THE SPECIFICITY OF PEPSIN

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All the hitherto available information regarding the enzymatic action of pepsin has been obtained through the use of proteins as substrates. Much of this information must be regarded as tentative as long as no substrate of simple, well known structure can be provided for the study of pepsin action. It is the purpose of the present communication to report the finding of such substrates for swine pepsin.

The compound carbobenzoxy-l-glutamyl-l-tyrosine (I) is hydrolyzed to carbobenzoxyglutamic acid and tyrosine in the presence of crystalline swine pepsin. The hydrolysis occurs optimally at about pH 4 (Fig. 1). At pH 1.8 to 2, which is generally accepted as the optimum for pepsin, the hydrolysis of substrate (I) occurs rather slowly. Repeated recrystallization of the pepsin preparation did not appreciably alter its activity towards substrate (I). On the other hand, inactivation of pepsin at pH 8.0, followed by readjustment of the activity to pH 4.0, resulted in loss of the hydrolytic activity toward the substrate. The possibility that the splitting of substrate (I) by crystalline pepsin might be due to the presence of another enzyme of the cathepsin type is unlikely, since neither cysteine nor hydrogen peroxide had any effect on the rate of hydrolysis. Furthermore, there was no demonstrable carboxypeptidase activity in the enzyme preparations employed. The results of all these experiments justify the conclusion that the splitting of carbobenzoxyglutamyltyrosine is attributable to the action of pepsin itself (Table I).

The specificity of the pepsin action at pH 4 was investigated by means of other synthetic compounds differing from carbo-
Specificity of Pepsin

benzoxylglutamyltyrosine to a greater or lesser degree. Substitution of the tyrosine portion of the peptide by various amino acids showed that while carbobenzoxy-l-glutamyl-l-phenylalanine

\[
\text{COOH} \\
\text{CH}_{2} \quad \text{C}_{6}\text{H}_{4}\cdot \text{OH} \\
\text{CH}_{2} \quad \text{CH}_{3}
\]

\[
\text{C}_{6}\text{H}_{5}\cdot \text{CH}_{2}\cdot \text{O} \cdot \text{CO} - \text{NH} \cdot \text{CH} \cdot \text{CO} \quad \text{NH} \cdot \text{CH} \cdot \text{COOH}
\]

(I)

was split fairly rapidly by pepsin with an optimum at about pH 4.5 (cf. Fig. 1), carbobenzoxy-l-glutamyl-Z-diiodotyrosine, carbobenzoxy-l-glutamyl-Z-glutamic acid, and carbobenzoxy-l-glutamyl-
Fig. 1. pH dependence of hydrolysis of synthetic substrates by crystalline pepsin. Curve A, carboxbenzoxyl-L-glutamyl-L-tyrosine; Curve B, carboxbenzoxyl-L-glutamyl-L-phenylalanine. Enzyme concentration, 2.4 mg. of pepsin N per cc.

**TABLE I**

*Hydrolysis of Carboxbenzoxylglutamyltyrosine with Crystalline Pepsin*

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>Time</th>
<th>Hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hrs.</td>
<td>per cent</td>
</tr>
<tr>
<td>Twice crystallized pepsin, 1.4 mg. protein N per cc.</td>
<td>24</td>
<td>53</td>
</tr>
<tr>
<td>&quot; &quot; &quot; inactivated at pH 8 and readjusted to pH 4</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>4 times crystallized pepsin, 1.5 mg. protein N per cc.</td>
<td>24</td>
<td>54</td>
</tr>
<tr>
<td>Twice crystallized pepsin, 1.4 mg. protein N per cc.</td>
<td>22.5</td>
<td>51</td>
</tr>
<tr>
<td>&quot; &quot; &quot; &quot; + cysteine (0.005 mM per cc.)</td>
<td>40.5</td>
<td>72</td>
</tr>
<tr>
<td>22.5</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>46.5</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>Twice crystallized pepsin, 0.8 mg. protein N per cc.</td>
<td>24</td>
<td>33</td>
</tr>
<tr>
<td>&quot; &quot; &quot; &quot; + hydrogen peroxide (0.2 mM per cc.)</td>
<td>24</td>
<td>35</td>
</tr>
</tbody>
</table>

* pH 4.0.
Specificity of Pepsin

glycine were resistant to pepsin action (Table II). On the other hand, substitution of the glutamic acid residue indicated that both carbobenzyoxglycyl-1-tyrosine and carbobenzoxy-l-tyrosyl-l-tyrosine were split by pepsin at a rather slow rate. All the peptides which were found in these experiments to be hydrolyzed by swine

**Table II**

*Behavior of Synthetic Substrates toward Crystalline Pepsin*

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Time (hrs)</th>
<th>Hydrolysis (per cent)</th>
<th>Isolation of products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbobenzyox-l-glutamyl-l-tyrosine</td>
<td>6</td>
<td>18</td>
<td>Carbobenzyox-l-glutamic acid</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>81†</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>Carbobenzyox-l-glutamyl-l-phenylalanine</td>
<td>24</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>93</td>
<td></td>
</tr>
<tr>
<td>Carbobenzyox-l-glutamyl-l-diiodotyrosine</td>
<td>24</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Carbobenzyox-l-glutamyl-l-glutamic acid</td>
<td>24</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Carbobenzyox-l-glutamylglycine</td>
<td>24</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Carbobenzyox-l-tyrosyl-l-tyrosine</td>
<td>24</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Carbobenzyoxglycyl-l-tyrosine</td>
<td>24</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>43</td>
<td></td>
</tr>
</tbody>
</table>

* pH 4.0; 1.6 mg. of pepsin N per cc.
† Beginning of tyrosine crystallization. If the reaction is allowed to proceed, about 50 per cent of the liberated tyrosine crystallizes out.

pepsin contain the aromatic amino acid residues tyrosine or phenylalanine. In the most sensitive substrates, tyrosine is combined with glutamic acid. On the basis of these experiments one may infer that the sensitivity of a peptide bond toward pepsin depends upon the nature of both amino acid residues which participate in the peptide bond that is hydrolyzed. However, there
are still other structural details of the substrate molecule which influence its sensitivity towards pepsin.

All the previously mentioned synthetic substrates for pepsin contain a free α-carboxyl and a free γ-carboxyl close to the peptide bond which is hydrolyzed by the enzyme. Substitution of the α-carboxyl of substrate (I) to yield carbobenzyoxyl-l-glutammoyl-l-tyrosineamide (II) strongly depresses the rate of hydrolysis but

Table III

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Time (hrs)</th>
<th>Hydrolysis (%)</th>
<th>Isolation of products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbobenzyoxyl-l-glutamyyl-l-tyrosine</td>
<td>24</td>
<td>53</td>
<td>Carbobenzyoxyl-l-glutamylic acid</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>Carbobenzyoxyl-l-glutamyyl-l-tyrosineamide</td>
<td>43</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>68</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Carbobenzyoxyl-l-glutaminyl-l-tyrosineamide</td>
<td>43</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>68</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Carbobenzyoxyl-l-glutamyyl-l-tyrosylglycine</td>
<td>24</td>
<td>39</td>
<td>Carbobenzyoxyl-l-glutamylic acid</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>68</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>Carbobenzyoxylglycyl-l-glutamyyl-l-tyrosine</td>
<td>24</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>l-Glutamyyl-l-tyrosine</td>
<td>74</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Glycyl-l-glutamyyl-l-tyrosine</td>
<td>24</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>Benzyoxyl-l-tyrosine</td>
<td>48</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Chloracetyl-l-tyrosine</td>
<td>24</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

* pH 4.0; 1.4 mg. of pepsin N per cc.
† Tyrosine crystallization.
‡ An experiment at pH 7.0 also showed no hydrolysis of this substrate by the pepsin preparation.

does not eliminate it entirely. However, if both carboxyls are masked to give carbobenzyoxyl-l-glutaminyl-l-tyrosineamide, the product is entirely resistant to pepsin action (Table III). Furthermore, substitution of only the γ-carboxyl, as in carbobenzyoxyl-l-glutaminyl-l-phenylalanine, results in a slowing down of the hydrolysis, when compared with the hydrolysis of the acid containing two carboxyls (Table IV). One may conclude, therefore, that the presence of free carboxyls is favorable to the action
of pepsin if other structural requisites for the enzymatic action are fulfilled. In order to subject this conclusion to still another test, carbobenzoxy-\textit{l}-glutamyl-\textit{l}-tyrosylglycine (III) was investigated. The latter possesses a free \textit{\alpha}-carboxyl but at a greater distance from the glutamyltyrosine linkage than in (I). However, the rate of peptic hydrolysis of (III) is only slightly diminished when compared with that of (I); it is, however, remarkably increased when compared with that of the amide (II).

These results showing the favorable influence of free carboxyls lead to the expectation that pepsin action is inhibited by the presence, in the substrate, of a free amino group in close proximity to the peptide bond. \textit{l}-Glutamyl-\textit{l}-tyrosine is completely resistant to the action of pepsin. Furthermore, the following compounds containing basic groups were tested for lability toward pepsin, with completely negative results at \textit{pH} 2 and \textit{pH} 4: benzoyl-\textit{l}-lysineamide, benzoylglycyl-\textit{l}-lysineamide, benzoyl-\textit{l}-histidineamide, benzoylglycyl-\textit{l}-histidineamide, benzoyl-\textit{l}-arginineamide. However, the free tripeptide glycyl-\textit{l}-glutamyl-\textit{l}-tyrosine (IV) is split by pepsin with remarkable ease (Table III) between the glutamyl and tyrosine residues.

The specificity of pepsin is sensitive not only to the nature of the amino acids which participate in the peptide bond of the substrate, but also to the sequence of these amino acids. As an

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Time</th>
<th>Hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbobenzoxy-\textit{l}-glutamyl-\textit{l}-phenylalanine</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>46</td>
<td>38</td>
</tr>
<tr>
<td>Carbobenzoxy-\textit{l}-glutamyl-\textit{d}-phenylalanine</td>
<td>24</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>46</td>
<td>3</td>
</tr>
<tr>
<td>Carbobenzoxy-\textit{l}-glutaminyl-\textit{l}-phenylalanine</td>
<td>20</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>44</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>92</td>
<td>50</td>
</tr>
<tr>
<td>Carbobenzoxy-\textit{l}-phenylalanyl-\textit{l}-glutamic acid</td>
<td>20</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>44</td>
<td>20</td>
</tr>
</tbody>
</table>

* \textit{pH} 4.2; 1.4 mg. of pepsin N per cc.
example, carbobenzoxy-L-phenylalanyl-L-glutamic acid is hy-
drolyzed much more slowly than is carbobenzoxy-L-glutamyl-L-
phenylalanine (Table IV).

The optical selectivity of pepsin is demonstrated in the resistance
of carbobenzoxy-L-glutamyl-D-phenylalanine to the hydrolytic
action of the enzyme (Table IV).

A few years ago Calvery and Schock (1) found that pepsin
liberates tyrosine from egg albumin. It was rather difficult to
reconcile this observation with the generally accepted opinion that
all polypeptides were resistant to pepsin action. The hypothesis
was discussed that, during the pepsin action on egg albumin,
there might be formed peptides containing tyrosine in a special,
hitherto unknown, and extremely labile combination (2). Hy-
potheses of this kind now become unnecessary, since it has been
shown that pepsin splits peptide linkages of simple peptides, such
as glycylglutamyltyrosine, with the liberation of tyrosine. The
latter process is comparable to the formation of tyrosine during
the pepsin action on egg albumin. Our experiments have demon-
strated that pepsin is not restricted to the splitting of centrally
located peptide bonds but also hydrolyzes terminal peptide bonds
if they are situated at the carboxyl end of the peptide chain.
The formation of tyrosine during the peptic digestion of a genuine
protein probably proceeds in such a manner that at first the
carboxyl group of tyrosine is liberated and that afterwards the
peptide bond involving the amino group of tyrosine is hydrolyzed.

The enzymes that digest genuine proteins are usually classified
as pepsinases, tryptases, or papainases on the basis of the pH
optimum of their rather complex action on proteins (3). It is
assumed that pepsin has its optimum around pH 1.8,1 while the
optimum of the papainases was found to depend upon the nature
of the substrates and to vary from pH 3 to pH 10. However,
the experiments with synthetic substrates have demonstrated the
pepsin action to depend largely upon the structure of the substrate

1 Dyckerhoff and Tewes (4) observed that the splitting of casein and
gelatin by pepsin is, under certain conditions, more rapid at pH 4 than at
pH 2. However, Northrop (5) has shown that the pH determinations
performed by these authors by means of the indicator method were erro-
neous and that their experiments were actually carried out at about pH
2.35 and not at pH 4.
and to extend deeply into the range which was hitherto regarded as being reserved for the papainases. Consequently, it is not possible any longer to base the differentiation between pepsin and papainases on the assumption that pepsin has a single characteristic pH optimum. Another consequence is the following. If it is desired to investigate whether a given peptide or peptide derivative can be hydrolyzed by pepsin, a series of tests at different pH values must be performed. The splitting of carbobenzoxyglutamylphenylalanine by pepsin would never have been observed if experiments had been performed only at pH 1.8.

The preference shown by pepsin to peptides containing glutamic acid in addition to tyrosine or phenylalanine may partly explain the rapid and extensive hydrolysis by pepsin of proteins such as edestin, cascin, and egg albumin (6) which contain many aminodicarboxylic acid residues as well as tyrosine and phenylalanine. The slow peptic hydrolysis of gelatin (6) corresponds to a very low content of aromatic amino acids. Protamines with a very high content of basic amino acids are either resistant to pepsin or attacked very slowly by the enzyme. This is in accord with the fact that no peptide derivative of well established structure and containing a basic amino acid as constituent is known to be digested by pepsin. Before a final conclusion is reached, however, it seems desirable to study a greater number of peptides which contain the residues of basic amino acids, and especially those peptides that contain the residues of basic amino acids as well as of aromatic amino acids.

Pepsin is not the only proteinase of the gastrointestinal tract that is specifically adapted to the hydrolysis of substrates containing tyrosine or phenylalanine. Chymotrypsin exhibits a similar preference towards the residues of these aromatic amino acids (7). Nevertheless, both enzymes exhibit distinctly different specificities. For example, pepsin hydrolyzes carbobenzoxyglutamyltyrosine between the two amino acid residues, thus demonstrating that it is not inhibited by an α-carboxyl in close proximity to the peptide bond which is attacked. Chymotrypsin does not hydrolyze the previously mentioned substrate; the enzyme does, however, hydrolyze carbobenzoxytyrosylglycineamide between the tyrosine and the glycine residues. Carbo-

1 The behavior of peptides containing aspartic acid has not been investigated as yet.
benzoxytyrosylglycine is not attacked by chymotrypsin. Thus chymotrypsin is, in contrast to swine pepsin, highly sensitive towards the α-carboxyl. However, both enzymes are similar in that they do not require a basic group within their respective substrates. Therefore, the enzymatic hydrolysis of, for example, carboxbenzoxylglutamyltyrosine by pepsin represents the interaction of an acidic enzyme and an acidic substrate.

The availability of synthetic substrates for pepsin having only one sensitive peptide bond permits a more precise study to be made of the kinetics of peptic hydrolysis and the quantitative estimation of pepsin in biological fluids. Furthermore, it permits a comparative study of the relative specificities of pepsins of various animal species to be undertaken.

The authors wish to express their thanks to Mr. S. Nagy who performed the analyses reported in this paper.

**EXPERIMENTAL**

**Carbobenzoxy-L-Glutamyl-L-Tyrosine**—This compound was prepared as described in (8).

The enzymatic hydrolysate by pepsin of 554 mg. of this substance was filtered from a small amount of crystalline material which had separated during the reaction (15 mg. of tyrosine), concentrated to a small volume, and acidified to Congo red. The resulting oil was extracted with ethyl acetate. The ethyl acetate extract was dried and concentrated. Addition of petroleum ether resulted in the crystallization of a material (175 mg.) which after recrystallization from ethyl acetate-petroleum ether melted at 116°. When mixed with an authentic sample of carboxbenzoxyl-\(l\)-glutamic acid, the melting point was 118°. The aqueous layer from the ethyl acetate extraction was adjusted to pH 4 and cooled strongly. Tyrosine crystals separated out (32 mg.). Calculated for tyrosine, 7.7 per cent NH₃-N; found, 7.9 per cent NH₃-N.

**Carbobenzoxy-L-Glutamyl-L-Phenylalanine**

**Carbobenzoxy-L-Glutamyl-L-Phenylalanine Ethyl Ester**—To an ethyl acetate solution of phenylalanine ethyl ester (prepared

*The glutamic acid residue in this compound and in the subsequent glutamyl peptides is linked to the next amino acid through the α-carboxyl group.*
Specificity of Pepsin

from 5 gm. of l-phenylalanine) 3.5 gm. of carbobenzyox glutamic acid anhydride were added to a faintly alkaline reaction. The reaction mixture was allowed to stand for 3 hours, washed with dilute hydrochloric acid and water, and concentrated down. The resulting material was recrystallized from hot ethyl acetate. Yield, 4.2 gm. M.p., 144°.

\[ C_{4}H_{4}O_{2}N_{2} \] Calculated: C 63.1, H 6.1, N 6.1
456.5 Found: " 62.8, " 5.9, " 6.1

Carbobenzyox-l- Glutamyl-l-Phenylalanine—1.1 gm. of the above ester were shaken with 5 cc. of n NaOH for 15 minutes. On acidification to Congo red there separated a syrup which quickly crystallized. After recrystallization from ethyl alcohol-water the material melted at 162°. Yield, 0.8 gm.

\[ C_{3}H_{2}O_{2}N_{2} \] Calculated: C 61.7, H 5.6, N 6.5
428.4 Found: " 61.6, " 5.7, " 6.6
\[ \alpha \] = +12.2° (2.4% in n NaOH)

Carbobenzyox-l- Glutamyl-1-Phenylalanine

Carbobenzyox-l- Glutamyl-1-Phenylalanine Ethyl Ester—This compound was prepared in the same manner as was the l form. M.p., 131°.

\[ C_{4}H_{4}O_{2}N_{2} \] Calculated: C 63.1, H 6.1, N 6.1
456.5 Found: " 62.8, " 6.1, " 6.3

Carbobenzyox-l- Glutamyl-d-Phenylalanine—This compound was prepared in the same manner as was the l form. M.p., 122°.

\[ C_{3}H_{2}O_{2}N_{2} \] Calculated: C 61.7, H 5.6, N 6.5
428.4 Found: " 61.4, " 5.7, " 6.4
\[ \alpha \] = -21.5° (2.4% in n NaOH)

Carbobenzyox-l- Glutamyl-l-Diiodotyrosine—1.7 gm. of carboxybengxy glutamy tyrosine were dissolved in 25 cc. of concentrated ammonium hydroxide and 8 cc. of n I₂-KI solution were added dropwise with shaking. On acidification to Congo red there resulted a gelatinous precipitate which was filtered off, washed with water, and dried. The pure diiodo compound (1.4 gm.) was obtained by crystallization from hot alcohol. M.p., 188°.

\[ C_{12}H_{10}O_{2}N_{2}I_{2} \] Calculated: C 37.9, H 3.2, N 4.0, I 30.5
696.3 Found: " 38.0, " 3.3, " 3.8, " 36.3
The same substance was also obtained by coupling carbo-
benzoxyglutamic acid anhydride with diiodotyrosine methyl
ester and saponifying the resulting ester.

Carbobenzoxy-l-Glutamyl-l-Tyrosineamide—1 gm. of the cor-
responding ester (1) was dissolved in 10 cc. of methyl alcohol satu-
rated with ammonia at 0°. After 2 days at room temperature,
the solution was evaporated down and the residue dissolved in
dilute bicarbonate solution. The substance obtained on acidifying
with dilute hydrochloric acid was recrystallized from dioxane-

\[ \text{C}_{11} \text{H}_{16} \text{O}_7 \text{N}_4 \]
Calculated. C 59.6, H 5.7, N 9.5
443.4 Found. " 59.4, " 5.8, " 9.4

Carbobenzoxy-l-Glutaminyl-l-Tyrosineamide—2.1 gm. of carbo-
benzoxyglutamyltyrosine ethyl ester were esterified in the usual
manner with methyl alcohol saturated with hydrogen chloride.
The syrupy ester obtained on evaporation was treated with a
methyl alcohol solution of dry ammonia. The diamide crystal-
lized out on allowing the reaction mixture to stand at room tem-
perature. Yield, 1.8 gm. M.p., about 240°.

\[ \text{C}_{21} \text{H}_{26} \text{O}_7 \text{N}_4 \]
Calculated. C 59.7, H 5.9, N 12.7
442.5 Found. " 59.4, " 6.1, " 12.6

Carbobenzoxy-1-Glutamyl-L-Tyrosylglycine

Carbobenzoxy-1-Glutamyl-L-Tyrosine Hydrazide—4.7 gm. of the
corresponding ethyl ester (1) were treated with 1.5 cc. of hydrazine
hydrate in 5 cc. of absolute alcohol. Slight warming gave a clear
solution and on standing overnight the hydrazide separated out.
Yield, 4.5 gm. On recrystallization from absolute alcohol the
substance melted at 194°.

\[ \text{C}_{21} \text{H}_{26} \text{O}_7 \text{N}_4 \]
Calculated. C 57.6, H 5.7, N 12.2
458.5 Found. " 57.4, " 5.6, " 11.9

Carbobenzoxy-l-Glutamyl-L-Tyrosylglycine Ethyl Ester—2.3 gm.
of the above hydrazide were suspended in 25 cc. of ice-cold water,
dissolved by the addition of 6 cc. of concentrated hydrochloric
acid, and converted to the azide by the addition of 0.5 gm. of
sodium nitrite. The azide was extracted with ethyl acetate
and the extract was washed repeatedly with ice-cold water. The
Specificity of Pepsin

dry azide solution was then added to a dry ether solution of glycine ethyl ester (from 5 gm. of the hydrochloride). After 24 hours the reaction mixture was washed with dilute hydrochloric acid and water. On evaporation of the ether-ethyl acetate layer, 1.7 gm. of the expected product were obtained. After recrystallization from dioxane-ether the melting point was 193–194°.

\[ C_{16}H_{25}O_{3}N_{4} \text{ Calculated. C 58.9, H 5.9, N 7.9} \]
\[ 529.5 \text{ Found. } " 58.6, " 5.9, " 8.0 \]

Carbobenzoxy-l-Glutamyl-l-Tyrosylglycine—2.5 gm. of the above ester were suspended in absolute alcohol and treated with 14 cc. of \( N \) \( \text{NaOH} \). The resulting solution was left at 20° for 1 hour, acidified to Congo red with \( N \) hydrochloric acid, and concentrated down. The syrup which separated crystallized on standing. Yield, 2.0 gm. The material was air-dried for analysis. A sample dried in \( \text{vacuo} \) at 100° for 6 hours over \( \text{P}_{2}\text{O}_{5} \) melted at 182°.

\[ C_{24}H_{33}O_{5}N_{4} \cdot H_{2}O \text{ Calculated. C 55.5, H 5.6, N 8.1, H}_{2}O 3.5 \]
\[ 519.5 \text{ Found. } " 55.7, " 5.4, " 8.2, " 3.4 \]

The enzymatic hydrolysate by papain of 250 mg. of this substance was concentrated to a small volume and acidified to Congo red. The resulting oil was extracted with ethyl acetate. The ethyl acetate layer was washed with water and then extracted with dilute bicarbonate solution. The bicarbonate extract was acidified to give a syrup which crystallized on standing in the ice chest. The dry material (86 mg.) melted at 118–120° and gave a mixed melting point of 119° with an authentic sample of carbobenzoxy-l-glutamic acid.

Carbobenzoxy-l-glutamic acid

C_{12}H_{16}O_{4}N. \text{ Calculated. C 55.5, H 5.4, N 5.0} \]
\[ 281.2 \text{ Found. } " 55.3, " 5.3, " 5.0 \]

Carbobenzoxyglycyl-l-Glutamyl-l-Tyrosine

l-Glutamyl-l-Tyrosine Ethyl Ester—4.7 gm. of carbobenzoxy-l-glutamyl-l-tyrosine ethyl ester were hydrogenated in alcohol in the usual manner. Yield, 3.2 gm. The substance was recrystallized from hot alcohol. M.p., 144°.

\[ C_{14}H_{28}O_{4}N_{3} \text{ Calculated. C 56.7, H 6.6, N 8.3} \]
\[ 338.4 \text{ Found. } " 56.4, " 6.4, " 8.2 \]
Carbobenzyglycyl-1-Glutamyl-1-Tyrosine Diethyl Ester—3.1 gm. of glutamyltyrosine ester were esterified twice in the usual manner with ethanol-HCl. The syrupy diethyl ester hydrochloride was converted to the free ester with potassium carbonate. To the ethyl acetate solution of the free ester, 2.3 gm. of carbbenzoxyglycyl chloride were added in two portions with shaking. The reaction mixture was then shaken with a dilute bicarbonate solution. The ethyl acetate layer was then washed successively with water, dilute hydrochloric acid, and water. Crystallization occurred when the dried ethyl acetate solution was concentrated down. Yield, 4.3 gm. The substance was recrystallized from ethyl acetate. M.p., 169°.

\[
\text{C}_{13}\text{H}_{13}\text{O}_{2}\text{N}_{4}. \quad \text{Calculated. C 60.3, H 6.3, N 7.5}
\]

\[
\text{557.6} \quad \text{Found. “ 60.3, “ 6.4, “ 7.6}
\]

Carbobenzyglycyl-1-Glutamyl-1-Tyrosine—2.8 gm. of the diethyl ester were suspended in 25 cc. of ethanol and with cooling there were slowly added 15 cc. of \(\text{N} \text{NaOH}\). After 1 hour, 16 cc. of \(\text{N} \text{HCl}\) were added (Congo red acidity). The material which crystallized out on standing was filtered off and air-dried. Yield, 2.1 gm. A sample dried in vacuo at 100° for 5 hours over \(\text{P}_{2}\text{O}_{5}\) melted at 173°.

\[
\text{C}_{17}\text{H}_{17}\text{O}_{5}\text{N}_{4}. \quad \text{H}_{2}\text{O}. \quad \text{Calculated. C 55.5, H 5.6, N 8.1, \text{H}_{2}\text{O} 3.5}
\]

\[
\text{519.5} \quad \text{Found. “ 55.3, “ 5.6, “ 8.0, “ 3.7}
\]

During the enzymatic hydrolysis of 130 mg. of this substance by pepsin a crystalline precipitate separated out. It was filtered off and dried (20 mg.). Calculated for tyrosine, 7.7 per cent of \(\text{NH}_{\text{2}}\text{N}\); found, 7.8 per cent of \(\text{NH}_{\text{2}}\text{N}\).

Glycyl-1-Glutamyl-1-Tyrosine—1 gm. of the carbbenzoxy compound was hydrogenated in the usual manner. The tripeptide crystallized out on evaporation of the filtrate. Yield, 0.6 gm.

\[
\text{C}_{18}\text{H}_{18}\text{O}_{2}\text{N}_{4}.2\text{H}_{2}\text{O}. \quad \text{Calculated. C 46.6, H 6.4, N 10.2, \text{H}_{2}\text{O} 10.9}
\]

\[
\text{412.3} \quad \text{Found. “ 46.8, “ 6.4, “ 10.2, “ 10.9}
\]

Carbobenzyglycyl-1-Glutaminyl-1-Phenylalanine

Carbobenzoxy-L-Glutaminyl-1-Phenylalanine Ethyl Ester—2.15 gm. of carbobenzoxyglutamylphenylalanine ethyl ester were suspended in 35 cc. of dry chloroform. 1.1 gm. of \(\text{PCl}_{5}\) were added, followed
Specificity of Pepsin

by shaking for 5 minutes at 0° and subsequent removal of the HCl by evacuation for 10 minutes. The solution was then added with cooling to an ethereal solution of ammonia. After the solution had stood at room temperature for 1 hour, the precipitate was filtered off, washed thoroughly with cold water, and dried. Yield, 1.5 gm. M.p., 138°.

\[ \text{C}_{10}H_{10}O_{4}N_{2} \]
Calculated. C 63.3, H 6.4, N 9.2
Found. " 63.4, " 6.4, " 9.1

Carbobenzyoxy-l-Glutaminyl-l-Phenylalanine — 1.15 gm. of the above ester were dissolved in 60 cc. of alcohol and 2.5 cc. of \( \text{N} \) \( \text{NaOH} \) were added. The solution was acidified to Congo red after standing 1 hour at room temperature. The substance crystallized out upon concentration under reduced pressure. Yield, 0.9 gm. M.p., 180°.

\[ \text{C}_{10}H_{10}O_{4}N_{2} \]
Calculated. C 61.8, H 5.9, N 9.8
Found. " 61.9, " 6.1, " 9.7

Carbobenzyoxy-l-Phenylalanyl-l-Glutamic Acid

Carbobenzyoxy-l-Phenylalanyl-l-Glutamic Acid Diethyl Ester — 3.5 gm. of carbobenzyoxy-l-phenylalanyl chloride were added to a dry ether solution of glutamic acid diethyl ester (prepared from 6 gm. of the hydrochloride). The mixture was allowed to stand at room temperature for 1 hour and washed with dilute hydrochloric acid, water, and bicarbonate solution. The dried ethereal solution was concentrated, yielding a crystalline product. Yield, 3.7 gm. M.p., 115°.

\[ \text{C}_{19}H_{19}O_{7}N_{2} \]
Calculated. C 64.4, H 6.7, N 5.8
Found. " 64.5, " 6.7, " 5.8

Carbobenzyoxy-l-Phenylalanyl-l-Glutamic Acid — 2.4 gm. of the above ester were shaken with a mixture of 10 cc. of \( \text{N} \) \( \text{NaOH} \) and 10 cc. of alcohol. After 1 hour the solution was acidified to Congo red with \( \text{N} \) hydrochloric acid and concentrated under reduced pressure. The resulting crystals were filtered off and washed with cold water. Yield, 1.5 gm. M.p., 180°.

\[ \text{C}_{19}H_{19}O_{7}N_{2} \]
Calculated. C 61.7, H 5.6, N 6.5
Found. " 61.5, " 5.7, " 6.5
Carbobenzoxy-L-Glutamyl-L-Glutamic Acid—This compound was prepared as described in (8).

Carbobenzoxy-L-Tyrosyl-L-Tyrosine—This compound was prepared as described in (8).

L-Glutamyl-L-Tyrosine—This compound was prepared as described in (8).

Carbobenzoxy-L-Glutamylglycine—This compound was prepared as described in (9).

Carbobenzoxyglycyl-L-Tyrosine—This compound was prepared as described in (7).

Enzymatic Studies

The crystalline pepsin was prepared according to the directions of Philpot (10). Unless otherwise stated, twice crystallized preparations were employed. Solutions of the enzyme were made up in acetate buffer of pH 4.0.

The concentration of the synthetic substrates was 0.05 mM per cc. in all cases. The solutions were buffered by 0.1 M acetate buffers. The temperature in all cases was 40°. The extent of hydrolysis was followed by the determination of the amino nitrogen liberated in the Van Slyke microvolumetric apparatus. Enzyme blanks and controls to test the lability of the substrates in the absence of pepsin were performed throughout these investigations.

BIBLIOGRAPHY

THE SPECIFICITY OF PEPSIN
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