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Transpeptidation and the α-Chymotrypsin-Catalyzed Hydrolysis of α-Amino Acid Esters, Hydroxamides, Amides, and Hydrazides*

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Transpeptidation reactions have been shown to be of negligible, or minor, importance in the α-chymotrypsin-catalyzed hydrolysis of L-tyrosine methyl ester, hydroxamide, amide, and hydrazide, under conditions normally employed in kinetic studies. The kinetics of the hydrolytic reactions and their dependence upon pH can be interpreted in terms of participation of two substrate species, a less reactive α-amino acid derivative and its more reactive conjugate acid.

The apparent pH optima for the α-chymotrypsin-catalyzed hydrolysis of α-amino acid esters, hydroxamides, and hydrazides lie in a more acid region than those of the corresponding α-*N*-acyl derivatives. Results obtained with α-*N*-acylated and nonacylated derivatives of L-tyrosine (Kaufman *et al.*, 1949; Jansen *et al.*, 1951; Parks and Plaut, 1953; Schwert and Takenaka, 1955; Lutwack *et al.*, 1957) are summarized in Table I.

TABLE I

pH OPTIMA FOR α-CHYMOTRYPSIN-CATALYZED HYDROLYSES OF SOME L-TYROSINE DERIVATIVES

—CH(CH₂C₆H₄-OH)CO—	—OC₂H₅	—NH₂	—NHNH₂	—NHOH
H_2N—	6.2–7.0[a,b]		7.05[f]	6.95[f]
CH_3CONH—	8.0–8.2[a,c]	7.90[f]	7.95[f]	7.60[f]
C_6H_5CONH—	7.8[d,e]	7.8[d,e]	8.0[f]	
β-(C_5H_4N)CONH—		7.90[f]	7.80[f]	

[a] Jansen *et al.* (1951). [b] Schwert and Takenaka (1955). [c] Parks and Plaut (1953). [d] Kaufman *et al.* (1949). [e] In 30% methanol. [f] Lutwack *et al.* (1957).

Foster *et al.* (1954) offered an explanation for the difference in pH optima of L-tyrosinhydroxamide and α-*N*-acetyl-L-tyrosinhydroxamide based upon the assumption that hydrolysis at the α-amino acid carboxyl function is the predominating reaction. Since the pH optima for uncharged substrates occur at pH 7.9 ± 0.1, it can be assumed that the enzyme is protonated at this pH in a manner that is optimal for catalyzing the hydrolysis of these substrates. The lower pH optimum of α-*N*-acetyl-L-tyrosinhydroxamide can be accounted for in terms of partial ionization of the latter substrate to the less reactive hydroxamate anion (Hogness and Niemann, 1953). However, the amino group of L-tyrosinhydroxamide, conjugate acid $pK_A' \cong 7.0$, would be predominately unprotonated at pH values much greater than 7. With an increase in concentration of the unprotonated species, assumed to be less reactive than the protonated or α-*N*-acylated-L-tyrosinhydroxamide, as the pH is increased, the pH optima would be shifted to a more acidic region than observed for the α-*N*-acyl derivative. This argument is equally applicable to the ester, amide, and hydrazide.

Schwert (1955) suggested that "it seems more probable that the reduction in apparent reaction velocity at higher pH values is attributable to transpeptidation onto the uncharged α-amino group." Support for this view is provided by observations that show that the extent of transpeptidation increases with increasing pH throughout the region of interest, *i.e.*, pH 6 to 8 (Johnston *et al.*, 1950a,b; Brenner *et al.*, 1950; Lestrovaya and Mardashev, 1956). Thus, there are three reactions to consider:

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(1) hydrolysis of protonated substrate; (2) hydrolysis of unprotonated substrate; and (3) transpeptidation onto unprotonated substrate.

$$H_3^{\oplus}NCHRCOY + H_2O \longrightarrow H_3^{\oplus}NCHRCO_2^{\ominus} + H^{\oplus} + HY \quad (1)$$

$$H_2NCHRCOY + H_2O \longrightarrow H_3^{\oplus}NCHRCO_2^{\ominus} + HY \quad (2)$$

$$2H_2NCHRCOY \longrightarrow H_2NCHRCONHCHRCOY + HY \quad (3)$$

As the pH is increased, reactions of the unprotonated substrate, equations 2 or 3, will become more important. If the rate of either of these reactions is equal to or greater than that of the protonated substrate, equation 1, the observed velocity would not be expected to decrease before pH 7.9 is reached, but would appear to decrease if the rate of liberation of protons were followed. Since the pH optima for the nonacylated derivatives are near pH 7.0, as determined either by the rate of disappearance of substrate or by the formation of products other than acid, the protonated molecules, equation 1, react faster than the unprotonated ones, equations 2 and 3. The explanation of Foster *et al.* (1954) requires reaction 2 to be slower than reaction 1, and the transpeptidation reaction, equation 3, to be much slower than either hydrolysis reaction. Schwert's (1955) alternative hypothesis requires reaction 3 to have a rate greater than that of reaction 2, but not much less than that of reaction 1. With L-tyrosinhydroxamide (Foster *et al.*, 1954) and L-tyrosinhydrazide (Lutwack *et al.* 1957) the sum of reactions 1, 2, and 3 was determined. Thus, in these instances the proposal of Schwert (1955) cannot explain the decrease in reactivity with increasing pH unless reactions 2 and 3 are slower than reaction 1, the condition upon which Foster *et al.* (1954) base their hypothesis.

Goldenberg *et al.* (1951) observed that ethyl-L-leucinate exhibits a pH optimum at pH 6.8 when the rate of liberation of acid is followed and *ca.* 7.2 when the rate of disappearance of ester is determined. Halsey *et al.* (1954) suggested that this difference in pH optima is probably due to a transpeptidation reaction. However, Lutwack *et al.* (1957) pointed out that hydrolysis of the unprotonated substrate, equation 2, would not be detected by the former procedure, thus providing an alternative explanation which predicts the direction of the shift in the pH optimum. Since neither transpeptidation, equation 3, nor hydrolysis of the unprotonated substrate, equation 2, can be detected near pH 7 by determining the rate of liberation of acid, either or both of these reactions could account for the shift in pH optimum.

The ability of α-chymotrypsin to catalyze the synthesis of peptides and peptide-like bonds is well established. Protein digests can be transformed into insoluble "plasteins" (Lundsteen, 1938; Tauber, 1949, 1951; Haurowitz and Horowitz, 1955), presumably via repeated transpeptidation reactions (Horowitz and Haurowitz, 1959). Peptides have been synthesized from acylated α-amino acids and α-amino acid anilides (Bergmann and Fruton, 1938, 1944; Janssen *et al.*, 1953) or phenylhydrazine (Schuller and Niemann, 1952) and from α-amino acid esters and amines (Brenner *et al.*, 1950; Tauber, 1952; Kaganova and Orehovich, 1954; Lestrovaya and Mardashev, 1956). Some of these reactions exhibit pH optima near pH 7 and others near pH 9 or higher.

Schuller and Niemann (1952) found that several acylated α-amino acids did not react with phenylhydrazine at pH 7.9, the pH optimum for hydrolysis of the corresponding amides, but gave the phenylhydrazides with pH optima between pH 5.5 and 6.5. The observation that α-*N*-benzoyl-L-tyrosylglycinamide exchanges its glycinamide moiety with N^{15}-glycinamide but α-*N*-benzoyl-L-tyrosinamide exchanges its amide moiety with $N^{15}H_3$ only to a limited extent at the same pH, 7.6, has been attributed to the difference in the pK_A' values of the conjugate acids of glycinamide, 7.9, and ammonia, 9.4 (Johnston *et al.*, 1950; Fruton *et al.*, 1951).

In an explanation of the apparent absence of reactivity of *N*-alkyl-L-phenylalanine ethyl esters with α-chymotrypsin, Kuk-Meiri and Lichtenstein (1957, 1960) suggested that nonacylated α-amino acid derivatives had to be converted to dipeptides before they could be hydrolyzed. Although Tauber (1952) reported that α-chymotrypsin converts L-phenylalanine ethyl ester into L-phenylalanyl-L-phenylalanine ethyl ester, with a pH optimum of 8.6, he also noted that this dipeptide ester was not hydrolyzed at pH 7.7. Since the pH optimum for the hydrolysis of phenylalanine ethyl ester occurs at *ca.* pH 6.4 (Parks and Plaut, 1953; Goldenberg and Goldenberg, 1950) it is difficult to reconcile the hypothesis of Kuk-Meiri and Lichtenstein (1957, 1960) with the available data.

The reaction products of α-chymotrypsin and L-tyrosinamide, and other compounds, at pH 7.8 were examined chromatographically by Blau and Waley (1954) with particular reference to the rate of disappearance of L-tyrosinamide and appearance of L-tyrosine and L-tyrosyl-L-tyrosine. Initially, formation of L-tyrosine was favored over that of the dipeptide, but after several hours the relative abundance of the two species was inverted. It was suggested (Blau and Waley, 1954) that transpeptidation and rapid hydrolysis of an L-tyrosyl-L-tyrosinamide intermediate was responsible for the observed behavior. However, this interpretation conflicts with the observations of Tauber (1952) noted above.

Lestrovaya and Mardashev (1956) systematically examined the reaction products of α-chymotrypsin and the ethyl esters and amides of L-phenylalanine and L-tyrosine and found that the amount of peptide formed decreased rapidly with decreasing concentration of enzyme. Peptide formation was barely detectable at 4 mg of enzyme per ml, a concentration higher than that usually employed in kinetic studies and five times less than that used by Blau and Waley (1954).

The preceding observations suggest that transpeptidation is generally associated with higher enzyme concentrations or longer reaction periods than normally employed in evaluation of kinetic constants of the hydrolytic reactions. In a study conducted under the latter conditions, McDonald and Balls (1956) noted that α-chymotrypsin and ethyl-L-tyrosinate in aqueous 1-butanol at pH 6.2 gave, after 1 hour, approximately equal amounts of L-

tyrosine, ethyl-L-tyrosinate, and butyl L-tyrosinate. However, in the absence of 1-butanol the only components present were L-tyrosine and ethyl-L-tyrosinate, *i.e.*, no peptide was formed unless its R_F value was the same as that of L-tyrosine or its ethyl ester.

While it appears unlikely that transpeptidation is an important factor in kinetic studies involving hydrolysis of nonacylated α-amino acid derivatives, the evidence is ambiguous. Therefore, it was decided to synthesize the methyl ester, hydroxamide, amide, and hydrazide of L-tyrosyl-L-tyrosine and to use them as reference compounds in a chromatographic study of the reaction products arising from the interaction of α- and δ-chymotrypsin with the corresponding L-tyrosine derivatives.

Experimental

Methyl-L-tyrosinate.—The hydrochloride of this compound, m.p. 187–189°, was prepared from L-tyrosine and methanol in 95–98% yields by the procedure of Brenner and Huber (1953). The hydrochloride, dissolved in a minimum amount of water and brought to pH 8–9 with N aqueous sodium hydroxide, gave 95% of the chloride free ester, m.p. 134–135°. Fischer and Schrauth (1907) give as m.p. 135–136°.

Ethyl L-tyrosinate Hydrochloride.—This compound, m.p. 166–168°, was prepared, in 90–96% yields, as described above. Rohmann (1897) gave as m.p. 166°.

L-*Tyrosinhydroxamide.*—Reaction of hydroxylamine and methyl-L-tyrosinate as described by Foster, Jennings, and Niemann (1954) gave the hydroxamide, m.p. 163–164°, with decomposition.

L-*Tyrosinamide.*—Ammonolysis of methyl-L-tyrosinate gave the amide, m.p. 154–156°, in 73% yield. Blau and Waley (1954) and Koenigs and Mylo (1908) report as m.p. 153–154°.

L-*Tyrosinhydrazide.*—This compound, m.p. 193–195°, was prepared from hydrazine hydrate and methyl-L-tyrosinate by the procedure of Curtius (1917). Curtius reports m.p. 195.5 and Lutwack *et al.* (1957) m.p. 193–194°.

Ethyl N-*Carbobenzyloxy-L-tyrosinate.*—Acylation of ethyl L-tyrosinate with carbobenzyloxy chloride by the method of Bergmann and Zervas (1932) gave 70% of the desired product, m.p. 78–78.5°. Esterification of *N*-carbobenzyloxy-L-tyrosine, m. p. 91–93°, by the procedure of Brenner and Huber (1953) gave 75% of product, m.p. 75–78°.

N-*Carbobenzyloxy-L-tyrosinhydrazide.*—Reaction of ethyl *N*-carbobenzyloxy-L-tyrosinate and hydrazine hydrate as described by Harington and Pitt-Rivers (1944) gave 80–92% of the hydrazide, m.p. 221–223°, after recrystallization from ethanol. Harington and Pitt-Rivers report m.p. 220–221°.

N-*Carbobenzyloxy-L-tyrosyl-L-tyrosine Methyl Ester.*—This compound, m.p. 175–176° after recrystallization from aqueous acetone, was prepared as described by Sheehan and Hess (1955) from equimolar quantities of carbobenzyloxy-L-tyrosine, methyl-L-tyrosinate, and *N,N'*-dicyclohexylcarbodiimide dissolved in tetrahydrofuran. It was also obtained by the procedure of Bergmann and Fruton (1937) from *N*-carbobenzyloxy-L-tyrosinhydrazide and methyl-L-tyrosinate and by esterification of *N*-carbobenzyloxy-L-tyrosyl-L-tyrosine recovered as a by-product of the preceding syntheses, as directed by Barkdoll and Ross (1944).

Methyl L-Tyrosyl-L-tyrosinate Hydrochloride.—This compound, m.p. 210–211° after recrystallization from a mixture of methanol and ethyl ether, was obtained as a by-product of the esterification of *N*-carbobenzyloxy-L-tyrosyl-L-tyrosine. Barkdoll and Ross (1944) report m.p. 210°.

L-*Tyrosyl-L-tyrosinhydroxamide Hydrochloride.*—A methanolic solution of methyl *N*-carbobenzyloxy-L-tyrosyl-L-tyrosinate was heated for 15 minutes under refluxing conditions with an excess of hydroxylamine, the solvent removed *in vacuo*, the residue taken up in a small amount of methanol, and sufficient water added to cause the separation of an oil. This oily product was triturated with anhydrous ethyl ether to give a colorless precipitate, m.p. 162.5–163.5°, which gave a positive reaction with a solution of ferric chloride in aqueous hydrochloric acid. This product was suspended in 0.18 N methanolic hydrogen chloride and hydrogenolyzed over palladium black until evolution of carbon dioxide had ceased. The catalyst was removed and anhydrous ethyl ether added to the solution to give a hygroscopic colorless precipitate. This product was soluble in water, gave a positive ferric chloride reaction and only a single spot when chromatographed, and developed with diazotized sulfanilamide. Satisfactory analyses could not be obtained because of the hygroscopic nature of the product.

N-*Carbobenzyloxy-L-tyrosyl-L-tyrosinamide.*—Condensation of *N*-carbobenzyloxy-L-tyrosine with L-tyrosinamide and *N,N'*-dicyclohexylcarbodiimide, as described for the corresponding methyl ester, gave *N*-carbobenzyloxy-L-tyrosyl-L-tyrosinamide, m.p. 189–191°, in 50% yield.

Anal. Calcd. for $C_{26}H_{27}N_3O_6$(477.5): C, 65.4; H, 5.7; N, 8.8. Found: C, 65.3; H, 5.9; N, 8.6.

L-*Tyrosyl-L-tyrosinamide Hydrochloride.*—Hydrogen was passed through a stirred suspension of 2.0 g of *N*-carbobenzyloxy-L-tyrosyl-L-tyrosinamide and 0.65 g of palladium black in 60 ml of 0.18 N methanolic hydrogen chloride until evolution of carbon dioxide had ceased. The catalyst was removed, anhydrous ethyl ether was added to the solution, and the precipitate was collected, recrystallized from a mixture of methanol and ethyl ether, and dried at 78° over phosphorus pentoxide to give the dipeptide amide hydrochloride, m.p. 237–240°.

Anal. Calcd. for $C_{18}H_{22}N_3O_4Cl$(380): C, 56.9; H, 5.8. Found: C, 56.5; H, 5.6.

N-Carbobenzyloxy-L-tyrosinhydrazide.—A solution of 2.3 g of methyl *N*-carbobenzyloxy-L-tyrosyl-L-tyrosinate and 0.77 ml of hydrazine hydrate in 50 ml of methanol was heated under refluxing conditions for 15 minutes, the solution cooled to room temperature, and the product allowed to crystallize. A first crop of 1.3 g and a second crop of 1.0 g of the hydrazide, m.p. 246–248°, was obtained. Barkdoll and Ross (1944) report m.p. 246°.

Anal. Calcd. for $C_{26}H_{28}N_4O_6$(492.5): C, 63.4; H, 5.7; N, 11.4. Found: C, 63.0; H, 5.6; N, 11.3.

L - *Tyrosyl* - L - *tyrosinhydrazide Dihydrochloride.*—Decarbobenzoxylation, as described for the amide, gave 77% of the hydrazide dihydrochloride, m.p. 222–223°, after recrystallization from a mixture of methanol and ethyl ether and drying *in vacuo* over phosphorus pentoxide.

Anal. Calcd. for $C_{18}H_{26}N_4O_6Cl_2$(449): C, 48.1; H, 5.8; N, 12.5. Found: C, 48.4; H, 5.7; N, 12.0.

L-*Tyrosyl*-L-*tyrosine Anhydride.*—Equivalent amounts of methanolic sodium methoxide were added to separate methanolic solutions of methyl L-tyrosyl-L-tyrosinate hydrochloride and hydroxylamine hydrochloride, the precipitated sodium chloride was removed from each solution, and the two solutions were mixed and stored at 4° for 18 hours. The crystalline precipitate was collected and dried *in vacuo* over phosphorous pentoxide to give the anhydride, m.p. 278–281°, $[\alpha]_D^{25}$ −234 ± 4° (c, 1.73% in 1 N aqueous sodium hydroxide). Fischer and Schrauth (1907) report m.p. 277–280° and $[\alpha]_D^{25}$ −223.8° (c, 2.4% in dilute sodium hydroxide).

L-*Tyrosyl*-L-*tyrosine.*—An aqueous solution of methyl L-tyrosyl-L-tyrosinate hydrochloride was adjusted to pH 10 by addition of 1 N aqueous sodium hydroxide, allowed to stand at 25° for 1 hour, and acidified, and absolute ethanol was added to the solution to precipitate the dipeptide, m.p. *ca.* 300°, with decomposition, $[\alpha]_D^{25}$ −12.1° (c, 1.16% in 1 N aqueous hydrochloric acid). This product gave but a single spot when chromatographed. Bergmann *et al.* (1934) report m.p. > 240 and $[\alpha]_D^{19}$ − 30.1 (in water with one equivalent of hydrochloric acid).

Enzymes.—The salt-free, bovine α-chymotrypsin was an Armour preparation, lot No. T-97207. The salt-free δ-chymotrypsin was a Nutritional Biochemicals Corp. preparation, lot No. 6714. Stock solutions 10 times the concentrations indicated in Table II were used to make up the reaction mixtures.

Reaction Mixtures and Chromatography.—In general, the reaction mixtures were prepared from 8 ml of substrate stock solution, 1 ml of a tris(hydroxymethyl)aminomethane–hydrochloric acid buffer, and 1 ml of enzyme stock solution, all of which were of appropriate concentration to give the final concentrations indicated in Table II. The pH was adjusted to pH 7.0 with 2 N aqueous hydrochloric acid both before and after the enzyme stock solution was added and again determined after 24 hours. All experiments were conducted at 25–27°.

At 0, 1, 2, 4, and 24 hours, 10, 6, 24, and 10 μl aliquots of the L-tyrosine methyl ester, amide, hydrazide, and hydroxamide reaction mixtures, respectively, were removed and applied to Whatman No. 1 paper with a 2-μl pipet. The paper strips were then developed with the solvents indicated in Table III. The reagent used to spray the chromatograms was prepared by mixing a 1% solution of sulfanilamide in 10% (v/v) aqueous hydrochloric acid and a 5% aqueous solution of sodium nitrite followed by extraction of the diazotized sulfanilamide into 1-butanol (Block *et al.*, 1952). After the chromatograms had been sprayed and dried they were again sprayed with one-half saturated aqueous sodium carbonate to develop the peach-colored spots which were viewed in both visible and ultraviolet light. Further details are given in Table III, where the results of all experiments have been collected.

Control experiments with no enzyme added showed only the initial substrate to be present. Neither α- or δ-chymotrypsin gave a discrete spot. When samples of L-tyrosyl-L-tyrosinamide of diminishing size were chromatographed, it was found that as little as 0.2 μg could be detected. In no case did the pH of a reaction mixture change by more than 0.1 of a pH unit over a 24-hour period.

TABLE II

REACTION SYSTEMS FOR CHROMATOGRAPHIC EXPERIMENTS

No.	Substrate	[S]	Chymotrypsin	[E][a]	THAM[b]
1	Methyl	0.020 M	α	0.40	0.02 M
2	Tyrosinate	0.020 M	α	0.004	0.02 M
3		0.020 M	α	0.004	0.02 M
4		0.020 M	δ	0.004	0.02 M
5	Tyrosinamide	0.024 M	α	0.22	0.02 M
6		0.024 M	δ	0.22	0.02 M
7		0.050 M	α	1.37	0.02 M
8		0.050 M	δ	1.37	0.02 M
9	Tyrosinhydrazide	0.004 M	α	1.37	0.02 M
10		0.004 M	δ	1.37	0.02 M
11	Tyrosinhydroxamide	0.013 M	α	0.68	0.20 M
12		0.013 M	δ	0.68	0.20 M

[a] Enzyme concentration in mg. protein/ml. To convert to mg. protein nitrogen/ml. multiply by 0.1475. [b] Concentration of tris-(hydroxymethyl)aminomethane.

RESULTS

The reactions of L-tyrosine methyl ester, hydroxamide, amide, and hydrazide with chymotrypsin were in general conducted with the highest substrate and enzyme concentrations used for evaluation of their kinetic constants, and at their respective pH optima, in order to maximize, within the limits just stated, the possibility of observing transpeptidation reactions. Since glycyl-L-phenylalaninamide was found to give different products with δ-chymotrypsin than with α-chymotrypsin (Hakin *et al.*, 1960), separate experiments were conducted with both enzymes. However, with the substrates used in this study no difference between the α- and δ-chymotrypsin–catalyzed reactions could be detected.

Representative data for the different substrates are given in Table III and below.

Methyl L-*tyrosinate.*—With an enzyme concentration of 0.4 mg/ml, methyl L-tyrosinate was completely hydrolyzed within 5 minutes, during which L-tyrosine precipitated from the reaction mixture. However, with an enzyme concentration of 0.004 mg/ml the density of the methyl L-tyrosinate spot diminished with increasing time of reaction, whereas the L-tyrosine spot reached a saturation density within 1 hour, since L-tyrosine had started to precipitate from the reaction mixture. The precipitate was collected and dissolved in water, and the solution was chromatographed and found to contain only L-tyrosine. The spot with R_F = 0.62 was barely detectable, ≤0.2 μg, and its density did not change with time. The R_F value of this spot did not coincide with those of any of the reference compounds but was closest to that of L-tyrosyl-L-

TABLE III

CHROMATOGRAPHY OF REACTION PRODUCTS OF L-TYROSINE DERIVATIVES WITH CHYMOTRYPSIN AT pH 7.0 AND 25–27°

Experiments[a]					Reference Compounds	
No.	Substrate[b]	Solvent No.[c]	Spot[d] Density	R_F and Spot[e] Size	R_F and Spot[e] Size	Compound
3, 4	Tyr-OCH_3	1	+++	0.49 ± 0.03	0.49 ± 0.02	Tyr
			±	0.62 ± 0.02	0.72 ± 0.02	Tyr-Tyr
			++	0.80 ± 0.03	0.79 ± 0.04	Tyr-OCH_3
			−		0.87 ± 0.02	Tyr Anhydride
			−		0.88 ± 0.04	Tyr-Tyr-OCH_3
7, 8	Tyr-NH_2	1	++	0.49 ± 0.03	0.49 ± 0.02	Tyr
			+++	0.59 ± 0.04	0.61 ± 0.02	Tyr-NH_2
			±	0.72 ± 0.03	0.72 ± 0.02	Tyr-Tyr
			±	0.75[f] ± 0.04	0.76 ± 0.03	Tyr-Tyr-NH_2
			−		0.87 ± 0.02	Tyr Anhydride
9, 10	Tyr-$NHNH_2$	1	++	0.48 ± 0.02	0.49 ± 0.02	Tyr
			−		0.72 ± 0.02	Tyr-Tyr
			+	0.75 ± 0.06	0.76 ± 0.08	Tyr-$NHNH_2$
			−		0.87 ± 0.05	Tyr-Tyr-$NHNH_2$
			−		0.87 ± 0.02	Tyr Anhydride
9, 10	Tyr-$NHNH_2$	2	−		0.72 ± 0.03	Tyr-Tyr-$NHNH_2$
			−		0.73 ± 0.03	Tyr-Tyr
			−		0.76 ± 0.02	Tyr Anhydride
			+	0.79 ± 0.03	0.79 ± 0.03	Tyr
			+	0.78 ± 0.06	0.79 ± 0.07	Tyr-$NHNH_2$
9, 10	Tyr-$NHNH_2$	3	++	0.24 ± 0.02	0.25 ± 0.02	Tyr
			±	0.47[g] ± 0.03	0.48 ± 0.03	Tyr-Tyr
			+	0.64 ± 0.13	0.68 ± 0.13	Tyr-$NHNH_2$
			−		0.78 ± 0.16	Tyr-Tyr-$NHNH_2$
11, 12	Tyr-NHOH	1	+++	0.50 ± 0.03	0.49 ± 0.02	Tyr
			++	0.71 ± 0.04	0.72 ± 0.02	Tyr-Tyr
			−		0.78 ± 0.03	Tyr-Tyr-NHOH
			+	0.79 ± 0.05	0.79 ± 0.11	Tyr-NHOH
			−		0.87 ± 0.02	Tyr Anhydride
11, 12	Tyr-NHOH	4	−		0.55 ± 0.05	Tyr-Tyr-NHOH
			+	0.51[h] ± 0.11	0.56 ± 0.08	Tyr-NHOH
			−		0.65 ± 0.02	Tyr Anhydride
			++	0.79 ± 0.03	0.79 ± 0.04	Tyr
			++	0.83 ± 0.02	0.83 ± 0.03	Tyr-Tyr
11, 12	Tyr-NHOH	3	++	0.25 ± 0.02	0.25 ± 0.02	Tyr
			+	0.48 ± 0.03	0.48 ± 0.03	Tyr-Tyr
			−		0.63 ± 0.03	Tyr-Tyr-NHOH
			++	0.75 ± 0.11	0.74 ± 0.10	Tyr-NHOH

[a] The reaction mixtures are described in Table II. [b] The following abbreviations are used: Tyr = tyrosine, Tyr-OCH_3 = methyl tyrosinate, Tyr-NH_2 = tyrosinamide, Tyr-$NHNH_2$ = tyrosinhydrazide, Tyr-NHOH = tyrosinhydroxamide, Tyr-Tyr = tyrosyltyrosine, etc. [c] Solvents: (1) butanol–acetic acid–pyridine–water (30:6:20:24), (2) 2 N HCl saturated with butanol, (3) pyridine–isoamyl alcohol–water (37:43:20), and (4) water half-saturated with butanol. [d] Spot densities indicated as strong = +++, medium = ++, weak = +, marginal = ±, negative = −. [e] In R_F units. [f] This spot appeared on only one chromatogram after 36 hours of reaction. [g] This end of the R_F 0.64 spot appeared slightly darker in ultraviolet light. [h] The R_F value varied from 0.65 to 0.43, and the spot was barely detectable after 2 hours.

tyrosine. However, the very low concentration of the compound of R_F = 0.62 and its lack of variation with time of reaction suggested that it was of negligible importance in the chymotrypsin-catalyzed hydrolysis of methyl L-tyrosinate.

L-Tyrosinamide.—At the lower substrate and enzyme concentrations of experiments 5 and 6 (Table II), only L-tyrosine and L-tyrosinamide were detected even after 10 hours. However, at 0.05 M substrate and 1.37 mg enzyme/ml (experiments 7 and 8) a barely detectable L-tyrosyl-L-tyrosine spot appeared at 1 hour and slowly increased until it almost equaled at 24 hours and surpassed at 36 hours the density of the L-tyrosine spot. The suggestion of a spot at R_F = 0.75, that appeared at 36 hours corresponds to L-tyrosyl-L-tyrosinamide, but could be a higher peptide derivative. These latter results agree with those of Blau and Waley (1954), but at low enzyme and substrate concentrations the formation of L-tyrosyl-L-tyrosine is of negligible importance during the time, less than 4 hours, normally taken for kinetic studies.

L-Tyrosinhydrazide.—When chromatographed with either solvent no. 1 or 2 (Table III), the reaction products gave only two spots, corresponding to L-tyrosine and L-tyrosinhydrazide. However, the spot size, or "tailing," of the L-tyrosinhydrazide spot was so large with both solvents that the presence of L-tyrosyl-L-tyrosine could not be excluded. On the chromatograms developed with 2 N HCl saturated with butanol, solvent No. 2, L-tyrosine could be seen as a light fluorescent spot within the darker L-tyrosinhydrazide spot when viewed under ultraviolet light. When developed with solvent No. 3 the L-tyrosinhydrazide spot again was large; however, the tailing increased after zero time. This additional tail appeared very slightly darker than the rest of the spot under ultraviolet light, indicating the possible presence of another compound with an R_F value near 0.47. Thus, the presence of trace amounts of L-tyrosyl-L-tyrosine is again indicated, but, as with methyl L-tyrosinate, at very low concentrations which did not change with time.

L-Tyrosinhydroxamide.—L-Tyrosine, L-tyrosinhydroxamide, and L-tyrosyl-L-tyrosine were easily discernible on the chromatograms with each of the solvents used. However, the possible presence of L-tyrosyl-L-tyrosinhydroxamide could only be ruled

out when solvent No. 3 was used as the developer. L-Tyrosinhydroxamide was essentially gone at 4 hours, but the pH did not change significantly because of the high buffer concentration. The L-tyrosyl-L-tyrosine spot was definitely present at 15 minutes and continued to increase in density with increasing time, being about one-third that of the L-tyrosine spot at 4 hours, but had almost disappeared at 24 hours. Thus, a significant proportion of the reaction of L-tyrosinhydroxamide with α- or δ-chymotrypsin, under conditions tending to optimize detection of transpeptidation, results in formation of L-tyrosyl-L-tyrosine in addition to simple hydrolysis.

Discussion

The results obtained with L-tyrosine methyl ester, amide, and hydrazide are in agreement with those of McDonald and Balls (1956) and of Lestrovaya and Mardashev (1956). The peptide detected by the latter investigators as a reaction product of L-tyrosinamide and high concentrations of α-chymotrypsin may be identified as L-tyrosyl-L-tyrosine, since the reported R_F value is the same as that found in our studies, in which the same paper and developer were used. However, our results have shown that under the conditions usually employed in kinetic studies, insignificant amounts of L-tyrosyl-L-tyrosine are formed from L-tyrosine methyl ester and hydrazide and also from the amide for reaction periods of less than 4 hours.

The possibility of a peptide intermediate during hydrolysis (Blau and Waley, 1954; Kuk-Meiri and Lichtenstein, 1957, 1960) must be considered. The results obtained by Blau and Waley (1954) and with L-tyrosinhydroxamide demonstrate that L-tyrosyl-L-tyrosine is hydrolyzed very slowly by α-chymotrypsin. Since this peptide was present only in trace amounts in the experiments with the methyl ester, hydrazide, and amide, its rate of formation cannot be much faster than that of its hydrolysis and, consequently, is too slow to be of significance in the hydrolysis reaction. Therefore, L-tyrosyl-L-tyrosine can be excluded as an important intermediate unless significant quantities of this dipeptide are present in the reaction mixture.

The other possibility is that a dipeptide derivative, *e.g.*, L-tyrosyl-L-tyrosinamide, is an intermediate. Since none of the dipeptide derivatives corresponding to the four substrates investigated were detected in the reaction mixtures, these dipeptide derivatives could be present only in low steady-state concentrations. A calculation based upon the data of Blau and Waley (1954) shows that L-tyrosyl-L-tyrosinamide disappears from the reaction mixture about sixty times faster than L-tyrosinamide. However, these investigators found that L-tyrosyl-L-tyrosinamide when incubated with α-chymotrypsin gives primarily L-tyrosinamide and L-tyrosyl-L-tyrosine, in a ratio of about 2:1, and very little L-tyrosine. Therefore, the expected major degradation product from any dipeptide derivative is L-tyrosyl-L-tyrosine, which should be detectable, for the reasons given above, if this reaction pathway is of any significance. Since neither L-tyrosyl-L-tyrosine nor a L-tyrosyl-L-tyrosine derivative was found in any but trace amounts, it may be concluded that peptide derivatives are not significant intermediates, and, hence, transpeptidation is of negligible importance during the chymotrypsin-catalyzed hydrolysis of tyrosine methyl ester, amide, or hydrazide when studied under conditions used for evaluation of kinetic constants of the hydrolytic reactions.

L-Tyrosinhydroxamide cannot be included with the above three substrates, since L-tyrosyl-L-tyrosine was detected after 15 minutes and increased in concentration until the L-tyrosinhydroxamide was exhausted. Again, no other peptide derivative was found. However, the amount of L-tyrosine was substantially greater than the amount of L-tyrosyl-L-tyrosine, so that the transpeptidation reaction was minor, but not negligible, with respect to the hydrolysis reaction.

L-Tyrosine amide and hydroxamide probably form L-tyrosyl-L-tyrosine in a similar manner but on a different time scale. Blau and Waley (1954) proposed equations 4 and 5 to explain the formation of L-tyrosyl-L-tyrosine and its predominance over L-tyrosine after several hours.

$$2NH_2CHRCO{-}NH_2 \longrightarrow NH_2CHRCO{-}NHCHRCO{-}NH_2 + NH_3 \quad (4)$$

$$NH_2CHRCO{-}NHCHRCO{-}NH_2 + \overset{\oplus}{N}H_3CHRCO_2^{\ominus} \longrightarrow NH_2CHRCO{-}NHCHRCO_2H + NH_2CHRCO{-}NH_2 \quad (5)$$

The direct participation of L-tyrosine seems unlikely in the light of several investigations which have shown that blocking the carboxyl function as the ester, amide, etc., greatly enhances the rate of peptide formation when these derivatives are either donors (Haurowitz and Horowitz, 1955; Horowitz and Haurowitz, 1959) or acceptors (Kaganova and Orehovich, 1954) of acyl groups. An alternative to reaction 5 would, therefore, be a transpeptidation reaction between L-tyrosyl-L-tyrosinamide and L-tyrosinamide to give L-tyrosyl-L-tyrosyl-L-tryosinamide and ammonia with subsequent hydrolysis to L-tyrosyl-L-tyrosine and L-tyrosinamide. This reaction is analogous to the formation of α-*N*-benzoyl-L-tyrosylglycinamide from α-*N*-benzoyl-L-tyrosinamide and glycinamide (Johnston *et al.*, 1950; Fruton *et al.*, 1951).

The results of this investigation demonstrate that transpeptidation is not a significant factor in the α-chymotrypsin–catalyzed hydrolysis of L-tyrosine methyl ester, amide, and hydrazide under conditions ordinarily used in kinetic studies of these reactions. With the hydroxamide transpeptidation could intervene but even under the most unfavorable circumstances its contribution would be minor and incapable of causing substantial error in kinetic studies. Thus, the proposal that the differences in the pH optima of the α-*N*-acylated and nonacylated α-amino acid derivatives is due to a transpeptidation reaction (Schwert, 1955) has been refuted and support has been provided for the alternative hypothesis that the difference is a consequence of a change in relative concentrations of the less reactive α-amino acid derivative and its more reactive conjugate acid (Foster *et al.*, 1954; Lutwack *et al.*, 1957).

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