Pig Liver Esterase. Reactions with Alcohols, Structure–Reactivity Correlations, and the Acyl-Enzyme Intermediate*

P. Greenzaid and W. P. Jencks†

ABSTRACT: Methanol and other weakly acidic alcohols are highly effective nucleophiles toward the acyl group in reactions catalyzed by a pig liver esterase preparation. The total rate of phenyl acetate disappearance is increased 5.5-fold by 0.5 M methanol with little change in the hydrolysis rate; the less reactive *p*-nitrophenyl and 2,4-dinitrophenyl acetates show progressively smaller rate increases. The hydrolysis of alkyl acetates is inhibited by methanol, although methanol increases the total rate of ethyl thiolacetate disappearance. The more slowly hydrolyzed thiol ester substrate, acetamidoethyl thiolacetate, exhibits no increase in total reaction rate in the presence of methanol. These results support the acyl-enzyme mechanism, with partitioning of the intermediate between water and methanol. However, differences in methanolysis/hydrolysis ratios for different substrates, differential inactivation of different activities with diethyl *p*-nitrophenyl phosphate and the kinetics of enzyme inactivation by bis(*p*-nitrophenyl) phosphate show that the enzyme preparation contains at least two types of active site. The nucleophilic reactivity of alcohols is decreased by electron-withdrawing substituents, whereas the reactivity of aliphatic esters is not affected by electron-withdrawing substituents in the alcohol; these results are in accord with the known effect of substituents on the equilibrium constants of the reactions. The substituent effects in the enzymatic reaction suggest the development of a positive charge on the attacking alcohol, but little change in the charge of the leaving alcohol compared to the ester, and are consistent with acid catalysis in the transition state for ester hydrolysis. The free energy of hydrolysis of the acetyl-enzyme intermediate formed from three different esters was estimated to be approximately +0.4 kcal mole.

We report here a study of the reactions of a series of acetate esters with water and alcohols catalyzed by a preparation of pig liver esterase. Pig liver esterase is a serine enzyme with two active sites on each molecule, which dissociates into active half-molecules in the presence of dilute acid or concentrated salts (Krisch, 1966; Horgan *et al.*, 1966a,b; Barker and Jencks, 1969a). The purpose of these experiments was to investigate the mechanism of catalysis and the rate-determining step of these reactions by determining the effects of nucleophiles on the rates of hydrolysis and alcoholysis of rapidly and slowly reacting ester substrates and by estimating the charge distribution in the transition state by measurement of substituent effects in the alcohol and ester. It is known that certain mammalian esterases will catalyze acyl group transfer from esters to amino acid derivatives and other amines (Lipmann and Tuttle, 1950; Benohr and Krisch, 1967; Franz and Krisch, 1968; Goldberg and Fruton, 1969) and Adler and Kistiakowsky (1962) have interpreted the noncompetitive inhibition of the hydrolysis of methyl butyrate by methanol (50% at 0.5 M methanol) in terms of a two-step reaction mechanism, *e.g.*, the rate-determining formation of an acyl-enzyme intermediate. A preliminary communication describing some of this work has appeared (Greenzaid and Jencks, 1970).

Experimental Section

Ester substrates were generally commercial preparations which were redistilled or recrystallized before use. Trifluoroethyl acetate was prepared by the method of Bruice *et al.* (1962). Phenyl thiolacetate was prepared according to the method of O'Neill *et al.* (1961). Cyclohexylmethyl acetate was prepared from refluxing alcohol and acetic anhydride in the presence of a trace of sulfuric acid, washed, dried, and distilled (bp 108° (40 mm)). Acetamidoethyl thiolacetate (*N*-2-mercaptoethylacetamide acetate, *N*,*S*-diacetylcysteamine) was prepared as described by Gerstein and Jencks (1964) and was crystallized as a hygroscopic solid (mp 31–33°) from ether–petroleum ether (bp 37–46°). Phenyl acetate was redistilled; a sample that was further purified by the method of Jencks and Gilchrist (1968) gave the same kinetic results as the unpurified material. Saturated solutions of 2,4-dinitrophenyl acetate were prepared by vigorously stirring an excess of the solid in water at 65° for 15 sec, followed by rapid cooling to room temperature, and filtration after stirring for an additional 5 min. Concentrations were determined from the absorbance at 360 nm of a sample in alkali, based on ϵ 14,700. The ester undergoes hydrolysis upon standing and samples used for kinetic experiments contained from 4 to 15% free dinitrophenol. Solutions of *p*-nitrophenyl acetate at concentrations up to 3.2 mM were prepared in the same way, but most solutions were prepared by prolonged stirring at room temperature. Concentrations were determined by spectrophotometric examination at 400 nm in alkali, based on ϵ 18,320 (Kézdy and Bender, 1962).

Methoxyethanol, chloroethanol, trifluoroethanol, and propyn-2-ol were redistilled. Dioxane was distilled after refluxing over sodium. Stock solutions of Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid), Aldrich) were prepared shortly before use in 0.14 M potassium phosphate buffers, pH 7.4, containing 10^{-4} M EDTA. A preparation of diethyl *p*-nitrophenyl phosphate (K & K Laboratories) was found to be contaminated with a large amount of *p*-nitrophenol; however, it was shown that after alkaline hydrolysis the preparation had no effect on enzyme activity. The concentra-

* Publication No. 760 from the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154. *Received October 15, 1970.* This work was supported by grants from the National Science Foundation (GB 5648) and the National Institute of Child Health and Human Development of the National Institutes of Health (HD 01247). P. G. was a Public Health Service Fellow of the National Institute of General Medical Sciences (National Institutes of Health).

† To whom to address correspondence.

tion of ester was estimated from the absorbance at 400 nm in alkali of aliquots before and after hydrolysis in 1 M sodium hydroxide for 10 min at room temperature. Glass-distilled water was used throughout.

Liver esterase was purified by the method of Horgan *et al.* (1966a, 1969b). The enzyme was assayed during purification with 0.28 mM *p*-nitrophenyl acetate in 0.033 M potassium phosphate buffer (pH 7.37) at 25°. The final chromatography step with CM-Sephadex C-50 was carried out with a 5 × 80 cm column and 2-l. volumes for establishing the elution gradient. A shoulder of activity and A_{230} (fraction A) was eluted before the peak fraction B. Fraction B was further purified by chromatography on a small (approximately 25 ml) DEAE (Whatman 22) column eluted with a gradient composed of 100 ml of 0.02 M potassium phosphate buffer (pH 7.4) and 100 ml of buffer containing 0.25 M sodium chloride. The peak (20% yield, 1.3-fold purification) was precipitated with ammonium sulfate and stored at −15° in 0.02 M potassium phosphate buffer (pH 7.4). The activity of this enzyme was determined against 1.5 mM phenyl acetate as a standard in each kinetic experiment and was found to remain constant, ±10%, over a period of 1.5 years. Enzyme concentrations and kinetic experiments were based upon this standard assay. Concentrations and turnover numbers are based upon a molecular weight of 163,000 and absorbance (1 mg/ml) of 1.305 at 280 nm (Horgan *et al.*, 1966a).

The stock solution of 1.6×10^{-5} M enzyme was diluted 16-fold and stored at 0–4° for up to 1 month. Aliquots of this solution were diluted 2- to 15-fold on the day of use before further dilution into the reaction mixture. All dilutions were carried out with 0.02 M potassium phosphate buffer (pH 7.4).

Spectrophotometric experiments were carried out by following the release of phenol or phenolate ion at its absorption maximum with a Zeiss PMQ II or Gilford 2000 spectrophotometer fitted with thermostated cell compartments. The reaction was started by adding 0.02–0.20 ml of enzyme solution to a final reaction volume of 3 ml containing 0.033 M potassium phosphate buffer (pH 7.37).

Acid release was followed at pH 7.37 in a 20-ml reaction mixture containing 0.01 M potassium chloride with a Radiometer TTTlC titrator with magnetic stirring at 25°. A gentle stream of nitrogen was passed over the surface except for reactions of highly volatile esters. The titrant was 0.005–0.02 M sodium hydroxide. Boiled water was used if the sodium hydroxide concentration was less than 0.01 M. The reaction was always started by the addition of 0.04–0.20 ml of enzyme solution.

In a number of experiments with methanol in the reaction mixture aliquots were removed from the titration solution and monitored for the total reaction rate by spectrophotometric measurement of phenol release; the subsequent rate of acid release was corrected for the resulting volume change. Dinitrophenol release from dinitrophenyl acetate was monitored at 360 nm 30 sec after a 6-fold dilution of an aliquot into 0.01 M phosphate buffer (pH 7.4). The titrator and spectrophotometric measurements were standardized against each other in experiments carried out in the absence of methanol, taking into account the second mole of acid released from dinitrophenol ionization. The rate of acetic acid release in the presence of methanol was obtained by subtracting the rate of dinitrophenol release from the total rate of acid release. Rates were corrected for nonenzymatic hydrolysis which was measured under the same experimental conditions. *p*-Nitrophenol liberation from *p*-nitrophenol acetate was measured at 348 nm, the isosbestic point of *p*-nitrophenol and its anion, with aliquots quenched in 0.5 M acetic acid, using an extinction coefficient of 5150 based upon the difference in the extinction coefficients of *p*-nitrophenol and *p*-nitrophenol acetate at this wavelength (*cf.* Bergmann *et al.*, 1958). The observed rate of acid liberation was corrected for the acid liberated from *p*-nitrophenol, which was found to be 65% ionized at pH 7.37. Phenol liberation from phenyl acetate was measured in the same manner at 270 nm, based upon ϵ 1480. The total rate of reaction of ethyl thiolacetate was determined from the change in absorption at 412 nm in the presence of 0.36 mM Ellman's reagent and 10^{-4} M EDTA based upon ϵ 13,600 (Ellman, 1959). The rate was found to be independent of the concentration of Ellman's reagent over the range 0.16–0.64 mM. The rate of acid liberation was generally measured in the titrator in the absence of Ellman's reagent because acid liberation from the 2-nitro-5-thiobenzoate and the strong inhibition of hydrolysis by methanol interfered with measurements of the rate of acetic acid release in the presence of methanol. However, it was shown that the rate of acid liberation from hydrolysis in the absence of methanol was the same in the presence and absence of Ellman's reagent and the activation of the total reaction rate, measured on aliquots removed from the titrator and read at 412 nm, was found to be the same as in the spectrophotometric experiments. The rate of acid release from the hydrolysis of acetamidoethyl thiolacetate was measured in the titrator and the total reaction rate was measured with aliquots which were diluted 8-fold into a solution of 0.37 mM Ellman's reagent in 0.01 M phosphate buffer, pH 7.8, containing 10^{-4} M EDTA and read immediately at 412 nm. Argon was bubbled through the reaction mixture for 10 min before the addition of enzyme.

Reactions were generally followed for from 3 to 10 min. Initial rates were measured with up to 10% reaction. Reactions measured by removal of aliquots were followed for up to 20% reaction (phenyl acetate up to 30%). A falling off of the rate with time was occasionally noted, especially for reactions of methyl acetate and trifluoroethyl acetate, and in the presence of high methanol concentrations; the rates were measured from the initial slopes of these curves. Occasional lags were seen for reasons which were not apparent, especially with acetamidoethyl thiolacetate; however, the rates after the initial lag period (approximately 1 min) were found to be the same as in other runs which exhibited no lag. The rates were found to be linear with respect to enzyme concentration in the range 0.17×10^{-9} to 1.7×10^{-9} M for the hydrolysis of *p*-nitrophenyl acetate. The reproducibility with *p*-nitrophenyl acetate was somewhat less than that with the other substrates. It was shown that the rate of hydrolysis of any methyl acetate that was formed as a reaction product during the hydrolysis of other esters in the presence of methanol was sufficiently slow that it did not contribute to the observed overall rate of hydrolysis, measured by acid release.

Results

Purification and Properties. Our kinetic studies were carried out with liver esterase fraction B, purified by a slight modification of the procedure of Horgan *et al.* (1966a). This preparation was found to give more stable and reproducible kinetic behavior than enzyme prepared according to the Adler and Kistiakowsky (1961) procedure, which was used for earlier experiments in this laboratory (Barker and Jencks,

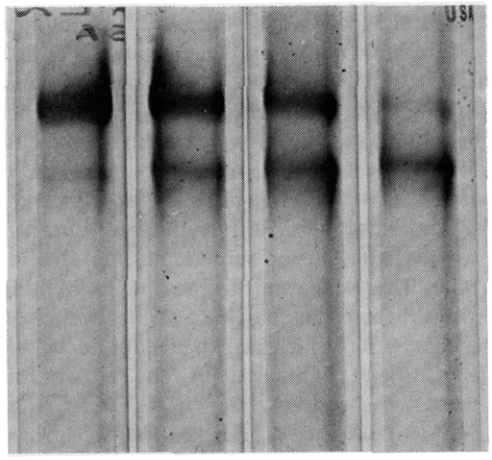


FIGURE 1: Gel electrophoresis of esterase (1.6×10^{-9} M) incubated in 0.02 M potassium phosphate buffer (pH 7.4) at 23° for (from left to right) 5 min, 2 hr, 5 hr, and 22 hr before electrophoresis.

1969b). However, we also obtained a second esterase, fraction A, which was not reported in the original purification procedure of Horgan *et al.* (1966a) and which exhibits a different substrate specificity (Barker and Jencks, 1969a) and much less stable kinetic behavior compared to fraction B. All three enzymes have very similar or identical physical properties. The sedimentation constants, $s^{5\ mg/ml}_{20,w}$, were found to be 7.75, 7.75, and 7.74 S for fraction A, fraction B, and the Adler and Kistiakowsky enzyme, respectively. Immunologically the three enzymes are very similar by complement fixation assay, but show small, definite differences (Barker and Jencks, 1969a).

Fraction B was found to be homogeneous upon electrophoresis in 7.5% polyacrylamide gel containing 0.05 M Tris–0.38 M glycine buffer (pH 8.5) of a sample initially 0.2 mg/ml, followed by staining for protein with Amido Black. At an initial concentration of 1.6×10^{-9} M the enzyme is also almost homogeneous if subjected to electrophoresis immediately after dilution, but exhibits progressive dissociation to a faster moving band, presumably the half-molecule, if it is allowed to stand at this concentration before electrophoresis; after 22 hr the dissociation is almost complete (Figure 1). The positions of the bands of the dilute enzyme were determined by staining for enzymatic activity according to the procedure of Holmes and Masters (1967). It should be noted that these multiple bands could easily be misinterpreted as evidence for the existence of isozymes if the effects of enzyme concentration and of dissociation time were not examined.

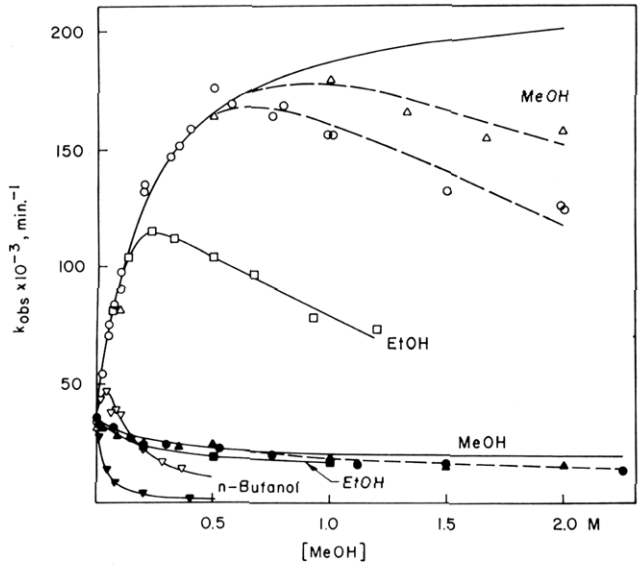


FIGURE 2: The effect of methanol, ethanol, and 1-butanol on the total reaction rate (open symbols, upper curves) and hydrolysis rate (closed symbols, lower curves) of phenyl acetate in the presence of 1.4×10^{-9} M esterase at 25°, pH 7.37. The phenyl acetate concentration was 3.0 mM (triangles) or 1.5 mM (all other points). The solid lines for the methanol reaction are theoretical curves calculated as described in the text.

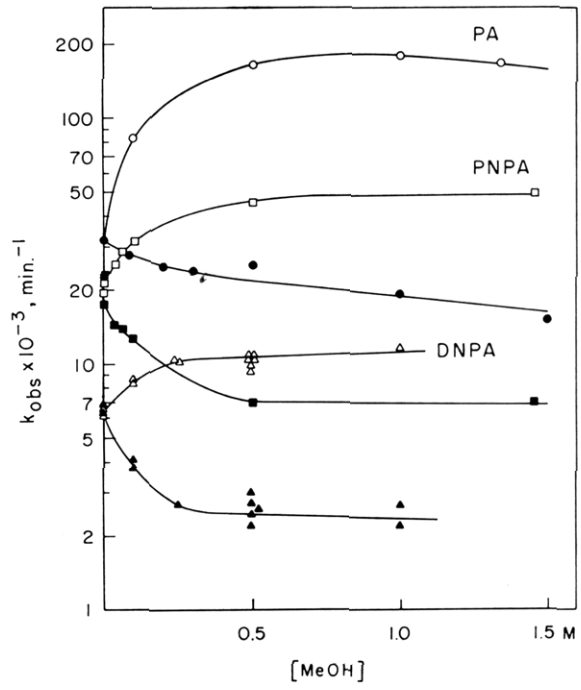


FIGURE 3: The effect of methanol on the total reaction rate (open symbols, upper line) and hydrolysis rate (closed symbols, lower line) of phenyl acetate (3.0 mM), *p*-nitrophenyl acetate (1.0 mM), and dinitrophenyl acetate (0.8 mM), in the presence of methanol at 25°, pH 7.37. The phenyl acetate results are from separate titrimetric and spectrophotometric experiments; the other reactions are based on spectrophotometric examination of aliquots taken from a reaction mixture in the titrator. The enzyme concentrations were 1.4×10^{-9}, 0.72×10^{-9}, and 2.5×10^{-9} M for phenyl acetate, PNPA, and DNPA,[1] respectively.

Reactions with Nucleophilic Reagents. Low concentrations of methanol cause a marked increase in the total rate of the esterase-catalyzed cleavage of phenyl acetate (Figure 2). As the methanol concentration is increased the rate levels off at a maximum 5.5-fold larger than the rate in the absence of methanol, and at still higher concentrations there is a slow decrease in rate which is largely or entirely caused by competitive inhibition, since it is approximately half as large at 3.0 as at 1.5 mM phenyl acetate concentration. The increase in total rate is accompanied by a small initial decrease in hydrolysis rate, measured by the rate of acetic acid release, followed by a levelling off, and a further smaller decrease comparable to that observed for the total rate. The difference between the total and hydrolysis rates represents the conversion of phenyl acetate into methyl acetate by reaction with methanol acting as a nucleophile. The ratio of methanolysis to hydrolysis was found to be independent of enzyme con-

TABLE I: Effect of Alcohols and Modifiers on Esterase Activity at pH 7.37, 25°.

Reagent	M	k_{obsd}/k_0[a] PNPA 0.55 mM	Phenyl Acetate 1.5 mM	DNPA 0.80 mM	Methyl Acetate 25 mM
Ethanol	0.1	1.6			
	0.5	2.2			
1-Butanol	0.033	1.10			
	0.167	0.38			
Acetone	0.1	1.1	1.0		0.33
	0.2				0.17[b]
	0.3			1.6	
	0.5	0.97			
Ethylene glycol	0.1	0.93			
	0.5	1.1			
Acetonitrile	0.1	1.3		0.9	
	0.2		1.0[c]		0.69
	0.5	1.5	1.1[c]	1.4	
	1.0		0.95		
	2.0		0.51		
Benzene	0.0045	1.2			
	0.0067		1.2		
	0.01				0.84
Dimethylformamide	0.1	1.30			
	0.5	1.25			
Dioxane	0.1	1.2	1.0	1.2	
	0.2				0.40
	0.5	0.92	1.1	1.4	
Dimethyl sulfoxide	0.1	1.15			
	0.5	1.3			
Aniline	0.1	1.0			
	0.5	0.80			
Methylammonium chloride	0.02	1.0[d]	0.94		
	0.09		0.83		
N-Methylhydroxylamine	0.02		0.85[d]		
	0.002		0.87[d]		
Methoxyamine	0.075	1.16[d]			
Acetamide	0.5	0.96			
Phenol	0.0015		0.84		
	0.005	0.75[d]			
	0.010	0.45[d]			
	0.02	0.25[d]			
Trifluoroethanol	0.02	1.1[d]			
	0.10	1.05[d]			
	0.50	0.32[d]			
	0.033				
Methyl acetate	0.025		0.81		
	0.10		0.82		

[a] Ratio of observed rates in the presence and absence of the indicated modifier. [b] 0.44 at 100 mM ester. [c] 0.95 and 1.0 at 3 mM ester. [d] Corrected for nonenzymatic reaction.

:entration in the range 8.5×10^{-10}–1.7×10^{-9} M and of)henyl acetate concentration in the range 0.2–6 mM. With :thanol the rate increase is smaller, a maximum of 3.2-fold, ınd the inhibition at higher concentrations is larger. With -butanol an initial rate increase is followed by a marked nhibition of both the total and hydrolysis rates at low .lcohol concentrations (Figure 2). *p*-Methoxyphenyl acetate ındergoes hydrolysis at the same rate as phenyl acetate and exhibits the same 5.5-fold activation in the presence of 0.6 M methanol.

The effects of methanol on the total and hydrolysis rates of phenyl acetate, *p*-nitrophenyl acetate (PNPA),[1] and 2,4-dinitrophenyl acetate (DNPA) are plotted on a semi-

[1] Abbreviations used are: PNPA, *p*-nitrophenyl acetate; DNPA, 2,4-dinitrophenyl acetate.

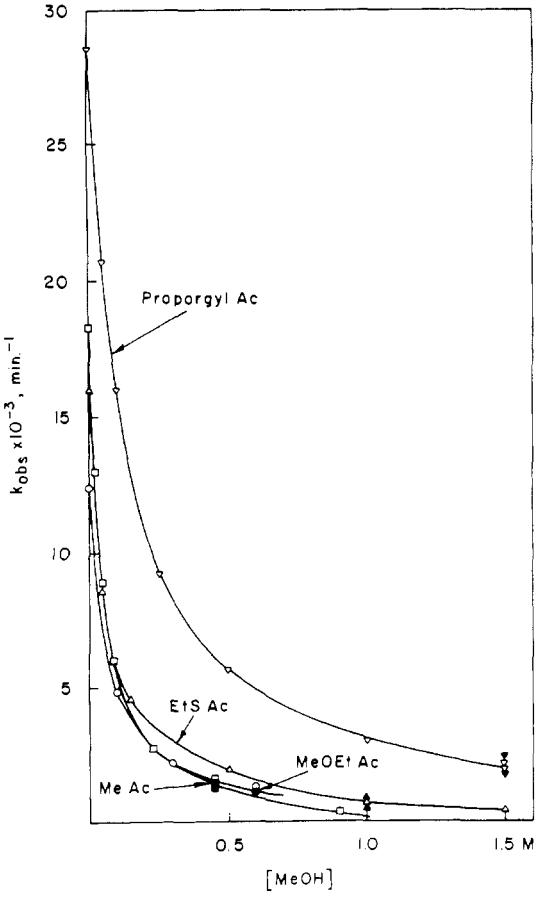


FIGURE 4: Effect of methanol on the esterase-catalyzed hydrolysis of aliphatic esters at 25°, pH 7.37. Propargyl acetate, ▽ (11 mM), ▼ (6.2 and 19 mM), [enzyme] 1.7–5.0 × 10^{-9} M; methyl acetate, □ (25 mM), ■ (15 and 50 mM), [enzyme] 2.5–10 × 10^{-9} M; ethyl thiolacetate, △ (20 mM), ▲ (10 and 40 mM), [enzyme] 2.5–10 × 10^{-9} M; methoxyethyl acetate, ○ (19 mM), ● (12 mM), [enzyme] 3.4 × 10^{-9} M.

logarithmic scale in Figure 3. The pattern is similar for each substrate, but the slower reaction of PNPA exhibits a smaller rate increase (2.5-fold) and a greater inhibition of hydrolysis compared to phenyl acetate and the still slower reaction of DNPA exhibits a still smaller increase in total rate (1.7-fold) and a still larger inhibition of hydrolysis.

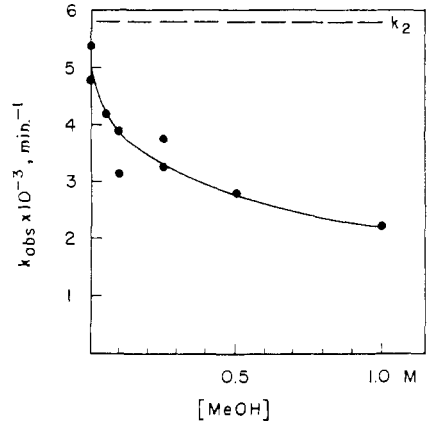


FIGURE 5: Effect of methanol on the rate of hydrolysis of 9 mM benzyl acetate catalyzed by 5.5 × 10^{-9} M esterase at 25°, pH 7.37.

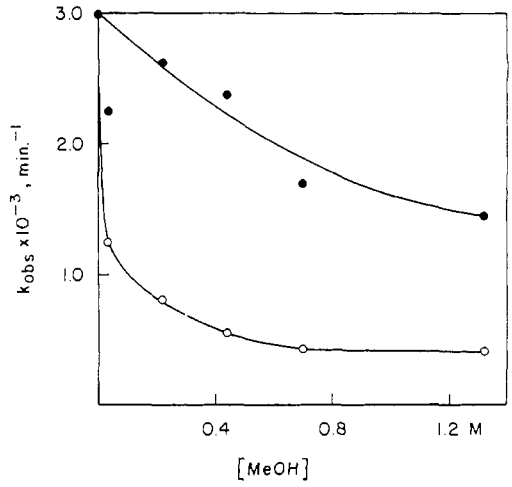


FIGURE 6: Effect of methanol on the total rate (●) and the hydrolysis (○) of acetamidoethyl thiolacetate catalyzed by 7.6 × 10^{-9} M esterase at 25°, pH 7.37, in the presence of 10^{-4} M EDTA. The total rate was measured from aliquots of the reaction mixture assayed for thiol release with Ellman's reagent.

The effects of a number of small molecules on the esterase-catalyzed decomposition of several ester substrates are summarized in Table I. Except for alcohols, which can act as nucleophiles, the effects are generally small. The slower reactions of DNPA and, to a lesser extent, PNPA, appear to be more susceptible to activation by molecules such as acetone and acetonitrile than is phenyl acetate. The susceptibility of the enzyme toward activation by small molecules other than alcohols is considerably less than that of the Adler and Kistiakowsky enzyme (Barker and Jencks, 1969b).

In contrast to the hydrolysis of phenyl acetates, which levels off at a constant rate with increasing methanol concentration, hydrolysis of the smaller aliphatic acetates, methyl acetate, methoxyethyl acetate, ethyl thiolacetate, and propargyl acetate, is more sharply inhibited by methanol and the inhibition approaches completion with increasing methanol concentration (Figure 4). In the presence of 1 M methanol, for example, the hydrolysis rate of PNPA is reduced to 30% whereas that of methyl acetate is reduced to <2% of the value in the absence of methanol. Experiments at different ester concentrations indicate that little, if any, of this inhibition may be attributed to competitive inhibition by methanol. The methanolysis in the presence of 1 M methanol was found to be independent of ethyl thiolacetate concentration between 10 and 40 mM.

The relatively slow hydrolysis of benzyl acetate exhibits a leveling off of the inhibition of hydrolysis by methanol at about 50% of the original rate in the presence of 1 M methanol (Figure 5). The total rate of disappearance of acetamidoethyl thiolacetate, a thiol ester with a turnover number approximately one-fourth that of ethyl thiolacetate, shows no initial increase in the presence of methanol (Figure 6). The hydrolysis rate shows a sharp initial drop followed by a levelling off.

A detailed interpretation of these results would be facilitated by knowledge of the effect of methanol on the total rate of reaction of small aliphatic acetates. This was determined in the case of ethyl thiolacetate by measuring the total rate of thiol release with Ellman's reagent at a series of ester concentrations (Figure 7). Methanol causes an initial increase in the observed total rate of somewhat less than 2-fold,

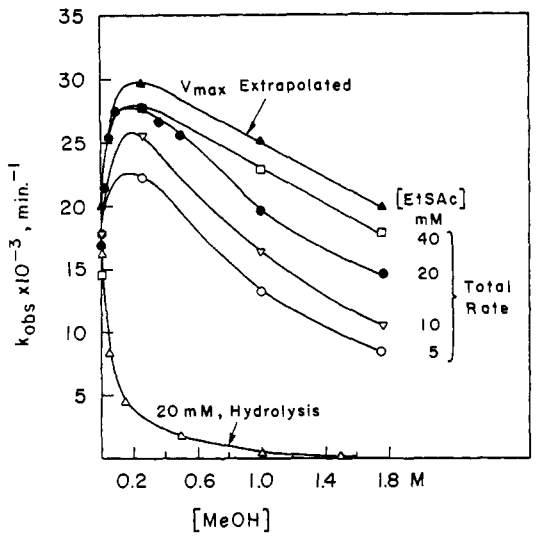


FIGURE 7: The effect of methanol on the total reaction rate (upper curves) and hydrolysis rate (lower curve) of ethyl thiolacetate at 25°, pH 7.37. Upper curve: different thiol ester concentrations measured in the presence of 0.36 mM Ellman's reagent and 0.033 M phosphate buffer, 7×10^{-10} M [enzyme]. The upper curve is extrapolated to infinite ester concentration. Lower curve measured in the titrator, 0.01 M potassium chloride, 2.5×10^{-9} M [enzyme].

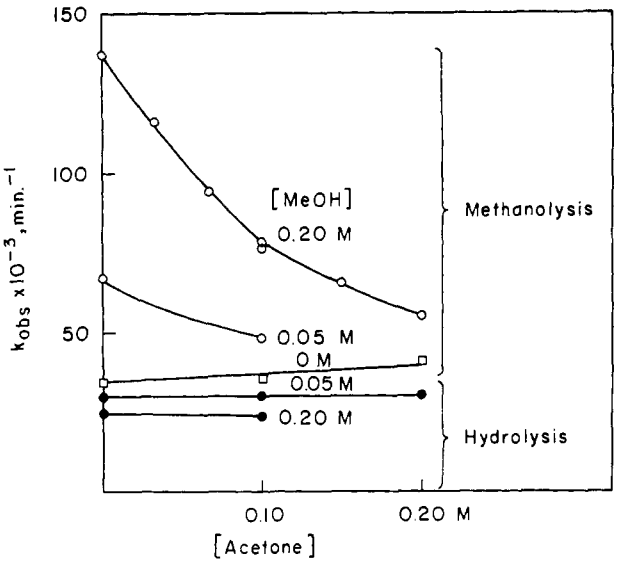


FIGURE 8: Effect of acetone on the methanolysis (○) and the hydrolysis (● □) of 3 mM phenyl acetate catalyzed by 0.8–2.2 × 10^{-9} M esterase at pH 7.37, 25°.

followed by an inhibition of the reaction that is more severe at lower ester concentrations; this is accompanied by a sharp inhibition of hydrolysis that approaches completion with increasing methanol concentration. The peculiar shape of the curves at low methanol concentrations is a consequence of inhibition of the rate by high substrate concentrations in the absence of methanol.

The esterase-catalyzed reaction of 3 mM phenyl acetate with methanol is inhibited by acetone (Figure 8). However, the same concentrations of acetone have little effect on the rate of hydrolysis either in the presence or in the absence of methanol, so that this inhibition is specific for the methanolysis reaction. A similar inhibition of methanolysis was observed with dioxane, acetonitrile, methyl acetate, and trifluoroethanol (neither of the last two compounds undergoes appreciable reaction under the conditions of these experiments). The esterase-catalyzed reaction of 1.5 mM phenyl acetate with ethanol is blocked by butanol–0.04 M 1-butanol was found to cause a 75% increase in the rate of phenyl acetate disappearance and 0.23 M ethanol causes a 330% increase, but the two alcohols together give only a 70% increase.

Inactivation by Phosphorylating Agents. Partial inactivation of liver esterase by diethyl *p*-nitrophenyl phosphate (E-600) causes a greater loss of activity for the methanolysis of phenyl acetate than for its hydrolysis (Figure 9). The activity for ethyl thiolacetate hydrolysis exhibits the same relatively high sensitivity to inactivation as phenyl acetate methanolysis. The differential inactivation results in a decrease in the amount of methanol activation (the increase in the total rate in the presence of 0.5 M methanol) from a factor of 4.9 to 1.6 for phenyl acetate; there is no significant change in the corresponding ratio of 1.6 for ethyl thiolacetate. Similar results were obtained with enzyme purified by the Adler and Kistiakowsky (1961) procedure.[2] Inactivation by bis(*p*-nitrophenyl) phosphate does not cause a large change in the relative activities toward these different reactions, but was found not to follow first-order kinetics, confirming the results obtained by Heymann and Krisch (1967) with a different enzyme preparation.

Structure and Reactivity. Kinetic parameters for a series of acetate esters and thiol esters and for ethyl butyrate are summarized in Table II. In a few cases substrate inhibition or activation was observed and approximate K_s' or K_m^H values for interaction with a second molecule of substrate are noted

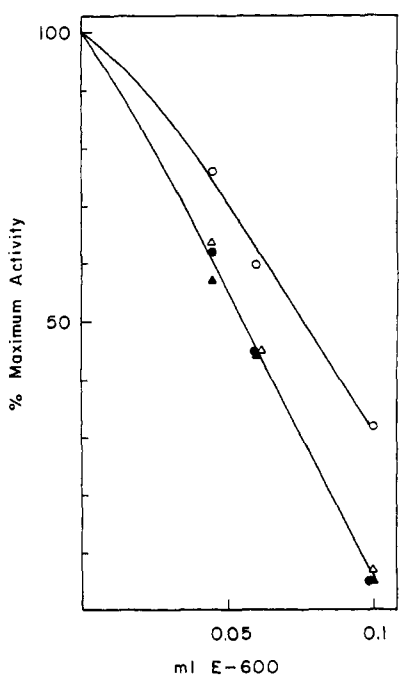


FIGURE 9: Inactivation of liver esterase (0.5 ml of 1.6×10^{-8} M) by diethyl *p*-nitrophenyl phosphate (E-600, approximately 10^{-5} M). Upper line: ○, hydrolysis of 3 mM phenyl acetate. Lower line: △, hydrolysis of 20 mM ethyl thiolacetate; ●, methanolysis of phenyl acetate in 0.5 M methanol; ▲, methanolysis of ethyl thiolacetate in 0.2 M methanol.

[2] L. Goldsmith and W. P. Jencks, unpublished experiments.

TABLE II: Kinetic Constants for Liver Esterase at 25°, pH 7.37, as Determined by Ultraviolet Spectroscopy[a] and Automatic Titration.[b]

Acetate Ester	$V_{max} \times 10^{-3}$ [c,d] (min^{-1})	K_m (mM)	[E][e] $\times 10^9$ (M)	No. of Points	Concn Range (mM)	K_s' for Substrate Inhibn[e] (mM)	Method
Phenyl	36[f,g]	0.09	0.54	12	0.08–15	20	Titration
p-Nitrophenyl	20[h,i]	0.031	0.60	26	0.01–3.2		Ultraviolet
2,4-Dinitrophenyl	7[g]	0.06	1.9	20	0.015–1.2		Ultraviolet
p-Methoxyphenyl	36	0.19	1.9	11	0.012–4.7		Ultraviolet
in 0.60 M MeOH	195	0.61	0.64	8	0.17–4.4		Ultraviolet
Phenyl thiol-	41[f]	0.04	0.74	14	0.025–2.5	4	Titration[j]
Methyl	19	2.0	2.1	11	1–20		Titration
Ethyl	17	0.6[e]	2.1	8	0.25–15		Titration
Ethyl thiol-	19[f]	0.63	2.1	7	0.2–18		Titration
	20[f]	0.77	0.71	8	0.3–40	140	Ultraviolet
In 1 M MeOH	25	3.1	0.71	5	0.57–40		Ultraviolet
n-Butyl	2.9[e,k]	0.2[e]	12	7	0.22–15		Titration
n-Butyl thiol-	13	0.20	3.3	9	0.15–4.1		Titration
Chloroethyl	16	0.15	2.5	10	0.10–25		Titration
Methoxyethyl	13	0.98	3.4	8	0.4–25		Titration
Propargyl	29[f]	0.15	1.7	17	0.1–19	90	Titration
Trifluoroethyl	23[e]	0.45[e]	1.2	8	0.25–10.0		Titration
Benzyl	5.1[g]	0.55	5.5	15	0.15–9.0		Titration
Acetamidoethyl thiol-	4.8[h,l]	13	6.1	9	1.2–95		Titration
Cyclohexylmethyl	1.5[m]	0.48	8.1	6	0.05–2.0		Titration
Ethyl butyrate	32[h,n]	0.08[e]	0.69	21	0.125–12.5		Titration

[a] In the presence of 0.033 M phosphate buffer. [b] In the presence of 0.01 M KCl. [c] Per mole of enzyme (two active sites). [d] Standardized with 1.5 mM phenyl acetate, k_{obsd} = 34,000 min^{-1}. [e] Approximate value(s). [f] Substrate inhibition. [g] Calculated from the measured K_m and rate constants determined at one substrate concentration in a number of different experiments. [h] Substrate activation. [i] $V_{max}^{H} = 25 \times 10^3$ min^{-1}, $K_m^{H} = 0.20$ mM.[e] [j] Corrected for acid liberation from C_6H_5SH (pK = 6.3). [k] Based on 2.3–15 mM (four points); the double-reciprocal plot was not linear at lower concentrations. [l] $V_{max}^{H} = 5.6 \times 10^3$ min^{-1}, K_m^{H} = 24 mM. [m] Probable substrate inhibition of 20% observed at 2 mM. [n] $V_{max}^{H} = 51 \times 10^3$ min^{-1}, $K_m^{H} = 0.62$ mM.

in these cases.[3] In addition to the esters listed in Table II, 5 mM cyclohexyl acetate was examined and found to have a turnover number of 300 min^{-1} and 10 mM γ-butyrolactone was found to have a turnover number of <100 min^{-1}.

The effects of dilute solutions of alcohols on the rate of the esterase-catalyzed disappearance of phenyl acetate are shown in Figure 10. All of the alcohols examined cause a rate increase except for ethylene glycol, which has little effect, and trifluoroethanol, which inhibits. This inhibition is noncompetitive—0.4 M trifluoroethanol was found to cause 54 ± 2% inhibition over the range 1 to 6 mM phenyl acetate.

Discussion

Liver esterase is a highly effective catalyst—the turnover number of our enzyme preparation for phenyl acetate in the presence of 1 M methanol is 180,000 min^{-1}, based on enzyme molarity, or 90,000 min^{-1}, based on the total concentration of active sites. The turnover number of 17,000 min^{-1} for ethyl acetate is some 2×10^{12}–10^{13} faster than the uncatalyzed, pH-independent rate of hydrolysis of ethyl acetate and is 2×10^{10} faster than the hydrolysis of ethyl acetate caused by hydroxide ion at pH 7. These rate constants are of the same order of magnitude as the rate constants for diffusion-controlled proton transfer near neutrality and raise the question of how the proton produced in the hydrolysis reaction is transferred to the solvent, a problem that is even more significant for the still faster reaction catalyzed by acetylcholinesterase. The rate of proton transfer from a protonated base, such as imidazolium ion, to the solvent at neutrality is approximately 10^3 sec^{-1} under optimum conditions (Eigen *et al.*, 1960), so that the proton-transfer step could become kinetically significant in these reactions. One possible mechanism for the proton removal is that the neutral carboxylic acid is the initial product of the enzyme-catalyzed hydrolysis and that the proton dissociates from the enzyme while still attached to the acid, so that equilibration of the proton with the solvent does not involve the enzyme directly (*cf.* Kaiser and Lo, 1969).

It might be expected, by analogy with other serine hydrolases, that reactions of acyl compounds catalyzed by liver esterase would proceed through an acyl-enzyme intermediate according to eq 1, which includes a rapid binding step with a dissociation constant K_s, an acylation step k_2, hydrolysis of the acyl-enzyme k_3, and reaction of the acyl-enzyme with

[3] The K_s' value is the inhibition constant for inhibition by a second mole of substrate, and K_m^{H} and V_{max}^{H} refer to the Michaelis constant and maximum velocity, respectively, for the reaction at high substrate concentrations when substrate activation is observed (Adler and Kistiakowsky, 1962).

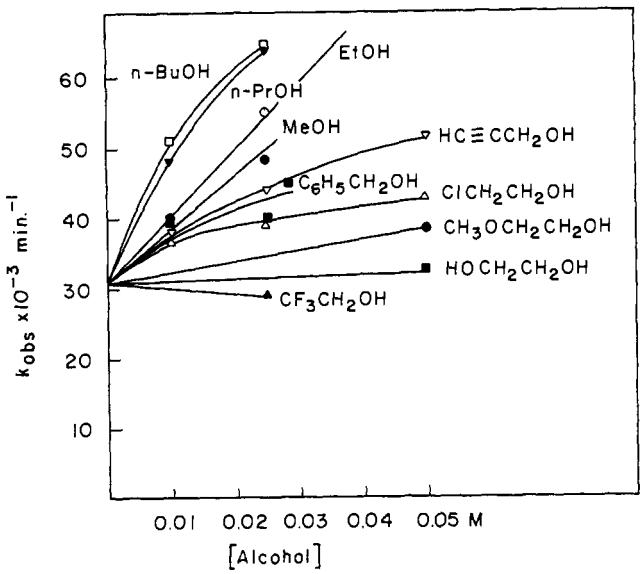


FIGURE 10: Effect of alcohols on the total reaction rate of 3 mM phenyl acetate at 25°, pH 7.37.

$$AcX + E \underset{K_s}{\rightleftharpoons} AcX\cdot E \xrightarrow{k_2} \begin{matrix} \xrightarrow{k_3} AcOH + E \\ Ac\text{-}E + X \\ \xrightarrow{k_4[N]} AcN + E \end{matrix} \qquad (1)$$

a nucleophile, such as methanol, k_4[N]. One reason for preferring an acyl-enzyme mechanism for this and related enzymes follows from the geometry of the reaction. Nucleophilic attack on an acyl compound occurs perpendicular to the plane of the sp^2 carbonyl carbon atom to give a transition state or intermediate that approaches tetrahedral geometry, followed by formation of a new planar acyl compound and expulsion of the leaving group from the side opposite to that of the original attack (Figure 11). This demands that the leaving and entering groups be on opposite sides of the plane defined by the acyl compound, so that in a single displacement mechanism in which the substrate lies upon the surface of the enzyme, attack by a nucleophile from the exterior will be hindered by interference by the mass of the enzyme with the displacement of the leaving group into the interior. This problem is avoided in a "double-displacement" mechanism (Koshland, 1954) in which a group in the enzyme acts successively as a nucleophile and leaving group and the leaving and entering groups of the substrate and product can react on the exterior side of the acyl compound. This mechanism is less likely for an acyl-transferring enzyme in which specific sites for the attacking and leaving groups are required; for such enzymes the reaction can occur by a "single-displacement" mechanism either with a compulsory order of binding of substrates in a "sandwich" in which one nucleophile binds to the enzyme below the plane of the acyl compound, or with binding of the acyl group perpendicular to the surface of the enzyme so that entering and leaving groups may react from the two sides. The acyl-enzyme mechanism, by utilizing a nucleophile that is covalently bound to the enzyme, also has the advantage of a minimal negative entropy requirement for the binding and optimal positioning of one nucleophile.

Previous work has not established that esterase-catalyzed

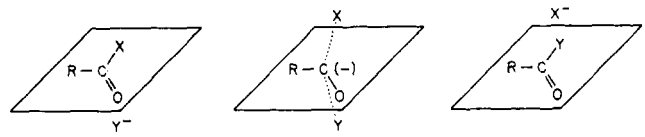


FIGURE 11: Geometry of an acyl-transfer reaction.

reactions of ordinary substrates proceed through an acyl-enzyme, although the formation of inactive phosphoryl- and dimethylcarbamoyl-enzymes has been shown (Horgan *et al.*, 1969a; Krisch, 1966; Heymann and Krisch, 1967) and Stoops *et al.* (1969) have noted that the similar turnover numbers for ethyl and phenyl butyrates are consistent with an acyl-enzyme intermediate. However, a recent, independent series of experiments carried out by Goldberg and Fruton (1970), utilizing a similar approach to that reported here but with amino acids as acyl acceptors, has provided strong kinetic evidence for the acyl-enzyme mechanism in reactions catalyzed by beef liver esterase.

The two limiting types of behavior according to eq 1 for esterase-catalyzed cleavage of a substrate in the presence of increasing concentrations of a nucleophile, such as methanol, are illustrated in Figure 12A,B. If the acylation step is fast, so that acyl-enzyme can be supplied as fast as it reacts further, the total rate of ester disappearance will increase linearly and the rate of hydrolysis will remain constant with increasing methanol concentration (Figure 12A). If the acylation step is slow, then the total rate of substrate disappearance cannot increase, and added methanol will cause an increase in methanolysis at the expense of hydrolysis, with a constant total rate of substrate disappearance (Figure 12B). If k_2 and k_3 are comparable in magnitude, then both k_2 and k_3 will be partly rate determining for the hydrolysis rate in the

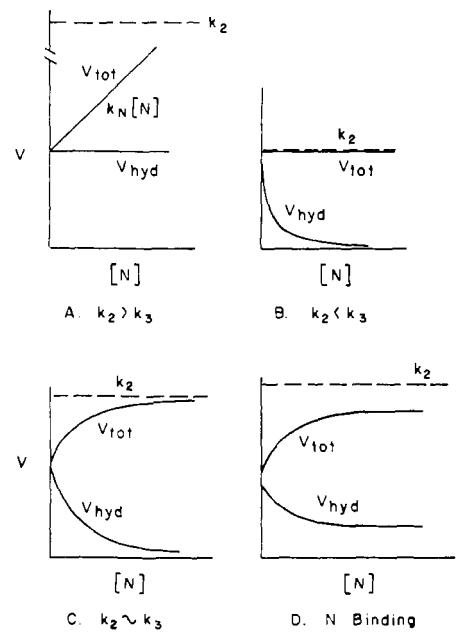


FIGURE 12: The expected effect of an added nucleophile, N, such as methanol, on esterase-catalyzed total and hydrolysis rates of an ester (A) when acylation (k_2) is fast and deacylation (k_3 and k_4[N]) is rate determining, (B) when acylation is rate determining, (C) when acylation and deacylation occur at similar rates, and (D) as in step C, but with a binding site that becomes saturated with methanol or with more than a single type of active site.

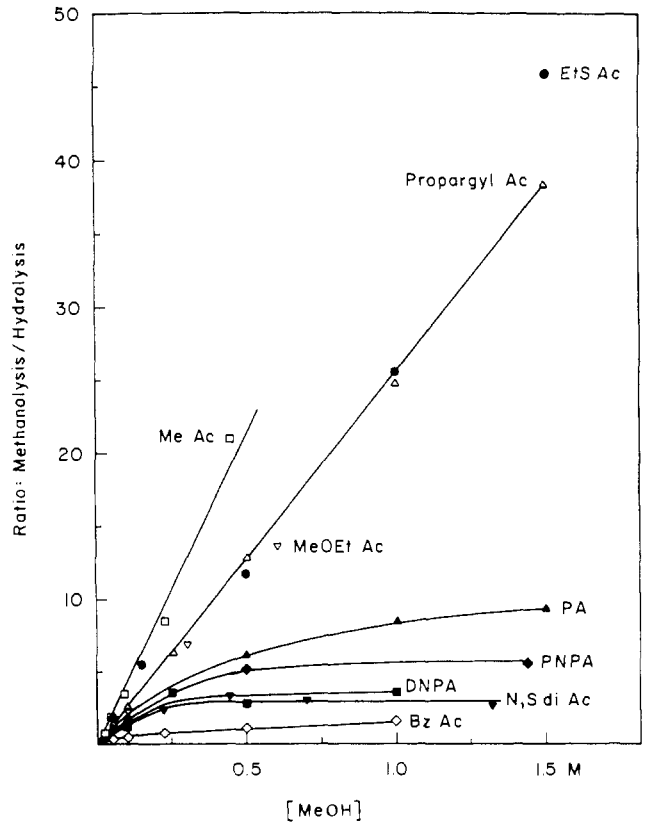


FIGURE 13: Ratios of methanolysis to hydrolysis in the reactions of a series of esters catalyzed by esterase at 25°, pH 7.37. Closed symbols: based on measured total and hydrolysis rates. Open symbols: based on measured hydrolysis rates and calculated total rates. The esters and enzyme concentrations are as follows: 25 mM methyl acetate, 2.5×10^{-9} M; 20 mM ethyl thiolacetate, $0.7–2.5 \times 10^{-9}$ M; 11 mM propargyl acetate, 1.7×10^{-9} M; 19 mM methoxyethyl acetate, 3.4×10^{-9} M; 3.0 mM phenyl acetate, $1.4–1.7 \times 10^{-9}$ M; 1.0 mM PNPA, 0.7×10^{-9} M; 0.8 mM DNPA, 2.5×10^{-9} M; 25 mM *N*-2-acetamidoethiol thiolacetate; 7.6×10^{-9} M; 9 mM benzyl acetate, 5.5×10^{-9} M.

absence of methanol and the addition of methanol will increase the total rate until it is equal to the rate of acylation k_2 and will eventually decrease the hydrolysis rate to zero (Figure 12C).

The enzyme preparation used in these experiments, purified by the method of Horgan *et al.* (1966a), is particularly suitable for an examination of the effects of methanol because of its high sensitivity toward this and other alcohols (Figure 2), so that the kinetic behavior of these nucleophiles may be measured with minimal interference from nonspecific solvent effects caused by high concentrations of alcohols. The overall rate of phenyl acetate disappearance, for example, is increased twofold by 0.05 M methanol, to give equal rates of hydrolysis and methanolysis, whereas with esterase purified by the method of Adler and Kistiakowsky (1961) the rate is increased by only 30% under the same conditions and with other enzymes, such as chymotrypsin, methanol concentrations on the order of 0.5–1.0 M are generally required in order to obtain equal rates of hydrolysis and methanolysis (Bender and Glasson, 1960; Bender *et al.*, 1964).

In several respects the esterase-catalyzed reactions of esters in the presence of methanol are in accord with the behavior expected according to the acyl-enzyme mechanism of eq 1. Phenyl acetate is the most reactive of the series of substituted phenyl acetates and its total rate of disappearance is increased up to 5.5-fold by added methanol, as would be expected if the hydrolysis step k_3 is largely rate determining in the absence of methanol and the large value of k_2 permits a considerable increase in the overall rate (Figure 3). The inhibition of hydrolysis by low concentrations of methanol is small, as expected if deacylation is largely rate determining. Since the hydrolysis of other esters is slower than that of phenyl acetate, k_3 cannot be rate determining for these esters and the acylation step k_2 must be at least partially rate determining. The smaller rate of acylation requires that the overall rate can be increased less and that hydrolysis will be inhibited more as methanol is added to react with the acyl-enzyme. These expectations are borne out in the observed progressively smaller rate increases and enhanced inhibition of hydrolysis with PNPA and DNPA compared to phenyl acetate in the presence of methanol (Figure 3).

In the aliphatic ester series the rate of hydrolysis of a series of substrates is inhibited at low methanol concentrations and approaches zero with increasing methanol concentration, as expected if the acylation step becomes rate determining and the acyl-enzyme intermediate is trapped by reaction with methanol (Figure 4). In the case of ethyl thiolacetate the overall rate, determined by the rate of thiol release, increases by somewhat less than twofold in the presence of methanol, as expected if k_2 and k_3 are both partially rate determining for hydrolysis in the absence of methanol and k_2 becomes rate determining in the presence of methanol (Figure 12C); the hydrolysis is inhibited by low concentrations of methanol as in the case of the other simple aliphatic esters (Figure 7). For the much slower reaction of acetamidoethyl thiolacetate the acylation step must be almost entirely rate determining and, as expected, there is no increase in overall rate upon the addition of methanol (Figure 6).

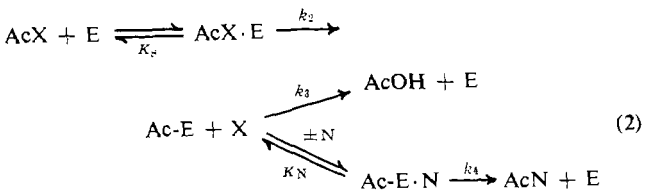
These results provide strong evidence in favor of a two-step reaction mechanism, presumably the acyl-enzyme mechanism, in which one step (k_2) is dependent on the nature of the leaving group of the ester and occurs at a rate that is independent of the concentration of nucleophile and the other step involves a partitioning of the acyl group between water (k_3) and an added nucleophile (k_4). However, several pieces of evidence are not consistent with a single, common acyl-enzyme intermediate according to eq 1. First, the rates of hydrolysis of substituted phenyl acetates, benzyl acetate, and acetamidoethyl thiolacetate do not approach zero when the methanol concentration is increased sufficiently so that k_2 has presumably become rate determining, as required by the mechanism of eq 1 (Figure 12C), but instead level off at a constant value (Figure 12D). Second, the ratio of the rates of methanolysis and hydrolysis must be the same for the reaction of a common acyl-enzyme intermediate with a given concentration of methanol regardless of the nature of the leaving group from which the acyl-enzyme was formed (*cf.* Epand and Wilson, 1963), but these ratios vary widely for the different esters examined in this work, as shown in Figure 13. The solid points in this figure represent directly measured ratios with esters for which both the total and hydrolysis rates were determined, while the open circles are based on the observed inhibition of hydrolysis and total rates estimated from the rate constants obtained with other esters.

The levelling off of the hydrolysis rates and methanolysis/hydrolysis ratios in the presence of methanol may be explained by postulating a methanol binding site (eq 2) which becomes saturated at high concentrations of methanol so that the rate of methanolysis and the methanolysis/hydrolysis ratio

TABLE III: Equilibrium Constants for Acetyl-Enzyme Formation from Esters, AcOR, at 25°.

R	V_{max}/K_m = $(k_2/K_s) \times 10^{-6}$ M^{-1} min^{-1}	$k_{ROH} \times 10^{-5}$ M^{-1} min^{-1}	K_1[a]	K_{form}[b] (M^{-1})	ΔF_{hyd}[c] (k_{cal})
CH_3CH_2	28	9.0	31	1.9	0.38
$CH_3OCH_2CH_2$	13	1.52	87	2.3	0.49
$ClCH_2CH_2$	107	5.0	210	1.8	0.33
				Av 2.0	0.40

[a] $K_1 = (k_2/K_s)/k_{ROH}$ for acetyl-enzyme formation from the ester. [b] K_{form} for acetyl-enyzme formation from acetic acid. [c] For hydrolysis of the acetyl-enzyme, based on activity of pure water = 1.0.

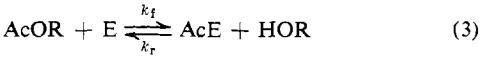


(2)

become independent of methanol concentration; such a site could also explain the specific inhibition of methanolysis by acetone and dioxane (Greenzaid and Jencks, 1970). Indeed, a site for methanol and other alcohols must almost certainly exist, in view of the 1000-fold greater molar reactivity of methanol compared to water and the increase in the nucleophilic reactivity of alcohols with increasing alkyl chain length. The solid lines for the phenyl acetate data in Figure 2 were calculated from kinetic constants and steady state rate equations derived from eq 2 and provide a satisfactory fit to the observed data, making allowance for competitive inhibition of phenyl acetate binding at high methanol concentrations and a small deviation at zero methanol concentration caused by substrate inhibition. However, another explanation must be sought for the different methanolysis/hydrolysis ratios that are observed with different esters.

The differential inactivation by diethyl *p*-nitrophenyl phosphate of the methanolysis and hydrolysis reactions of phenyl acetate and of the reactions of phenyl acetate and ethyl thiolacetate (Figure 9) shows that the enzyme contains at least two different types of active sites; similar results have been obtained with enzyme purified according to the method of Adler and Kistiakowsky (1961).[2] One type of site catalyzes the hydrolysis of substituted phenyl acetates and probably of the relatively large substrates benzyl acetate and acetamidoethyl thiolacetate; this site is relatively resistant to phosphorylation. Another type of site catalyzes the hydrolysis and methanolysis of the smaller alkyl acetates and also has some activity toward the larger acetates; the reaction of methanol with the acetyl-enzyme formed from phenyl acetates, which is the microscopic reverse of the first step in the hydrolysis of methyl acetate, also occurs at this site. This site is more sensitive to inhibition by acetone and to inactivation by diethyl *p*-nitrophenyl phosphate. There is a considerable body of physical and immunological evidence for the homogeneity of this and other esterase preparations (Augusteyn *et al.*, 1969; Horgan *et al.*, 1969a,b; Barker and Jencks, 1969a; Pahlich *et al.*, 1969, and references therein), but it is known that different preparative methods give esterases from hog liver with significantly different kinetic properties (Barker and Jencks, 1969a,b) and Heymann and Krisch (1967) have shown that the inactivation of their preparation by bis(*p*-nitrophenyl) phosphate is not first order with respect to enzyme, an observation we have confirmed with our enzyme preparations. Furthermore, partial inactivation of apparently homogeneous enzyme prepared by the Adler and Kistiakowsky procedure by acid, base, guanidine hydrochloride, thiocyanate, or sarcosyl gave a preparation with approximately 20% of the hydrolysis and 70% of the original methanolysis activity toward phenyl acetate and an increase in the activation by methanol of the total rate of phenyl acetate disappearance from 2- to 5-fold, similar to that of the enzyme used in the experiments reported here.[2] It is not known at this time whether the different types of sites exist in the same or different enzyme molecules, nor whether they represent sites which differ with respect to intrinsic structure or the presence of bound modifier molecules. It must be concluded that the existence of an acyl-enzyme intermediate has been demonstrated by the experiments reported here only for the site at which methanol acts as an effective nucleophile.

Equilibria. According to the principle of microscopic reversibility, the reaction of an alkyl ester with enzyme to form acyl-enzyme and the reaction of the corresponding alcohol with the acyl-enzyme to form the ester must pass through the same transition state. The equilibrium constant for this reaction (k_1, eq 3 and 4) may be estimated from the second-order rate constants for the reaction in the forward and reverse directions, k_f and k_r (*cf.* Epand and Wilson, 1964). The second-order rate constant for the formation of



(3)

$$K_1 = \frac{k_f}{k_r} = \frac{k_2/K_s}{k_{ROH}} = \frac{V_{max}/K_m}{k_{ROH}} \quad (4)$$

acetyl-enzyme from an ester in dilute solution is k_2/K_s which is equal to V_{max}/K_m (Bender and Kézdy, 1965). The second-order rate constants, k_{ROH}, for the reaction of alcohol with the acetyl-enzyme may be estimated from the initial slopes of plots of turnover number against alcohol concentration under conditions in which the deacylation step in the reaction with phenyl acetate is almost completely rate determining (Figure 10). The equilibrium constants so obtained (Table III) indicate that acetyl-enzyme formation from ethyl acetate is favored by a factor of approximately 30; from more "energy-rich" esters the factor is correspondingly larger. The equilibrium constant, K_{form}, for the formation of acetyl-

enzyme from acetic acid (eq 5), based on the convention that the activity of pure water is 1.0, was calculated from the known equilibrium constants for the formation of ethyl acetate, methoxyethyl acetate, and chloroethyl acetate (eq 6, Jencks and Gilchrist, 1964). The resulting value of K_{form} =

$$AcOH + E \overset{K_{form}}{\rightleftharpoons} AcE \quad (5)$$

$$AcOH + HOR \rightleftharpoons AcOR \quad (6)$$

2.0 ± 0.3 indicates that acetyl-enzyme should be formed spontaneously from nonionized acetic acid; the free energy of hydrolysis of the acetyl-enzyme is +0.4 kcal/mole. The fact that the same equilibrium constant was obtained from the rate constants for the reactions in the two directions of three different esters and alcohols at low concentrations indicates that the treatment is internally consistent and is also consistent with the action of a single active site in these reactions—if different active sites with different rate or binding constants were involved in the reactions with different esters and alcohols, such agreement would not be expected.

The apparent free energy of hydrolysis for the acetyl-enzyme of +0.4 kcal/mole may be compared to that for the acetate ester of a protected serine residue of approximately −3.5 kcal/mole (Inward and Jencks, 1965);[4] the difference of some 3.9 kcal must be ascribed to noncovalent interaction of the acetyl group with neighboring groups in the active site. This is already a large free energy for the binding of an acetyl group and the fact that much larger values would be required to explain the equilibrium if the acyl-enzyme were a high-energy acylimidazole or acid anhydride serves to rule out these structures for the acetyl-enzyme intermediate.

Structure–Reactivity Relationships. A number of scattered observations in the literature indicate that catalysis of ester hydrolysis by various esterases exhibits only a small dependence on the chemical reactivity or the thermodynamic stability of the substrate. Striking examples of this are evident in the similar reactivities of ethyl and phenyl butyrate (Stoops *et al.*, 1969) and of oxygen and thiol esters (Malhotra and Philip, 1966); the latter report also describes a correlation of the maximal rates of hydrolysis of a series of substituted phenyl acetates catalyzed by a goat intestinal esterase with Hammett σ values to give a ρ of −0.16. This behavior is in marked contrast to the hydrolysis of esters by chymotrypsin, which catalyzes the hydrolysis of *p*-nitrophenyl esters many orders of magnitude more rapidly than ethyl esters, and suggests that there is at least a quantitative difference in the mechanisms of catalysis by these two classes of enzyme.

The original objective of this work was to examine the reactivity of a series of oxygen nucleophiles in esterase-catalyzed reactions, for comparison to structure–reactivity data obtained previously for the reactivity of these nucleophiles toward furoylchymotrypsin (Inward and Jencks, 1965). The effects of variations in the structure of the alcohol on the reaction rate may be discussed in terms of either the free or the bound substrate and for the reaction in either direction, starting with a series of esters which undergo nucleophilic attack by a group on the enzyme (presumably the serine hydroxyl group), or with a series of alcohols which attack the acyl-enzyme; *i.e.*, the properties of the transition state for the catalyzed reaction may be evaluated relative to those of both the starting materials and the products of the hydrolysis reaction. The rate constants for hydrolysis of substituted phenyl esters cannot be compared to those for reactions of alkyl esters, in view of the evidence that different active sites are involved in these reactions, but it is probable that the hydrolysis of simple alkyl esters and the alcoholysis of the acetyl-enzyme formed from phenyl acetate are catalyzed by the same active site, as indicated in the previous section.

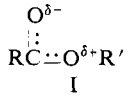
The polar substituent effects on the reactivity of alcohols under nonsaturating conditions provide a measure of the change in the charge on the hydroxyl oxygen atom in going from the free alcohol in solution to the transition state. The observed order of nucleophilic reactivity is 1-butanol > 1-propanol > ethanol > methanol > propargyl alcohol > chloroethanol > methoxyethanol > ethylene glycol > trifluoroethanol (Figure 10). This order suggests that the polar effect is superimposed on an effect which may be attributed to interaction of the alcohol with a hydrophobic site on the enzyme, as has been observed for chymotrypsin (McDonald and Balls, 1956; Inward and Jencks, 1965); there is an increased reactivity with increasing length of the hydrocarbon chain and a decreased reactivity with hydrophilic substituents, such as the hydroxyl group. Although these data do not permit an exact quantitative interpretation, it is clear that the reactivity of the alcohols *decreases* with electron-withdrawing substituents and increased acidity. This is the opposite of what is observed for the base-catalyzed nonenzymatic reactions of alcohols near neutrality and is also different from the behavior of chymotrypsin. Ethanol and trifluoroethanol exhibit similar reactivities toward furoylchymotrypsin, for example, whereas in the esterase-catalyzed reactions the 4000-fold greater acidity of trifluoroethanol is accompanied by a decrease in reactivity of 100-fold or more compared to ethanol. This result provides evidence that a partial positive charge is developed on the hydroxyl oxygen atom when a free alcohol reaches the transition state in the esterase-catalyzed reaction.

On the other hand, there is no significant effect of polar substituents in the alcohol on the reactivity of esterase toward bound aliphatic esters (Table II)—the V_{max} values for ethyl and trifluoroethyl acetates, for example, do not differ significantly. According to the mechanism of eq 1, this means that the k_2 values for formation of the acyl-enzyme do not change significantly with changing polar substituents. The K_m values, although more scattered than the V_{max} values, also show no trend with changing polar substituents (Table II). This means that there is no indication of an effect of polar substituents on the binding of esters to the enzyme; any such effect on K_s should also appear in K_m in view of the similar rate constants for the different esters. There is also no significant effect of polar substituents on the rate constants for reaction of the free esters from dilute solution, $V_{max}/K_m = k_2/K_s$. It may be concluded from these results that there is essentially no change in the charge on the alcohol oxygen atom of the ester in going from either the free or the bound ester to the transition state of the esterase-catalyzed reaction.

The relationship between these effects of substituents on the rate of the reaction in the forward and reverse directions (eq 3) is given by the effect on the equilibrium constant, $K_{eq} = k_f/k_r$. Acetyl group transfer from a series of esters to a common acceptor is thermodynamically favored by electron-withdrawing substituents, with a β value of 0.70

[4] The free energies of hydrolysis of the furoyl-enzyme and a furoyl-serine ester at pH 7.0 reported by Inward and Jencks (1965) are incorrect because of an error in the calculation of the free energy of ionization of furoic acid; the correct values are −6070 and −8820 cal per mole, respectively.

relating log K and the pK of the alcohol (Gerstein and Jencks, 1964). The reason for this effect is that the inductive and resonance effects of the strongly electron-attracting carbonyl group induce the development of a partial positive charge in the alcoholic oxygen atom of the ester that is destabilized by electron-withdrawing substituents (I). This effect on the

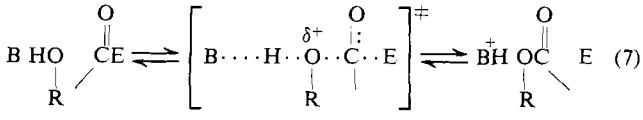


I

equilibrium constant explains the observation that the esterase-catalyzed reactions of aliphatic alcohols are inhibited by electron-withdrawing substituents whereas the reactions of the corresponding esters are insensitive to polar substituents. Assuming that the equilibrium constants are the same for the free and bound esters, very approximate β values of 0 and 0.7 may be assigned to the rate constants for reactions of the ester and alcohol, respectively.

These substituent effects must be accommodated by any proposed mechanism for the catalytic activity of esterase. The most probable chemical explanation is that the observed effects reflect a partial or complete cancellation of substituent effects on proton transfer to or from the alcoholic oxygen atom and on the cleavage or formation of the bond between this atom and the carbonyl carbon atom.

Starting with the alcohol and not taking a position as to whether formation or breakdown of a tetrahedral intermediate is rate determining, the β value of approximately 0.7 suggests that there is a relatively small amount of proton abstraction in the transition state, so that positive charge development from the developing oxygen–carbon bond more than compensates for the increase of electron density caused by the removal of a proton by a catalyzing base (eq 7).



(7)

Starting with the ester, the β value near zero suggests that the donation of a proton from a catalyzing acid must be evenly balanced with the amount of carbon–oxygen bond cleavage, so that there is no net change in the charge on the alcohol oxygen atom. An alternative possibility is that the transition state consists of an essentially unperturbed ester and that the substituent effect on the reactivity of alcohols simply reflects the effect on the equilibrium constant for ester formation. This would be the case, for example, if the rate-determining step for the formation and breakdown of the acyl-enzyme were a conformational change of the enzyme·ester complex.

In contrast to the esterase-catalyzed reactions, the absence of an effect of polar substituents on the reactivity of alcohols in chymotrypsin-catalyzed reactions means that there is essentially no change in the charge on the oxygen atom of the attacking *alcohol* upon reaching the transition state in these reactions. This has been interpreted in terms of compensating substituent effects on proton removal and on oxygen–carbon-bond formation, *i.e.*, assistance to the reaction by general base catalysis; similar results and conclusions hold for the reactions of aliphatic amines (Inward and Jencks, 1965). The increased reactivity of anilides containing electron-donating substituents supports the complementary conclusion for the reverse reaction—*i.e.*, that proton donation to the leaving amine is important (Inagami *et al.*, 1969; Parker and Wang, 1968)—but the magnitude of the effect is much larger than predicted from the results with the aliphatic amines and suggests either that the transition states for the reactions involving aliphatic and aromatic amines are significantly different or that there is a substituent effect on the binding of substrate to the enzyme; it is of interest that the correlation disappears if it is based upon the reaction rates of unbound anilides (Inagami *et al.*, 1969).

There are a few further conclusions that may be drawn from the effects of substituents on substrates for esterase-catalyzed reactions. The values of $V_{max}/K_m = k_2/K_s$ for the reactions of thiols, trifluoroethanol, and phenols with the acetyl-enzyme may be estimated from the observed rates of hydrolysis of the acetate esters of these compounds and the equilibrium constants for eq 5, obtained from the known free energies of hydrolysis of the esters (eq 5 and 6, Jencks and Gilchrist, 1964; Gerstein and Jencks, 1964). Such calculations indicate that the rate constants for the reactions of these nucleophiles are small or negligible compared to rates of hydrolysis, as a consequence of the energy-rich nature of the esters that would be formed as the products of the reactions. In the thiolester reactions the thermodynamic instability of the esters may provide a rate-enhancing effect sufficient to overcome the unfavorable effect of the low basicity of sulfur, which would be expected to diminish the effectiveness of catalysis by proton donation to the leaving sulfur atom (eq 7).

Increased alkyl chain length or the addition of polarizable substituents increases the strength of interaction of esters with the enzyme, as manifested in the decreasing K_m values in the series methyl, ethyl, butyl, chloroethyl, and propargyl acetate; the same phenomenon has been noted above for the corresponding alcohols. The cyclohexane ring provides a weaker and less favorable interaction with the active site compared to the benzene ring of phenyl esters—the low reaction rates of cyclohexyl and cyclohexylmethyl acetates, and also of butyl and benzyl acetates and acetamidoethyl thiolacetate, suggest that the relatively large leaving groups of these esters bring about a structurally unfavorable interaction of the ester with the active site that results in a less effective catalysis of their hydrolysis. The very low reactivity of γ-butyrolactone makes it unlikely that the enzyme binds and cleaves the cis form of esters (Bruice, 1961).

Acknowledgments

We are grateful to David Barker for his help in purification of the enzyme and to David Jencks for assistance in the inactivation experiments.

References

Adler, A. J., and Kistiakowsky, G. B. (1961), *J. Biol. Chem. 236*, 3240.

Adler, A. J., and Kistiakowsky, G. B. (1962), *J. Amer. Chem. Soc. 84*, 695.

Augusteyn, R. C., de Jersey, J., Webb, E. C., and Zerner, B. (1969), *Biochim. Biophys. Acta 171*, 128.

Barker, D. L., and Jencks, W. P. (1969a), *Biochemistry 8*, 3879.

Barker, D. L., and Jencks, W. P. (1969b), *Biochemistry 8*, 3890.

Bender, M. L., Clement, G. E., Gunter, C. R., and Kézdy, F. J. (1964), *J. Amer. Chem. Soc. 86*, 3697.

Bender, M. L., and Glasson, W. A. (1960), *J. Amer. Chem. Soc. 82*, 3336.
Bender, M. L., and Kézdy, F. J. (1965), *Annu. Rev. Biochem. 34*, 49.
Benöhr, H. C., and Krisch, K. (1967), *Hoppe-Seyler's Z. Physiol. Chem. 348*, 1102.
Bergmann, F., Rimon, S., and Segal, R. (1958), *Biochem. J. 68*, 493.
Bruice, T. C. (1961), *J. Polymer Sci. 49*, 100.
Bruice, T. C., Fife, T. H., Bruno, J. J., and Brandon, N. E. (1962), *Biochemistry 1*, 7.
Eigen, M., Hammes, G. G., and Kustin, K. (1960), *J. Amer. Chem. Soc. 82*, 3482.
Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82*, 70.
Epand, R. M., and Wilson, I. B. (1963), *J. Biol. Chem. 238*, 1718.
Epand, R. M., and Wilson, I. B. (1964), *J. Biol. Chem. 239*, 4145.
Franz, W., and Krisch, K. (1968), *Hoppe-Seyler's Z. Physiol. Chem. 349*, 1413.
Gerstein, J., and Jencks, W. P. (1964), *J. Amer. Chem. Soc. 86*, 4655.
Goldberg, M. I., and Fruton, J. S. (1969), *Biochemistry 8*, 86.
Goldberg, M. I., and Fruton, J. S. (1970), *Biochemistry 9*, 3371.
Greenzaid, P., and Jencks, W. P. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 407.
Heymann, E., and Krisch, K. (1967), *Hoppe-Seyler's Z. Physiol. Chem. 348*, 609.
Holmes, R. S., and Masters, C. J. (1967), *Biochim. Biophys. Acta 132*, 379.
Horgan, D. J., Dunstone, J. R., Stoops, J. K., Webb, E. C., and Zerner, B. (1969a), *Biochemistry 8*, 2006.
Horgan, D. J., Stoops, J. K., Webb, E. C., and Zerner, B. (1969b), *Biochemistry 8*, 2000.
Horgan, D. J., Webb, E. C., and Zerner, B. (1966a), *Biochem. Biophys. Res. Commun. 23*, 18.
Horgan, D. J., Webb, E. C., and Zerner, B. (1966b), *Biochem. Biophys. Res. Commun. 23*, 23.
Inagami, T., Patchornik, A., and York, S. S. (1969), *J. Biochem. (Tokyo) 65*, 809.
Inward, P. W., and Jencks, W. P. (1965), *J. Biol. Chem. 240*, 1986.
Jencks, W. P., and Gilchrist, M. (1964), *J. Amer. Chem. Soc. 86*, 4651.
Jencks, W. P., and Gilchrist, M. (1968), *J. Amer. Chem. Soc. 90*, 2622.
Kaiser, E. T., and Lo, K.-W. (1969), *J. Amer. Chem. Soc. 91*, 4912.
Kézdy, F., and Bender, M. L. (1962), *Biochemistry 1*, 1097.
Koshland, D. E., Jr. (1954), *in* Mechanisms of Enzyme Action, McElroy, W. D., and Glass, B., Ed., Baltimore, Md., Johns Hopkins, p 608.
Krisch, K. (1966), *Biochim. Biophys. Acta 122*, 265.
Lipmann, F., and Tuttle, L. C. (1950), *Biochim. Biophys. Acta 4*, 301.
Malhotra, O. P., and Philip, G. (1966), *Biochem. Z. 346*, 386.
McDonald, C. E., and Balls, A. K. (1956), *J. Biol. Chem. 221*, 993.
O'Neill, J. J., Kohl, H., and Epstein, J. (1961), *Biochem. Pharmacol. 8*, 399.
Pahlich, E., Krisch, K., and Borner, K. (1969), *Hoppe-Seyler's Z. Physiol. Chem. 350*, 173.
Parker, L., and Wang, J. H. (1968), *J. Biol. Chem. 243*, 3729.
Stoops, J. K., Horgan, D. J., Runnegar, M. T. C., de Jersey, J., Webb, E. C., and Zerner, B. (1969), *Biochemistry 8*, 2026.

Peptidoglutaminase. Enzymes for Selective Deamidation of γ-Amide of Peptide-Bound Glutamine*

Mamoru Kikuchi,† Hiroshi Hayashida, Eiichi Nakano, and Kenji Sakaguchi

ABSTRACT: Two different glutaminases have been separated and partially purified from a soil isolate microorganism identified as a strain of *Bacillus circulans*. These enzymes have been named peptidoglutaminases because of their specificity for catalyzing the hydrolysis of the γ-amide group of peptide-bound glutamine. Peptidoglutaminase I was specific for derivatives substituted at the α-amino group of L-glutamine such as carbobenzoxy-L-glutamine, glycyl-L-glutamine, L-leucylglycyl-L-glutamine, and *N*-acetyl-L-glutamine but was inactive toward L-glutamine derivatives substituted at the carboxyl position or at both α-amino and carboxyl positions Peptidoglutaminase II was specific for hydrolysis of the γ amide group of L-glutamine residues substituted at the car boxyl position or both the α-amino and carboxyl groups (*e.g.*, L-glutaminylglycine or L-phenylalanyl-L-glutaminyl-glycine).

Both enzymes catalyzed the deamidation of free L-glutamine poorly and had no activity toward asparagine, asparagine derivatives, or other amino acid amides. No peptidase activity was associated with either peptidoglutaminase.

Glutaminases (L-glutamine amidohydrolase, EC 3.5.1.2) are widely distributed in animals, plants, yeasts, and bacteria (Hartman, 1968; Meister, 1955; Meister *et al.*, 1955; Roberts, 1960). The specificity of previously reported glutaminases was restricted to free glutamine or some derivatives such as methyl-DL-glutamine. Our aim was to find an enzyme which could

* From the Noda Institute for Scientific Research, Noda-Shi, Chiba-Ken, Japan. *Received September 2, 1970.*

† To whom to address correspondence at: College of General Education, University of Tokyo, Tokyo, Japan. *Received September 2, 1970.* This work was supported in part by the Central Research Laboratories of Kikkoman Shoyu Co., Ltd.