presence of an inhibitor, 2′-guanosine monophosphate, show that over the pH region 5–9 fewer groups ionize in the complex than in the free protein. The difference titration curve for ribonuclease T_1 and its 2′-guanosine monophosphate complex has a maximal value of *ca.* 1 mole of hydrogen ion/mole of protein at pH 6.3, which can be interpreted as reflecting a shift in the pK of one group from 4.9 to 7.8, and therefore as suggesting that one carboxyl group, presumably Glu-58, is masked by binding of the inhibitor. The pH at which abnormal tyrosines become exposed to the solvent through unfolding of the molecule shifts toward higher pH in the presence of inhibitor; binding of 2′-guanosine monophosphate apparently stabilizes the native conformation of ribonuclease T_1. One or both of the normal tyrosines may interact with 2′-guanosine monophosphate.

Ribonuclease T_1 (RNase T_1) is a globular enzyme that specifically hydrolyzes guanosine 2′,3′-cyclic phosphate and the 3′-phosphodiester linkages of guanosine nucleotides in RNA (Egami *et al.*, 1964). Takahashi (1965) has determined the sequence of the single chain of 104 amino acid residues and the location of the two disulfide bridges. It should be of interest to compare the properties of this protein with those of the ribonuclease of bovine pancreas, which exhibits different substrate specificity (Carter and Cohn, 1950; Schmidt *et al.*, 1951) and is probably not homologous.

The following hydrogen ion titration experiments explore the environments of the ionizable groups of RNase T_1. An improved hydrogen electrode, which does not require bubbling of the titration solution (Iida and Imai, 1969), was preferred

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