Cryoscopic molecular weight determinations of the polymers was made by using a Beckmann thermometer and a Hershberg melting point apparatus. *p*-Nitrotoluene, m.p. 51.7°, K_f 0.78, was used as solvent.

Catalytic hydrogenation of N,N-divinylaniline was performed in a volumetric microhydrogenation apparatus. Raney nickel, 10% palladium-on-charcoal and platinum dioxide were all successfully used as catalyst. Either absolute ethanol or diglyme were used as solvent.

The product isolated from catalytic hydrogenation of N,N-divinylaniline formed a picrate salt as yellow needles, m.p. 140°. The melting point was identical to that of diethylaniline picrate prepared in the same way (lit.[13] 142°). Mixing the two picrates did not cause a lowering of the melting point.

Attempted preparation of the picrate of N,N-divinylaniline by the same method gave only a brown resinous material melting over a range of 114° to 125°.

Determination of residual double bonds in polymers and copolymers by catalytic hydrogenation was made according to the same general procedures as in the hydrogenation of the monomer. Diglyme was used as solvent in most cases on account of the solubility of the polymers. The polymers were introduced to the solvent–catalyst mixture after the system had been allowed to come to equilibrium either by dropping the preweighed sample from the sample container inside the vessel or by injecting into the system a measured quantity of the diglyme solution of the polymer. The latter method was necessary for many fluffy or powdery samples which could not be weighed properly in the sample container. Diglyme was prepurified to remove peroxide and kept under a nitrogen atmosphere. In each case the reaction was allowed to run at least 18 hours to ensure completion of the reaction.

The infrared spectrum of N,N-divinylaniline showed the pertinent peaks as shown in Table XVII.

TABLE XVII

INFRARED ABSORPTION BANDS FOR N,N-DIVINYLANILINE

Wave length, μ	Intensity	Wave length, μ	Intensity
3.30	Weak	8.32	V. strong
6.03sh	Medium	8.96	Weak
6.10sh	Strong	9.35	Weak
6.20sh	V. strong	9.45	Weak
6.28	V. strong	9.80	Weak
6.69	Strong	10.22sh	Medium
7.08	Medium	10.37	V. strong
7.30	Medium	10.62	Weak
7.42	Strong	12.30	V. strong
7.62	Strong	13.10	Strong
7.80	Strong	14.35	V. strong
8.06	V. strong	14.80	Medium

The disappearance or diminishing of the strong shoulders in the 6.0 to 6.2 μ region of the spectra of polymers and copolymers leaving only a sharp band at 6.25 μ similar to that of diethylaniline was used as a criterion for the reduced double bond content in addition to the catalytic hydrogenation analyses.

(13) R. L. Shriner and R. C. Fuson, "Systematic Identification of Organic Compounds," John Wiley and Sons, Inc., New York, N. Y., 3rd. edition, 1948, p. 242.

COMMUNICATIONS TO THE EDITOR

A COMPARISON OF THE ACYL-ENZYME INTERMEDIATES, CINNAMOYL-TRYPSIN AND CINNAMOYL-α-CHYMOTRYPSIN[1]

Sir:

The reaction of N-*trans*-cinnamoylimidazole with α-chymotrypsin proceeds by a mechanism involving a preliminary adsorptive step followed by two catalytic steps, acylation of the enzyme producing cinnamoyl-α-chymotrypsin then deacylation of this intermediate to give cinnamate and regenerate the enzyme.[2,3] It has now been demonstrated that the trypsin-catalyzed hydrolysis of N-*trans*-cinnamoylimidazole proceeds in the same fashion, and it is possible to compare the spectrophotometric and kinetic behavior of the two acyl-enzyme intermediates, cinnamoyl-trypsin and cinnamoyl-α-chymotrypsin.

The rates of acylation of trypsin and α-chymotrypsin by N-*trans*-cinnamoylimidazole and the rates of deacylation of cinnamoyl-trypsin and of cinnamoyl-α-chymotrypsin have been determined spectrophotometrically (Table I). The data in Table I indicate that at concentrations of enzyme which are equal to or greater than that of the substrate and at pH's around 4 or 5, the rate of acylation is much greater than that of deacylation for α-chymotrypsin, and reasonably greater for trypsin. This result indicates that it should be possible to prepare and observe reasonably stable acyl-enzymes at those pH's. Cinnamoyl-α-chymotrypsin can be prepared at pH 4 in a quantitative fashion by the stoichiometric reaction of N-*trans*-cinnamoylimidazole and α-chymotrypsin, and can be kept for hours at pH 4 with only slight decomposition. The difference spectrum of cinnamoyl-α-chymotrypsin *vs.* α-chymotrypsin is shown in Fig. 1.[4] The trypsin-catalyzed hydrolysis of N-*trans*-cinnamoylimidazole at pH 5.2 has been carried out at an [enzyme]/[substrate] ratio 23.7. Under these conditions the acylation and deacylation reactions can be considered to be two consecutive first-order reactions[2] in which the first step is 39 times as fast as the second. The maximum concentration of the intermediate, cinnamoyl-trypsin, formed transiently then can be calculated to be 90.0% of the initial substrate concentration. It then was possible to obtain a difference spectrum of cinnamoyl-trypsin *vs.* trypsin at the time of maximum concentration of the intermediate, correcting for the 2.3% of reactant and the 7.7% of product present. The difference spectrum of the intermediate also was computed from observations on a solution containing

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

(2) M. L. Bender and B. Zerner, *J. Am. Chem. Soc.*, **83**, 2391 (1961).

(3) M. L. Bender, G. R. Schonbaum, G. A. Hamilton and B. Zerner, *ibid.*, **83**, 1255 (1961).

(4) (a) In both difference spectra the absorbance due to the enzyme has been subtracted directly by an equivalent concentration of the appropriate enzyme in the reference cell of the spectrophotometer. (b) The cinnamoyl-α-chymotrypsin difference spectrum omits consideration of the small bumps observable in the enlarged view of the maximum shown in M. L. Bender, G. R. Schonbaum and G. A. Hamilton, *J. Polymer Sci.*, **49**, 75 (1961).

TABLE I

A COMPARISON OF THE ACYLATION AND DEACYLATION REACTIONS OF TRYPSIN AND α-CHYMOTRYPSIN USING N-*trans*-CINNAMOYLIMIDAZOLE AS SUBSTRATE AT 25.0°

Parameter	Trypsin[a]	α-Chymotrypsin[b]
$k_{acylation}$ at *p*H 5.2[d] (l./mole sec.)	63.4[c]	12×10^3
$k_{deacylation}$ at *p*H 5.2[e] (sec.$^{-1}$)	1.69×10^{-4}	1.4×10^{-4}
λ_{max} of cinnamoyl-enzyme (mμ)	296	292
ϵ_{max} of cinnamoyl-enzyme	19,300	17,700
k_{OH} of cinnamoyl-enzyme in 7.74 *M* urea (l./mole sec.)	4.5×10^{-2}	4.1×10^{-2}
pK_a' of deacylation of cinnamoyl-enzyme	7.3	7.15

[a] 1.6% acetonitrile–water, tris–acetic acid buffer. [b] 1.6% acetonitrile–water, acetate buffer. [c] Assuming molecular weight of trypsin is 24,000 and using protein absorbance (optical factor = 0.694) as a measure of concentration. [d] Determined by the disappearance of the substrate. [e] Determined by the disappearance of the cinnamoyl-enzyme and/or by the appearance of cinnamate ion.

77.7% of acyl-enzyme, 20.6% of reactant and 1.7% of product. The agreement between the two spectra was better than 2%. The difference spectrum of cinnamoyl-trypsin *vs.* trypsin based on the average of these two calculations is shown in Fig. 1.[4a]

The spectrophotometric and kinetic results of the trypsin-catalyzed hydrolysis of N-*trans*-cinnamoylimidazole give direct experimental proof of the two-step catalytic mechanism, and support the earlier indirect kinetic evidence of Schwert and Eisenberg[5] and of Stewart and Ouellet.[6a]

The acylation of trypsin by N-*trans*-cinnamoylimidazole is considerably slower than that of α-chymotrypsin. It is possible that the difference in (second-order) acylation constants results from a difference in the adsorptive equilibrium constants and not in the rate constants of acylation themselves.[6b]

The rate constants for the deacylation of cinnamoyl-trypsin and cinnamoyl-α-chymotrypsin are very similar in magnitude. The effects of pH on the deacylation rate constants are also similar.[7] The similarity of cinnamoyl-trypsin and cinnamoyl-α-chymotrypsin is further seen in a comparison of the spectra of these compounds, and a comparison of the alkaline hydrolytic rate constants of these compounds in 7.74 *M* urea. The spectra of these intermediates cannot be analyzed from a structural point of view because of our ignorance of the effect of the enzyme environment on the spectrum. However, superficially the two compounds are quite similar to each other. When the two intermediates are converted to ordinary esters by the denaturing solvent, 7.74 *M* urea, they act kinetically similar to each other and to the model compound O-cinnamoyl-N-acetylserinamide.[3] Thus it appears that cinnamoyl-trypsin, like cinnamoyl-α-chymotrypsin, is an ester of a serine moiety of the enzyme.[3]

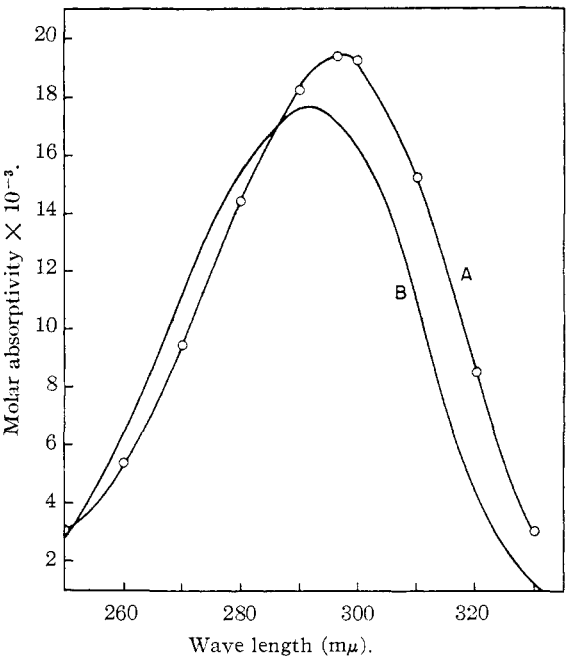

Fig. 1.—Difference spectra of cinnamoyl-trypsin, A, and cinnamoyl-α-chymotrypsin, B; see text for details.

Although the specificities of trypsin and α-chymotrypsin may differ from each other, the mechanism of their catalytic action appears to be the same. Trypsin and α-chymotrypsin are similar with respect to biological origin, molecular weight, types of substrates on which they act, the presence of a single active site per molecule, the presence of a DFP-inhibitable serine hydroxyl group in this active site, and a proton of the peptide sequence surrounding this active site. To these similarities can now be added similarities with respect to the stepwise catalytic sequence, pH dependence of the catalytic action and similarities in the spectral and kinetic behavior of the acyl-enzyme intermediate. The formation of an acyl-enzyme intermediate now has been demonstrated for two related serine proteinases[10]; it is not unreasonable to extrapolate this mechanism to all enzymes of this family.

(5) G. W. Schwert and M. A. Eisenberg, *J. Biol. Chem.*, **179**, 665 (1949).

(6) (a) J. A. Stewart and L. Ouellet, *Can. J. Chem.*, **37**, 751 (1959). (b) These authors found that the first-order acylation constants for the α-chymotrypsin- and trypsin-catalyzed hydrolyses of *p*-nitrophenyl acetate were similar.

(7) The trypsin-catalyzed hydrolysis of N-α-benzoyl-L-arginine ethyl ester depends on a group with an apparent pK_a of 6.25[8] or 6.02[9] in water at 25°. While the apparent pK_a found here does not quantitatively agree with these results, the findings are similar and indicate that the process being observed here is related to those observed with specific substrates.

(8) H. Gutfreund, *Trans. Faraday Soc.*, **51**, 441 (1955).

(9) T. Inagami and J. M. Sturtevant, *Biochim. et Biophys. Acta*, **38**, 64 (1960).

(10) B. S. Hartley, *Ann. Revs. Biochem.*, **29**, 45 (1960).

(11) Alfred P. Sloan Foundation Research Fellow.

(12) National Institutes of Health Postdoctoral Research Fellow.

DEPARTMENT OF CHEMISTRY
NORTHWESTERN UNIVERSITY
EVANSTON, ILLINOIS

MYRON L. BENDER[11]
E. T. KAISER[12]
BURT ZERNER

RECEIVED OCTOBER 2, 1961

HEPTALENIUM ION

Sir:

We wish to report the synthesis of 1-heptalenium fluoroborate (I) by a four-step sequence from 1,5-naphthalenedicarboxylic acid utilizing the ring enlargement route developed by Nelson, Fassnacht and Piper[1] for the preparation of cycloheptatrienes.

(1) N. A. Nelson, J. H. Fassnacht and J. U. Piper, *J. Am. Chem. Soc.*, **83**, 206 (1961).