SPECIFICITY OF CATHEPSIN C*

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(Received for publication, May 31, 1955)

Cathepsin C, one of the proteinases found in animal tissues (1), has been extensively purified from beef spleen (2). Earlier studies showed that at pH 5 crude preparations of this enzyme catalyze the hydrolysis of dipeptide amides at the CO—NH₂ bond (3), and of the ester linkage of dipeptide esters (4). With suitable substrates, the predominant reaction near pH 5 is hydrolysis, whereas near pH 7.5 the enzyme catalyzes replacement reactions leading to the polymerization of the dipeptide units, and yielding long chain peptides (3, 5).

In the present investigation, an attempt was made to define more precisely the effect, on the rate of enzymic action, of changes in the structure of substrates of cathepsin C. It had been demonstrated previously that the enzyme is specific in its action on dipeptide esters (or amides) containing two α-amino acid residues; thus glycyl-L-phenylalanine ethyl ester is readily hydrolyzed at pH 5, whereas β-alanyl-L-phenylalanine ethyl ester is resistant under comparable experimental conditions (4). Also, it had been shown that several amino acid esters were not attacked, and that acylation of the free α-amino group of a sensitive dipeptide amide blocks enzyme action (4). Hence the “backbone” specificity (6) of cathepsin C was considered to be as given in the accompanying formula, where X is either NH₂ or an alkoxy group.

\[
\begin{array}{c}
\text{R} \\
\text{NH₂CHCO} \\
\text{NHCHCO—X} \\
\text{R'}
\end{array}
\]

Cathepsin C does not appear to exhibit absolute “side chain” specificity in regard to the nature of the R and R' groups, although the best substrates found previously are those in which the R group is hydrogen or a small aliphatic side chain (methyl, hydroxymethyl), and the R' group is the benzyl or p-hydroxybenzyl side chain of L-phenylalanine or of L-tyrosine, respectively. (The corresponding derivatives of D-phenylalanine are resistant (5).) Thus, glycyl-L-phenylalaninamide or glycyl-L-tyrosina-

* This study was aided by grants from the American Cancer Society (on recommendation of the Committee on Growth of the National Research Council) and from the Rockefeller Foundation.

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mide or the corresponding ethyl esters are suitable substrates. It should be added, however, that glycylglycine ethyl ester and glycyl-L-leucine ethyl ester\(^1\) are also readily hydrolyzed by a highly purified preparation of cathepsin C.

To investigate further the structural requirements in substrates of cathepsin C, a series of dipeptide amides and esters was prepared in which the \(R'\) group was the side chain of L-tyrosine or L-phenylalanine, and other structural features of the compounds were varied.

Attention may be given first to the action of cathepsin C at pH 5, since here it is possible to study the rate of hydrolysis under conditions in which the extent of transamidation is negligible and the substrates are stable in the absence of added enzyme. The data are summarized in Table I. It will be noted that, in general, the comparable amides and esters are hydrolyzed at similar rates, although in some instances the esters are cleaved more rapidly. Thus, glycyl-L-tyrosinamide and glycyl-L-tyrosine ethyl ester are hydrolyzed at approximately the same rate at pH 5, whereas sarcosyl-L-tyrosinamide is split more slowly than is sarcosyl-L-tyrosine ethyl ester. It was found previously (4) that crude preparations of cathepsin C act more rapidly on glycylglycine ethyl ester than on glycylglycinamide.

A further point of general interest is that the action of cathepsin C is similar on pairs of substrates that differ only in having an L-phenylalanine or an L-tyrosine residue at the sensitive linkage. As may be seen from the data in Table I, methylation of the phenolic hydroxyl group of the L-tyrosine residue also does not alter the rate of enzymic action.

The replacement of the glycyl residue of glycyl-L-tyrosinamide, glycyl-L-phenylalaninamide, glycyl-L-tyrosine ethyl ester, or glycyl-L-phenylalanine ethyl ester by a sarcosyl residue in all cases leads to a marked diminution of the rate of enzymic hydrolysis at pH 5. It may be concluded, therefore, that the enzymic action is inhibited by the introduction of a methyl group at the terminal \(\alpha\)-amino group of substrates such as glycyl-L-tyrosinamide. However, the slower hydrolysis of the sarcosyl compounds cannot be interpreted to indicate that a primary \(\alpha\)-amino group is essential for the action of cathepsin C, since the replacement of the glycyl residue of glycyl-L-tyrosinamide or glycyl-L-tyrosine ethyl ester by an L-prolyl residue gives substrates that are hydrolyzed at essentially the same rate as the glycyl compounds, although the prolylphenylalanine derivatives are split somewhat more slowly than is glycyl-L-phenylalaninamide or glycyl-L-phenylalanine ethyl ester. Since the \(N\)-dimethylglycyl compounds were not available, it cannot be stated at present whether the enzyme action is limited to substrates that have an \(N\)-terminal amino or

\(^1\) M. J. Mycek and J. S. Fruton, unpublished observations.
imino acid. As noted above, acylation of the terminal α-amino group blocks the action of the enzyme. In this connection, it may be added that acetyl-L-phenylalanine ethyl ester is resistant to the hydrolytic action of cathepsin C at pH 5 (Table I).

TABLE I

Action of Cathepsin C on Peptide Amides and Peptide Esters near pH 5

The first column denotes the N-terminal amino acid and other substituents on the compounds indicated at the top of the next four columns. The experimental conditions were as given in the experimental section.

<table>
<thead>
<tr>
<th>Substituent</th>
<th>Hydrolysis in 2 hrs.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1-Tyrosinamide</td>
</tr>
<tr>
<td>Glycyl-</td>
<td>19</td>
</tr>
<tr>
<td>Glycyl-p-methoxy-</td>
<td>21</td>
</tr>
<tr>
<td>Sarcosyl-</td>
<td>3</td>
</tr>
<tr>
<td>L-Prolyl-</td>
<td>16</td>
</tr>
<tr>
<td>Glycyl-N-methyl-</td>
<td>0†</td>
</tr>
<tr>
<td>L-Alanyl-</td>
<td>12</td>
</tr>
<tr>
<td>D-Alanyl-</td>
<td></td>
</tr>
<tr>
<td>α-Aminoisobutyryl-</td>
<td>6</td>
</tr>
<tr>
<td>L-Seryl-</td>
<td>12</td>
</tr>
<tr>
<td>L-Leucyl-</td>
<td></td>
</tr>
<tr>
<td>D-Leucyl-</td>
<td></td>
</tr>
<tr>
<td>L-Lysyl-</td>
<td>0</td>
</tr>
<tr>
<td>e-Benzoyl-L-lysyl-</td>
<td>2</td>
</tr>
<tr>
<td>Glycylglycyl-</td>
<td>0</td>
</tr>
</tbody>
</table>

* L-Prolyl-L-phenylalanine crystallized.
† This value was determined by means of the Grassmann-Heyde titration method.
‡ Tested with 1.4 units of cathepsin C, and the measured rate divided by 4 to facilitate comparison.
§ Tested in the presence of 30 per cent methanol; under these conditions, glycyl-L-phenylalanine ethyl ester was hydrolyzed at a rate of 6 μmoles per hour.

It was of interest to find that glycyl-N-methyl-L-phenylalaninamide is resistant to the action of cathepsin C, demonstrating that the introduction of a methyl group at the peptide bond between the 2 α-amino acid residues blocks the hydrolysis of the CO—NH₂ linkage. Thus, although the enzyme does not catalyze the hydrolysis of the bond joining the 2 amino acid residues, substitution of the N atom of this bond decisively interferes with the action of cathepsin C.
In studying the effect of changes in the "side chain" of the N-terminal amino acid on the rate of enzymic action, the glycyl residues of the standard substrates were replaced by the following: L-alanyl, D-alanyl, \( \alpha \)-aminoisobutyryl, L-seryl, L-leucyl, D-leucyl, L-lysyl, \( \epsilon \)-acetyl-L-lysyl, \( \epsilon \)-benzoyl-L-lysyl. All the resulting compounds were hydrolyzed more slowly than the corresponding glycyl compounds or were resistant to hydrolysis (Table I). Although replacement of the glycyl residue of glycyl-L-tyrosinamide or glycyl-L-phenylalaninamide by an L-alanyl or L-seryl residue diminishes only slightly the rate of enzyme action, the introduction of the side chain of a larger L-amino acid residue, as in L-leucyl-L-phenylalanine ethyl ester, causes a marked decrease in the rate. With a bulkier neutral side chain such as \( \epsilon \)-acetylamino-n-butyl or \( \epsilon \)-benzoylamino-n-butyl on the N-terminal residue, the rate of hydrolysis at pH 5 is decreased even further. It is of interest that, although the \( \epsilon \)-acetyllysyl derivatives are hydrolyzed slowly under the conditions of these experiments, the corresponding lysyl compounds are resistant to enzyme action. Apparently, the presence of the free \( \epsilon \)-amino group in the substrate makes the amide or ester bond less sensitive to attack by cathepsin C. It should be added, however, that, under the usual experimental conditions, substances such as L-lysyl-L-tyrosinamide or L-lysyl-L-phenylalaninamide are not inhibitors of cathepsin C when glycyl-L-tyrosinamide or glycyl-L-phenylalaninamide are substrates (Table II). Also, L-lysyl-L-tyrosine ethyl ester is not hydrolyzed to an appreciable extent by cathepsin C at the linkage between the lysyl and tyrosyl residues, to form free lysine; this was demonstrated by paper chromatography with n-butanol-acetic acid-water (8:2:1) as the solvent.

Experiments were also performed with many of the above derivatives of glycyl-L-tyrosinamide, glycyl-L-phenylalaninamide, glycyl-L-tyrosine ethyl ester, and glycyl-L-phenylalanine ethyl ester to determine their susceptibility to the action of cathepsin C at pH values near 7.5. At this pH, at least three possible reactions may occur: enzyme-catalyzed hydrolysis of the sensitive linkage, enzyme-catalyzed replacement reactions, and non-enzymic cyclization to form diketopiperazines. As judged by the rate of ammonia liberation from dipeptide amides or the disappearance of dipeptide esters, the amides are more stable in the absence of added enzyme at pH 7.5 than are the corresponding esters (Table III). It will be noted from Table III that, if allowance is made for the non-enzymic reaction of the compounds tested at pH 7.5, the relative effects of cathepsin C at this pH are roughly comparable to those observed at pH 5. This is concordant with the view (3) that the catalysis of transamidation at pH 7.5 is effected by the same enzyme that catalyzes hydrolysis at pH 5. Among the compounds listed in Table III, and not previously tested with cathepsin C at pH 7.5, the only ones which appeared to give an insoluble polymer
analogous to that obtained from glycyl-L-phenylalaninamide or glycyl-L-tyrosinamide (3, 5) were glycyl-O-methyl-L-tyrosinamide and ε-acetylyl-L-lysyl-L-phenylalaninamide; with the last named compound, the transamidation reaction was slow and the extent of precipitation of presumed polymer was very slight.

It will be noted from Table II that, when glycyl-L-phenylalaninamide or glycyl-L-tyrosinamide was subjected to the action of cathepsin C at pH 7.6

**Table II**

*Effect of Lysyl Peptides on Action of Cathepsin C*

All reaction flasks contained either 0.05 M glycyl-L-tyrosinamide or 0.05 M glycyl-L-phenylalaninamide. L-Lysyl-L-phenylalaninamide or L-lysyl-L-tyrosinamide was added at a concentration of 0.05 M. In the experiments with glycyl-L-tyrosinamide, 0.2 cathepsin unit was present per 2 ml.; with glycyl-L-phenylalaninamide, 0.16 unit was used. The other experimental conditions were as given in the experimental section.

<table>
<thead>
<tr>
<th>Substances present</th>
<th>Ammonia liberation per ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 5.1</td>
</tr>
<tr>
<td></td>
<td>2 hrs.</td>
</tr>
<tr>
<td>Glycyl-L-tyrosinamide</td>
<td>12</td>
</tr>
<tr>
<td>Glycyl-L-tyrosinamide + L-lysyl-L-phenylalaninamide</td>
<td>12</td>
</tr>
<tr>
<td>Glycyl-L-tyrosinamide + L-lysyl-L-tyrosinamide</td>
<td>12</td>
</tr>
<tr>
<td>Glycyl-L-phenylalaninamide</td>
<td>10</td>
</tr>
<tr>
<td>Glycyl-L-phenylalaninamide + L-lysyl-L-tyrosinamide</td>
<td>11</td>
</tr>
<tr>
<td>Glycyl-L-phenylalaninamide + L-lysyl-L-tyrosinamide</td>
<td>9</td>
</tr>
</tbody>
</table>

* A gelatinous precipitate was present.

The rate of ammonia liberation in the presence of L-lysyl-L-phenylalaninamide or L-lysyl-L-tyrosinamide, the rate of ammonia liberation was considerably greater than that observed in the control experiments. Also, the appearance of the polymeric peptides formed from glycyl-L-phenylalaninamide and glycyl-L-tyrosinamide was delayed in the presence of the lysyl compounds. These observations are analogous to those made previously (3) upon the addition of substances such as L-argininamide, and indicate that, although the lysyl compounds are not substrates of cathepsin C, they can serve as replacement agents in transamidation reactions catalyzed by the enzyme. Since the pK' values of the α- and ε-amino groups of these compounds are approximately 8 and
TABLE III

Action of Cathepsin C on Peptide Amides and Peptide Esters near pH 7.5

The data are given in micromoles of ammonia liberated or ester disappeared per 2 ml. of reaction mixture. The numbers in parentheses denote the extent of reaction in the absence of added enzyme. Where no figures are given for control experiments, the compound was stable in the absence of enzyme. Except where otherwise indicated, 0.35 cathepsin unit was used per 2 ml. of reaction mixture. The other experimental conditions were as given in the experimental section.

<table>
<thead>
<tr>
<th>Compound tested</th>
<th>Extent of reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 hr.</td>
</tr>
<tr>
<td></td>
<td>μmoles</td>
</tr>
<tr>
<td>Glycyl-L-tyrosinamide</td>
<td>32*</td>
</tr>
<tr>
<td>Glycyl-L-tyrosine ethyl ester</td>
<td>51 (26)</td>
</tr>
<tr>
<td>Sarcosyl-L-tyrosinamide</td>
<td>8</td>
</tr>
<tr>
<td>Sarcosyl-L-phenylalanine amide</td>
<td>8</td>
</tr>
<tr>
<td>Sarcosyl-L-tyrosine ethyl ester</td>
<td>22 (17)</td>
</tr>
<tr>
<td>Sarcosyl-L-phenylalanine ethyl ester</td>
<td>19 (13)</td>
</tr>
<tr>
<td>L-Prolyl-L-tyrosinamide</td>
<td>31†</td>
</tr>
<tr>
<td>L-Prolyl-L-phenylalaninamide</td>
<td>21‡</td>
</tr>
<tr>
<td>L-Prolyl-L-tyrosine ethyl ester</td>
<td>19 (5)</td>
</tr>
<tr>
<td>L-Prolyl-L-phenylalanine ethyl ester</td>
<td>10 (4)</td>
</tr>
<tr>
<td>α-Aminoisobutyryl-L-tyrosine ethyl ester</td>
<td>12 (7)</td>
</tr>
<tr>
<td>α-Aminoisobutyryl-L-phenylalanine ethyl ester</td>
<td></td>
</tr>
<tr>
<td>L-Lysyl-L-tyrosinamide</td>
<td></td>
</tr>
<tr>
<td>L-Lysyl-L-phenylalaninamide</td>
<td></td>
</tr>
<tr>
<td>L-Lysyl-L-tyrosine ethyl ester</td>
<td>8 (9)</td>
</tr>
<tr>
<td>L-Lysyl-L-phenylalanine ethyl ester</td>
<td>8 (9)</td>
</tr>
<tr>
<td>e-Acetyl-L-lysyl-L-phenylalaninamide</td>
<td>15 (13)</td>
</tr>
<tr>
<td>e-Acetyl-L-lysyl-L-phenylalanine ethyl ester</td>
<td></td>
</tr>
<tr>
<td>L-Lysyl-L-tyrosine ethyl ester</td>
<td>10 (6)</td>
</tr>
<tr>
<td>e-Acetyl-L-lysyl-L-phenylalanine ethyl ester</td>
<td></td>
</tr>
<tr>
<td>Glycylglycyl-L-phenylalanine ethyl ester</td>
<td>3</td>
</tr>
</tbody>
</table>

* A gelatinous precipitate was present.
† This reaction was followed by the Conway and Grassmann-Heyde methods, and the ammonia liberation was equivalent to the increase in titratable acidity.
‡ L-Prolyl-L-phenylalanine crystallized from the reaction mixture.
§ Diketopiperazine crystallized from the reaction mixture.

10.5, respectively, it may be expected that such transamination reactions largely involve the participation of the α-amino group of the lysyl residue.

Of special interest was the finding that the dipeptide derivatives con-
taining an N-terminal proline, although rapidly attacked by cathepsin C at pH 7.5, did not undergo any measurable transamidation. In the case of the derivatives of L-prolyl-L-phenylalanine, a crystalline product appeared during the enzyme reaction, and was identified as the sparingly soluble free dipeptide (7). When the action of cathepsin C on L-prolyl-L-tyrosinamide was followed at pH 7.5 both by measurement of ammonia liberation and by titration with alcoholic KOH, the ammonia liberation was stoichiometrically equivalent to the increase in titratable acidity. As shown previously (3), when transamidation occurs with substrates such as glycy1-L-phenylalaninamide, the extent of ammonia liberation is greater than the increase in titratable acidity. It may be concluded, therefore, that the action of cathepsin C on the prolyl dipeptide derivatives tested is restricted to hydrolysis, and that these prolyl compounds do not serve as replacement agents to a measurable extent. In this connection, it may be added that other studies in this laboratory have shown that, in the action of papain on carbobenzoxyglycinamide at pH 7.5, dipeptides such as L-leucylglycine are very effective replacement agents, whereas L-prolylglycine is completely ineffective in this regard. The failure of prolyl compounds to serve as replacement agents may be due, in part, to the fact that the pK' of the immonium group in such compounds is near 9 (8), although steric factors may also be involved.

The data in Table III also are of interest in relation to the relative stability of dipeptide derivatives at pH 7.5, in the absence of added enzyme. The fact that the prolyl derivatives are more stable than the comparable glycy1 or sarcosyl compounds is noteworthy in view of the known tendency of compounds such as glycy1-L-proline, L-prolyl-L-proline, and glycy1-L-prolinamide to undergo cyclization with great ease (9). It would appear that the formation of diketopiperazines is favored by the presence of a proline residue in an amino acylprolyl compound rather than at the N-terminal position.

A number of compounds were not tested at pH 7.5, because they were sparingly soluble in water at this pH. They were ε-acetyl-L-lysyl-L-tyrosinamide, all the ε-benzoyl derivatives of the lysine compounds, L-leucyl-L-phenylalanine ethyl ester, and D-leucyl-L-phenylalanine ethyl ester.

**DISCUSSION**

The studies described in the present communication, together with earlier work on cathepsin C, justify the conclusion that this enzyme is adapted to the hydrolysis of amide or ester bonds of dipeptide amides or dipeptide esters containing two α-amino acid residues. Sufficient data are not available about the action of cathepsin C on CO—NH bonds joining two α-amino acids to permit the conclusion that this enzyme can cleave dipep-
tide units from the amino end of a long peptide chain; studies on the action of cathepsin C on proteins and long chain synthetic peptides are in progress. If the specificity observed with synthetic substrates applies to the action of the enzyme on proteins, cathepsin C may prove to be a useful addition to the proteolytic enzymes used as reagents for the selective cleavage of proteins. From the studies reported in this paper, it may be expected that cathepsin C would be more effective in attacking peptide chains in which the N-terminal amino acid is proline or one that bears a small aliphatic side chain (as in glycine, alanine, serine).

One may venture to interpret the effect, on the rate of enzyme action, of structural changes in substrates of cathepsin C in terms of a hypothesis concerning the stereochemistry of the enzyme-substrate interaction. It seems likely that the enzyme requires, in its substrates, a free terminal \(\alpha\)-amino or \(\alpha\)-imino group, and it may be assumed that the combination of cathepsin C with a substrate involves such a group, as well as the carbonyl group that participates in the sensitive linkage. The inability of cathepsin C to hydrolyze \(\beta\)-alanyl-L-phenylalanine ethyl ester (4) indicates that the distance between the terminal amino group and the sensitive carbonyl group is critical for enzymic action. It may be surmised that, in the combination of the enzyme with the substrate in a manner leading to catalysis, the portion of the substrate molecule between (and including) these groups is held in a specific conformation. One possibility is that, in the enzyme-substrate complex, this portion of the substrate molecule is in a form tending toward a hexagon, as shown in Fig. 1, a.

If it is assumed that the "active center" of the enzyme interacts with the substrate by approaching it from above the plane of the hexagon (as drawn in Fig. 1, a), then it seems possible to interpret several aspects of the specificity of cathepsin C. It may be expected that the presence of \(D\)-amino acid residues at the N-terminal position (Fig. 1, b), or contributing the \(R'\) group (Fig. 1, c), would tend to prevent a close fit of enzyme and substrate. Thus \(D\)-leucyl-L-phenylalanine ethyl ester is hydrolyzed very slowly (Table I), and glycyl-\(D\)-phenylalaninamide is resistant to enzyme action (5). If the side chain group is small, as in \(D\)-alanine, hydrolysis of \(D\)-alanyl-L-tyrosine ethyl ester is observed, but this is slower than the hydrolysis of L-alanyl-L-tyrosine ethyl ester (Table I). It is perhaps significant that \(D\)-alanyl-L-tyrosine ethyl ester is hydrolyzed at approximately the same rate as is \(\alpha\)-aminoisobutyryl-L-tyrosine ethyl ester (Table I), which has methyl groups projecting above and below the plane of the hexagon (Fig. 1, d).

The effect of large \(R\) groups of N-terminal L-amino acid residues in decreasing the sensitivity of substrates of cathepsin C may perhaps be attributed to steric hindrance of the enzyme-substrate interaction as a con-
sequence of the free rotation about the bonds of the side chain (e.g., of the 
ε-acetyl-L-lysyl residue). However, where such free rotation is not pos-
sible, as in L-prolyl, the approach of the enzyme may not be hindered, thus 
explaining the ready hydrolysis of the prolyl compounds. It may be sur-
mised that the slower hydrolysis of the sarcosyl compounds, as compared 
to the analogous L-prolyl compounds, is a consequence of steric hindrance 
caused by an N-methyl group that can assume a position above the plane 
of the postulated hexagon. The inability of cathepsin C to act on glycyl-
N-methyl-L-phenylalaninamide may be due to steric hindrance caused by 
substitution at the CO—NH bond between the 2 amino acid residues. 
This substitution may interfere with the folding of the peptide chain into a 
conformation required for successful interaction with the enzyme, and may

\[
\begin{align*}
\text{(a)} & \quad \text{R CO-NH R'} \\
\text{(b)} & \quad \text{R CO-NH R'} \\
\text{(c)} & \quad \text{R CO-NH R'} \\
\text{(d)} & \quad \text{R CO-NH R'}
\end{align*}
\]

Fig. 1. Postulated folding of dipeptide derivatives in their interaction with 
cathepsin C. The heavy lines denote bonds in front of the plane of the 
paper, whereas the light lines denote bonds behind the plane of the paper. The 
dash lines represent the bond cleaved by the enzyme. a, a dipeptide derivative com-
posed of two L-amino acid residues; b, a dipeptide derivative in which the N-termi-
nal amino acid has a D configuration; c, a dipeptide derivative in which the R' group 
belongs to a D-amino acid; d, a derivative of α-aminoisobutyryl-L-tyrosine.

prevent an interaction, by hydrogen bond formation, with the CO—NH 
group of the substrate.

Experiments now in progress are designed to determine the magnitudes 
of \((k_2 + k_3)/k_1\) and \(k_3\) in the reactions

\[
E + S \xrightarrow{k_1} ES \xrightarrow{k_3} E + \text{products}
\]

where \(E\) is cathepsin C and the substrate \((S)\) is one of the series of dipep-
tide derivatives found to be hydrolyzed by the enzyme. It may be hoped 
that such data on the effect of structural changes in the substrate on the 
relative magnitude of \(K_m\) and \(k_3\) will throw further light on the mecha-
nism of cathepsin C action.

EXPERIMENTAL

In the conduct of the enzyme experiments, the pH was maintained at 
P\(H\) 5.0 to 5.2 with 0.1 M citrate buffer and at P\(H\) 7.4 to 7.6 with 0.1 M
phosphate buffer. Cysteine (0.004 M) was used as activator. The cathepsin C preparation was kindly provided by Dr. de la Haba, and had a specific activity [C. U.]_{mg. N} = 175, in the terms defined previously (1). In most of the experiments reported in this communication, the enzyme concentration was 0.35 cathepsin unit per 2 ml. of reaction mixture; in some instances, as indicated in Table I, the enzyme concentration was increased to 1.4 units per 2 ml. The substrate concentration was 0.05 M in all cases. The temperature was maintained at 37.0°. The rate of enzyme action on the peptide amides was followed by measurement of the extent of ammonia liberation in Conway microdiffusion vessels (10). The rate of disappearance of the peptide esters was followed by a modification of the hydroxamic acid method of Hestrin (4, 11). In several experiments, the Grassmann-Heyde titration method (12) also was employed. In all cases, control experiments were performed to determine the lability of the substrate, in the absence of added enzyme, at the pH and temperature of the enzyme test. Where no data are given for such control experiments, the substrates were found to be stable during the period of the enzyme experiment.

To facilitate comparison of the rates of hydrolysis of the various compounds tested near pH 5, the data in Table I denote the micromoles of substrate cleaved per ml. during the first 2 hours of the incubation, although samples were withdrawn for analysis at other time intervals (1 hour, 4 hours) as well. It was found in most cases that, during the initial 2 hour interval, and at the initial substrate concentration of 0.05 M, the rate at pH 5 was linear with time within the precision of the analytical methods employed. It was assumed that the extent of transamidation at pH 5 was sufficiently small so that it could be neglected (3).

Glycyl-L-tyrosinamide acetate, glycyl-L-phenylalaninamide acetate, L-alanyl-L-tyrosinamide acetate, and glycylglycyl-L-phenylalanine ethyl ester acetate were prepared as described previously (5). The synthesis of glycyl-L-phenylalanine ethyl ester hydrochloride and L-seryl-L-tyrosinamide hydrochloride also has been described (4).

For the synthesis of the other compounds tested as substrates, the general method of Vaughan and Osato (13) was used. The coupling reaction was usually performed by first preparing at -5° the requisite mixed anhydride from the carbobenzoxy amino acid (0.01 mole) and isobutylchlorocarbonate (0.01 mole) in the presence of triethylamine (0.01 mole) and toluene (20 ml.). After 15 minutes, a chilled mixture of the amino acid ethyl ester hydrochloride (0.01 mole), triethylamine (0.01 mole), and chloroform (20 ml.) was added. The reaction mixture was left at room temperature overnight, washed with water, dilute bicarbonate solution, and water; the organic layer was dried with exsiccated Na₂SO₄, and concentrated in vacuo to yield the desired carbobenzoxy dipeptide ester. For conversion of the
ester to the corresponding amide, the compound (about 0.01 mole) was dissolved in 25 ml. of methanol previously saturated with dry NH$_3$ at 0°, and the solution was kept at room temperature for 2 days. The solution was then concentrated in vacuo to give the carbobenzoxy dipeptide amide. The removal of the carbobenzoxy group was performed in the usual manner by catalytic hydrogenolysis in the presence of palladium-black and the equivalent amount of 10 N hydrochloric or of glacial acetic acid; ethanol was used as the solvent.

**Glycyl-L-tyrosine Ethyl Ester Hydrochloride**—This compound was prepared by hydrogenolysis of 1 gm. of the carbobenzoxy derivative (14). Yield, 0.6 gm.; [α]$^\text{D}_2$ +17.1° (2 per cent in water).

C$_{12}$H$_{19}$O$_4$N$_4$Cl (302.8). Calculated, N 9.3; found, N 9.5

The acetate of this dipeptide ester has been described previously (4). **Glycyl-O-methyl-L-tyrosinamide Acetate**—3 gm. of O-methyl-L-tyrosine (15) were converted to the ethyl ester hydrochloride in the usual manner. Yield, 92 per cent; m.p., 202°; [α]$^\text{D}_2$ -4.9° (2 per cent in water).

C$_{12}$H$_{19}$O$_4$N$_4$Cl (259.7). Calculated, N 5.4; found, N 5.4

The coupling reaction gave an oily carbobenzoxy dipeptide ester, which was converted to the crystalline amide with an over-all yield of 61 per cent; m.p., 132°.

C$_{16}$H$_{15}$O$_7$N$_4$ (385.4). Calculated, N 10.9; found, N 10.8

Hydrogenolysis gave the dipeptide amide acetate; yield, 63 per cent; [α]$^\text{D}_2$ +39.5° (2 per cent in water).

C$_{14}$H$_{17}$O$_4$N$_4$ (311.3). Calculated, N 13.5; found, N 13.4

**Sarcosyl-L-tyrosinamide Hydrochloride**—Sarcosine (0.1 mole) was carbobenzyolated in the usual manner to give the oily carbobenzoxy sarcosine in a yield of 97 per cent. The coupling reaction gave the carbobenzoxy dipeptide ester in a yield of 64 per cent; m.p., 136°.

C$_{22}$H$_{26}$O$_8$N$_4$ (414.5). Calculated, N 6.8; found, N 6.7

The amide was obtained in a yield of 85 per cent; m.p., 122–124°.

C$_{20}$H$_{24}$O$_4$N$_4$ (385.4). Calculated, N 10.9; found, N 11.0

Hydrogenolysis of 1.3 gm. of the amide gave the desired product in a yield of 87 per cent; [α]$^\text{D}_2$ +31.5° (2 per cent in water).

C$_{12}$H$_{18}$O$_4$N$_4$Cl (287.8). Calculated, N 14.6; found, N 14.3

**Sarcosyl-L-tyrosine Ethyl Ester Hydrochloride**—Hydrogenolysis of 2 gm. of the carbobenzoxy compound gave the product in 96 per cent yield. The
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Substance was recrystallized from methanol-ether; [α]$_D^{22}$ +11.0° (2 per cent in water).

C$_{14}$H$_{15}$O$_3$N$_2$Cl (316.8). Calculated, N 8.8; found, N 8.8

Sarcosyl-L-phenylalaninamide Hydrochloride—The carbobenzoxy dipeptide ester was obtained as an oil, and was converted to the amide (over-all yield, 49 per cent); m.p., 134°.

C$_{28}$H$_{32}$O$_3$N$_3$ (369.4). Calculated, N 11.4; found, N 11.2

Hydrogenolysis gave the dipeptide amide hydrochloride in 84 per cent yield; [α]$_D^{22}$ +25.4° (2 per cent in water).

C$_{12}$H$_{18}$O$_2$N$_2$Cl (271.8). Calculated, N 15.5; found, N 15.2

Sarcosyl-L-phenylalanine Ethyl Ester Hydrochloride—Hydrogenolysis of the oily carbobenzoxy compound gave the product in 82 per cent yield; [α]$_D^{22}$ +4.7° (2 per cent in water).

C$_{14}$H$_{21}$O$_3$N$_2$Cl (300.8). Calculated, N 9.3; found, N 9.3

1-Prolyl-L-tyrosinamide Hydrochloride—The oily carbobenzoxy dipeptide ester was converted to the amide (over-all yield, 52 per cent); m.p., 172–173°.

C$_{17}$H$_{14}$O$_3$N$_2$ (411.5). Calculated, N 10.2; found, N 10.0

Hydrogenolysis gave the dipeptide amide in non-crystalline form (yield, 99 per cent). Despite repeated precipitation from methanol-ether, the substance could not be obtained in a completely pure state.

C$_{14}$H$_{23}$O$_3$N$_2$Cl (313.8). Calculated, N 13.4; found, N 12.7

1-Prolyl-L-tyrosine Ethyl Ester Hydrochloride—Hydrogenolysis of the oily carbobenzoxy compound gave a non-crystalline solid in 80 per cent yield; [α]$_D^{22}$ -29.3° (2 per cent in water).

C$_{16}$H$_{29}$O$_3$N$_2$Cl (342.8). Calculated, N 8.2; found, N 7.9

1-Prolyl-L-phenylalaninamide Hydrochloride—The oily carbobenzoxy dipeptide ester was converted to the amide (over-all yield, 68 per cent); m.p., 180–181°.

C$_{22}$H$_{32}$O$_3$N$_2$ (395.5). Calculated, N 10.6; found, N 10.4

Hydrogenolysis gave a non-crystalline product; yield, 94 per cent; [α]$_D^{22}$ -15.8° (2 per cent in water).

C$_{14}$H$_{25}$O$_2$N$_2$Cl (297.7). Calculated, N 14.1; found, N 13.8

1-Prolyl-L-phenylalanine Ethyl Ester Hydrochloride—Hydrogenolysis of
the oily carbobenzoxy compound gave the desired product in crystalline form; yield, 75 per cent; \([\alpha]_D^{22} -40.9^\circ\) (2 per cent in water).

\[ \text{C}_{14}\text{H}_{30}\text{O}_2\text{N}_3\text{Cl} \] (326.8). Calculated, N 8.6; found, N 8.5

When 32.7 mg. of this substance were subjected to enzymic hydrolysis by cathepsin C at pH 5 under the usual conditions, a crystalline precipitate appeared within 90 minutes. After 4 hours, 55 per cent of the ester had disappeared, as judged by the hydroxamic acid method, and the crystals were filtered off, washed with water, and dried. Yield, 12.5 mg.; m.p., 242–244°, with decomposition. This product is L-prolyl-L-phenylalanine (7).

\[ \text{C}_{14}\text{H}_{19}\text{O}_3\text{N} \] (262.3). Calculated, N 10.7; found, N 10.8

Glycyl-N-methyl-L-phenylalaninamide Hydrochloride—N-Methyl-L-phenylalanine was prepared from 3.3 gm. of D-phenylalanine in a manner similar to that described for the conversion of L-methione to N-methyl-L-methionine (16). Yield, 1.9 gm. (53 per cent); \([\alpha]_D^{18} +48.4^\circ\) (2 per cent in 0.13 N NaOH). Fischer (17) has reported \([\alpha]_D^{18} +49.7^\circ\) (2 per cent in 0.1 N NaOH) for this compound. It was converted in the usual manner to the ethyl ester hydrochloride. Yield, 96 per cent; m.p., 133°; \([\alpha]_D^{22} +7.8^\circ\) (2 per cent in water).

\[ \text{C}_{12}\text{H}_{18}\text{O}_2\text{N}_2\text{Cl} \] (243.7). Calculated, N 5.7; found, N 5.3

The oily carbobenzoxy dipeptide ester was converted to the amide which crystallized with difficulty. Over-all yield, 43 per cent; m.p., 130°.

\[ \text{C}_{29}\text{H}_{25}\text{O}_4\text{N}_3 \] (369.4). Calculated, N 11.4; found, N 11.7

Hydrogenolysis gave the dipeptide amide hydrochloride, which was recrystallized from a mixture of methanol, acetone, and ethyl acetate. Yield, 61 per cent; \([\alpha]_D^{22} -19.6^\circ\) (2 per cent in water).

\[ \text{C}_{15}\text{H}_{15}\text{O}_2\text{N}_3\text{Cl} \] (271.8). Calculated, N 15.5; found, N 15.5

This compound was found to decompose with the liberation of ammonia when in contact with the carbonate used in the Conway method.

Glycyl-N-methyl-L-phenylalanine Ethyl Ester Hydrochloride—Hydrogenolysis of the oily carbobenzoxy compound gave the product in poor yield (25 per cent).

\[ \text{C}_{14}\text{H}_{15}\text{O}_2\text{N}_3\text{Cl} \] (300.8). Calculated, N 9.3; found, N 9.3

L-Alanyl-L-tyrosine Ethyl Ester Hydrochloride—Hydrogenolysis of the carbobenzoxy compound (18) gave the desired product in 85 per cent yield; \([\alpha]_D^{25} +6.4^\circ\) (2 per cent in water).

\[ \text{C}_{14}\text{H}_{17}\text{O}_2\text{N}_3\text{Cl} \] (316.8). Calculated, N 8.8; found, N 8.6
d-Alanyl-L-tyrosine Ethyl Ester Hydrochloride—The coupling product was obtained in 78 per cent yield; m.p., 105–107°.

\[ C_{23}H_{42}O_{3}N_8 \] (414.5). Calculated, N 6.8; found, N 6.7

Hydrogenolysis gave the dipeptide ester hydrochloride in 90 per cent yield; \( [\alpha]_D^{25} +4.9^\circ \) (2 per cent in water).

\[ C_{14}H_{21}O_{4}N_2Cl \] (316.8). Calculated, N 8.8; found, N 8.5

\( \alpha \)-Aminoisobutyryl-L-tyrosine Ethyl Ester Hydrochloride—Carbobenzoxy-\( \alpha \)-aminoisobutyric acid was prepared in the usual manner; yield, 53 per cent; m.p., 64–66°.

\[ CH_{16}O_{4}N \] (237.3). Calculated, N 5.9; found, N 5.8

The oily carbobenzoxy dipeptide ester was subjected to hydrogenolysis, yielding a non-crystalline solid in 45 per cent yield; \( [\alpha]_D^{25} -9.1^\circ \) (2 per cent in water).

\[ C_{15}H_{22}O_{3}N_2 \] (330.8). Calculated, N 8.5; found, N 8.4

\( \alpha \)-Aminoisobutyryl-L-phenylalanine Ethyl Ester Hydrochloride—The coupling product was obtained in 35 per cent yield; m.p., 94–95°.

\[ C_{25}H_{28}O_{3}N_2 \] (412.5). Calculated, N 6.8; found, N 6.8

Hydrogenolysis gave the dipeptide ester hydrochloride in non-crystalline form. Yield, 77 per cent; \( [\alpha]_D^{25} -18.1^\circ \) (2 per cent in water).

\[ C_{14}H_{21}O_{4}N_2Cl \] (314.8). Calculated, N 8.9; found, N 9.0

On incubation of 31.5 mg. of this compound dissolved in 2 ml. at pH 7.5 and 37° for 4 hours, 7.6 mg. of a crystalline precipitate were obtained (m.p., 290–292°, decomposition); its nitrogen analysis agrees with that of \( \alpha \)-aminoisobutyryl-L-phenylalanine diketopiperazine.

\[ C_{12}H_{18}O_{3}N_2 \] (232.3). Calculated, N 12.1; found, N 12.2

L-Leucyl-L-phenylalaninamide Acetate—The coupling reaction gave an oily carbobenzoxy dipeptide ester, which was converted to the amide (overall yield, 80 per cent); m.p., 176–178°.

\[ C_{22}H_{29}O_{4}N_2 \] (411.5). Calculated, N 10.2; found, N 10.1

Hydrogenolysis gave the dipeptide amide acetate in a yield of 53 per cent.

\[ C_{17}H_{27}O_{4}N_3 \] (337.4). Calculated, N 12.5; found, N 12.3

L-Leucyl-L-phenylalanine Ethyl Ester Hydrochloride—Hydrogenolysis of the oily carbobenzoxy compound gave the desired product in an overall yield of 45 per cent; \( [\alpha]_D^{22} -3.8^\circ \) (2 per cent in water).

\[ C_{17}H_{27}O_{4}N_2Cl \] (342.9). Calculated, N 8.2; found, N 8.2
d-Leucyl-L-phenylalanine Ethyl Ester Hydrochloride—The carbobenzyox
dipeptide ester was obtained in crystalline form (yield, 46 per cent); m.p.,
114–115°.

\[
C_{23}H_{32}O_{14}N_2 \quad (440.5). \quad \text{Calculated, N 6.4; found, N 6.4}
\]

Hydrogenolysis gave the dipeptide ester hydrochloride in a yield of 90 per
cent; \( [\alpha]^2_D + 43.3^\circ \) (2 per cent in water).

\[
C_{12}H_{27}O_{14}N_2Cl \quad (342.9). \quad \text{Calculated, N 8.2; found, N 8.1}
\]

L-Lysyl-L-tyrosinamide Dihydrobromide—The dicarbobenzyox
dipeptide ester was obtained as a product melting at 106–107°; yield, 73 per cent.

\[
C_{23}H_{35}O_{14}N_3 \quad (605.7). \quad \text{Calculated, N 6.9; found, N 7.1}
\]
The ester was converted nearly quantitatively to the amide; m.p., 183° (19).

\[
C_{16}H_{26}O_{14}N_4 \quad (576.7). \quad \text{Calculated, N 9.7; found, N 9.5}
\]
The decarbobenzoxylation was effected by treatment with HBr-acetic
acid (20); yield, 92 per cent; \( [\alpha]^2_D + 17.3^\circ \) (2 per cent in water).

\[
C_{12}H_{26}O_{14}N_4Br_2 \quad (470.2). \quad \text{Calculated, N 11.9; found, N 11.6}
\]

L-Lysyl-L-tyrosine Ethyl Ester Dihydrochloride—Hydrogenolysis of the
carbobenzyox compound gave this substance in 97 per cent yield; \( [\alpha]^2_D + 16.9^\circ \) (2 per cent in water).

\[
C_{17}H_{38}O_{14}N_4Cl_2 \quad (410.3). \quad \text{Calculated, N 10.2; found, N 10.0}
\]

L-Lysyl-L-phenylalaninamide Dihydrochloride—The dicarbobenzyox
dipeptide ester was obtained in a yield of 65 per cent; m.p., 131°.

\[
C_{33}H_{39}O_{14}N_3 \quad (589.7). \quad \text{Calculated, N 7.1; found, N 7.2}
\]
The corresponding amide was obtained in a yield of 95 per cent; m.p., 170°.

\[
C_{21}H_{36}O_{14}N_4 \quad (560.7). \quad \text{Calculated, N 10.0; found, N 9.7}
\]

Hydrogenolysis gave the desired dipeptide amide dihydrochloride in a
yield of 89 per cent; \( [\alpha]^2_D + 38.0^\circ \) (2 per cent in water).

\[
C_{17}H_{35}O_{14}N_4Cl_2 \quad (365.3). \quad \text{Calculated, N 15.3; found, N 15.1}
\]

L-Lysyl-L-phenylalanine Ethyl Ester Dihydrochloride—Hydrogenolysis of
the carbobenzyox compound gave this product in 98 per cent yield; \( [\alpha]^2_D + 14.5^\circ \) (2 per cent in water).

\[
C_{17}H_{32}O_{14}N_4Cl_2 \quad (394.3). \quad \text{Calculated, N 10.6; found, N 10.6}
\]

ε-Acetyl-L-lysyl-L-tyrosinamide Hydrochloride—ε-Acetyl-L-lysine (21) was
carbobenzyoxylated in the usual manner to yield an oily product. The
coupling reaction gave the desired compound in 78 per cent yield; m.p., 165°.

\[
\text{C}_{27}\text{H}_{32}\text{O}_{7}\text{N}_{4} \quad (513.6). \quad \text{Calculated, N 8.2; found, N 7.9}
\]

The acylated dipeptide ester was converted to the amide with a yield of 93 per cent; m.p., 235°.

\[
\text{C}_{28}\text{H}_{35}\text{O}_{4}\text{N}_{4} \quad (484.6). \quad \text{Calculated, N 11.6; found, N 11.3}
\]

Hydrogenolysis gave the desired product in 97 per cent yield; \([\alpha]_{D}^{22} +17.5° \) (1 per cent in water).

\[
\text{C}_{19}\text{H}_{27}\text{O}_{3}\text{N}_{4}\text{Cl} \quad (415.9). \quad \text{Calculated, N 10.1; found, N 9.8}
\]

\(\varepsilon\)-Acetyl-1-lysyl-1-tyrosine Ethyl Ester Hydrochloride—Hydrogenolysis of the carbobenzyloxy compound gave this product with a yield of 85 per cent; \([\alpha]_{D}^{22} +13.2° \) (1 per cent in water).

\[
\text{C}_{18}\text{H}_{30}\text{O}_{6}\text{N}_{4}\text{Cl} \quad (370.9). \quad \text{Calculated, N 10.5; found, N 10.5}
\]

When 2 ml. of a 0.05 M solution of this substance were kept at pH 7.5 and 37° for 24 hours, 8.7 mg. of a crystalline compound separated; m.p., 238–239°. Its nitrogen analysis agrees with that of the diketopiperazine.

\[
\text{C}_{18}\text{H}_{28}\text{O}_{4}\text{N}_{3} \quad (317.4). \quad \text{Calculated, N 13.2; found, N 12.9}
\]

\(\varepsilon\)-Benzoyl-L-lysyl-L-tyrosinamide Hydrochloride—\(\varepsilon\)-Benzoyl-L-lysine was prepared by the method of Kurtz (22) and carbobenzyoxylated in the usual manner to yield an oily product (yield, 96 per cent). The coupling product also could not be crystallized and was converted to the amide (over-all yield, 54 per cent); m.p., 215–216°.

\[
\text{C}_{28}\text{H}_{34}\text{O}_{4}\text{N}_{4} \quad (546.6). \quad \text{Calculated, N 10.2; found, N 10.0}
\]
Hydrogenolysis gave the benzoyl dipeptide ester as a non-crystalline solid in a yield of 86 per cent; \([\alpha]_{D}^{22} +32.6^\circ\) (1 per cent in water). The analysis was not satisfactory.

\[\text{C}_{22}\text{H}_{19}\text{O}_{4}\text{N}_{4}\text{Cl} (449.0). \text{Calculated, N 12.5; found, N 11.8}\]

\( \varepsilon\)-Benzoyl-\(L\)-lysyl-\(L\)-tyrosine Ethyl Ester Hydrochloride—Hydrogenolysis of the carbobenzyox compound gave the product in a yield of 95 per cent; \([\alpha]_{D}^{22} +24.3^\circ\) (1 per cent in water).

\[\text{C}_{22}\text{H}_{20}\text{O}_{4}\text{N}_{4}\text{Cl} (478.0). \text{Calculated, N 8.8; found, N 8.6}\]

\( \varepsilon\)-Benzoyl-\(L\)-lysyl-\(L\)-phenylalaninamide Hydrochloride—The coupling product was obtained in a yield of 69 per cent; m.p., 140°.

\[\text{C}_{22}\text{H}_{19}\text{O}_{4}\text{N}_{4} \text{(559.7). Calculated, N 7.5; found, N 7.5}\]

The amide was prepared with a yield of 82 per cent; m.p., 210°.

\[\text{C}_{26}\text{H}_{20}\text{O}_{4}\text{N}_{4} \text{(590.6). Calculated, N 10.6; found, N 10.6}\]

Hydrogenolysis gave the desired product as a non-crystalline solid; yield, 88 per cent; \([\alpha]_{D}^{22} +30.5^\circ\) (1 per cent in water).

\[\text{C}_{22}\text{H}_{19}\text{O}_{4}\text{N}_{4}\text{Cl} (433.0). \text{Calculated, N 12.9; found, N 12.6}\]

\( \varepsilon\)-Benzoyl-\(L\)-lysyl-\(L\)-phenylalanine Ethyl Ester Hydrochloride—Hydrogenolysis of the carbobenzyox compound gave the product in a yield of 93 per cent; \([\alpha]_{D}^{22} +21.3^\circ\) (1 per cent in water).

\[\text{C}_{22}\text{H}_{20}\text{O}_{4}\text{N}_{4}\text{Cl} (462.0). \text{Calculated, N 9.1; found, N 8.8}\]

Acetyl-\(L\)-phenylalanine Ethyl Ester—This substance was prepared by the treatment of acetyl-\(L\)-phenylalanine with HCl in absolute ethanol. Yield, 85 per cent; m.p., 93–94°.

\[\text{C}_{12}\text{H}_{17}\text{O}_{3}\text{N}(235.3). \text{Calculated, N 6.0; found, N 5.9}\]

The authors wish to acknowledge with gratitude the valuable assistance of Mrs. M. E. Radford and Miss M. J. Mycek.

**SUMMARY**

A study of the action of purified beef spleen cathepsin C on a series of dipeptide amides and dipeptide esters at pH 5 has shown that the rate of hydrolysis of the amide or ester bond depends on the structure and configuration of both amino acid residues present in the compound. The presence of \(\alpha\)-amino acid residues in the substrate renders the sensitive bond more resistant to hydrolysis by cathepsin C. The rate of action of the enzyme is also decreased by the presence of large side chain groups (isobutyl, \(\varepsilon\)-acetylamino-\(n\)-butyl) in the \(N\)-terminal \(L\)-amino acid residue of the substrate. Although methylation of the terminal \(\alpha\)-amino groups of a
substrate such as glycyl-L-tyrosinamide diminishes the rate of hydrolysis, comparable derivatives of L-prolyl dipeptides are hydrolyzed rapidly. The effect of structural changes in substrates of cathepsin is evident also at pH 7.5, where transamidation reactions can occur. The substances resistant to hydrolysis at pH 5 do not participate as substrates in polymerization reactions at pH 7.5. The L-prolyl dipeptide esters and amides, though hydrolyzed at pH 7.5, do not form polymers, since these substrates do not appear to be active as replacement agents.

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