On the Spontaneous and α-Chymotrypsin-Catalyzed Hydrolysis of 4-*cis*-Benzylidene-2-phenyloxazolin-5-one. Catalysis by Tris(hydroxymethyl)aminomethane*

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ABSTRACT: 4-*cis*-Benzylidene-2-phenyloxazolin-5-one and *N*-acetyl-L-valine *p*-nitrophenyl ester have been considered as substrates for α-chymotrypsin. Results obtained indicate the desirability of activated derivatives of *N*-acylamino acids as substrates in specificity studies. The reactions of Tris with 4-*cis*-benzylidene-2-phenyloxazolin-5-one and the corresponding acyl-enzyme, α-benzamido-*cis*-cinnamoyl-α-chymotrypsin, have been investigated. In both cases, the Tris amide of α-benzamido-*cis*-cinnamic acid (λ_{max} 282 mμ) is the final product, and reaction occurs through an intermediate species (λ_{max} 285 mμ). This intermediate is relatively stable, and was isolated and examined. Evidence obtained suggests that Tris intervention in these systems produces the Tris *ester* of α-benzamido-*cis*-cinnamic acid as the intermediate species. This ester is subsequently converted to the Tris *amide* in both the enzymatic and nonenzymatic systems.

4-*cis*-Benzylidine-2-phenyloxazolin-5-one was synthesized by Plöchl in 1883 and proved to be the forerunner of the class of compounds now called unsaturated oxazolinones. The utility of unsaturated oxazolinones as intermediates in organic syntheses is well established (Carter, 1946). However, only qualitative data on the rates of hydrolysis, alcoholysis, and aminolysis of unsaturated oxazolinones have previously been available.

Saturated oxazolinones were found to be useful substrates in studies of the mechanism of action and specificity of proteolytic enzymes (de Jersey *et al.*, 1966; de Jersey and Zerner, 1969). Brocklehurst and Williamson (1966) investigated the α-chymotrypsin-catalyzed hydrolysis of methyl α-benzamido-*cis*-cinnamate, and compared the kinetic constants obtained with published constants for methyl cinnamate and *N*-acetyl-L-valine methyl ester. Consequently, 4-*cis*-benzylidene-2-phenyloxazolin-5-one and *N*-acetyl-L-valine *p*-nitrophenyl ester have been considered as activated derivatives to allow accurate specificity comparisons to be made.

Brocklehurst and Williamson (1967) also studied the α-chymotrypsin-catalyzed hydrolysis of 4-*cis*-benzylidene-2-phenyloxazolin-5-one and postulated an N→O acyl shift to rationalize the data obtained. Since evidence for such a shift in an α-chymotrypsin-catalyzed hydrolysis would constitute an important advance in our knowledge of the mechanism of the catalytic process, the hydrolysis of 4-*cis*-benzylidene-2-phenyloxazolin-5-one by α-chymotrypsin was reinvestigated.

In the present work, it has been shown that the results of Brocklehurst and Williamson (1967) may be explained in terms of the effect of Tris on the deacylation of α-benzamido-*cis*-cinnamoyl-α-chymotrypsin. The nature of the reactions of Tris with 4-*cis*-benzylidene-2-phenyloxazolin-5-one and with α-benzamido-*cis*-cinnamoyl-α-chymotrypsin has been investigated.

Experimental Section

Materials. 4-*cis*-Benzylidene-2-phenyloxazolin-5-one was prepared by the reaction of benzaldehyde with hippuric acid in the presence of acetic anhydride, and recrystallized from benzene, mp 166°, lit. (Gillespie and Snyder, 1934) mp 167°. α-Benzamido-*cis*-cinnamic acid and methyl α-benzamido-*cis*-cinnamate were prepared by the hydrolysis and methanolysis of 4-*cis*-benzylidene-2-phenyloxazolin-5-one. α-Benzamido-*cis*-cinnamic acid had mp 227–228°, lit. (Gillespie and Snyder, 1934) mp 224–236°. Methyl α-benzamido-*cis*-cinnamate was recrystallized from benzene–petroleum ether (bp 60–80°) mp 142°, lit. (Kochetkov *et al.*, 1959) mp 139–141°. The Tris amide of α-benzamido-*cis*-cinnamic acid was prepared as follows. 4-*cis*-Benzylidene-2-phenyloxazolin-5-one (2 g) in 70 ml of acetonitrile was added to 1 g of Tris in 10 ml of water and the mixture was stirred at room temperature until solution was complete (6 hr). The solvent was removed under reduced pressure, yielding an oily solid which was extracted with Tris buffer (pH 8.6) and with water. Recrystallization from chloroform–petroleum ether (three times) and from ethyl acetate–hexane gave a colorless product, mp 185–186° dec. *Anal.*[1] Calcd for $C_{20}H_{22}N_2O_5$:

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[1] Microanalyses were performed by the Australian Microanalytical Service, Melbourne, Victoria, and by the Microanalytical Service of the Department of Chemistry, University of Queensland.

C, 64.85; H, 5.99; N, 7.56; O, 21.60. Found: C, 64.21; H, 5.91; N, 7.50; O, 21.10. *N*-Acetyl-L-valine *p*-nitrophenyl ester was prepared by the general method of Ingles *et al.* (1966). After recrystallization from chloroform–hexane, *N*-acetyl-L-valine *p*-nitrophenyl ester had mp 75–76°, $[\alpha]_D^{25}$ − 45.1° (*c* 1, chloroform), and gave 100.6% of the calculated amount of *p*-nitrophenol on hydrolysis. Optical purity was established by the rapid complete hydrolysis by α-chymotrypsin. *Anal.* Calcd for $C_{13}H_{16}N_2O_5$: C, 55.71; H, 5.75; N, 9.99. Found: C, 55.72; H, 6.14; N, 9.91.

N-trans-Cinnamoylimidazole, *p*-nitrophenyl acetate, 4,4-dimethyl-2-phenyloxazolin-5-one, α-chymotrypsin (three-times crystallized), and acetonitrile were obtained as listed previously (de Jersey *et al.*, 1969). Tris (Trizma Base, Reagent Grade) was obtained from Sigma Chemical Co. and D_2O (>99.8%) from Koch-Light Laboratories (England). Dioxane was purified by distillation, followed by chromatography on basic alumina (Dasler and Bauer, 1946), and *N*-ethylmorpholine (Eastman) was redistilled before use. Glycine ethyl ester hydrochloride was prepared by refluxing glycine with ethanol saturated with HCl, and was recrystallized from ethanol–ether, mp 143°, lit. (Curtius and Goebel, 1888) mp 144°. Other buffer components were analytical grade reagents. Equivalent Tris buffers at half-neutralization were prepared in H_2O and D_2O using weighed amounts of Tris and 10 N HCl. Ionic strength was kept constant by addition of KCl. The pD's of the buffers in D_2O were calculated using the equation, pD = meter pH + 0.40 (Lumry *et al.*, 1951; Zerner and Bender, 1961).

Methods. Ultraviolet spectrophotometric measurements were made at 25 ± 0.1° using a Cary 14 recording spectrophotometer as described previously (de Jersey *et al.*, 1969), with substrates made up in acetonitrile. The loss of 4-*cis*-benzylidene-2-phenyloxazolin-5-one was followed at 363 mμ (Δε 36,000) or at 380 mμ (Δε 25,000), and the hydrolyses of *p*-nitrophenyl acetate and *N*-acetyl-L-valine *p*-nitrophenyl ester were followed at 400 mμ. The spontaneous hydrolysis of 4,4-dimethyl-2-phenyloxazolin-5-one was observed at 250 mμ. The low solubility of 4-*cis*-benzylidene-2-phenyloxazolin-5-one necessitated the addition of organic solvents to the buffers. Consequently, the reaction mixtures used contained 10 or 20% (v/v) acetonitrile or 4.8% (v/v) dioxane, the limiting solubility of 4-*cis*-benzylidene-2-phenyloxazolin-5-one in such systems being ~10^{-5} M. α-Chymotrypsin solutions were titrated with *N-trans*-cinnamoylimidazole (Schonbaum *et al.*, 1961) before and after a series of kinetic runs.

Kinetic constants, k_{cat} and K_m, for the α-chymotrypsin-catalyzed hydrolyses of 4-*cis*-benzylidene-2-phenyloxazolin-5-one and *N*-acetyl-L-valine *p*-nitrophenyl ester, determined under steady-state conditions ($[S]_0 \gg [E]_0$), were evaluated from data for Lineweaver–Burk plots by the method of least squares. *N*-Benzoylaminoisobutyryl-α-chymotrypsin was prepared by reaction of 4,4-dimethyl-2-phenyloxazolin-5-one with a slight excess of α-chymotrypsin in 0.01 M acetate buffers (pH 5.0 or pD 5.5) (de Jersey and Zerner, 1969). Aliquots of acyl-enzyme were then added to the Tris buffers, to which *p*-nitrophenyl acetate had previously been added to give a concentration of 6.5×10^{-4} M. The rate of release of *p*-nitrophenol increased until a zero-order rate was attained, indicating that deacylation was complete. First-order rate constants for the deacylation of *N*-benzoylaminoisobutyryl-α-chymotrypsin in the various Tris buffers were thus obtained. The α-chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate was followed at a single substrate concentration (6.5×10^{-4} M).

Infrared spectra of methyl α-benzamido-*cis*-cinnamate and the Tris amide of α-benzamido-*cis*-cinnamic acid were determined on a Perkin-Elmer 237 spectrophotometer, using Nujol mulls and NaCl optics.

Results

Enzymatic Hydrolyses of 4-cis-Benzylidene-2-phenyloxazolin-5-one and N-Acetyl-L-valine p-Nitrophenyl Ester. The hydrolysis of 4-*cis*-benzylidene-2-phenyloxazolin-5-one by α-chymotrypsin was first studied in 0.1 M Tris buffers (pH 7.9) containing 20% (v/v) acetonitrile or 4.8% (v/v) dioxane at 25°; values of k_{cat} (0.31 ± 0.03 sec^{-1}) and K_m(7×10^{-6} M) were obtained. Burst experiments in 0.1 M acetate buffer (pH 5.34) containing 20% (v/v) acetonitrile were performed with [4-*cis*-benzylidene-2-phenyloxazolin-5-one]$_0$ = 6.9×10^{-6} M and $[E]_0 = 1.7 \times 10^{-6}$ M. Under these conditions, an initial rapid decrease in absorbance (or burst) equal to the enzyme concentration was observed. Considering the three-step mechanism proposed for α-chymotrypsin-catalyzed hydrolyses (eq 1),[2] the equivalence of the size

$$E + S \underset{}{\overset{K_S}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} \underset{+\ P_1}{ES'} \xrightarrow{k_{+3}} \underset{+\ P_2}{E} \qquad (1)$$

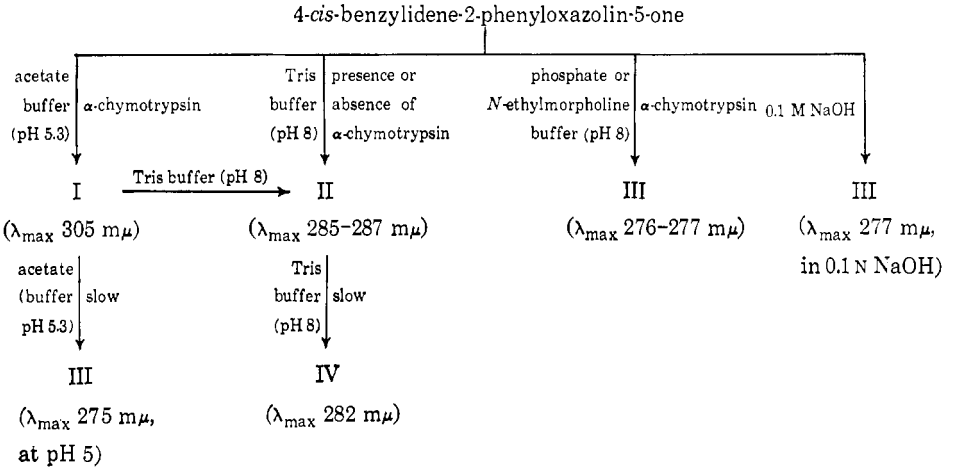
of the burst to the enzyme concentration indicates that $k_{+2} \gg k_{+3}$ and that $[S]_0 \gg K_m$ (Ouellet and Stewart, 1959). Observation of the hydrolysis of 4-*cis*-benzylidene-2-phenyloxazolin-5-one by α-chymotrypsin at pH 5.34 gave values of $k_{cat} = 2.5 \times 10^{-4}$ sec^{-1} and $K_m \leq 10^{-6}$ M. By measuring the size of the burst at low substrate concentrations, K_m was estimated at ~2×10^{-7} M. The α-chymotrypsin-catalyzed hydrolysis of 4-*cis*-benzylidene-2-phenyloxazolin-5-one in 0.1 M phosphate buffer (pH 7.93) containing 10% (v/v) acetonitrile was examined, giving values of 0.033 sec^{-1} and 4×10^{-7} M for k_{cat} and K_m.

The hydrolysis of *N*-acetyl-L-valine *p*-nitrophenyl ester was followed in 0.1 M phosphate buffer (pH 6.96). Values of 0.17 sec^{-1} and 2.7×10^{-4} M for k_{cat} and K_m were obtained.

Spectra of the Hydrolysis Products of 4-cis-Benzylidene-2-phenyloxazolin-5-one. Ultraviolet absorption spectra of the products of spontaneous and α-chymotrypsin-catalyzed hydrolyses of 4-*cis*-benzylidene-2-phenyloxazolin-5-one under various conditions were determined. The results obtained are summarized in Scheme I. Species II is formed from 4-*cis*-benzylidene-2-

[2] For oxazolinones, which may be regarded as internal esters, there is no P_1.

SCHEME I



phenyloxazolin-5-one in Tris buffers in the presence or absence of α-chymotrypsin, with $[S]_0 < [E]_0$, or with $[S]_0 > [E]_0$. Species II is also formed when species I, prepared from 4-*cis*-benzylidene-2-phenyloxazolin-5-one and α-chymotrypsin at pH 5.3, is added to Tris buffer. After α-chymotrypsin has reacted with 4-*cis*-benzylidene-2-phenyloxazolin-5-one (in excess) to form species II, the enzyme is fully active when assayed against *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester.

Spectra of α-benzamido-*cis*-cinnamate ion in 0.1 M acetate buffer (pH 5.3; 20% (v/v) acetonitrile), in 0.1 M Tris buffer (pH 8.0; 10% (v/v) acetonitrile), and in 0.1 N NaOH have maxima at 275, 276, and 277 mμ, respectively. Spectra of methyl α-benzamido-*cis*-cinnamate and free α-benzamido-*cis*-cinnamic acid in 0.1 N HCl both show λ_{max} values of 285 mμ. The apparent pK_a of α-benzamido-*cis*-cinnamic acid, measured by spectrophotometric titration at 315 mμ, was found to be 3.8. The spectrum of the Tris amide of α-benzamido-*cis*-cinnamic acid in 0.1 M Tris buffer (pH 8.2) containing 10% (v/v) acetonitrile had λ_{max} 282 mμ (ε 18,800). The Tris amide is stable under these conditions.

The infrared spectrum of the Tris amide of α-benzamido-*cis*-cinnamic acid had absorption maxima at 1657 (sh), 1649 (sh), 1642, 1612, 1530, and 1290 cm^{-1} which could be ascribed to the two amide groups present in the molecule. The infrared spectrum of methyl α-benzamido-*cis*-cinnamate had absorption maxima at 1720, 1657 (sh), 1649 (sh), 1642, and 1290 cm^{-1} (sh). The 1720-cm^{-1} absorption is therefore due to the ester carbonyl group, while the common maxima at 1657, 1649, 1642, and 1290 cm^{-1} are presumably associated with the benzamido carbonyl group.

Effects of Tris and D_2O on α-Chymotrypsin-Catalyzed Hydrolyses. Kinetic constants obtained from a study of the α-chymotrypsin-catalyzed hydrolysis of 4-*cis*-benzylidene-2-phenyloxazolin-5-one in a series of Tris buffers in H_2O (pH 8.02 ± 0.02), and in a similar series in D_2O (pD 8.53 ± 0.02), are listed in Table I. For comparative purposes, the effects of Tris and D_2O on the α-chymotrypsin-catalyzed hydrolyses of 4,4-dimethyl-2-phenyloxazolin-5-one and *p*-nitrophenyl acetate were determined. Figure 1 shows a plot of k_{cat} *vs.* [Tris] for hydrolyses of 4-*cis*-benzylidene-2-phenyloxazolin-5-one and 4,4-dimethyl-2-phenyloxazolin-5-one in the aqueous buffers. Second-order rate constants for Tris catalysis of deacylation, k_T, were obtained from Figure 1 and similar plots, and are listed in Table II. Values of k_T are expressed in terms of the concentration of Tris free base.

Spontaneous Hydrolysis of 4-cis-Benzylidene-2-phenyloxazolin-5-one. The hydrolysis of 4-*cis*-benzylidene-2-phenyloxazolin-5-one was followed in the same Tris buffers as were used for the enzymatic hydrolyses. With an initial 4-*cis*-benzylidene-2-phenyloxazolin-5-one concentration of 8×10^{-7} M, first-order kinetics were obeyed when the decrease in oxazolinone concentration was followed at 380 mμ. Second-order rate constants for Tris catalysis were obtained from plots of k_{obsd} *vs.* [Tris] and are included in Table II. Similarly, values of k_T in the spontaneous hydrolyses of 4,4-dimethyl-2-phenyloxazolin-5-one and *p*-nitrophenyl acetate were determined (Table II). In all cases, plots of k_{obsd} *vs.* [Tris] were linear. The rate constants for the decrease in [4-*cis*-benzylidene-2-phenyloxazolin-5-one] (measured at 380 mμ) also measure the rates of formation of species II. When the reaction of 4-*cis*-benzylidene-2-phenyloxazolin-5-one in Tris buffers is observed at 310 mμ, the rapid formation and slower decay of species II is observed (Figure 2). The effects of [Tris] on the rate of formation of species II (measured at 380 mμ) and on the rate of conversion of species II into species IV (measured at 310 mμ) were determined (Figure 3). Because of the smallness of the absorbance change corresponding to the formation of species IV, and the slowness of the reaction (Figure 2), rate constants could not be evaluated with accuracy. The spontaneous hydrolysis of 4-*cis*-benzylidene-2-phenyloxazolin-5-one in 0.1 M phosphate and *N*-ethylmorpholine buffers at pH 8 was found to occur very slowly. With an initial [4-*cis*-benzylidene-2-phenyloxazolin-5-one] of 8.5×10^{-6} M, the decrease in absorbance at 380 mμ was not first order. Glycine ethyl ester proved to react rapidly with 4-*cis*-benzylidene-2-phenyloxazolin-5-one, giving a stable product with λ_{max} 282 mμ. In 0.18 M glycine ethyl ester–HCl buffer (pH 7.70), k_{obsd} was measured at $1.7_2 \times 10^{-2}$ sec^{-1}.

Effect of α-Chymotrypsin on the Conversion of Species II into Species IV. The conversion of species II into

TABLE I: Kinetic Constants for the α-Chymotrypsin-Catalyzed Hydrolysis of 4-*cis*-Benzylidene-2-phenyloxazolin-5-one in Tris buffers at 25°.[a]

[Tris][b] (M)	Solvent[c]	k_{cat} (sec^{-1})	K_m (μM)
0.10	H_2O	0.73	2.9_3
	D_2O	0.35	2.1_5
0.05	H_2O	0.48	2.4_1
	D_2O	0.20	1.3_6
0.035	H_2O	0.41	2.3_3
	D_2O	0.18	1.2_7
0.02	H_2O	0.27	1.4_9
	D_2O	0.12	1.1_2
0.01	H_2O	0.16_5	0.95
	D_2O	0.06	0.49

[a] $[E]_0 = 0.42_4$ μM; [4-*cis*-benzylidene-2-phenyloxazolin-5-one]$_0$ varied from 1 to 10 μM. [b] This concentration refers to the total concentration of Tris; buffers at half-neutralization (pH 8.02 ± 0.02 or pD 8.53 ± 0.02). [c] Reaction mixtures contained 12.1% (v/v) acetonitrile.

TABLE II: Spontaneous and α-Chymotrypsin-Catalyzed Hydrolyses in Tris Buffers in Water and Deuterium Oxide at 25°.

Substrate	$k_T^{H_2O}$[a] (M^{-1} sec^{-1})	$k_T^{D_2O}$[a] (M^{-1} sec^{-1})	$k_T^{H_2O}/k_T^{D_2O}$
α-Chymotrypsin-catalyzed hydrolyses			
4-*cis*-Benzylidene-2-phenyloxazolin-5-one	18.4	9.0	2.0_5
4,4-Dimethyl-2-phenyloxazolin-5-one	0.098	0.03_8	2.5
p-Nitrophenyl acetate	0.090	0.05_0	1.8
Spontaneous hydrolyses			
4-*cis*-Benzylidene-2-phenyloxazolin-5-one	0.040	0.036	1.1
4,4-Dimethyl-2-phenyloxazolin-5-one	0.0009_5	0.0009_2	1.0_3
p-Nitrophenyl acetate	0.00040	0.00038	1.0_5

[a] The second-order rate constants refer to the concentration of Tris free base. The ten Tris buffers in H_2O and D_2O listed in Table I were used in all these experiments.

species IV was found to be catalyzed by α-chymotrypsin. With initial concentrations of α-chymotrypsin and 4-*cis*-benzylidene-2-phenyloxazolin-5-one equal to 1.40×10^{-5} and 8.3×10^{-6} M, respectively, the reaction was followed in a series of Tris buffers (0.1–0.8 M, pH 8.25). Observation at 310 mμ was commenced when the absorbance at 380 mμ (due to 4-*cis*-benzylidene-2-phenyloxazolin-5-one) had fallen to zero. The initial rate of decrease in absorbance at 310 mμ, corrected for the rate in the absence of enzyme, was found to be independent of Tris concentration. The product of the reaction of α-chymotrypsin with species II had λ_{max} 282 mμ. The effect of enzyme concentration on the conversion of species II into species IV (in 0.1 M Tris buffer; [4-*cis*-benzylidene-2-phenyloxazolin-5-one]$_0$ = 8.3×10^{-6} M) was determined at a range of enzyme concentrations from 1.4×10^{-6} to 1.4×10^{-5} M. The initial rate of reaction, measured at 310 mμ, was found to be proportional to the enzyme concentration.

Reactions of Isolated Species II. Species II was isolated as follows: 500 μl of 4.35×10^{-3} M 4-*cis*-benzylidene-2-phenyloxazolin-5-one was added with stirring to 80 ml of 0.8 M Tris buffer (pH 8.02) and 20 ml of acetonitrile, and the decrease in absorbance at 363 mμ was followed. After 800 sec, the absorbance had fallen to ~2% of the initial absorbance, and the reaction mixture was extracted rapidly with a 100-ml and two 50-ml portions of chloroform. The combined chloroform extracts were washed with three 10-ml portions of water, and then extracted with five 50-ml portions of water. The aqueous extracts were combined and concentrated to 40 ml under reduced pressure, extracted with 20 ml of chloroform, and then taken to dryness. The residue was dissolved in ~0.5 ml of water and con-

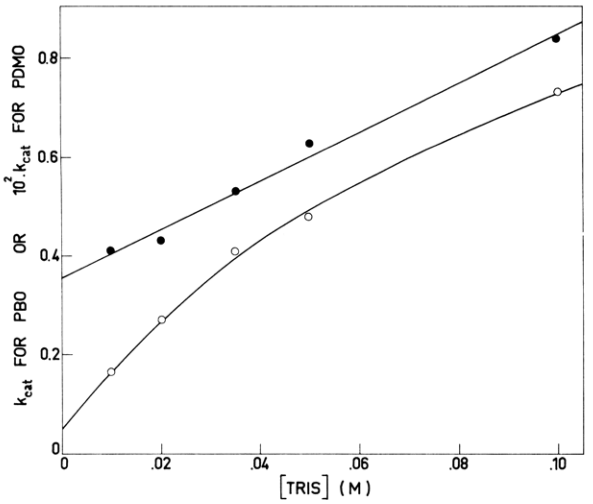


FIGURE 1: Effect of Tris concentration on the catalytic rate constants for the α-chymotrypsin-catalyzed hydrolyses of 4-*cis*-benzylidene-2-phenyloxazolin-5-one (○—○—○) and 4,4-dimethyl-2-phenyloxazolin-5-one (●—●—●) at 25°. Aqueous Tris buffers (pH 8.02); units of k_{cat}, sec^{-1}.

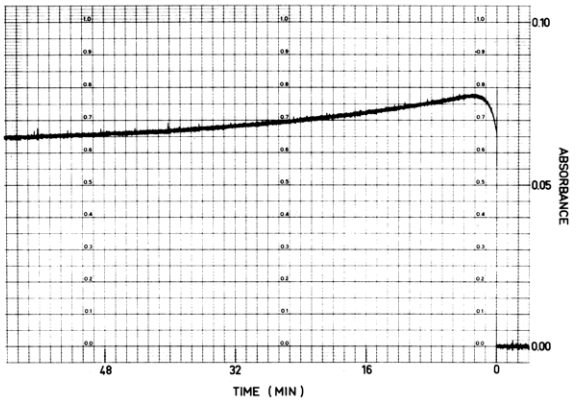


FIGURE 2: Progress curve for the reaction of 4-*cis*-benzylidene-2-phenyloxazolin-5-one in 0.8 M Tris buffer (pH 8.27) containing 10% (v/v) acetonitrile at 25°. $[S]_0 = 8.6 \times 10^{-6}$ M; reaction observed at 310 mμ.

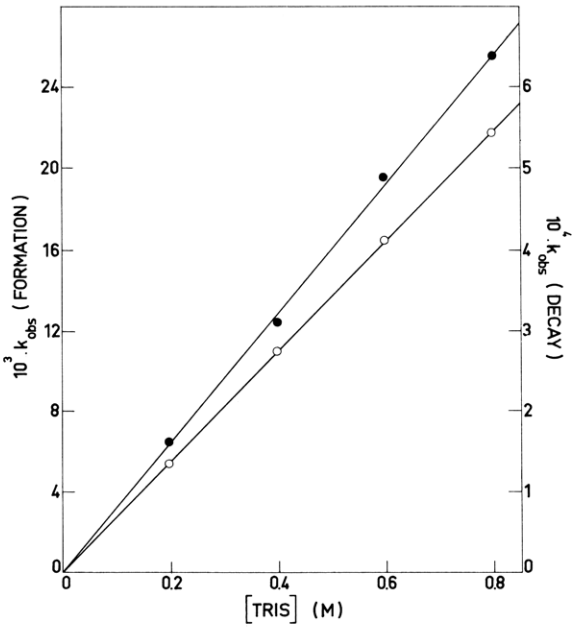


FIGURE 3: Effect of Tris concentration on the apparent first-order rate constants for the formation of species II from 4-*cis*-benzylidene-2-phenyloxazolin-5-one (○—○—○) and for the conversion of species II to species IV (●—●—●) at 25°. Rate constants measured in Tris buffers (pH 8.26 ± 0.01) containing 10% (v/v) acetonitrile; $[S]_0 = 8.6 \times 10^{-6}$ M; units of k_{obsd}, sec^{-1}.

tained about 1 μmole of material (based on the absorbance at 285 mμ). The isolated material was examined rapidly, since a slow spontaneous decomposition was found to occur.

The spectrum of isolated species II in 0.1 M phosphate buffer (pH 7.00) had λ_{max} 287 mμ. Isolated species II reacted rapidly with α-chymotrypsin in 2 mM phosphate buffer (pH 7.2) to give a product with λ_{max} 277–288 mμ (species III). When the reaction was followed at 310 mμ, a small increase in absorbance was followed by a decrease, behavior consistent with the formation of a small amount of acyl-enzyme. The rate of reaction of species II with α-chymotrypsin in phosphate buffer (pH 7) was much greater than the rate of the corresponding reaction in Tris buffers (pH 8.2). Under conditions where species II is rapidly converted into species III, the Tris amide of α-benzamido-*cis*-cinnamic acid is stable. Isolated species II does not react rapidly with glycine ethyl ester, being apparently unchanged after 20 min in 0.18 M glycine ethyl ester–HCl buffer (pH 7.70). Paper chromatography in 1-butanol–pyridine–acetic acid–water (15:10:3:12, v/v) was used to investigate further the nature of species II. Isolated species II was found to be free of Tris, which gave a yellow color with ninhydrin, and ran with an R_F of 0.45. Species II ran at the solvent front, as did α-benzamido-*cis*-cinnamic acid and its Tris amide. After hydrolysis of species II by α-chymotrypsin in 0.1 M phosphate buffer (pH 7.00), the reaction mixture was lyophilized and subjected to chromatography as before. In this way, Tris was shown to be released on hydrolysis of species II. In 0.1 N NaOH, the spectrum of isolated species II had λ_{max} 297 mμ, which decreased to 281 mμ in 60 hr. Spectra of methyl α-benzamido-*cis*-cinnamate and the Tris amide of α-benzamido-*cis*-cinnamic acid in 0.1 N NaOH had $\lambda_{max} \sim$ 304 mμ and 298 mμ, respectively. Under these conditions methyl α-benzamido-*cis*-cinnamate has a half-life of 246 sec at 25°, while the Tris amide has a similar stability to that of isolated species II.

Discussion

4-cis-Benzylidene-2-phenyloxazolin-5-one and N-Acetyl-L-valine p-Nitrophenyl Ester as Activated Substrates. The specificity of the deacylation step of α-chymotrypsin-catalyzed hydrolyses has been investigated using activated esters as substrates (Bender *et al.*, 1964b). For such activated esters, it may be shown that k_{cat} is a direct measure of k_{+3} (*e.g.*, by burst experi-

ments). For methyl esters, however, k_{cat} is often a complex constant. Solution of eq 1 under steady-state conditions gives $k_{cat} = k_{+2}k_{+3}/(k_{+2} + k_{+3})$ and $K_m = (k_{+3}/(k_{+2} + k_{+3}))K_s$ (Gutfreund and Sturtevant, 1956).

4-*cis*-Benzylidene-2-phenyloxazolin-5-one, an activated derivative of α-benzamido-*cis*-cinnamic acid,[3] has several advantages over methyl α-benzamido-*cis*-cinnamate, a stable ester of the same acid, as a substrate in specificity studies. Firstly, 4-*cis*-benzylidene-2-phenyloxazolin-5-one has very desirable spectral characteristics ($\Delta\epsilon$ at 363 mμ = 36,000). Secondly, burst experiments at pH 5.34 indicate that $k_{+2} \gg k_{+3}$. Thirdly, the extremely low K_m (expected if $k_{+2} \gg k_{+3}$) allows the use of substrate concentrations in the range of K_m. Brocklehurst and Williamson (1966) reported values of k_{cat} (0.1 sec^{-1}) and K_m (2.4×10^{-2} M) for the α-chymotrypsin-catalyzed hydrolysis of methyl α-benzamido-*cis*-cinnamate in phosphate buffer (pH 7.9) containing 4.8% (v/v) dioxane, at 25°. However, the highest substrate concentration used was 8.4×10^{-4} M, casting doubt on the accuracy of the reported k_{cat}. Extrapolation of the k_{cat}'s for 4-*cis*-benzylidene-2-phenyloxazolin-5-one hydrolysis to zero [Tris] (Figure 1) gives a value of ~0.04 sec^{-1} at pH 8.02. In phosphate buffer (pH 7.93), a value of 0.033 sec^{-1} for k_{cat} was obtained, in agreement with the previous result, but in contrast with the k_{cat} of 0.10 sec^{-1} obtained by Brocklehurst and Williamson (1966) for the hydrolysis of methyl α-benzamido-*cis*-cinnamate.

Comparison of the measured K_m's for 4-*cis*-benzylidene-2-phenyloxazolin-5-one and methyl α-benzamido-*cis*-cinnamate gives a value of the ratio k_{+2}/k_{+3} for 4-*cis*-benzylidene-2-phenyloxazolin-5-one of ~10^5, if it is assumed that K_s values for 4-*cis*-benzylidene-2-phenyloxazolin-5-one and methyl α-benzamido-*cis*-cinnamate are similar. The magnitude of this ratio shows the magnitude of the error which may be involved if the term k_{+3}/k_{+2} in the expression for K_m is neglected. Gutfreund (1955) estimated k_{+1}, the rate of formation of ES, for the trypsin–*N*-benzoyl-L-arginine ethyl ester system. However, in calculating k_{+1} to be $\geq 4 \times 10^6$ M^{-1} sec^{-1}, it was assumed that $K_m = (k_{-1} + k_{+2})/k_{+1} = K_m^{BH}$, rather than $(k_{+3}/(k_{+2} + k_{+3}))K_m^{BH}$. The measured K_m for *N*-benzoyl-L-arginine amide (2×10^{-3} M) is likely to be a better estimate of K_m^{BH} than is the K_m for *N*-benzoyl-L-arginine ethyl ester (10^{-5} M) (Zerner and Bender, 1964). Substitution in Gutfreund's calculation gives a limiting value of $k_{+1} \geq 10^5$ M^{-1} sec^{-1}. This considerably weakens the argument that $k_{-1} \gg k_{+2}$ and therefore that $K_m^{BH} = K_s = k_{-1}/k_{+1}$.

Hein and Niemann (1961) determined k_{cat} and K_m for the α-chymotrypsin-catalyzed hydrolyses of a family of *N*-acetyl-L-valine derivatives at pH 7.9. In the present work, the α-chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-valine *p*-nitrophenyl ester was observed at pH 6.96 since at higher pH, the formation of oxazolinone may have complicated the experiment (de Jersey and Zerner, 1969). Assuming a pK_a' of 6.86 for the catalytically important group (Bender *et al.*, 1964a), the value of k_{cat} measured at pH 6.96 (0.17 sec^{-1}) gives a value of 0.28 sec^{-1} at pH 7.9. This result is completely consistent with the conclusion that $k_{cat} = k_{+3}$ (Zerner and Bender, 1964). For *N*-acetyl-L-valine methyl ester, $k_{cat} = 0.15$ sec^{-1} and therefore, $k_{+2} = 0.32$ sec^{-1} and $k_{+3} = 0.28$ sec^{-1}.

Although α-benzamido-*cis*-cinnamoyl-α-chymotrypsin possesses the β-aromatic substituent and the *N*-acyl group characteristic of specific substrates, it is a relatively stable acyl-enzyme: $k_{+3} \sim 0.04$ sec^{-1}, compared with ~200 sec^{-1} for *N*-benzoyl-L-tyrosyl-α-chymotrypsin (Gutfreund and Hammond, 1959). *N*-Benzoyl-D-tyrosyl-α-chymotrypsin is similarly stable, with $k_{+3} \sim 0.15$ sec^{-1} (de Jersey and Zerner, 1969).

Nature of the Products of 4-cis-Benzylidene-2-phenyloxazolin-5-one Hydrolysis. Species II (λ_{max} 285–287 mμ) is the first product of the α-chymotrypsin-catalyzed hydrolysis of 4-*cis*-benzylidene-2-phenyloxazolin-5-one in Tris buffers (pH 8). At pH 5, species I (λ_{max} 305 mμ) is formed. Brocklehurst and Williamson (1967) proposed that species I and II are both acyl-enzymes.

The present observations of the reactions of 4-*cis*-benzylidene-2-phenyloxazolin-5-one establish that species II is not an acyl-enzyme, since it is formed in the spontaneous reaction of 4-*cis*-benzylidene-2-phenyloxazolin-5-one in Tris buffers as well as in the α-chymotrypsin-catalyzed reaction. Further, α-chymotrypsin is fully active in a rate assay against *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester in the presence of species II. The burst experiment at pH 5.3, and the observation of Brocklehurst and Williamson (1967) that formation of species I prevents reaction of the enzyme with *N-trans*-cinnamoylimidazole, conclusively demonstrate that species I (λ_{max} 305 mμ) is α-benzamido-*cis*-cinnamoyl-α-chymotrypsin. The λ_{max} is not inconsistent with the identification of the acyl-enzyme as a serine ester (Bender *et al.*, 1962). Species III is clearly α-benzamido-*cis*-cinnamate ion, while species IV is the Tris amide of α-benzamido-*cis*-cinnamic acid. Therefore, the results reported by Brocklehurst and Williamson (1967) do not constitute evidence for an N→O acyl shift in the α-chymotrypsin-catalyzed hydrolysis of 4-*cis*-benzylidene-2-phenyloxazolin-5-one. The rate constants measured by these workers refer to the slow reactions of Scheme I.

The Effect of Tris on the α-Chymotrypsin-Catalyzed Hydrolysis of 4-cis-Benzylidene-2-phenyloxazolin-5-one. The central importance of Tris in explaining the observed phenomena was shown by studying the hydrolysis of 4-*cis*-benzylidene-2-phenyloxazolin-5-one in phosphate and *N*-ethylmorpholine buffers at pH 8. In both cases, the first observable product of the α-chymotrypsin-catalyzed hydrolyses was α-benzamido-*cis*-cinnamate ion. The effect of [Tris] on the kinetic constants for 4-*cis*-benzylidene-2-phenyloxazolin-5-one hydrolysis (Table I) is largely explained as an effect on the deacylation reaction. Thus, k_{cat} (which measures k_{+3}) and K_m

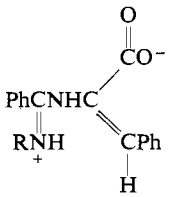
[3] Although the absolute configurations of the geometrical isomers of 4-benzylidene-2-phenyloxazolin-5-one have not been definitely established, the isomer with mp 166° is here assumed to be the *cis* form (*cf.* Brocklehurst and Williamson, 1967).

(which is proportional to k_{+3}/k_{+2}) both increase as [Tris] increases. Tris has a much greater effect on the deacylation of α-benzamido-*cis*-cinnamoyl-α-chymotrypsin than on the deacylation of the other two acyl-enzymes examined. Thus, k_T for α-benzamido-*cis*-cinnamoyl-α-chymotrypsin is ~200 times k_T for benzoyl-aminoisobutyryl-α-chymotrypsin and acetyl-α-chymotrypsin. Such differences in the magnitude of Tris catalysis indicate that even if two compounds are compared as substrates for α-chymotrypsin in the same Tris buffer, the k_{+3} ratio obtained may not reflect the ratio at zero concentration of Tris (see Figure 1 for an example). Although plots of k_{cat} against [Tris] are linear for 4,4-dimethyl-2-phenyloxazolin-5-one and *p*-nitrophenyl acetate, the plot for 4-*cis*-benzylidene-2-phenyloxazolin-5-one shows a saturation effect (Figure 1). A plot of $1/k_{cat}$ against 1/[Tris] is linear, and assuming that this saturation effect indicates the formation of a Michaelis complex, then the K_m for Tris obtained from the reciprocal plot is 0.05 M. Another possible explanation for the saturation effect would be that k_{+2} is no longer much greater than k_{+3} at high [Tris]. This explanation seems unlikely since K_m is still very low in the 0.1 M Tris buffer (3 μM).

Faller and Sturtevant (1966) first studied the effect of Tris on the hydrolysis of acetyl-α-chymotrypsin in Tris buffers at pH 7.0 containing 10% (v/v) dioxane. Fife and Milstien (1967) measured the effect of Tris on the deacylation of butyryl-α-chymotrypsin at pH 8.80, obtaining a k_T of 0.06 M^{-1} sec^{-1} in terms of Tris free base. This value is similar to the present value of k_T for Tris catalysis of the deacylation of acetyl-α-chymotrypsin (0.09 M^{-1} sec^{-1}). Oliver and Viswanatha (1968) observed that Tris catalyses the deacylation of *N-trans*-cinnamoyl-trypsin and that the free base is the catalytic species. These workers reacted Tris with *N-trans*-cinnamoylimidazole and isolated a compound with mp 190° and λ_{max} 279 mμ. They stated that the mass spectrum was consistent with the compound being an *O*-cinnamoyl derivative, *i.e.*, an ester of *trans*-cinnamic acid. In the present work, the final product of the reaction of Tris with 4-*cis*-benzylidene-2-phenyloxazolin-5-one has been identified as the Tris amide of α-benzamido-*cis*-cinnamic acid. The infrared spectrum indicates the absence of an ester carbonyl group (by comparison with the infrared spectrum of methyl α-benzamido-*cis*-cinnamate). Further, the λ_{max} (282 mμ) bears the same relationship to the λ_{max} of methyl α-benzamido-*cis*-cinnamate (285 mμ) as does the λ_{max} of cinnamamide (276 mμ) to the λ_{max} of methyl cinnamate (279.5 mμ) (B. Zerner, unpublished result).

Effect of Tris on the Spontaneous Hydrolysis of 4-cis-Benzylidene-2-phenyloxazolin-5-one. The values of k_T for the spontaneous hydrolyses of 4-*cis*-benzylidene-2-phenyloxazolin-5-one, 4,4-dimethyl-2-phenyloxazolin-5-one, and *p*-nitrophenyl acetate (Table II) indicate that Tris reacts with 4-*cis*-benzylidene-2-phenyloxazolin-5-one much more efficiently than with 4,4-dimethyl-2-phenyloxazolin-5-one or *p*-nitrophenyl acetate. The formation of species II and its conversion into species IV are both first order in [Tris] (Figure 3), supporting the direct participation of Tris in both reactions.

The Nature of Species II. An oxazolinone may react (nonenzymatically) in two ways with nucleophiles. For example, Leplawy *et al.* (1960) found that the hindered nucleophile, methyl α-aminoisobutyrate, reacts with 4,4-dimethyloxazolin-5-one in benzene by nucleophilic attack at C-2, giving the ring-opened amidine as product. When glycine ethyl ester is the nucleophile, reaction occurs principally at C-5 (and presumably also at carbon 2). It would appear possible, therefore, that the *nonenzymatic* reactions of 4-*cis*-benzylidene-2-phenyloxazolin-5-one could be accounted for by nucleophilic attack of the hindered nucleophile, Tris, at C-2, to give the amidine

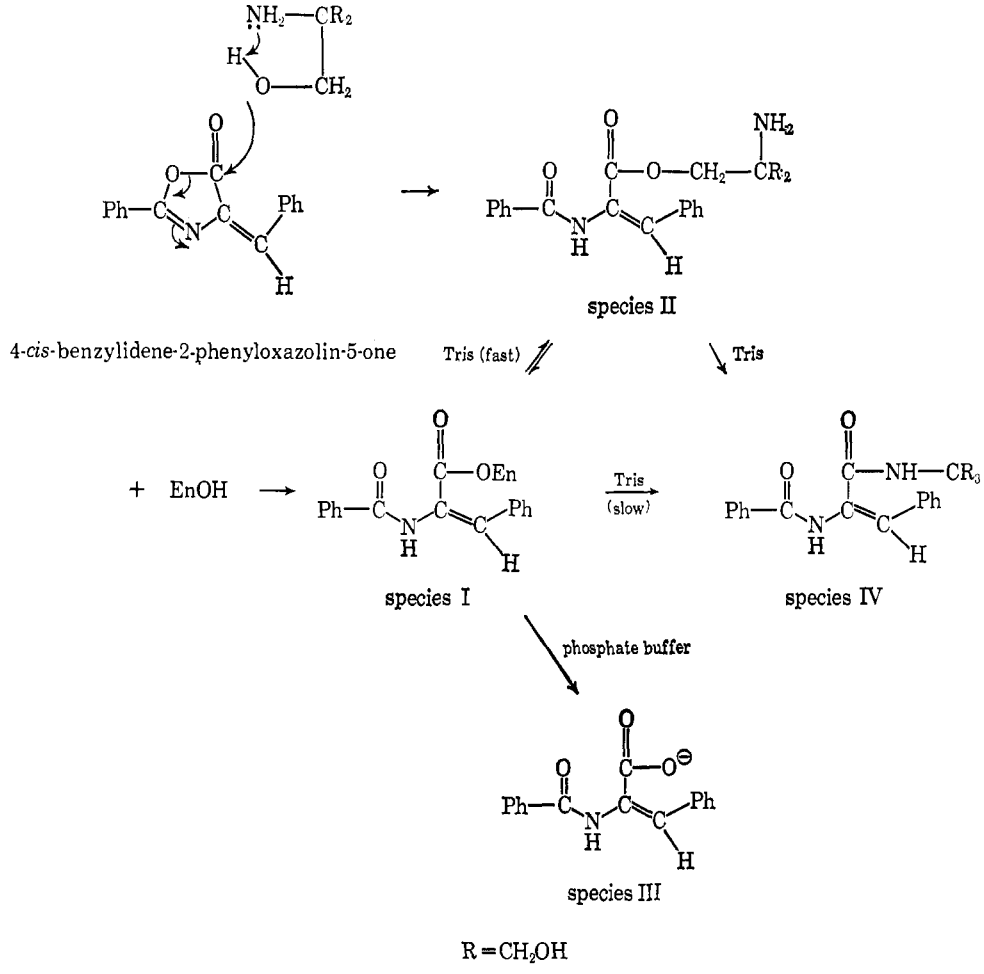


Further, this would be in accord with the assumed *cis* configuration of the phenyl group and the carbonyl carbon (C-5) of 4-*cis*-benzylidene-2-phenyloxazolin-5-one. It should be noted, however, that the most recent evidence favors the *trans* configuration for the isomer of 4-benzylidene-2-phenyloxazolin-5-one used in this study (Kochetkov *et al.*, 1960; but *cf.* Filler, 1965).

Detailed consideration of the experimental evidence, however, supports Scheme II as the mechanism of Tris intervention in the nonenzymatic and enzymatic reactions of 4-*cis*-benzylidene-2-phenyloxazolin-5-one (*cf.* de Jersey and Zerner, 1967). In Scheme II, it is proposed that species II is an ester formed by nucleophilic attack of a Tris hydroxyl group, which is subject to intramolecular base catalysis by the amino group. Simple nucleophilic attack by a Tris hydroxyl group seems unlikely when the relative nucleophilicities of amino and hydroxyl groups are compared. The conversion of species II into species IV could occur by intramolecular or intermolecular aminolysis.

The evidence which supports this scheme is now summarized. (i) An intermediate similar to species II was not observed in the reaction of glycine ethyl ester with 4-*cis*-benzylidene-2-phenyloxazolin-5-one. Such an intermediate is not possible according to Scheme II, but is not excluded by nucleophilic attack at C-2. Nucleophilic attack by the amino group of Tris would be subject to steric hindrance by the hydroxymethyl groups. Such steric hindrance would be greatly reduced in glycine ethyl ester and the product of the reaction of 4-*cis*-benzylidene-2-phenyloxazolin-5-one with glycine ethyl ester (λ_{max} 282 mμ) is reasonably identified at the corresponding amide. That species II is not an amidine is indicated by its λ_{max} (285–287 mμ), since the amidine should resemble α-benzamido-*cis*-cinnamate ion. (ii) The compound isolated by Oliver and Viswanatha (1968) by reaction of *N-trans*-cinnamoylimidazole with Tris is a Tris ester of cinnamic acid. Jencks and Carriuolo (1960b) had previously reported similar results with acetylimidazole and also from the reaction of Tris with *p*-nitrophenyl acetate. They proposed that reaction

SCHEME II



proceeds by intramolecular general base catalysis (*cf.* Scheme II). Bruice and York (1961), however, argued that the reaction of *p*-nitrophenyl acetate with Tris at pH 8 is an uncomplicated aminolysis, giving the *N*-acetyl derivative of Tris.

That the product of Tris intervention in the deacylation of *trans*-cinnamoyl-trypsin is relatively stable to catalysis by α-chymotrypsin (Oliver and Viswanatha, 1968), whereas species II is easily hydrolyzed by α-chymotrypsin in phosphate buffer, is readily accounted for in terms of the "specificity" of methyl cinnamate and methyl α-benzamido-*cis*-cinnamate. (iii) The observed values of $k_T^{H_2O}/k_T^{D_2O}$ (Table III), while apparently consistent with nucleophilic attack, are not inconsistent with Scheme II, since Jencks and Carriuolo (1960a) observed that the general-base-catalyzed aminolysis of phenyl esters by amines exhibits only a small isotope effect (glycine, 1.1; ammonia, 1.5). (iv) The behavior of isolated species II in 0.1 N NaOH is readily explained in terms of Scheme II, since an O→N shift has been found to occur rapidly under alkaline conditions (Pierce and Lunsford, 1951). In base, then, the Tris ester could rearrange rapidly to the amide (λ_{max} 298 mμ; *cf.* species II, λ_{max} 297 mμ) which is only slowly hydrolyzed (λ_{max} 281 mμ after 60 hr). (v) Catalysis by Tris of the conversion of species II to species IV (Figure 3) is readily understood if Tris acts as a general base catalyst of the intramolecular aminolysis. However, it should be noted that part of the observed catalysis may be due to a salt effect, since ionic strength was not held constant in this experiment (Figure 3). (vi) Catalysis by α-chymotrypsin of the formation of species IV is probably due to the presence of a small amount of species I (the acyl-enzyme) at equilibrium. This catalysis is independent of [Tris], as expected from Scheme II in the presence of moderate concentrations of Tris.

These considerations show that Scheme II adequately accounts for the experimental observations. Tris may react with other oxazolinones and acyl-α-chymotrypsins to give intermediates similar to species II. Tris certainly has a large catalytic effect on the rate of ring opening of 2-phenyloxazolin-5-one (de Jersey *et al.*, 1969) and on the deacylation of hippuryl-α-chymotrypsin (J. de Jersey, A. A. Kortt, and B. Zerner, unpublished results). However, spectral observation of species II was facilitated by the conjugated double-bond system of α-benzamido-*cis*-cinnamic acid, and even in this system, spectral changes are quite small. Therefore, the generality of the proposed series of reactions may be difficult to establish.

These experiments do not comment critically on the configuration of the 4-benzylidene-2-phenyloxazolin-5-one isomer used in this work. However, they do point up further complications in the use of activated esters

as substrates. The reactions of oxazolinones (hindered and not hindered at C-5) with nucleophiles (hindered and unhindered) will be further investigated to define these reactions in aqueous solution.

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