14-epicassaidate 2-acetate in 1 ml of acetic acid and 1 ml of ethyl acetate at 0° was treated with approximately four times the theoretical quantity of ozone. This solution was allowed to stand cold for 20 min and was then stirred for 30 min with 0.4 g of powdered zinc at room temperature. Filtration and concentration of the filtrate under reduced pressure afforded an oily residue which was purified on a silica-coated chromatoplate using pure ether for development.

The less polar of the two ultraviolet-absorbing bands from the plate was the less intense of the two bands and afforded ~0.5 mg of **2β-hydroxy-8β-methylpodocarpane-7,9-dione acetate (6)** which had an $R_f$ value (on silica with 100% ether) and an infrared spectrum identical with those of an authentic sample.

The more polar band from the chromatoplate contained the major product from the reaction, **2β,9β-dihydroxy-8β-methylpodocarpan-7-one 2-acetate (18)**. This oil, shown to be 95–97% pure by tlc, was dissolved in 2 ml of methanol and 0.5 ml of 2 *N* aqueous sodium hydroxide, and the solution was heated under reflux for 1.5 hr. It was concentrated by warming in a stream of nitrogen, and the residue was extracted three times with ether. The crude product from these extracts was purified on a silica chromatoplate developed with tetrahydrofuran–methylene dichloride (1:9). The main band from the chromatoplate was extracted, and the oily product from it was dissolved in 3 drops of tetrahydrofuran. Hexane was added in increments with boiling until the tetrahydrofuran was substantially all removed. Cooling caused precipitation of an amorphous solid which crystallized when seeded with **2β,9β-dihydroxy-8α-methylpodocarpan-7-one (4)**. The perhaps 0.5 mg of crystalline product melted at 176–181° (authentic **4** melts at 180–182°), and its infrared spectrum was superimposable upon that of authentic 2β,9β-dihydroxy-8α-methylpodocarpan-7-one. The $R_f$ value of this product on a silica chromatoplate was also identical with that of **4**.

**2β,9α-Dihydroxy-8β-methylpodocarpan-7-one (21) from Methyl 7,14-Diepicassaidate 3-Acetate (20).** Methyl 7,14-diepicassaidate 3-acetate (15 mg) was subjected to ozonolysis in the manner just described for methyl 14-epicassaidate 3-acetate (**17**). The less polar of the two ultraviolet-absorbing bands contained the minor product (1.5 mg) which was recrystallized from ether–pentane to give colorless plates, mp 148–150° and undepressed upon admixture with an authentic sample of **2β-hydroxy-8β-methylpodocarpane-7,9-dione acetate (6)**. The infrared spectra and $R_f$ values for the two compounds were identical.

The more polar band contained the major product, presumably **2β,9α-dihydroxy-8β-methylpodocarpan-7-one 2-acetate** (acetate of **21**), which was an oil with an $R_f$ value greater than that of 2β,9β-dihydroxy-8α-methylpodocarpan-7-one 2-acetate (**3**). Its infrared spectrum was quite sharp and compatible with the assigned structure. This oily acetate (4.5 mg) was then hydrolyzed by heating it with 2 ml of methanol and 0.5 ml of 2 *N* aqueous sodium hydroxide for 1.5 hr. Concentration of the reaction under nitrogen followed by extraction of the product with ether gave oily **2β-9α-dihydroxy-8β-methylpodocarpan-7-one (21)** which was >98% pure at this stage of purification. The remaining impurity was removed on a chromatoplate using tetrahydrofuran–methylene dichloride (1:9) for development. The pure oil showed an infrared spectrum compatible with the assigned structure (**21**) and definitely different from that of 2β,9β-dihydroxy-8α-methylpodocarpan-7-one (**19** = **4**).

**Acknowledgment.** The authors are indebted to Dr. Ross Pitcher, Varian Associates Application Laboratory, Pittsburgh, Pa., for the 100-Mc nmr spectra. They further express appreciation to the Analytical and Physical Laboratory personnel at this Institute for analytical and spectral determinations.

# The Preparation and Properties of *trans*-Cinnamoyl-Papain[1]

**Lewis J. Brubacher and Myron L. Bender**

*Contribution from the Division of Biochemistry, Department of Chemistry, Northwestern University, Evanston, Illinois 60201.* *Received August 25, 1966*

**Abstract:** *trans*-Cinnamoyl-papain was prepared from the reaction of excess *trans*-cinnamoylimidazole with papain at pH 3.43 followed by gel filtration to remove the excess substrate and products. The difference spectrum of *trans*-cinnamoyl-papain *vs.* papain was determined by an indirect method since this acyl-enzyme is not stable under the conditions of its preparation. The deacylation of *trans*-cinnamoyl-papain is first order in acyl-enzyme and is identical with the rate of appearance of *trans*-cinnamic acid. The rate of the deacylation reaction is dependent on a basic group of $pK_a = 4.69$. In deuterium oxide, the rate is reduced 3.35-fold and the $pK_a$ of the base controlling the reaction is raised 0.34 p*K* unit. Added nucleophiles including both amines and alcohols increase the rate of disappearance of the acyl-enzyme by competing with water for the acyl-enzyme. The second-order rate constants for the reactions of nucleophiles with the acyl-enzyme are larger for amines than for the corresponding alcohols; furthermore, they are dependent on the structure of the alkyl group of the nucleophile. The basicity of the nucleophile appears to be of little importance. This last observation together with the $D_2O$ effect suggest that the group of $pK_a = 4.69$ acts as a general basic catalyst in deacylation.

In 1937 Weiss suggested that papain and other sulfhydryl enzymes catalyze certain hydrolytic and synthetic reactions *via* the intermediate formation of an acyl-thiolenzyme.[2] This suggestion, however, did not fall on fertile ground; only when the acyl-enzyme hypothesis was again suggested[3] in the early 1950's was it seriously tested and found to be valid for several proteolytic enzymes, particularly chymotrypsin.[4]

After a series of kinetic studies on papain-catalyzed reactions Smith and co-workers proposed that the acyl-enzyme mechanism applies to papain,[5] by analogy to another sulfhydryl enzyme, glyoxalase.[6] Gutfreund applied the same hypothesis to ficin-catalyzed reactions

(1) This research was supported by grants from the National Institutes of Health.

(2) J. Weiss, *Chem. Ind.* (London), 685 (1937).

(3) I. B. Wilson, F. Bergmann, and D. Nachmansohn, *J. Biol. Chem.*, **186**, 781 (1950).

(4) M. L. Bender and F. J. Kézdy, *J. Am. Chem. Soc.*, **86**, 3704 (1964).

(5) E. L. Smith, B. J. Finkle, and A. Stockell, *Discussions Faraday Soc.*, 20, 96 (1955).

(6) E. Racker in "The Mechanism of Enzyme Action," W. D. McElroy, and B. Glass, Ed., Johns Hopkins Press, Baltimore, Md., 1954.

Table I. Nucleophiles Used in the Deacylation of *trans*-Cinnamoyl-Papain

| Nucleophile | Source | $n^{25}$D or mp, °C Obsd | $n^{25}$D or mp, °C Lit. | p$K_a$° Obsd | p$K_a$° Lit. |
|---|---|---|---|---|---|
| Ammonia | Baker reagent grade | | | 9.26 | 9.21[a] |
| Methylamine | Fisher reagent grade | | | 10.56 | 10.62[a] |
| Trimethylamine | Eastman White Label | | | 9.79 | 9.76[a] |
| Morpholine | *a* | 1.4512 | 1.4545 (20)[d] | 8.55 | 8.36[a] |
| Benzylamine hydrochloride | *b* | 262–263 | | 9.45 | 9.34[a] |
| Benzyl alcohol | Fisher reagent grade | 1.5363 | 1.53955 (20)[e] | | |
| Glycinamide hydrochloride | Mann, lot K1083 | 187–188[r] | 186–189[t] | 7.83 | 7.93[p] |
| L-Tryptophanamide hydrochloride | Cyclo I, lot K-3082 | 251–253 dec | 254–255[q] | 7.60 | 7.5[q] |
| D-Tryptophanamide hydrochloride | Cyclo I, lot V-1205 | 255–256 dec | 254–255[q] | 7.64 | 7.5[q] |
| Tryptamine hydrochloride | Mann, M. A. | 252.5–254.5 dec | 251–253[f] | 10.10 | |
| Tryptophol | Aldrich[c] | 58–59 | 59[g] | | |
| Aminoacetonitrile bisulfate | Aldrich | 121–127 | 121[h] | | 5.3[l] |
| Methoxyamine hydrochloride | Eastman White Label | 128–142 | 149–150[i] | | 4.60[m] |
| Methyl L-tryptophanate hydrochloride | Cyclo I, lot K-3071 | 214.5–215.5 dec | 214[j] | 7.32 | |
| Methyl D-tryptophanate hydrochloride | Cyclo II, lot V-1202 | 214–216 dec | | 7.32 | |
| Imidazole | Eastman White Label | 88–90 | 90[k] | | 6.95[m] |
| 2-Phenylethylamine | Eastman White Label | 1.5296 | 1.575[d] | 9.88 | 9.83[a] |
| 2-Phenylethyl alcohol | Eastman White Label | 1.5299 | 1.5267 (18)[d] | | 9.78[n] |

[a] Redistilled. [b] Prepared by passage of HCl through an ethereal solution of benzylamine. [c] Recrystallized from dry *n*-hexane. [d] R. C. Weast, Ed., "Handbook of Chemistry and Physics," 46th ed, Chemical Rubber Co., Cleveland, Ohio, 1965. [e] C. J. Weast, Ed., "International Critical Tables," McGraw-Hill Book Co., Inc., New York, N. Y., 1933. [f] R. W. Jackson, *J. Biol. Chem.*, **84**, 1 (1929). [g] F. Ehrlich, *Ber.*, **45**, 883 (1912). [h] H. Stephen, *J. Chem. Soc.*, 871 (1931). [i] L. Semper and L. Lichtenstadt, *Ber.*, **51**, 928 (1918). [j] E. Abderhalden and M. Kempe, *Z. Physiol. Chem.*, **52**, 207 (1907). [k] G. Dedichen, *Ber.*, **39**, 1831 (1906). [l] S. Soloway and H. Lipschitz, *J. Org. Chem.*, **23**, 613 (1958). [m] T. C. Bruice and G. L. Schmir, *J. Am. Chem. Soc.*, **80**, 148 (1958). [n] M. M. Tuckerman, J. R. Mayer, and F. C. Nachod, *ibid.*, **81**, 92 (1959). [o] $\mu$ = 0.100. [p] H. R. Almond, Jr., R. J. Kerr, and C. Niemann, *J. Am. Chem. Soc.*, **81**, 2856 (1959). [q] H. T. Huang and C. Niemann, *ibid.*, **73**, 3223 (1951). [r] Recrystallized from ethanol–water. [s] H. K. Hall, Jr., *J. Am. Chem. Soc.*, **79**, 5441 (1957). [t] P. Bergell and H. Wulfing, *Z. Physiol. Chem.*, **64**, 348 (1910).

to explain the identical rates of hydrolysis of the ethyl ester and amide of $\alpha$-N-benzoyl-L-arginine.[7]

Recently the direct observations of two acyl-papains were reported, *trans*-cinnamoyl-papain[8] and thionohippuryl-papain.[9] This paper gives a complete report of the preparation and characterization of *trans*-cinnamoyl-papain. The availability of an isolated acyl-enzyme has made possible a systematic study of a single step in papain-catalyzed reactions, namely the deacylation step. The results of the effect of pH, deuterium oxide, and added nucleophiles on deacylation are presented.

## Experimental Section

**Materials.** The water used throughout this work in the preparation of solutions was doubly distilled, the second distillation being performed in a Corning all-glass distillation apparatus, Model AG-2, following passage of the water through a mixed-bed, ion-exchange column containing Amberlite MB-3. All solid buffers and acids were of reagent or CP grade.

*trans*-Cinnamoylimidazole was prepared by the published method[10] and recrystallized several times from dry *n*-hexane, mp 133.2–133.8°. It was always freshly crystallized before use.

S-*trans*-Cinnamoylcysteine was prepared from *trans*-cinnamoyl chloride and cysteine according to the method used by Zervas, *et al.*[11] A low yield of 2% was obtained due to the rapid hydrolysis of the acid chloride to give *trans*-cinnamic acid which was identified as a major water-insoluble product. The melting behavior of S-*trans*-cinnamoylcysteine, 144–145° (heating rate 1°/30 sec) and 164–167° (heating rate 1°/2 sec), is typical of a zwitterionic compound. After vigorous basic hydrolysis of S-*trans*-cinnamoylcysteine (3 days at 98° in 0.5 *M* NaOH), the reaction mixture exhibits an ultraviolet spectrum with $\lambda_{max}$ 269.5 m$\mu$ in base and 278.5 m$\mu$ in acid, which is typical of *trans*-cinnamic acid. The infrared spectrum of S-*trans*-cinnamoylcysteine shows a peak at 1680 cm$^{-1}$ which is typical of a thiol ester.[12] Furthermore, a facile S → N shift of the *trans*-cinnamoyl group occurs at high pH. All this evidence indicates that the product is indeed S-*trans*-cinnamoylcysteine, $[\alpha]^{22}$D −27 ± 4° (*c* 0.414, 1 *M* HCl). *Anal.* Calcd for $C_{12}H_{13}NO_3S$: C, 57.35; H, 5.21; N, 5.57; S, 12.76. Found: C, 57.30; H, 5.30; N, 5.34; S, 12.69.[13]

$\alpha$-N-*trans*-Cinnamoyltryptophanamide was synthesized as follows. An aqueous solution of L-tryptophanamide–HCl, neutralized and buffered to pH 8.6, was added at room temperature to an anhydrous ether solution containing a 20% excess of *trans*-cinnamoyl chloride. The mixture was stirred vigorously for 7 min while adding 1 equiv of $Na_2CO_3$. During an additional 35-min stirring period, a precipitate formed which was filtered off and added to the ether layer. The ethereal suspension, after washing, was stripped of solvent to yield 400 mg of crude product (58%). Recrystallization from ethanol–water gave a gelatinous precipitate, the filtrate later yielding a small amount of crystalline material of mp 202–203°. *Anal.* Calcd for $C_{20}H_{19}N_3O_2$: C, 72.05; H, 5.74; N, 12.60. Found: C, 70.00; H, 5.76; N, 12.39. Calcd for $C_{20}H_{19}N_3O_2 \cdot 0.5H_2O$: C, 70.16; H, 5.89; N, 12.27.

The nucleophiles used in the deacylation of *trans*-cinnamoyl-papain are listed in Table I which gives also the source, melting point, and p$K_a$ of the conjugate acid. The p$K_a$ values were determined by measuring the pH of a solution 0.0100 *M* in nucleophile ($\mu$ = 0.100) which was exactly half-neutralized by added standard acid or base.

**Papain.** Twice-crystallized papain was purchased from the Worthington Biochemical Corp. and converted to the more soluble mercuripapain according to the following typical procedure.[14] A papain suspension (1 g) in pH 4.5 acetate buffer (total volume 36 ml) was made 0.03 *M* in cysteine and after about 30 min centrifuged at 10,000*g* for 20 min at 2°. The sedimented papain was washed with 2 ml of distilled water, centrifuged, and dissolved in 14 ml of 0.01 *M* $HgCl_2$ giving a total volume of 20.0 ml. Sufficient 95% ethanol was added to give a total volume of 30.0 ml, and the

(7) H. Gutfreund, *Discussions Faraday Soc.*, **20**, 167 (1955).
(8) M. L. Bender and L. J. Brubacher, *J. Am. Chem. Soc.*, **86**, 5333 (1964).
(9) G. Lowe and A. Williams, *Proc. Chem. Soc.*, 140 (1964).
(10) G. R. Schonbaum, B. Zerner, and M. L. Bender, *J. Biol. Chem.*, **236**, 2930 (1961).
(11) L. Zervas, I. Photaki, and N. Ghelis, *J. Am. Chem. Soc.*, **85**, 1337 (1963).
(12) R. A. Nyquist and W. J. Potts, *Spectrochim. Acta*, **15**, 514 (1959).
(13) Analysis performed by MicroTech Laboratories, Skokie, Ill.
(14) M. C. Easley, personal communication.

solution was centrifuged to remove a small amount of undissolved material. The supernatant was made up to 40.0 ml total volume by adding 95% ethanol and allowed to crystallize at 2°. Over a period of 1 week additional increments of 95% ethanol were added to bring the final ethanol concentration up to 70%. Examination of the mercuripapain crystals under 440× magnification showed plates as long as 2–3 cm; sheaves, which are several plates joined together at a common line, were also observed.[15] The mercuripapain crystals were dissolved in 14 ml of 0.05 *M* acetate buffer, pH 5.2, $\mu$ = 0.038, to give a mercuripapain solution approximately $1.5 \times 10^{-3}$ *M* in papain. This represents about a 50% recovery of the initial material. The mercuripapain solutions could be stored at 2° for several months without noticeable loss of activity upon activation by removal of mercury.

Mercuripapain was activated to papain by gently shaking 2–3 ml of the mercuripapain solution with two volumes of a toluene solution of 4-methylbenzenethiol (10 mg/ml) for 5 min.[16] (The mercury is apparently complexed by the thiol and extracted into the toluene layer.) The slightly cloudy aqueous layer was gel filtered through a freshly prepared Sephadex G-25 column (1.5 × 12 cm) fitted with a circle of filter paper on top and equilibrated with 0.05 *M* acetate buffer, pH 5.2, $\mu$ = 0.038. The papain appeared between 8 and 15 ml of eluate volume. Vials of five to ten drops each were collected within this range, assayed by measuring the absorbance at 280 m$\mu$, and combined to give an active papain stock solution of the desired concentration, generally $4–6 \times 10^{-4}$ *M* total protein, based on $\epsilon_{280}$ 51,100 and a molecular weight of 20,700.[17] This solution was stored at 2° in a volumetric flask flushed with nitrogen and sealed with Parafilm. When in use the stoppered flask was kept in ice. A buffer blank carried through this same activation procedure contained no detectable amounts of activator or toluene.

**Spectrum of *trans*-Cinnamoyl-Papain.** Since a large excess of *trans*-cinnamoylimidazole must be used in order to acylate papain to a high degree, the excess substrate and products were removed by Sephadex filtration before observing the spectrum of *trans*-cinnamoyl-papain. To 0.700 ml of 0.05 *M* acetate buffer, $\mu$ = 0.100, pH 3.43, was added 0.500 ml of a papain solution of concentration $3.68 \times 10^{-4}$ *M*. At zero time, 50 $\mu$l of *trans*-cinnamoylimidazole ($1.0 \times 10^{-2}$ *M* in purified acetonitrile[18]) was added. After 300 sec the reaction mixture was placed on a 1.6 × 6.3 cm column of Sephadex G-25, which had been previously equilibrated with the same acetate buffer, and eluted. (Later calculations showed that the enzyme was about 60% acylated.) The eluate fraction between 4.8 and 8.3 ml contains the major part of the partially acylated enzyme. It was placed in a bath at 25.0°. A 0.250-ml portion of the eluate was added to 2.25 ml of the pH 3.43 acetate buffer in a 1.00-cm cell in the sample compartment of the Cary 14 PM recording spectrophotometer. A reference cell containing an approximately equal concentration of enzyme was prepared directly from stock enzyme solution. Spectra were taken from 390 to 240 m$\mu$ at 7-min intervals over a period of 3.5 hr (the deacylation half-life is 1 hr) using the 0.0–0.2 slide wire.

Meanwhile, at three different times, a 1.00-ml aliquot of the eluate was prepared for *trans*-cinnamic acid analysis by gel filtration using Sephadex G-25. (Distilled water was used as the eluent since it elutes aromatic acids faster and with less dilution than do electrolyte solutions.[19]) The *trans*-cinnamic acid fraction was analyzed by differential spectrophotometric analysis at 295 and 280 m$\mu$ so as to correct for the small amount of autolysis peptides present. A test analysis of a solution containing papain and a known amount of *trans*-cinnamic acid gave 100.2% recovery of the acid. The concentrations of *trans*-cinnamic acid, $C_t$, obtained in this way, were plotted against $e^{-k_3 t}$, where $k_3$ ($=1.91 \times 10^{-4}$ sec$^{-1}$) was determined from the repeated spectra. The plot was indeed linear and yielded values of $C_\infty$ (intercept) and $C_0$ (slope plus intercept) which were used in the rearranged first-order relationship of eq 1 to calculate $C_t$ at any other desired time.

$$C_t = C_\infty - (C_\infty - C_0)e^{-k_3 t} \qquad (1)$$

**Deacylation Kinetics.** A 1.6 × 6.5 cm Sephadex G-25 column was used in preparing stock solutions of *trans*-cinnamoyl-papain for the deacylation experiments. It was washed with 0.01 *M* formate buffer ($\mu$ = 0.100, pH 3.3–3.5) and stored and used in a 4° room. To 1.50 ml of stock papain solution ($3 \times 10^{-4}$ *M* in 0.05 *M* acetate buffer, pH 4, 25°) were added three 50-$\mu$l aliquots of $1 \times 10^{-2}$ *M* *trans*-cinnamoylimidazole, one each at $t$ = 0, 120, and 210 sec. At $t$ = 300 sec, the solution was placed on the cold Sephadex column and eluted; the 6.60- to 8.00-ml eluate fraction was collected as the acyl-enzyme stock solution and stored in ice. (The substrate and reaction products were shown to elute later.) Deacylation runs were performed by adding 100 $\mu$l of the acyl-enzyme stock solution to either 2.00 or 3.00 ml of the desired buffer at 25.0 ± 0.2° and following the disappearance of acyl-enzyme at 330 m$\mu$ using the 0.0–0.2 slide wire. The acyl-enzyme in the 100-$\mu$l pipet was warmed by hand to approximately room temperature before adding it to the cell. The pH of the cell contents was measured immediately after the reaction was complete using a Radiometer type PHM4c pH meter which was standardized with phthalate or borate buffers.[20]

For the deacylation experiments in $D_2O$ a stock solution of *trans*-cinnamoyl-papain in $D_2O$ was prepared using the same procedure as above except that the Sephadex column was prewashed with 35 ml of 0.05 *M* acetate buffer made up in $D_2O$, $\mu$ = 0.100, pD 3.92. The Sephadex filtration then serves both to remove excess substrate and to replace the $H_2O$ solvent by $D_2O$. $D_2O$ buffer solutions for the kinetic experiments were prepared using glacial acetic acid and solid anhydrous sodium acetate, $Na_2HPO_4$, and $KH_2PO_4$. $D_2O$ was Volk Lot No. 20280, 99.64% enrichment. The pD at the end of the reaction was determined by adding 0.40 to the pH meter reading.[21]

## Results

**Difference Spectrum of *trans*-Cinnamoyl-Papain *vs.* Papain.** The difference spectrum of *trans*-cinnamoyl-$\alpha$-chymotrypsin *vs.* chymotrypsin was obtained directly, since $\alpha$-chymotrypsin reacts rapidly and stoichiometrically with equimolar *trans*-cinnamoylimidazole, and the rate of deacylation is very slow.[22] In contrast, *trans*-cinnamoyl-papain deacylates at a rate comparable to the rate of acylation by *trans*-cinnamoylimidazole. Consequently, 100% acylation is never achieved, and the exact concentration of acyl-enzyme is not known directly. Therefore, the difference spectrum of *trans*-cinnamoyl-papain *vs.* papain had to be determined indirectly.

Spectra of a solution of deacylating *trans*-cinnamoyl-papain were taken repeatedly for three and one-half half-lives of the acyl-enzyme. In any of the spectra the observed absorbance, $A_{\lambda,t}$, at any particular wavelength and time is a function of the absorbances of three species according to eq 2 where $\epsilon_\lambda^E$, $\epsilon_\lambda^C$, and $\Delta\epsilon_\lambda^{CP}$ are the molar absorptivities of enzyme, *trans*-cinnamic acid, and *trans*-cinnamoyl-papain *vs.* papain, respectively; $E$ is the concentration of total enzyme not compensated for by the enzyme in the reference cell, and $C_t$ and $CP_t$ are the concentrations of *trans*-cinnamic acid and *trans*-cinnamoyl-papain, respectively, at time $t$. The value of $CP_t$ is equal to the concentration of *trans*-cinnamic acid which appears in the time interval between $t$ and $\infty$. Combining eq 2 with the form it takes when $t = \infty$ gives eq 3 in which $\Delta\epsilon_\lambda^{CP}$ is the only unknown. Values of $A_{\lambda,t}$ were taken from

$$A_{\lambda,t} = \epsilon_\lambda^E(E) + \epsilon_\lambda^C(C_t) + \Delta\epsilon_\lambda^{CP}(CP_t) \qquad (2)$$

$$\Delta\epsilon_\lambda^{CP} = \frac{A_{\lambda,t} - A_{\lambda,\infty}}{C_\infty - C_t} + \epsilon_\lambda^C \qquad (3)$$

the early spectra and values of $C_t$ were calculated from

(15) J. R. Kimmel and E. L. Smith, *J. Biol. Chem.*, 207, 515 (1954).
(16) M. Soejima and K. Shimura, *J. Biochem.* (Tokyo), **49**, 260 (1961).
(17) M. L. Bender, *et al.*, *J. Am. Chem. Soc.*, **88**, 5890 (1966).
(18) G. L. Lewis and C. P. Smyth, *J. Chem. Phys.*, 7, 1085 (1939).
(19) B. Gellote, *J. Chromatog.*, 3, 330 (1960).
(20) R. G. Bates, *J. Res. Natl. Bur. Std.*, **66A**, 179 (1962).
(21) P. K. Glasoe and F. A. Long, *J. Phys. Chem.*, **64**, 188 (1960).
(22) M. L. Bender, G. R. Schonbaum, and B. Zerner, *J. Am. Chem. Soc.*, **84**, 2540 (1962).

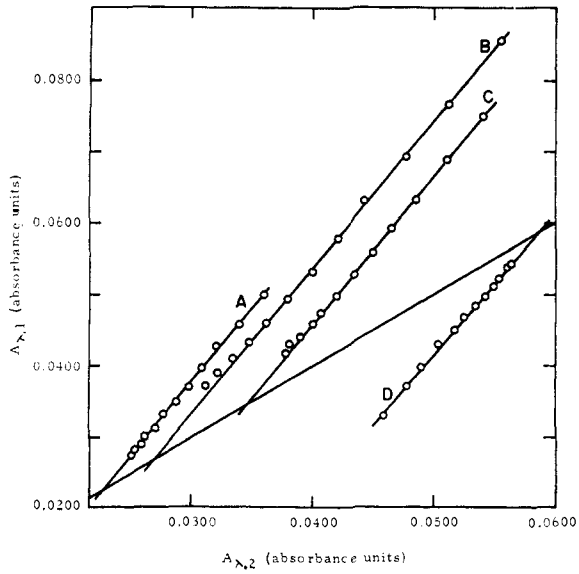


Figure 1. A plot of data obtained from repeated difference spectra of *trans*-cinnamoyl-papain *vs.* papain according to eq 4: A, 350 mμ; B, 330 mμ; C, 310 mμ; D, 285 mμ. The unlettered line is the bisectrix.

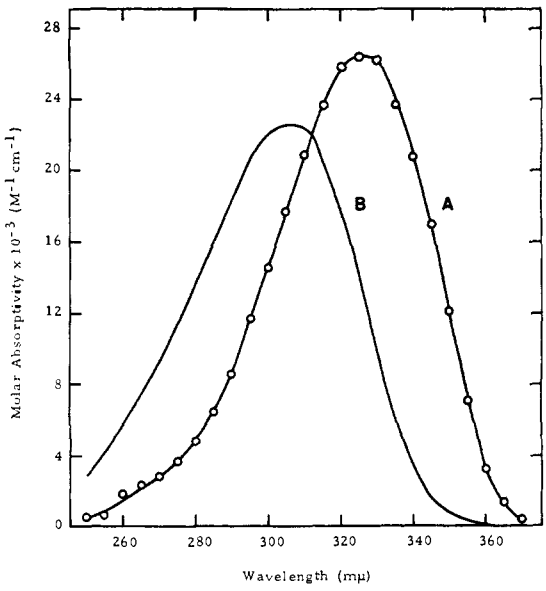


Figure 2. A, spectrum of *trans*-cinnamoyl-papain *vs.* papain (0.05 *M* acetate, $\mu$ = 0.100, pH 3.43). B, spectrum of S-*trans*-cinnamoylcysteine (0.009 *M* HCl, pH 2.1).

eq 1. Due to enzyme denaturation and/or autolysis, a good infinity spectrum could not be obtained. Hence values of $A_{\lambda,\infty}$ had to be determined indirectly. The infinity reading of a first-order reaction may be obtained from a Guggenheim plot[23] or from eq 4 due to Kézdy, *et al.*,[24] and later to Swinburne.[25] In eq 4, $A_{\lambda,1}$ and $A_{\lambda,2}$ are two absorbances (for a particular wavelength) separated by a constant time interval $\Delta t$. Since spectra of deacylating *trans*-cinnamoyl-papain were taken at 7-min intervals, it was possible

$$A_{\lambda,1} = A_{\lambda,\infty}(1 - e^{k_3\Delta t}) + A_{\lambda,2}e^{k_3\Delta t} \quad (4)$$

to compile sets of $A_{\lambda,1}$ and $A_{\lambda,2}$ values separated by a constant time interval of 3780 sec (approximately one half-life). These data were plotted according to eq 4 for wavelengths at 5-mμ intervals in the region between 270 and 370 mμ (see Figure 1). $A_{\lambda,\infty}$ is the point at which the extended straight line through the data points intersects the bisectrix (the line of unit slope which passes through the origin). This method was preferred here over the Guggenheim method because of the ease with which $A_{\lambda,\infty}$ may be determined, among other reasons.[25] The average value of $k_3$ from the seven plots between 320 and 350 mμ using eq 4 was 1.91 ± 0.04 × $10^{-4}$ $sec^{-1}$. Below 270 mμ where enzyme autolysis has the greatest spectral effect, the absorbances from the spectra taken during the first half-life were plotted *vs.* $e^{-0.000191t}$ according to an equation analogous to eq 1; $A_{\lambda,\infty}$ is the ordinate intercept of the straight line drawn through the points.

Values of $\epsilon_\lambda^C$ in eq 3 were determined from a spectrum of 4.15 × $10^{-6}$ *M trans*-cinnamic acid in the presence of 6.10 × $10^{-6}$ *M* papain *vs.* 6.10 × $10^{-6}$ *M* papain (0.05 *M* acetate, $\mu$ = 0.100, pH 3.47). These were approximately the same concentrations as in the difference spectrum experiment, and no perturbation of the spectrum was observed such as is seen at higher concentrations of *trans*-cinnamic acid and enzyme.[26]

Five sets of molar absorptivities for *trans*-cinnamoyl-papain *vs.* papain, one set from each of the first five spectra taken, were calculated using eq 3 and averaged. The results are listed in Table II and plotted in Figure 2.

When α-N-benzoyl-L-arginine ethyl ester is added to a solution containing *trans*-cinnamoyl-papain, papain activity toward this substrate reappears at the same rate as *trans*-cinnamoyl-papain deacylates.[17] Furthermore, when a solution containing *trans*-cinnamoylimidazole and mercuripapain is allowed to react for 5 min and then gel filtered on Sephadex G-25, the spectrum of the mercuripapain fraction is identical with that of normal mercuripapain and shows no change over a period of 4 hr. These two pieces of evidence indicate that *trans*-cinnamoylimidazole reacts at the active site of papain and only at the active site.

**Deacylation in $H_2O$.** The deacylation of *trans*-cinnamoyl-papain in $H_2O$ is first order in acyl-enzyme, the disappearance of which was followed at 330 mμ. The first-order plots, some of which are shown in Figure 3, were linear to at least 90% reaction. The appearance of *trans*-cinnamic acid as followed at 265 mμ for two reactions at pH 4.43 and pH 5.07 gave first-order rate constants which agreed to within 6% of those determined at 330 mμ. The deacylation rate was shown to be unaffected upon increasing the ionic strength above 0.100, or changing the buffer concentration at pH 4.8 (acetate) and at pH 10.1 (borate). Maleate and biphthalate buffers were found to inhibit deacylation.

(23) E. A. Guggenheim, *Phil. Mag.*, 2, 538 (1926).
(24) F. J. Kézdy, J. Jaz, and A. Bruylants, *Bull. Soc. Chim. Belges*, 67, 687 (1958).
(25) E. S. Swinburne, *J. Chem. Soc.*, 2371 (1960).

(26) The spectrum of 5.10 × $10^{-5}$ *M trans*-cinnamic acid in the presence of 6.68 × $10^{-5}$ *M* papain (total protein) *vs.* 6.68 × $10^{-5}$ *M* papain, exhibits abnormally low absorbance in the region fom 295 to 260 mμ. A plot of the difference between the molar absorptivities for this perturbed spectrum and the unperturbed molar absorptivities for *trans*-cinnamic acid shows a symmetrical difference spectrum with $\lambda_{max}$ 277 mμ and $\epsilon_{max}$ 6400 (0.05 *M* acetate, $\mu$ = 0.300, pH 5.1). This perturbation could be a perturbation of either the *trans*-cinnamic acid spectrum or of the papain spectrum.

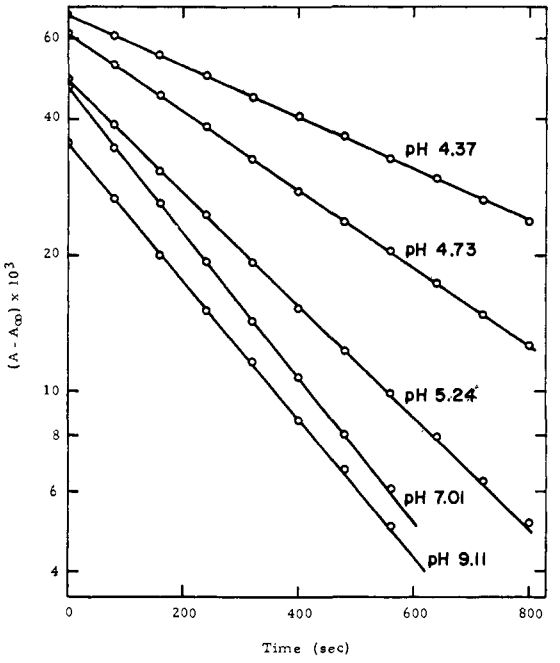


Figure 3. Typical first-order plots of the deacylation reactions of *trans*-cinnamoyl-papain in $H_2O$; $\mu$ = 0.100, 25.0°.

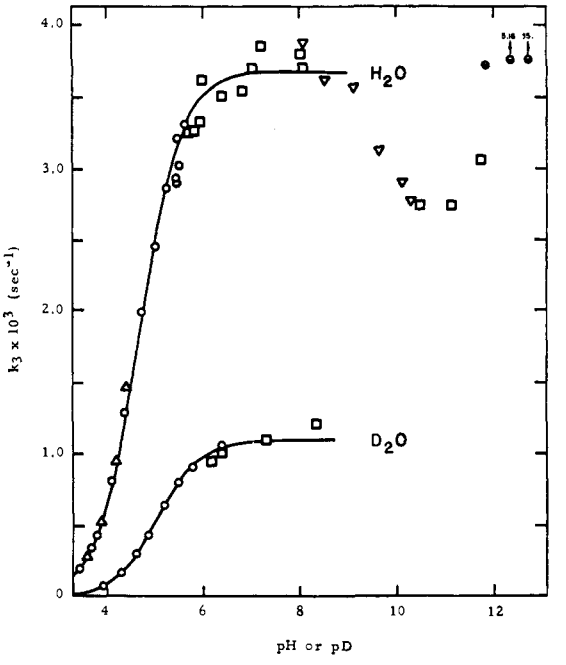


Figure 4. Effect of pH (or pD) on the deacylation of *trans*-cinnamoyl-papain: △, 0.05 *M* formate; ○, 0.05 *M* acetate; □, 0.033 *M* phosphate (0.017 *M*, pH 10.5 to 11.7); ▽, 0.0125 *M* borate; ◒, sodium hydroxide; $\mu$ = 0.100, 25.0°. The theoetical (solid) curves assume dependence of deacylation on a single basic group; $H_2O$, p$K_a$ = 4.69 and $k_3$(lim) = 3.68 × $10^{-3}$ $sec^{-1}$; $D_2O$, p$K_a$ = 5.03 and $k_3$(lim) = 1.10 × $10^{-3}$ $sec^{-1}$.

The dependence of the deacylation rate constant on pH is shown in Figure 4. Using the method of Brubacher and Kézdy,[27] a p$K$ of 4.69 and a $k_3$(lim) of 3.68 × $10^{-3}$ $sec^{-1}$ were determined for the region below pH 9.2. The reason for the valley in the pH–rate profile between pH 9.2 and 12 is not apparent. It is not a buffer or ionic strength effect. The rapid rise above pH 11 is no doubt due to direct hydroxide ion attack on the denatured acyl-enzyme

It should be noted that in all the deacylation reactions which were followed to completion at 330 m$\mu$ the infinity absorbance was about 10% of the initial absorbance and not zero as one might expect since neither the enzyme nor *trans*-cinnamic acid should absorb appreciably at 330 m$\mu$. The reason for this remains unexplained. If there is a second reaction it is very slow indeed. A deacylation reaction at pH 5.16 was followed for 24 half-lives. From the 11th to the 24th half-life, a period of 1 hr, the infinity absorbance decreased by only 0.0005 absorbance unit, which was less than 4% of the remaining absorbance and could simply be instrumental drift.

**Deacylation in $D_2O$.** The deacylation reactions in $D_2O$ gave good linear first-order plots. In Figure 4 is shown the pD dependence of the deacylation rate constant. The data are fitted to a curve of p$K$ = 5.03 and $k_3$(lim) = 1.10 × $10^{-3}$ $sec^{-1}$. Thus the rate in $H_2O$ is 3.35-fold greater than in $D_2O$, and the p$K$ of the base controlling the reaction is 0.34 pH unit lower. These results are similar to those for the deacylation of various acyl-$\alpha$-chymotrypsins.[28,29]

One reaction was performed in which 100 $\mu$l of concentrated acyl-enzyme in $D_2O$ was added to 3.00 ml of an $H_2O$ buffer (0.05 *M* acetate, $\mu$ = 0.100, pH 5.09). The deacylation rate was first order throughout the whole reaction with a rate constant of 2.77 × $10^{-3}$

**Table II.** The Difference Spectrum of *trans*-Cinnamoyl-Papain *vs.* Papain[a]

| Wavelength, m$\mu$ | Molar absorptivity,[b] $M^{-1}$ $cm^{-1}$ |
|---|---|
| 370 | 470 |
| 365 | 1,410 |
| 360 | 3,310 |
| 355 | 7,120 |
| 350 | 12,180 |
| 345 | 17,010 |
| 340 | 20,820 |
| 335 | 23,750 |
| 330 | 26,240 |
| 325 | 26,440 |
| 320 | 25,850 |
| 315 | 23,670 |
| 310 | 20,910 |
| 305 | 17,740 |
| 300 | 14,580 |
| 295 | 11,720 |
| 290 | 8,610 |
| 285 | 6,500 |
| 280 | 4,830 |
| 275 | 3,710 |
| 270 | 2,860 |
| 265 | 2,400 |
| 260 | 1,910 |
| 255 | 680 |
| 250 | 590 |
| 245 | −680 |
| 240 | +530 |

[a] 0.05 *M* acetate buffer, $\mu$ = 0.100, pH 3.43, 25.0 ± 0.1°. [b] Uncertainty is ±3% above 300 m$\mu$ and ±500$M^{-1}$ $cm^{-1}$ below 300 m$\mu$.

(27) L. J. Brubacher and F. J. Kézdy, unpublished procedure utilizing a generalized plot of log ($k/k$(lim)) *vs.* pH − p$K$.

(28) M. L. Bender and G. A. Hamilton, *J. Am. Chem. Soc.*, **84**, 2570 (1962).

(29) M. L. Bender, G. E. Clement, F. J. Kézdy, and H. d'A. Heck, *ibid.*, **86**, 3680 (1964).

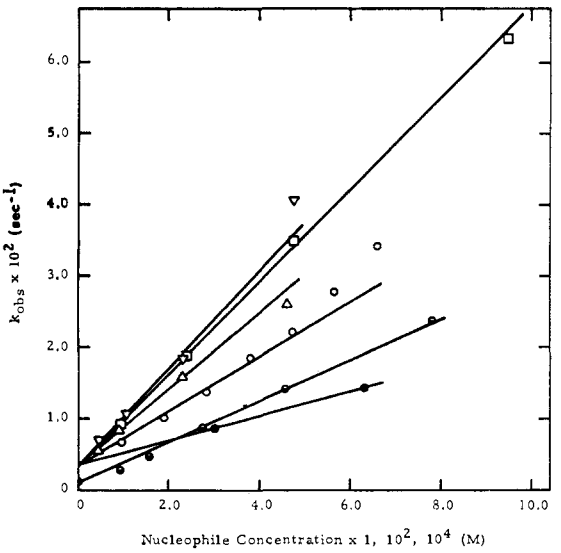


Figure 5. The effect of nucleophile concentration for the observed first-order rate constant in the deacylation of *trans*-cinnamoyl-papain. See footnotes *a*, *b*, and *c* of Table III for conditions. Abscissa is multiplied by $10^4$ for tryptamine, ▽, and L-tryptophanamide, □; by $10^2$ for glycinamide, △, D-tryptophamamide, ◒, and ammonia, ●; and by unity for methanol, ○. The D-tryptophanamide experiments were done at 13.7° which accounts for the lower intercept with this nucleophile.

$sec^{-1}$ which agrees within 5% of that calculated for a normal reaction in $H_2O$ at that pH. Thus there is either no conformational change of the acyl-enzyme in $D_2O$ which affects deacylation, or else the conformational change is a rapidly reversible one which is complete within less than 10 sec.

**Deacylation in the Presence of Added Nucleophiles.** When *trans*-cinnamoyl-papain is added to an aqueous buffer solution containing an added nucleophile, N, it can undergo first-order deacylation by either of two paths as shown in eq 5 where $k_{3,II}$ and $k_{4,II}$ are the second-order rate constants for water and added nucleophile, respectively.[30] Equation 5 implies that the acyl-enzyme will deacylate with a first-order rate constant, $k_{obsd}$, given by eq 6 where $k_3$ is the first-order deacylation rate constant in buffer with no

$$\begin{array}{l} \mathrm{O} \\ \| \\ \mathrm{RCE} - \end{array} \begin{cases} \xrightarrow{k_{3,II}(H_2O)} \begin{array}{l} \mathrm{O} \\ \| \\ \mathrm{RCO^-} \end{array} + \mathrm{E} \\ \xrightarrow{k_{4,II}(N)} \begin{array}{l} \mathrm{O} \\ \| \\ \mathrm{RCN} \end{array} + \mathrm{E} \end{cases} \quad (5)$$

$$k_{obsd} = k_{3,II}(H_2O) + k_{4,II}(N) = k_3 + k_{4,II}(N) \quad (6)$$

added nucleophile, assuming that neither $k_{3,II}$ nor the water concentration is significantly changed by the added nucleophile. Except in the case of methanol the concentration of added nucleophile was sufficiently small that the water concentration was not appreciably decreased. In the case of L-tryptophanamide it is shown below that $k_{3,II}$ is not significantly perturbed by the presence of the nucleophile.

The rate of disappearance of *trans*-cinnamoyl-papain in the presence of added nucleophiles gave linear first-order plots up to at least 90% reaction for reactions with freshly prepared acyl-enzyme. Data from reactions using acyl-enzyme more than a few hours old showed some curvature in the first-order plots and are not included in the data reported here. The values of $k_{obsd}$ for several nucleophiles are plotted as a function of nucleophile concentration in Figure 5.

In the reactions with increasing methanol concentrations an approximate spectrophotometric analysis of the final reaction mixture indicated that methyl *trans*-cinnamate was present in the expected amount to within ±25%. In a turnover reaction of *trans*-cinnamoylimidazole with papain in the presence of L-tryptophanamide, α-N-*trans*-cinnamoyl-L-tryptophanamide was produced in 78 ± 5% yield as determined spectrophotometrically. This compares well with the expected value of 76.3% as calculated from the known kinetic constants and concentrations. Thus L-tryptophanamide (and presumably the other nucleophiles as well) definitely acts as a nucleophile, and in addition it can be said that $k_{3,II}$ is not perturbed more than 70%, if at all, by the presence of L-tryptophanamide.

The results for the nucleophiles studied are compiled in Table III. With a few exceptions this work was done at pH 9.1 where the deacylation in water is on the plateau of Figure 4 while most of the amines are in the basic form. The value of the second-order deacylation rate constant in column six, $k_{4,II}$, is the slope of the corresponding line in Figure 5 or, for those cases where only a single nucleophile concentration was studied, it was calculated by dividing ($k_{obsd} - k_3$) by the nucleophile concentration.

The values of $k_{4,II}$ for methylamine and L-tryptophanamide determined at pH 10 are slightly lower than those at pH 9 which indicates that *trans*-cinnamoyl-papain is less reactive toward added nucleophiles in this pH region as it also is toward water (see Figure 4). Both values of $k_{4,II}$ for methylamine were calculated assuming that only the free base is involved (2.8% at pH 9.01 and 53.4% at pH 10.62); the good agreement between the two values validates that assumption.

The strikingly large value of $k_{4,II}$ for L-tryptophanamide as compared to glycinamide, which has a similar $pK_a$, suggested the possibility of nucleophile binding. D-Tryptophanamide was studied in an attempt to observe saturation of the acyl-enzyme, but, as Figure 5 shows, up to a concentration of 0.078 *M* D-tryptophanamide no saturation is observed. At concentrations of 0.14 and 0.46 *M* D-tryptophanamide, precipitation occurred when the *trans*-cinnamoyl-papain was added to the nucleophile solution.

The nonreactivity of imidazole is real. The posisibility that imidazole reacts with *trans*-cinnamoyl-papain and then reacylates the enzyme with no apparent deacylation is ruled out since the rate of acylation of papain by *trans*-cinnamoylimidazole is slow, particularly so at this pH; hence some *trans*-cinnamoylimidazole, if formed, would escape from the enzyme surface to the solution where it would be spontaneously hydrolyzed with a half-life of approximately 500 sec, and net catalysis by imidazole would be observed.

According to the data of Table III, methanol is a 59-fold better nucleophile than water. Thus in this respect *trans*-cinnamoyl-papain is similar to *trans*-cinnamoyl-α-chymotrypsin for which methanol is

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

Table III. Deacylation of *trans*-Cinnamoyl-Papain in the Presence of Amine and Alcohol Nucleophiles[a]

| Nucleophile | p$K_a$ | pH of expt | Concn range (free base) × $10^3$, $M$ | No. of detn | $k_{4,11}$, $M^{-1}$ sec$^{-1}$ | $k_{4,11}^{RNH_2}/k_{4,11}^{ROH}$ |
|---|---|---|---|---|---|---|
| Ammonia | 9.26 | 9.18 | 30–63 | 2 | 0.172 | 2590 |
| Water | | | | | 0.0000665 | |
| Methylamine | 10.56 | 10.62 | 2.23 | 1 | 10.5 | |
| | | 9.01 | 1.13 | 1 | 12.4 | 3150 |
| Methanol | 15.5[d] | 7.7–8.2[b] | 950–6600 | 7 | 0.00394[f] | |
| Trimethylamine | 9.79 | 10.77 | 23.0 | 1 | 0.029 | |
| Morpholine | 8.55 | 10.48 | 30.0 | 1 | 0.044 | |
| Glycinamide | 7.83 | 9.2 | 4.5–45.0 | 4 | 0.533 ± 0.027 | |
| L-Tryptophanamide | 7.60 | 10.28 | 0.86 | 1 | 42.0 | |
| | | 9.09 | 0.1–1.0 | 4 | 64.5 ± 1.4 | |
| D-Tryptophanamide[c] | 7.64 | 9.0 | 10–80 | 5 | 0.283[c] | |
| Tryptamine | 10.10 | 9.09 | 0.05–0.5 | 4 | 67.8 ± 7.3 | 151 |
| Tryptophol | | 9.12 | 20.2 | 1 | 0.448 | |
| 2-Phenylethylamine | 9.88 | 9.15 | 0.296 | 1 | 34.1 | 500 |
| 2-Phenylethanol | 15.8[e] | 9.10 | 39.8 | 1 | 0.068 | |
| Methyl L-tryptophanate | 7.32 | 9.06 | 0.902 | 1 | 91.7 | |
| Methyl D-tryptophanate | 7.32 | 9.07 | 10.27 | 1 | 0.543 | |
| Benzylamine | 9.45 | 9.10 | 1.11 | 1 | 17.8 | 39.3 |
| Benzyl alcohol | 15.6[e] | 9.15 | 291.0 | 1 | 0.453 | |
| Aminoacetonitrile | 5.3 | 9.06 | 5.20 | 1 | 0.476 | |
| Methoxyamine | 4.60 | 9.10 | 4.89 | 1 | 0.890 | |
| Imidazole | 6.95 | 9.09 | 9.76 | 1 | <0.03 | |

[a] 0.0125 $M$ borate buffer, $\mu$ = 0.100, 25.0 ± 0.1°. [b] pH increases as concentration of methanol increases; 0.033 $M$ phosphate buffer, $\mu$ = 0.100, 25.0 ± 0.1°. [c] 0.0125 $M$ borate buffer, $\mu$ = 0.525, 13.7 ± 0.2°. [d] P. Ballinger and F. A. Long, *J. Am. Chem. Soc.*, **82**, 795 (1960). [e] Calculated from $\rho$ = 1.42 for substituted methyl alcohols (footnote *d*) and $\sigma^*$ values of R. W. Taft, *ibid.*, **75**, 4236 (1953). [f] The slope in Figure 5 of 0.00379 $M^{-1}$ sec$^{-1}$ was corrected for the calculated decrease in $k_3$ due to the decrease in ($H_2O$).

a 76-fold better nucleophile than water.[30] The deacylation of *trans*-cinnamoyl-papain in 4.71 $M$ methanol studied between pH 3.5 and 7.7 is dependent upon an ionizable basic group of p$K$ = 4.60 which agrees well with the p$K$ of 4.69 found in water.

## Discussion

**The Chemical Nature of *trans*-Cinnamoyl-Papain.** The best evidence for the existence of an intermediate in a chemical reaction is the actual observation of a species with properties different from either reactants or products, and whose formation and decomposition can quantitatively account for the over-all kinetics. In the present work an intermediate has been observed spectrophotometrically in the papain-catalyzed hydrolysis of *trans*-cinnamoylimidazole. Initial evidence for its existence was the observation that a reacting system showed, at 350 mμ, an absorbance which first increased slightly to a maximum and then decreased to zero. The isolated intermediate has the characteristic strong absorbance of the *trans*-cinnamoyl group ($\epsilon_{max}$ 26,500) and a $\lambda_{max}$ of 326 mμ which is significantly different from both that of the reactant (*trans*-cinnamoylimidazole, $\lambda_{max}$ 307 mμ and that of the product (*trans*-cinnamate ion, $\lambda_{max}$ 269 mμ). In this intermediate the *trans*-cinnamoyl moiety remains associated with the papain molecule throughout the course of Sephadex filtration both when the enzyme is in the native state and in the denatured state; thus the *trans*-cinnamoyl group is probably covalently bonded to the enzyme. The competition experiment in which α-N-benzoyl-L-arginine ethyl ester was added to the solution of *trans*-cinnamoyl-papain showed that the *trans*-cinnamoyl group, although introduced into the papain molecule by a nonspecific substrate, is located at the same site which catalyzes the hydrolysis of a specific substrate.

The most probable type of attachment between the *trans*-cinnamoyl moiety and papain is the covalent bond formed when an appropriate nucleophilic group on the enzyme displaces imidazole, an excellent leaving group, from the substrate *trans*-cinnamoylimidazole. The possible nucleophilic groups contained in the amino acid side chains of papain are the OH groups of serine, threonine, and tyrosine, the imidazole group of histidine, the carboxylate groups of aspartic and glutamic acids, and the SH group of cysteine. Nucleophilic attack by the SH group to give a thiol ester is most probable since (1) a free SH group is known to be essential to the activity of papain,[31] and (2) dependence on an ionizable group of p$K_a$ ~ 8.4, which is typical of an SH group, is observed in acylation[32] but not in deacylation. This latter observation rules out carboxylate, imidazole, and the OH groups of serine, threonine, and tyrosine as the site of acylation unless the p$K_a$ values of these groups are significantly perturbed.

The sensitivity of *trans*-cinnamoyl-papain to nucleophilic attack by amines is strong chemical evidence in support of the suggestion that *trans*-cinnamoyl-papain is a thiol ester. Thiol esters are known to be more susceptible to attack by nitrogen nucleophiles than are oxygen esters.[33,34] Connors and Bender have studied the alkaline hydrolysis and *n*-butylaminolysis of ethyl *p*-nitrobenzoate and ethyl *p*-nitrothiolbenzoate.[35] The aminolysis of the thiol ester was shown to

(31) E. L. Smith and J. R. Kimmel in "The Enzymes," Vol. 4, P. D. Boyer, H. Lardy, and K. Myrback, Ed., Academic Press Inc., New York, N. Y., 1960.
(32) M. L. Bender and L. J. Brubacher, *J. Am. Chem. Soc.*, **88**, 5880 (1966).
(33) T. C. Bruice in "Organic Sulfur Compounds," Vol. 1, N. Kharasch, Ed., Pergamon Press, New York, N. Y., 1961.
(34) E. M. Kosower, "Molecular Biochemistry," McGraw-Hill Book Co., Inc., New York, N. Y., 1962.
(35) K. A. Connors and M. L. Bender, *J. Org. Chem.*, **26**, 2498 (1961).

follow the rate law of eq 7. The ratio $k_S/k_O$ for the various terms of eq 7 were found to be: $k_1$, 0.83;

$$-\text{d(ester)}/dt = [k_1(OH^-) + k_2(RNH_2) + k_3(RNH_2)^2 + k_4(RNH_2)(OH^-)](\text{ester}) \quad (7)$$

$k_2$, $\geqslant 150$; $k_3$, $\geqslant 1400$; $k_4$, $\geqslant 1400$. These ratios show that *n*-butylamine is a much better nucleophile toward the thiol ester than toward the oxygen ester, both in the base-catalyzed and the uncatalyzed terms. The similarity of the hydroxide ion catalyzed rates for oxygen and thiol esters holds in general.[33] A further corroboration of the generalization that thiol esters are more susceptible than oxygen esters to attack by nitrogen nucleophiles is the fact that the hydrolysis of ethyl thiolacetate is catalyzed by imidazole,[36] whereas the rate of hydrolysis of ethyl acetate is unaffected by 1 *M* imidazole.[37] If *trans*-cinnamoyl-papain is a thiol ester, then *trans*-cinnamoyl-α-chymotrypsin may be considered its oxygen ester analog. In Table IV are compared the nucleophilic rate constants for two pairs of nucleophiles. Consider the methanol–methylamine pair. The 670-fold greater reactivity of methylamine toward *trans*-cinnamoyl-papain than toward *trans*-cinnamoyl-α-chymotrypsin clearly supports the identification of *trans*-cinnamoyl-papain as a thiol ester. An identical argument can be based on the water–ammonia pair. The thiol ester nature of acyl-papains explains the well-known ability of papain to catalyze peptide bond syntheses and transamidation reactions,[38-44] since these reactions involve attack by amine nucleophiles.

Table IV. Comparison of Nucleophilic Rates in the Deacylation of *trans*-Cinnamoyl-Papain and *trans*-Cinnamoyl-α-chymotrypsin

| Nucleophile | $k_{4,II}$ Actual | $k_{4,II}$ Normalized |
|---|---|---|
| | Cinnamoyl-papain | |
| Water | 0.30 | 1[c] |
| Ammonia | >143.0[a] | >480 |
| | Cinnamoyl-α-chymotrypsin | |
| Methanol | 0.23 | 1[c] |
| Methylamine | 155.0[b] | 670 |

[a] Based on the observed absence of effect of 1 *M* ammonia on the deacylation of *trans*-cinnamoyl-α-chymotrypsin; unpublished experiments of Dr. C. R. Gunter. [b] Based on experiments by Dr. J. Feder on *trans*-cinnamoyl-α-chymotrypsin. [c] To correct for reactivity differences due to differences in binding of the nucleophiles to the two acyl-enzymes, the methanol and water rate ratios are normalized to unity.

A model compound for *trans*-cinnamoyl-papain is S-*trans*-cinnamoylcysteine. It is significant that the $\lambda_{max}$ of *trans*-cinnamoyl-papain is shifted to a longer wavelength (lower energy) than the $\lambda_{max}$ of the model compound. This same pattern of behavior is observed in other *trans*-cinnamoyl-enzymes as shown in Table V. For four *trans*-cinnamoyl-enzymes the energy of the electronic transition in the *trans*-cinnamoyl moiety is 4.2 ± 1.5 kcal/mole less than for the appropriate model compound. This similarity for the four enzymes suggests that the cause of the perturbation of the $\lambda_{max}$ of the *trans*-cinnamoyl group in papain is similar to that in trypsin, α-chymotrypsin, and subtilisin. The cause may be a common environment. Upon denaturation of *trans*-cinnamoyl-papain by 4.8 *M* guanidinium chloride $\lambda_{max}$ is found to decrease to between 301 and 309 mμ,[45] a range which encompasses the $\lambda_{max}$ of the model compound and confirms the identification of *trans*-cinnamoyl-papain as a thiol ester.

Table V. Difference Spectra of Some *trans*-Cinnamoyl-Enzymes and Model *trans*-Cinnamic Acid Derivatives[a]

| *trans*-Cinnamoyl- | $\lambda_{max}$ mμ | $\lambda_{max}$ cm$^{-1}$ | $\epsilon_{max}$ | $\lambda_{max}$ (model) − $\lambda_{max}$ cm$^{-1}$ | kcal/mole |
|---|---|---|---|---|---|
| Papain[b] | 326 | 30,675 | 26,500 | 2000 | 5.7 |
| α-Chymotrypsin[c] | 292 | 34,247 | 17,700 | 1280 | 3.7 |
| Trypsin[d] | 296 | 33,784 | 19,400 | 1740 | 5.0 |
| Subtilisin[e] | 289 | 34,602 | 21,000 | 920 | 2.6 |
| Subtilisin[f] | 290 | 34,483 | 17,000 | 1040 | 3.0 |
| N-Acetylserinamide[g] | 281.5 | 35,524 | 24,300 | ... | ... |
| Cysteine[h] | 306 | 32,680 | 22,600 | ... | ... |

[a] 1.6% acetonitrile–water (v/v); pH 4; 25°. [b] No acetonitrile; pH 3.43. See Figure 2. [c] Reference 22. [d] M. L. Bender and E. T. Kaiser, *J. Am. Chem. Soc.*, **84**, 2556 (1962). [e] Unpublished observations of M. L. Begué, Northwestern University. [f] S. A. Bernhard, S. J. Lau, and H. Noller, *Biochemistry*, **4**, 1108 (1965). [g] 10% acetonitrile–water (v/v), ref 22. [h] pH 2.1, see Figure 2.

The possibility that *trans*-cinnamoyl-papain is an acylimidazole type of compound is ruled out by the much greater reactivity of methylamine as compared to methanol, namely, 3150-fold. Experiments with *trans*-cinnamoylimidazole indicate that methylamine is only 40-fold more reactive toward this model compound than is methanol. A similar comparison of the reactivities of methylamine and methanol toward S-*trans*-cinnamoylcysteine cannot be made because of the very rapid S → N shift of the *trans*-cinnamoyl group.

**The $D_2O$ Effect.** A large $D_2O$ effect such as is seen for *trans*-cinnamoyl-papain has commonly been interpreted to imply general basic catalysis rather than nucleophilic catalysis in the rate-determining step.[46] However, in view of the recent criticisms of the validity of this criterion,[47] and the fact that this criterion has been developed for oxygen esters not thiol esters, additional evidence that the deacylation of *trans*-cinnamoyl-papain is general base catalyzed is desirable.

**The Effect of Added Nucleophiles.** The second-order rate constants, $k_{4,II}$, for the reaction between the amine nucleophiles of Table III and *trans*-cinnamoyl-papain are plotted in Figure 6 as a function of p$K_a$. Clearly there is no simple relationship between $k_{4,II}$ and p$K_a$; in fact, since L-tryptophanamide

(36) M. L. Bender and B. W. Turnquest, *J. Am. Chem. Soc.*, 79, 1656 (1957).
(37) J. F. Kirsch and W. P. Jencks, *ibid.*, 86, 833 (1964).
(38) M. Bergmann and H. Fraenkel-Conrat, *J. Biol. Chem.*, 119, 707 (1937).
(39) M. Bergmann and O. K. Behrens, *ibid.*, 124, 1 (1938).
(40) S. W. Fox and M. Winitz, *Arch. Biochem. Biophys.*, 35, 419 (1952).
(41) T. Murachi, *ibid.*, 61, 461, 468 (1956).
(42) D. M. Kirschenbaum, *ibid.*, 103, 249 (1963).
(43) Y. P. Dowmont and J. S. Fruton, *J. Biol. Chem.*, 185, 629 (1950).
(44) M. J. Mycek and J. S. Fruton, *ibid.*, 226, 165 (1957).
(45) The two limits correspond, respectively, to spectra taken in the presence and absence of the denaturing agent.
(46) M. L. Bender, E. J. Pollock, and M. C. Neveu, *J. Am. Chem. Soc.*, 84, 595 (1962).
(47) R. L. Blakeley, Ph.D. Thesis, Harvard University, 1964.

and tryptamine, which are of quite similar structure but different basicity, have almost identical $k_{4,II}$ values, basicity appears to have little influence on nucleophilicity (*vide infra*). This result is in contrast to the observed linear dependence of the nucleophilic rate constant on $pK_a$ (slope = 0.8) for the reaction of various amines with *p*-nitrophenyl acetate.[48,49]

For a number of amine nucleophiles in the deacylation of furoyl-α-chymotrypsin a similar insensitivity of nucleophilicity to basicity is observed.[50] It is of interest to compare rate constants for pairs of amines that are common to both studies. Toward furoyl-α-chymotrypsin methylamine is a sixfold better nucleophile than ammonia, whereas toward *trans*-cinnamoyl-papain methylamine is a 70-fold better nucleophile than ammonia. A similar comparison for the methylamine–morpholine pair gives a ratio of 36:1 toward furoyl-α-chymotrypsin and a ratio of 280:1 toward *trans*-cinnamoyl-papain. Apparently *trans*-cinnamoyl-papain is more selective in its reactions with added nucleophiles than furoyl-α-chymotrypsin.

The particularly large rate enhancement shown by certain nucleophiles in the deacylation of *trans*-cinnamoyl-papain is also interesting. L-Tryptophanamide, for example, has a second-order rate constant more than a million-fold greater than water. A part of this large enhancement is no doubt due to the greater reactivity of nitrogen nucleophiles than oxygen nucleophiles toward thiol esters (*vide supra*). However, other factors seem to be involved; glycinamide, which has essentially the same $pK_a$ as L-tryptophanamide, is a poorer nucleophile by a factor of more than 100. This comparison and the above observation that *trans*-cinnamoyl-papain is more selective than furoyl-α-chymotrypsin toward nucleophiles suggest the possibility that the attacking nucleophile binds to *trans*-cinnamoyl-papain in an equilibrium step before nucleophilic attack occurs. When binding occurs, it may be shown that $k_{4,II} = k_4/K_{n,app}$; $K_{n,app} = K_n(1 + (W_0/K_w))$ where $W_0$ is the water concentration, $K_w$ and $K_n$ are the dissociation constants of the acyl-enzyme–water complex and the acyl-enzyme–nucleophile complex, respectively, and $k_4$ is the first-order intramolecular rate constant for deacylation. Thus the values of $k_{4,II}$ in Table III depend not only on the nucleophilicity but also the binding ability of the attacking nucleophile.

Since the $K_s$ for benzyl α-N-benzyloxycarbonyl-L-lysinate is 20-fold smaller than the $K_s$ for the corresponding methyl ester,[32] it would appear that the leaving groups of these esters contribute to the binding of the substrate to the enzyme. If deacylation is the reverse of acylation, the principle of microscopic reversibility requires then that there also be binding of the nucleophile in deacylation. Additional support for nucleophile binding in deacylation is the low reactivity of D-tryptophan derivatives compared to the corresponding L isomers (see Table III). Fruton and co-workers[43,44] have made extensive studies on transamidation reactions using dipeptides as nucleophiles. They also have concluded that papain exhibits specificity toward the nucleophile.[44] Thus the 100-fold difference between the $k_{4,II}$ values for L-tryptophanamide and glycinamide reflects

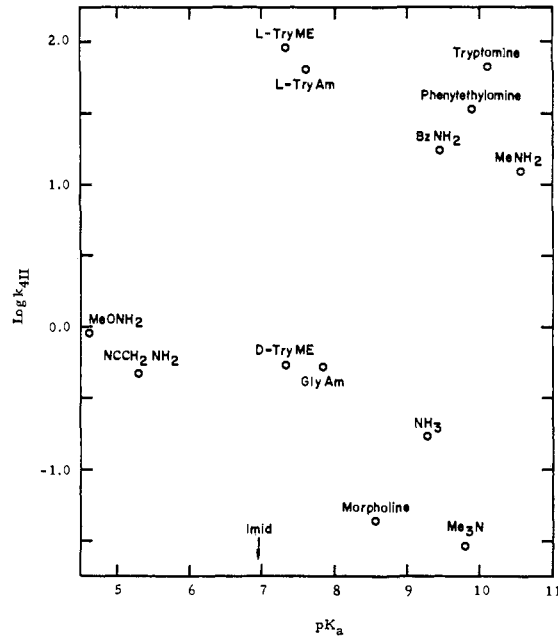


Figure 6. Log $k_{4,II}$ *vs.* $pK_a$ for several amine nucleophiles in the deacylation of *trans*-cinnamoyl-papain (data of Table III).

the better binding ability of the former since the two compounds should have similar inherent nucleophilicities because of the similarity in $pK_a$.

It is difficult in general to separate the contribution of binding from the contribution of basicity in the over-all reactivity of the nucleophiles. However, a comparison of L-tryptophanamide with tryptamine is instructive. The similarity in structure implies that the binding effect should be approximately the same for both. (The polar amide group in L-tryptophanamide apparently contributes little to the binding since replacing it with a less polar methoxy group has essentially no effect on the value of $k_{4,II}$; *cf.* methyl L-tryptophanate.) Thus the effect of basicity is small, if present at all, since the observed $k_{4,II}$ values are the same for these two nucleophiles of differing basicity.

Although the rate constants do not reflect dependence on basicity, this is not to say that basicity is not a factor in determining nucleophilicity, but rather that the contribution of basicity to nucleophilicity is counteracted by an effect which is inversely dependent on basicity. Such an effect could be the tendency of the nucleophile to release a proton to a general base in the transition state. The less basic the nucleophile, the more readily it should release the proton; conversely, the less basic the nucleophile the poorer the nucleophile. These two effects will tend to cancel. Hence the apparent lack of dependence of over-all nucleophilicity on basicity suggests that general basic catalysis occurs in the deacylation of *trans*-cinnamoyl-papain. The same conclusion has been given in the case of furoyl-α-chymotrypsin.[50]

Thus both the nucleophile and the $D_2O$ studies imply general basic catalysis and the two results taken together give strong evidence for general basic catalysis in the deacylation step.

If nucleophilic catalysis by imidazole also occurs as has been suggested,[51] one is forced to postulate a

(48) T. C. Bruice and R. Lapinski, *J. Am. Chem. Soc.*, 80, 2265 (1958).
(49) W. P. Jencks and J. Carriuolo, *ibid.*, 82, 1778 (1960).
(50) P. W. Inward and W. P. Jencks, *J. Biol. Chem.*, 240, 1986 (1965).
(51) G. Lowe and A. Williams, *Biochem. J.*, 96, 194 (1965).

mechanism in which imidazole (from histidine) attacks the thiol ester to form a new acyl-enzyme (acyl-imidazole), followed by a general base catalyzed nucleophilic attack by a bound nucleophile ($H_2O$ or added nucleophile) in which the general base is a group other than the nucleophilic imidazole. This possibility may be ruled out, however. If the first (nucleophilic) step is rate determining, deacylation would not show a kinetic dependence on the concentration of added nucleophile, contrary to experiment; if the second (general base catalyzed) step is rate determining, the intermediate (acyl-imidazole) should be observable, yet no such intermediate has been observed. Nucleophilic catalysis by imidazole may also be ruled out on the basis of the high reactivity of amine nucleophiles compared to alcohol nucleophiles towards *trans*-cinnamoyl-papain. If such catalysis occurred, the added nucleophiles would actually be attacking an acyl-imidazole, toward which amines and alcohols do not show such widely differing reactivities (*vide supra*).

# The Kinetics and Mechanism of Papain-Catalyzed Hydrolyses[1]

**Myron L. Bender and Lewis J. Brubacher**

*Contribution from the Division of Biochemistry, Department of Chemistry, Northwestern University, Evanston, Illinois 60201.* *Received August 25, 1966*

**Abstract:** The kinetics of the papain-catalyzed hydrolysis of the *p*-nitrophenyl, benzyl, and methyl esters of α-N-benzyloxycarbonyl-L-lysine have been studied as a function of pH at 25° and are consistent with the two-step mechanism involving an acyl-enzyme intermediate. The complex rate constant, $k_{cat}$, is dependent on an ionizable basic group of $pK_a = 3.5$; $k_{cat}/K_m$ shows a bell-shaped pH dependence. The acylation and deacylation rate constants and the binding constants were determined for each ester. Differences in $K_s$ suggest that the leaving group is involved in binding. In the hydrolyses of *p*-nitrophenyl ε-N-formyl-α-N-benzyloxycarbonyl-L-lysinate and *p*-nitrophenyl α-N-acetyl-L-tryptophanatate $k_{cat}$ is dependent on ionizable basic groups of $pK_a = 3.96$ and 4.70, respectively. The rate of inhibition of papain by L-1-chloro-3-tosylamido-4-phenyl-2-butanone is dependent on an ionizable basic group of $pK_a = 8.28$, presumably the thiolate group of a cysteine residue. Amino acid analysis of the alkylated papain shows no loss of histidine. The mechanistic implications of these results and the results of other workers are discussed.

Several investigators have concluded that papain, like α-chymotrypsin, catalyzes hydrolytic reactions by the mechanism of eq 1.[2-5] The rate of disappearance of substrate, $v$, is given by eq 2, and eq 3 and 4 relate the complex constants $k_{cat}$ and $K_m$ to the rate constants of eq 1.[6,7]

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' + P_1 \xrightarrow{k_3} E + P_2 \quad (1)$$

$$v = k_{cat}(E)_0(S)/((S) + K_m) \quad (2)$$

$$k_{cat} = k_2k_3/(k_2 + k_3) \quad (3)$$

$$K_m = k_3K_s/(k_2 + k_3); \; K_s = k_{-1}/k_1 \quad (4)$$

We have found that the *p*-nitrophenyl, benzyl, and methyl esters of α-N-benzyloxycarbonyl-L-lysine are the best substrates yet found for papain, and it was of interest to determine whether the kinetics of their hydrolyses are consistent with eq 1. The results are reported here along with some studies on the inhibition of papain by L-1-chloro-3-tosylamido-4-phenyl-2-butanone (TPCK).[8]

## Experimental Section

**Materials.** Papain, buffer materials, and routine procedures have been described in the preceding paper.[9] The normality of papain stock solutions was determined using α-N-benzoyl-L-arginine ethyl ester as a secondary standard according to the published procedure.[10] The *p*-nitrophenyl,[11] benzyl,[12] and methyl,[12] esters of Z-lysine, and the *p*-nitrophenyl ester of α-N-acetyl-DL-tryptophan[13] have been described in previous papers from this laboratory. Stock solutions of the lysine substrates were made up in acetonitrile with 5–10% distilled water added to increase solubility. No spontaneous hydrolysis occurred in these solutions when stored at 2° for a period of several weeks. Stock solutions of *p*-nitrophenyl α-N-acetyl-DL-tryptophanate were prepared using anhydrous acetonitrile.

TPCK was purchased from the Cyclo Chemical Corp. and recrystallized from 95% ethanol–water by Dr. R. L. Blakeley; mp 106–107° (lit.[14] 102–103°).

**Kinetic Measurements. Esters of Z-Lysine.** Reactions were followed spectrophotometrically using the Cary Model 14 CM recording spectrophotometer. Wavelengths used were: methyl ester, 224 mμ ($\Delta\epsilon$ −73.0 at neutral pH); benzyl ester, 236 mμ ($\Delta\epsilon$ −38.9 at neutral pH); and *p*-nitrophenyl ester, 340 mμ below pH 7, 400 mμ above pH 7. In experiments with the benzyl and methyl esters, a 1.1 neutral density screen was placed in the reference light path when using the 0.0–2.0 slide wire, a 0.5 neutral density screen when using the 0.0–0.2 slide wire (benzyl ester only). Reactions were generally performed as follows. Substrate (50 μl)

(1) This research was supported by grants from the National Institutes of Health.

(2) E. L. Smith, *J. Biol. Chem.*, **233**, 1392 (1958).

(3) J. R. Whitaker and M. L. Bender, *J. Am. Chem. Soc.*, 87, 2728 (1965).

(4) G. Lowe and A. Williams, *Biochem. J.*, **96**, 199 (1965).

(5) J. F. Kirsch and M. Igelström, *Biochemistry*, **5**, 783 (1966).

(6) Except for $k_{cat}$ the symbols used for the kinetic constants are those recommended by the International Union of Biochemistry, "Enzyme Nomenclature," Elsevier Publishing Co., Amsterdam, 1965, p 49.

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

(8) Abbreviations used in this paper are: TPCK, L-1-chloro-3-tosylamido-4-phenyl-2-butanone; Z, benzyloxycarbonyl substituted at the α-amino position of L-amino acids.

(9) L. J. Brubacher and M. L. Bender, *J. Am. Chem. Soc.*, **88**, 5871 (1966).

(10) M. L. Bender, *et al.*, *ibid.*, **88**, 5890 (1966).

(11) M. L. Bender, J. V. Killheffer, Jr., and R. W. Roeske, *Biochem. Biophys. Res. Commun.*, **19**, 161 (1965).

(12) M. L. Bender and F. J. Kézdy, *J. Am. Chem. Soc.*, **87**, 4954 (1965).

(13) B. Zerner, R. P. M. Bond, and M. L. Bender, *ibid.*, **86**, 3674 (1964).

(14) G. Schoellman and E. Shaw, *Biochemistry*, **2**, 252 (1963).