The Chymotrypsin-catalyzed Hydrolysis and Synthesis of N-Acetyl-L-tyrosine Hydroxamic Acid*

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The formation of an acyl-enzyme intermediate has been demonstrated in the chymotrypsin-catalyzed hydrolysis of activated acyl compounds (1) such as acyl chlorides, p-nitrophenyl esters, anhydrides, and acylamidazoles, as well as with alkyl esters of cinnamic acid (2) and N-acetyltrypophan (3). There is, however, no direct evidence indicating that a similar intermediate is formed in the hydrolysis of unactivated peptide-like substrates, such as amides, peptides, hydroxamic acids, or acylhydrazides. Bernhard, Coles, and Nowell (4) have concluded from a kinetic argument that the chymotrypsin-catalyzed hydrolysis and hydrolylaminoysis of methyl hippurate do not proceed through an acyl-enzyme intermediate, and Bender and Glasson (5) have reported results similar to those upon which Bernhard et al. base their conclusion, for the hydrolysis and methanolysis of N-acetyl-L-phenylalanine methyl ester. Recently, Epand and Wilson (6) have shown that a number of different hippurate esters give the same yield of hydroxamic acid in the presence of chymotrypsin and a constant concentration of hydroxylamine. If it is assumed that a direct reaction of hydroxylamine with different enzyme-bound esters would give different yields of hydroxamic acid, this is evidence that a common acyl-enzyme intermediate is formed in these reactions.

The formation of an acyl-enzyme intermediate in the hydrolysis of peptide-like substrates would be expected to be difficult, because the thermodynamic barrier to formation of an acyl-enzyme involving an ester linkage would probably prevent the accumulation of detectable quantities of this intermediate. Furthermore, enzymatic hydrolysis of unactivated peptide-like substrates has been found to occur only with substrates which would be expected to form extremely labile acyl-enzymes, as estimated from the rates of enzymatic hydrolysis of the corresponding p-nitrophenyl and alkyl esters. The formation of an acyl-enzyme in the reaction of chymotrypsin with unactivated substrates could, however, be demonstrated if this intermediate could be trapped by an acyl group acceptor which could compete effectively with water. Since hydroxylamine has been found to be highly reactive with activated acyl groups (7, 8) and has previously been used to trap the acyl group of isolated acyl-enzymes (9-11), it was used as a trapping agent for the postulated acyl-enzyme formed from the unactivated chymotrypsin substrates, N-acetyl-L-tyrosine hydroxamic acid and N-acetyl-L-tyrosine. In a preliminary communication it was reported that the amount of trapping by hydroxylamine during the hydrolysis of N-acetyl-L-tyrosine hydroxamic acid in the presence of hydroxylamine did not appear to be consistent with the acyl-enzyme mechanism, as it is ordinarily formulated, for the hydrolysis of this compound (12). Subsequently it was shown independently in several laboratories that the quantitative comparisons upon which this conclusion was based are not valid, because the yield of hydroxamic acid in the reference reaction, the hydrolysis of N-acetyl-L-tyrosine ethyl ester in the presence of hydroxylamine, is dependent on the concentration of enzyme as well as hydroxylamine (13-15). When the comparison is made at a constant enzyme concentration and at a concentration of N-acetyl-L-tyrosine hydroxamic acid well above the K_m for this substrate, a difference is still found between the observed yields of hydroxamic acid and those predicted by the acyl-enzyme hypothesis (13), but the differences are considerably smaller than those originally reported (12). A full account of these experiments and of a complementary series of experiments dealing with the chymotrypsin-catalyzed synthesis of N-acetyl-L-tyrosine hydroxamic acid from N-acetyl-L-tyrosine and hydroxylamine is presented here. In addition, the results of some experiments which were carried out to determine the chemical behavior of a known acyl-enzyme, furanyl-chymotrypsin, are reported.

EXPERIMENTAL PROCEDURE

Materials—The ethyl esters of N-acetyl-L-tyrosine, N-acetyl-L-tryptophan, m.p. 105-106° (2), and N-acetyl-L-phenylalanine, m.p. 89.5-91° (16), were obtained from Mann Research Laboratories. Different preparations of ATE exhibited melting points of 79-80° and 96-98°; the reported melting point is 79-80° (17). Both preparations were enzymatically active, and enzymatically active ATH was synthesized from both preparations. N-Acetyl-

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1 Epand and Wilson report that the observed rate of the chymotrypsin-catalyzed hydrolysis of methyl hippurate is slower than the hydrolysis of other hippurate esters and, therefore, slower than the rate of deacylation of hippuryl-chymotrypsin if the reaction proceeds through an acyl-enzyme (6). The slow step of methyl hippurate hydrolysis must therefore be the formation of the acyl-enzyme. On the other hand, Bernhard et al. have reported that the over-all rate of methyl hippurate disappearance is increased several fold in the presence of hydroxylamine (4). If these reactions proceed through an acyl-enzyme, this result requires that the deacylation step be rate-determining, since the over-all rate is increased by increasing the rate of reaction with an acyl acceptor. This apparent inconsistency has not been explained.
l-tyrosine, m.p. 150-151.5°, was obtained from the Cyclo Chemical Corporation. N-Acetyl-l-tyrosine hydroxamic acid, sodium salt, was synthesized from the corresponding ethyl ester (18) and was crystallized from methanol-water (approximately 10:1), m.p. 191-191.5° with decomposition. Analysis gave C 50.35, H 5.10, N 10.43; theoretical: C 50.77, H 5.03, N 10.76. Fururylhydroxamic acid, m.p. 124-124.5°, was synthesized by the method of Jones and Hurd (10) with the use of methyl furate, prepared from fururyl chloride and sodium methoxide, as the starting material, and was crystallized twice from water. Fururylimidazole was synthesized by the method described previously (20). p-Nitrophenyl acetate, m.p. 77.5-79.5°, was synthesized by the method of Clistawin (21) and recrystallized from diethyl ether. Three times crystallized, salt-free a-chymotrypsin (Lot 6010B) was obtained from the Worthington Biochemical Corporation measured spectrophotometrically at 540 nm as the ferric hydroximate complex after the addition of 4.0 ml of a solution of 20% ferric chloride in 1.4 M HCl. Alkaline hydroxylamine solution (1 ml) was then added, and the ferric hydroxamate complex was measured spectrophotometrically. The yields of the corresponding hydroxamic acids could not be determined quantitatively, because the pure compounds were not available for use as standards. However, a rough estimate of the amount of hydroxamic acid formation was made by assuming that the yields in the nonenzymatic reactions of these esters with alkaline hydroxylamine were the same as those with ATE under the same conditions. That this assumption is approximately correct in the case of acetylphenylalanine ethyl ester is suggested by the fact that the yield of hydroxamic acid in the enzymatic reaction, calculated on this basis, levels off and begins to approach 100% as the hydroxylamine concentration is raised to 3.0 M.

The rate of hydrolysis of ATE was followed by measuring the amount of remaining ester in aliquots of the reaction mixture with a slight modification of the Hestrin alkaline hydroxylamine procedure (22). Aliquots (1 ml) were removed from the reaction mixture and added to 1.0 ml of a solution which was freshly prepared by mixing 2 parts of 3.5 M NaOH with 1 part of 4.0 M hydroxylamine hydrochloride (alkaline hydroxylamine solution). The acid-hydroxamic acid formed was measured spectrophotometrically at 540 mμ as the ferric hydroxamate complex after the addition of 4.0 ml of a solution of 20% FeCl₃·6H₂O in 1.4 M HCl. The ester concentration was determined by reference to a weighed standard solution of ATE which was treated in an identical manner. The rate of synthesis of ATH and the final yield of hydroxamic acid from the enzymatic hydroxylaminolysis of ATE at enzyme concentrations of 1.9 to 2.5 × 10⁻⁴ M were determined by adding 1.0-ml aliquots of the reaction mixture to 4.0 ml of a solution of 20% ferric chloride in 1.4 M HCl. Alkaline hydroxylamine solution (1 ml) was then added, and the ferric hydroxamate concentration was determined by reference to a weighed standard solution of ATE which was made up in an identical reaction mixture without the enzyme. The rate of ester disappearance during the simultaneous hydrolysis and hydroxylaminolysis of ATE was determined by measuring the amount of remaining ester in aliquots of the reaction mixture by the Hestrin alkaline hydroxylamine procedure described above, correcting for the amount of hydroxamic acid formed enzymatically. It should be noted that the yield of hydroxamic acid from ATE in the Hestrin alkaline hydroxylamine assay is low and depends upon the hydroxylamine concentration. The yield was found to be 74% when 1.0 ml of a solution of ATE in 3.45 M hydroxylamine (98% free base) was added to 1.0 ml of alkaline hydroxylamine solution. In addition, the color intensity of the ferric hydroxamate complex is dependent upon the acid concentration, and it is therefore necessary to adjust all ferric chloride and alkaline hydroxylaminolysis reaction mixtures to the same final hydroxylamine and hydrogen ion concentrations.

The yield of ATH in experiments in which rates were not measured was determined by adding 0.5 ml aliquot of the reaction mixture to 2.0 ml of 20% ferric chloride in 1.4 M HCl. In each case it was shown that the reactions with ATE had proceeded to completion and that no significant amount of ATE hydrolysis had occurred during the period of observation by analysis of aliquots taken at several time intervals and by calculation from the rates of ATE and ATH disappearance measured in separate experiments. With the more concentrated enzyme solutions the reaction was complete in 30 seconds, at the time the first aliquot was taken.

The rates and products of the chymotrypsin-catalyzed hydroxylaminolysis of N-acetyl-L-tryptophan ethyl ester and N-acetyl-L-phenylalanine ethyl ester were measured by adding 1.0-ml aliquots of the reaction mixture to 4.0 ml of a solution of 20% ferric chloride in 1.4 M HCl. Alkaline hydroxylamine solution (1 ml) was then added, and the ferric hydroxamate complex was measured spectrophotometrically. The yields of the corresponding hydroxamic acids could not be determined quantitatively, because the pure compounds were not available for use as standards. However, a rough estimate of the amount of hydroxamic acid formation was made by assuming that the yields in the nonenzymatic reactions of these esters with alkaline hydroxylamine were the same as those with ATE under the same conditions. That this assumption is approximately correct in the case of acetylphenylalanine ethyl ester is suggested by the fact that the yield of hydroxamic acid in the enzymatic reaction, calculated on this basis, levels off and begins to approach 100% as the hydroxylamine concentration is raised to 3.0 M.

The rate of hydrolysis of ATH was followed by the ferric chloride assay. In a typical assay, 0.08-ml aliquots were removed from a reaction mixture containing 0.1 M substrate and were added to 10.0 ml of a solution of 20% ferric chloride in 1.4 M HCl. Reactions were followed approximately 20 to 47% toward completion, and the rates were zero order for the time period in which they were followed. The pH of the reactions was determined in a diluted aliquot at the beginning or end of the kinetic run, or both, or by following the pH in a reaction mixture identical with the one used for kinetic study.

The rate of ATH synthesis was followed by adding 0.20-ml aliquots of the reaction mixture to 1.0 ml of a 20% ferric chloride solution containing 1.4 M HCl. The quenched reaction mixtures were then adjusted to the same hydroxylamine and hydrogen ion concentration by addition of neutralized hydroxylamine hydrochloride or water, or both. Precipitated protein and ATE were removed by filtering the solutions through glass wool prior to spectrophotometric measurement of the ferric hydroxamate complex at 540 mμ. The hydroxamic acid concentration was determined by reference to a standard solution of ATH which was made up in a reaction mixture similar to that used for kinetic measurements, except that chymotrypsin and ATE were omitted. The readings were corrected for the absorption at 540 mμ of ATE in the presence of ferric chloride.

Fururyl-chymotrypsin—Fururyl-chymotrypsin was synthesized by allowing a stoichiometric amount of fururylimidazole to react
with chymotrypsin at 25° in 0.01 M acetate buffer at pH 4.67, for at least 1 hour. Synthesis of furoyl-chymotrypsin occurs in approximately 15 minutes under these conditions, as measured by the disappearance of furoylimidazole absorption at 290 m\u20131, but the longer incubation was carried out to allow any residual furoylimidazole to hydrolyze nonenzymatically. The rate constant for the nonenzymatic hydrolysis of furoylimidazole under these conditions is 5.3 \times 10^{-2} \text{ min}^{-1} (measured spectrophotometrically at 283 m\u20131), and since acylation consumes most of the furoylimidazole, there is no furoylimidazole remaining when the kinetic studies with furoyl-chymotrypsin are carried out. The extent of acylation was determined by measuring the catalytic activity of an aliquot of the acyl-enzyme solution with p-nitrophenyl acetate, by the method which has been described previously (20), except that 0.1 M phosphate buffer, pH 7.4, was used in the assay mixture. The enzyme was found to be 80 to 90\% acylated under these conditions.

The rate of deacylation of furoyl-chymotrypsin was followed by measuring the disappearance of the difference spectrum of the acyl-enzyme against an identical solution containing unacylated chymotrypsin as a blank. Although the \( \lambda_{\text{max}} \) of the difference spectrum of furoyl-chymotrypsin is at 255 m\u20131, the total change in absorbance in the formation of furoic acid anion (\( \lambda_{\text{max}} = 245 \text{ m}\u20131 \)) is greatest at 265 m\u20131. The difference spectrum of the products of deacylation was recorded at the end of the kinetic run. Since the enzyme was only 80 to 90\% acylated by furoylimidazole, the difference spectra are contaminated by absorption resulting from the small amount of furoic acid formed nonenzymatically.

**Product of Deacylation of Furoyl-chymotrypsin in 0.042 M Hydroxylamine**—Deacylation was carried out at 25° in 0.08 M phosphate buffer, pH 7.4, in the presence of 0.042 M hydroxylamine. The reaction mixture, containing the deacylation product, was deproteinized by the addition of trichloroacetic or perchloric acid to a final concentration of 0.86 M and 1.0 M, respectively, and the mixture was then centrifuged in a clinical centrifuge for 10 minutes and passed through glass wool to remove all traces of precipitated protein. In a number of instances, more than one centrifugation was necessary to remove all of the protein. An aliquot of the filtrate was then allowed to react for 30 minutes with 2.0 M hydroxylamine (56\% free base). It was shown that under these conditions an furoylhydroxylamine (formed instantaneously in the reaction of 0.2 M hydroxylamine, 96\% free base, with 2.9 \times 10^{-3} M furoylimidazole) is converted to furoylhydroxamic acid. The furoylhydroxamic acid formed in this reaction, as well as that formed directly from the reaction of hydroxylamine with furoyl-chymotrypsin, was measured spectrophotometrically as the ferric chloride complex at 540 m\u20131. The difference between the amounts of furoylhydroxamic acid found before and after reaction with concentrated neutral hydroxylamine represents the amount of furoylhydroxylamine formed in the reaction of hydroxylamine with the acyl-enzyme. Since the color of the furoylhydroxamic-ferric chloride complex is unstable, the absorbance of all assay mixtures, including a sample of pure furoylhydroxamic acid which was treated in an identical manner, was measured at a standard time interval (generally 15 minutes) after the addition of ferric chloride.

**RESULTS**

**Reaction of Hydroxylamine with N-Acetyl-L-tyrosine Ethyl Ester**—The chymotrypsin-catalyzed reaction of hydroxylamine with ATE in the presence of 1.9 to 2.5 \times 10^{-4} M enzyme was followed by simultaneously measuring the appearance of hydroxamic acid and the disappearance of ester. Since the rate of ester disappearance is the sum of the rates of hydrolysis and hydroxylaminolysis, the hydrolytic rate can be determined by subtracting the rate of hydroxylaminolysis from the rate of ester disappearance. The results of a typical experiment are presented in Fig. 1. The rates of hydroxylaminolysis and ester disappearance follow zero order kinetics until the reactions are almost complete. Inhibition by the products of the reaction is not significant under these conditions, since the final concentration of products is low (initial ATE concentration, 5.10 \times 10^{-3} M). The first order rate constants for the hydrolysis and hydroxylaminolysis of ATE, obtained by dividing the observed zero order rates by the enzyme concentration, were summarized in Table 1 for a number of different experimental conditions.

The partitioning of ATE between water and hydroxylamine in the presence of 1.9 to 2.5 \times 10^{-4} M enzyme can be determined by measuring the fraction of the initially present ester which has been converted to hydroxamic acid at the end of the experiments or by comparing the individual rate constants for hydrolysis and hydroxylaminolysis. The ratio of the rate constants for the two reactions should predict the ratio of the products formed and, as shown in Table 1, there is good agreement between the two methods of calculation. The yield of hydroxamic acid at this enzyme concentration varies from 50\% at 0.8 M hydroxylamine to 80\% at 3.0 M concentration. The nonenzymatic hydroxylaminolysis of ATE (cf. Anderson (24)) and the enzymatic hydrolysis of ATH were shown to be negligible under the conditions of these reactions.

The rate of the enzymatic hydroxylaminolysis of ATE in the presence of 1.9 to 2.5 \times 10^{-4} M enzyme increases with increasing
in the pH range in which the rate of enzymatic hydrolysis is pH-

-independent, the rate constant for the hydrolysis of ATE remains

-amine concentration does not cause a large decrease in the rate

-portion of the initial ATE which was converted to the hydroxamic

-ation of the initial ATE which was converted to the hydroxamic

-nylaminolysis cannot be measured at high enzyme concen-

- of ATE at a constant hydroxylamine concentration is de-

- the rate constant for the hydrolysis of ATE remains

- constant at approximately 150 sec⁻¹ in the presence of hydroxyl-

- yield of ATH to decrease with increasing pH at a

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- Hydroxylaminolysis

| NH₂OH | pH | Ionic strengtha | ktotalb | k⁺c | k⁻d | Hydrox-

- Hydroxylaminolysis

| NH₂OH | pH | Ionic strengtha | ktotalb | k⁺c | k⁻d | Hydrox-

- Hydroxylaminolysis

- standard was maintained with NaCl or KCl.

- ktotal = khydrolysis + khydroxylaminolysis.

- Rate of hydroxylaminolysis.

- Rate of hydrolysis.

- ATEfinal/AEItial.

- Percentage of hydroxylaminolysis = khydroxylaminolysis/ktotal.

- In the absence of Tris buffer.

- Initial ATE concentration was 5.47 × 10⁻³ M.

- hydroxylaminolysis, and no indication of a saturation of the enzyme with respect to acyl group acceptor was observed with hydroxylamine concentrations up to 3.0 M. This is illustrated in Fig. 2, which also shows that increasing the hydroxylamine concentration does not cause a large decrease in the rate of hydrolysis occurring during the hydroxylaminolysis. Thus, in the pH range in which the rate of enzymatic hydrolysis is pH-

- independent, the rate constant for the hydrolysis of ATE remains

- constant at approximately 150 sec⁻¹ in the presence of hydroxyl-

- yield of ATH obtained in the reaction of 2.0 × 10⁻³ M enzyme was unchanged when the reaction was carried out in a silicon-treated test tube (pH 8.2, ionic strength 0.8, 0.2 M Tris). The yield of ATH was also not significantly affected by the presence of 0.1 M AT (1.5 × 10⁻¹ and 2.0 × 10⁻³ M enzyme, 0.8 M hydroxylamine, pH 7.74, ionic strength 2.8, 0.2 M Tris, initial ATE 5.0 × 10⁻³ M; and 4.9 × 10⁻⁵ M enzyme, 2.8 M hydroxylamine, pH 7.03, ionic strength 2.9, initial ATE 5.1 × 10⁻³ M). Preincubation of 2.3 × 10⁻³ and 3.0 × 10⁻³ M enzyme with 1.2 M hydroxylamine at pH 7.7 and 25° for 10 or 100 minutes, respectively, did not affect the yield of ATH upon subsequent reaction with ATE. There is a tendency for the yield of ATH to decrease with increasing pH at a
given hydroxylamine and enzyme concentration (Table I).

- The yield of hydroxamic acid in the reaction of ATE with 0.8 M hydroxylamine in the presence of 3.6 × 10⁻⁴ M chymotrypsin at pH 8.28 was found not to be significantly affected by a 4-fold variation of ATE concentration; yields of 39, 36, and 35% were obtained with 0.0026, 0.0051, and 0.011 M ATE, respectively (0.2 M Tris, ionic strength 0.9). It was found that in the presence of Tris buffer the disappearance of hydroxylamine-reactive material did not proceed entirely to completion, which suggests that a small fraction of the ester acylates Tris. In the presence of 0.2 M Tris at pH 8.0, for example, the hydroxylamine-reactive material at the end of the experiment was 4 to 7% of the starting ester. However, this reaction does not appear to interfere with the measurement of the partitioning of ATE between hydroxylamine and water, because similar results were obtained in the presence and absence of Tris buffer (Table I).

- Hydroxylamine is a bifunctional nucleophile which can react with activated acyl groups to form both hydroxamic acids and

- at 25°. Reaction conditions are given in Table I.
Products of chymotrypsin-catalyzed reaction of hydroxylamine with N-acetyl-L-tryptophan ethyl ester and N-acetyl-L-phenylalanine ethyl ester at 25°

<table>
<thead>
<tr>
<th>Compound</th>
<th>NH₃OH</th>
<th>Ionic strength</th>
<th>pH</th>
<th>Absorbance change at 540 mÅ</th>
<th>Hydroxy-</th>
<th>Rate (absorbance change per 10 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>aminolys</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>y</td>
<td></td>
</tr>
<tr>
<td>N-Acetyl-L-tryptophan ethyl ester</td>
<td>x</td>
<td></td>
<td>2.65</td>
<td>8.15</td>
<td>0.238</td>
<td>0.044</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>2.55</td>
<td>8.10</td>
<td>0.360</td>
<td>0.118</td>
<td>31a</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>3.15</td>
<td>8.15</td>
<td>0.283</td>
<td>0.178</td>
<td>47a</td>
</tr>
<tr>
<td>N-Acetyl-L-phenylalanine ethyl ester</td>
<td>0.8</td>
<td>2.66</td>
<td>7.68</td>
<td>0.370</td>
<td>0.215</td>
<td>51a</td>
</tr>
<tr>
<td></td>
<td>1.8</td>
<td>2.87</td>
<td>7.30</td>
<td>0.425</td>
<td>0.455</td>
<td>73a</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>3.13</td>
<td>7.58</td>
<td>0.468</td>
<td>0.529</td>
<td>85a</td>
</tr>
</tbody>
</table>

* Substrate concentration at zero time was 2.03 x 10⁻⁵ M (Kₘ = 9 x 10⁻⁵ M) (26); enzyme concentration, 7.3 x 10⁻⁸ M.

** Assuming that 100% conversion of ester produces an optical density of 0.377 (see “Experimental Procedure”).

* Substrate concentration at zero time was 3.00 x 10⁻³ M (Kₘ = 1.1 x 10⁻³ M) (27); enzyme concentration, 4.0 x 10⁻⁸ M.

* Assuming that 100% conversion of ester produces an optical density of 0.622 (see “Experimental Procedure”).

O-acetylhydroxylamines (25). The O-acetylhydroxylamine compounds are slowly converted to hydroxamic acid by further reaction with concentrated hydroxylamine. An investigation of the initial product of the hydroxylaminolysis of ATE was therefore carried out to determine whether there is a rapid accumulation of the O-acetylhydroxylamine compound, which is subsequently converted to hydroxamic acid via an enzymatic or nonenzymatic pathway. A typical reaction mixture used for studying the hydroxylaminolysis of ATE (0.2 M Tris buffer, pH 8.2) was analyzed for O-acetyl-L-tyrosylhydroxylamine after the reaction had been allowed to proceed approximately 5% toward completion. The enzymatic reaction was stopped by the addition of 1.0 M HCl to pH 5.5, following by precipitation of protein with trichloroacetic acid at a final concentration of 0.26 M. The deproteinized solution was incubated with 2.0 M hydroxylamine, pH 6.0, for 30 minutes at 25°, to convert any O-acetylhydroxylamine to the corresponding hydroxamic acid (28). No increase in hydroxamic acid was found under these conditions, and it was concluded that extensive accumulation of O-acetyl-L-tyrosylhydroxylamine does not occur under the conditions of this experiment.

Reaction of Hydroxylamine with N-Acetyl-L-tryptophan Ethyl Ester and N-Acetyl-L-phenylalanine Ethyl Ester—Since hydroxylamine is several orders of magnitude more reactive than water with activated acyl groups (25), it was unexpected that such high hydroxylamine concentrations would be required to compete effectively with water for the postulated acetylhydroxyl-enzyme intermediate formed during the hydrolysis of ATE. In order to determine whether this was the result of some peculiarity of ATE, the reactivity of hydroxylamine with N-acetyl-L-tryptophan ethyl ester and N-acetyl-L-phenylalanine ethyl ester was studied under conditions identical with those used for the experiments with ATE (Table II). As was observed with ATE, the final yield of hydroxamic acid and the rate of hydroxamic acid formation are greater at 3.0 M than at 1.8 M hydroxylamine, which shows that these esters are not completely hydroxylaminolized by hydroxylamine concentrations below 1.8 M. Exact estimates of the yield of hydroxamic acid formed from these esters cannot be made, since pure samples of the hydroxamic acids were not available. The results are, however, similar to those obtained with ATE, which undergoes about 80% hydroxylaminolysis in 3.0 M hydroxylamine at approximately the same enzyme concentration. Furthermore, as was observed with ATE, there is no indication from the rates of hydroxamic acid appearance that the enzyme is becoming saturated with respect to the acyl group acceptor at hydroxylamine concentrations up to 3.0 M.

Hydrolysis of N-Acetyl-L-tryosine Hydroxamic Acid in Presence of Hydroxylamine—Hydroxylaminolysis of the postulated acetyltyrosyl-chymotrypsin intermediate formed during the hydrolysis of ATH was measured by studying the effect of hydroxylamine on the initial rate of ATH hydrolysis in the presence and absence of hydroxylamine, under otherwise identical conditions. The results are given in Table III, and data from a typical rate measurement are shown in Fig. 3. The rate of ATH hydrolysis is decreased in the presence of hydroxylamine. However, as shown in the last two columns of Table III, the rate decreases do not

<table>
<thead>
<tr>
<th>Table III</th>
<th>Effect of hydroxylamine on rate of chymotrypsin-catalyzed hydrolysis of 0.10 M N-acetyl-L-tryosine hydroxamic acid at 25°</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>N-acetyl-L-tryosine ethyl ester</td>
</tr>
<tr>
<td>0.8</td>
<td>2.6</td>
</tr>
<tr>
<td>0.8</td>
<td>1.0</td>
</tr>
<tr>
<td>1.8</td>
<td>2.14</td>
</tr>
<tr>
<td>1.8</td>
<td>2.14</td>
</tr>
<tr>
<td>1.8</td>
<td>2.14</td>
</tr>
<tr>
<td>1.8</td>
<td>2.16</td>
</tr>
<tr>
<td>3.0</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>3.0</td>
<td>3.0 ± 0.3</td>
</tr>
</tbody>
</table>

* Maintained with NaCl or KCl.

- pH values were measured in a diluted aliquot at the end of the reaction. The values given are ±0.06 pH unit.
- Rate constants were calculated from the observed zero order rate data divided by the enzyme concentration.
- Calculated for hydrolysis via an acyl-enzyme intermediate from the results obtained in the reaction of ATE with hydroxylamine catalyzed by 1.5 x 10⁻³ M chymotrypsin (Table I).
- This value is ±0.09 pH unit.

Substrate concentration was 0.125 M in 0.4 M Tris; enzyme concentration was 7.5 x 10⁻⁸ M.
precisely parallel the trapping of the postulated acetyltirosyl
enzyme intermediate formed from ATE under the same exper-
nmental conditions. The differences are small, but appear to be
greater than the experimental error of the measurements.

The effect of hydroxylamine on the kinetic parameters of the
chymotrypsin-catalyzed hydrolysis of ATH is shown in Fig. 4. The
$V_{\text{max}}$ is decreased from 2.55 to 1.56 sec$^{-1}$, and the $K_m$ is
increased from 0.024 to 0.031 m in the presence of 1.8 m hydroxy-
lamine. Hydroxylamine therefore produces a mixed type of in-
hibition. The difference between the $K_m$ value reported here
for the hydrolysis of ATH in the absence of hydroxylamine and
the value reported by Foster and Niemann (28) (0.043 m) was
found to be due to an effect of ionic strength. Because of the ef-
ect of hydroxylamine on the $K_m$ for ATH, the observed inhibi-
tion of ATH hydrolysis by hydroxylamine is larger at substrate
concentrations at which the enzyme is not saturated with sub-
strate. Thus, while $V_{\text{max}}$ is decreased 34%, the observed rate of
hydrolysis of 0.01 m ATH is decreased 44% by 1.8 m hydroxy-
lamine.

Although the hydrolysis of ATH is reversible, there is not a
significant amount of reversal of the hydrolytic reaction under
the conditions used for studying the effect of hydroxylamine on
the rate of hydrolysis of ATH (Table III), since in the most unfa-
favorable case, the ATH concentration at the end of the kinetic
run was $5.5 \times 10^{-3}$ m and the calculated equilibrium concen-
tration of ATH under the same conditions is $5.2 \times 10^{-4}$ m (29).

**Kinetics of Synthesis of N-Acetyl-L-tyrosine Hydroxamic Acid—**

The results of two experiments in which the effect of hydroxyla-
mine concentration on the rate of chymotrypsin-catalyzed syn-
thesis of ATH from AT was determined are shown in Fig. 5,
Curves A and B. The rate constants were obtained from the
initial linear segment of runs in which the reaction proceeded 8 to
90% to the equilibrium, as calculated from the equilibrium con-
stant for ATH synthesis (29). The rate of ATH formation in-
creases linearly, within experimental error, with increasing hy-
droxylamine concentration up to 3.0 m. Although a complete
kinetic analysis was not carried out, the reaction shows little de-
pendence on the concentration of AT under these experimental
conditions; the rate in the presence of 3.0 m hydroxylamine was
found to be decreased only 16% when the concentration of AT
was decreased from 0.56 to 0.126 m. The rate constant for the
chymotrypsin-catalyzed synthesis of ATH is $3.18 \times 10^{-3}$ sec$^{-1}$
in the presence of 1.6 m hydroxylamine at 25° and pH 6.98. The
nonenzymatic synthesis of ATH from AT and hydroxylamine
proceeded at a detectable rate and contributed approximately
10% to the observed rates of ATH synthesis in the presence of
chymotrypsin (Fig. 5, Curve D).

**Reaction of Furoyl-chymotrypsin with Hydroxylamine, Ammonia,
Ethanol, and Methanol—** Furoyl-chymotrypsin was synthesized
in 80 to 90% yield by allowing a stoichiometric amount of furoyl-
imidazole to react with chymotrypsin in 0.01 m acetic buffer,
pH 4.67, for 1 hour at 25°. The extent of acylation at the com-
pletion of this reaction was determined by measuring the amount
of $p$-nitrophenol released upon acylation of the remaining free
enzyme with $p$-nitrophenyl acetate, by the method which has
been described previously (20). The rate of deacylation of
furoyl-chymotrypsin was measured by the disappearance of the
difference spectrum of the acyl-enzyme at 265 m$\mu$ against an identical solution containing free chymotrypsin as a

\* M. Goldlust and R. Hammerschlag, unpublished experiments.

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**Fig. 4.** Dependence on substrate concentration of the rate of
hydrolysis of N-acetyl-L-tyrosine hydroxamic acid catalyzed by
$1.32 \times 10^{-3}$ m chymotrypsin in the presence and absence of 1.8 m
dydroxylamine at 25°. The reaction mixture contained Tris
buffer, 0.2 m, pH 8.19 ± 0.03; ionic strength was maintained at
2.15 with NaCl.

**Fig. 3.** Effect of 1.8 m hydroxylamine on the rate of hydrolysis
of N-acetyl-L-tyrosine hydroxamic acid by $1.3 \times 10^{-3}$ m chymo-
trypsin at 25°. The initial ATH concentration was 0.1 m; 0.4 m
Tris buffer, pH 8.17 ± 0.03; ionic strength maintained at 2.15
with NaCl. ●, in the absence of hydroxylamine, $k = 1.78$ sec$^{-1}$;
○, in the presence of 1.8 m hydroxylamine, $k = 1.20$ sec$^{-1}$.
Fig. 5. Rate of synthesis of N-acetyl-L-tyrosine hydroxamic acid catalyzed by 4.9 × 10⁻⁴ M chymotrypsin as a function of hydroxylamine concentration at pH 6.97 ± 0.03. Curve A and B: experimental results; the initial N-acetyl-L-tyrosine concentration was 0.25 M; ionic strength was maintained at 3.2 by the addition of NaCl. Curve C: calculated curve for ATH synthesis via an acyl-enzyme intermediate; to be compared with Curve A (see “Discussion”). The value of \( k_f \cdot [\text{RCOOH} \cdot \cdot \cdot \text{Enz}] / k_i \) was obtained from Equation 4 and the initial slope of Curve A (at a hydroxylamine concentration at which \( k_i \gg k_f [\text{NH}_2\text{OH}] \)), and was found to be 1.92 × 10⁻⁴ μmole of ATH min⁻¹ 2.0 ml⁻¹ M⁻¹ for 4.9 × 10⁻⁴ M enzyme under the experimental conditions described above. The value of \( k_f / k_i = 0.42 \) was obtained from the data of Table 1. Curve C was then plotted from the rearranged form of Equation 4, rate = \( k_f \cdot [\text{RCOOH} \cdot \cdot \cdot \text{Enz}] / (1 + k_i / k_f [\text{NH}_2\text{OH}] ) \). Curve D: nonenzymatic synthesis of ATH under identical conditions.

Rate of decylation is increased 6.9-fold in 0.025 M hydroxylamine at pH 7.4. The rate of decylation of furoyl-chymotrypsin as a function of hydroxylamine concentration is shown in Fig. 7.

Both furoylhydroxamic acid and O-furoylhydroxylamine were shown to be products of the decylation in the presence of dilute hydroxylamine. O-Furoylhydroxylamine was determined by analysis of aliquots of the deproteinized reaction mixture for hydroxamic acid before and after a further incubation with concentrated neutral hydroxylamine, which converts the O-acylhydroxylamine compound to the hydroxamic acid (25). In a typical experiment, 1.86 μmoles of furoyl-chymotrypsin were incubated with 0.042 M hydroxylamine, pH 7.4, for either 3 or 10 minutes. Decylation would be expected to be virtually complete within 3 minutes under these conditions, and analysis of the reaction mixture revealed that 0.41 and 0.57 μmole of hydroxamic acid were formed in 3 and 10 minutes, respectively. After further reaction of a deproteinized aliquot of the reaction mixtures with concent-

Fig. 6. Deacylation of 5.2 × 10⁻⁵ M furoyl-chymotrypsin in the presence and absence of 8.0 × 10⁻³ M hydroxylamine at 25° in phosphate buffer, pH 7.32. Decylation in the absence of hydroxylamine is shown on the lower time scale and by the open circles; \( k = 6.76 \times 10^{-2} \text{ min}^{-1} \). Decylation in the presence of hydroxylamine is shown on the upper time scale and by the crosses; \( k = 2.08 \times 10^{-1} \text{ min}^{-1} \).
tratned neutral hydroxylamine, 0.86 and 0.88 μmole of hydroxylamine were found in the 3- and 10-minute aliquots, indicating that 0.45 and 0.31 μmole of O-furoylhydroxylamine were present after 3 and 10 minutes of deacylation, respectively. O-Furoylhydroxylamine (prepared from furoylimidazole and 0.2 M hydroxylamine at pH 7) was recovered in 80% yield when subjected to the conditions used for the assay of the products of the enzymatic reaction. These values, therefore, are not an exact measure of the amount of O-furoylhydroxylamine formed in the enzymatic reaction, and it may only be concluded that the amount of O-furoylhydroxylamine is as large or larger than the amount of furoylhydroxamic acid formed in the initial reaction. Upon further incubation in the presence of enzyme, there is a decrease in the concentration of O-furoylhydroxylamine and an increase in the concentration of furoylhydroxamic acid. Since the nonenzymatic reaction is very slow under these experimental conditions, this suggests that the enzyme catalyzes the conversion of O-furoylhydroxylamine to furoylhydroxamic acid. No enzymatic hydrolysis of 1.3 × 10⁻³ M furoylhydroxamic acid was detected in 51 hours in the presence of 4.9 × 10⁻⁴ M enzyme at pH 7.90 (0.1 M Tris buffer, ionic strength 0.54, 8.0 × 10⁻⁴ M EDTA).

The reactions of methanol and ethanol with furoyl-chymotrypsin were followed spectrophotometrically at 265 μM in the same manner (Fig. 7). These compounds are considerably more effective nucleophilic reagents than water on a molar basis, although they are far less reactive than hydroxylamine. The rate of deacylation is linearly related to the methanol concentration up to 1.0 M and to the ethanol concentration up to 1.4 M; i.e., there is no evidence for saturation of the enzyme with respect to acyl group acceptor up to these concentrations of alcohol.

The results of preliminary studies of the deacylation of furoyl-chymotrypsin in the presence of ammonia are shown in Table IV. Although the rate of furoyl-chymotrypsin disappearance is increased in the presence of ammonia, it is evident that this compound is a much less effective nucleophilic reagent toward furoyl-chymotrypsin than is hydroxylamine.

The absorption maxima of furoyl-chymotrypsin, of the products of deacylation of furoyl-chymotrypsin in the presence of the above nucleophilic reagents, and of reference furoyl derivatives are compared in Table V. The spectra of the reaction products are shifted by the presence of absorption due to furoate anion, which is formed by hydrolysis of furoylimidazole during enzyme acylation as well as by concurrent hydrolysis during furoylchymotrypsin deacylation. The results do not permit a quantitative interpretation, but do indicate that products other than furoate anion, presumably the corresponding ester or amide, are formed during the deacylation of the acyl-enzyme in the presence of methanol, ethanol, hydroxylamine, and ammonia.

### TABLE IV

<table>
<thead>
<tr>
<th>Ammonia/mol</th>
<th>pH</th>
<th>k/min⁻¹</th>
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<tbody>
<tr>
<td>0.0</td>
<td>8.00</td>
<td>0.10</td>
</tr>
<tr>
<td>0.25</td>
<td>9.71</td>
<td>0.20</td>
</tr>
<tr>
<td>0.5</td>
<td>9.81</td>
<td>0.30</td>
</tr>
<tr>
<td>0.5</td>
<td>9.77</td>
<td>0.36</td>
</tr>
<tr>
<td>1.0</td>
<td>6.64</td>
<td>0.56</td>
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<tr>
<td>1.15</td>
<td>9.77</td>
<td>0.76</td>
</tr>
</tbody>
</table>

* Sum of all ionic species in solution.

### TABLE V

<table>
<thead>
<tr>
<th>Compound</th>
<th>λmax (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Furoyl-chymotrypsin</td>
<td>256</td>
</tr>
<tr>
<td>Product of deacylation in H₂O</td>
<td>245</td>
</tr>
<tr>
<td>Potassium furoate</td>
<td>254</td>
</tr>
<tr>
<td>Product of deacylation in 0.99 M methanol</td>
<td>251.5</td>
</tr>
<tr>
<td>Product of deacylation in 1.03 M ethanol</td>
<td>250</td>
</tr>
<tr>
<td>Methyl furoate</td>
<td>255</td>
</tr>
<tr>
<td>Product of deacylation in 0.025 M hydroxylamine</td>
<td>256</td>
</tr>
<tr>
<td>M-Furoylhydroxylamine</td>
<td>254-256</td>
</tr>
<tr>
<td>Furoylhydroxamic acid</td>
<td>254</td>
</tr>
<tr>
<td>Furoylhydroxamic acid in the presence of chymotrypsin</td>
<td>254.5</td>
</tr>
<tr>
<td>Product of deacylation in 0.25 M ammonia, pH 9.7</td>
<td>248.4</td>
</tr>
</tbody>
</table>

* Difference spectrum against the same concentration of free enzyme. Enzyme concentration was 4.1 to 3.4 × 10⁻⁴ M.

* O-Furoylhydroxylamine was synthesized by allowing 0.21 M hydroxylamine (97% free base) to react with 3.7 × 10⁻³ M furoylimidazole for 5 minutes.

* The spectrum was measured at pH 9.7.

**DISCUSSION**

Attempts to Trap Acyl-enzyme Intermediate: Ester and Hydroxamic Acid Hydrolysis—The rationale for the experiments reported here is the principle that a given intermediate in a reaction, even if it does not accumulate in detectable amounts, must react in the same way regardless of the precursors from which it was formed. Thus, an acetyl L-tyrosyl enzyme intermediate should be partitioned in the same manner between two acyl group acceptors, such as water and hydroxylamine, whether it is formed from an ester, an amide, or some other derivative of acetyl-L-tyrosine. Failure to observe such constant behavior of an acyl-enzyme intermediate is difficult to reconcile with the acyl-enzyme hypothesis and suggests that other reaction paths should be considered.

Acetyl-L-tyrosine ethyl ester was chosen as a reference compound to determine the behavior of the presumed acetyl-L-tyrosyl-enzyme. It was assumed that the hydrolysis of ATE proceeds through an acyl-enzyme intermediate, although this has not been directly demonstrated. There is, however, strong evidence that the chymotrypsin-catalyzed reactions of simple alkyl esters of N-acetyl-L-tryptophan (3) and cinnamic acid (2) proceed through an acyl-enzyme intermediate. The relative reactivity of the presumed acetyl-tyrosyl-enzyme with water and hydroxylamine was determined by measuring the product ratios, the rates of hydrolysis, and the rates of hydroxylaminolysis as a function of hydroxylamine concentration (Table I). These reactions are shown schematically in Equation 1.
It the hydrolysis of acetyl-L-tyrosine hydroxamic acid proceeds through the same acyl-enzyme intermediate as that of ATE, then the acyl enzyme should react in the same manner with hydroxylamine and water. In the particular case of ATH, the result of the reaction of the acyl enzyme with hydroxylamine would be to regenerate ATH. The slow, rate-determining step of the chymotrypsin-catalyzed hydrolysis of ATH through an acyl-enzyme intermediate must be the formation of the acyl-enzyme, because the hydrolysis of ATE, and hence the hydrolysis of the acyl-enzyme, proceeds 100 times faster than the hydrolysis of ATH. Therefore, any molecules of acyl-enzyme which react with hydroxylamine to re-form ATE do not undergo hydrolysis, and the result of the reaction with hydroxylamine would be a decrease in the observed rate of ATH hydrolysis. The amount of this decrease at a given hydroxylamine concentration may be predicted from the partitioning of the acyl-enzyme between water and hydroxylamine, measured in the experiments with ATE. For example, the ATE experiments show that the postulated acyl-enzyme gives 44% ATH in the presence of 1.8 m hydroxylamine, so that the hydrolysis of ATH should be decreased 44% by 1.8 m hydroxylamine.

This argument may be expressed quantitatively with the steady state rate equation for the hydrolysis of ATH in the presence of hydroxylamine under conditions in which the enzyme is saturated with ATH.

\[
\text{Rate} = \frac{k_2 [\text{Enz}]}{k_3 + k_{-1} [\text{NH}_2\text{OH}]} 
\]

When the rates of hydrolysis and hydroxylaminolysis are equal, \(k_3 = k_{-1} [\text{NH}_2\text{OH}]\) and the observed rate of ATH hydrolysis = \(k_2 [\text{Enz}]/2\), or one-half of the rate in the absence of hydroxylamine, in agreement with the less formal treatment above. As the hydroxylamine concentration is increased, \(k_{-1} [\text{NH}_2\text{OH}]\) will increase proportionally, and the rate of hydrolysis will be correspondingly decreased.

Our first experiments showed a wide discrepancy between the predicted and observed inhibition of ATH hydrolysis by hydroxylamine, and it was concluded that the results could not be explained by the usual schemes for the hydrolysis of both ATE and ATH through the same acyl-enzyme intermediate (12). These experiments were carried out at higher enzyme concentrations for the determinations of the rate of ATH hydrolysis than for the determination of the partitioning of the presumed acyl-enzyme formed from ATE, because of the much slower rate of hydrolysis of ATE than of ATE, and on the assumption that the partitioning of the acyl-enzyme intermediate should not be dependent upon the enzyme concentration. It was subsequently shown independently in several laboratories (13-15) that the partitioning in the reaction with ATE is, in fact, dependent upon the enzyme concentration, thereby invalidating these comparisons. If the reactions with ATE and ATH are carried out at the same enzyme concentration, the differences between the observed inhibition of ATH hydrolysis by hydroxylamine and that predicted from the experiments with ATE are considerably smaller. In the presence of 0.8, 1.8, and 2.0 m hydroxylamine, the range of observed inhibition of ATH hydrolysis is 14 to 20, 28 to 39, and 40 to 41%, compared to calculated values of 27, 44, and 58%, respectively, for the acyl-enzyme mechanism. These differences appear to be larger than the experimental error of the determinations and, therefore, offer no support for the acyl enzyme hypothesis; however, they are not sufficiently large to be put forward as conclusive evidence against the acyl-enzyme hypothesis.

Epand and Wilson (14) have reported that the observed inhibition of ATH hydrolysis by hydroxylamine at low ATH concentration agrees closely with that predicted from the experiments with ATE. Their experimental data are in close agreement with our results at low ATH concentration. However, a more valid comparison may be made at ATH concentrations larger than the \(K_m\) of ATH, because the effect of hydroxylamine on the \(K_m\) of ATH (Fig. 4) will cause a further decrease in the rate of ATH hydrolysis by inhibiting the binding of ATH to the enzyme at low substrate concentrations. Thus, the observed inhibition at low substrate concentrations reflects an inhibition of ATH binding as well as any possible effect of hydroxylamine in the partitioning of an acyl-enzyme intermediate.6

The unexpected effect of enzyme concentration on the reactions of ATE with water and hydroxylamine is itself of some interest and demands particular caution in the comparison of other results obtained with chymotrypsin at different enzyme concentrations. The reason for this effect is unknown. A dimerization or polymerization of the enzyme at high enzyme concentrations provides a possible explanation for the results if it is assumed that the polymer and monomer acyl-enzymes display different relative susceptibilities to attack by water and by hydroxylamine. It is not known whether chymotrypsin is aggregated under the conditions of these experiments, but it has been reported that acetyl-chymotrypsin undergoes aggregation at pH values up to 9.0 and that acetyl-chymotrypsin is more liable to aggregation than is free chymotrypsin (30).

Kędz, Clement, and Bender (15) and Epand and Wilson (14) have suggested that the concentration effect results from a competition between free enzyme and hydroxylamine for reaction with an O-acylhydroxylamine which may be formed from acetyl-L-tyrosyl-enzyme and hydroxylamine. Since the reaction of an O-acylhydroxylamine with hydroxylamine would give only ATH, while that with the enzyme would give both ATH and AT, a differing concentration of free enzyme could affect the yield of ATH in the over-all reaction. If this explanation is correct, it would appear that with enzyme concentrations of over \(10^{-4}\) m in the presence of 0.8 m hydroxylamine all of the reaction goes by the enzymatic pathway, since there is no further effect on the yield of ATH if the enzyme concentration is increased to over \(10^{-4}\) m (Table 1).

The rate of chymotrypsin-catalyzed hydroxamic acid formation from ATE and related compounds increases with increasing

6 Epand and Wilson have subsequently studied the reaction at higher substrate concentrations and have observed an inhibition of ATH hydrolysis by hydroxylamine which is in agreement with our results under similar conditions and is somewhat less than predicted by the acyl-enzyme mechanism (personal communication).
Hydroxylamine concentration and reaches values larger than the rate of ATE hydrolysis in the absence of hydroxylamine (Tables 1 and II; Fig. 2). In the case of the ATE reactions, this is not accompanied by any measurable decrease in the rate of ATE hydrolysis, within the quite appreciable error of the hydrolysis rate determinations in the presence of concentrated hydroxylamine (Fig. 2). Hydroxamic acid formation is, therefore, a reaction which is superimposed on the hydrolysis reaction, rather than one which occurs at the expense of hydrolysis. If these reactions occur through an acyl-enzyme intermediate, this requires that the rate-determining step be the reaction of the acyl-enzyme with the acyl acceptor (water or hydroxylamine), and that the rate of acyl-enzyme formation be considerably faster than the rate of its hydrolysis. If this were not the case, the rate of hydroxamic acid formation could not increase linearly with hydroxylamine concentration and the increase in the rate of hydroxamic acid formation would cause a decrease in the rate of hydrolysis when the rate of acyl-enzyme formation was no longer able to keep up with the rate of deacylation. This conclusion indicates that the $K_a$ for ATE is not a true equilibrium constant and is consistent with the finding of Bernhard and Gutfreund (31) that the $K_a$ for ATE is different for hydrolysis and for hydroxaminolysis. An unexplained difference between our results and those of Bernhard and Gutfreund is the report of these workers that the rate of hydroxamic acid formation from ATE is not linear with respect to hydroxylamine concentration (32). If deacylation of the acyl-enzyme is rate-determining for hydrolysis of esters of N-acetyl-L-tyrosine, it follows that the maximum rates of hydrolysis of different esters should be identical. This point has not yet been tested experimentally.

If the hydrolysis of ATH proceeds by a direct attack of water rather than through an acyl-enzyme intermediate, it is necessary to account for the fact that the hydrolysis of ATE is inhibited by hydroxylamine while, within the error of the hydrolysis rate determinations, there is not a large inhibition of ATE hydrolysis by hydroxylamine. No definitive explanation of this difference is available, but it is perhaps not unreasonable to expect that highly concentrated solutions of hydroxylamine would be inhibitory to chymotrypsin. A number of small molecules, including diethylformamide, acetonitrile, dioxane, pyridine, and diethyl ether, some of which are similar to portions of known chymotrypsin substrates, are inhibitors for chymotrypsin (33-36). Although the effects of these inhibitors are usually larger on $K_a$ than on $V_{max}$, significant effects on both parameters are found (33, 34). It would not be surprising that the hydrolysis of ATE is less susceptible to such inhibition, if ATE hydrolysis proceeds through an acyl-enzyme and most of the enzyme is in the acylated form, in view of reports that acyl- and diisopropylphosphorylchymotrypsin are less susceptible to denaturation than free chymotrypsin (37-39).

Hydroxamic Acid Synthesis Clearer results were obtained from a study of the initial rates of chymotrypsin-catalyzed ATH synthesis from AT and hydroxylamine. The equilibrium constant for this reaction is sufficiently favorable for ATH synthesis that hydroxamic acid formation may be measured readily at pH values near neutrality in the presence of moderately concentrated hydroxylamine (29). The rate of enzyme-catalyzed hydroxamic acid synthesis shows little or no deviation from a linear increase with increasing hydroxylamine concentration (Fig. 5). This means that there is a hydroxylamine molecule in the activated complex for this reaction, i.e. that the rate-determining step involves an attack of hydroxylamine on either an acyl-enzyme or enzyme bound AT itself. If the reaction proceeds through an acyl-enzyme, this result requires that the formation of acyl-enzyme from AT, which is in the form of the carboxylate ion at the pH of these experiments, must occur in a rapid, pre-equilibrium step, followed by a slow reaction with hydroxylamine at concentrations up to $3\text{M}$. If the formation of the acyl-enzyme were partly or wholly rate-determining, then as the hydroxylamine concentration was raised a point would be reached at which every mole of acyl-enzyme that was formed would react rapidly with hydroxylamine, and a further increase in hydroxylamine concentration would not result in a further increase in the rate of hydroxamic acid formation. It would be surprising on chemical grounds if a carboxylate ion could form an ester with the hydroxyl group of a serine residue in a rapid, reversible reaction, followed by a slow reaction with $3\text{M}$ hydroxylamine. The requirement for acylation to occur in a rapid equilibrium step is that the rate of the back-reaction of the acyl-enzyme with water be much faster than the reaction with hydroxylamine. The authors know of no chemical reaction of an acyl group in which $3\text{M}$ hydroxylamine does not react faster than water; if this is not the case in the enzymatic reaction, the enzyme must specifically activate water for attack on the acyl group. A somewhat similar situation obtains in the chymotrypsin-catalyzed condensation of N-benzoyl-L-tyrosine with glycylglycine to form N-benzoyl-L-tyrosylglycylglycine, which proceeds at a rate proportional to the concentration of glycylglycine (40). As pointed out by Gawron et al., this requires that the deacylation step be rate-determining in this reaction also, if it proceeds through an acyl enzyme. Again, it is difficult to understand how an ester, with a free energy of hydrolysis of 5 to 8 kcal per mole near neutral pH (41), may be formed in a rapid equilibrium, followed by a rate-determining reaction with the acyl acceptor. The requirement that water react with the acyl-enzyme much faster than $3\text{M}$ hydroxylamine is not supported by the results obtained with ATE, if it is assumed that the reactions of this compound proceed through an acyl enzyme intermediate. In $3\text{M}$ hydroxylamine, ATE gives a 56% yield of ATH at the same enzyme concentration and pH as in the ATH synthesis experiments. Thus, the rates of hydrolysis and hydroxaminolysis of the presumed acyl-enzyme intermediate are approximately equal and the acyl-enzyme cannot be formed in a rapid equilibrium from AT under these conditions. The situation may be treated quantitatively as follows. The scheme for ATH synthesis via an acyl-enzyme is shown in Equation 3, and the corresponding steady state rate equation, in Equation 4. It is
assumed that $K_1$ and $k_i$ and, therefore, the rate of acyl-enzyme formation are independent of hydroxylamine concentration. The concentration of AT used in the synthetic experiments (0.25 m) is well above the binding constant for AT ($K_a = 0.115$ m) (23), but the enzyme is probably not saturated with the free acid form of AT, which would presumably be the reactive species in the acylation step.

$$\text{RCOOH} + \text{Enz} \xrightarrow{K_2} \text{RCOOH} \cdot \text{Enz} \xrightarrow{k_1} \text{RCOOH} \cdot \text{NH}_2\text{OH} \xrightarrow{k_2} \text{RCOOH} \cdot \text{Enz} \xrightarrow{K_p} \text{RCOOH} + \text{Enz}$$

Equation 4 predicts that the rate of ATH synthesis will depart from linearity with respect to hydroxylamine concentration as the term $k_2[NH_2OH]$ becomes appreciable compared to $k_1$. When $k_2[NH_2OH]$ and $k_1$ are equal, half of the molecules of acyl-enzyme that are formed will react with hydroxylamine to give ATH and half will react with water to regenerate AT; therefore, a linear relationship would be expected under these conditions. The observed initial rates were linear with time and that the concentration of AT is well above the binding constant for ATH (3.1 x 10^-3 M), while the concentration of AT is well above the binding constant for AT; therefore, very little binding and hydrolysis of ATH would be expected to occur under these conditions.

If the synthesis of ATH proceeds by a pathway which does not involve an acyl enzyme intermediate, it would be expected from the principle of microscopic reversibility that the hydrolysis of ATH also would occur by a pathway which does not involve an acyl-enzyme intermediate.

Bender and co-workers have obtained evidence that N-acetyl-tryptophan reacts with chymotrypsin to form N-acetyl-tryptophanyl-enzyme. If a similar reaction occurs with AT, the data of Fig. 5 suggest that hydroxylamine may react with enzyme-bound AT before as well as after acyl-enzyme formation; i.e. hydroxylamine may compete with the serine hydroxyl group for the acyl group which has been activated by the enzyme. In this treatment it has been assumed that the back-reaction, $k_2[NH_2OH]/k_1$, is not significant under the conditions of the experiments shown in Fig. 5. This is to be expected from the facts that the observed initial rates were linear with time and that the rates were followed to a maximum of about 30% toward their equilibrium positions. Furthermore, the maximum concentrations of ATH formed in the course of the experiments (1.0 to 2.9 x 10^-3 M) are well below the $K_m$ for ATH (3.1 x 10^-3 M), while the concentration of AT is well above the binding constant for AT; therefore, very little binding and hydrolysis of ATH would be expected to occur under these conditions.

It is of interest that the rate of ATH synthesis from AT (3.2 x 10^{-2} sec^{-1}) in the presence of 1.0 m hydroxylamine at pH 6.98 is of the same order of magnitude as the rates of two other chymotrypsin-catalyzed reactions in which carboxylic ions are the substrate: the exchange of $^{18}O$ from H$_2$O into the carboxyl group of N-acetyl-3,5-dibromo-L-tyrosine (4.4 x 10^{-2} sec^{-1} at pH 7.2 and 20° (42)) and the corresponding reaction with N-acetyl-L-tryptophan (3.0 x 10^{-2} sec^{-1} at pH 7.9 and 25° (43)). These rates are also of the same order as the rates of hydrolysis of the corresponding anilides (43, 44). The calculated maximal rate of the chymotrypsin-catalyzed condensation of N-benzoyl-L-tyrosine with 1 m glycylanilide is 2.5 sec^{-1} at pH 6.5 and 23°; however, experimental measurements were made only up to 0.09 m glycylanilide$_2$ which would give a rate constant of 0.23 sec^{-1} (40).

Reactions of Furagly-chymotrypsin with Ethanol, Methanol, Ammonia, and Hydroxylamine — In order to study the behavior of a known acyl-enzyme, for comparison with the hypothetical acyl-enzyme formed in the chymotrypsin-catalyzed reactions of acetyl-L-tyrosine derivatives, the rate of disappearance of furagly-chymotrypsin in the presence of a series of nucleophilic reagents was examined. As has previously been demonstrated for other chymotrypsin reactions (11, 5, 45), methanol and ethanol are considerably more effective acyl acceptors, on a molar basis, than is water. The reactivity of ammonia is approximately the same as that of ethanol, but is less than that of methanol. The high reactivity of hydroxylamine, a 0.025 m solution of which causes a 6.9-fold increase in the rate of acyl-enzyme disappearance, is in striking contrast to the rather low reactivity of hydroxylamine in reaction with AT.
the chymotrypsin-catalyzed solvolysis of ATE. In the latter reaction, nearly 3.0 M hydroxylamine is required to make the rate of hydroxamic acid formation equal to the rate of hydrolysis, in the presence of more than 10^{-4} M enzyme. N-Acetyl-l-phenylalanine ethyl ester and N-acetyl-l-tryptophan ethyl ester share this relatively low reactivity (Table II), while methyl hippurate has an intermediate reactivity with hydroxylamine (4, 6). From the very limited data available, it would appear that hydroxylamine is much more reactive toward "abnormal," unreactive substrates than it is toward normal substrates for chymotrypsin. The difference probably does not reflect the presence or absence of an acyl-enzyme intermediate, since there is evidence that the hydrolysis of N-acetyl-l-tryptophan ethyl ester proceeds through an acyl-enzyme intermediate (3). The rapid reaction with furoyl-chymotrypsin reflects partly the formation of O-furoylhydroxylamine, but the rate of hydroxamic acid formation is also very rapid. Nitrogen nucleophiles such as hydroxylamine and glycine have previously been shown to be highly reactive in base-catalyzed reactions with N, O-diacylserinamide, a model for the active site of the acyl-enzyme (46).

On the other hand, methanol does not appear to have an unusually low reactivity toward reactive chymotrypsin substrates, since the rates of hydrolysis and methanolysis are approximately equal with N-acetyl-l-phenylalanine methyl ester (5) and furoyl-chymotrypsin in 0.5 M and 0.12 M methanol, respectively.

It has been reported that the reaction of trimethylacetylchymotrypsin with hydroxylamine gives a low yield of hydroxamic acid (9, 10). The demonstration that O-acetylhydroxylamine is formed in the reaction of furoyl-chymotrypsin with hydroxylamine suggests that O-acetylhydroxylamine formation is responsible for the low yield of hydroxamic acid obtained in this reaction. Epan and Wilson have reported in a preliminary communication (14) that O-hippurylhydroxylamine is both formed and hydrolyzed in chymotrypsin-catalyzed reactions.

**Question of Acyl Acceptor Site on Chymotrypsin**—Bernard et al. (4) have reported that the rate of chymotrypsin-catalyzed hydroaminolysis of methyl hippurate levels off with increasing hydroxylamine concentration, and suggest that this behavior is due to a saturation with hydroxylamine of a binding site for the nucleophilic reagent which acts as an acceptor for the acyl group. From the variation of the rate with hydroxylamine concentration, a $K_m$ value of 0.23 M was calculated. Hydroxylamine also was found to inhibit methyl hippurate hydrolysis, and the $K_i$ value of 0.20 M for this inhibition agrees well with the $K_m$. Bender and Glasson (5) have reported a similar inhibition of the hydrolysis of N-acetyl-l-phenylalanine methyl ester by low concentrations of methanol, which was attributed to binding of methanol to a water site.

The data reported here provide no evidence for a binding site for either hydroxylamine or methanol in chymotrypsin-catalyzed reactions of N-acetyl-l-tyrosine derivatives or in reactions of furoyl-chymotrypsin. The rates of hydroxamic acid formation from ATE and from AT are both close to linearity with respect to hydroxylamine concentration up to 3.0 M hydroxylamine, which requires that if there is a binding site for hydroxylamine, the $K_m$ must be considerably larger than 3.0 M. Furthermore, there is no large inhibition of ATE hydrolysis by hydroxylamine, which might suggest a competitive binding of water and hydroxylamine. The reason for the difference between these results and those of Bernhard is not obvious; unless the kinetics of chymotrypsin-catalyzed reactions is considerably more complicated than has generally been supposed, it would not be expected that the binding constant for an acyl acceptor would differ by more than an order of magnitude in the presence of different acyl groups. Similarly, the rates of deacylation of furoyl-chymotrypsin show no indication of a leveling off with increasing methanol, ethanol, and ammonia concentrations up to approximately 1.0 M, which indicates that any binding of these acyl acceptors must be weak if it exists at all. Foster (45) has shown that the rate of methanolysis of acetyl-chymotrypsin is linear with respect to methanol concentration up to 2.5 M without inhibition of the rate of hydrolysis, and Bender et al. (47) have shown that the rate of deacylation of cinnaamoyl-chymotrypsin is linear with respect to methanol concentration up to 6 M.

The relative reactivities of these acyl group acceptors toward furoyl-chymotrypsin are in some respects similar to their reactivities toward the activated acyl group of p-nitrophenyl acetate. As is the case in base-catalyzed reactions with p-nitrophenyl acetate, methanol and ethanol are considerably more reactive than water, and methanol is more reactive than ethanol (48). In both reactions, hydroxylamine, in spite of its 2000-fold lower basicity, is approximately 10 times more reactive than ammonia (8). Further experiments are in progress to define more exactly the structural requirements for nucleophilic reactivity toward furoyl-chymotrypsin.

In spite of this negative evidence, there is strong evidence that some acyl acceptors do bind to the active site of chymotrypsin. McDonald and Balls (11) have shown that the rates of reaction of acetylchymotrypsin with alcohols increase with increasing chain length of the alcohol and that the reactivity of heptanol-1 is 15 times greater than that of ethanol. Since it would not be expected that there would be a large difference in the chemical reactivity of alcohols of increasing chain length, this result is difficult to explain without invoking a specific binding of the longer chain alcohols. The fact that (+)-butan-2-ol is acetylated in preference to (+)-butan-2-ol in the presence of chymotrypsin and p-nitrophenyl acetate also suggests that there is a specific binding site, or at least a stereospecific steric requirement, for this acyl acceptor (49).

**SUMMARY**

The rates of chymotrypsin-catalyzed hydrolysis and hydroxylaminolysis of derivatives of N-acetyl-L-tyrosine have been compared in an attempt to determine whether the different reactions proceed through a common acyl-enzyme intermediate. If it is assumed that the reactions of N-acetyl-l-tyrosine ethyl ester proceed through an acyl-enzyme, the inhibition of the hydrolysis of N-acetyl-l-tyrosine hydroxamic acid by hydroxylamine appears to be slightly less than predicted by the acyl-enzyme hypothesis, but the differences are not large enough to warrant a firm conclusion. The rate of synthesis of N-acetyl-l-tyrosine hydroxamic acid from N-acetyl-l-tyrosine and hydroxylamine does not deviate significantly from linearity with respect to hydroxylamine concentration up to 3.0 M hydroxylamine. We have not been able to account for this result in terms of the acyl-enzyme hypothesis.

The rate of hydroxamic acid formation from N-acetyl-l-tyrosine ethyl ester is linear with respect to hydroxylamine concentration, and hydroxylamine does not cause a large decrease in the rate of hydrolysis of this substrate at concentrations of up to 3.0 M. The rate of deacylation of furoyl-chymotrypsin in the presence of methanol, ethanol, ammonia, and hydroxylamine
were determined. No evidence was obtained for the existence of a binding site for any of these acyl acceptors. Both furfuryldihydroxamic acid and O-furoylhydroxylamine are products of the reaction of furfurylchymotrypsin with hydroxylamine.

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