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Kinetics of Pig Liver Esterase Catalysis

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The rates of pig liver esterase catalyzed hydrolysis of simple aliphatic esters were measured by applying the *p*H-stat technique to unbuffered solutions over a wide range of *p*H and of substrate concentration. An enzyme preparation containing a single esterase was employed. The observed kinetics do not obey the Michaelis–Menten equation and can be explained either by substrate activation or by identical interacting sites. The *p*H dependence indicates that both a histidine residue and a dicarboxylic amino acid residue may be involved in catalysis by the enzyme. Studies of the effects of added electrolytes failed to reveal any electrostatic interaction between enzyme and substrate. Data obtained from addition of the reaction products (alcohol and acid) show that the product alcohol is released from the enzyme before the product acid during the course of the hydrolysis and that the undissociated acid behaves kinetically like the substrate for the reverse (ester synthesis) reaction. The esters methyl *n*-butyrate, ethyl *n*-butyrate and methyl chloroacetate are compared as substrates. Limits are set upon the values of some specific rate constants. Pig liver esterase is found to be more active at low *p*H than is horse liver esterase and shows greater deviations from Michaelis–Menten kinetics.

Introduction

Liver esterase, which catalyzes the hydrolysis of aliphatic esters, is of interest largely for the opportunity it affords to compare its action upon related simple substrates. The kinetic behavior of pig liver esterase as a function of substrate concentration never has been investigated in an environment free from the inhibitory effects[1] of buffers and of *p*H indicators.

On the other hand, several careful studies have been made of horse liver esterase.[2–5] With methyl *n*-butyrate as substrate (S) the kinetics of the horse enzyme were found to obey the Michaelis–Menten equation $v = V_m[S]/(K_M + [S])$, when $[S] > 1$ mM and *p*H ≤ 9,[2,5] but to deviate from the equation when $[S] < 0.3$ mM and *p*H = 10.2.[4] These kinetic studies employed esterase preparations which, although purified, were heterogeneous.[6]

Although Craig and Kistiakowsky[5] found a sigmoidal plot for V_m *vs.* *p*H, with no free acid group manifest in the *p*H dependence of the horse enzyme's activity, Rozengart, *et al.*,[7] claimed that pig liver esterase exhibits a bell-shaped activity *vs.* *p*H curve, sharply peaked at *p*H 8.

A pig liver esterase preparation containing only one esterase was obtained[8] for use in the present work, so that any unusual kinetic results could not be attributed to a mixture of enzymes. The pig enzyme was employed so that species differences between the kinetics shown by the pig and the horse esterases could be determined. The experiments of C. and K.[5] were extended to lower [S] in order to search for non-Michaelis–Menten behavior, and *p*H studies were undertaken to resolve the uncertainty in the *p*H dependence of liver esterases. Information about the enzymatic mechanism was obtained from the kinetic effects of added electrolytes and reaction products.

Experimental

Method.—The rates were measured with a sensitive manual *p*H-stat method which already has been described[5]; the acid formed during the hydrolysis reaction was continuously titrated with standard alkali while the *p*H was held constant by means of a potentiometric circuit. A saturated calomel reference electrode, similar to the one used by C. and K.,[5] but equipped with a capillary salt bridge filled with stagnant KCl solution, was found to eliminate the difference in liquid junction potentials between the

(1) R. Ammon and M. Jaarma, in "The Enzymes," ed. J. B. Sumner and K. Myrbäck, Vol. I, 1st Ed., Academic Press, Inc., New York, N. Y., 1950, Part 1, Chapter 9.

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

(3) B. H. J. Hofstee, *ibid.*, **207**, 211, 219 (1954).

(4) G. B. Kistiakowsky and P. C. Mangelsdorf, Jr., *J. Am. Chem. Soc.*, **78**, 2964 (1956).

(5) N. C. Craig and G. B. Kistiakowsky, *ibid.*, **80**, 1574 (1958).

(6) W. M. Connors, A. Pihl, A. L. Dounce and E. Stotz, *J. Biol. Chem.*, **184**, 29 (1950).

(7) V. I. Rozengart, C. A. Kibardin, E. I. Bernardelli and P. A. Finogenov, *Doklady Akad. Nauk S.S.S.R.*, **82**, 293 (1952).

(8) A. J. Adler and G. B. Kistiakowsky, *J. Biol. Chem.*, (1962).

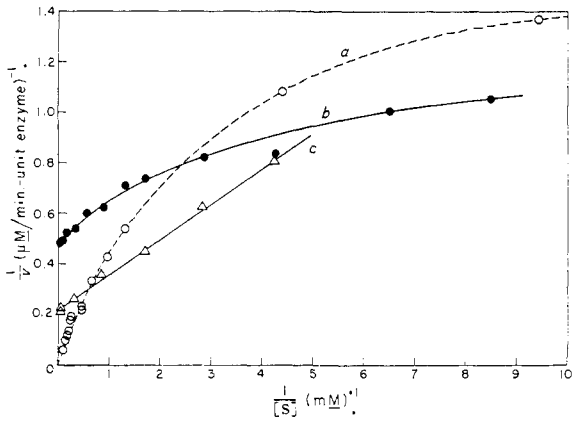

Fig. 1.—Typical Lineweaver–Burk plots for pig liver esterase: 1a (O) methyl chloroacetate at *p*H 7; 1b (●) methyl *n*-butyrate at *p*H 7; with all values of $1/v$ multiplied by 5; 1c (△) methyl *n*-butyrate at *p*H 5 with added 494 m*M* methanol.

reaction mixtures and the buffers used to standardize the *p*H-stat.

Most runs were made in buffer-free solutions at 25.0° in the presence of 0.01 *M* electrolyte (usually KCl), which was found not to affect the rates. Measurements were extended to low substrate concentrations by the use of low enzyme concentrations, which permitted initial rates to be determined before substrate was depleted.

Materials.—Pig liver esterase, prepared by a procedure described elsewhere,[8] was not subjected to the final purification step of column chromatography, since this step has been shown[8] not to affect the kinetic properties of the enzyme preparation. The enzyme stock solution was stored for nearly 2 years at −25°. Diluted aliquots were kept at 2° for periods of up to 2 weeks. Neither denaturation nor change in kinetics was observed during either type of storage. One enzyme "unit" was defined as that amount which hydrolyzed methyl *n*-butyrate at a rate of 10 micromoles per liter per minute in a solution 0.01 *M* in KCl and 14.7 m*M* in ester at 25° and *p*H 8. The concentration of esterase present in a run ranged from 0.1 to 100 units, or from about 10^{-11} to 10^{-8} *M*.[9]

The horse liver esterase preparation used was that of C. and K.,[5] which had lost 15% of its activity during 2.5 years of storage at 2°.

The substrates methyl and ethyl *n*-butyrates, and also *n*-butyric and formic acids, were Eastman White Label reagents; methyl chloroacetate was a Columbia product. All esters were repurified.[5] Methanol was Fisher Reagent, absolute. The maximum impurities[5] present in these organic compounds were: water, 0.1%; organics, < 0.1%; acid, <0.1%, except for ≤0.2% in methyl chloroacetate. Dilute HCl was added to stock methyl chloroacetate solutions in order to prevent basic hydrolysis.

All organic chemicals were reagent grade.

Treatment and Precision of Data.—Runs lasted 3 to 20 min. Initial rates were determined from plots of Δt *vs.* time, where Δt is the time required for the neutralization of a known increment of alkali. The values given for enzymatic reaction rates, v, in units of micromoles per liter per minute per unit of enzyme, have been corrected for incomplete dissociation of product acid and for hydroxide-catalyzed hydrolysis (usually less than 5% of the total rate). Published rate constants, when available, were used for the latter calculation and could be reproduced experimentally.

Most values of kinetic parameters were determined graphically. However, several series of data were fitted to the Michaelis–Menten equation or to a similar expression (see Discussion) by a least-squares analysis which assumed that [S] was exactly known and that the percentage standard deviation of v, s_v, was a constant for each series. From such an analysis s_v was found to be 3% for methyl *n*-butyrate at *p*H 7. However, because of experimental difficulties under conditions of low *p*H, very high *p*H, very low [S], or added product acid, the precision was sometimes much worse. For example, the value of s_v was 10% for methyl *n*-butyrate at *p*H 5 in the presence of 247 m*M* methanol and 8.8 m*M* *n*-butyric acid.

(9) The approximate concentration of pig liver esterase was calculated from the estimated molecular weight[8] and from dry weight analyses performed on the twice-chromatographed enzyme by Schwarzkopf Microanalytical Laboratory, Woodside, N. Y.

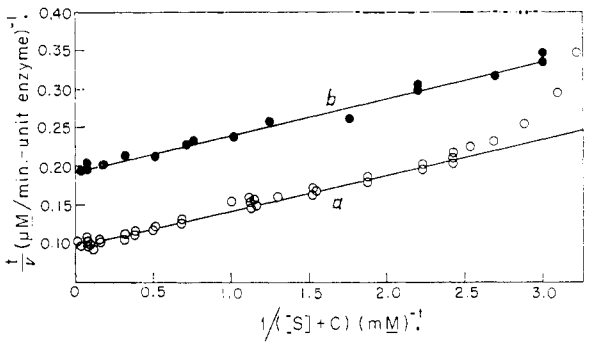

Fig. 2.—Typical analytical plots of rate data for methyl *n*-butyrate; the solid lines are derived from eq. 1 and the parameters of Table I: 2a (O) *p*H 8, C = 0.30; 2b (●) *p*H 6, C = 0.22, with all points displaced upward on the $1/v$ axis by 0.1 unit.

Results and Discussion

Dependence of Rates upon Substrate Concentration.—The effect of substrate concentration upon the kinetic behavior of pig liver esterase was investigated[10] over a range of *p*H with methyl *n*-butyrate, ethyl *n*-butyrate and methyl chloroacetate as substrates. Typical Lineweaver–Burk ($1/v$ *vs.* $1/[S]$) plots are given in Fig. 1. The nonlinearity of these plots for the chloroacetate ester, and for the butyrates at *p*H above 5 and ester concentration below about 0.6 m*M*, indicates that the data fail to adhere to the Michaelis–Menten equation. Such deviations have been observed for other enzymes, among them urease,[11] fumarase[12] and horse liver esterase.[3,4]

Butyrate Esters.—The following 3-parameter equation, which reduces to the Michaelis–Menten equation when $C = 0$, was found to fit the butyrate rates for [S] ≥ 0.1 m*M*

$$v = V([S] + C)/(K + [S] + C) \qquad (1)$$

Typical results, graphed in the form of a linearization of eq. 1, are shown in Fig. 2; the empirical constants obtained are listed in Table I. The deviation from eq. 1 which occurs at 0.01 mM < [S] < 0.1 mM (Fig. 2a) will be discussed later.

Methyl Chloroacetate.—The simple linear equation

$$v = a[S] + b \qquad (2)$$

was found to represent all the chloroacetate data. A sample analytical plot (Fig. 3) and the derived empirical constants, a and b (Table I), are given.

Interpretation in Terms of Mechanism.—Under the same conditions (*p*H 8, methyl *n*-butyrate concentration ranging from 0.1 to 0.6 m*M*) for which

(10) Details of the experiments, results and interpretations presented in this paper may be found in the Ph.D. thesis of A. J. Adler, Radcliffe College, 1960.

(11) G. B. Kistiakowsky and A. J. Rosenberg, *J. Am. Chem. Soc.*, **74**, 5020 (1952).

(12) R. A. Alberty, V. Massey, C. Frieden and A. R. Fuhlbrigge, *ibid.*, **76**, 2485 (1954).

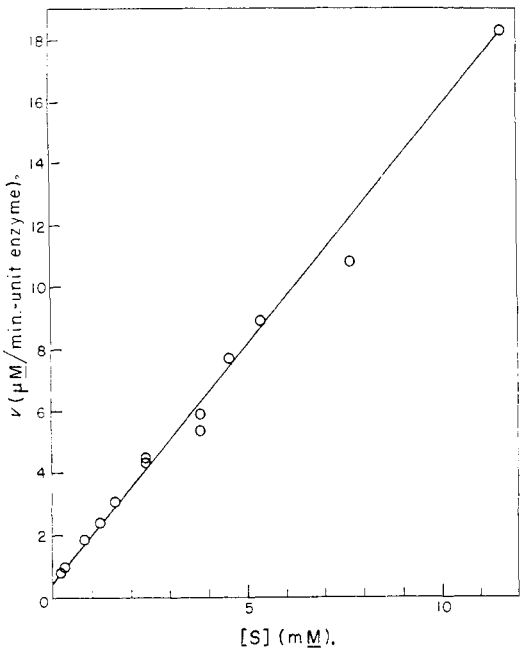

Fig. 3.—Typical analytical plot of rate data for methyl chloroacetate; *p*H, 7; the solid line is derived from eq. 2 and the parameters of Table I.

the pig liver esterase data exhibit large deviations from Michaelis–Menten kinetics, the data taken for horse liver esterase (see later) yield a linear Lineweaver–Burk plot. Therefore, the non-Michaelis–Menten kinetics shown by the pig esterase cannot be blamed upon a possible systematic error in the experimental method; this behavior must be intrinsic to the enzyme.

In general, deviations from Michaelis–Menten kinetics can be expected whenever the enzymatic reaction mechanism provides more than one parallel pathway for the formation of product from substrate, provided that the relative rates of these pathways change as the substrate concentration is varied. Several such mechanisms are: (A) activation of the enzyme by a second molecule of substrate ester, (B) identical active sites which interact so that the presence of substrate bound at one site changes the kinetic parameters of the neighboring site,[11] (C) 2 (or more) different enzymes, (D) 2 (or more) non-identical active sites on the same enzyme molecule, and (E) a modifier mechanism.[13]

Steady-state kinetic measurements cannot, by themselves, permit a choice among these mechanisms, all of which result in rate expressions of the form

$$v = (a'[S]^n + b'[S]^{n-1} + \ldots + l'[S])/([S]^n + m'[S]^{n-1} + \ldots + z'[S]) \quad (3)$$

The empirical rate expressions are consistent with eq. 3. Equation 3 reduces to eq. 1 when [S] is high enough so that $(a'[S]^n + b'[S]^{n-1})$ and $([S]^n + m'[S]^{n-1})$ are, respectively, much greater than the sum of all other terms in the numerator and in the denominator of eq. 3; similar conditions

(13) J. Z. Hearon, S. A. Bernhard, S. L. Friess, D. J. Botts and M. F. Morales, in "The Enzymes," ed. P. D. Boyer, H. Lardy and K. Myrbäck, Vol. I, 2nd Ed., Academic Press, Inc., New York, N. Y., 1959, Chapter 2, p. 67 ff.

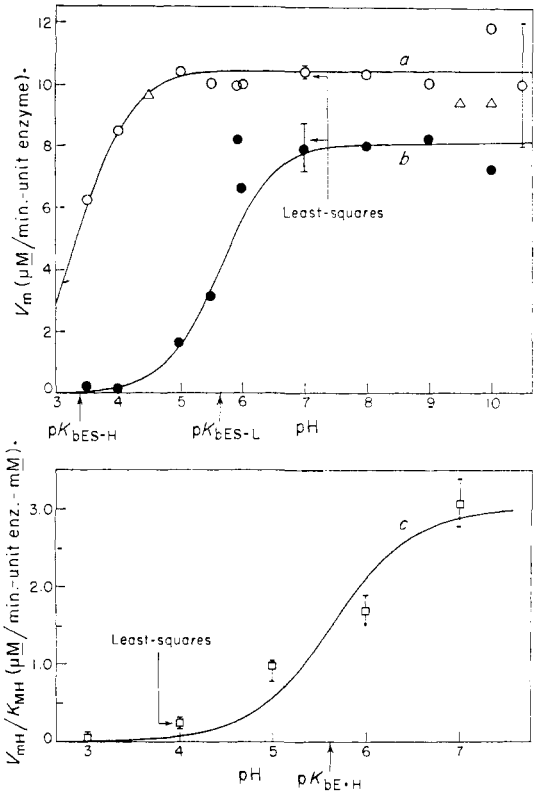

Fig. 4.—*p*H dependence of pig liver esterase activity: 4a, V_{mH} for methyl *n*-butyrate, (○) V_{mH}, Δv of individual runs at [S] = 44 m*M*; 4b (●) V_{mL} for methyl *n*-butyrate; 4c (□) V_{mH}/K_{MH} for methyl chloroacetate. "Least-squares": Values and standard deviations from least-squares analyses. The solid lines are theoretical titration curves for an ionizing basic group.

can be found for the empirical eq. 2. The empirical parameters of Table I can be given mechanistic interpretations through identification with the constants of eq. 3.

Biochemical evidence[8] can be used to show that mechanism C is invalid for pig liver esterase, that D is improbable, and that either A or B is possible. Since no suitable modifier is likely to be present,[10] E probably is not responsible for the observed kinetics. Although mechanisms A and B appear equally reasonable, it was found that A provided no method of obtaining mechanistically meaningful constants (such as V_m) from the data, even under simplifying assumptions. Therefore, B was chosen for analysis of the data.

In the Appendix the mechanism for 2 interacting sites (eq. 7) is presented along with the equations (9–11) employed to derive from the data Michaelis–Menten parameters for each site. V_{mL} and K_{ML} refer to the form of pig liver esterase responsible for the low-[S] catalytic pathway, that is, the form in which only one active site is occupied with substrate; V_{mH} and K_{MH} are V_m and K_M for the high-[S] form, with both sites occupied.

pH Dependence of Pig Liver Esterase.—The effect of *p*H upon the kinetic parameters of an ampholytic enzymatic active site obeying the Michaelis–Menten equation is given by[14]

(14) R. A. Alberty, *Advances in Enzymol.*, **17**, 1 (1956).

TABLE I

EMPIRICAL CONSTANTS FOR PIG LIVER ESTERASE CATALYZED HYDROLYSIS

pH	V(μM/min. unit enzyme)	K(mM)	C(mM)	Range of [S] (mM)
		Methyl *n*-butyrate		
3.5	6.25	1.47	$\leqslant 0.05$	0.59 to 39.0
4.0	8.48	1.11	$\leqslant .01$	.12 to 42.9
4.5[a]	9.6	...	..	44.2
5.0	10.4	0.714	.06	.12 to 44.2
5.5	10.1	.654	.12	.12 to 5.9
5.92	10.0	.442	.24	.12 to 14.7
6.0	10.1	.455	.22	.12 to 44.2
7.0[b]	10.42 ± 1.6%	.439 ± 4.1%	.27 ± 9.3%	.12 to 44.2
8.0	10.3	.467	.30	.012 to 58.7
9.0	10.0	.414	.29	.12 to 44.2
9.5[a]	9.4	...	..	44.2
10.0	11.8	.455	.20	.12 to 42.8
10.0[a]	9.4	...	..	44.2
10.5	8–12	...	..	.12 to 2.9
		Ethyl *n*-butyrate		
7.0	11.7	0.44	0.27	0.10 to 12.7

pH	a(μM/min. unit enzyme mM)	b(μM/min. unit enzyme)	Range of [S] (mM)
	Methyl chloroacetate		
3.0	0.025	0.185	1.52 to 75.8
4.0[b]	.118 ± 9.6%	.190 ± 20.1%	0.76 to 18.9
5.0	.49	.25	.76 to 22.7
6.0	.84	.40	.076 to 18.9
7.0	1.53	.70	.11 to 11.4

[a] Single run at 44 mM ester. [b] Values and standard deviations from least-squares analysis.

$$V_m = V_m'/\left(1 + \frac{[H^+]}{K_{bes}} + \frac{K_{aes}}{[H^+]}\right),$$

$$\frac{V_m}{K_m} = \frac{V_m'}{K_m'}\Big/\left(1 + \frac{[H^+]}{K_{be}} + \frac{K_{ae}}{[H^+]}\right) \quad (4)$$

where

$$K_{ae} = [H^+][E^-]/[EH],\ K_{aes} = [H^+][ES^-]/[EHS]$$
$$K_{be} = [H^+][EH]/[EH_2^+],\ K_{bes} = [H^+][EHS]/[EH_2S^+] \quad (5)$$

V_m' and K_M' are independent of *p*H and represent V_m and K_M for the catalytically active ionic forms of the enzyme (EH and EHS). Equation 4 shows that plots of V_m and of V_m/K_M *vs.* *p*H should have the form of titration curves.

Figure 4 presents the results obtained when several of the kinetic parameters derived from the data by the assumption of an interacting-site mechanism are graphed as a function of *p*H. Such plots were drawn also with V_{mH}/K_{MH} for methyl *n*-butyrate and with V_{mL} for methyl chloroacetate. In each case the form of the curve is sigmoidal, not bell-shaped; only values of pK_b are apparent in the *p*H range of the data. Thus, the *p*H dependence of pig liver esterase is similar to that of the horse enzyme under similar well-defined conditions.[5] The bell-shaped *p*H dependence which has been claimed[7] for the pig enzyme probably is an artifact due to the effect of buffers or of other inhibitors.

The good fit of the experimental points in Fig. 4 to theoretical titration curves for a basic group can, perhaps, be taken as a justification for the choice of the interacting-site mechanism, since the derived kinetic parameters show the *p*H dependence appropriate for values of V_m and of V_m/K_M.

In Table II are given the *p*H-independent kinetic parameters, defined by eq. 4 and obtained from the asymptotes of Fig. 4 or of similar curves, as well as the pK values for the ionizing groups at the active site of pig liver esterase. The pK values, defined by eq. 5, were taken to be the midpoints of the appropriate titration curves.

TABLE II

*p*H-INDEPENDENT PARAMETERS

	Methyl *n*-butyrate	Ethyl *n*-butyrate	Methyl chloroacetate
	Mechanistically meaningful kinetic parameters		
V_{mH}' (μM/min. enz. unit)	10.4 ± 0.3	11.7[d]	$\geqslant 55$[e]
V_{mL}' (μM/min. enz. unit)	8.1 ± .3	8.8[d]	1.4 ± 0.4
K_{MH}' (mM)	0.36 ± .02	0.36[d]	$\geqslant 21$.[e]
K'_{ML} (mM)	.01 ± .001[c]	...	$\leqslant 0.04$[e]
k_{OH}[a] (M^{-1} sec.$^{-1}$)	.103	0.053	112
	Ionization constants of active site		
pK_{bE-H}	3.97 ± 0.15	...	5.6 ± 0.3
pK_{bES-H}	3.38 ± .07	...	.....
pK_{bES-L}	5.68 ± .15	...	5.3 ± 0.5
pK_{aE-H}	$\geqslant 11$	...	>8
pK_{aES-H}	$\geqslant 11.2$	...	.....
pK_{aES-L}	$\geqslant 11$	...	>8
pK_a of acyl moiety[b] of substrate	4.82	4.82	2.86

[a] Specific rate constant for hydroxide-catalyzed hydrolysis. [b] Ionization constants of *n*-butyric and chloroacetic acids. [c] Obtained by assuming that the high-*p*H plateau is reached for K_{ML} by *p*H 8. [d] Obtained by assuming that the high-*p*H plateau is reached by *p*H 7 for ethyl butyrate (as it is for methyl butyrate). [e] Obtained by using limits placed upon K_{MH} and K_{ML} (see Appendix).

A comparison of the pK values in Table II with those of ionizing groups in proteins[15] shows that

(15) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids, and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943, p. 445.

the pK_a values for pig liver esterase can be accounted for if the only acidic group required in catalysis is the very weakly acidic hydroxyl of a serine residue. Serine is believed to be necessary for the action of liver esterases.[8,16] Some of the pK_b values of Table II seem to implicate the imidazole group of a histidine residue (pK 5.6 to 7.0) as the basic group at the active site of pig liver esterase, whereas the other pK_b values are within the usual range for the carboxyl of an aspartic or glutamic acid residue (pK 3.0 to 4.7). There are several hydrolytic enzymes, including chymotrypsin,[17] whose pH dependence has been totally accounted for by the assumption of histidine at the active site, even though these enzymes, like liver esterase,[16] are known to have dicarboxylic acid residues adjacent to the active serine.[18]

A possible explanation for the complex pH dependence shown by pig liver esterase activity is that both imidazole and COO^- are required for catalysis. Evidence will be presented later to show that a sequence of at least 2 first-order steps has to be postulated in enzymatic hydrolysis by the esterase. If imidazole were required for some of these steps and COO^- for others, then plots of V_m and of V_m/K_M *vs.* pH would be titration curves.[19] However, the apparent value of pK_b read from each curve would be a function of the pK of each basic group taking part in any step of the reaction and of all specific rate constants involved. Each experimentally determined pK_b for each substrate could have a value ranging anywhere from 3.0 to 7.0, depending upon which reaction steps were the slowest ones under the conditions of the experiments. For the interacting-site mechanism no correlation could be expected among pK_{bE-H}, pK_{bES-H} pK_{bE-L} and pK_{bES-L} for the same substrate, nor among values of pK_b for different substrates (with the exception of pK_{bE-L} which should be the same for all substrates but which, unfortunately, could not be measured here). If this explanation for the pH dependence is correct, then pig liver esterase would be the first histidine–serine enzyme shown to have a function for the dibasic acid at its active site. The COO^- would take part both in the initial binding of E to S (as shown by pK_{bE-H} for methyl *n*-butyrate) and in subsequent first-order steps (as shown by pK_{bES-H}).

Comparison of Substrates. Ethyl *n*-Butyrate *vs.* Methyl *n*-Butyrate.—Table II shows that, although $K_{MH'}$ is the same for both esters, $V_{mH'}$ is somewhat greater for the ethyl ester.[20] This finding indicates that the additional methylene group in the alkyl part of the ethyl ester does not result in a more strongly bound ES complex, although the methylene does appear to accelerate the unimolecular steps of the reaction, perhaps through hydrophobic bonding to the enzyme. If the reaction pathway proceeds *via* an acyl-enzyme intermediate, then the hydrolysis of this intermediate cannot, under the conditions of the experiments, be the rate-determining step of the reaction; if it were, then V_m should depend only upon the acyl part of the ester.

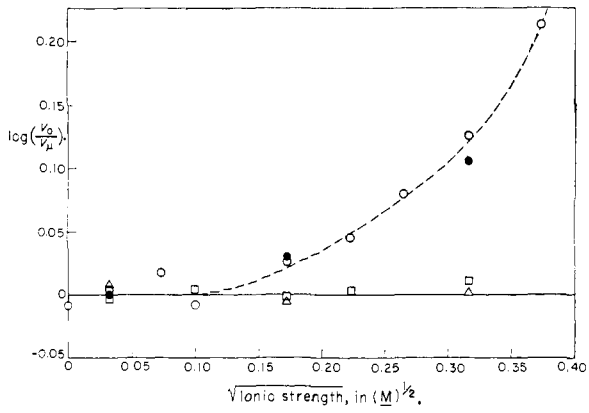

Fig. 5.—Effect of added electrolytes upon rates of hydrolysis of methyl *n*-butyrate; v_0 = enzymatic rate in the absence of added salt.

Symbol	pH	[S](mM)	Added salt	v_0 (μM/min. unit)
○	5	0.59	KCl	5.80
●	5	5.9	KCl	9.95
△	5	0.59	Na_2SO_4	5.80
□	8	0.59	KCl	6.50

Methyl Chloroacetate *vs.* Methyl *n*-Butyrate.—Two well-known effects which the electron-withdrawing chlorine atom has upon its compounds are illustrated by the entries in Table II for k_{OH^-} and for $pK_{a,acyl}$. The qualitative correlation between $V_{mH'}$ and k_{OH^-} for the two methyl esters under consideration suggests that a nucleophilic attack may occur as part of the mechanism of pig liver esterase catalysis. A comparison of the values of $K_{MH'}$ reveals that the chloroacetate ES complex is much looser than the butyrate complex, even though the chloroacetate is hydrolyzed more rapidly at high [S]; there is no evidence here for the "promotion" which has been proposed for acetylcholinesterase.[21] No correlation is found between the pK_b values for ES complexes and the pK values for the acid moieties of the substrate esters.

Effect of Added Electrolytes.—The effect of added salts upon the pig liver esterase catalyzed hydrolysis of methyl *n*-butyrate is shown in Fig. 5. The form of the plot was chosen as a test for the presence of a primary kinetic salt effect. If charges z_S and z_E are carried by the substrate and by the active site of the enzyme, respectively, during any interaction of S with E slow enough to influence the over-all reaction rate, then $\log (v_0/v_\mu = -1.018 z_E z_S \sqrt{\mu}$, and a plot of $\log (v_0/v_\mu)$ *versus* $\sqrt{\mu}$ should be a straight line of slope $= -1.018 z_E z_S$. Here v_0 and v_μ are the rates at ionic strength zero (obtained by extrapolation) and ionic

(16) H. S. Jansz, C. H. Posthumus and J. A. Cohen, *Biochim. et Biophys. Acta*, **33**, 387, 396 (1959).

(17) H. Gutfreund and J. M. Sturtevant, *Biochem. J.*, **63**, 656 (1956).

(18) The only hydrolytic enzyme whose pH dependence appears to reflect a COO^- group is papain, which has a type of active site different from that of liver esterase. See J. R. Kimmel and E. R. Smith, *Advances in Enzymol.*, **19**, 267 (1957).

(19) L. Peller and R. A. Alberty, *J. Am. Chem. Soc.*, **81**, 5907 (1959).

(20) This can be compared to Hofstee's finding that each additional methylene group in *n*-fatty-acid esters of *m*-hydroxybenzoic acid increases V_m, for catalysis by horse liver esterase, by a factor of 2.5.

(21) I. B. Wilson, in "The Mechanism of Enzyme Action," ed. W. D. McElroy and B. Glass, The Johns Hopkins Press, Baltimore, Md., 1954, p. 642 ff.

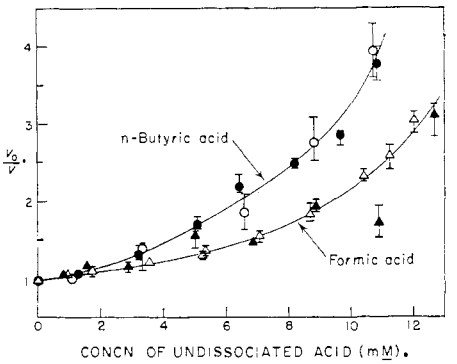

Fig. 6.—Kinetic effect of undissociated aliphatic acids upon hydrolysis of methyl *n*-butyrate; v_0 = enzymatic rate in the absence of added acid. All runs were made with [S] = 1.18 m*M*: ○, constant *p*H 5.0, varied total concn. *n*-butyric acid = 0 to 29.2 m*M*; ●, varied *p*H 3.7 to 5.7, constant total concn. *n*-butyric acid = 11.68 m*M*; △, constant *p*H 4.0, varied total concn. formic acid = 0 to 37.1 m*M*; ▲, varied *p*H 3.3 to 5.0, constant total concn. formic acid = 17.67 m*M*.

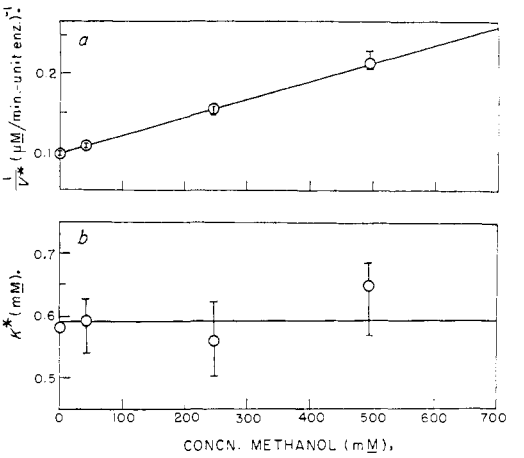

Fig. 7.—Effect of methanol upon kinetic parameters for hydrolysis of methyl *n*-butyrate; *p*H 5 for all data; no added product acid: 7a, effect upon $1/V^*$; 7b, effect upon K^*.

strength μ, respectively, under otherwise identical reaction conditions. The value of the constant is that for aqueous solutions at 25°.

The inhibition by KCl observed at *p*H 5 cannot be interpreted as a simple ionic strength effect, since additions of KCl and of Na_2SO_4, at the same μ and *p*H, do not result in the same v_0/v_μ, and since the plot for KCl at *p*H 5 is not linear. It can be concluded from the △ and □ data that z_E and/or z_S equal zero at *p*H 5 and at *p*H 8. That is, if a non-zero charge is held by the group on the enzyme (say, COO^-) which is helping to catalyze a given step of the reaction, then the charge held by the substrate (or enzyme-substrate intermediate) which is being attacked in the step must be zero. This conclusion seems reasonable, for neither an ester molecule nor an acyl-enzyme intermediate would be expected to carry a charge.

The specific inhibition caused by KCl at *p*H 5 can be quantitatively interpreted as noncompetitive inhibition,[14] with K_{KCl} = [E][KCl]/[E − KCl] = [ES][KCl]/[ES − KCl] = 0.44 *M*. From the fact that KCl inhibits at *p*H 5 but not at *p*H 8, one may speculate that the chloride ion, but not the potassium ion, binds to the enzyme near the active site.

Effect of Added Reaction Products.—Information has been obtained about the reaction mechanism through observation of the effects upon the rates of ester hydrolysis produced by the addition of varying concentrations of product acid and/or alcohol. These changes in rate, which can be represented experimentally as product inhibition may be interpreted as resulting from steps of the reverse reaction proceeding while the forward reaction rate is being measured.

Substrate of the Reverse (Ester Synthesis) Reaction.—Preliminary experiments revealed that *n*-butyric acid inhibits the hydrolysis of its methyl ester much more strongly at *p*H 5 than at *p*H 8, suggesting that the undissociated acid may be a more effective inhibitor than the butyrate anion. If true, this result would mean that the acidic substrate for the reverse (synthesis) reaction is the free acid, not the anion.

The ○ and ● data of Fig. 6 show that the amount of inhibition produced by *n*-butyric acid over a range of *p*H and of total acid concentration (free acid plus acid anion concentrations) appears to be correlated with just one parameter, the concentration of undissociated butyric acid, regardless of large variations in the concentration of the acid anion. The normalization of each rate accomplished by dividing it by v_0 (the uninhibited rate at the same *p*H) eliminates from consideration the variation of K_m and of V_M with changing *p*H. The results of butyrate inhibition experiments can have either of two explanations: First, free butyric acid (pK_a = 4.8) may be the only ionic form capable of inhibiting the enzyme. Or, second, the reverse reaction (that is, the inhibition) may require the presence on the enzyme of the acid form of an ionizing group with pK_a about 4.8, a group which was not detected in the *p*H dependence of V_m or K_M for the forward reaction. In the second case, both the free acid and the anionic forms of butyrate could act equally well as inhibitors.

The formic acid data of Fig. 6 were taken in order to provide a choice between these alternatives. The finding that the △ and ▲ results coincide shows that the first explanation is the correct one; the *p*H dependence of formate inhibition can be totally explained by assuming that the undissociated form of an acid of pK_a 3.7 (formic acid) inhibits pig liver esterase but not by assuming that the presence of an acid group of pK_a 4.8 is required on the enzyme for inhibition to take place.

Thus it can be concluded that the only acidic species which goes onto the esterase to act as substrate for the synthesis reaction is the undissociated acid molecule.

The Sequence of Steps in the Hydrolysis Reaction.—A study was made of the kinetic effects of added methanol and/or *n*-butyric acid under such conditions that the data fitted the Michaelis–Menten equation (*p*H 5, methyl *n*-butyrate concentration ≥ 0.24 m*M*). Mechanistically, the

linear Lineweaver–Burk plots obtained (see Fig. 1c for an example) indicate that the hydrolyses reported here were catalyzed by the high-[S] form of pig liver esterase only, so that only one reaction pathway needs to be considered in any mechanism proposed to explain the data. The effects of the added reaction products upon the kinetic parameters, V^* and K^*, operationally defined in the appendix, are shown in Figs. 7 and 8 and can be summarized by

$$1/V^* = 1/V_m + C_1[\text{methanol}]; \quad K^* = K_M \text{ with added methanol}$$

$$V^* = V_m;\ K^* = K_M + C_2[\text{butyric acid}], \quad \text{with added butyric acid} \quad (6)$$

$$V^* = V_p^*;\ K^* = K_p^* + C_3[\text{butyric acid}], \quad \text{with both products present}$$

Two mechanisms for enzymatic hydrolysis are considered in the Appendix. Mechanism 12 postulates that there is only one reaction intermediate, the Michaelis complex, and that both reaction products are given off in the same step. A comparison of the results of eq. 6 with the predictions of eq. 14 shows that this simplest possible reversible Michaelis–Menten mechanism is not valid here and that at least a second intermediate, EA, must be postulated, as in mechanism 15. One can see, by comparing eq. 6 to eqs. 17, 19 and 20, that all the data are consistent with the two-intermediate mechanism, provided—and only provided—that P_1 is methanol and P_2 is butyric acid.

Thus in the sequence of reaction steps by which an ester is hydrolyzed by pig liver esterase, first the product alcohol is released from the enzyme, and then the product acid is set free. The intermediate EA, which was a necessary assumption, may well, therefore, be an acyl-enzyme complex, although the existence of such a complex has not been proven for liver esterase.

The results of the present research cannot provide a choice among mechanism 15 or a three-intermediate mechanism in which the third step (k_5 step of mechanism 15) results in a Michaelis complex E–P_2 and in which a fourth step is required before P_2 is set free, or an even longer sequence of reaction steps. All are compatible with the data. However, mechanism 15 probably is too simple. Work with chymotrypsin[22] has shown that the role of water in the hydrolysis reaction is analogous to that of the alcohol in the ester synthesis reaction. The three-intermediate mechanism, but not mechanism 15, allows for this symmetry.

Fig. 7b shows that K^* is independent of $[P_1]$ (methanol concentration) for methyl *n*-butyrate at *p*H 5. The Appendix predicts for mechanism 15 that if K^* is independent of $[P_1]$, then $K^* = K_M = k_2/k_1 = [E][S]/[ES]$. This conclusion is true also for the three-intermediate case. Therefore, under these conditions, K_M can be approximated by a dissociation constant, and $k_2 >> k_3$. This inequality need not, however, be valid for other substrates or even for other values of *p*H.

Limits on the Values of Some Specific Rate Constants.—Although the data are not sufficient to permit evaluation of any of the specific rate constants involved in the multiple-intermediate enzymatic mechanism, limits may be assigned to the values of some rate constants. Only the parameters for the high-substrate concentration form of pig liver esterase acting upon methyl *n*-butyrate will be considered here, since the interpretation of V_{mH} is fairly unambiguous; in fact, the empirical constant V equals a maximum velocity for the high-[S] form of the enzyme for several other mechanisms in addition to the interacting-site mechanism. From inequalities derived by Peller and Alberty[19] and applied here to V_{mH}' and K_{MH}' for methyl *n*-butyrate, $V_{mH}'/[E]_0 = 10^3$ sec.$^{-1} \leq k_{(i+1)}$, and $V_{mH}'/[E]_0K_{MH}' = 2 \times 10^6\ M^{-1}$ sec.$^{-1} < k_1$, where k_1 is the rate constant for the bimolecular step in the hydrolysis reaction (for example, k_1 in the two-intermediate mechanism of the Appendix), and $k_{(i+1)}$ is the constant for any unimolecular step in the hydrolysis reaction (k_3 or k_5 in the same mechanism).

The "turnover number" of 10^3 per second is comparable to those for several enzymes more specific than liver esterase. Since $K_{MH} = k_2/k_1$ for pig liver esterase, at least at *p*H 5, k_1 may be much larger than the lower limit given above. For a diffusion-controlled bimolecular step of an enzymatic reaction k_1 has been calculated[23] to be 10^8–$10^9\ M^{-1}$ sec.$^{-1}$. Therefore, the lower limit set on k_1 for pig liver esterase is consistent with diffusion control, although other types of rate limitation in the bimolecular step certainly are not excluded.

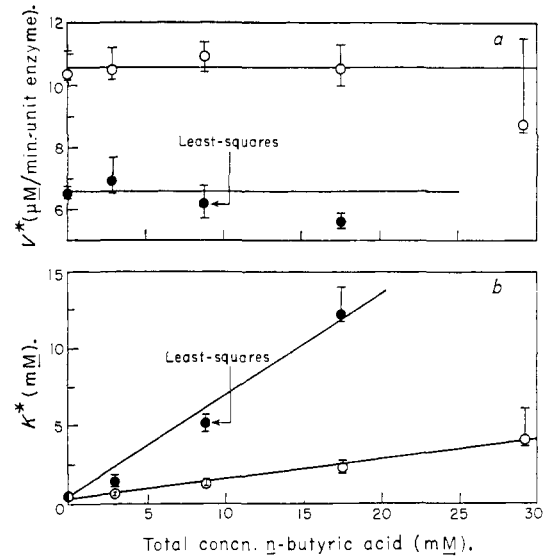

Fig. 8.—Effect of *n*-butyric acid upon kinetic parameters for hydrolysis of methyl *n*-butyrate; *p*H 5 for all data: O, concn. methanol = 0; ●, concn. methanol = 247 m*M*. 8a, effect upon V^*; 8b, effect upon K^*.

Comparison of Horse and Pig Liver Esterases.—In the present research some of the experiments previously performed[4,5] with horse liver esterase acting on methyl *n*-butyrate were repeated and extended. The results confirmed the finding that the horse enzyme obeys the Michaelis–Menten equation at *p*H 8[5] even at [S] as low as 0.1 m*M*, al-

(22) M. L. Bender and W. A. Glasson, *J. Am. Chem. Soc.*, **82**, 3336 (1960).

(23) R. A. Alberty and G. G. Hammes, *J. Phys. Chem.*, **62**, 154 (1958).

though the Lineweaver–Burk plot at pH 10 curves appreciably at [S] $\leq$ 0.3 mM.[4]

There are several species differences apparent in the kinetics of the two mammalian liver esterases as the enzymes hydrolyze methyl n-butyrate: (i) The substrate is somewhat more tightly bound to the pig enzyme ($K_{MH}' = 0.36$ mM) than to the horse enzyme (2.5 mM). (ii) The pig esterase ($pK_{bE-H} = 4.0$, $pK_{bES-H} = 3.4$) is strikingly more active at low pH than is the horse enzyme ($pK_{bE-H} = 7.2$, $pK_{bES-H} = 4.7$). (iii) Some pH-independent kinetic parameters of horse liver esterase are: $V_{mH}' = 11.2$, $V_{mL}' = 0.12\ \mu M$/min.-unit enzyme; $K_{ML}' \leq 0.01$ mM. A comparison of these data with Table II shows that, although both enzymes exhibit deviations from Michaelis–Menten behavior, the deviation of pig liver esterase is more pronounced and begins at a much lower pH (pH $\simeq$ 5.5 instead of $8 < p\text{H} < 10$) than that of horse liver esterase.

Appendix

Kinetic Consequences of Some Enzymatic Mechanisms. Interacting Sites.—For the mechanism

$$\begin{array}{ccccc} E_2 + S & \underset{k_2}{\overset{2k_1}{\rightleftharpoons}} & E_2S & \xrightarrow{k_3} & E_2 + P \\ & & + & & \\ & & S & & \\ & & k_4 \downarrow & \uparrow 2k_5 & \\ & & E_2S_2 & \xrightarrow{2k_6} & E_2S + P \end{array} \qquad (7)$$

the steady-state rate is expressed as a function of substrate concentration by

$$v = \frac{V_{mH}[S]^2 + V_{mL}K_{MH}[S]}{[S]^2 + 2K_{MH}[S] + K_{ML}K_{MH}} \qquad (8)$$

where

$V_{mL} = 2k_3\,[E]_0$ $\qquad$ $K_{ML} = (k_2 + k_3)/k_1$
$V_{mH} = 2k_6\,[E]_0$ $\qquad$ $K_{MH} = (k_5 + k_6)/k_4$
$[E]_0$ = total enzyme concentration

The empirical parameters of Table I can be identified with mechanistically meaningful parameters as

(i) For methyl and ethyl butyrate:

$$V_{mH} = V,\ K_{MH} = \frac{K + C}{2},\ V_{mL} = \frac{2VC}{K + C} \qquad (9)$$

From data at pH 8, [methyl butyrate] < 0.1 mM:

$$K_{ML} = \left(\frac{v_{\text{extrapolated from eq. 1}}}{v_{\text{experimental}}} - 1\right)\left(\frac{[S]^2 + 2K_{MH}[S]}{K_{MH}}\right) \qquad (10)$$

(ii) For methyl chloroacetate:

$$\frac{V_{mH}}{K_{MH}} = 2a,\quad V_{mL} = 2b$$
$$K_{MH} \gg [S]/2,\quad K_{ML} \ll 2[S] \qquad (11)$$

Thus, by means of this interacting-site mechanism, 3 mechanistically meaningful constants (and 4 at pH 8) can be obtained for methyl n-butyrate. Two meaningful constants are to be had for methyl chloroacetate: upper or lower limits can be set on 2 more by substituting in the above inequalities the highest or lowest [S] used for data, multiplied by a factor determined by the precision of the data.

Effects of Added Reaction Products upon Rates.—Two reversible single-pathway mechanisms for hydrolytic action are presented which differ in the number of reaction intermediates postulated. V^* and K^* stand for the kinetic parameters defined empirically by the intercept and by the slope/intercept ratio of a $1/v$ *vs.* $1/[S]$ plot. These parameters can be defined whenever such a plot is linear, even in the presence of reaction products. V^* and K^* are not necessarily equivalent to V_m and K_M, which represent the customary maximum velocity and Michaelis constant determined for the hydrolysis reaction in the absence of products.

One intermediate EX

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EX \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E + P \qquad (12)$$

where P stands for all reaction products, here alcohol and acid, which must come off the enzyme together in this mechanism. The complete rate expression is:

$$v = \frac{(k_1k_3[S] - k_2k_4[P])\,[E]_0}{k_1[S] + k_4[P] + k_2 + k_3} \qquad (13)$$

When one or the other product, but not both, is present, in concentration [P], then

$$V^* = V_m$$
$$K^* = K_M + \frac{k_4}{k_1}[P] \qquad (14)$$

Therefore, when *either* product is added, V_m must remain constant, although K^* can increase.

Two intermediates, ES and EA

$$\begin{array}{ccccc} E + S & \underset{k_2}{\overset{k_1}{\rightleftharpoons}} & ES & \underset{k_4}{\overset{k_3}{\rightleftharpoons}} & EA + P_1 \\ & & & & \\ & & +H_2O\ \ k_5 \downarrow & \uparrow k_6 & \\ & & & & E + P_2 \end{array} \qquad (15)$$

where P_1, P_2 are alcohol, acid, but not necessarily in that order.

The rate is given by

$$v = \frac{(k_1k_3k_5[S] - k_2k_4k_6[P_1][P_2])[E]_0}{(k_1k_3 + k_1k_5 + k_1k_4[P_1])[S] + (k_2k_6 + k_3k_6 + k_4k_6[P_1])[P_2] + (k_2k_5 + k_3k_5 + k_2k_4[P_1])} \qquad (16)$$

Case 1: $[P_1] \neq 0$, $[P_2] = 0$. Rate expressions equivalent to eq. 17 have been derived previously,[24,25] for glucose-6-phosphatase

$$\frac{1}{V^*} = \frac{1}{V_m} + \frac{k_4}{k_3k_5[E]_0}[P_1] \qquad (17)$$

$$K^* = \frac{(k_2 + k_3)k_5 + k_2k_4[P_1]}{(k_2 + k_5)k_1 + k_1k_4[P_1]}$$

$$\lim_{[P_1]\to\infty} K^* = \frac{k_2}{k_1} \qquad (18)$$

Thus, this mechanism predicts that $1/V^*$ should be a linear function of $[P_1]$ but that K^* is a complicated function of $[P_1]$ which can increase, decrease or remain constant, depending upon the values of the rate constants. However, in the special case that $dK^*/d[P_1] = 0$ for all $[P_1]$, then $K^* = k_2/k_1$.

Case 2: $[P_1] = 0$, $[P_2] \neq 0$. Then

$$V^* = V_m$$
$$K^* = K_M + \frac{k_6K_M}{k_5}[P_2] \qquad (19)$$

The predictions here are that V_m should remain constant and that K^* should be a linear function of $[P_2]$.

Case 3: $[P_1]$ = constant = $p \neq 0$, $[P_2] \neq 0$. When both products are present, eq. 16 can be cast into the Michaelis–Menten form only provided that $k_1k_3k_5\,[S] >> k_2k_4k_6\,[P_1]\,[P_2]$, in which case

(24) H. L. Segal, *J. Am. Chem. Soc.*, **81**, 4047 (1959).
(25) L. F. Haas and W. L. Byrne, *ibid.*, **82**, 947 (1960).

$$V^* = V_p^*$$

$$K^* = K_p + \frac{k_6(k_2 + k_3 + k_4 p)}{k_1(k_3 + k_5 + k_4 p)}[P_2] \qquad (20)$$

where V_p^*, K_p^* are the V^*, K^* obtained when $[P_1] = p$, $[P_2] = 0$. Equation 20 is of the same form as eq. 19.

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Photochemical Reactions of Thiobenzophenones[1]

BY GERALD OSTER, LOUIS CITAREL[2] AND MURRAY GOODMAN

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Irradiation of thioketones with visible light transforms them quantitatively to their oxygen analogs. The quantum yield of the reaction is less the greater the dark stability of the compound in air. For thiobenzophenone the dependence of quantum yield on oxygen concentration indicates that the reactive excited species has a lifetime of at least 1.5×10^{-7} sec. Near ultraviolet light transforms thiobenzophenone in the absence of oxygen into benzhydryl mercaptan, benzhydryl disulfide and tribenzhydryl tetrasulfide. This reaction takes place best with solvents which have easily extractable hydrogens and is inhibited by oxygen. Photolysis of thioketones proceeds in saturated hydrocarbon solvents if far ultraviolet light is employed. The absorption bands of thiobenzophenone in ethanol at 315 mμ and 595 mμ are further separated for thiobenzophenones with electron releasing substituents. The position of the two bands is influenced by the nature of the solvent.

Introduction

Thioketones differ strikingly from their oxygen analogs in that they are colored (usually blue) and highly reactive. Their ultraviolet spectra also offer some interesting differences from their oxygen counterparts. The photochemistry of benzophenone recently has been described in detail.[3] One purpose of the present work is to compare the photochemical reactions of thiobenzophenone with those of benzophenone. The thio compounds have widely separated absorption bands and hence allow the selective excitation of a single band without interference from other overlapping bands. As a consequence, one might expect differences in photochemical behavior depending on the wave length region used for excitation. The second purpose of the present work is to describe the effect of substituents on both the visible and ultraviolet spectra of substituted aromatic thiones.

Experimental

Materials.—The thioketones were prepared by treatment of an ethanolic solution of the corresponding ketone with H_2S in the presence of HCl.[4] The ketones were obtained either from Eastman or synthesized and purified by well known procedures. The thioketones were purified by chromatography over Florisil (Floridin Co., Tallahassee, Florida).[5] Nitrogen–oxygen gas mixtures were supplied by Matheson Company.

Benzhydryl disulfide was prepared by the procedure of Staudinger and Freudenberger[6] in 63% yield. The synthesis of benzhydryl mercaptan was accomplished by two different procedures. In the first method thiobenzophenone (5.0 g., 0.025 mole) was dissolved in anhydrous ether and reduced *via* the usual lithium aluminum hydride reduction. During the reaction the solution color turned from a deep blue to a pink. The complex was decomposed by the slow addition of water and the aqueous phase separated from the organic phase. The aqueous phase was extracted further with two 50 ml. portions of ether and the ether phases combined. The ether was removed by distillation leaving an oily residue which was dried over magnesium sulfate. The oily material was distilled under reduced pressure to yield 3.5 g. (70%) of a colorless oil, b.p. 138° at 2.5 mm., $n^{25}D$ 1.6159 (reported $n^{25}D$ 1.6159[7]). In the second method, this compound was prepared by the procedure described by Suter.[7]

Procedures.—Visible and ultraviolet spectra were determined in absolute ethanol at room temperature using a DU spectrophotometer which was modified to be automatically recording (Process Instruments, Inc.).

For the visible light photochemistry the solutions were irradiated with nearly monochromatic light of 588 mμ achieved by an interference filter (Bausch and Lomb) placed in front of a 500 watt tungsten lamp projector (Bell and Howell, type TDC). The change in transmission was followed by the use of a red-sensitive RCA 1P22 photomultiplier tube whose output was amplified with an Aminco photometer instrument and recorded as a function of time on a Leeds and Northrup Speedomax recorder. Gases previously equilibrated with ethanol were bubbled through the solutions. The concentrations of the thioketones were adjusted to give an optical density of unity at 588 mμ. The reaction also was followed by determining the ultraviolet spectrum of a 100-fold dilution of a 1 ml. aliquot drawn at intervals from the solution being irradiated. The intensity of the light falling on the solution was determined with a calibrated thermopile (Eppley Laboratories, Newport, R. I.).

For the near ultraviolet light photochemistry two lamps were employed. To follow the reaction, we used an AH-4 (General Electric) high pressure mercury lamp with a Wood's glass filter to isolate the 365 mμ line and a $CuSO_4$ filter[8] to remove the deep red component. The change in transmission at 365 mμ was followed on the recorder as described above but now a RCA 1P28 photomultiplier was used. In order to obtain large amounts of products produced by near ultraviolet light a Hanovia S-100 intermediate pressure mercury lamp was employed. This lamp in the form of a straight tube was inserted in a water-cooled (de-ionized water cooled externally) quartz jacket fitted with a flint glass filter to absorb ultraviolet radiation below 300 mμ. The lamp and quartz jacket were incorporated in an apparatus designed to allow for the irradiation of a sample under inert atmosphere and with facilities to draw off samples at any desired time interval. The reaction chamber can contain 700 ml. of solution. If desired one can isolate any particular spectral line by circulating the appropriate light

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(1) Presented at the 135th National Meeting of the American Chemical Society, Boston, April 1959. Taken in part from the Ph.D. thesis of Louis Citarel, Polytechnic Institute of Brooklyn, June 1960.

(2) Thiokol Research Fellow 1958–1959.

(3) J. N. Pitts, Jr., R. L. Letsinger, R. P. Taylor, J. M. Patterson, G. Recktenwald and R. B. Martin, *J. Am. Chem. Soc.*, **81**, 1068 (1959).

(4) H. Staudinger and H. Freudenberger, *Ber.*, **61**, 1576 (1928).

(5) R. H. Abeles, R. F. Hutton and F. H. Westheimer, *J. Am. Chem. Soc.*, **79**, 712 (1957).

(6) H. Staudinger and H. Freudenberger, *Organic Syntheses*, **11**, 94 (1931).

(7) M. M. Klenk, C. M. Suter and S. Archer, *J. Am. Chem. Soc.*, **70**, 3846 (1948).

(8) E. J. Bowen, "Chemical Aspects of Light," 2nd Ed., Oxford, 1947, Appendix II.