THE SYNTHESIS AND THE ENZYMATIC DEGRADATION OF 
\( l\)-TYROSYL-\( l\)-LYSYL-\( l\)-GLUTAMYLL-\( l\)-TYROSINE

BY ALBERT A. PLENTL* AND IRVINE H. PAGE†

WITH THE COLLABORATION OF F. R. VAN ABELE

(From the Lilly Laboratory for Clinical Research, Indianapolis City Hospital, Indianapolis)

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The formation of angiotonin from the serum protein fraction \( \alpha_2 \)-globulin and renin (1, 2) as well as its inactivation by proteolytic enzymes suggests that this pressor substance contains peptide linkages. Preliminary studies and interpretations (3) of the action of crystalline proteolytic enzymes led to the conclusion that angiotonin contains groupings of which the simplest representation is afforded by

\[
\text{Aromatic} \quad \text{Basic} \quad \text{Acid} \quad \text{Aromatic}
\]

\[
\text{NH}_2 - \text{CH} - \text{CO} - \text{NH} - \text{CH} - \text{CO} - \text{NH} - \text{CH} - \text{CO} - \text{NH} - \text{CH} - \text{COOH}
\]

The object of this investigation was to prepare a tetrapeptide similar in structure which would not only be of interest for the study of the kinetics of enzyme action but might serve as a model for degradation experiments on naturally occurring polypeptides. A comparison of the rates of hydrolysis of such a synthetic tetrapeptide with angiotonin might give some valuable information concerning the structure of this pressor substance.

Since the rate of hydrolysis of a polypeptide by a well defined proteolytic enzyme appears to be a function of the structure of the substrate as well as of the general nature of the enzyme, a comparison of the rate of hydrolysis might be used for identification purposes. A compound containing amino acids in the arrangement outlined above should be susceptible to hydrolysis by carboxypeptidase, trypsin, chymotrypsin, pepsin, and, if there exists any structural similarity to angiotonin, the rates of hydrolysis by these enzymes should be comparable.

The amino acids tyrosine, lysine, and glutamic acid were selected for the synthesis, because a number of necessary intermediates and methods for their syntheses are known. The synthesis herein reported consists of a condensation of two dipeptides whose functional groups were protected, followed by removal of the protecting groups by standard methods.

The first dipeptide was prepared by a condensation of O-acetyl-N-car-
boc-tyrosine chloride (I) with ε-carbobenzoxy-L-lysine methyl ester (II). Minor variations in the procedure resulted in two distinctly different condensation products, one melting at 156° and the other at 138°. Both substances yielded the same hydrazide on reaction with hydrazine hydrate. Since there is no possibility of a racemization, a structural difference other than asymmetry must be assumed to explain this phenomenon.

The condensation of O-acetyl-N-carbobenzoxytyrosyl chloride and the ε-carbobenzyxlysine methyl ester is carried out in such a way that the acid chloride is added to an excess of the basic ester, so that during the whole experiment the medium is slightly alkaline. It therefore offers the possibility for hydrolysis of very susceptible ester linkages such as the acetyl group on the phenolic hydroxyl of the tyrosine, leaving the carboxylic ester of the lysine portion intact. The conversion of the ester (III) to the hydrazide is carried out in an even stronger alkaline medium, thus converting both the acetylated as well as the deacetylated compound into the same hydrazide.

The compound with a melting point of 156° gave an analytical value of 8.06 per cent of acetyl; the compound with a melting point of 138° gave 2.84 per cent. The theoretical values for the acetylated and deacetylated esters are 6.80 and 0.00 respectively. The suspicion that the discrepancies might be due to the presence of the carbobenzoxy group was confirmed by acetyl analyses on model substances (Table I).

An attempt was made to prepare the other moiety of the desired tetrapeptide by esterifying carbobenzoxy-L-glutamyl-L-tyrosine monoethyl ester, followed by removal of the carbobenzoxy group. The esterification proceeded satisfactorily and the expected carbobenzoxy diethyl ester was isolated in good yield. On treatment with palladium and hydrogen in

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**Table I**

Comparison of Acetyl Determinations on Carbobenzoxy Compounds with and without Acetyl Groups

<table>
<thead>
<tr>
<th>Compound</th>
<th>Formula</th>
<th>% acetyl (COCH₃)</th>
<th>Calculated</th>
<th>Found</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Carbobenzoxy-L-tyrosine ethyl ester</td>
<td>C₁₉H₂₀O₄N₂</td>
<td>None</td>
<td>2.46</td>
<td></td>
</tr>
<tr>
<td>N-Carbobenzoxy-L-tyrosine</td>
<td>C₁₁H₁₂O₂N</td>
<td>11.08</td>
<td>2.06</td>
<td></td>
</tr>
<tr>
<td>O-Acetyl-N-carbobenzoxy-L-tyrosine</td>
<td>C₂₁H₂₀O₄N</td>
<td>12.20</td>
<td>8.06</td>
<td></td>
</tr>
<tr>
<td>O-Acetyl-N-carbobenzoxy-L-tyrosyl-N-</td>
<td>C₁₄H₁₃O₄N₃</td>
<td>6.80</td>
<td>8.06</td>
<td></td>
</tr>
<tr>
<td>carbobenzoxy-L-lysine methyl ester, m.p. 138°</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-Carbobenzoxy-L-tyrosyl-N-carbobenzoxy-</td>
<td>C₂₂H₂₄O₈N₄</td>
<td>None</td>
<td>2.84</td>
<td></td>
</tr>
<tr>
<td>L-lysine methyl ester, m.p. 156°</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
(I) R = H

(VII) R = COCH₃

Hydrazide (IV) Free diester (VII)

Cbzo-\text{NH-CH-CO-NH-CH-COOMe}

Cbzo-\text{NH-CH-CO-NH-CH-COOEt}

(VIII)

Cbzo-\text{NH-CH-CO-NH-CH-CO-NH-CH-COOMe}

Cbzo-\text{NH-CH-CO-NH-CH-COOEt}

(IX)

\text{NH}_2-\text{CH-CO-NH-CH-CO-NH-CH-CO-NH-CH-CO-OH}

\text{L-Tyrosyl-L-lysyl-L-glutamyl-L-tyrosine}
alcoholic solution, an ill defined, though crystalline substance was obtained. Analytical data suggested it to be a mixture of \( \text{l-glutamyl-l-tyrosine mono-} \) and diethyl esters. This procedure was therefore discarded in favor of the method of Fruton and Bergmann (4) who prepared the diethyl ester as an intermediate in the synthesis of glycyl-\( \text{l-glutamyl-l-tyrosine} \). The ester was not isolated in crystalline form.

The hydrazide of the tyrosyllysine moiety, when allowed to react with nitrous acid in acetic acid solution, gave a crystalline azide which was immediately treated with \( \text{l-glutamyl-l-tyrosine diethyl ester} \) (VII) in ethyl acetate solution. Although the crude condensation product was obtained in good yield, an analytically pure material was difficult to prepare. The purity of this tetrapeptide derivative appeared to be particularly important, because the anticipated removal of the protecting groups would yield a water-soluble compound whose molecular weight could not be determined by the conventional micromethods. Upon repeated recrystallization from various solvent mixtures and finally from pure ethyl alcohol the compound was obtained in pure form; its solubility in organic solvents and camphor rendered it suitable for the determination of its molecular weight by the Rast method (5). These values were in good agreement with theory. This, together with the correct analytical figures for carbon, hydrogen, and nitrogen as well as ethoxyl and the absence of amino nitrogen may be taken as evidence that condensation to the tetrapeptide had taken place.

Saponification of VIII under mild conditions followed by removal of the two carbobenzoxy groups by catalytic hydrogenation with palladium black proceeded without difficulty and gave the desired \( \text{l-tyrosyl-l-lysyl-l-glutamyl-l-tyrosine} \) as the tetrahydrate. The final compound was optically active. It did not affect the arterial blood pressure of a pithed cat when injected in doses of 20 mg. per kilo.

Since this compound contains two aromatic, one basic and one dicarboxylic amino acid, it should be sensitive to hydrolysis by a number of proteolytic enzymes. The structural detail of a substrate necessary to render it susceptible to hydrolysis by proteolytic enzymes has been carefully investigated by Bergmann and his associates. The indispensable groups of substrates for carboxypeptidases according to Bergmann (6) are

\[
\text{R} \quad \ldots \text{NH--CH--COOH}
\]

\[(X)\]

where \( \text{R} \) is a hydroxybenzyl or benzyl radical. The tetrapeptide which we have synthesized contains such an arrangement (Structure \( \Delta \), Fig. 1) and hence should be attacked by carboxypeptidase and the hydrolysis should
A. A. PLENTL AND I. H. PAGE

**Fig. 1.** Structural formula for \(I\)-tyrosyl-\(I\)-lysyl-\(I\)-glutamyl-\(I\)-tyrosine illustrating the necessary arrangements for hydrolysis by proteolytic enzymes: \(A\), carboxypeptidase; \(B\), pepsin; \(C\), trypsin; \(D\), chymotrypsin.

**Fig. 2.** The course of hydrolysis of \(I\)-tyrosyl-\(I\)-lysyl-\(I\)-glutamyl-\(I\)-tyrosine by carboxypeptidase. The enzyme concentration was 0.0067 mg. of N per cc. of test solution; the pH was maintained at 7.8 by means of 0.1 M phosphate buffer. Incubation temperature 36°.

proceed with the liberation of 1 molecule of tyrosine. The resulting tetrapeptide, with a glutamic acid residue in terminal position, no longer contains the requisite groups in the backbone and may be expected to resist
further hydrolysis by this enzyme. Although Hofmann and Bergmann (7) found that a compound containing glutamic acid in terminal position can be hydrolyzed by carboxypeptidase, it should be noted that an exceedingly high concentration of the enzyme was necessary in order to hydrolyze carbobenzyoxglycyl-l-glutamic acid (7). These authors employed almost 100 times as much enzyme for the hydrolysis of carbobenzyoxglycyl-l-glutamic acid as we have used in our experiments. Inspection of Fig. 2 will reveal that exactly 1 mole of amino acid nitrogen has been liberated after 3 hours incubation of the tetrapeptide with carboxypeptidase with no further increase during the ensuing 9 hours. The concentration of carboxypeptidase was thereupon increased 50-fold, when a significant rise in amino nitrogen was observed, indicating that hydrolysis of the tripeptide had taken place, probably with formation of tyrosine, lysine, and glutamic acid. The hydrolysis of tyrosyllysylglutamytyrosine by crystalline carboxypeptidase thus proceeded as expected and fully confirmed the specificity requirements for this enzyme, as advanced by Bergmann (6).

The specificity requirements for pepsin (6) call for the following arrangement of amino acids in the substrate molecule

\[ \text{RI} \quad \text{R2} \]

\[\text{\textunderscore NH\textunderscore CH\textunderscore CO\textunderscore NH\textunderscore CH} \ldots \]  

Where \( R_2 \) must be a benzyl- or hydroxybenzyl radical and \( R_1 \) an inert or preferably dicarboxylic amino acid residue such as glutamic acid. Since our tetrapeptide contains such atomic groups in its backbone and side chain (Structure B, Fig. 1), it should be sensitive to peptic digestion, and this is borne out by the experimental data reproduced in Fig. 3. The increase in amino nitrogen on incubation with pepsin corresponds to one peptide bond with no further increase on prolonged digestion. It must be concluded that pepsin attacks the substrate on the same sensitive peptide bond as does carboxypeptidase; hence both liberate 1 mole of tyrosine.

Contrary to our expectations, the residual tripeptide, tyrosyllysylglutamic acid, was entirely resistant to trypsinic action, even in exceedingly high concentrations of the enzyme. The tripeptide contains the requisite groups (Structure C, Fig. 1) for typical trypsin substrates (6); i.e., the amino acid residue \( R \) in XII should be a lysyl

\[ R \]

\[\text{\textunderscore CO\textunderscore NH\textunderscore CH\textunderscore CO\textunderscore NH} \ldots \]  

or arginyl radical with its amino and carboxyl groups involved in a peptide linkage. Since the concentration of both enzymes, pepsin and trypsin, was
Fig. 3. The course of hydrolysis of \( l \)-tyrosyl-\( l \)-lysyl-\( l \)-glutamyl-\( l \)-tyrosine by pepsin, followed by trypsin. The enzyme concentration was 0.495 mg. of pepsin N and 0.094 mg. of trypsin N per cc. of test solution. During peptic digestion the pH was maintained at 3.0 by means of citrate buffer and adjusted to pH 7.8 after the addition of trypsin. The enzyme blank was determined on a solution of pepsin and trypsin in the same concentration as the test solution and maintained at pH 7.8. Incubation temperature 36°.

Fig. 4. The course of hydrolysis of \( l \)-tyrosyl-\( l \)-lysyl-\( l \)-glutamyl-\( l \)-tyrosine by trypsin and chymotrypsin. The enzyme concentration was 0.094 mg. of trypsin and 0.233 mg. of chymotrypsin N per cc. of test solution; pH 7.8 and incubation temperature 36°.
quite high, the inertness of the tripeptide to tryptic hydrolysis \(\text{(i.e., after peptic digestion of the tetrapeptide)}\) might conceivably be attributed to an inhibiting effect of digestion products resulting from the action of trypsin on pepsin. That a number of polypeptides are formed in appreciable amount is indicated by the constantly increasing enzyme blank (Fig. 3) after the addition of trypsin to the tripeptide-tyrosine-pepsin mixture. In order to avoid such complications the action of crystalline trypsin on the tetrapeptide, \text{i.e.} without previous pepsin digestion, was investigated (Fig. 4). Although the rate of hydrolysis was quite slow compared to carboxypeptidase or pepsin, this substrate was hydrolyzed at a speed comparable to other typical trypsin substrates such as benzoyl-l-lysinamide \(6\) and has the advantage that the course of the hydrolysis can be followed by amino nitrogen determinations rather than titration of the liberated carboxyl groups.

Fruton and Bergmann \(8\) investigated the action of chymotrypsin on a number of synthetic substrates and found it to exhibit the phenomenon of multiple specificity. Chymotrypsin can act as a carbonyl proteinase (endopeptidase) or aminopeptidase (exopeptidase) requiring an aromatic amino acid whose carboxyl or amino group or both must be involved in a peptide linkage. The necessary structural detail is given by either XIII or XIV

\[
\begin{align*}
\text{(XIII)} &\quad R
\quad \text{NH}_2-\text{CH}--\text{CO}--\text{NH}... \\
\text{NH}-\text{CH}--\text{CO}--\text{NH}... \\
\text{(XIV)} &
\end{align*}
\]

where R must be a benzyl or hydroxybenzyl radical. Since tyrosyl-l-lysyl-l-glutamyl-l-tyrosine contains one such grouping (Structure D, Fig. 1), chymotrypsin must exert at least its aminopeptidase effect; \text{i.e., liberate 1 mole of tyrosine from 1 mole of the tetrapeptide. The increase in amino nitrogen with time obtained by chymotryptic digestion of the tetrapeptide is reproduced in Fig. 4. As anticipated, 1 mole of amino nitrogen was liberated during digestion. This amount of amino nitrogen must be attributed to tyrosine if the enzyme action is due to exopeptidase activity. If, on the other hand, the enzyme should exhibit endopeptidase activity, two dipeptides would be formed. An \(\alpha\)-aminocarboxyl determination according to the ninhydrin method of Van Slyke, Dillon, Mac-Fadyen, and Hamilton \(9\) after 96 hours digestion gave a value which corresponds to 97 per cent of the amino nitrogen liberated during the reaction. This may therefore serve as evidence that chymotrypsin had hydrolyzed the first peptide bond (between the tyrosine and lysine amino acid residues) and hence had acted as an exopeptidase.
The hydrolysis of the tetrapeptide by the four crystalline enzymes conforms with the concept that all proteolytic enzymes hydrolyze the peptide linkage but that it must be in a specific and proper environment of amino acid residues. The inactivation of angiotonin by proteolytic enzymes suggested in a qualitative way that it contains at least three peptide linkages in an environment of amino acid residues similar to L-tyrosyl-L-lysyl-L-glutamyl-L-tyrosine. It was hoped that a quantitative study of the action of crystalline enzymes on this tetrapeptide might yield some additional information regarding the "environment" of the peptide linkages in angiotonin.

**Table II**

Comparison of Action of Crystalline Proteolytic Enzymes on Angiotonin and L-Tyrosyl-L-lysyl-L-glutamyl-L-tyrosine

<table>
<thead>
<tr>
<th></th>
<th>Carboxypeptidase</th>
<th>Chymotrypsin</th>
<th>Trypsin</th>
<th>Pepsin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Angiotonin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein N per cc. test solution, mg.</td>
<td>0.0060</td>
<td>0.0097</td>
<td>0.0018</td>
<td>0.053</td>
</tr>
<tr>
<td>pH</td>
<td>7.4</td>
<td>7.1</td>
<td>7.2</td>
<td>4.5</td>
</tr>
<tr>
<td>(K^*)</td>
<td>0.030</td>
<td>0.036</td>
<td>0.016</td>
<td>0.042</td>
</tr>
<tr>
<td>(C^\dagger)</td>
<td>5.00</td>
<td>3.7</td>
<td>86.0</td>
<td>0.87</td>
</tr>
<tr>
<td><strong>L-Tyrosyl-L-lysyl-L-glutamyl-L-tyrosine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein N per cc. test solution, mg.</td>
<td>0.0067</td>
<td>0.233</td>
<td>0.094</td>
<td>0.495</td>
</tr>
<tr>
<td>pH</td>
<td>7.8</td>
<td>7.8</td>
<td>7.8</td>
<td>3.0</td>
</tr>
<tr>
<td>(K^*)</td>
<td>0.0016</td>
<td>0.000104</td>
<td>0.000303</td>
<td>0.00182</td>
</tr>
<tr>
<td>(C^\dagger)</td>
<td>0.24</td>
<td>0.00045</td>
<td>0.00323</td>
<td>0.036</td>
</tr>
</tbody>
</table>

\[ * K = \frac{2.3}{t} \log \frac{100}{100 - \% \text{ hydrolysis}}; \]  \( t = \) incubation time in minutes.

\[ \dagger C = \frac{\text{mg. protein N per cc. test solution}}{K} \]

Reaction constants and proteolytic coefficients were calculated from the various data of Figs. 2 to 4 and compared to the corresponding values previously reported (3) (Table II). A strict comparison between the two sets of data is not possible, since the proteolytic coefficients for both substrates and the four enzymes should be known over the whole pH range. Nevertheless, a few tentative conclusions can be drawn. Although the proteolytic coefficients for pepsin and carboxypeptidase are more than 10 times greater for angiotonin than for the tetrapeptide, this does not necessarily indicate a difference in structure of the substrate. The values for
the action of trypsin and chymotrypsin on these two substrates are sufficiently wide to suggest a fundamental difference. The extreme sensitivity of angiotonin to the latter two enzymes must be due to a structural arrangement which had not as yet been realized in any synthetic substrate. Particularly the peptide linkage in angiotonin which is sensitive to tryptic action must be distinctly different from Structure C, Fig. 1. Since the substitution of arginine for lysine would only slightly more than double the value of the proteolytic coefficient (6), this sensitivity of angiotonin to trypsin possibly might be attributed to a new basic amino acid. Another possibility is that specificity requirements for trypsin have been too narrowly defined and must be broadened.

The data on chymotrypsin do not lend themselves to such a comparison, since the proteolytic coefficient recorded in Table I for this enzyme refers only to its exopeptidase activity. It is conceivable, though unlikely (3), that chymotrypsin acts on angiotonin as an endopeptidase and an interpretation of the data on this enzyme should therefore be postponed until a suitable substrate becomes available.

EXPERIMENTAL

O-Acetyl-N-carbobenzoxy-l-tyrosyl Chloride (I)—This compound was prepared from O-acetyl-N-carbobenzoxy-l-tyrosine and phosphorus pentachloride according the method of Bergmann, Zervas, Salzmann, and Schleich (10).

$\varepsilon$-Carbobenzoxy-l-lysine Methyl Ester (II)—To a solution of 4.5 gm. of $\varepsilon$-carbobenzoxy-l-lysine methyl ester hydrochloride (11) in 10 cc. of water were added 50 cc. of ether and the mixture cooled in an ice-salt bath. Anhydrous potassium carbonate was slowly added with shaking until the aqueous portion became a paste (12). The free ester was obtained by extraction of the paste with ether, and the ethereal solution diluted with an equal volume of ethyl acetate and dried over anhydrous potassium carbonate at 0°.

O-Acetyl-N-carbobenzoxy-l-tyrosyl-$\varepsilon$-carbobenzoxy-l-lysine Methyl Ester (III)—3.5 gm. of O-acetyl-N-carbobenzoxy-l-tyrosyl chloride in dry ether suspension were added in two portions with shaking to the above ether-acetate solution of the free carbobenzoxy-l-lysine methyl ester. After addition of the first portion, $\varepsilon$-carbobenzoxy-l-lysine methyl ester hydrochloride crystallized from the solution. The mixture was allowed to stand 5 minutes and the second portion of acid chloride added together with an aqueous solution of 1.7 gm. of potassium carbonate. The mixture was shaken for 15 minutes, transferred to a separatory funnel, and a few cc. of pyridine added to decompose the excess acid chloride. 200 cc. of water and 200 cc. of ether were added and the mixture shaken vigorously. O-Acetyl-
N-carbobenzoxy-\(l\)-tyrosyl-\(e\)-carbobenzoxy-\(l\)-lysine methyl ester separated as a flocculent precipitate between the two layers. It was collected by centrifugation and washed with dilute hydrochloric acid, bicarbonate solution, and water. Yield, about 4.0 gm. On recrystallization from methyl alcohol, the substance melted at 138° (uncorrected).

\[
\text{C}_{18}\text{H}_{25}\text{O}_5\text{N}_4. \text{ Calculated. } C \ 64.45, H \ 6.16, N \ 6.63, -\text{COCH}_3 \ 6.80 \\
633 \quad \text{Found. } " \ 64.63, " \ 6.17, " \ 6.90, " \ 8.06 \\
\]  

In several runs, a product was obtained which after recrystallization from methyl alcohol melted at 156°. In view of certain considerations discussed in the theoretical part of this paper, this compound appears to be the deacetylated ester.

\[
\text{C}_{32}\text{H}_{36}\text{O}_8\text{N}_3. \text{ Calculated. } C \ 65.02, H \ 6.12, N \ 7.12, -\text{COCH}_3 \ 0.00 \\
590 \quad \text{Found. } " \ 64.62, " \ 6.24, " \ 6.32, " \ 2.84 \\
\]

\(N\)-Carbobenzoxy-\(l\)-tyrosyl-\(e\)-carbobenzoxy-\(l\)-lysine Hydrazide (IV)—2.0 gm. of the acetyl dicarbobenzoxytyrosyl-\(l\)-lysyl methyl ester dissolved in warm methyl alcohol were treated with 0.6 cc. of hydrazine hydrate. On standing at room temperature overnight, 1.5 gm. of the crystalline hydrazide separated. For analysis, the substance was purified by solution in hot methyl alcohol and concentrated in vacuo until crystallization occurred; m.p. 210°.

\[
\text{C}_{31}\text{H}_{36}\text{O}_7\text{N}_5. \text{ Calculated. } C \ 62.94, H \ 6.10, N \ 11.85 \\
590 \quad \text{Found. } " \ 62.47, " \ 6.09, " \ 11.48 \\
\]

The hydrazide prepared from the higher melting ester (m.p. 156°), presumably the deacetylated compound, also melted at 210°. No depression in melting point was noted with a mixture of the two hydrazides.

\(N\)-Carbobenzyoxy-\(l\)-tyrosyl-\(e\)-carbobenzoxy-\(l\)-lysyl-\(l\)-glutamyl-\(l\)-tyrosine Di-ethyl Ester (VIII)—1.5 gm. of \(N\)-carbobenzyoxy-\(l\)-tyrosyl-\(e\)-carbobenzoxy-\(l\)-lysyl hydrazide were ground in a mortar with 20 cc. portions of warm 75 per cent acetic acid. 225 cc. of this acid were required to produce a clear solution. After filtering, 113 cc. of water were carefully added to bring the acetic acid concentration to 50 per cent, and yet maintain complete solution. It was cooled to 4° and treated with an aqueous solution of 210 mg. of sodium nitrite. The azide crystallized out on standing in the ice bath for 10 minutes. The reaction mixture was diluted with 300 cc. of ice-cold water and extracted with cold ethyl acetate. The ethyl acetate solution was washed with ice water, cold bicarbonate solution, and again
with water. After drying over anhydrous magnesium sulfate at 0°, the solution was filtered directly into an ethyl acetate solution of L-glutamyl-L-tyrosine diethyl ester prepared from 1.6 gm. of the monoester. On standing overnight a small amount of a gelatinous precipitate formed. This was removed by filtration and the clear ethyl acetate solution thoroughly washed with water, dilute hydrochloric acid, aqueous bicarbonate, and again with water. On concentration of the dried ethyl acetate solution, crystallization occurred. Yield about 1.0 gm. The substance was recrystallized from alcohol-ethyl acetate mixtures and 95 per cent ethyl alcohol; m.p. 204°.

\[
\text{C}_{46}\text{H}_{38}\text{O}_{12}\text{N}_{4}. \text{ Calculated. } \text{C} 63.57, \text{H} 6.38, \text{N} 7.57, \text{OC}_{2}\text{H}_{6} 9.72, \text{NH}_{3}\text{N} 0.00
\]
\[
\text{Found. } \text{C} 63.21, \text{H} 6.44, \text{N} 7.57, \text{OC}_{2}\text{H}_{6} 9.50, \text{NH}_{3}\text{N} 0.01
\]
\[
\text{Mol. wt., calculated, 925}
\]

1-Tyrosyl-L-lysyl-L-glutamyl-L-tyrosine (IX)—230 mg. of the dicarboxybenzoyl tetrapeptide ester were dissolved in 15 cc. of 1 N NaOH by shaking at room temperature for 1 hour. A small amount of insoluble material was removed by filtration and the cooled filtrate acidified to Congo red with dilute hydrochloric acid. The product was collected by centrifugation, washed with water, dissolved in dilute sodium carbonate solution, and reprecipitated with hydrochloric acid. The wet material was washed with water, dissolved in 25 cc. of alcohol, and hydrogenated in the presence of palladium black and a drop of glacial acetic acid. A few drops of water were added occasionally to keep the product in solution. When hydrogenation was complete, the catalyst was filtered off and the solution concentrated in vacuo. A crystalline product was obtained by repeated addition and removal of absolute methyl alcohol. The residue was recrystallized from an ethyl alcohol-water mixture and dried at 60° over phosphorus pentoxide for 1 hour. On concentration of all mother liquors and recrystallization from the same solvent mixture, a total yield of 160 mg. was obtained.

\[
\text{C}_{29}\text{H}_{39}\text{O}_{9}\text{N}_{4}. \text{ Calculated. } \text{C} 51.70, \text{H} 6.95, \text{N} 10.55, \text{NH}_{3}\text{N} 4.16
\]
\[
\text{Found. } \text{C} 51.62, \text{H} 6.60, \text{N} 10.09, \text{NH}_{3}\text{N} 4.20
\]

The 4 molecules of water of crystallization could not be determined in the usual manner because of the instability of the peptide at temperatures high enough to remove the water quantitatively. The evidence for the presence of 4 molecules of water rests in the ratio of total N to NH_{3}N as well as in the results of the enzymatic studies described below.
Enzymatic Studies

Enzymes—Carboxypeptidase was prepared according to Anson (13) and recrystallized four times. Crystalline pepsin, trypsin, and chymotrypsin were prepared according to the directions of Northrop (14). All enzyme preparations had previously been tested for their activity, typical synthetic substrates and angiotonin being used (3, 6).

Incubation—A standard solution of the tetrapeptide was prepared by dissolving 139 mg. in 25 cc. of water. For each of the experiments recorded in Figs. 2 to 4, 5.00 cc. of the standard solution were diluted to 10.00 cc. with the desired buffer containing the requisite amount of enzyme. The final substrate concentration was therefore 0.1 mM, which corresponds to 0.0585 mg. of amino nitrogen per cc. of test solution for each amino group. Since there are two free amino groups in the original substrate, the blank value in each experiment was 0.117 mg. of NH₃-N per cc.

0.1 M citrate or phosphate buffers were used to maintain the acidity at the desired value.

The course of the hydrolysis was followed by the nitrous acid method of Van Slyke and the amino acid carboxyl in the chymotrypsin experiment was determined by the ninhydrin method of Van Slyke, Dillon, MacFayden, and Hamilton (9).

The authors are indebted to Miss Doris Brown for her valued assistance.

SUMMARY

The tetrapeptide, tyrosyllysylglutamyltyrosine, has been synthesized by a method which establishes its structure. It was accomplished by condensation of the two dipeptides, tyrosyllysine and glutamyltyrosine, whose functional groups were protected. These protecting groups were removed, freeing the desired optically active tetrapeptide in good yield. This compound did not affect the arterial blood pressure of a pithed cat in doses of 20 mg. per kilo of body weight.

The tetrapeptide was subjected to enzymatic hydrolysis by crystalline carboxypeptidase, chymotrypsin, trypsin, and pepsin. It was hydrolyzed by these four enzymes at the expected rate, thus supporting the specificity requirements for proteolytic enzymes as suggested by Bergmann and his associates.

The rate of hydrolysis under the influence of carboxypeptidase and pepsin was similar to the rate of inactivation of angiotonin by the same enzymes. Chymotrypsin and trypsin hydrolyzed the tetrapeptide very much more slowly than angiotonin. It was suggested that the sensitivity of angiotonin to tryptic hydrolysis is due to an as yet unknown amino acid arrangement.
BIBLIOGRAPHY

THE SYNTHESIS AND THE ENZYMATIC DEGRADATION OF l-TYROSYL-l-LYSYL-l-GLUTAMYL-l-TYROSINE

Albert A. Plentl and Irvine H. Page