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Carboxylesterases (EC 3.1.1). Kinetic Studies on Carboxylesterases*

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ABSTRACT: Kinetic evidence has been obtained which is consistent with the formation of an acyl-enzyme intermediate in the hydrolysis of butyrate esters catalyzed by pig liver carboxylesterase. A study of the pig liver carboxylesterase-catalyzed hydrolysis of phenyl butyrate revealed activation by substrate and by modifiers such as benzene. This activation has been interpreted in terms of a classical kinetic scheme involving sites for substrate and modifier on a single enzyme molecule.

For a series of activated esters, the catalytic rate constants for the carboxylesterase-catalyzed hydrolyses were found to be relatively insensitive to changes in the acyl group, indicating that binding is not responsible for the high reactivity of these systems. Kinetic and other data suggest that there are possibly some different elements of essential chemistry between the esterases and the proteinases.

Mammalian liver carboxylesterases (EC 3.1.1) are highly efficient catalysts of ester hydrolysis. The enzymes differ markedly from the serine proteinases in that high reactivity is exhibited toward simple aliphatic esters. For example, the k_{cat} for the pig liver carboxylesterase-catalyzed hydrolysis of *p*-nitrophenyl acetate is $\sim 10^5$ times the k_{cat} for the same reaction catalyzed by α-chymotrypsin (Horgan *et al.*, 1966). However, α-chymotrypsin exhibits a similarly high reactivity toward "specific" substrates, *e.g.*, *N*-acetyl-L-tyrosine ethyl ester. Since, for these substrates, it has been shown that both the acylation and deacylation steps are sensitive to changes in the acyl moiety, several workers have postulated that a major part of this high reactivity results from the binding of the acyl moiety to the active site (Hein and Niemann, 1962; Koshland, 1962; Bender *et al.*, 1964a).

Investigations of the specificity of carboxylesterases have also shown that changes in the acyl and alcohol moieties affect reactivity. Webb (1964) has extensively studied the specificity of horse liver carboxylesterase. There was only a threefold change in reactivity of acetate and butyrate esters when the chain length of the alcohol moiety was varied from C_1 to C_8. Esters of the C_4 alcohol exhibited the highest reactivity. By contrast, the reactivity of the ethyl ester of *n*-alkyl aliphatic acids increased 14-fold from the acetate to the valerate. Longer chain acyl groups resulted in a small decrease in reactivity. The best substrates were found to contain

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TABLE I: Physical Constants of Substrates.

Acyl Group	Alcohol Group	Mp (°C)
Butyryl	Ethyl	Colorless liquid[a]
Butyryl	Phenyl	Colorless liquid[b]
Butyryl	*o*-Nitrophenyl	Yellow liquid[b]
Butyryl	*m*-Nitrophenyl	Yellow liquid[b]
Butyryl	2,4-Dinitrophenyl	Yellow liquid[c]
Acetyl	Phenyl	Colorless liquid[b]
Acetyl	*p*-Nitrophenyl	77.5–78[b,d]
Acetyl	2,4-Dinitrophenyl	71–72[b,e]
Formyl	Phenyl	Colorless liquid[b]
Formyl	*p*-Nitrophenyl	71.8–72.8[b,f]
Propionyl	Phenyl	Colorless liquid[b,g]
Valeryl	Phenyl	Colorless liquid[b]
Trimethylacetyl	Phenyl	Colorless liquid[b]
L-Tyrosine·HCl	Ethyl	164.0–165.0[h]
D-Tyrosine·HCl	Ethyl	164.0–165.0[i]
N-Benzyloxycarbonyl-L-tyrosine	*p*-Nitrophenyl	156.5–157.0[j]
N-Benzyloxycarbonyl-D-tyrosine	*p*-Nitrophenyl	156.8–157.2[k]
Hippuryl	*p*-Nitrophenyl	170[l]
Hippuryl	*p*-Nitrothiophenyl	166[m]

[a] Aldrich (Milwaukee) preparation. Gas chromatography indicated that the compound was greater than 99% pure. [b] Spectral analysis demonstrates that the compound contained less than 1 mole % of the corresponding phenol. [c] Compare Huggins and Lapides (1947). The compound contained 5.5 mole per cent 2,4-dinitrophenol. [d] Lit. (Bender and Nakamura, 1962) mp 77.5–78°. [e] Lit. (Bender and Turnquest, 1957) mp 71.0–71.5°. [f] Lit. (Stevens and van Es, 1964) mp 71–72°. [g] Eastman product was purified by the method described for nitrophenyl butyrates. [h] $[\alpha]_D^{21}$ 57.6° (*c* 0.33, pyridine). Lit. (Schwert and Takenaka, 1955) mp 165–166°. [i] $[\alpha]_D^{21}$ −58.6° (*c*. 0.37, pyridine). [j] $[\alpha]_D^{22}$ −7.72° (*c* 3.3, acetone). Lit. (Zerner *et al.*, 1964) $[\alpha]_D^{28}$ −8.5° (*c* 3, acetone), mp 158–160°. [k] $[\alpha]_D^{22}$ 7.65° (*c* 2.9, acetone). [l] Lit. (McDonald and Balls, 1957) mp 171°. [m] Lit. (de Jersey *et al.*, 1966) mp 166°.

nonpolar groups at both ends of the molecule. Webb proposed that these results (together with inhibition studies) indicate that van der Waals' forces must play a major role in enzyme–substrate binding.

Hofstee (1954) has shown for a series of straight-chain fatty acid esters of *m*-hydroxybenzoic acid that increases in the acyl carbon chain result in an increase in reactivity up to ten carbon atoms, with each additional carbon atom doubling the rate. Crude horse liver enzyme preparations were used in this study.

Similar specificity studies to those have been made by Malhotra and Philip (1966) with an esterase purified from goat intestinal mucosa, using *p*-nitrophenyl esters. Their work further supports the proposition that hydrophobic interactions play an important part in the carboxylesterase-catalyzed hydrolysis of esters, both in respect of binding of the substrate and of esterolytic activity.

Non-Michaelis–Menten kinetics have been reported for carboxylesterases. Burch (1954) found that the hydrolysis of methyl butyrate by horse liver carboxylesterase was complex. Adler and Kistiakowsky (1962) reported substrate activation (methyl and ethyl butyrates and methyl chloroacetate) for the pig liver enzyme and two interacting sites were postulated to explain these observations. Similar results were obtained by Barker and Jencks (1967) and Levy and Ocken (1967). Barker and Jencks found that 0.5 M LiBr, which stabilizes the monomeric form of pig liver carboxylesterase, reduced the substrate activation shown by Hofstee-type substrates.

We have investigated the kinetic behavior of carboxylesterases to obtain information on: (i) the effect of structure on the reactivity of the substrate; (ii) the effect of high substrate concentration on reaction; (iii) the comparative behavior of various carboxylesterases; and (iv) the mechanism of action of these enzymes.

Experimental Section

Reagents. All salts and buffers were reagent grade. Buffer solutions were filtered through a sintered-glass funnel to remove lint. Benzene (Baker) was reported to contain 0.05% (v/v) water. Hexane (British Drug Houses) was redistilled and the fraction bp 67–70° was collected.

Pig and ox liver carboxylesterases were purified as previously described (Horgan *et al.*, 1969a; Runnegar *et al.*, 1969). Sheep liver carboxylesterase was a purified sample (K. Scott, M. T. C. Runnegar, and B. Zerner, unpublished results). The specific activity of the pig liver enzyme used varied: 447 (~78% pure), 505 (~89%), and 569 (~100%). The specific activity of the

TABLE II: Hydrolysis of Butyric Acid Esters by "Fast" and "Slow" Ox, Sheep, and Pig Liver Carboxylesterases at 25°.

Butyrate	k_{OH} (M^{-1} sec^{-1})[a]	k_{cat} (sec^{-1}), 10^5K_m (M)			
		"Fast" Ox[b]	"Slow" Ox[b]	Sheep[b]	Pig
Phenyl	0.69	160, 17	200, 25	105, 14	519, 10[c]
p-Nitrophenyl	5.9	110, 22	110, 34	100, 20	367, 14[c]
					372, 23[d]
o-Nitrophenyl	2.8	210, 6	240, 8	89, 17	348, 17[d]
m-Nitrophenyl	3.2	60, 16	65, 19	77, 10	345, 25[d]
2,4-Dinitrophenyl	23				233, 11[c,e]
Ethyl					551, 50[f]
Phenyl					520, 27[f]

[a] Determined in standard sodium hydroxide containing 1.2% acetonitrile. [b] 0.1 M phosphate (pH 7.45), 12.8% acetonitrile. [c] 0.05 M phosphate (pH 7.42), 12.8% acetonitrile. [d] 0.15 M Tris (pH 8.1), 12.8% acetonitrile. [e] Similar depression has been found with 2,4-dinitrophenyl acetate. [f] pH-Stat measurement, pH 7.5.

ox liver carboxylesterase was 77 ("fast") and 73 ("slow"), and of the sheep liver enzyme was 218. α-Chymotrypsin (three-times crystallized) was obtained from the Worthington Biochemical Corp. Electric eel acetylcholinesterase (type III, 20,000 units/2 ml, Sigma Chemical Co.) was dialyzed against 0.1 M NaCl–10^{-4} M phosphate buffer (pH 7) before use.

The normalities of the enzyme solutions were determined as previously described (Horgan *et al.*, 1969b; Runnegar *et al.*, 1969; Schonbaum *et al.*, 1961; Bender *et al.*, 1966). These normalities were used in the calculation of k_{cat}.

Bovine serum albumin was a crystallized, lyophilized preparation from the Sigma Chemical Co.

Preparation of Substrates. Phenyl acetate, *p*-nitrophenyl acetate, phenyl butyrate, *o*-, *m*-, and *p*-nitrophenyl butyrates, 2,4-dinitrophenyl butyrate, phenyl valerate, and phenyl trimethylacetate were prepared from the corresponding acid anhydride or chloride in dry pyridine as has been described for the synthesis of *p*-nitrophenyl butyrate (Horgan *et al.*, 1969b). 2,4-Dinitrophenyl acetate, *p*-nitrophenyl hippurate, and *p*-nitrophenyl thiolhippurate were synthesized by reacting equimolar quantities of the phenol, acid, and dicyclohexylcarbodiimide in ethyl acetate. Phenyl and *p*-nitrophenyl formates were prepared by the method of Stevens and van Es (1964). L- and D-tyrosine ethyl esters were prepared by the procedure of Fischer (1901) and were twice crystallized from ethanol–ether. *N*-Benzyloxycarbonyl-D-tyrosine was prepared from *N*-benzyloxycarbonyl-D-tyrosine ethyl ester (Zerner *et al.*, 1964). The ester (2 g) was allowed to react at room temperature for 48 hr in a solution of 42 ml of ethanol and 100 ml of 0.1 N NaOH. The reaction mixture was extracted with six 20-ml portions of chloroform. The water layer was acidified with 1.5 ml of 4 N HCl and stirred. An oil separated which on scratching rapidly gave crystals. The product was recrystallized by dissolving it in the minimum amount of sodium acetate solution (anhydrous sodium acetate, 1 g/100 ml) followed by the slow addition of 1 N HCl: yield 1.1 g, mp 91.0–92.3°, $[\alpha]_D^{23}$ − 9.94° (*c* 2, acetic acid). The L isomer is reported to have $[\alpha]_D^{29}$ 10.3 ± 0.5° (*c* 3.7, acetic acid) (Iselin and Schwyzer, 1960). *N*-Benzyloxycarbonyl-D-tyrosine *p*-nitrophenyl ester was prepared by reacting equimolar quantities of *N*-benzyloxycarbonyl-D-tyrosine, *p*-nitrophenol, and dicyclohexylcarbodiimide for 1 hr at 0°. The ester was purified by the method of Zerner *et al.* (1964).

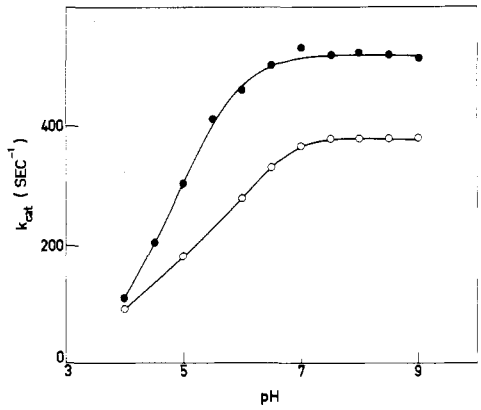


FIGURE 1: pH dependence of k_{cat} for the pig liver carboxylesterase-catalyzed hydrolyses of phenyl butyrate (●) and *p*-nitrophenyl butyrate (○) in 10% acetonitrile at 25°.

Physical constants of the esters are summarized in Table I.

Rate Measurements. Rate measurements were made at 25.0 ± 0.1° using a Cary 14 recording spectrophotometer, or at 25.0 and 38.0 ± 0.1°, using a recording pH-stat (Radiometer TTT1c, SBR2c and SBU1a, Copenhagen) (Horgan *et al.*, 1969a).

In the spectrophotometric assays, an aliquot (100, 75, 50, 25, or 20 μl) of the substrate in acetonitrile was added to 3–4 ml of the buffer solution in a 1-cm cell. Alternately, water solutions of esters were used. To 3–4 ml of these solutions, a 200-μl aliquot of concentrated buffer was added to obtain the desired pH and salt concentration.

The maximum velocity, *V*, and the Michaelis constant, K_m, were obtained from Lineweaver–Burk or

TABLE III: Effect of Acyl Group on Reactivity.[a]

Substrate	Acetylcholinesterase k_{cat} (sec^{-1})	Carboxylesterase (pig) k_{cat} (sec^{-1})	Carboxylesterase (ox) k_{cat} (sec^{-1})	Substrate	α-Chymotrypsin k_{cat} (sec^{-1})
Phenyl formate	13,000[b]	660[c]		*p*-Nitrophenyl formate	~0.7[d]
Phenyl acetate	18,000[e]	380[e]	300[e]	*p*-Nitrophenyl acetate	6.8×10^{-3} [f]
Phenyl propionate	18,000[e]				
Phenyl butyrate		519[g]	200[h]	*p*-Nitrophenyl butyrate	7.7×10^{-3} [i]
Phenyl valerate		860[j]		*p*-Nitrophenyl valerate	3.8×10^{-2} [i]
Phenyl trimethyl-acetate			6.3[k]	*p*-Nitrophenyl trimethyl-acetate	1.3×10^{-4} [l]
2-Phenyl-4,4-dimethyl-oxazolin-5-one			25[m]	2-Phenyl-4,4-dimethyl-oxazolin-5-one	1.1×10^{-3} [m]

[a] 25°. [b] 0.042 M phosphate, 0.10 M NaCl (pH 7.05), and 0.8% acetonitrile. [c] 0.11 M phosphate (pH 6.59) and 0.8% acetonitrile. [d] The value of k_{cat} (pH 8) has been calculated from a substrate-independent rate following a stoichiometric "burst" at pH 5.33 (in 0.14 M NaCl, 0.058 M acetate, and 1.5% acetonitrile) assuming k_{cat} depends upon a pK_a' of 6.85. [e] The esterases exhibited substrate inhibition in a substrate concentration range of 10^{-3}–10^{-2} M; 0.18 M NaCl and 0.053 M Tris (pH 8.30). [f] Bender *et al.* (1964b), at pH 8 in 1.6% acetonitrile. [g] 0.05 M phosphate (pH 7.42) and 12.8% acetonitrile. [h] 0.01 M phosphate (pH 7.45) and 12.8% acetonitrile. [i] Cane and Wetlaufer (1966), from deacylation rate constant of the isolated acyl-enzyme (pH 8.1). [j] 0.037 M phosphate (pH 7.62) and 5% acetonitrile. [k] 0.1 M phosphate (pH 7.45) and 3% acetonitrile. *p*-Nitrophenyl hippurate and *p*-nitrophenyl thiolhippurate were also shown to have k_{cat}'s of this order of magnitude. [l] Bender (1962), at pH 7.9. [m] de Jersey *et al.* (1966), at pH 7.0 in 3% acetonitrile.

TABLE IV: Comparison of Rate Constants for α-Chymotrypsin- and Carboxylesterase-Catalyzed Hydrolyses.[a]

Substrate	Pig Liver Carboxylesterase k_{cat} (sec^{-1})	α-Chymotrypsin k_{cat} (sec^{-1})
L-Tyrosine ethyl ester	71[b,c]	39[b,d]
D-Tyrosine ethyl ester	71[b,c]	Very low[b,d]
Cbz-L-tyrosine *p*-nitrophenyl ester	24[e]	128[f]
Cbz-D-tyrosine *p*-nitrophenyl ester	0.9[e]	0.2[g]

[a] 25°. [b] Rates determined in 0.2 M NaCl using a pH-Stat. [c] pH 6.2. [d] pH 6.0. [e] 0.041 M phosphate (pH 7.2), 8.6% acetonitrile. [f] 0.072 M phosphate (pH 7.68), 8.5% acetonitrile. [g] 0.072 M phosphate (pH 7.75) and 8.4% acetonitrile.

Eadie analysis of the data using the method of least squares.

Gel Filtration. The major experimental details have been described (Horgan *et al.*, 1969a). A column of Sephadex G-200 (1.2 × 107 cm) was equilibrated at 4° (3 weeks) with 0.032 M Tris buffer (pH 7.50). The bed length remained constant throughout the experiments and the void volume measured (Blue Dextran 2000, Pharmacia) was 50.4–50.8 ml. The column was loaded in *individual* experiments with 2-ml aliquots in the following sequence: Blue Dextran 2000 (0.2%), bovine serum albumin (5 mg/ml), and two solutions of pig liver carboxylesterase (specific activity 560, 0.8 μg/ml; specific activity 520, 2.8 mg/ml). Elution profiles were determined by measuring the absorbance at 280 mμ or by a rate assay.

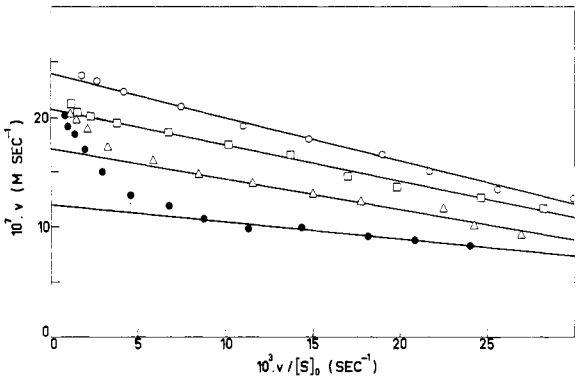


FIGURE 2: Effect of increasing benzene concentration on the pig liver carboxylesterase-catalyzed hydrolysis of phenyl butyrate at 25°. [Benzene] = zero (●), 1.2 mM (△), 1.7 mM (□), and 8.4 mM (○).

An exactly similar series of experiments was then done in which the column buffer contained ~0.01 M benzene (carboxylesterase specific activity 520; 0.98 μg/ml and 2.8 mg/ml).

Results

pH–Rate Profile. The pH–rate profiles for the pig liver carboxylesterase-catalyzed hydrolysis of phenyl and *p*-nitrophenyl butyrates are shown in Figure 1. The pK'_a's obtained by taking the pH values at half maximum activity are 4.8 and 5.1 for phenyl and *p*-nitrophenyl butyrates, respectively.

Comparative Activities. A comparison of k_{cat} values for a series of butyrate esters and for various carboxylesterases is given in Table II.

Effect of Acyl Group on Reactivity. The enzyme-catalyzed rates of hydrolysis of a series of phenyl esters were determined. The enzymes used in this study were ox and pig liver carboxylesterases and acetylcholinesterase (Table III). Data for the α-chymotrypsin-catalyzed hydrolysis of a corresponding series of *p*-nitrophenyl esters are also included in Table III.

Hydrolysis of D- *and* L-*Esters.* The data in Table IV show a comparison of k_{cat} values for some D- and L-substrate pairs obtained with pig liver carboxylesterase and the corresponding data for the α-chymotrypsin-catalyzed reactions.

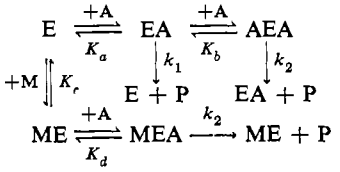
Substrate Activation. In the absence of organic solvent, the hydrolysis of phenyl butyrate catalyzed by pig liver carboxylesterase exhibits substrate activation (Figure 2). Michaelis–Menten kinetics are obtained over the phenyl butyrate concentration range (30–100 μM) but substrate activation is observed above 100 μM. Also shown in Figure 2 is the effect of various concentrations of benzene on the reaction. As the concentration of benzene increases, the maximum velocity increases until Michaelis–Menten kinetics are observed over the entire substrate concentration range. The values of V and K_m obtained at the highest benzene concentration are approximately twice as large as the values obtained in the absence of benzene. In addition, the substrate activation curve extrapolates to the value of V determined at the highest benzene concentration. The kinetic scheme shown in Scheme I is consistent with these results. In this scheme, the enzyme

TABLE V: Effect of Benzene on V and K_m for Phenyl Butyrate.[a]

[Benzene] (mM)	V (μM sec^{-1})	K_m (μM)
0	1.2	15
1.19	1.7	28
1.67	2.1	34
3.98	2.3	38
6.33	2.3	35
8.44	2.4	40

[a] 0.032 M Tris and 0.075 M NaCl (pH 7.84) at 25°. $[E]_0 = 3.9 \times 10^{-9}$ N (pig liver carboxylesterase of specific activity 447). $[A]_0$ = 2.12–0.034 mM.

SCHEME I



substrate complex (EA) decomposes with a rate constant k_1. The modifier- and substrate-activated complexes (MEA and AEA) are postulated to decompose with the same rate constant k_2, which is larger than k_1. The steady-state equations for this kinetic scheme in the presence and absence of modifier are given in eq 1 and 2.

$$v = k_2[E]_0[A] \times \frac{k_1K_b/k_2 + K_aK_b[M]/K_cK_d + [A]}{K_aK_b(1 + [M]/K_c) + K_b[A](1 + K_a[M]/K_cK_d) + [A]^2} \quad (1)$$

$$v = [E]_0[A]\frac{k_1K_b + k_2[A]}{K_aK_b + K_b[A] + [A]^2} \quad (2)$$

Under certain conditions (see below), these rate expressions simplify to Michaelis–Menten form, so that it is possible to test this kinetic scheme and to calculate all the rate and apparent dissociation constants. At low substrate concentrations, in the absence of modifier, Michaelis–Menten kinetics are observed and eq 2 simplifies to

$$v = \frac{k_1[E]_0[A]}{K_a + [A]} \quad (3)$$

from which $k_1[E]_0$ and K_a may be evaluated. When the modifier site is saturated, eq 1 also simplifies to Michaelis–Menten form (eq 4).

$$v = \frac{k_2[E]_0[A]}{K_d + [A]} \quad (4)$$

Hence $k_2[E]_0$ and K_d may be evaluated. Equation 4 requires that at saturating concentrations of modifier,

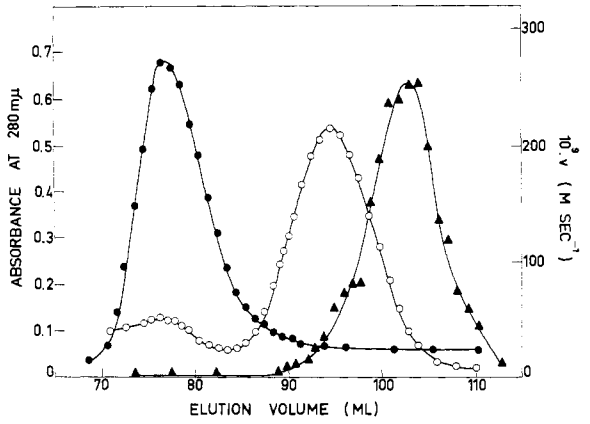


FIGURE 3: Gel filtration on Sephadex G-200 in 0.032 M Tris (pH 7.50), ~0.01 M in benzene. (▲—▲—▲) Pig liver enzyme applied at 0.98 μg/ml; (●—●—●) pig liver enzyme applied at 2.8 mg/ml; (○—○—○) bovine serum albumin applied at 5 mg/ml.

TABLE VI: Calculation of K_b at Different Concentrations of Phenyl Butyrate.

K_b (mM)	[A] (mM)	v (μM sec^{-1})
0.99	2.12	2.02
1.14	1.72	1.92
1.10	1.31	1.85
1.13	0.86	1.71
1.44	0.52	1.49
K_b (av) = 1.09 mM[a]		

[a] Average value over the substrate concentration range 2.12–0.86 mM.

TABLE VII: Rate and Apparent Dissociation Constants for Pig Liver Carboxylesterase-Catalyzed Hydrolysis of Phenyl Butyrate.

$k_1[E]_0$	1.2 μM sec^{-1}
$k_2[E]_0$	2.4 μM sec^{-1}
K_a	15 μM
K_b	1090 μM
K_c	720–300 μM
K_d	40 μM

TABLE VIII: Effect of Acetonitrile on V and K_m.[a]

[Acetonitrile] (mM)	V (μM sec^{-1})	K_m (μM)
10	1.4	20
200	2.3	28
670	3.0	42
1340	3.0	73

[a] Conditions: pH 7.88; otherwise as in footnote *a*, Table V.

both V and K_m become independent of modifier concentration. Table V shows that this condition is satisfied over the benzene concentration range 4–8.4 mM. Using the values of k_1, k_2, and K_a determined in the manner described above, K_b may be calculated, and these results are shown in Table VI. The fact that the calculated value of K_b is independent of the initial rate over the high substrate concentration range demonstrates that the complexes AEA and MEA decompose at the same rate.

The value of K_c may be similarly calculated at low benzene concentrations and gives the values 0.72 mM at 1.19 mM benzene, and 0.30 at 1.67 mM benzene (K_d taken as 40 μM, Table V). The large uncertainty in K_c reflects the small activation observed. The kinetic parameters determined are summarized in Table VII.

The effect of acetonitrile on the pig liver carboxylesterase–phenyl butyrate system has also been investigated and these results are given in Table VIII.

Gel Filtration. The results of the gel filtration experiment on Sephadex G-200 (Figure 3) demonstrate that the pig liver carboxylesterase is monomeric in ~0.01 M benzene solution under the conditions of the experiment. The elution profile is virtually identical with that obtained in the absence of benzene (see Horgan *et al.*, 1969b).

Discussion

Acyl-enzyme Intermediate. The spectrophotometric observation of a "burst" in the titration of carboxylesterases with nitrophenyl dimethylcarbamates (Horgan *et al.*, 1969b; Runnegar *et al.*, 1969) demonstrates that a dimethylcarbamoyl-enzyme intermediate is formed. This result is consistent with the kinetic scheme shown in eq 5, where ES is the enzyme–substrate complex;

$$E + S \overset{K_S}{\rightleftharpoons} ES \xrightarrow{k_{+2}} \underset{+\ P_1}{ES'} \xrightarrow{k_{+3}} E + P_2 \qquad (5)$$

ES′, the acyl-enzyme; P_1, the alcohol (phenol) moiety; and P_2, the acid moiety of the substrate; k_{+2} and k_{+3} are the rate constants for acylation and deacylation, and K_s is the substrate constant. The high values of k_{cat} for good substrates (Table II), coupled with the fact that the carboxylesterases are unstable at low pH, have so far precluded the direct demonstration of the formation of an acyl-enzyme intermediate. The kinetic consequences of eq 5 have been considered previously (Zerner and Bender, 1964).

The data of Table II are not inconsistent with the (largely) rate-limiting deacylation of a butyryl-enzyme. The small degree of discrimination between ethyl and phenyl butyrates supports this argument, as do the other data, provided that the acylation rate constant is decreased with nitro substitution on the aromatic ring. That this is not an unreasonable assumption is shown by the sensitivity of the ox enzymes to the position of the nitro substituents (*cf.* Runnegar *et al.*, 1969). Further, this sensitivity would appear to arise from a steric rather than from a polar effect. The comparative be-

havior of the ox, sheep, and pig enzymes may be partially understood in terms of their active-site sequences (Augusteyn *et al.*, 1969).

Further support for rate-limiting deacylation may be found in the data of Table VIII. For simplicity, the acyl-enzyme has been omitted from Scheme I. If there is a rate-limiting decomposition of a butyryl-enzyme, then the values of K_a and K_d must be less than the values of K_b and K_c, respectively, if the assumption is made that all true dissociation constants in the system are of the same order of magnitude. The K_b/K_a ratio of $\sim$73 and the K_c/K_d ratio of $\sim$18–7.5 are in accord with this argument. Further, the *absolute* values of K_a, K_b, K_c, and K_d are also in accord with this proposition. The fact that in the present work AEA and MEA decompose at the same rate suggests that a *single* active site is modified by the binding of a modifier molecule (A or M). The results of Levy and Ocken (1967) with inhibitors in the closely related ethyl butyrate system also clearly support a single active site. If deacylation is *totally* rate limiting, $K_d/K_a = k_2(E)_0/k_1(E)_0 = 2$, for the phenyl butyrate system. The measured ratio of 2.67 is thus interpreted to be in more than fortuitous agreement. Moreover, it should be noted that if the measured constants were true dissociation constants, one would reasonably have expected K_b and K_d to be of the same order of magnitude, whereas $K_b/K_d = 27$. We shall return to a consideration of the acyl-enzyme later in this paper.

Substrate Activation. The non-Michaelis–Menten kinetics exhibited by phenyl butyrate are adequately accounted for by Scheme I. Phenyl butyrate was chosen as a substrate to reexamine the original observation of Adler and Kistiakowsky (1962) because of the greater precision with which it can be investigated over a wide substrate concentration range. That benzene does not alter the state of the enzyme on the time scale of the gel filtration experiment (Figure 3) has been demonstrated, but this alone does not rule out the possibility of interacting sites, since the dimer does not dissociate rapidly on the time scale of the kinetics (Blakeley *et al.*, 1969). Nonetheless the Cary traces were strictly linear. Moreover, there was no decrease in substrate activation after standing in enzyme solution (0.2 μg/ml, 0.02 M acetate, pH 3.57, 0.5 M NaCl) (*cf.* Barker and Jencks, 1967) until 90% of the original activity was lost. The partially denatured enzyme was dialyzed against 0.032 M Tris (pH 7.59), 0.5 M in NaCl. No decrease in substrate activation was observed. It would appear, therefore, that an isomerization scheme (Monod *et al.*, 1965) or a polymerization scheme (Nichol *et al.*, 1967) need not be invoked to account for the data. The hydrolysis of ethyl butyrate (pig liver enzyme) has been investigated over the substrate concentration range 0.8–25 mM, yielding a linear Eadie plot. These data, therefore, do not comment on the results of Adler and Kistiakowsky (1962). Further, since acetonitrile (Table VIII) has a similar effect to benzene (although it is inhibitory at higher concentrations), the effect of substrate activation has probably been blanketed in the present work.

A Comparison of Esterases and Proteinases. Examination of Table III reveals that acetyl-α-chymotrypsin is an activated ester *vis-à-vis* ethyl acetate, the rate enhancement being $\sim 6 \times 10^4$ (calculated at pH 8). Acetyl carboxylesterases show a further rate enhancement by a factor of $\sim 10^5$ over acetyl-α-chymotrypsin. We postulate that this rate enhancement is significant, and in view of the similarity of the active-site sequences (Sanger, 1963; Augusteyn *et al.*, 1969), that these results support at least some different elements of essential chemistry for the esterases and proteinases.

For the formyl- and acetyl-enzymes, where the size of the acyl group is minimal, the acyl derivatives of the esterases are 10^3–10^6 times as reactive as the corresponding proteinase derivatives. Further, this large difference in reactivity is still present in a comparison of the trimethylacetyl- and *N*-benzoylaminoisobutyryl-enzymes, indicating that the differing reactivities are not due to a binding phenomenon. Bender *et al.* (1964a) have proposed that the high reactivity of "specific" substrates of the proteinases is the result of a "rigidification" of the acylserine intermediate. This hypothesis clearly cannot be extended to the carboxylesterases.

An *O*-acetylserine intermediate has been convincingly demonstrated in the α-chymotrypsin-catalyzed hydrolysis of activated acetate esters (Oosterbaan and van Adrichem, 1958). For the "specific" substrates of α-chymotrypsin, the participation of an acyl-enzyme has again been demonstrated, and this intermediate has been equated to an *O*-acylserine intermediate. Indeed, Shalitin and Brown (1966) have reported the isolation of a "specific" *O*-acylserine intermediate.

Diisopropyl phosphorofluoridate phosphorylates the active-site serine of both the esterases and the proteinases, leading to inactive enzymes, and the work of Weiner *et al.* (1966) emphasizes the central importance of serine in the proteinases.

Table IV shows a comparison of k_{cat} values for some D- and L-substrate pairs. It is clear that pig liver carboxylesterase exhibits some specificity, but that this is much less marked than that shown by α-chymotrypsin. Indeed, *N*-benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester behaves rather as a hindered substrate for the carboxylesterase. It should be noted that *N*-benzyloxycarbonyl-D-tyrosine *p*-nitrophenyl ester exhibits a "burst" under the conditions of the α-chymotrypsin experiment, and it has already been shown that *N*-benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester gives a similar "burst" with α-chymotrypsin at low pH (Kézdy *et al.*, 1964). The "essential role" for serine in the catalytic function of the proteinases appears to be well established (Weiner *et al.*, 1966) and has been further supported in our present study of oxazolinones and hippuryl-chymotrypsin (de Jersey *et al.*, 1969; de Jersey and Zerner, 1969a,b). While there is reasonable support in the present work for the (largely) rate-limiting deacylation of a butyryl-enzyme, our results do not comment on the generality of such a reaction, or on the nature of the intermediate. Further, acetonitrile may well be complicating some of the kinetic measurements and many more data are required adequately to define these systems kinetically. Nonetheless, if the intermediate is a serine ester, it is an exceedingly

reactive ester *vis-à-vis* the corresponding ester in chymotrypsin-catalyzed reactions. The liver esterases are similar in esterolytic activity (within two orders of magnitude) to acetylcholinesterase. The possibility exists, therefore, that there may be different chemistry available to this intermediate. Further support for differing elements of chemistry comes from the pH dependence of the pig liver enzyme, but this cannot be regarded as definitive. Again, ox and pig liver carboxylesterase are rapidly inactivated by the model hydroxylating system: hydroxylamine, cupric ion, oxygen (Runnegar *et al.*, 1968) under conditions where chymotrypsin is not affected.

On the other hand, if the esterases and proteinases enjoy the same chemistry, it may be possible to "activate" the sterically limited acyl-proteinases (*e.g.*, formyl-chymotrypsin) to a level comparable with that of the esterases.

Further work in this laboratory will be directed toward a detailed examination of the similarities and differences of the esterases and proteinases.

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