Papain-Catalyzed Reactions of Esters with Alcohols. The Nature of the Rate-Determining Step*

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ABSTRACT: The simultaneous rates of the papain-catalyzed hydrolysis and ethanolysis of ethyl hippurate were determined in water and in ethanol-^{14}C-water. The kinetics of this system closely follows predictions made from a steady-state treatment of a mechanistic model which includes as its key feature a covalent acyl-enzyme intermediate (eq 3-5). While the rate of hydrolysis of this compound is only slightly reduced in 1.72 M (*ca.* 10%, v/v) ethanol, the total rate of acyl transfer is increased by 40%, an observation supporting previously drawn conclusions that the rate of deacylation of the acyl-enzyme is a factor in the rate-determining step for this reaction. The values of K_m for both the hydrolysis and transesterification reactions are increased by more than the 40% predicted by the simple kinetic model implying the existence of a pronounced solvent effect on the enzyme–substrate binding constant. The ratio of the second-order rate constants for nucleophilic attack on the acyl-enzyme, $k_{ethanol}/k_{water}$, is equal to 15.5 ± 0.5 at saturating concentrations of ester. Similarly, the rate of release of *p*-nitrophenol from *p*-nitrophenyl hippurate is increased approximately twofold by the addition of ethanol to 10% (v/v) and by nearly a factor of 3 in the same concentration of methanol. The apparent values of K_m for this substrate are also increased, a fact which tends to mask the increase in V_{max} at subsaturating levels of substrate. The increase in the rate of *p*-nitrophenol production is accompanied by the formation of ethyl hippurate in approximate proportion to the amount of ethanol present in solution. Quantitative calculations for this substrate are complicated by the fact that organic solvents increase the rate of hydrolysis of *p*-nitrophenyl hippurate. Direct measurements of the rate of formation of hippuric acid and of that of *p*-nitrophenol under near-identical conditions gave $k_{ethanol}/k_{water}$ = 12 ± 2, a value which compares reasonably well with that measured for ethyl hippurate under the same conditions. This identity is consistent with, but does not require, a common acyl-enzyme. A number of observations emanating from the present work as well as from other studies in the literature cannot be entirely accommodated by the simple kinetic scheme usually written for proteases. They do, however, fit satisfactorily into a slightly expanded model which has an additional kinetically important rate constant, probably representing the rate of alcohol departure from the enzyme surface (eq 6–10).

Recent studies from several laboratories have lent support to the original suggestion of Smith *et al.* (1955) that the papain-catalyzed hydrolysis reactions of acyl derivatives proceed through the intermediate formation of an acyl-enzyme formed from the acyl moiety of the substrate and a reactive sulfhydryl group on the enzyme. It has been possible to spectrophotometrically detect the intermediate acyl-enzyme formed by the substrate methyl thionohippurate (Lowe and Williams, 1965a), and to isolate the cinnamoyl-enzyme made by reacting the enzyme with cinnamoyl-imidazole (Brubacher and Bender, 1966). It has also been shown that the maximal velocities observed for series of esters sharing common acyl groups are essentially independent of the alcohol fractions indicating a common rate-determining step in the over-all reaction which is most likely the hydrolysis of an acyl-enzyme (Lowe and Williams, 1965b; Kirsch and Igelström, 1966; Bender and Brubacher, 1966). These experiments are also consistent with a mechanism in which the rate-determining step is either a slow conformational change (Kirsch and Katchalski, 1965) or the release of the hydrolyzed acyl moiety from the enzyme.

The over-all pathway for the papain-catalyzed hydrolysis of esters is customarily analyzed in terms of the scheme suggested by Gutfreund and Sturtevant (1956) for the chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate

$$E + S \underset{}{\overset{K_S}{\rightleftharpoons}} \underset{\displaystyle P_1}{\underset{+}{ES_1}} \xrightarrow{k_2} ES_2 \xrightarrow{k_3} E + P_2 \qquad (1)$$

where ES_1 is the noncovalent enzyme–substrate complex, ES_2 an acyl-enzyme, P_1 the alcohol moiety of the ester, and P_2 the carboxylic acid. If the slow step in ester hydrolysis is indeed measured by the rate of nucleophilic attack of water upon the acyl-enzyme, then this rate should be increased by replacing part of the

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water with a more effective nucleophile, but this expectation should not be realized for a rate-determining step in the over-all reaction involving either a conformational change in the enzyme or release of the final product. The experiments of Glazer (1966) which demonstrated that papain catalyzes transesterification reactions suggested that methanol and ethanol might be used for this purpose. In addition to predicting that the total rate of disappearance of substrate would be increased by the addition of alcohol, the mechanism demands that the partitioning of the acyl-enzyme, as expressed by the ratio of v(hydrolysis)/v(alcoholysis) should be independent of the leaving group of the starting ester.

In order to differentiate between these possibilities, the reactions of HEE[1] and HNP with papain were studied in the presence of fixed ratios of alcohol to water.

Experimental Section

Materials. HEE was prepared according to the method of Kirsch and Katchalski (1965), mp 59.5–60.5°, lit. (Curtius, 1902) mp 60.5°. Synthesis of HNP was carried out in a similar manner using a slight excess of acid over *p*-nitrophenol in pyridine solvent. Dropwise addition of thionyl chloride at 0° yielded the ester, mp 169–170°, after recrystallization from ethanol (lit. (McDonald and Balls, 1957) mp 167–168 and 170–181° (Lowe and Williams, 1965b)).

Three different preparations of papain were used in these experiments: (A) Worthington Biochemical Corp., lot no. 5591, twice recrystallized; (B) Worthington Biochemical Corp., lot no. 5629, twice recrystallized and further twice recrystallized in this laboratory by sodium chloride fractionation at neutral pH according to the method of Ebata and Yasunobu (1962); and (C) enzyme crystallized from dry papaya latex (Wallerstein Co.) essentially according to the method of Kimmel and Smith (1954) using the modification of Masuda (1959). The activities of the various enzyme preparations differed when assayed with either benzyloxycarbonylglycine, *p*-nitrophenyl ester, or HEE; therefore, rate constants obtained for different batches of enzyme acting on a given substrate cannot be compared directly. All samples were checked for purity by disc electrophoresis (Reisfield *et al.*, 1962) at pH 4.5 and each was judged to be greater than 95% homogeneous. Enzyme concentrations were calculated on the basis of optical density measurements at 280 mμ using an extinction coefficient of 5.05×10^4 M^{-1} cm^{-1} and a molecular weight of 21,000.

Acetonitrile and 2,6-lutidine were distilled and water was glass distilled from dilute alkaline potassium permanganate. Rossville absolute ethanol, Merck reagent grade methanol, Nutritional Biochemical Corp. L-cysteine hydrochloride, and Eastman White Label 2-mercaptoethanol were used without further purification. Ethanol-1-^{14}C was obtained from New England Nuclear Corp.

Measurements of radioactivity were carried out with a Packard Tri-Carb liquid scintillation spectrometer, Model 3003. Samples were counted in 10 ml of a solution containing 4 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene per l. of toluene (Hall and Cocking, 1965) to *ca.* 1% counting error. All measurements were corrected to disintegrations per minute. The commercial sample of ethanol had a reported specific activity of 4.60 mc/mmole and showed a single peak on vapor phase chromatographic analysis, using an 8-ft column, 20% Carbowax 4000 on Chromosorb-P, at 90°. The material was diluted with absolute ethanol to a final specific activity of $1.72 \pm 0.02 \times 10^6$ dpm/mmole. The radiopurity of this diluted sample was determined by conversion to HEE through reaction with hippuryl chloride (Research Organic Chemicals Co.) in the presence of pyridine. The specific activity of the HEE obtained was identical with that of the ethanol-1-^{14}C used.

Enzyme Kinetics. Three methods were employed for the activation of papain:[2] (1) addition of the enzyme to the assay mixture containing ME (Kirsch and Katchalski, 1965); (2) preactivation of the enzyme under conditions of dilute enzyme and cysteine essentially according to the method of Kirsch and Igelström (1966), except that activation was carried out in the presence of 0.012 M citrate at pH 6.0; and (3) preactivation in solutions containing concentrated enzyme and cysteine at room temperature (24 ± 1°) and pH 5.8. These activation mixtures contained 0.010 M citrate buffer, 1.0×10^{-3} M EDTA, 0.040 M cysteine, and *ca.* 1×10^{-4} M papain. Maximal activity under these conditions was attained within 5 min and was constant for 5 hr. Under the conditions of the assay used, which was the hydrolysis of HEE described below, the activity was unchanged over at least 40 min and the relative concentrations of papain and cysteine used in the assay mixture had no effect[3] on this observation.

Hydrolysis experiments were carried out at pH 6.0 and 40° by automatic titration using the instrumentation described by Kirsch and Igelström (1966). Reactions were initiated by the addition of a small volume of the enzyme (*ca.* 2% of the total volume) to the thermostated vessel after complete thermal and hydrogen

[1] Abbreviations used: HEE, ethyl hippurate; HNP, *p*-nitrophenyl hippurate; ME, 2-mercaptoethanol.

[2] Some samples of papain had considerable activity (*ca.* 40% of total) in the absence of activator, a phenomenon previously reported by Sanner and Pihl (1963). The enzymes, however, were always used under conditions of full activation by either ME or cysteine. In addition, one source of papain (C) was used over a long period of time and showed a gradual loss in total activity presumably owing to autolysis. Experiments designed to measure the relative rates of hydrolysis and ethanolysis for either HNP or HEE were always carried out on the same day. Comparisons involving absolute rates, however, between HNP and HEE made on this batch of papain required making allowances for this loss which varied from 4 to 19%.

[3] The rates were insensitive to cysteine concentration within the range 4×10^{-4} to 3×10^{-3} M at papain concentrations of 5.6×10^{-7} or 1.1×10^{-6} M.

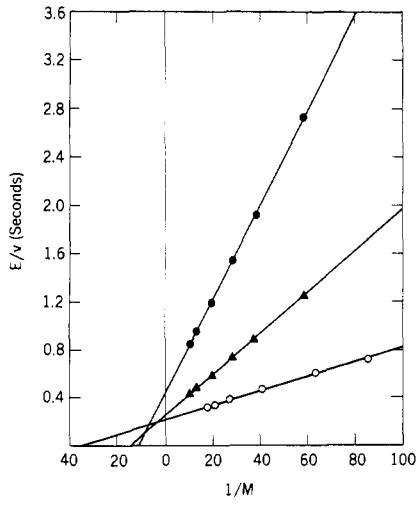

FIGURE 1: The reactions of papain with HEE at pH 6.0 and 40°. The reaction mixtures contained 0.30 M KCl, 5.0×10^{-4} M EDTA, 1.3 M CH_3CN, 1.5×10^{-3} M cysteine, and 1.3×10^{-6} M papain (batch C, activation method 3). (○) Hydrolysis in the absence of ethanol; (▲) hydrolysis in 1.72 M ethanol; (●) ethanolysis in 1.72 M ethanol.

ion equilibrium had been achieved. There was no detectable hydrolysis of HEE before introduction of the enzyme. The assay mixtures contained 0.30 M potassium chloride and 5.0×10^{-4} M EDTA in all cases. The solutions also contained acetonitrile, ethanol, cysteine, or ME, and papain as indicated. The base used as titrant was standardized 0.1 or 0.05 N potassium hydroxide. Initial rates of hydrolysis were measured from the continuous titration curve from the first 4% of hydrolysis of the substrate.

The rates of incorporation of radioactivity were measured on the same samples from which hydrolysis data had been obtained in ethanol-1-^{14}C. The reactions were quenched by rapid decantation of the assay mixture and extraction of the HEE into methylene chloride. The organic solution was then washed repeatedly with water, saturated sodium bicarbonate, and water, and dried over anhydrous magnesium sulfate. The solvent was removed under a stream of nitrogen and the residue recrystallized from a carbon–tetrachloride–low-boiling petroleum ether (bp 30–60°) mixture to constant specific activity. All samples were vacuum dried over phosphorus pentoxide before counting. Much less than 1% of the ethanol incorporation rate was observed in the absence of enzyme.

Titragraphic measurements of the rates of hydrolysis of HNP were carried out essentially as described for HEE at pH 6.0 and 40°. Because of the appreciable rate of hydrolysis in the absence of the enzyme, these reactions were initiated by the addition of the ester in an acetonitrile solution, amounting to 1.25% of the total assay volume, which brought the final concentration of acetonitrile to 1.3 M. All reaction mixtures contained 0.30 M potassium chloride and 5.0×10^{-4} M EDTA. Ethanol, when present, was 0.86, 1.72, or 2.58 M as indicated. The final concentrations of cysteine and papain (batch C activated by method 3) were 2.5×10^{-4} and 5.70×10^{-7} or 5.95×10^{-7} M, respectively. The observed rates of hydrolysis were corrected for both the nonenzymatic rates and also for the titration of the *p*-nitrophenol released at pH 6.0. The fractional contributions to the final hippuric acid concentration from the nonezymatic pathway were evaluated by graphical integration of plots of rates of hydrolysis *vs.* per cent reaction assuming the blank rates followed *pseudo*-first-order kinetics and the enzymatic reactions obeyed the Michaelis–Menten rate law. The initial velocities at time zero were measured directly, and the values of K_m were obtained from the spectrophotometric measurements. It was determined by this method that about 8.5% of the total hippuric acid originated nonenzymatically.

Spectrophotometric measurements using HNP were carried out using a Zeiss PMQ II spectrophotometer equipped with a water-jacketed cuvet block. Changes in per cent transmittance were recorded with a Varian G-14 recorder. The appearance of *p*-nitrophenol was monitored at 317 or 340 mμ and the rates were calculated as described previously (Kirsch and Igelström, 1966). Prior to initiation of the kinetic run, the stock enzyme solution, which was activated at room temperature, was mixed with the appropriate solutions which had been kept at 40°. After thermal equilibrium had been established, final, minor adjustments were made to bring the pH values to 6.0. Aliquots of these solutions were withdrawn for spectrophotometric assays. No decrease in enzyme activity occurred over the period of time in which the enzyme was maintained at 40° under these conditions. All reactions were initiated by the addition of HNP in an acetonitrile solution which was 1% of the total volume, unless otherwise indicated, to give in all cases a final concentration of 1.3 M acetonitrile. This addition of the HNP solution changed the pH by less than 0.02 unit. Enzymatic reaction velocities were corrected for the appropriate blanks. The assay solutions contained 0.010 M buffer (citrate, phosphate, or 2,6-lutidine), 5.0×10^{-4} M EDTA, potassium chloride to adjust the ionic strength to 0.3, and cysteine and papain as indicated. Organic solvents other than 1.3 M acetonitrile are given where relevant. The kinetic parameters $V_{max}/[E]$ and K_m were evaluated as described by Kirsch and Igelström (1966) using a weighted least-squares method.

Results

Reactions with Ethyl Hippurate. The rates of papain-catalyzed hydrolysis and ethanolysis of HEE at several different initial substrate concentrations are shown in Figure 1 and summarized in Table I. The addition of ethanol to the reaction medium significantly increases the value of K_m for the hydrolytic reaction. Similarly, the magnitude of K_m for the ethanolysis reaction increases with increasing concentrations of

TABLE I: The Reactions of Papain with Ethyl Hippurate.

[Ethanol-1-^{14}C] (M)	V_{max}/[E] (sec^{-1})	V_{max}/[E][H_2O] (M^{-1} sec^{-1} × 10^2)	V_{max}/[E][ROH] (M^{-1} sec^{-1} × 10^2)	K_m (mM)
0[a]	4.71 ± 0.14[b]	9.18 ± 0.27[c]		29.3 ± 1.9[b]
1.72[a]	4.19 ± 0.071[b]	9.16 ± 0.27[c]		70.9 ± 2.2[b]
1.72[a]	2.37 ± 0.06[d]		138 ± 3[d]	92.1 ± 3.9[d]
0.86[e]	0.65 ± 0.06[d]		76 ± 7[d,f]	43 ± 6[d,f]
1.72[e]	1.44 ± 0.18[d]		84 ± 10[d,g]	88 ± 15[d,g]

[a] Conditions as described in Figure 1. Values of V_{max} and K_m were determined from six concentrations of HEE unless otherwise noted. [b] Hydrolysis reactions. [c] The concentration of water is taken as 55 M × 93.3% (1.3 M acetonitrile, *ca.* 6.7%, v/v) for no ethanol and 55 M × 83.3% (1.3 M acetonitrile, *ca.* 6.7%, v/v, and 1.72 M ethanol, *ca.* 10%, v/v). [d] Ethanolysis reactions. [e] Papain, batch A, activated by method 1. The assay mixture contained 0.30 M KCl, 5.0 × 10^{-4} M EDTA, 5.0 × 10^{-3} M ME, and 6.9 or 7.2 × 10^{-7} M papain. HEE was isolated as described in the Experimental Section except that quenching was immediately preceded by the addition of carrier HEE. [f] Determined from four concentrations of HEE. [g] Determined from five concentrations of HEE.

ethanol. It should also be noted that the values of K_m for the hydrolysis and ethanolysis reactions in 1.72 M ethanol do appear to be significantly different.

The maximal velocities of hydrolysis are identical in 0.0 and 1.72 M ethanol when allowance is made for the reduced concentration of water in the latter solution. The same dependence of nucleophile concentration is also observed in the ethanolysis reaction.

A comparison of the relative rates of reaction of ethanol and water with HEE as a function of substrate concentration is shown in Table II. The changing relative rates reflect the fact noted above that the K_m values for the reaction are not identical. The $k_{ethanol}/k_{water}$ ratio is fairly insensitive to enzyme batch and method of activation used.

TABLE II: Comparison of the Papain-Catalyzed Rates of Hydrolysis and Ethanolysis of Ethyl Hippurate as a Function of Varying Substrate Concentration.[a]

Concn of HEE (mM)	Rate of Hydrolysis × 10^5 (mmoles/min ml)	Rate of Ethanolysis × 10^5 (mmoles/min ml)	$k_{ethanolysis}$[b]/$k_{hydrolysis}$
94.0	19.1	9.50	13.3
72.9	16.9	8.42	13.3
49.9	13.9	6.71	12.9
35.3	11.0	5.16	12.6
26.0	9.14	4.17	12.2
17.0	6.46	2.93	12.1
∞[c]	33.5 ± 0.6	18.9 ± 0.5	15.1 ± 0.5

[a] The reactions were carried out in 1.72 M ethanol. The conditions are as described in Figure 1. [b] (v_i(ethanolysis)/[EtOH])/(v_i(hydrolysis)/[H_2O]). [c] Extrapolated.

Reactions with p-Nitrophenyl Hippurate. The reactions of HNP with papain were monitored both by automatic titration and spectrophotometrically. A typical set of titration experiments is shown in Figure 2 and the results are summarized in Table III. The ratios of $k_{ethanol}/k_{water}$ obtained for HNP given in Table III can be compared with those given in Table II for HEE in 1.72 M ethanol, since the same batch and method

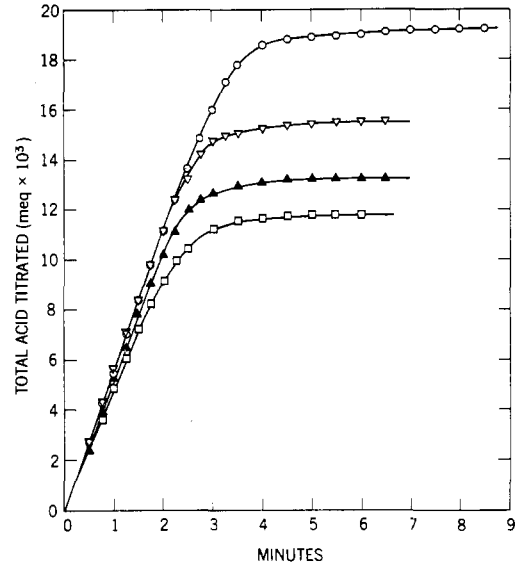

FIGURE 2: The papain-catalyzed hydrolysis of HNP at pH 6.0 and 40° as a function of ethanol concentration. The reaction mixtures contained 0.30 M KCl, 5.0 × 10^{-4} M EDTA, 1.3 M acetonitrile, 2.5 × 10^{-4} M cysteine, and 5.70 × 10^{-7} M papain (batch C, activated by method 3) and 3.91 × 10^{-4} M HNP in a total volume of 40 ml. The hydrolysis reaction was measured in: (○) no ethanol, (▽) 0.86 M ethanol, (▲) 1.72 M ethanol, and (□) 2.58 M ethanol.

TABLE III: The Reactions of Papain with *p*-Nitrophenyl Hippurate in Varying Concentrations of Ethanol.[a]

$[C_2H_5OH]$ (M)	Detn No.	Hippuric Acid Produced[b] (M × 10^4)	[HEE] Produced[c]/[Hippuric Acid] Produced	$k_{ethanolysis}$[d]/$k_{hydrolysis}$	Rate of Hippuric Acid Production,[e,b] $v_i/[E][H_2O]$ (M^{-1} sec^{-1})
0	3	3.58	0		0.067
0.86	1	2.87	0.25	14.2	0.070
1.72	4	2.49	0.44	11.8	0.072
2.58	1	2.29	0.56	9.4	0.066

[a] The conditions are as described in Figure 2. [b] Corrected for nonenzymatic values and for nitrophenol ionization. [c] Assuming that the decrease in the amount of hippuric acid formed from that produced in the absence of ethanol is due to the formation of HEE. The rate of hydrolysis of HEE under these conditions is very slow compared to the rate of reaction of HNP. [d] Calculated from the values in column 4. [e] Determined from the corrected initial slopes in Figure 2.

TABLE IV: The Reactions of Papain with *p*-Nitrophenyl Hippurate under Varying Solvent Conditions.[a]

Solvent (M)	$V_{max}/[E]$[b] (sec^{-1})	$V_{max}/[E][H_2O]$ (M^{-1} sec^{-1} × 10^2)	K_m (mM)	$V_{max}/[E][ROH]$ (M^{-1} sec^{-1} × 10^2)	k_{ROH}/k_{H_2O}
Acetonitrile (1.3)	3.78 ± 0.26	7.37 ± 0.51	0.0241 ± 0.0006		
Acetonitrile (3.2)	4.52 ± 0.10	9.87 ± 0.22	0.0492 ± 0.0026		
Acetonitrile (1.3) plus ethanol (1.7)	7.85 ± 0.31		0.0854 ± 0.0063	260[c]	36[c]
				194 ± 19[d]	20 ± 2[d]
Acetonitrile (1.3) plus methanol (2.5)	12.01 ± 0.52		0.111 ± 0.008	345[c]	47[c]
				300 ± 21[d]	30 ± 2[d]

[a] Reaction conditions as in Figure 3. [b] For the total rate of appearance of *p*-nitrophenol. [c] Calculated using the value of $V_{max}/[E][H_2O]$ obtained from the determination of 1.3 M acetonitrile. [d] Using the value of $V_{max}/[E][H_2O]$ from the determination in 3.2 M acetonitrile.

of activation of papain was used in each case. The ratio for HNP appears to be influenced to some degree by the ethanol concentration.

The papain-catalyzed rates of release of *p*-nitrophenol from HNP are shown in Figure 3 and the results are collected in Table IV. While citrate was the buffer most commonly used, it can be seen that low concentrations of other buffers (phosphate and lutidine) do not appreciably affect the kinetic parameters (Table V). It is evident from Table IV that increasing concentrations of organic solvent increase the values of K_m as is the case with HEE. It is interesting to note that the values of $V_{max}/[E][H_2O]$ are increased somewhat by raising the concentration of acetonitrile from 1.3 to 3.2 M. The ratios calculated for $k_{alcohol}/k_{water}$ in Table IV, therefore, are only approximations due to the dependence of the rates of *hydrolysis* on solvent composition and the consequent difficulty in isolating the hydrolysis velocities from the total reaction with the spectrophotometric assay. An attempt was made to circumvent these problems by measuring the rate of hydrolysis by automatic titration followed by a measurement of the total rate of *p*-nitrophenol release under the same conditions.[4] The only difference in the two assay conditions was the use of 0.30 M potassium chloride in the titration method and 0.25 M potassium chloride plus 0.010 M citrate buffer in the spectrophotometric measurements. The value of $v_i/[E]$ thus obtained for the hydrolysis of HNP, corrected for the nonenzymatic rate and for the ionization of *p*-nitrophenol, was 3.45 ± 0.09 sec^{-1} (five determinations), giving 0.0753 ± 0.0020 M^{-1} sec^{-1} for the second-order rate constant. The total rate of *p*-nitrophenol release, measured at 340 mμ, was 4.96 ± 0.14 sec^{-1} (five determinations). The ratio of rate of formation of [HEE]/[hippuric acid], therefore, is (1.51 ± 0.23)/(3.45 ± 0.09) = 0.44 ± 0.07, giving a value of $k_{ethanol}/k_{water}$ of 12 ± 2. This figure compares

[4] Each reaction mixture contained 1.3 M CH_3CN, 5.0×10^{-4} M EDTA, 2.5×10^{-4} M cysteine, 5.95×10^{-7} M papain (batch C, activation method 3), 4×10^{-4} M HNP, and 1.7 M EtOH.

TABLE V: The Reaction of *p*-Nitrophenyl Hippurate with Papain as a Function of Different Buffering Agents.[a]

Buffer (M)	Concn of Ethanol (M)	V_{max}/[E] (sec^{-1})	V_{max}/[E] Cor[b] (sec^{-1})	V_{max}/[E][H_2O] Cor[b] (M^{-1} sec^{-1} × 10^2)	K_m (mM)	Substrate Range (mM)
Citrate (0.010)	0	3.16 ± 0.13	3.36 ± 0.14	6.55 ± 0.27	0.033 ± 0.007	0.271–0.090
Citrate (0.010)	1.72	5.86 ± 0.22	6.25 ± 0.23		0.096 ± 0.011	0.406–0.090
Phosphate (0.010)	0	2.57 ± 0.10	3.06 ± 0.12	5.96 ± 0.23	0.023 ± 0.007	0.409–0.102
Phosphate (0.010)	1.72	5.25 ± 0.26	6.26 ± 0.31		0.089 ± 0.014	0.409–0.102
Lutidine (0.010)	0	2.69 ± 0.10	3.28 ± 0.12	6.39 ± 0.23	0.032 ± 0.007	0.336–0.099

[a] The reactions were carried out at pH 6.0 and 40°. Rates were measured by following the appearance of *p*-nitrophenol at 340 mμ. Each reaction mixture contained 5.0 × 10^{-4} M EDTA, 1.3 M acetonitrile, 0.010 M buffer as indicated, and potassium chloride to give an ionic strength of 0.3 M. The cysteine and papain concentrations for citrate, phosphate, and lutidine were, respectively, 2.5, 2.7, and 2.7 × 10^{-4} M (cysteine) and 5.95, 6.07, and 6.27 × 10^{-7} M (papain, batch C activated by method 3). [b] Corrected for the time-dependent loss in enzyme activity (footnote 2).

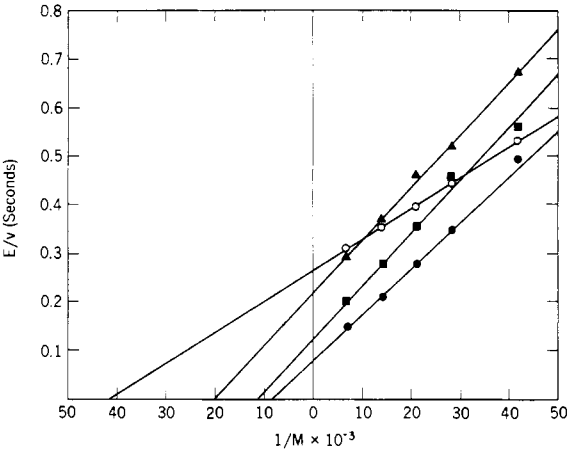

FIGURE 3: The reactions of papain with HNP at pH 6.1 and 40° corrected for the nonenzymatic reaction. The reaction mixtures contained 0.25 M KCl, 0.010 M citrate buffer, 5.0 × 10^{-4} M EDTA, 3.5 × 10^{-4} M cysteine, and 2.50 × 10^{-7} M papain (batch B, activation method 2). The rate of appearance of *p*-nitrophenol was measured at 317 mμ: (○) 1.3 M CH_3CN, (▲) 3.2 M CH_3CN, (■) 1.3 M CH_3CN plus 1.7 M C_2H_5OH, and (●) 1.3 M CH_3CN plus 2.5 M CH_3OH.

well with those obtained in Table III where the method of calculation was by measurement of the differences in end-point production of hippuric acid rather than by rates.

Discussion

Transesterification Reactions. Equation 1 can be expanded to include transesterification reactions

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES_1 \xrightarrow{k_2} ES_2 + P_1 \begin{cases} \xrightarrow{k_3[H_2O]} E + P_2 \\ \xrightarrow{k_4[ROH]} E + P_3 \end{cases} \quad (2)$$

where P_3 is the transesterified substrate. In the case of an exchange reaction, $k_4 = k_{-2}$ and P_3 and S are identical.

The values of K_m and k_{cat} derived from this model using the steady-state assumption are

$$K_m = \frac{(k_3[H_2O] + k_4[ROH])K_s}{k_2 + k_3[H_2O] + k_4[ROH]} \quad (3)$$

For hydrolysis

$$k_{cat} = \frac{k_2 k_3[H_2O]}{k_2 + k_3[H_2O] + k_4[ROH]} \quad (4)$$

For transesterification

$$k_{cat} = \frac{k_2 k_4[ROH]}{k_2 + k_3[H_2O] + k_4[ROH]} \quad (5)$$

This treatment gives identical expressions for K_m, for hydrolysis and alcoholysis reactions.

A number of investigations have previously been made on methanol–water competition experiments with chymotrypsin. Applewhite *et al.* (1958) in studying the hydrolysis of methyl hippurate noted that k_{cat} decreased more than sixfold and K_m increased by a factor of 3 when 2.5 M methanol was introduced into the reaction mixture. Several nonhydroxylic solvents effected similar changes in K_m, but did not change k_{cat}. Clement and Bender (1963) similarly found that acetonitrile increased K_m, but not k_2, k_3, or k_{cat} in the chymotrypsin-catalyzed reactions of *N*-acetyl-L-tryptophan methyl ester. They later attempted to employ reciprocal linear forms of eq 3–5 in order to obtain all the rate and equilibrium constants for the pathway through solvent variation (Bender *et al.*, 1964). The interpretation of this treatment is complicated by the solvent dependence of the enzyme–substrate dissociation constant (K_s) and by the possible solvent dependence of some of the catalytic constants. Inward and Jencks (1965) have shown that the rate of *hydrolysis* of furoyl-chymotrypsin is increased by aliphatic alcohols and Faller and Sturtevant (1966) have demonstrated a similar effect for acetyl-chymotrypsin.

The rate constant for the hydrolysis of HEE is not reduced by the addition of 1.7 M ethanol (Table I); a result which in the absence of other factors would indicate that $k_2 \gg k_3(H_2O) + k_4(EtOH)$. The results with HNP (see below), however, demonstrate that organic solvents cause a substantial increase in k_{cat} and therefore make it difficult to come to firm conclusions about the relative magnitudes of the terms in the denominator of eq 3–5 for HEE. The total rate of acyl transfer at infinite substrate concentration as measured by the sum $k_3[H_2O] + k_4[EtOH]$ is 40% greater in 1.7 M ethanol than in the absence of added alcohol. This number substituted into eq 3 predicts that K_m would increase from 0.029 to 0.041 M. The observed values of K_m in 1.7 M ethanol are 0.071 and 0.092 M for the hydrolysis and ethanolysis reactions, respectively. The difference can most likely be at least partially attributed to an increase in K_s mediated by the organic solvent. Equation 3 further requires that K_m(hydrolysis) equals K_m(ethanolysis) at any fixed solvent composition. The values actually obtained for these constants do appear, within the precision of the experiment, to be significantly different. It is not clear whether these differences represent an undetected systematic error in the experiments or require a modification of the kinetic model, such as the one considered below. The value of k_{EtOH}/k_{H_2O} is 15.1 ± 0.5 at saturating HEE and decreases at lower concentrations (Table II).

Lake and Lowe (1966) have recently reported that the rate of *p*-nitrophenol release from HNP is not increased by added methanol at concentrations of up to 2.0 M when measured at 0.065 mM HNP,[5] and have concluded that the rate-determining step in the deacylation of hippuryl-papain is not a hydrolytic step. The results documented in Figure 3 and Table IV show clearly that the rate of *p*-nitrophenol release is significantly increased by alcohol. The experimental conditions in the present case differ slightly from those of Lake and Lowe in that all solutions contained a minimum of 6.7% (v/v) CH_3CN, reactions were studied at 40°, and the papain was activated with cysteine; while the previous investigators used no organic solvent other than methanol, a temperature of 35°, and thiocresol-activated papain. The effect of increasing the concentration of acetonitrile from 1.3 to 3.2 M is to increase k_{cat} by 34% and K_m twofold. Further large increases in both K_m and V_{max} are observed when either ethanol or methanol is used to replace a fraction of the CH_3CN while maintaining a total concentration of organic solvent of 16.7% (v/v). The addition of ethanol leads to the formation of HEE (Figure 2) in approximate proportion to the amount of alcohol present (Table III). The best estimate of the ratio k_{EtOH}/k_{H_2O} from the spectrophotometric determinations is 20 ± 2 (Table IV). This value does not compare very well with that obtained by direct measurement of products or by pH-Stat measurements (see Results). The apparent reason for this is attributed to the uncertainty in the rate constant for hydrolysis in ethanol solution. The model described by eq 2 requires that the partitioning ratio of the acyl-enzyme be independent of the leaving group of the substrate. This was observed within experimental error where direct measurements of both products or of both rates were made. Identical ratios of k_{EtOH}/k_{H_2O} do not by themselves prove the existence of a common intermediate along the pathway, since such an identity might only represent the relative nucleophilicity of the reagents in direct enzyme-catalyzed displacement reactions on the two substrates. The value of k_{MeOH}/k_{H_2O} is 30, and can be compared to a ratio of 59 obtained by Brubacher and Bender (1966) for cinnamoyl-papain. A large part of the discrepancy between the results reported here and the conclusions of Lake and Lowe (1966) can be attributed to the large solvent-mediated increase in K_m which masks the catalytic effect of alcohol in increasing the rates at subsaturating concentrations of HNP. On the assumption that the larger increase in K_m can be assigned to a solvent effect on K_s, it can be calculated from eq 3 that K_s (16.7% CH_3CN) = 1.5 K_s (6.7% CH_3CN). This figure gives consistent results when combined with the experimentally determined values for K_m and k_{cat} in 6.7% CH_3CN plus 10% EtOH and used in eq 3, thus indicating that the bulk solvent effect of 10% CH_3CN and 10% EtOH on K_s is about the same. One observation that is extremely difficult to

[5] Dr. Lowe has informed us that this was the concentration actually used.

reconcile with the relatively simple mechanism described in eq 2 is that k_{cat} for HNP in the absence of alcohol is only 70% of the corresponding rate constant for HEE (Tables I and V). Similarly, Lowe and Williams (1965b) found k_{cat}(HNP) equals 0.65 k_{cat}(HEE). Since the rate of acylation of the enzyme by HNP should be greater than the corresponding constant for HEE, eq 3 demands that k_{cat}(HNP) $\geq$ k_{cat}(HEE). The expected relationship between nitrophenyl and ethyl esters was, in fact, obtained for carbobenzoxyglycine substrates (Kirsch and Igelström, 1966). The total rates of acyl transfer for the two esters of hippuric acid are, however, virtually identical in 10% ethanol (Tables I and V).

The Kinetics of Leaving Group Dissociation. It is not obvious that simple alcohols such as methanol or ethanol should be more nucleophilic than water toward an acyl-enzyme. Inward and Jencks (1965) have shown that the rate of deacylation of furoyl-chymotrypsin is quite independent of the pK_a of the attacking alcohol, although all the alcohols studied were more than two orders of magnitude more reactive than water. Brubacher and Bender (1966) found that the rate constants for deacylation of cinnamoyl-papain as mediated by water, methanol, and benzyl alcohol were 0.0000665, 0.00394, and 0.453 M^{-1} sec^{-1}, respectively. Recent experiments by Schechter and Berger (1967) have shown that the rates of hydrolysis of DL-alanine oligopeptides of known composition are sensitive to the stereochemical configuration of an amino acid that is one or two residues removed toward the C terminus from the peptide bond being cleaved, and Brubacher and Bender (1967) have further demonstrated that the relative rate constants for the reactions of tetra-, tri-, di-, and monoglycine amides with cinnamoyl-papain are 50, 37, 19, and 1.0, respectively. These experiments clearly indicate some degree of interaction between the incoming nucleophile and by analogy with the leaving group of the substrate, with the enzyme, although in no case has it been possible to demonstrate saturation of the acyl-enzyme with increasing concentrations of nucleophile. The active site around the reactive sulfhydryl group appears to be a cleft, large enough to accommodate a substrate several amino acids in length (Drenth *et al.*, 1967). Interestingly, no such cleft appears in chymotrypsin (Matthews *et al.*, 1967). The question of whether or not the binding of the leaving group is reflected *significantly* in K_s is at present unresolved. The previously reported correlations of $1/K_m$ with k_{OH^-} for esters of carbobenzoxyglycine were considered to provide evidence that K_s varied very little throughout the series since k_{OH^-} should be a measure of the susceptibility of the substrates to nucleophilic attack, while K_s should be primarily a function of Van der Waals interactions (Kirsch and Igelström, 1966). The success of this correlation does not require that the leaving group not contribute to K_s, but rather that the variation in such a contribution be small compared to the variation in k_2. Bender and Brubacher (1966), on the other hand, have concluded that substrate reactivity for esters of *N*-α-benzyloxycarbonyl-L-lysine is expressed primarily in K_s, and only slightly in k_2; a result that is much more difficult to rationalize chemically.

In summary at this point it is emphasized that the general scheme embodied in eq 2–5 is directly supported by the following. (1) The total rate of substrate utilization is increased for both HNP and HEE in added alcohol, and most of this additional rate can be accounted for in terms of the appearance of transesterified products. (2) The partitioning ratio (k_{EtOH}/k_{H_2O}) is independent of the substrate. Point 1 further indicates that the rate-determining step involves the deacylation of the acyl-enzyme. The solvent effect on K_s, while not predicted by this mechanism, does not conflict with it. On the other hand, this model does not satisfactorily account for the large increase in the rate of hydrolysis of HNP mediated by the addition of organic solvent, for the fact that k_{cat} for HNP is less than the corresponding value for HEE in the absence of alcohol but equal to it in 10% (v/v) ethanol, nor for the demonstrated interaction of leaving groups with the enzyme.

Many of these discordant results can be more satisfactorily accommodated in terms of the scheme described in eq 6, which is an expansion of eq 2, allowing one additional kinetically important step for the departure of the leaving group from the enzyme surface.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES_1 \xrightarrow{k_2} ES_{2x} \xrightarrow{k_{2x}} \begin{matrix} ES_2 \\ + \\ P_1 \end{matrix} \xrightarrow{k_4'} E + P_2' \quad (6)$$

The terms, ES_1, ES_2, and P_1 have the same significance as in eq 1 and 2. The symbols k_1 and k_{-1} represent the rate constants for the formation and dissociation of ES_1, ES_{2x} is an acyl-enzyme still associated with the leaving group of the original substrate, k_{2x} is the rate constant for the dissociation of P_1, and k_4' equals $k_3[H_2O] + k_4[N]/K_N$, where N is any acyl acceptor other than H_2O and K_N is the enzyme–nucleophile dissociation constant. The sum of hydrolyzed and other transacylated products is represented by P_2'. The steady-state solution for eq 6 leads to

$$K_m = \frac{k_4'k_{2x}(k_{-1} + k_2)}{k_1(k_{2x}k_4' + k_2k_{2x} + k_2k_4')} \quad (7)$$

$$k_{cat} = \frac{k_2k_{2x}k_4'}{k_{2x}k_4' + k_2k_{2x} + k_2k_4'} \quad (8)$$

In the limiting case of $k_{2x} \gg k_4'$ or k_2, and $k_{-1} \gg k_2$, eq 7 reduces to eq 3, and eq 8 to eq 4 plus eq 5. The requirement that the departure of the leaving group from the enzyme surface be kinetically significant is, therefore, that k_{2x} is not very much greater than either k_4' or k_2. Since typical values of k_4' are in the range

of 5 sec^{-1}, k_{2r} should be less than *ca.* 100 sec^{-1}.[6] As saturation of the enzyme with nucleophile has not been observed, it may be assumed that the dissociation constant is at least 1 M, a figure which leads to expected rate constants for the association of the enzyme with the nucleophile of 100 M^{-1} sec^{-1}. This figure is several orders of magnitude less than those commonly obtained for rates of enzyme–substrate association (Eigen and Hammes, 1963) and may reflect, among other factors, a slow conformational change accompanying this step or perhaps more simply that most of the acceptor sites are competitively occupied by water. In the latter case, the free enzyme might also have a molecule of water bound to the acyl acceptor site, which must dissociate before the enzyme can combine with substrate.

For nitrophenyl esters, $k_2 \gg k_4'$ and eq 7 and 8 reduce to

$$K_m = \frac{k_4'k_{2r}(k_2 + k_{-1})}{k_1k_2(k_{2r} + k_4')} \quad (9)$$

$$k_{cat} = \frac{k_{2r}k_4'}{k_{2r} + k_4'} \quad (10)$$

and the locus of the activation effect of acetonitrile on k_{cat} can be on either k_4' or k_{2r} or both. The addition of organic solvents should decrease the activity coefficient of *p*-nitrophenol in the aqueous phase, and in the absence of other factors would therefore increase k_{2r}. The ratio $(k_2 + k_{-1})/k_1$ must also increase, however, in order to account for the entire observed increase in K_m. The observation that k_{cat} for HNP is less than the corresponding rate constant for HEE when both reactions were measured in the absence of ethanol can be understood in terms of the rate of release of nitrophenol from the enzyme surface being less than the rate of release of ethanol in low concentrations of organic solvents. In high organic solvent concentrations, the k_{2r} terms increase so that k_{cat} approaches k_4' and the observed rates for the two substrates merge.

The evidence supporting the kinetic importance of the rate constant introduced in eq 6 can possibly be explained in other ways. It appears worthwhile to introduce consideration of this term at this point because of the clear inability of the model schematicized in eq 2 to accommodate much of the present body of experimental data.

[6] This figure also represents an upper limit for k_{-1} if the leaving group on the intact substrate occupies the same binding site with a similar ΔF_1, before fission. Values of k_2 are most likely greater than this (Kirsch and Igelström, 1966). In the event that such binding does obtain, the usual assumption that K_s represents a true equilibrium constant would not be tenable.

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