Binding Sites for Substrate Leaving Groups and Added Nucleophiles in Papain-Catalyzed Hydrolyses*

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ABSTRACT: The interactions of some alcohol and amine nucleophiles in the papain-catalyzed hydrolysis of the *p*-nitrophenyl esters of *N*-acetyl-L-tryptophan and *N*-CBz-L-Lys have been investigated. The effects of the added nucleophile on the kinetic parameters and product distribution were found to vary with the structure of the nucleophile, and both activation and inhibition could be observed with respect to the rate of disappearance of substrate. The results for the addition of *n*-alkyl alcohols could be interpreted in terms of simple partition of the acyl-enzyme between water and alcohol *only* for the smallest members of the series. However, all the results could be satisfactorily explained by a scheme in which the added modifier binds to the free enzyme, enzyme–substrate complex, and acyl-enzyme. Evidence is presented for the existence of two different modes of binding for the added nucleophile, one which may also overlap that of the substrate leaving group, the other which interferes with substrate binding.

The dependence of k_{cat}/K_m upon the nucleophile concentration, and the noncompetitive inhibition by 2-pentanone are interpreted in terms of a ternary enzyme–substrate–nucleophile complex. The increased binding affinity of the added nucleophile with lengthening of the apolar chain in the case of the aliphatic alcohols suggests hydrophobic interactions to be the major force responsible for binding. The reactivity of the alcohol toward the acyl-enzyme apparently decreases with increasing chain length, whereas the formation of the ternary complex increases the rate of acylation with the more apolar nucleophiles. The results support the existence of an acyl-enzyme intermediate, with general base catalysis involved in deacylation. The specificity of binding for the added modifier suggests the possibility of using papain as a catalyst in asymmetric synthesis of acyl derivatives, and in peptide synthesis.

Substantial evidence indicates that papain- (EC 3.4.4.10) catalyzed hydrolyses proceed *via* an acyl-enzyme intermediate (eq 1) (Lowe and Williams, 1964; Husain and Lowe, 1965; Bender and Brubacher, 1964; Kirsch and Igelstrom, 1966)

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} \underset{+\ P_1}{ES'} \xrightarrow{k_3 W} E + P_2 \qquad (1)$$

where ES is the noncovalent enzyme–substrate complex, ES′ is the acyl-enzyme formed by nucleophilic attack of cysteine-25 of the enzyme on the substrate, P_1 is the substrate leaving group, P_2 is the carboxylic acid derivative from the substrate, and W represents water. Water is not unique in the deacylation; for example, replacement by amine or alcohol derivatives may result in changes in the rate and products of the reaction. Thus the ability of papain to catalyze transesterification and transamidation reactions has been known for some time (Glazer, 1966; Tollin and Fox, 1957; Mycek and Fruton, 1957). The addition of either amines or alcohols increased the rate of deacylation of the nonspecific acyl-enzyme *trans*-cinnamoyl-papain (Brubacher and Bender, 1966). The second-order rate constants for the deacylation were found to be larger for amines than for the corresponding alcohols, and dependent upon the structure of the nucleophile. The lack of obvious relationship between the basicity of the nucleophile and its effect on deacylation suggested that the added nucleophile might bind to the enzyme prior to participating in the deacylation.

Conflicting results have been reported from investigations of the effect of methanol and ethanol on the papain-catalyzed hydrolysis of esters of hippuric acid. Lake and Lowe (1966) found that k_{cat} for the release of *p*-nitrophenol in the papain-catalyzed hydrolysis of *p*-nitrophenyl hippurate was independent of methanol concentration in the range 0–2.0 M methanol, while k_{cat} for the release of hippuric acid from methyl hippurate was inversely proportional to the methanol concentration. A rate-limiting conformational change, or nucleophilic attack, in addition to the reactions shown in eq 1, was proposed to account for the results. Henry and Kirsch (1967) found that methanol and ethanol increased both the rate of release of *p*-nitrophenol and K_m in the papain-catalyzed hydrolysis of *p*-nitrophenyl hippurate. Methanol and ethanol caused increases in K_m, but no change in k_{cat} (when allowance was made for the decreased concentration of water) in the papain-catalyzed hydrolysis of ethyl hippurate. A scheme similar to eq 1, but with an additional kinetically important step, namely, the release from the enzyme of the alcohol moiety of the ester substrate, was postulated. In the papain-catalyzed hydrolysis of nitrophenyl esters of CBz-Gly, the addition of ethanol has been found to have no effect on the acylation when determined either by presteady-state or steady-state parameters (Hubbard and Kirsch, 1968).

Much is still unknown about the detailed processes involved in the acylation and deacylation steps in papain-catalyzed hydrolyses. For example, it is not clear whether the catalytic group involved in deacylation is a carboxyl or

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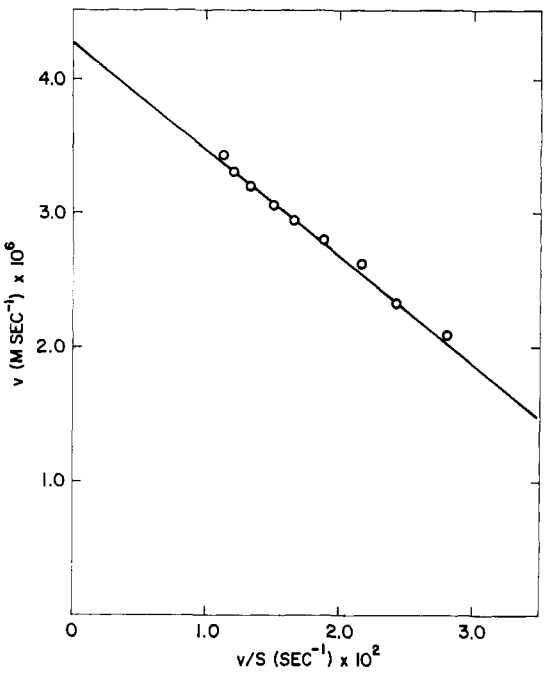


FIGURE 1: Typical plot of v *vs.* v/S for 0.75 M 1-butanol and *p*-nitrophenyl *N*-acetyl-L-tryptophanate.

imidazole, and whether it acts as a general base or nucleophile. Similarly in the acylation step, the dependence of k_2 and K_s upon the substrate structure is not clear (Hubbard and Kirsch, 1968; Bender and Whitaker, 1965; Brubacher, and Bender 1966). The potential utility of added nucleophiles to elucidate the mechanism of action of papain has not yet been fully investigated. In particular, the effects of nucleophiles of different structure and reactivity on the reaction with specific substrates (where the possibility for nonproductive binding is reduced) have not been studied in detail. For example, in the hydrolysis of a specific substrate in which $k_2 > k_3$, an added nucleophile should change the rate-limiting step when the nucleophile is at sufficiently high concentration. Thus by extrapolation to infinite concentration one should be able to obtain the value of k_2. (Ideally the only effect of the nucleophile is to increase the deacylation rate by competing with water.) This should provide a simple measure of the effect of variations in substrate structure and pH on k_2 (and thus on K_s). By comparing the effects of corresponding amines and alcohols, confirmation of a thiolester intermediate should be obtained, since model systems (Connors and Bender, 1961) predict that an amine will react faster than the corresponding alcohol toward a thiolester. The results from such experiments may also provide information on whether the deacylation involves general base or nucleophilic catalysis (nucleophilic catalysis by carboxyl has recently been ruled out by Williams and Whitaker (1968)). If the added nucleophile binds to the enzyme, then the effect of varying the structure of the added reagent should reflect the specificity of binding sites in the active center. In particular, evidence for the existence of a specific site for the substrate leaving group should be observed if such a site exists, since the added nucleophiles resemble the leaving group of the substrate. For these reasons a systematic investigation has been undertaken to determine the effects of added alcohols and amines of widely varying structure on the reactions of specific substrates with papain. The initial results of this investigation are reported herein.

Experimental Section

Materials. *p*-Nitrophenyl *N*-acetyl-L-tryptophanate from Cyclo Chemical Co. (lots G1847 and P2297) was recrystallized several times from ethyl acetate–hexane, mp 135–136° (>94% L isomer as determined by papain hydrolysis). *p*-Nitrophenyl CBz-L-lysinate (Cyclo lot M2135) was recrystallized from 3% ethanol–acetonitrile, mp 150–151°. Methyl *N*-acetyl-L-tryptophanate (Cyclo lot K4228) was recrystallized from ethyl acetate–hexane, mp 153.5–154°. *N*-Benzoyl-L-arginine ethyl ester hydrochloride (Mann lot M2024), mp 131–132°, was used without further purification. *N*-Acetyl-L-tryptophan (Cyclo lot R5560) was recrystallized from tetrahydrofuran–ethyl acetate, mp 235–237° dec. L-Tryptophanamide hydrochloride from Cyclo (lot K5579) was recrystallized from ethanol–water–acetone, mp 261° dec.

Mercuripapain, Worthington Biochemical Corp. (lots 7KA and 8GA), was activated by the method of Brubacher (1966) using *p*-toluenethiol in toluene followed by gel filtration on Sephadex G-25. The activated papain was stored as a stock solution at 1° under a nitrogen atmosphere in sodium acetate buffer at pH 5.2. The molarity of the enzyme solution was determined using *N*-Bz-L-ArgEt according to the method of Brubacher (1966).

The water was doubly distilled and deionized. All buffer materials were reagent grade. Mallinckrodt Nanograde acetonitrile was used without further purification. Methanol (reagent grade from Baker and Adamson), ethanol (absolute from U. S. Industrial Chemicals), 1-propanol (Matheson, Coleman & Bell), 1-butanol (Fisher), 1-pentanol (Eastman), and 2-pentanone (Aldrich) were distilled prior to use (the central 60% of the distillate was kept). *p*-Toluenethiol and EDTA, from Eastman, were used without further purification.

Kinetic Measurements. The steady-state parameters k_{cat} ($=V_{max}/E_0$) and K_m were determined from initial velocity measurements at varying substrate concentrations or from complete reaction curves from the least-mean-squares plot of v *vs.* v/S (Bender *et al.*, 1964). Maximum substrate concentrations were $\geq 3K_m$. Corrections were applied for spontaneous hydrolysis of the substrate. The results for several experiments were determined by both methods and excellent agreement between them was obtained. No nonrandom deviations from Michaelis–Menten kinetics were observed. Figure 1 shows a typical plot of v *vs.* v/S. The experimental parameters $k_{cat}^{P_1}$ and $k_{cat}^{P_2}$ have been defined as follows: $k_{cat}^{P_1} = k_{cat}$ for the release of P_1, $k_{cat}^{P_2} = k_{cat}$ for the release of P_2.

All the reactions with added alcohols and the appropriate controls were measured at pH 7.0, I = 0.5 M, and acetonitrile + alcohol concentration = 10% (v/v), *i.e.*, 50 M water. The experiments with L-tryptophanamide were carried out at pH 7.3 and 7.9 with I = 0.6 M, and those with methylamine at pH 8.1 with I = 0.8 M.

Spectrophotometric measurements of the release of *p*-nitrophenol were made on a Cary 14 instrument at 347.6 nm at 25°. Solutions of phosphate buffer, potassium chloride, EDTA, acetonitrile, and the appropriate alcohol, and papain

TABLE I: The Effect of Methanol on the Papain-Catalyzed Hydrolysis of *p*-Nitrophenyl *N*-Acetyl-L-tryptophanate.[a]

[Methanol] (M)	0	0.5	1.0	1.5	2.0
$k_{cat}^{P_1}$ (sec^{-1})	3.54	3.80	4.17	4.36	4.63
$k_{cat}^{P_2}$ (sec^{-1})	3.63	3.67	3.70	3.60	3.63
K_m (M × 10^4)[b]	1.00	1.12	1.19	1.37	1.27
c	0.94	1.05	1.07	1.25	1.23
$k_{cat}^{P_1}/K_m$[d,e]	3.54	3.40	3.51	3.30	3.63
f	3.50	3.55	3.42	3.47	3.47

[a] Experimental conditions are given in the text. [b] Determined spectrophotometrically. [c] Determined titrimetrically. [d] $sec^{-1}\,M^{-1} \times 10^{-4}$. [e] [S] > K_m. [f] [S] < K_m.

stock solution were made to give the following conditions: pH 7.0, I = 0.5 M, EDTA = 1×10^{-5} M, acetonitrile + alcohol 10% (v/v), and papain = 3×10^{-7}–10^{-6} M. After thermal equilibration, substrate was added from an acetonitrile stock solution. Final volumes were 3.10 ml. The spontaneous rates of substrate hydrolysis were determined separately under the same conditions.

Titrimetric measurements were performed using a Radiometer pH meter 26, Titrator II, Titrigraph SBR2c, and SBU-1 syringe assembly. Solutions of potassium chloride, acetonitrile, EDTA, alcohol, and papain were prepared to give the following final conditions: I = 0.5 M, acetonitrile + alcohol 10% (v/v), 1×10^{-5} M EDTA and 3×10^{-7}–10^{-6} M papain. After thermal equilibration, the pH was adjusted to pH 7.0 by the addition of the titrant (potassium hydroxide 2×10^{-2} M) and the substrate was added to give a final volume of 15.3 ml. The reaction was performed under an atmosphere of nitrogen. The pK_a for *p*-nitrophenol under these conditions was found to be 7.18 ± 0.02, and was used to make corrections for base uptake due to *p*-nitrophenol.

Product analyses for the esters resulting from alcohol participation were determined from the decrease in the total uptake of base in the pH-Stat experiments. For methyl *N*-acetyl-L-tryptophanate, the product analysis was also performed by raising the pH of the completed reaction solution to pH 10 with 2.0 M sodium carbonate, and extracting with 5-ml portions of dichloromethane. After drying the extract over anhydrous magnesium sulfate, the ultraviolet spectrum was obtained, and the absorption at 280 nm was compared with that of a standard curve. The latter was obtained by adding known amounts of methyl *N*-acetyl-L-tryptophanate to similar solutions of enzyme, *p*-nitrophenol, and *N*-acetyl-L-tryptophan, and extracting by the same procedure. From these data the concentration of methyl *N*-acetyl-L-tryptophanate was obtained. Close agreement between the two methods was found.

Results

The effect of methanol on the papain-catalyzed hydrolysis of *p*-nitrophenyl *N*-acetyl-L-tryptophanate was studied by determining its effect on k_{cat} and K_m. In these experiments the release of *p*-nitrophenol was measured spectrophotometrically, and the release of *N*-acetyl-L-tryptophan titrimetrically. The value of k_{cat}/K_m was also calculated from the apparent first-order reaction with [S] ≪ K_m. The results for methanol concentrations to 2.0 M are shown in Table I. At higher concentrations of methanol, k_{cat} leveled off and K_m began to increase. Since high concentrations of methanol affect the solvent properties and thus may lead to secondary solvent effects (Bender *et al.*, 1964), the effect of an inert organic solvent was investigated. Table II shows the effect of acetonitrile on the k_{cat}/K_m ratio. There is negligible effect to a concentration of 10% acetonitrile, where the dielectric constant is similar to that of 2.0 M methanol. Above this value, k_{cat}/K_m decreases sharply. Effects on k_{cat} and K_m were similar when determined by measuring the initial velocities and by using the "one-run" technique. The ester product methyl *N*-acetyl-L-tryptophanate had a negligible effect on the reaction at concentrations to and exceeding the concentration of that formed in the reaction (Table III). That methyl *N*-acetyl-L-tryptophanate was indeed formed during the reaction was confirmed by extraction and quantitative spectrophotometric determination of the concentration of the methyl ester by comparison with an authentic sample. This product analysis was in good agreement with that calculated from the decrease in the total uptake of base in the pH-Stat experiments (Figure 2). Accurate values of k_{cat} and K_m were not obtained for the papain-catalyzed hydrolysis of methyl *N*-acetyl-L-tryptophanate because the reaction was too slow. The value of k_{cat}/K_m for this compound was found to be 3.39 $sec^{-1}\,M^{-1}$, and that of $K_m \geq 3 \times 10^{-3}$ M, under the conditions indicated in the Experimental Section.

The effects of ethanol, 1-propanol, 2-propanol, 1-butanol, and 1-pentanol on k_{cat} and K_m for the release of *p*-nitrophenol and *N*-acetyl-L-tryptophan are shown in Tables IV–VII. The results range from an increase in k_{cat} and K_m and decrease

TABLE II: The Effect of Acetonitrile on the Papain-Catalyzed Hydrolysis of *p*-Nitrophenyl *N*-Acetyl-L-tryptophanate.[a]

Acetonitrile (v/v)	0	4	8	12	16	20
k_{cat}/K_m[b]	3.25	3.37	3.45	2.93	2.31	1.82

[a] The conditions are given in the Experimental Section. [b] $sec^{-1}\,M^{-1} \times 10^{-4}$.

TABLE III: The Effect of Methyl *N*-Acetyl-L-tryptophanate on the Papain-Catalyzed Hydrolysis of *p*-Nitrophenyl *N*-Acetyl-L-tryptophanate.[a]

[Me-*N*-Ac-L-Trp][b] (M)	0	1.24 × 10^{-3}	1.24 × 10^{-4}	1.24 × 10^{-5}	1.24 × 10^{-6}
V_{max}[c]	4.29	4.31	4.36	4.44	4.32
K_m[d]	0.98	0.94	1.03	1.01	1.00

[a] The conditions are given in the Experimental Section. [b] Methyl *N*-acetyl-L-tryptophanate. [c] M $sec^{-1} \times 10^6$. [d] M × 10^4.

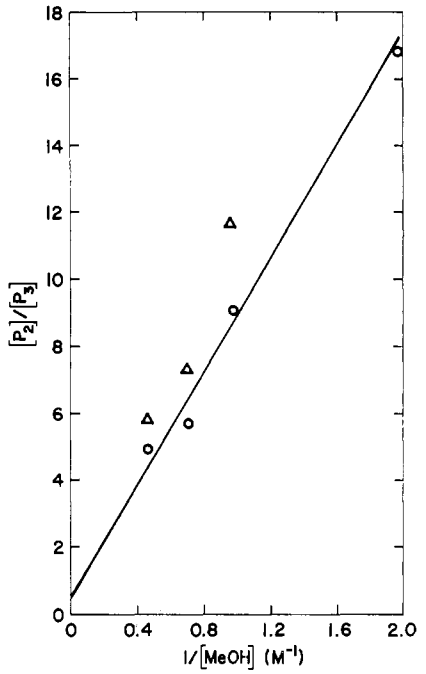


FIGURE 2: Product ratio for the participation of methanol in the papain-catalyzed hydrolysis of *p*-nitrophenyl *N*-acetyl-L-tryptophanate. (○) From pH-Stat experiments, (△) from product isolation.

TABLE IV: The Effect of Ethanol on the Papain-Catalyzed Hydrolysis of *p*-Nitrophenyl *N*-Acetyl-L-tryptophanate.[a]

[Ethanol] (M)	0	0.37	0.69	1.02	1.37
$k_{cat}^{P_1}$ (sec^{-1})	3.46	3.60	3.87	4.00	3.98
$k_{cat}^{P_2}$ (sec^{-1})	3.45	3.57	3.58	3.79	3.78
K_m (M × 10^4)[b]	0.94	0.99	1.10	1.13	1.16
c	1.00	1.11	1.10	1.30	1.44
$k_{cat}^{P_1}/K_m$[d,e]	3.68	3.63	3.52	3.34	3.43
f	3.50	3.49	3.41	3.46	3.45

[a] The conditions are given in the Experimental Section. [b] Determined spectrophotometrically. [c] Determined titrimetrically. [d] $sec^{-1}M^{-1} \times 10^{-4}$. [e] [S] > K_m. [f] [S] < K_m.

in k_{cat}/K_m with increasing ethanol concentration, to a decrease in k_{cat} and K_m and an increase in k_{cat}/K_m with increasing concentrations of pentanol. The addition of 2-pentanone (0.3 M) decreased k_{cat} from 3.48 to 2.49 sec^{-1}, while K_m remained constant. The simultaneous effect of 1-pentanol and 2-propanol is shown in Figure 3.

The effect of a number of amines was also investigated. The high pK_a values for most of these amines result in a mixture of the free-base and protonated forms in the pH range of maximum activity of papain. Preliminary investigations indicated that different effects on k_{cat} and K_m and k_{cat}/K_m were obtained with different amines. For example, L-tryptophanamide[1] caused an increase in each of the parameters as shown in Table VIII, whereas methylamine caused a decrease in k_{cat} and K_m, with their ratio remaining constant (Table IX).

In the case of L-tryptophanamide, the product resulting

[1] The increased rate of hydrolysis of *p*-nitrophenyl *N*-acetyl-L-tryptophanate in the presence of L-tryptophanamide was first observed by Dr. A. Thomson in this laboratory.

TABLE V: The Effect of 1- and 2-Propanol on the Papain-Catalyzed Hydrolysis of *p*-Nitrophenyl *N*-Acetyl-L-tryptophanate.[a]

[1-Propanol] (M)	0	0.27	0.54	0.80	1.07
$k_{cat}^{P_1}$ (sec^{-1})	3.42	3.50	3.55	3.64	3.54
$k_{cat}^{P_2}$ (sec^{-1})	3.56	3.50	3.44	3.36	3.15
K_m (M × 10^4)[b]	1.00	1.08	1.15	1.15	1.08
c	0.99	1.06	1.09	1.14	1.07
$k_{cat}^{P_1}/K_m$[d,e]	3.42	3.26	3.09	3.13	3.19
f	3.50	3.35	3.14	3.22	3.24
[2-Propanol] (M)	0.50	1.0			
$k_{cat}^{P_1}$ (sec^{-1})	3.63	3.60			
K_m (M × 10^4)[b]	1.10	1.15			
$k_{cat}^{P_1}/K_m$[d,e]	3.30	3.13			

[a] The conditions are given in the Experimental Section. [b] Determined spectrophotometrically. [c] Determined titrimetrically. [d] $sec^{-1}M^{-1} \times 10^{-4}$. [e] [S] > K_m. [f] [S] < K_m.

TABLE VI: The Effect of 1-Butanol on the Papain-Catalyzed Hydrolysis of *p*-Nitrophenyl *N*-Acetyl-L-tryptophanate.[a]

[1-Butanol] (M)	0	0.25	0.50	0.75	1.00
$k_{cat}^{P_1}$ (sec^{-1})	3.45	3.20	2.96	2.84	2.69
$k_{cat}^{P_2}$ (sec^{-1})	3.30	2.97	2.69	2.22	2.00
K_m (M × 10^4)[b]	0.96	0.88	0.80	0.76	0.67
c	0.94	0.87	0.73	0.66	0.62
$k_{cat}^{P_1}/K_m$[d,e]	3.60	3.63	3.69	3.80	4.03
f	3.50	3.57	3.59	3.75	3.75

[a] The conditions are given in the Experimental Section. [b] Determined spectrophotometrically. [c] Determined titrimetrically. [d] $sec^{-1}M^{-1} \times 10^{-4}$. [e] [S] > K_m. [f] [S] < K_m.

TABLE VII: The Effect of 1-Pentanol on the Papain-Catalyzed Hydrolysis of *p*-Nitrophenyl *N*-Acetyl-L-tryptophanate.[a]

[1-Pentanol] (M)	0	0.075	0.15	0.225	0.30
$k_{cat}^{P_1}$ (sec^{-1})	3.45	3.06	2.83	2.66	2.46
$k_{cat}^{P_2}$ (sec^{-1})	3.40	3.05	2.59	2.33	2.18
K_m (M × 10^4)[b]	0.96	0.81	0.72	0.62	0.55
c	0.94	0.82	0.74	0.62	0.59
$k_{cat}^{P_1}/K_m$[d,e]	3.60	3.80	3.95	4.27	4.50
f	3.50	3.64	3.79	4.15	4.53

[a] The conditions are given in the Experimental Section. [b] Determined spectrophotometrically. [c] Determined titrimetrically. [d] $sec^{-1}M^{-1} \times 10^{-4}$. [e] [S] > K_m. [f] [S] < K_m.

TABLE VIII: The Effect of *N*-Acetyl-L-tryptophanamide on the Papain-Catalyzed Hydrolysis of *p*-Nitrophenyl *N*-Acetyl-L-tryptophanate.[a]

pH 7.90				
[L-Tryptophanamide][b] (M)	0	0.67	3.35	6.67
$k_{cat}^{P_1}$ (sec^{-1})	3.38	5.70	15.30	23.5
K_m (M $\times 10^4$)	1.20	1.74	3.44	5.04
$k_{cat}^{P_1}/K_m$[c]	2.82	3.27	4.45	4.67
pH 7.30				
[L-Tryptophanamide][b] (M)	0	0.33	0.83	1.67
$k_{cat}^{P_1}$ (sec^{-1})	3.34	4.35	5.61	7.21
K_m (M $\times 10^4$)	1.25	1.27	1.31	1.45
$k_{cat}^{P_1}/K_m$[c]	2.69	3.44	4.33	5.01

[a] The conditions are given in the Experimental Section. [b] M $\times 10^2$, free-base concentration, based on $pK_a = 7.60$ (Brubacher, 1966). [c] $sec^{-1}M^{-1} \times 10^{-4}$.

TABLE IX: The Effect of Methylamine on the Papain-Catalyzed Hydrolysis of *p*-Nitrophenyl *N*-Acetyl-L-tryptophanate.[a]

[Methylamine][b] (M)	0	0.41	1.02	2.04	4.08	8.16
$k_{cat}^{P_1}$ (sec^{-1})	2.84	2.84	2.97	3.18	2.36	1.62
K_m (M $\times 10^4$)	1.23	1.21	1.30	1.56	0.91	0.65
$k_{cat}^{P_1}/K_m$[c]	2.31	2.35	2.29	2.04	2.59	2.50

[a] The conditions are given in the Experimental Section. [b] M $\times 10^2$, free-base concentration, based on $pK_a = 10.56$ (Brubacher, 1966). [c] $sec^{-1}M^{-1} \times 10^{-4}$.

from attack by the amide on the acyl-enzyme, *N*-acetyl-L-tryptophan-L-tryptophanamide, was identified chromatographically and could be isolated by gel filtration on Sephadex G-10. Further details on the effects of these and other amines will be reported later.

The effect of methanol on k_{cat} and K_m in the papain-catalyzed hydrolysis of *p*-nitrophenyl CBz-L-lysinate is shown in Table X.

Discussion

Brubacher and Bender (1966) have shown that the effect of added nucleophiles on the acyl-enzyme *trans*-cinnamoyl-papain can be interpreted by the scheme of eq 2, where ES′ is the acyl-enzyme, ES′W and ES′N are complexes involving noncovalently bound water and nucleophile, respectively,

$$\begin{array}{l} K_W[W] \rightleftharpoons ES'W \xrightarrow{k_3} E + P_2 \\ ES' \\ K_N[N] \rightleftharpoons ES'N \xrightarrow{k_4} E + P_3 \end{array} \qquad (2)$$

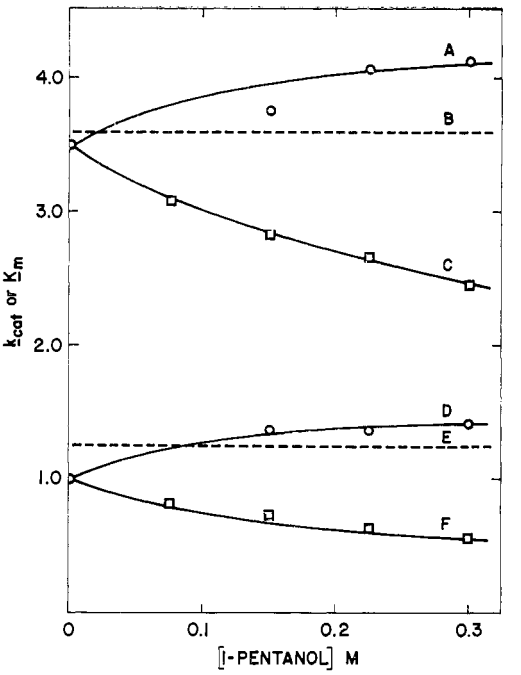


FIGURE 3: The effect of a mixture of 1-pentanol (0–0.3 M) and 2-propanol (1.0 M) on the papain-catalyzed hydrolysis of *p*-nitrophenyl *N*-acetyl-L-tryptophanate. (A) $k_{cat}^{P_1}$ (sec^{-1}). (B) $k_{cat}^{P_1}$ for 2-propanol (1.0 M) alone. (C) $k_{cat}^{P_1}$ (sec^{-1}) for 1-pentanol in the absence of 2-propanol. (D) K_m (M $\times 10^4$) for 1-pentanol and 2-propanol (1.0 M). (E) K_m (M $\times 10^4$) for 2-propanol (1.0 M). (F) K_m (M $\times 10^4$) for 1-pentanol in the absence of 2-propanol.

TABLE X: The Effect of Methanol on the Papain-Catalyzed Hydrolysis of *p*-Nitrophenyl CBz-L-lysinate.[a]

[Methanol] (M)	0	0.5	1.0	1.5	2.0
$k_{cat}^{P_1}$ (sec^{-1})	37.7	39.9	40.7	40.6	43.5
K_m (M $\times 10^6$)	3.16	3.42	3.54	3.65	4.09
$k_{cat}^{P_1}/K_m$[b]	1.19	1.16	1.15	1.12	1.07

[a] The conditions are given in the Experimental Section. [b] $sec^{-1}M^{-1} \times 10^{-7}$.

K_W and K_N are the dissociation constants for these complexes, k_3 and k_4 are the first-order rate constants for breakdown of the complexes, and P_2 and P_3 are the products resulting from attack on the acyl-enzyme by water and nucleophile, respectively. The extension of this type of investigation to the case of specific substrates of papain such as *p*-nitrophenyl *N*-acetyl-L-tryptophanate and *p*-nitrophenyl CBz-L-lysinate is more complicated, since the rate of deacylation is too fast to allow isolation of the acyl-enzyme in the pH range of maximum catalysis. The effect of added nucleophiles must therefore be studied either in the presence of the substrate and substrate leaving group (turnover conditions), or by rapid reaction techniques with either the substrate or the free-acid analog of the substrate (substrate minus the leaving group). The present study has been done using the former method. By using a fixed concentration of organic solvent, the con-

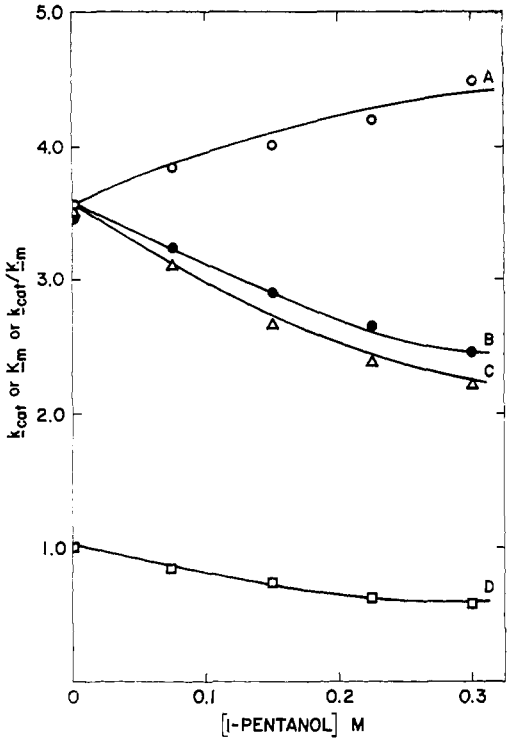


FIGURE 4: Comparison of the experimental results for the effect of 1-pentanol on the papain-catalyzed hydrolysis of *p*-nitrophenyl *N*-acetyl-L-tryptophanate with those calculated from Scheme I. The curves are drawn according to Scheme I with $\alpha = 1.67$, $\beta = 0$, $k_4 = 0.65$, and $K_n = 0.5$. (A) $k_{cat}^{P_1}/K_m$ (sec^{-1} $M^{-1} \times 10^{-4}$), (B) $k_{cat}^{P_1}$ (sec^{-1}), (C) k_{cat}^{P} (sec^{-1}), (D) K_m ($M \times 10^4$).

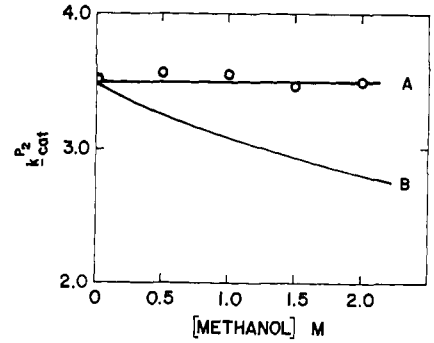


FIGURE 5: The effect of methanol on $k_{cat}^{P_2}$ for the papain-catalyzed hydrolysis of *p*-nitrophenyl *N*-acetyl-L-tryptophanate. Experimental values: (A) theoretical curve for $\beta = 1.0$ in Scheme I, (B) theoretical curve for $\beta = 0$ in Scheme I.

centration of water was kept constant, thus eliminating effects caused by varying concentrations of water.

Confirmation for the acyl-enzyme mechanism of eq 1 is found in the results for the addition of methanol (Table I). The addition of methanol causes an increase in both $k_{cat}^{P_1}$ and K_m, while the $k_{cat}^{P_1}/K_m$ ratio remains constant. These results are incompatible with a simple one-step scheme, since in such a case K_m would be independent of [N] and $k_{cat}^{P_1}/K_m$ would be dependent upon [N]. The possibility of a significant amount of the reaction occurring in the ES complex (bypassing the acyl-enzyme) has been considered. Based on the stoichiometric burst observed with papain and *p*-nitrophenyl *N*-acetyl-L-tryptophanate, and on the relative nucleophilicity of the added nucleophiles (to water, as measured by their effects on the rate of spontaneous hydrolysis), such an occurrence may be ruled out.

The reported results are based on experiments that were repeated at least three times. The values of k_{cat} and K_m obtained from spectrophotometric measurements usually showed a 3% range from the mean value, while those from titrimetric measurements were usually within a 5% range. All of the results have a confidence level of >99% as determined by the Student's t test. No nonrandom deviations from Michaelis–Menten kinetics were observed. The correlation coefficients for the Lineweaver–Burk plots were usually >0.999, while those for plots of *v vs. v/S* were usually >0.995. The standard deviations determined for the *v vs. v/S* plots were of the order of 1% for k_{cat}, and 3% for K_m for spectrophotometric measurements, and 2% for k_{cat} and 4% for K_m for titrimetric measurements. We feel that the experimentally determined values of k_{cat} and K_m are accurate within ±10%.

It has been shown that $k_2 \gg k_3$ for the papain-catalyzed hydrolysis of *p*-nitrophenyl *N*-acetyl-L-tryptophanate, since a stoichiometric burst is observable at low pH (Bender *et al.*, 1966). The k_2/k_3 ratio would be expected to have decreased by a factor of 2 at pH 7.0 (Brubacher and Bender, 1966). Thus in the absence of added nucleophile, the deacylation will be the rate-limiting step. As the concentration of added nucleophile is increased, the rate of deacylation will increase (provided the nucleophile participates in the deacylation) until it exceeds that of acylation. At this point the rate will become independent of [N] since the rate-limiting step will be acylation. The results for the addition of L-tryptophanamide to a concentration of 6×10^{-2} M (free base) confirm that $k_2 \gg k_3$, since $k_{cat}^{P_1}$ increases by an eightfold factor over k_3[W] without becoming independent of [N].

The increase in $k_{cat}^{P_1}$ together with the presence of acyl-nucleophile products is evidence for the participation of the added nucleophile in the deacylation. The lack of concurrence between the observed results (with the exception of methanol and possibly ethanol) and those expected from a simple partitioning of the acyl-enzyme between water and nucleophile suggests that the nucleophile is bound to the enzyme prior to participating in the deacylation. Since the observed effects on the kinetics of the reaction vary with the structure of the nucleophile and do not parallel its reactivity in similar nonenzymatic reactions, the enzyme must be exhibiting specificity toward the added reagent. In the case of the alcohols large differences in the effects on k_{cat} and K_m are observed for correspondingly small chemical changes in the added nucleophile. These results, when taken in conjunction with those obtained with *trans*-cinnamoyl-papain (Brubacher and Bender, 1966, 1967), indicate that the mode of binding to the enzyme results in specificity toward the added nucleophile.

From the postulated mechanistic identity of acylation and deacylation, and from the known restricted structural requirements of substrates, we may conclude that during the deacylation the added nucleophile is spatially oriented and positioned in a manner very similar to that of P_1 in the acylation reaction, and thus occupies the same site as P_1, the substrate leaving group.

Dependence of $k_{cat}^{P_1}/K_m$ upon [N] was observed for several of the nucleophiles; the results for 1-pentanol are shown in

SCHEME I

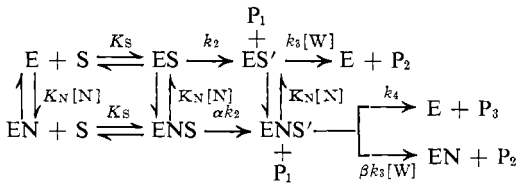


Figure 4. Such a dependence cannot be explained by participation of the nucleophile in the deacylation, but requires the interaction of the nucleophile to be prior to acyl-enzyme formation. Specifically, the results indicate the existence of a ternary enzyme–substrate–nucleophile complex. Such a complex implies binding of the nucleophile to the enzyme. Furthermore, the effect on the $k_{cat}^{P_1}/K_m$ ratio means that the bound nucleophile causes changes in the acylation rate and/or the dissociation constant of the substrate from the enzyme complex. Further evidence for the formation of such ternary complexes is found in the noncompetitive inhibition exhibited by 2-pentanone.

If the added nucleophile can bind to the acyl-enzyme and the enzyme–substrate complex, it would seem reasonable to expect that the nucleophile may also bind to the free enzyme. The formation of such a complex would result in competitive inhibition. The lack of observed saturation in the case of the addition of nucleophiles to *trans*-cinnamoyl-papain (Brubacher and Bender, 1966) suggests that the binding may be quite weak and therefore kinetically unobservable under the conditions used in the present study. The absence of an enzyme–nucleophile complex results in kinetic expressions that do not provide for an increase in K_m with increasing [N]. Thus the observed increase in K_m (see Table VIII) indicates the existence of such a complex.

The formation of an enzyme–substrate–nucleophile complex prior to acylation shows that the nucleophile can bind to the enzyme in the presence of the P_1 group of the substrate. One might therefore expect that in the deacylation, when water takes the place of P_1, that the nucleophile may also bind to the enzyme but not in the P_1 site. To achieve satisfactory agreement between the calculated and observed results it is necessary to include such an expression in the over-all reaction. For example, in the case of methanol it is possible to obtain kinetic parameters that give very good correlation between experimental and calculated values for $k_{cat}^{P_1}$ and K_m, either with or without such an additional reaction. However, only the expressions taking the reaction of water in the presence of the nucleophile into consideration give values of $k_{cat}^{P_2}$ that agree with the experimental values (Figure 5).

The simplest scheme accommodating all the observations is shown in Scheme I, where K_S and K_N are the dissociation constants for substrate and nucleophile from the enzyme complexes; α is a factor to account for any changes in the rate of acylation due to the added nucleophile; and β is a factor to account for any attack by water on the acyl-enzyme when the nucleophile is bound to the enzyme. The scheme has been simplified by the omission of a specific binding site for water. In the following expressions this could be taken into account by replacing [W] with [W]/K_W, where K_W is the dissociation constant for water from the acyl-enzyme–water complex. Furthermore, an additional step, that of dissociation of P_1 from the acyl-enzyme and acyl-enzyme–nucleophile complex, has been omitted for clarity. Since *p*-nitrophenol at concentrations $\leq[S_0]$ has been found to have a negligible effect on the papain-catalyzed hydrolysis of *p*-nitrophenyl *N*-acetyl-L-tryptophanate, it may be concluded that in the present experiments the concentration of the enzyme species containing bound P_1 was negligible. However, the concentration of the enzyme–P_1 complex(es) would be dependent upon the dissociation constant for P_1, as well as the concentration of P_1. If P_1 binds well to the enzyme, such a term could be significant. The expressions for the Michaelis parameters for Scheme I for the case where $k_2 \gg k_3[W] + k_4$ are seen in eq 3.

TABLE XI: The Effect of Alcohols on the Papain-Catalyzed Hydrolysis of *p*-Nitrophenyl *N*-Acetyl-L-tryptophanate.[a]

Alcohol	α	β	k_4 (sec^{-1})	K_N (M)	k_{ROH}/k_{H_2O}
Methanol	1.0	1.0	5.5	8.7	79
Ethanol	0.8	1.0	2.0	3.0	29
1-Propanol	0.85	0.8	1.0	1.45	14
1-Butanol	1.15	0.45	0.65	0.75	9
1-Pentanol	1.67	0	0.65	0.5	9

[a] From Scheme I.

$$k_{cat}^{P_1} = \frac{\frac{[N]^2}{K_N^2}(\alpha k_4 + \alpha\beta k_3[W]) + \frac{[N]}{K_N}(k_4 + \alpha k_3[W] + \beta k_3[W]) + k_3[W]}{\frac{[N]^2}{K_N^2} + \frac{[N]}{K_N}(\alpha + 1) + 1} \quad (3)$$

$$k_{cat}^{P_2} = \frac{\frac{[N]^2}{K_N}\alpha\beta k_3[W] + \frac{[N]}{K_N}(\alpha + \beta)k_3[W] + k_3[W]}{\frac{[N]^2}{K_N} + \frac{[N]}{K_N}(\alpha + 1) + 1} \quad (3a)$$

$$K_m = \frac{K_S[k_3[W] + \frac{[N]}{K_N}(\beta k_3[W] + k_4)]}{k_2\left[1 + \frac{[N]}{K_N}\alpha\right]} \quad (3b)$$

The ratio of products

$$\frac{[P_2]}{[P_3]} = \frac{\beta k_3[W]}{k_4} + \frac{k_3[W]K_N}{k_4}\frac{1}{[N]} \quad (3c)$$

There are thus four independent equations with four unknown parameters. By a curve-fitting procedure utilizing the expression and data for $k_{cat}^{P_1}$, values for these parameters were obtained and are shown in Table XI. Figure 6 shows the variation in these parameters with increasing chain length of the alcohol.

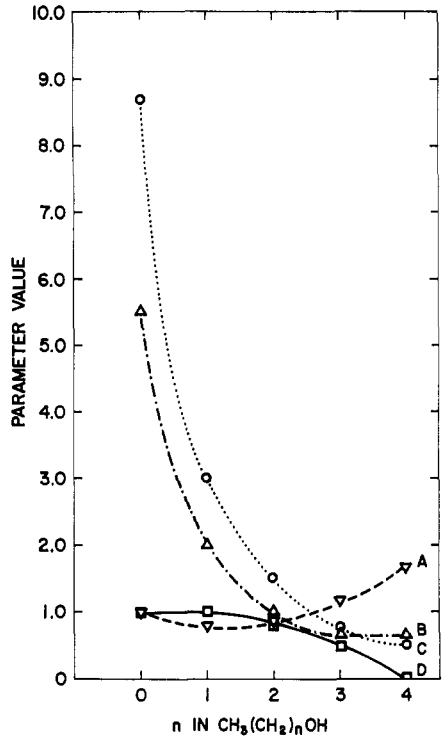


FIGURE 6: The effect of alcohol chain length on the values of the parameters of Scheme I. A = α, B = β, C = K_N, and D = β.

Very good correlations between the calculated curves and the experimental values for the effect of N on $k_{cat}^{P_1}$, $k_{cat}^{P_2}$, and K_m and their ratios were obtained, as seen in Figures 4 and 5. Table XII shows the calculated and experimental values for the product analysis plots. With the exception of ethanol, the experimental values agree within experimental error with the calculated values. The large experimental error in the P_2/P_3 ratio results from the division of a small difference between two large numbers by a large number. From our current knowledge of the rigorous steric requirements in the active center it seems unlikely that the situation can be quite as simple as suggested by Scheme I.

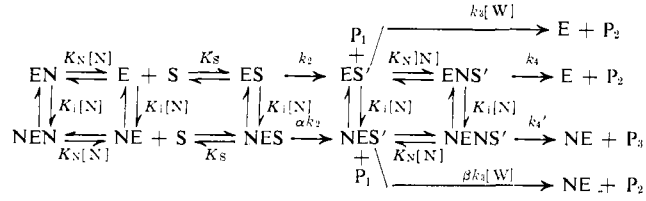
We have shown that in the deacylation the added nucleophile occupies the same area as P_1 in acylation; however, in the enzyme–substrate–nucleophile complex the nucleophile cannot occupy the P_1 site, since the leaving group is still present on the substrate. Thus there must be an additional area in the vicinity of the active center such that binding of a small molecule to it can perturb either the position of the substrate, or of the catalytically important group(s) of the enzyme, or both, resulting in a more (or less) favorable orientation for reaction. Evidence for the existence of two such modes of binding for the added nucleophile is found in the results of the competition experiment between 2-propanol and 1-pentanol (Figure 3). If there was only one significant binding site for the added nucleophile, one would expect that the simultaneous effect of the two alcohols would result in values of k_{cat} and K_m consistent with the concentrations and dissociation constants of the two alcohols, and in no case could the effect be greater than that caused by either alcohol alone. The observed synergistic effect suggests the presence on the enzyme of two binding sites for the added alcohols, one of which preferentially binds 1-pentanol, the other binds 2-propanol (*i.e.*, binding site specificity), such that the combined effect exceeds that of the individual alcohol. The effects of a nucleophile bound to this second site may manifest themselves as effects on either K_S (substrate binding) or k_2 (substrate reactivity), or both.

TABLE XII: Alcoholysis Rates and Dissociation Constants for the Effects of Alcohols on the Papain-Catalyzed Hydrolysis of *p*-Nitrophenyl *N*-Acetyl-L-tryptophanate.[a]

Alcohol	K_N/k_4 (M sec^{-1})	
	b	*c*
Methanol	2.0 ± 0.7	1.6
Ethanol	3.7 ± 1.0	1.5
1-Propanol	2.2 ± 0.6	1.5
1-Butanol	1.3 ± 0.3	1.2
1-Pentanol	0.6 ± 0.2	0.8

[a] According to Scheme I. [b] From $[P_2]/[P_3]$. [c] Calculated from the values of Table XI.

SCHEME II



If all this information is assembled as the basis for a reasonable chemical hypothesis, one must postulate the existence of certain species which are not kinetically important. Thus nonkinetic methods will probably be necessary to conclusively demonstrate their existence. The situation may be visualized as in Scheme II, where K_N is the dissociation constant for nucleophile bound to the P_1 site, and K_i is the dissociation constant for nucleophile bound in the other site. The expressions for k_{cat} and K_m for Scheme II are similar in form to those of Scheme I, but involve some additional terms. The parameters for Scheme I may be taken to represent the approximate values for those of Scheme II if K_i and K_N are of similar magnitude (*i.e.*, K_N (Scheme I) = $(K_i + K_N)/2$ (Scheme II) and k_4 (Scheme I) = $k_4' + k_4$ (Scheme II)). The value of K_i for noncompetitive inhibition by 2-pentanone is very similar to that observed for K_N for 1-pentanol in Scheme I and hence supports Schemes I and II.

Although experiments of the type reported here cannot provide conclusive proof that Scheme II is the actual mechanism, its ability to explain the observed results warrants the consideration of Scheme II as a useful working hypothesis, especially in the design of future experiments.

Evidence for multiple binding sites in papain has been reported by Schecter and Berger (1967) and Hill (1965). Binding of *N*-Bz-L-ArgEt to papain in a nonproductive mode has also been reported (Brocklehurst *et al.*, 1968), providing

further support for the concept of a binding site additional to the minimum necessary for "proper" substrate orientation. Proflavin has been found to bind to a hydrophobic region of ficin (a sulfhydryl protease with many similarities to papain) and to act as a noncompetitive activator in the hydrolysis of *N*-Bz-L-ArgEt (Hollaway, 1968). Such noncompetitive behavior also reflects the existence of a ternary enzyme–substrate–modifier complex. Data suggesting binding of urea to a site in the vicinity of the acyl moiety of the substrate has also been reported (Sluyterman, 1967). Although 66% methanol causes loss of activity, it causes no major conformational change (Sluyterman, 1967). In addition to the previously mentioned case of *trans*-cinnamoyl-papain, there are several other reported examples of enzymes exhibiting specificity toward added nucleophiles; for example, furoyl-chymotrypsin (Inward and Jencks, 1965), chymotrypsin (Ponzi and Hein, 1966), and trypsin (Seydoux *et al.*, 1969).

The experiments with the alcohols were performed at pH 7.0 where both k_{cat} and K_m are least sensitive to changes in pH (Brubacher, 1966). Similarly, solvent-mediated effects on the pK_a's of the catalytic groups of papain would be expected to be minimal in this pH range. It is very unlikely that such pK_a shifts would be responsible for the observed results, since Sluyterman (1967) has reported that 50% methanol causes only a very slight shift in the pH optimum of papain. This observation is in accord with that from the addition of alkylguanidines to the trypsin-catalyzed hydrolysis of ethyl *N*-acetylglycinate in which most of the activation observed is not due to the shift in pK_a (*ca.* 0.4 pH unit), but to an increase in the entropy of activation (Inagami and York, 1968).

As well as keeping the total concentration of organic solvent (acetonitrile + alcohol) constant, the changes in the dielectric constant of the medium were kept to a minimum (75.9 ± 0.4). Thus the observed effects of the alcohols cannot be caused by changes in the dielectric constant of the medium. According to Scheme I the dissociation constants for the binding of 1-butanol and 1-pentanol are the same; in other words, both show the same affinity for the binding site. The preferential partitioning of the more apolar alcohols to a hydrophobic area of the enzyme may consequently be ruled out, since the partitioning coefficients for 1-butanol and 1-pentanol between water and 1-octanol (an excellent model for partitioning to proteins) differ by a factor of 3 (Hansch *et al.*, 1968).

Certain trends in the results for the addition of the alcohols are apparent. The reactivity (as measured by k_4) of the alcohol toward the acyl-enzyme decreases with increasing chain length of the alcohol (Table XI). The observed change in reactivity with increasing chain length is in the opposite direction to that expected in the case of nucleophilic attack on a thiolester. We interpret this to indicate that steric or other effects predominate over the inductive effect in the attack on the acyl-enzyme. The dissociation constants, K_N, for the enzyme–nucleophilic species also show a decrease with increasing chain length of the alcohol (Table X). The increased binding with increasing apolar alcohol chains suggests that the major force involved in the binding is hydrophobic interaction. The present results do not allow positive identification of a specific binding site for water. However, extrapolation of the dissociation constants for the alcohols to zero chain length suggests that the dissociation constant for water would be quite large (>10 M). The values obtained for K_N for the alcohols from Scheme I cover a tenfold range from 0.75 to 8.7 M, and those for k_4 cover a similar range from 0.65 to 5.5 sec^{-1}, giving rise to alcoholysis/hydrolysis ratios from 9.3 (1-pentanol) to 79 (methanol) (Table XI). The methanolysis/hydrolysis ratio for *trans*-cinnamoyl-papain was found to be 59 (Brubacher and Bender, 1966). The following ratios have been reported for hippuryl-papain: k_{MeOH}/k_{H_2O} = 9.5 (Lake and Lowe, 1966); k_{MeOH}/k_{H_2O} = 30, and k_{EtOH}/k_{H_2O} = 12 to 15 (Henry and Kirsch, 1967). In Scheme I the decrease in β from 1 for methanol to 0 for 1-pentanol is taken to indicate that when a molecule of pentanol is bound to the enzyme, water is completely excluded from any position where it could attack the acyl-enzyme. However, with methanol, the mode of binding is such that even if a molecule of alcohol is bound, water is still able to react with the acyl-enzyme. The variation of α in Scheme I with chain length suggests that the binding of ethanol and 1-propanol in the second binding site causes changes in the active-site environment that result in a less favorable orientation of substrate and enzyme catalytic groups than in the absence of the alcohols. The effect of 1-butanol and 1-pentanol is in the opposite direction, *i.e.*, the groups are forced into more favorable positions.

The presence of binding sites for the added nucleophile should result in the observation of saturation phenomena at high nucleophile concentrations. Indeed, in the present work, the rates of change of k_{cat} and K_m with [N] do show some decreasing tendencies at high [N]. The values of the dissociation constants from Scheme I indicate that the lack of observed saturation in this reaction and in that with *trans*-cinnamoyl-papain (Brubacher and Bender, 1966) may be due to the use of added nucleophiles of insufficient binding affinity. Confirmation of these ideas must await experiments involving the addition of nucleophiles having higher affinity for the enzyme.

The interpretation of the results upon addition of L-tryptophanamide is complicated by the presence of both the free-base and protonated species. The protonated form may have some effect on the reaction since the results based on the concentration of free base at two different pH values are not identical (Table VIII).

The existence of a binding site for the substrate leaving group would be expected to result in a variation in K_S in a series of substrates with different leaving groups, but with the same acyl moiety. It would also be anticipated that the contribution to K_S would also vary with the acyl group. Thus the reported differences in substrate reactivity being due to k_2 in some instances and K_S in others are not irreconcilable (Hubbard and Kirsch, 1968; Brubacher and Bender, 1966). Investigations are continuing in order to answer some of the questions raised in the present study.

At the present time most evidence appears to favor general base catalysis in the deacylation step of papain, the general base being either imidazole or carboxylate (both of which are close to the reactive thiol (Drenth *et al.*, 1968)). An alternative possibility that does not yet seem to have been proposed involves general base catalysis by carboxylate on imidazole, which in turn acts as a general base in both acylation and deacylation, analogous to the case postulated for chymotrypsin (Blow *et al.*, 1969). Since the alcohols are completely protonated at the pH used in these experiments, their partici-

pation in the deacylation indicates that general base catalysis must be involved.

Conclusion

The results confirm the existence of an acyl-enzyme intermediate in the kinetically significant reaction pathway in the papain-catalyzed hydrolysis of *p*-nitrophenyl *N*-acetyl-L-tryptophanate. The added nucleophiles are bound by the acyl-enzyme and participate in the deacylation by a process involving general base catalysis. By analogy the substrate leaving group must also bind in this same mode and thus specificity may be exhibited toward the substrate leaving group. The added nucleophile can also bind in another mode in which it may affect the rate of acylation. An increase greater than eightfold in $k_{cat}^{P_1}$ caused by low concentrations of L-tryptophanamide (*ca.* 10^{-2} M free base) indicates the efficiency and specificity of the binding. Thus papain is unique among the commonly studied proteolytic enzymes since by virtue of such binding, which is not found with chymotrypsin, for example, it may have potential use in peptide synthesis. Furthermore the specificity of the two binding modes shown toward the added nucleophiles (for example, the competition experiment between 1-pentanol and 2-propanol) suggests the possibility of synthesizing optically active acyl derivatives from compounds such as 2-butanol or other asymmetric alcohols or amines. The results which are best interpreted by Schemes I and II, indicate the affinity of the enzyme for the added nucleophile to increase with increasing hydrophobic nature of the nucleophile. The reactivity of the added nucleophile appears to bear no relation to its basicity. In the case of added amines, the major effect is due to the free-base form; however, additional effects may be caused by the protonated species.

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