cavity of proper size to interact, either on the surface of the cycle or within the cavity, with aromatic groups of chymotrypsin substrates. This hypothesis receives support from the cyclodextrins,[11-13] cyclic substances which bind numerous aromatic compounds including the α-chymotrypsin substrate, N-acetyl-L-tyrosine ethyl ester,[13] *via* inclusion compounds. The cyclodextrins are thought to be doughnut-shaped molecules whose cavity is largely hydrophobic,[12] as is Fig. 1. Certainly Fig. 1 will explain the hydrophobic bonding which occurs in both α-chymotrypsin and trypsin. But in trypsin, some additional feature of the site must explain the ionic interaction between substrate and enzyme.

The bead model of α-chymotrypsin, shown in Fig. 2, was constructed on the basis of its amino acid sequence. In front of the white card are seen both components of the active site, the cycle bounded by Cys 42 to Cys 58 and the strand from Cys 191 to Cys 201. Serine 195 (↓), is almost directly above histidine 57 (↑). The remaining amino acids of the site presumably determine binding and specificity. The acyl group of the substrate may bind longitudinally across the cycle while the chemistry occurs at the top portion of the cycle. Figures 1 and 2 make many predictions concerning the structure of the active site, the mode of binding, and the mechanism of action of the enzyme which may be tested.

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The Acyl-enzyme Intermediate, *trans*-Cinnamoylpapain[1]

Sir:

α-Chymotrypsin, which contains a reactive alcoholic group, catalyzes the hydrolysis of cinnamate derivatives through the intermediate formation of *trans*-cinnamoyl-α-chymotrypsin, as has been shown spectrophotometrically.[2] In order to investigate the occurrence of acyl-enzyme intermediates in reactions catalyzed by enzymes presumed to contain a reactive thiol group,[3] we have investigated the catalysis of the hydrolysis of N-*trans*-cinnamoylimidazole by papain. We report our findings now, since the spectral observation of a dithioacylpapain intermediate in the papain-catalyzed hydrolysis of methyl thionohippurate was recently reported.[4]

When approximately equimolar papain and N-*trans*-cinnamoylimidazole were mixed at pH 5.2, the substrate absorption disappeared while a small maximum at ~325 mμ appeared; this absorption eventually disappeared leaving only the spectrum of cinnamate ion. Clearly a species involving the cinnamoyl group with λ_{max} near 325 mμ was present during the reaction. Since the activity of a *trans*-cinnamoylpapain solution toward α-N-benzoyl-L-arginine ethyl ester reappears at the same rate as *trans*-cinnamoylpapain deacylates, the cinnamoyl group must be attached to the active site. The cinnamoyl group is probably covalently attached to the enzyme rather than physically adsorbed because the species is formed slowly and survives Sephadex G-25 filtration.

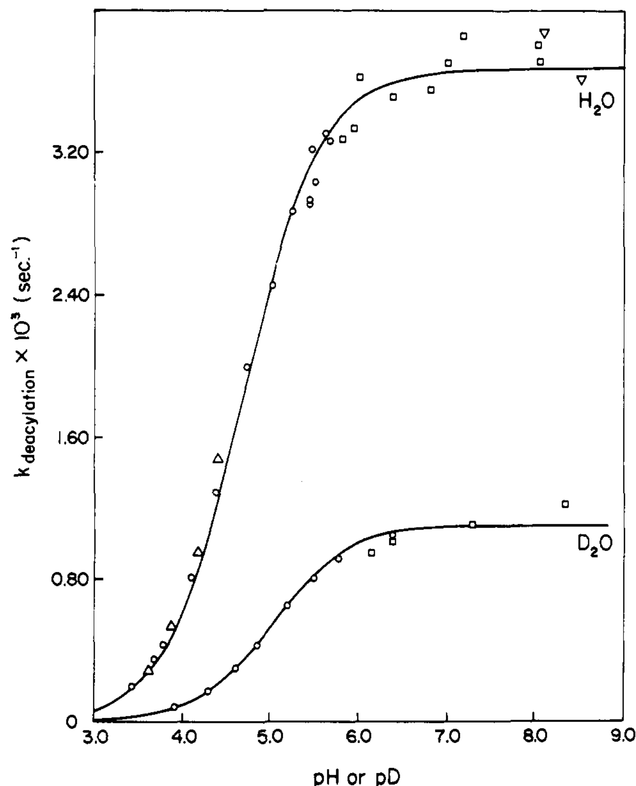

Fig. 1.—The effect of pH (or pD) on the deacylation of cinnamoylpapain. △ 0.05M formate; ○, 0.05 M acetate; □, 0.033 M phosphate; ▽, 0.0125 M borate; μ = 0.100, T = 25.0°. The solid lines are theoretical curves assuming dependence of rate constant on a single basic group; H_2O, pK_a = 4.69 and k^{lim} = 3.68 × 10^{-3} sec.$^{-1}$; D_2O, pK_a = 5.03, k^{lim} = 1.10 × 10^{-3} sec.$^{-1}$.

To prepare a solution of *trans*-cinnamoylpapain, a solution of papain (1.47 × 10^{-5} M)[5] was mixed with an excess of N-*trans*-cinnamoylimidazole (2.9 × 10^{-4} M) at pH 3.43 at 25°; after 300 sec., the excess substrate was removed by Sephadex filtration, prewashing the column with the desired buffer. The deacylation reactions, observed at 330 mμ, followed first-order kinetics over a range of enzyme (17-fold) and substrate (3-fold) concentrations. The rate constants were independent of buffer concentration and of ionic strength greater than μ = 0.10. The deacylation is dependent on a basic group of pK_a = 4.69 (Fig. 1). The deacylation of *trans*-cinnamoylpapain is affected by D_2O in the same manner as is the deacylation of cinnamoyl-α-chymotrypsin.[6] The pK_a of the basic group

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on which the reaction is dependent is perturbed by 0.34 pK units to pK_a = 5.03 in D_2O, and the rate constant is decreased by 3.35-fold in D_2O.

The following indirect method was used to obtain the difference spectrum of *trans*-cinnamoylpapain *vs.* papain, since this acyl-enzyme cannot be prepared quantitatively. A difference spectrum of a solution of the acyl-enzyme and enzyme (freed from excess substrate by Sephadex filtration) *vs.* enzyme was obtained using a Cary 14 PM spectrophotometer. The spectrum was obtained immediately after filtration and was repeated at 7 min. intervals until 3 to 4 half-lives had elapsed. By combining the spectral data and data on the concentration of cinnamic acid at various times, it was possible to calculate the difference spectrum of *trans*-cinnamoylpapain, using eq. 1, where $\epsilon_\lambda{}^{CP}$ is the (difference) molar absorptivity of

$$\epsilon_\lambda{}^{CP} - \epsilon_\lambda{}^{C} = \frac{A_\lambda{}^t - A_\lambda{}^\infty}{[C]^\infty - [C]^t} \quad (1)$$

the acyl-enzyme *vs.* enzyme at any wave length. Values of $A_\lambda{}^t$, the absorbance at time t and wave length λ, known to ±0.6%, were taken from the early spectra. Values of $A_\lambda{}^\infty$ were calculated from plots of the absorbance *vs.* time data from the repeated spectra.[7] Values of $[C]^t$, the concentration of cinnamic acid at time t, were found by spectrophotometric determination of cinnamic acid in aliquots of the reaction mixture; the cinnamic acid was separated from the acyl-enzyme and enzyme by Sephadex filtration and its concentration at various times was determined. $\epsilon_\lambda{}^C$ is the molar absorptivity of cinnamic acid at wave length λ.

The λ_{max} for the difference spectrum of *trans*-cinnamoylpapain *vs.* papain calculated on this basis, 326 mμ, is considerably higher than those of *trans*-cinnamoyl derivatives of three serine proteinases (Table I), indicating that *trans*-cinnamoylpapain is a different

TABLE I
DIFFERENCE SPECTRA OF SOME CINNAMOYL-ENZYMES AND CINNAMOYL ESTERS[a]

trans-Cinnamoyl-	λ_{max}, mμ	ϵ_{max}	$\lambda_{max} - \lambda_{max}$(model) m$\mu$	Δkcal.
Papain[d]	326[g]	26,500[c]	20	5.73
α-Chymotrypsin[2]	292[h]	17,700	10.5	3.65
Trypsin[i]	296	19,400	14.5	4.98
Subtilisin[b]	289	21,000	7.5	2.65
N-Acetylserinamide[e]	281.5	24,300	..	..
Cysteine[f]	306	22,600	..	..

[a] pH 4, 1.6% (v./v.) acetonitrile-water, 25°. [b] Unpublished observations of Dr. M. L. Begué. [c] Probable error in absorptivity is ±3%. [d] 0% CH_3CN, pH 3.43. [e] 10% CH_3CN. [f] pH 2.1. [g] 309 mμ after treatment with 4.8 M guanidinium chloride. [h] 282 mμ at 51°.[j] [i] M. L. Bender and E. T. Kaiser, *J. Am. Chem. Soc.*, **84**, 2556 (1962). [j] J. Mercuroff and G. P. Hess, *Biochem. Biophys. Res. Commun.*, 11, 283 (1963).

chemical species than the other three cinnamoyl-enzymes. Ruling out *trans*-cinnamoylimidazole because of the pH dependence of the deacylation (Fig. 1), the closest model compound of *trans*-cinnamoylpapain is the thiol ester S-cinnamoylcysteine,[8] which is found to have a λ_{max} of 306 mμ. Thus, the λ_{max} of *trans*-cinnamoylpapain is significantly higher than the λ_{max} of its model compound just as the λ_{max} of *trans*-cinnamoyl-α-chymotrypsin and the other acyl-serine proteinases are higher than the λ_{max} of the model compound, N-acetylserinamide. In energy terms, the electronic transition of the conjugated system is 4.2 ± 1.5 kcal./mole less in the cinnamoyl-enzyme than in the cinnamoyl ester, for all enzymes investigated. This common relationship supports the spectral assignment of *trans*-cinnamoylpapain as a thiol ester. This conclusion is supported by the fact that denaturation of *trans*-cinnamoylpapain in 4.8 M guanidinium chloride gives a λ_{max} of 309 mμ, almost identical with that of the model, S-*trans*-cinnamoylcysteine. Although an acylimidazole would be expected to acylate a thiol (papain) faster than an alcohol (α-chymotrypsin), this result is not found, presumably because of differing specificities of these two enzymes.

The common relationship between the absorption maxima of *trans*-cinnamoyl-enzymes of different chemistry and their model compounds (Table I) may result from a common interaction of the conjugated system and the protein.

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Daunomycin. I. The Structure of Daunomycinone

Sir:

Daunomycin, a metabolite of *Streptomyces peucetius*,[1] is an antibiotic which exhibits strong inhibition of the growth of a variety of experimental tumors.[2]

Daunomycin hydrochloride, $C_{27}H_{29}O_{10}N \cdot HCl$,[3] m.p. 188–190° dec., $[\alpha]_D$ +253° (c 0.15, methanol), displays an ultraviolet spectrum closely related to that of 1,4,5-trihydroxyanthraquinones,[4] the maxima in the visible region being less defined and shifted 5–10 mμ toward longer wave lengths when compared directly with the spectrum of helminthosporin. The quinoid chromophore is also proved by the ready reversible reduction on treatment with reducing agents and by polarographic behavior. Mild acid hydrolysis (0.2 N hydrochloric acid for 1 hr. at 90°) affords a red aglycone, daunomycinone, and a new amino sugar, daunosamine.[5]

Daunomycinone, $C_{21}H_{18}O_8$, m.p. 213–214°, $[\alpha]_D$ +193° (c 0.1, dioxane), one OCH_3, same electronic spectrum as the parent glycoside, yields tetracene, identified by its ultraviolet spectrum, on zinc dust distillation, thus suggesting the tetracyclic structure already found in the anthracyclines.[6] The presence of an aliphatic ketonic group is shown by the infrared absorption at 1718 cm.$^{-1}$ and by the ready formation of a 2,4-

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