[CONTRIBUTION FROM THE GIBBS CHEMICAL LABORATORY OF HARVARD UNIVERSITY]

Kinetics of Ester Hydrolysis by Horse Liver Esterase. II

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The hydrolysis of methyl *n*-butyrate was studied by the *p*H-stat technique over a *p*H range from 3.5 to 10 and a concentration range of 1.5 to 58 m*M* or less. Ethyl *n*-butyrate was studied over a narrower *p*H range. The substrate dependence of the rates was studied at *p*H 6 and 8 with methyl propionate, ethyl propionate, *n*-propyl propionate, methyl isobutyrate and ethyl isobutyrate. The dependence of V_{max} of methyl (and ethyl) *n*-butyrate on *p*H between 6 and 9 is very slight. At lower *p*H the activity decreases but is detectable even at *p*H 3.5, the activity–*p*H curve approaching sigmoid shape. The Michaelis constant increases with decreasing *p*H by more than an order of magnitude. No direct evidence of a two-site mechanism was obtained. The data on other esters also fit the simple Michaelis–Menten mechanism, the K_m's being larger at the lower of the two *p*H investigated and V_{max}'s being essentially the same. Inhibition by the products of the reactions, particularly butyric acid, was observed, which increases with the lowering of *p*H. These data are compared with the results of previous work on horse liver esterase and some of the inconsistencies resolved. The *p*H dependence of V_{max} agrees with that derived from the assumption that the active group undergoes acidic ionization, but the *p*H dependence of K_M does not agree completely with this treatment. A requirement of the general Michaelis–Menten mechanism for the *p*H dependence of V_{max}/K_M is found to be obeyed only approximately in the series of esters investigated. Also, if an acyl-enzyme intermediate is formed, its rate of decomposition cannot be the rate determining step.

Work was begun in this Laboratory on the kinetics of the non-specific enzyme liver esterase[1] to complement studies of the specific enzyme urease.[2] These studies of the hydrolysis of methyl *n*-butyrate and ethyl *n*-butyrate, over a thousand-fold concentration range at *p*H 10.2, were interpreted with a two-site or two-enzyme mechanism. For the same substrates Schwert and Glaid[3] found simple Michaelis–Menten behavior over a fivefold concentration range at *p*H 8. Both of these studies were conducted in unbuffered solutions at very low ionic strength, with a purified horse liver esterase preparation.[4] Conners, *et al.*,[4] also found Michaelis–Menten kinetics for the action of their enzyme preparation on methyl *n*-butyrate in strongly buffered solutions at *p*H 7.3. Christiansen and Graae,[5] using an extract from horse liver acetone powder, followed the hydrolysis of methyl valerate at *p*H 8 to nearly 100% reaction. They interpreted their findings in terms of an activated enzyme mechanism, much like that proposed by Medwedew,[6] adding a step involving the combination of the substrate with two molecules of enzyme. Hofstee,[7] also using an extract of horse liver acetone powder, found deviations from Michaelis–Menten kinetics at *p*H 8 for the hydrolysis of hydroxybenzoic acid esters of aliphatic acids.

For the *p*H dependence of liver esterase activity Connors, *et al.*,[4] found a bell-shaped curve with an optimum at *p*H 8, supporting previous work on liver esterases.[8] They used a single methyl *n*-butyrate concentration, 0.1 *M*, and a borate buffer concentration of 0.02 *M*. In contrast, K. and M.[1] suggested an activity *versus* *p*H curve with no maximum but of a sigmoid shape. There is clearly a great deal of uncertainty about the substrate and *p*H dependences of liver esterase activity.

Since Boursnell and Webb[9] have shown that horse liver esterase is irreversibly inhibited by antiesterases such as diisopropyl fluorophosphate, just as are acetyl cholinesterase, α-chymotrypsin and trypsin, it is reasonable to look for similarities in the kinetics. Wilson[10] has proposed a detailed, two-intermediate mechanism for acetylcholinesterase action. A similar mechanism has been proposed for α-chymotrypsin by Gutfreund and Sturtevant.[11] These mechanisms involve an acyl-enzyme compound as the second intermediate. The present study with a series of aliphatic esters, chosen for their water solubility and similarity in structure, was undertaken in an attempt to resolve the inconsistencies in the substrate and *p*H dependences of liver esterase activity, to examine the applicability of the two-intermediate mechanism and to compare the enzymatic catalysis with the well-understood acid and base catalysis.

Experimental Details

Although the conductometric method, which was previously used in this Laboratory, has excellent sensitivity in unbuffered, low ionic strength solutions, it is inconveniently non-linear at *p*H's near neutrality and is limited to low ionic strengths. It was therefore replaced for this research by a manual *p*H-stat method,[5] whereby the *p*H was maintained constant throughout an experiment by continuously adding standard alkali from a micrometer buret. The amount of titrant added measured the extent of the reaction.

A *p*H meter, having a sensitivity and stability of better than 0.003 *p*H unit, was constructed from a Type K potentiometer and a DuBridge–Brown type[12] d.c. amplifier. The amplifier, used with a galvanometer as a null detector, employed a FP 54 electrometer tube and served as a current amplifier with a gain of about 10^5. Its grid resistance was inserted between the glass electrode and the potentiometer, the output being fed to a Type E, L. and N. galvanometer. The electrometer tube was housed in a dehumidified brass case. Storage batteries were used as the power source.

The Beckman glass electrode (1190-80) was employed. Calomel electrodes with leak-type junctions gave fluctuating potentials in the rapidly stirred, low ionic strength solutions. An agar gel junction in an electrode design similar to one of

(1) G. B. Kistiakowsky and P. C. Mangelsdorf, Jr., THIS JOURNAL, **78**, 2964 (1956).

(2) G. B. Kistiakowsky and W. E. Thompson, *ibid.*, **78**, 4821 (1956).

(3) G. W. Schwert and A. J. Glaid, *J. Biol. Chem.*, **199**, 613 (1952).

(4) W. M. Connors, A. Pihl, A. L. Dounce and E. Stotz, *ibid.*, **184**, 29 (1950).

(5) J. A. Christiansen and J. Graae, *Acta Chem. Scand.*, **10**, 1258 (1956).

(6) G. Medwedew, *Enzymologia*, **2**, 1, 31, 53 (1937).

(7) B. H. J. Hofstee, *J. Biol. Chem.*, **207**, 211, 219 (1954).

(8) R. Amon and M. Jaarma, "The Enzymes," Vol. I, Part 1, ed. J. B. Sumner and K. Myrback, Academic Press, New York, N. Y., 1950.

(9) J. C. Boursnell and E. C. Webb, *Nature*, **164**, 875 (1949).

(10) I. B. Wilson, *Disc. Faraday Soc.*, **20**, 119 (1955).

(11) H. Gutfreund and J. M. Sturtevant, *Proc. Nat. Acad. Sci. U. S.*, **42**, 719 (1956).

(12) L. A. DuBridge and H. Brown, *Rev. Sci. Instr.*, **4**, 532 (1933).

Lingane[13] proved satisfactory. It was contained in a Pyrex tube of 12 mm. diameter which was closed at the bottom with sintered glass of medium porosity. Above it was a plug of 3% agar saturated with potassium chloride, and above the agar was saturated potassium chloride solution.

The reaction vessel was a 200-ml. tall-form beaker fitted with a rubber stopper in which were mounted the glass and calomel electrodes. Holes were provided for a nitrogen line, the micrometer buret, a microburet and a pipet to introduce the enzyme. The 150-ml. reaction mixtures were stirred with a Teflon-covered magnet bar.

The water-filled thermostat was regulated to ±0.02° at 25.0°.

A Gilmont micrometer buret (Manostat G 15401) of 1-ml. total capacity and 0.0002-ml. minimum scale division was used to deliver 0.01 *N* standard sodium hydroxide manually.

Solutions were prepared with CO_2-free water and stored in a CO_2-free box. Glass-distilled water was freed from carbon dioxide by purging with nitrogen until it had a *p*H of 7.0 ± 0.2 units.

Two enzyme preparations (no. 1 and 2) were made by the method of Connors, *et al.*[4] Their procedure was followed except in the last step, where concentration by evaporation from a dialysis bag was substituted for pressure filtration. The relative activity at each step agreed quite well with their report. The concentrated stock solution was stored at 1°. (A similar preparation made four years ago[1] and stored at 1° has retained nearly full activity.) Dilute enzyme stocks usually were prepared by diluting 0.10 ml. of the concentrated stock to 50 ml. with water equilibrated with atmospheric CO_2 and were stored at room temperature. The enzymatic activity of these solutions changed less than 2% in ten days. A unit of enzyme was defined as that amount which hydrolyzed 10 micromoles of ester per minute in a solution 0.010 *M* in potassium chloride and 14.5 m*M* in methyl *n*-butyrate at *p*H 8 and 25°.

The substrates, methyl-*n*-butyrate, ethyl *n*-butyrate, methyl propionate, ethyl propionate and *n*-propyl propionate were Eastman Kodak White Label reagents. Ethyl isobutyrate was obtained from the Verona Chemical Company. Methyl isobutyrate was synthesized by a standard procedure from White Label propionic acid and Merck absolute methanol. Where necessary, the esters were extracted with dilute sodium bicarbonate to remove acid, then dried with anhydrous magnesium sulfate and distilled. *n*-Propyl propionate was fractionally distilled to remove an unidentified impurity. The final purity of the esters was checked by refractive index, titration and gas chromatography. The refractive indices were within 0.0002 unit of the published values,[14] except for ethyl isobutyrate and *n*-propyl propionate which were within 0.0004 unit. Titration showed that acid was less than 0.05% for all the esters. As determined by gas chromatography, water was less than 0.1% for all the esters, and contaminating esters were less than 0.3% except in *n*-propyl propionate, where the impurity was less than 0.5%.

Stock substrate solutions were prepared daily volumetrically. Special care was taken to prevent loss of ester by evaporation. The extent of the hydrolysis of the esters in the stock solutions was less than 0.1% per day.

The 0.01 *N* standard alkali was prepared from CO_2-free water and CO_2-free sodium hydroxide, stored in a paraffin-lined bottle and standardized against Merck potassium acid phthalate. All other reagent solutions were prepared by weight from either Mallinckrodt or Merck analytical grade reagents. Beckman buffers were used to standardize the *p*H meter.

Rates were obtained graphically from the slopes of plots of ml. of base consumed *versus* time. The scatter of points, taken every 20 to 30 seconds, was such that initial slopes generally could be determined to better than 1% in individual runs at rates of the order of one micromole/minute.

Results

The influence of buffer ions and ionic strength was investigated briefly. Since Schwert and Glaid's[3] measurements were made in the presence of dilute phosphate buffer (apparently 0.0003 to 0.001 *M*), a series of enzymatic activity measurements were made with 42 m*M* methyl *n*-butyrate at *p*H 8 with phosphate concentrations varying from 0.005 to 0.008 *M*. No significant effect on the enzymatic rate was observed. Also, the presence of the equilibrium concentration of CO_2 was shown to be without effect since 0.001 *M* carbonate–bicarbonate did not affect the rate. No significant influence on the enzymatic activity was observed due to changes in the ionic strength from 0.001 to 0.016 *M* potassium chloride at *p*H 9 with 14.5 m*M* methyl *n*-butyrate. Thus, since the effect of 0.01 *M* ionic strength on the maximum rate appeared to be negligible and to define better the reaction conditions, all the measurements reported below were made in the presence of 0.010 *M* potassium chloride.

The concentration and *p*H dependence of the rate of enzymatic hydrolysis of methyl *n*-butyrate was studied over a wide range of *p*H. Figure 1 gives the

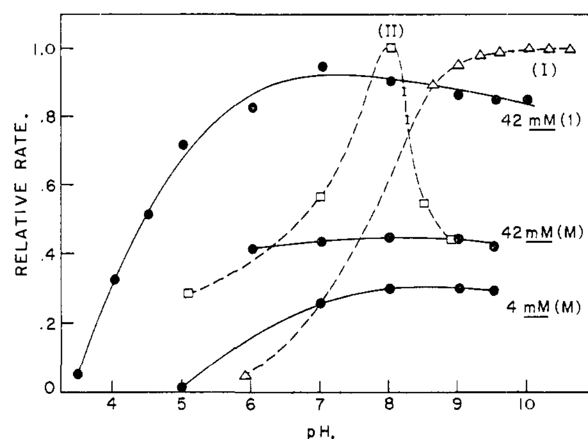


Fig. 1.—*p*H dependence of the rate of enzymatic hydrolysis of methyl *b*-butyrate: (1) enzyme preparation no. 1; (M) four year old preparation of Mangelsdorf; (I) dependence proposed by Kistiakowsky and Mangelsdorf; (II) curve proposed by Connors, *et al.* Curves (1) and (M) have been displaced downward for better clarity.

*p*H dependence measured at 42 m*M* ester for the authors' first enzyme preparation and at 42 and 4 m*M* ester for Mangelsdorf's preparation.[1] Since, as shown below, the authors' second enzyme preparation has the same *p*H dependence of activity, it is believed to be characteristic of liver esterase. A similar, nearly *p*H independent activity was observed for 18 m*M* ethyl *n*-butyrate between *p*H 6 and 9.5. Included on the graph in dotted lines are the bell-shaped curve reported by Connors, *et al.*,[4] and the sigmoid-shaped curve suggested by K. and M.[1] The results of the study of methyl *n*-butyrate hydrolysis by our second enzyme preparation at *p*H's 5, 6, 7, 8 and 9 are presented in Fig. 2 as a graph of the reciprocal of the initial rate ($1/v$) *versus* the reciprocal of the substrate concentration ($1/S$). Each point is an average of two or more determinations. They have been corrected for the hydroxide-catalyzed hydrolysis but have not been corrected for incomplete dissociation of product acid at low *p*H's.

The substrate dependence of the enzymatic ac-

(13) J. J. Lingane, "Electroanalytical Chemistry," Interscience Publishers, New York, N. Y., 1954, p. 209.

(14) A. I. Vogel, *J. Chem. Soc.*, 624 (1948).

TABLE I

CONCENTRATION DEPENDENCE OF LIVER ESTERASE-CATALYZED HYDROLYSIS FOR THE SERIES OF ESTERS AT pH 6 AND 8

Ethyl *n*-butyrate												
Initial ester concn. mM	0.307	0.611	0.917	1.22	1.84	3.05	4.57	6.07	7.61	10.6	15.2	30.3
Rate,[a] pH 6.06	..	..	..	..	..	2.83	3.32	3.64	3.60	3.98	4.02	4.11
Rate, pH 8.06	2.28	2.98	3.47	4.00	3.97	4.47	4.66	..	4.80	..	4.75	..
Methyl isobutyrate												
Initial ester concn. in mM	2.31	5.77	8.64	14.4	20.1	28.7	57.4	..	..	..	..	..
Rate, pH 6.06	..	5.20	6.27	7.35	7.92	8.28	9.38	..	..	..	..	..
Rate, pH 8.06	6.06	8.02	8.93	9.48	..	10.3	11.0	..	..	..	..	..
Ethyl isobutyrate												
Initial ester concn. in mM	0.302	0.601	0.902	1.20	2.10	3.00	4.49	5.98	7.48	14.9	29.8	..
Rate, pH 6.06	..	..	..	..	..	2.46	2.73	2.83	2.87	2.99	3.05	..
Rate, pH 8.06	2.21	2.87	3.09	3.44	3.70	3.71	3.78	..	3.75	..	..	..
Methyl propionate												
Initial ester concn. in mM	2.70	5.45	6.83	10.2	17.0	17.2	24.0	34.0	34.3	48.0	68.5	..
Rate, pH 6.06	..	..	..	0.894	..	1.55	2.05	..	2.80	3.21	3.93	..
Rate, pH 8.06	1.30	2.00	2.47	2.75	3.71	..	..	4.24	..	..	..	..
Ethyl propionate												
Initial ester concn. in mM	5.79	6.90	8.68	11.5	14.4	20.1	28.8	57.6	..	..	..	..
Rate, pH 6.06	0.871	1.01	1.14	1.27	1.32	1.46	1.61	1.66	..	..	..	..
Initial ester concn. in mM	0.702	1.16	1.40	1.74	2.45	3.50	5.24	5.79	8.73	14.4	28.8	57.6
Rate, pH 8.06	0.850	1.12	1.25	1.44	1.60	1.77	1.92	2.00	(1.95)	(1.98)	(1.85)	(1.81)
***n*-Propyl propionate**												
Initial ester concn. in mM	3.03	4.55	7.58	10.6	15.1	21.1	30.2	..	..	..	..	..
Rate, pH 6.06	3.04	3.51	4.02	4.28	4.42	4.49	4.53	..	..	..	..	..
Initial ester concn. in mM	0.304	0.370	0.455	0.609	0.913	1.22	2.13	3.03	4.55	7.58	15.1	..
Rate, pH 8.06	2.86	3.06	3.17	3.61	4.07	4.35	4.82	5.02	5.18	5.23	5.22	..

[a] Enzymatic rates in micromoles/liter-minute-unit enzyme. Maximum correction for non-enzymatic hydrolysis: 5.5% at 58 mM ethyl propionate. Range of % decomposition: minimum, 0.11% with 68.5 mM methyl propionate; maximum, 24% with 0.037 mM ethyl n-butyrate, 0.302 mM ethyl isobutyrate and 0.304 mM n-propyl propionate.

tivity with the other esters was studied at pH 6 and 8. At pH 5 the inhibition by product acid and incomplete dissociation of the acid obscure even initial rate determinations; at pH 9 the hydroxide-catalyzed hydrolysis interferes with the enzymatic catalysis. Table I summarizes the results. Most of the data in the table are averages of several determinations. Corrections for alkaline hydrolysis were calculated from published values. As with methyl n-butyrate, the enzymatic hydrolysis of these esters effectively followed Michaelis–Menten kinetics. Graphs of reciprocal rate *versus* reciprocal ester concentration were linear, except for ethyl propionate which showed substrate inhibition at high substrate concentrations.

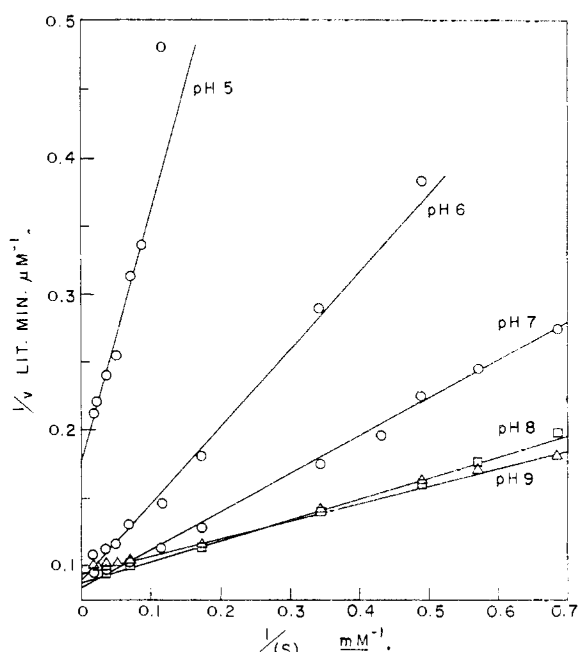


Fig. 2.—The dependence on concentration of the rate of hydrolysis of methyl n-butyrate at several pH.

Maximum rates and Michaelis constants for the esters at pH 6 and 8 are summarized in Table II. They have been corrected for incomplete dissociation of the product acid at pH 6 and hydroxide catalysis at pH 8. The usual linearization of the Michaelis–Menten type kinetic expression

$$1/v = (K_M/V_m)\ 1/(S) + 1/V_m$$

was used. Figure 3 displays the corrected constants for methyl n-butyrate at several pH. The constants for ethyl n-butyrate, methyl isobutyrate and ethyl propionate were evaluated by a weighted least squares procedure, with v/V_m as the weighting factor. For the other esters the constants were obtained graphically.

Accelerating initial rates or induction periods, which decreased in duration with increasing substrate concentration, were observed for methyl propionate hydrolysis at pH 8. Extrapolated to the time axis they extended to 30 seconds. No in-

duction periods were observed at *p*H 6. The observations at *p*H 8 could not be satisfactorily explained by the presence of an ester impurity since such would produce induction periods which increase in duration with increasing substrate concentration. They also could not be explained as initial, non-steady state kinetics since Schwert and Glaid's[3] estimate of the absolute rate constant for the breakdown of the enzyme–substrate complex predicts an induction period of less than 0.02 second. The rates obtained after the induction periods fitted, however, Michaelis–Menten kinetics.

TABLE II

MAXIMUM RATES, V_m, AND MICHAELIS CONSTANTS, K_M, FOR THE SERIES OF ESTERS AT *p*H 6 AND 8

Esters	*p*H 6.06 Cor.,[c] V_m	K_M, m*M*	*p*H 8.06 V_m[a]	K_M, m*M*
Methyl *n*-butyrate	11.6	6.24	11.2	1.72
Ethyl *n*-butyrate[b]	4.66	1.575	4.96	0.371
Methyl isobutyrate[b]	10.6	5.37	10.97	1.96
Ethyl isobutyrate	3.31	0.749	4.03	0.257
Methyl propionate	8.55	70.6	5.53	9.06
Ethyl propionate[b]	2.05	6.48	2.44	1.310
n-Propyl propionate	5.30	1.94	5.50	0.313

[a] Enzymatic rate in micromoles/liter-minute-unit enzyme. [b] The constants for these esters were obtained by least squares analysis. [c] Corrected for incomplete dissociation of product acid.

The observable activity of the enzyme at *p*H 3.5 raised the possibility of studying the esterification reaction. Consideration of the equilibrium constants for esterification ($K' = 0.048$)[15] and the dissociation of the acid shows that the *p*H change accompanying esterification above *p*H 3.5, where the enzyme is active, is given approximately by the product (K')(Alcohol). Thus, the sensitivity of the measurement is nearly independent of *p*H. For 5 *M* alcohol a total *p*H change of about 0.24 unit is to be expected. An attempt was made to observe this change at "*p*H" 5.9 with 5 *M* methanol, 0.1 *M* *n*-butyrate and five times the enzyme concentration usually employed in the hydrolysis experiments. No significant *p*H change was observed in 4 hr., and a precipitate indicated that the enzyme was probably not completely soluble in the 20% alcohol.

Strong inhibition by products was observed at *p*H 6 and below for all of the esters. For example, a noticeable decrease in rate was observed at *p*H 6 with 8.7 m*M* methyl *n*-butyrate after only 0.84% decomposition, and a 26% decrease in rate was observed with 2.9 m*M* ester after 2.5% decomposition. Larger decreases were observed at *p*H 5 for the same concentrations and per cent. decompositions. At *p*H 6 and 2.9 m*M* ester, the addition of 0.5 m*M* sodium butyrate caused a 25% decrease in rate whereas 0.5 m*M* methanol caused a 9% decrease in rate. Thus, at *p*H 6 the butyrate–butyric acid is the more potent inhibitor.

From the least squares analysis of the rates of hydrolysis of ethyl *n*-butyrate, methyl isobutyrate and ethyl propionate the average standard deviation in the rate measurements and in the maximum rates was found to be 2.2%, including the measurements of enzyme activity units. The standard deviation in the Michaelis constant (K_M) ranged from 3.0 to 6.8% with an average of 5.3%. The relative *p*H was known to better than 0.02 unit, being limited primarily by the standard buffers. An empirically determined *p*H correction of 0.06 unit was applied because of the difference in liquid junction potential in the standard buffers and the reacting solutions, due to stirring.

(15) "International Critical Tables," Vol. II, McGraw-Hill, Inc., New York, N. Y., 1930, p. 139.

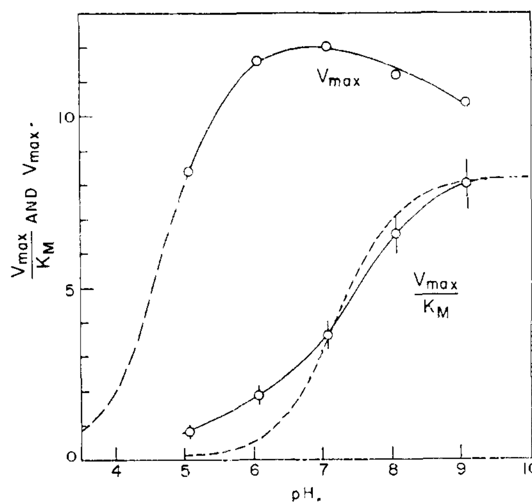


Fig. 3.—*p*H dependence of V_{max} and K_m for methyl *n*-butyrate: units of V_{max}, μM l.$^{-1}$ min.$^{-1}$ enzyme unit^{-1}; units of V_{max}/K_m, μM l.$^{-1}$ enzyme unit^{-1} mM^{-1}.

Some of the maximum rates and Michaelis constants for methyl *n*-butyrate and ethyl *n*-butyrate are compared in Table III with previous results in which a horse liver esterase prepared by the method of Connors, *et al.*, also was used. Weighted mean values are given for the previous measurements from this Laboratory. The differences at *p*H 8 between the results of this research and those of Schwert and Glaid may be due to the limited substrate concentration range investigated by them, to ionic strength effects, particularly on K_M, undisclosed by the present cursory ionic strength study at high substrate concentration, or to non-uniformity of the enzyme preparation. To test the possibility of such non-uniformity, the liver esterase preparation made four years ago[1] was investigated at *p*H 9 and 0.010 *M* ionic strength. The K_M of 1.83 m*M*, given in the table, was obtained. This differs significantly from the K_M of the more recent preparation. Evidently the enzyme preparation is not uniform; it may be a mixture of two enzymes, as suggested by the two-site hypothesis. The extremely divergent result of 22 m*M* for K_M obtained by Connors, *et al.*,[4] may be due, in part, to inhibition and/or ionic strength effects of the 0.025 *M* bicarbonate buffer used. It is noteworthy that the present results are the lowest values for K_M, indicating maximum freedom from inhibitors.

Discussion

As shown in Figs. 1 and 3 there is a marked contrast between the *p*H dependence observed in this

TABLE III

COMPARISON OF K_M'S OF SEVERAL RESEARCHES

	K_M for methyl n-butyrate			
	pH 7.4	pH 8	pH 9	pH 10.2
Mangelsdorf	....	......	......	2.46 mM
Schwert and Glaid	....	3.14 mM	......	......
Connors, *et al.*	22 mM	......	......	......
This research				
Preparation 2	2.7	1.72	1.30 mM	......
Mangelsdorf's preparation(M)....		......	1.83	......

	K_M for ethyl n-butyrate	
	pH 8	pH 10.2
Mangelsdorf	....	0.685 mM
Schwert and Glaid	0.683 mM	....
This research		
Preparation 2	0.371	....

	pH 8	
	S. and G.	This research
Ratio $\frac{V_m(\text{methyl } n\text{-butyrate})}{V_m(\text{ethyl } n\text{-butyrate})}$	1.93	2.26

research and the bell-shaped dependence found by Connors, *et al.*[4] There is also a significant difference between the location of the inflection point of the sigmoid-shaped curve presently observed and of the partly extrapolated curve reported earlier from this Laboratory. This latter discordance is not real, being the result of an improper assumption in the earlier work. The run at pH 5.9, on which the extrapolation was based, was made at a methyl n-butyrate concentration of 2.5 mM, which is of the order of the Michaelis constant. The present investigation of pH dependence shows that, although the maximum rate (V_m) is nearly constant from pH 10 to 5.9, the Michaelis constant (K_M) is a strong function of pH. Thus, the extrapolation which assumed K_M to be constant was unjustified. The marked contrast with the bell-shaped pH dependence reported by Connors, *et al.*, and others[8] may be the result of at least two factors. First, their activity–pH curve was not based on $V_{m's}$ but on the activity at a single substrate concentration, 0.1 M, where a pH-dependent substrate inhibition may be active as has now been observed for ethyl propionate. Second, their measurements were carried out in the presence of borate and mannitol buffers, which may have influenced the rate.

The unusual mechanism proposed by Christiansen and Graae[5] for the action of horse liver esterase on methyl valerate is not necessary to interpret their data. They fitted the results of four runs at the same enzyme concentration but different ester concentrations and four runs at nearly the same ester concentration but different enzyme concentrations, to an empirical equation

$$t = \frac{A(S)_0\alpha}{(E)_0} + B\sqrt{\frac{(S)_0}{(E)_0}}\left[\ln\frac{(1+\alpha)}{(1-\alpha)} - \sqrt{\alpha}\right]$$

where A and B are adjustable parameters and α is the degree of reaction. Their data can be fitted, however, to a simple Michaelis–Menten mechanism involving competitive inhibition by a product. If the four runs at nearly the same ester concentration are adjusted to the same enzyme concentration by multiplying the observed times by the enzyme concentration and then plotted as α *versus* time, the curves are superimposable within the apparent experimental error. This procedure indicates that the $\sqrt{1/(E)_0}$ dependence is not called for by the data. In addition, if their data for the four different ester concentrations are plotted as $(S)_0\alpha/t$ *versus* $[\ln(1-\alpha)]/t$, straight lines are obtained within the scatter of the experimental points. The slopes of these lines depend linearly on the initial ester concentration. Thus, the integrated Michaelis–Menten equation, which can be rearranged to[16]

$$\frac{(S)_0\alpha}{t} = C\frac{\ln(1-\alpha)}{t} + D$$

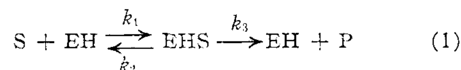
fits their data. The linear dependence of C on initial substrate concentration suggests product inhibition, presumably by the valerate. This fitting of their data to the conventional mechanism reduces the area of disagreement about liver esterase kinetics.

The hypothesis of a two-enzyme or two-site mechanism suggested by the earlier study[1] remains unsettled by the present research. The kinetic expression for a two-site Michaelis–Menten mechanism approaches a linear Lineweaver–Burk form at high substrate concentrations since it expands to

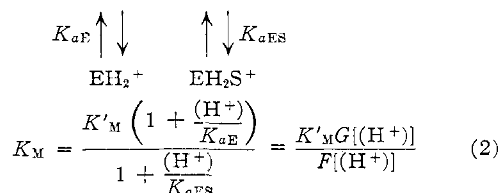
$$\frac{1}{v} = \frac{1}{V_1 + V_2} + \frac{K_1V_1 + K_2V_2}{(V_1+V_2)^2}\left[\frac{1}{(S)}\right] + [\ldots]\frac{1}{(S)^2} + \ldots$$

where subscripts 1 and 2 characterize the two sites. Because the pH-stat measurements were limited by lower sensitivity to higher ester concentrations, it may be that only the limiting, linear behavior of a two-site mechanism was resolved. The lack of quantitative agreement between the Michaelis constants determined for enzyme preparations (2) and (M) at pH 9 (Table III) suggests that a mixture of esterases is involved, rather than a single enzyme with two sites. This uncertainty about the uniformity of the enzyme must be borne in mind throughout the following discussion.

The concentration and pH dependence for methyl n-butyrate hydrolysis can be fitted to a simple Michaelis–Menten mechanism, including an acidic ionization of the active site. For the mechanism[16]



$$S + EH \underset{k_2}{\overset{k_1}{\rightleftarrows}} EHS \xrightarrow{k_3} EH + P \qquad (1)$$



K_{aE} (EH ⇄ EH_2^+), K_{aES} (EHS ⇄ EH_2S^+)

$$K_M = \frac{K'_M\left(1 + \frac{(H^+)}{K_{aE}}\right)}{1 + \frac{(H^+)}{K_{aES}}} = \frac{K'_M G[(H^+)]}{F[(H^+)]} \qquad (2)$$

and

$$V_m = \frac{V'_m}{1 + \frac{(H^+)}{K_{aES}}} = \frac{V'_m}{F[(H^+)]} \qquad (3)$$

In Fig. 3 the dotted portion of the V_m curve is a theoretical extrapolation based on $K_{aES} = 2.0 \times 10^{-5}$. It coincides with the experimental curve be-

(16) R. A. Alberty, "Enzyme Kinetics," "Advances in Enzymology," Vol. XVII, ed. F. F. Nord, Interscience Publishing Co., New York, N. Y., 1956.

TABLE IV

COMPARISONS OF pH DEPENDENCE OF ESTERASE CATALYSIS OF SEVERAL ESTERS AND OF ACID–BASE CATALYSIS

Ester	G $\frac{(V_m/K_M)\ pH\ 8}{(V_m/K_M)\ pH\ 6}$	Stand. dev., %	pH 8 $\frac{V_m\ (MeBu)}{V_m\ (x)}$	$\frac{k_{OH}\ (MeBu)}{k_{OH}\ (x)}$	$\frac{k_H\ (MeBu)}{k_H\ (x)}$
Methyl n-butyrate	3.51	..	(1.00)	(1.00)	(1.00)
Ethyl n-butyrate	4.52	7.9	2.26	1.96	0.925
Methyl isobutyrate	2.84	8.6	1.02	1.28	1.05
Ethyl isobutyrate	3.55	..	2.78	3.31	1.04
Methyl propionate	5.04	8.4	2.03	0.613	0.582
Ethyl propionate	5.89	..	4.59	1.13	.601
n-Propyl propionate	6.43	..	2.04	1.38	.581

tween pH 5 and 7. The low pH extrapolation is supported by the less detailed rate measurements (Fig. 1) in this pH region. The downward curvature of the V_m plot above pH 7 is probably an evidence of the involvement of a second acidic ionization in the active site. It is not possible, however, to fit as well a function $G[(H^+)]$ to the K_M/V_m curve. The dotted line in Fig. 3 represents the best possible fit, obtained with $K_{aE} = 6.3 \times 10^{-8}$. The deviation could be attributed to a more complex mechanism (*e.g.*, two-intermediate mechanism), non-uniformity of the enzyme or a pH dependent ionization constant due to an electrostatic effect, as was discussed in conjunction with urease.[2] The weakening of the implicated basic group, upon binding of the substrate, ($K_{aES} > K_{aE}$) fits with the interpretation that this group attracts the positive end of the carbonyl dipole of the ester in the initial complex.

Any mechanism such as that given in equation 1 involving several ionizations affecting the active site, if it can be fitted by a kinetic expression of the Michaelis–Menten form, has a pH dependence for V_m and K_M of the forms[16]

$$V_m = V'_m/F[(H^+)] \text{ and } K_M = K'_M G[(H^+)]/F[(H^+)]$$

Thus, for a multisubstrate enzyme, such as horse liver esterase, regardless of the substrate

$$\frac{(V_m/K_M)_{pH_1}}{(V_m/K_M)_{pH_2}} = \frac{G[(H^+)]_{pH_2}}{G[(H^+)]_{pH_1}} = G(H^+)_{12}$$

where G is characteristic only of the ionization of the active site on the free enzyme and is independent of the substrate. Values of G for the various esters studied are recorded in Table IV, along with the percentage deviations of those experimental data which were analyzed statistically. The spread of the values of G over a range of more than a factor of two is clearly outside the experimental uncertainty, which is less than 10%. Strictly speaking, thus, the theory is apparently inapplicable to the data. However, the variation in G is only a factor of two, while the kinetic constants involved spread over a much wider range. This suggests that there is no gross error in the application of the simple theory, and, if we are dealing with an enzyme mixture, that perhaps one enzyme predominates.

No kinetic evidence for an acyl–enzyme intermediate postulated by Wilson has been obtained, since if such were formed in a single step or if its breakdown were the rate determining step in a two-intermediate mechanism, then V_m would be the same for the various esters of the same acid. Reference to Table II shows that this is not so, except for methyl and n-propyl propionate.

If it is assumed that K_M at pH 8 is essentially independent of pH (or has approximately the same pH dependence for the various esters studied as was observed for methyl n-butyrate), then the data can be examined in two further respects. A comparison with the well-understood non-enzymatic hydrolysis is included in Table IV, where the maximum enzymatic rates at pH 8, relative to methyl n-butyrate, are compared with the relative rates of hydroxide and hydrogen ion catalysis. There is no correlation of the maximum rates with the latter which is essentially independent of the alcohol moiety of the ester. On the other hand, there is qualitative correlation between the maximum enzymatic rate and hydroxyl ion catalysis for methyl and ethyl esters of the same acid, suggesting a participation of basic catalysis in the enzymatic rate determining step of V_m.

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