NEW SYNTHETIC SUBSTRATES FOR PEPSIN

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All synthetic substrates for pepsin described heretofore (1-3) have been found to be hydrolyzed maximally near pH 4 at a relatively slow rate. It is now possible to describe synthetic substrates hydrolyzed much more rapidly by pepsin, with a pH optimum at 1.8 to 2.0, the same pH found to be optimal for proteins.

The previously described synthetic substrates were N-substituted peptides and some simple peptides in which a tyrosine or phenylalanine residue was linked to certain other amino acids such as glutamic acid (1), glycine (1), cysteine or cystine (2), or methionine (3). The uniform presence in these substrates of either a tyrosine or phenylalanine residue suggested the possibility of testing a peptide in which these two amino acids were linked to each other. Accordingly, acetyl-n-phenylalanyl-n-tyrosine and its iodinated derivative, acetyl-n-phenylalanyl-n-diiodotyrosine, were synthesized and exposed to the action of crystalline pepsin.\(^1\) Low concentrations of substrate and enzyme were used in these experiments, since these substrates had very low solubility at pH 2, and the concentration of enzyme had to be adjusted to the concentration of substrate and its rate of hydrolysis. Thus, 0.003 and 0.0005 mM of substrate per cc. were used respectively with 0.06 and 0.012 mg. of Pepsin N (concentrations much lower than Fruton and Bergmann (1) had used in their experiments, 0.05 mM substrate, and 1.4 to 2.4 mg. of Pepsin N). As may be seen from Fig. 1, both substrates were hydrolyzed, and both had a pH optimum at 1.8 to 2. At pH 2, hydrolysis was very rapid, the tyrosine peptide being 50 per cent hydrolyzed in 1 hour and the diiodotyrosine peptide 95 per cent in 15 minutes. The diiodotyrosine peptide was hydrolyzed much more rapidly at all pH values than was the tyrosine peptide. This would not have been expected from the observations of Fruton and Bergmann (1), who found carbobenzoxy-L-glutamyl-n-tyrosine to be hydrolyzed but the corresponding diiodotyrosine compound to be resistant to pepsin. Tyrosine and diiodotyrosine were isolated from the respective hydrolysates and identified by elementary analysis (cf. "Experimental").

To ascertain whether this rapid hydrolysis with an optimum rate at pH 2 occurs generally with dipeptides in which two aromatic amino acids are

\(^1\) Prepared from Cudahy pepsin according to Philpot (4).
linked to each other, a number of other \(N\)-substituted peptides of this nature were tested with pepsin. As shown in Table I, all were hydrolyzed quite rapidly, the rate being faster at pH 2 than at pH 3 or 4. In terms of side chain specificity it seems, therefore, that pepsin hydrolyzes optimally at pH 2 the bond linking two amino acids which carry the benzyl, hydroxybenzyl, or diiodohydroxybenzyl group\(^*\) as the side chain.

In order to test the stereospecificity of this reaction, several peptides in which an \(L\)-tyrosine or \(L\)-phenylalanine residue was replaced by the corresponding \(D\) enantiomorph were submitted to the action of pepsin. In no case was any hydrolysis observed (Table I). Hydrolysis was also completely inhibited when the phenolic group in \(N\)-carbobenzoxy-\(L\)-tyrosyl-\(L\)-phenylalanine was acetylated. Two dehydropeptides, formed as intermediates in the synthesis of some of the peptides described above, were also found to resist hydrolysis by pepsin (Table I). Finally, in order to investigate the susceptibility of a substance in which the function of the carboxyl group is suppressed, carbobenzoxy-\(L\)-phenylalaninyl-\(L\)-phenylalanine amide was incubated with pepsin. Because of the insolubility of this compound, a special effort was made (cf. "Experimental") to expose as great a portion of it as possible to the action of pepsin, and the incubation was prolonged for 6 days. No hydrolysis was perceptible. Nevertheless, conclusive proof for the necessity of a carboxyl group in this class of sub-

\[\text{Fig. 1. Hydrolysis of acetyl-}L\text{-phenylalanyl-}L\text{-diiodotyrosine and acetyl-}L\text{-phenylalanyl-}L\text{-tyrosine by pepsin at varying pH values. Curve a, acetyl-}L\text{-phenylalanyl-}L\text{-diiodotyrosine, 0.0005 mM per cc.; pepsin, 0.012 mg. of N per cc.; time of hydrolysis, 15 minutes. Curve b, acetyl-}L\text{-phenylalanyl-}L\text{-tyrosine, 0.003 mM per cc.; pepsin, 0.06 mg. of N per cc.; time of hydrolysis, 60 minutes.}\]

\(^*\) Not all the possible diiodotyrosine peptides of this nature have been tested. It seems possible that steric hindrance could interfere in the case of the diiodotyrosyldiiodotyrosine bond.
strate must await the availability of a more soluble substrate in which the carboxyl group is blocked.

Solubility tests made on the preparation of pepsin crystallized according to Philpot (4) showed it to contain more than one protein. Therefore Pepsin A of Herriott, Desreux, and Northrop (5), which has been shown to be homogeneous by phase rule solubility criteria, was prepared. This

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Action of Pepsin* on Various Synthetic Substrates†</th>
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</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>Concentration</td>
</tr>
<tr>
<td>N-Acetyl-L-tyrosyl-L-tyrosine</td>
<td>0.001</td>
</tr>
<tr>
<td>Acetyl-L-phenylalanyl-L-phenylalanine</td>
<td>0.0005</td>
</tr>
<tr>
<td>N-Carbobenzyloxy-L-tyrosyl-L-phenylalanine</td>
<td>0.0005</td>
</tr>
<tr>
<td>N-Acetyl-d-tyrosyl-L-tyrosine</td>
<td>0.004</td>
</tr>
<tr>
<td>Acetyl-d-phenylalanyl-L-tyrosine</td>
<td>0.004</td>
</tr>
<tr>
<td>Acetyl-d-phenylalanyl-L-diiodotyrosine</td>
<td>0.0005</td>
</tr>
<tr>
<td>N-Acetyldehydrotyrosyl-L-tyrosine</td>
<td>0.004</td>
</tr>
<tr>
<td>Acetylehydrophenylalanyl-L-tyrosine</td>
<td>0.004</td>
</tr>
<tr>
<td>N-Carbobenzyoxy-O-acetyl-L-tyrosyl-L-phenylalanine</td>
<td>0.002</td>
</tr>
<tr>
<td>Carbobenzyoxy-L-phenylalanyl-L-phenylalanine amide</td>
<td>0.002</td>
</tr>
</tbody>
</table>

* All experiments were conducted at 36.7° with a twice recrystallized sample of pepsin prepared from Cudahy pepsin according to Philpot (4).
† Data for acetyl-L-phenylalanyl-L-tyrosine and acetyl-L-phenylalanyl-L-diiodotyrosine are given in Fig. 1.
‡ Fruton and Bergmann (1) reported carbobenzyoxy-L-tyrosyl-L-tyrosine to be 8 per cent hydrolyzed in 24 hours at pH 4. No experiment at pH 2 was reported.
§ Calculated from a D.L. mixture on the assumption that equal quantities of the isomers were present.

Pepsin A hydrolyzed N-acetyl-L-tyrosyl-L-tyrosine, N-acetyl-L-phenylalanyl-L-tyrosine, and N-acetyl-L-phenylalanyl-L-diiodotyrosine even more rapidly than did the pepsin crystallized from Cudahy pepsin according to Philpot (4) (Table II).

Two questions are suggested by the fact that the conditions of hydrolysis used here differ from those used in the investigation of other synthetic substrates for pepsin: Is the greater speed of hydrolysis, observed here, more

* The kindness of Dr. R. M. Herriott in supplying another sample of Pepsin A for comparison is gratefully acknowledged.
due to the lower concentrations of substrate and enzyme that were used rather than to the different structure of the substrates? And could different conditions of hydrolysis account for the different pH optima that have been observed? To answer the first question, acetyl-L-phenylalanyl-L-tyrosine and carbobenzoxy-L-glutamyl-L-tyrosine (one of the most easily hydrolyzed of the previously known substrates) were hydrolyzed simultaneously under identical conditions (0.003 mM of substrate and 0.06 mg. of Pepsin N per cc. at pH 2). At the end of 1 hour the acetyl-L-phenylalanyl-L-tyrosine was 55 per cent hydrolyzed, but no trace of hydrolysis of the carbobenzoxy-L-glutamyl-L-tyrosine was detected. The incubation of the carbobenzoxy-L-glutamyl-L-tyrosine was continued for 48 hours, at which time it was found to be only 7 per cent hydrolyzed. It is evident, therefore, that the new synthetic substrates for pepsin are far more susceptible to the action of pepsin than are those previously described, the

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Substrate concen-</th>
<th>Pepsin concen-</th>
<th>Time</th>
<th>Per cent hydrolysis at pH 2.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl-L-phenylalanyl-L-diiodotyrosine</td>
<td>0.0005</td>
<td>0.012</td>
<td>15</td>
<td>100</td>
</tr>
<tr>
<td>Acetyl-L-phenylalanyl-L-tyrosine</td>
<td>0.003</td>
<td>0.06</td>
<td>60</td>
<td>56</td>
</tr>
<tr>
<td>N-Acetyl-L-tyrosyl-L-tyrosine</td>
<td>0.001</td>
<td>0.12</td>
<td>240</td>
<td>38</td>
</tr>
</tbody>
</table>

latter being hydrolyzed very slowly indeed under the conditions of hydrolysis that were used here. Variations in the speed of hydrolysis of the new synthetic substrates do occur, of course, with variation in the enzyme and substrate concentrations. Kinetic studies showing the extent of this variation and the factors controlling it are currently near completion. The results will be reported in another communication. It may be said, however, that the variation which has been observed is not great enough to account for the differences in speed of hydrolysis of the new and of the previously known substrates.

Studies to ascertain whether differences in pH optimum may occur under differing conditions of hydrolysis are also currently being made. A dependence of pH optimum on the concentrations of enzyme and substrate has been observed. For instance, when carbobenzoxy-L-glutamyl-L-tyrosine was hydrolyzed at a concentration of 0.002 mM per cc. by pepsin at a concentration of 0.06 mg. of N per cc., at pH 2, 3, and 4, the greatest degree of hydrolysis was found to be at pH 2, the least at 4 (Table I), instead of the greatest at 4 and the least at 2, as in the experiments of Fruton.
and Bergmann (1). Additional data with other substrates showing the reason for this shift in pH optimum will be presented in another communication.

It is convenient in describing the reactions of the various synthetic substrates for pepsin, now known, to divide them into two groups. This may be done on the basis of their structure; i.e., those in which a phenylalanine or tyrosine residue is linked to certain non-aromatic amino acids and those in which two aromatic amino acid residues are linked to each other. The first are hydrolyzed relatively slowly by pepsin. The pH optimum was found by Fruton and Bergmann to be near 4, but it has been shown here, for one of them, that under certain conditions of hydrolysis the pH optimum shifts to 2. Those in the second group are hydrolyzed very rapidly and the pH optimum is 1.8 to 2. Thus substrates in both groups may, under certain conditions of hydrolysis, be hydrolyzed at the same pH optimum as are proteins. The great speed at which substrates in the latter group are hydrolyzed suggests that, should two aromatic amino acid residues be linked to each other in the protein molecule, the bonds between them may be the primary sites at which pepsin acts upon the molecule.

EXPERIMENTAL

All compounds described below were recrystallized to constant melting point and, when optically active, to constant rotation prior to analysis.4

Acetyl-D-phenylalanyl-L-tyrosine—The method previously described for preparing this compound (6) was slightly modified as follows: Acetyl-dehydrophenylalanyl-L-tyrosine (20 gm.) was hydrogenated in 400 cc. of methyl alcohol with palladium-black as catalyst until the theoretical volume of hydrogen was absorbed. The palladium was filtered off, 400 cc. of water were added to the filtrate, and the mixture was cooled for 24 hours at 0°. The precipitate was filtered off (the filtrate being set aside) and recrystallized repeatedly from hot ethyl alcohol by the addition of water. Norit A was employed as the decolorizing agent. The melting point was 241°, slightly higher than 237°, reported previously (6). The specific rotation was +24.9 at 26° (2.5 per cent in pyridine); that previously reported was +25.1 at 20° (4 per cent in pyridine). This compound was shown by Bergmann, Stern, and Witte to be the D-L diastereomer.

Acetyl-L-phenylalanyl-L-tyrosine—The filtrate from the above was diluted with 400 cc. of water and cooled for another 24 hours at 0°. A second precipitate formed and was discarded, as it was found to contain a considerable quantity of the P-L diastereomer. The filtrate from this second precipitate was evaporated to dryness in vacuo, and the residue repeatedly

4 The author wishes to thank Dr. Adalbert Elek, who performed most of the analyses reported here.
SUBSTRATES FOR PEPsin

recrystallized from warm methyl alcohol (25 cc. per gm.) by the addition of an equal volume of water. Acetyl-L-phenylalanyl-L-tyrosine separated as triangular rods. Yield, 4 gm.; m.p. 230° (decomposition); $[\alpha]^25_0 = +14.5°$ (2 per cent in pyridine). For analysis the substance was dried in vacuo at 100°.

Acetyl-L-phenylalanyl-L-diiodotyrosine—Acetyl-L-phenylalanyl-L-tyrosine (11 gm.) was dissolved in 100 cc. of water and 150 cc. of concentrated NH$_2$OH and iodinated at 0° by the addition of 60 cc. of a 25 per cent potassium iodide solution containing 15 gm. of iodine. The resulting mixture was concentrated in vacuo at 45° until it gelatinized, and no longer smelled of ammonia. The jelly was removed by filtration and dissolved in warm glacial acetic acid. Upon cooling and adding water to the solution, the product separated as rosettes of needles and was recrystallized three times from hot 95 per cent alcohol by the addition of water. Yield, 12 gm.; m.p. 230° (decomposition); $[\alpha]^25_0 = +26.3°$ (2 per cent in pyridine).

Acetyl-D-phenylalanyl-L-diiodotyrosine—Acetyl-D-phenylalanyl-L-tyrosine (11.5 gm.) (m.p. 241–242°) was iodinated in the manner described above. The resulting solution was evaporated to dryness in vacuo at 45°, and the residue dissolved in boiling 50 per cent acetic acid. The addition of an equal volume of water caused the separation of acetyl-D-phenylalanyl-L-diiodotyrosine as clusters of needles. For recrystallization, it was dissolved in boiling 95 per cent alcohol and precipitated by the addition of 2 volumes of water. Norit A was employed as a decolorizing agent. Yield, 13 gm.; m.p. 215° (decomposition); $[\alpha]^25_0 = +6.8°$ (2 per cent in 95 per cent alcohol). For analysis the substance was dried at room temperature over P$_2$O$_5$ in a vacuum desiccator.
Azlactone of O,N-Diacetyldehydrotyrosine—In order to produce a purer product, the method previously described for the preparation of this compound (7) was modified as follows: 520 gm. of p-hydroxybenzaldehyde, 500 gm. of acetylglycine, 400 gm. of anhydrous sodium acetate, and 1.6 liters of acetic anhydride were heated on a boiling water bath for 1 hour. On cooling, bright yellow crystals of the azlactone separated. The azlactone was converted to acetamino-p-acetoxycinnamic acid by dissolving it in boiling glacial acetic acid and, while still hot, adding an equal volume of hot water. Recrystallization was effected in the same manner. Yield, 350 gm.; m.p. 232–233°.

C₁₃H₁₈O₆N. Calculated. C 59.3, H 5.0, N 5.3
263.1 Found. " 59.2, " 5.0, " 5.3

The acid was then reconverted to the azlactone by dissolving it in boiling acetic anhydride. Yield, 270 gm.; m.p. 137–138°.

C₁₃H₁₈O₆N. Calculated. C 63.6, H 4.5, N 5.7
245.1 Found. " 63.7, " 4.6, " 5.7

N-Acetyldehydrotyrosyl-L-tyrosine—A solution of L-tyrosine (36.2 gm.) in 1 liter of 0.2 N NaOH was added with constant stirring to a solution of 49 gm. of the azlactone of diacetyldehydrotyrosine in 600 cc. of acetone. The mixture was stirred for 2 hours and allowed to stand at room temperature overnight. N HCl (250 cc.) was added and the mixture concentrated in vacuo until a yellow gum separated. The gum was dissolved in water and 450 cc. of N NaOH were added. After standing overnight, 500 cc. of N HCl were added and the solution was concentrated in vacuo. Colorless needles separated and were recrystallized from water. Yield, 33 gm.; m.p. 231–232°. [α]D₂₀ = +36.7° (2 per cent in 95 per cent alcohol).

C₂₆H₃₀O₄N₂. Calculated. C 62.5, H 5.3, N 7.3
384 Found. " 62.4, " 5.3, " 7.2

N-Acetyl-D-tyrosyl-L-tyrosine—N-Acetyldehydrotyrosyl-L-tyrosine (15.3 gm.) was dissolved in 200 cc. of 95 per cent alcohol and hydrogenated as described in a previous section of this paper. The solution was filtered, evaporated to dryness in vacuo, and the residue dissolved in boiling water. After standing several hours, the crystals that had formed were filtered off. The filtrate was saved for the isolation of a possible second diastereomer (see below). The precipitate was dissolved in water with the aid of a little ammonium hydroxide, diluted to 300 cc., then adjusted to pH 2 with 10 per cent HCl. After long standing, short triangular prisms sepa-
rated and were purified by repeated crystallization from water. Yield, 3 gm.; m.p. 228°; \( [\alpha]_D^{25} = +31° \) (2 per cent in 95 per cent alcohol).

\[
C_{30}H_{32}O_8N_2. \text{ Calculated. } C 62.2, H 5.7, N 7.3 \\
386 \text{ Found. } " 62.3, " 5.6, " 7.3
\]

To ascertain which diastereomer had been obtained, 0.74 gm. of the compound was refluxed for 5.5 hours in boiling 24 per cent HCl. The solution was evaporated to dryness in vacuo at 45°C. Water was added and the solution again evaporated to dryness. The residue was dissolved in 19 cc. of water, the tyrosine precipitated with sodium acetate solution, filtered, and washed three times with water. It was dried in vacuo over P_2O_5 and solid NaOH. Yield, 0.65 gm. or 93 per cent; m.p. 305°; \( [\alpha]_D^{23} = 0° \). This yield of 93 per cent racemic tyrosine shows the compound to be the D-L diastereomer.

**Acetyl-L-tyrosyl-L-tyrosine**—The filtrate reserved as described in the preceding section was combined with the filtrate from the crystallization of the D-L diastereomer (see the preceding section) and evaporated to dryness in vacuo. The residue was dissolved in water and set aside for crystallization. After standing many days at 0°, a small yield of long pointed lustrous rods was obtained. They were repeatedly recrystallized from water. Yield, 2 gm.; m.p. 241°; \( [\alpha]_D^{23} = +19° \) (1.5 per cent in alcohol).

\[
C_{30}H_{32}O_8N_2. \text{ Calculated. } C 62.2, H 5.7, N 7.3 \\
386 \text{ Found. } " 62.3, " 5.6, " 7.3
\]

As the D-L diastereomer had been identified, this preparation, if pure, could be no other than the L-L diastereomer. Since the compound that had been identified as the D-L diastereomer was entirely resistant to pepsin, and this one was completely hydrolyzed, it is evident that the preparation was pure. The quantity of material at hand did not warrant its use for further proof of identity.

**Acetyl-DL-phenylalanyl-DL-phenylalanine, N-carbobenzoxy-O-acetyl-L-tyrosyl-L-phenylalanine ethyl ester, and carbobenzoxy-L-phenylalanyl-L-phenylalanine amide** were obtained in small amount from the collection of the late Dr. Max Bergmann.

**N-Carbobenzoxy-L-tyrosyl-L-phenylalanine**—As only a few mg. of the starting material N-carbobenzoxy-O-acetyl-L-tyrosyl-L-phenylalanine ethyl ester were available, N-carbobenzoxy-L-tyrosyl-L-phenylalanine was not isolated in the solid state. 10.64 mg. of the ester were dissolved in 0.1 cc. of 0.5 N NaOH and 2 cc. of acetone, and allowed to stand at room temperature overnight to remove the acetyl group from the tyrosyl radical and convert the ester to the acid. The solution was evaporated to dryness at room temperature, and the residue dissolved in 8 cc. of water. Aliquots of this solution were used in the hydrolytic experiments.
Carbobenzoxy-\textit{L}-\textit{glutamyl} \textit{L}-\textit{tyrosine}—This was prepared as previously described (1).

\textbf{Enzymatic Studies}

The crystalline pepsin was prepared from Cudahy pepsin according to the method of Philpot (4) and recrystallized twice. It was preserved under half saturated magnesium sulfate at 2°. Fresh solutions of it were prepared in 1.05 $\text{M}$ acetate buffer of pH 4.6 about every 7 days. Pepsin A was prepared by the fractionation procedure of Herriott, Desreux, and Northrop (5). It was filtered from the saturated magnesium sulfate solution as soon as prepared, dissolved in acetate buffer of pH 4.6, dialyzed overnight against acetate buffer of the same pH, and used immediately. The concentration of pepsin in the solution was determined with the aid of the micro-Kjeldahl procedure by calculating the difference between the total nitrogen and the non-protein nitrogen remaining in solution after precipitation of the protein with boiling 5 per cent trichloroacetic acid.

For the hydrolytic experiments the calculated quantity of substrate was brought into solution in a measured quantity of 0.5 $\text{n}$ NaOH solution. Varying amounts of water, acetate buffer, 0.5 $\text{n}$ HCl, and pepsin solution were added in predetermined quantities to produce the desired pH and pepsin concentration. The acetate which was required for buffering at pH 4.0 was also used at pH 3.0 and 2.0 so as to change the conditions of the experiment as little as possible. All experiments were carried out at 36.7°. Because of the very low solubility of the compounds, the concentration of substrate employed in each experiment was made low enough to avoid any possibility of precipitation during the experiment, and a concentration of pepsin was chosen which would insure a measurably slow rate of hydrolysis. In order to satisfy these conditions, the concentrations of both substrate and enzyme used in this work were much lower than those employed heretofore (1-3). On this account a sensitive method was required to measure the amino groups liberated during the enzymatic action. With only slight modification, the ninhydrin procedure of Moore and Stein (8) was found to meet this requirement. The ninhydrin solution was prepared afresh for each experiment and was never kept for more than a few hours. It was preserved at 0° when not in use. Extra alkali was added to the ninhydrin solution in a quantity sufficient to insure that, when 1 cc. of the reagent solution was added to the sample of the hydrolysate to be analyzed, the resulting mixture had a pH of 5.0. Between 0.1 and 0.5 cc. of either 0.5 or 1.0 $\text{n}$ NaOH was required per 50 cc. of ninhydrin solution. The exact amount depended upon the pH and the volume of the sample to be analyzed. Samples of the hydrolysate were pipetted into 1 cc. of the ninhydrin solution already measured into the colorimeter tubes. Fur-
ther enzymatic action was effectively prevented by the methyl cellosolve in this solution.

None of the substrates employed in this work contained an amino group. Hence all were ninhydrin-negative prior to hydrolysis. One of the products of hydrolysis was also ninhydrin-negative, and the other was a free amino acid. Some additional ninhydrin-positive material was formed, however, by the self-digestion of the pepsin. To correct for this, a pepsin control, without substrate, was run with each experiment at each pH value. The color factor of Moore and Stein for the particular amino acid liberated was then used to calculate the extent of hydrolysis.

The amide, being insoluble even in NaOH, had to be handled in a different manner from that used for the other substrates. It was dissolved in acetone and diluted with an equal volume of water. A sufficient amount of the amide was taken to make the resulting solution 0.001 m. Then 0.2 cc. aliquots were pipetted into a series of colorimeter tubes, and the solution was allowed to evaporate slowly so as to leave a thin layer of the amide spread over the surface of the tubes. 1 cc. of the pepsin solution at the required pH was added to each tube, and the tubes incubated for 6 days, one tube being withdrawn each day for determination of hydrolysis. The ninhydrin solution was added directly to each tube after the pH was adjusted to 5.0 by the addition of a calculated quantity of NaOH solution.

For the identification of tyrosine in the hydrolysate of acetyl-L-phenylalanyl-L-tyrosine, 300 mg. of the substrate were hydrolyzed in 200 cc. of pepsin solution (0.012 mg. of N per cc.) at pH 2. At the completion of hydrolysis the solution was adjusted to pH 5.0 and brought to the boiling point. The coagulated pepsin was removed by filtration, the solution concentrated by boiling (to about 30 cc.), and cooled to 0°. Needles of tyrosine (150 mg.) separated and were twice recrystallized from water. Yield, 63 mg.; m.p. 306°.

\[ \text{C}_{9} \text{H}_{17} \text{O}_{2} \text{N}. \quad \text{Calculated. C 59.7, H 6.1, N 7.7} \]

\[ \text{181} \quad \text{Found. C 59.6, H 6.1, N 7.6} \]

The identification of diiodotyrosine in the hydrolysate of acetyl-L-phenylalanyl-L-diiodotyrosine was carried out in a similar manner, except that the substrate (300 mg.), dissolved in alkali, was added a little at a time to the pepsin solution (followed by enough acid to maintain the pH at 2.0), and the filtrate, after removal of the coagulated pepsin, was concentrated in vacuo at 40°. The residue in this case was recrystallized by bringing it into solution in water with a few drops of 0.5 N NaOH, and precipitating it with a few drops of glacial acetic acid. Yield, 170 mg.; m.p. 204°.

\[ \text{C}_{9} \text{H}_{17} \text{O}_{2} \text{NI}_{2}. \quad \text{Calculated. C 25.0, H 2.1, N 3.2} \]

\[ \text{433} \quad \text{Found. C 25.2, H 2.1, N 3.1} \]
N-Substituted dipeptides containing two residues of the L forms of the aromatic amino acids, phenylalanine, tyrosine, or diiodotyrosine have been found to be hydrolyzed by pepsin. These peptides are hydrolyzed more rapidly than are any of the previously known synthetic substrates for pepsin. They have a pH optimum at 1.8 to 2, the same pH as is optimum for the action of pepsin on proteins. Pepsin A hydrolyzes them more rapidly than does pepsin crystallized from Cudahy pepsin, according to Philpot. Peptides containing the D forms of these amino acids, or tyrosyl peptides in which the phenolic group is blocked, are not hydrolyzed by pepsin. When carbobenzoxy-L-glutamyl-L-tyrosine was hydrolyzed at very low concentration with a small amount of pepsin, it also was found to be hydrolyzed maximally at pH 2. Its hydrolysis under these conditions was, however, exceedingly slow.

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