The Tryptic Hydrolysis of Pepsin

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THE TRYPTIC HYDROLYSIS OF PEPSIN

by

Frederick W. Pairent

A Thesis Submitted to the Faculty of the Graduate School of Loyola University in Partial Fulfillment of
the Requirements for the Degree of
Master of Science

June
1959
LIFE

Frederick W. Faivre was born in Philadelphia, Pennsylvania, January 18, 1932.

He was graduated from Southeast Catholic High School, Philadelphia, in June, 1949.

He received the degree of Bachelor of Science in Chemistry, from Saint Joseph's College, Philadelphia, in June, 1953. From May, 1952 to May, 1953, he was chairman of the Student Chapter of the American Chemical Society at Saint Joseph's College. He has been a member of the American Chemical Society since June, 1953.

During the period, January, 1954 to December, 1955, the author served with the United States Army. While on active duty, he received Medical Laboratory Technician training at Fort Sam Houston, Texas. Later he was attached to the toxicology section, of the USAFMD Medical Laboratory, Landstuhl, Germany.

He was employed as an analytical chemist in the Research and Development Department of Smith, Kline & French Laboratories, in Philadelphia, from February, 1956 to September, 1957.

He entered upon graduate studies in September, 1957, at Loyola University, Chicago, Illinois. During the summer
months of 1958, he was employed as a biochemist in the Honey Investigation Laboratory of the Eastern Utilization Research Branch of the Department of Agriculture, in Philadelphia.
ACKNOWLEDGMENT

The author wishes to express his gratitude to Doctor Martin B. Williamson, for his guidance during the work presented in this thesis. Also, to his wife, Constance, and to two friends, Mr. and Mrs. L. J. Banaszak, who rendered valuable aid in the preparation of the thesis.
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CHAPTER I

THE STRUCTURE OF PROTEINS

The importance of understanding any chemical reaction ultimately depends on a knowledge of the structure of the reactants involved. In the case of certain biochemical processes, proteins play an integral part either as reactants or as catalysts. To fully understand these chemical reactions, the chemical structure of the proteins involved must first be delineated.

Great strides have been made over the past several decades by chemists and enzymologists in the study of the physical and chemical properties of proteins. In spite of these advances, it is only very recently that structural information about proteins has accumulated to an extent sufficient to begin to permit a more direct and rational consideration of these properties in terms of detailed molecular architecture. Protein chemistry now stands on the threshold of a vast area of research, wherein the direct comparison of biological activity with structure will become commonplace. This present confidence, expressed by those studying the structure of proteins, stems for the most part from two major sources. First, the elegance and variety of physical methods now permit the experimental study of structural
features not even adequately established until recently. Second, the protein field now possesses the reassuring chemical background contributed by Sanger and his collaborators in their studies of insulin (71, 72, 74, 75, 77).

One of the greatest influences on the study of protein structure, and indeed on the entire field of chemistry, was the development by Martin and Synge (44), in 1941, of the entirely new technique of partition chromatography. The great problem in protein chemistry has always been to find methods of fractionating the extremely complex mixtures produced by the partial degradation of a protein. Older methods of fractional crystallization, and precipitation with various salts and other reagents, were usually inadequate to deal with these mixtures, therefore, methods of high resolving power were needed. Partition chromatography, especially in the form of paper chromatography, is such a method.

During the last few years, work in the field of protein chemistry has centered largely on the development of methods, so that this introduction will be more a consideration of techniques, than a discussion of results which are still rather few.

Amino Acids are organic compounds containing both carboxylic groups and amino groups. Proteins consist of chains of amino acids, connected through their amino and carboxylic groups, forming a polypeptide chain(s). Three types of polypeptide
chains are theoretically possible: straight chain, cyclic chain and branched chain. These may be illustrated by the following structures:

**STRAIGHT CHAIN:**

\[ \text{H}_2\text{N-} \text{RCH.CO-NH.R'CH.CO...} \]

**Characteristic:** At one end of the peptide is found an amino acid having a free alpha amino group (N-terminal); at the other end of the peptide is found an amino acid having a free carboxylic acid group alpha to the amino group (C-terminal).

**CYCLIC CHAIN:**

\[ \text{...NH.RCH.CO.NH.R'CH.CO...} \]

**Characteristic:** These peptides have neither N or C terminal groups.

**BRANCHED CHAIN:**

\[ \text{H}_2\text{N-} \text{RCH.CO...NHR.CH.CO...NH.R'CH.COOS} \]

\[ (\text{CH}_2)_4 \]

\[ \text{...NH.CCHR'.NH...OC.CHR.NH}_2 \]

**Characteristic:** These peptides contain more of one type of terminal amino acid than of the other. The illustration shown has two N-terminals and one C-terminal.

Any combination or multiple of these three basic forms is, of course, possible. The first two forms illustrated have been found to be the most common.
Some proteins are built up of two or more polypeptide chains, held together by a stable bond other than a peptide link. Such a bond is referred to as a "cross linkage". The disulfide bond of cystine is the most common type of "cross linkage". Here, two cystine residues are joined together (3-3) through their side chains. The less common phosphodiester bond is a much weaker link, and has been found in only a few proteins (66).

It has been long established that a protein molecule is built up of a chain or chains of alpha amino acids bound together by peptide bonds between their alpha amino and alpha carboxyl groups. The elucidation of the amino acid sequence of a protein can be accomplished by fairly easy methods of separation and analysis, such as, paper chromatography and "end group" analysis. Recently, numerous stepwise organic and enzymatic degradation methods have been introduced in an effort to increase the efficiency of amino acid sequence determination, especially in reference to larger protein molecules. An outline of the strategy summarizing the most popular approach to the determination of the sequence of amino acids will now be presented.

In the study of the structure of a protein, the first step after the molecular weight and purity of the protein has been established, is to determine its amino acid content. A paper chromatographic method developed by Levy (41), utilizes the 2,4-dinitrophenyl (DNP) derivatives of the amino acids, after
the protein is completely hydrolysed in dilute acid. The DNP-
amino acids are eluted from a two dimensional chromatogram after
development, and their concentrations are estimated colorimetrically.
This method works well only for small peptides and proteins
having relatively few amino acids.

Practically all the amino acid determinations reported
in the literature of the last four years, has been performed by
ion exchange chromatography on columns. This method has received
much attention since it was first developed by Moore and Stein (47)
in 1951. Since that time, the same investigators (48) have im-
proved the method to the point where separation of all the amino
acids is achieved by the use of a single column, and an automatic
recording device is arranged to measure the amino acid concen-
tration in the effluent fractions.

The terminal residues in proteins differ from the other
residues in the chain since they usually contain free alpha amino
or free carboxyl groups alpha to the amino group. This fact may
be used to identify them. The determination of the number of
these groups in a protein gives information as to the number and
arrangement of polypeptide chains in the molecule. However, with
some proteins containing N and C terminals, it may be that the
alpha carboxyl group is not free. This is shown to be the case
with the Melanophore Stimulating Hormone, in which the amino group
of the N-terminal is acetylated.
Methods for Identifying N-Terminal Amino Acids: The methods for identification of the N-terminal amino acids are in a much more satisfactory state of development than those for the C-terminal amino acids. A summary of the methods that have been most extensively applied to proteins are given in Table I. Methods 2, 4, and 5, are degradation methods which can be repeated on the residual peptide formed, after removal of the terminal amino acids.

Methods for Identifying C-Terminal Amino Acids: There are but three noteworthy methods for the determination of C-terminal residues in a protein. These are illustrated in Table II.

Tables I and II list only the fundamental reactions in use at present. It should be noted that many modifications of these methods exist.

Partial Hydrolysis of Proteins

Amino acid sequence determinations necessitate the breaking up of the protein into smaller peptide fragments. If a small peptide or protein is under consideration, partial acid hydrolysis (74, 75) of the molecule may be feasible. However, this non-specific hydrolysis procedure could hardly be used with large proteins. Both Sanger (76) and Desruelle (17) have discussed the acid hydrolysis of proteins in considerable detail and have pointed out the limitations of this method for the production of peptide fragments. Their discussions emphasize that the complexity of such hydrolysates makes the problem of adequate
### TABLE I

**METHODS FOR IDENTIFYING N-TERMINAL AMINO ACIDS**

<table>
<thead>
<tr>
<th>Method</th>
<th>Reagent</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. DNP (70,73)</td>
<td>Dinitrofluorobenzene (DNFB)</td>
<td>$\text{DNFB} + \text{NH}_2\text{-protein} \xrightarrow{\text{pH 8}} \text{DNFB-protein}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\xrightarrow{\text{dil. HCl}} \text{DNF-amo acids}$</td>
</tr>
<tr>
<td>2. PTC (19,20,23)</td>
<td>Phenylisothiocyanate</td>
<td>$\text{\theta-CN3} + \text{NH}_2\text{-protein} \xrightarrow{\text{pyridine}} \text{PTC-}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\xrightarrow{\text{dry HCl}} \text{protein} \xrightarrow{\text{phenylthiohydantoin}}$</td>
</tr>
<tr>
<td>3. Pipsyl (83)</td>
<td>$p$-iodophenylsulfonyl chloride</td>
<td>$\text{Pipsyl-Cl} + \text{NH}_2\text{-protein} \xrightarrow{\text{dil. HCl}} \text{pipsyl-}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\xrightarrow{\text{pipsyl-amo acids}} \text{protein}$</td>
</tr>
<tr>
<td>4. Thiocarbamate (40)</td>
<td>Carbon disulfide</td>
<td>$\text{CS}_2 + \text{NH}_2\text{-protein} \xrightarrow{\text{alkali}} \text{thiocar-}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\xrightarrow{\text{bamate protein}} \xrightarrow{\text{pH 3}} 2\text{-thiothiazol-}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\xrightarrow{\text{lid-5-one amino acid}} \text{protein}$</td>
</tr>
<tr>
<td>5. Aminopeptidase</td>
<td>Leucine aminopeptidase</td>
<td>Enzymatic cleavage of amino acids</td>
</tr>
<tr>
<td></td>
<td></td>
<td>having free alpha amino group</td>
</tr>
<tr>
<td>Method</td>
<td>Reagent</td>
<td>Reaction</td>
</tr>
<tr>
<td>----------------------</td>
<td>-----------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Reductive cleavage</td>
<td>(1) LiAlH$_4$</td>
<td>Protein–COOH + LiAlH$_4$ → Protein–OH dil HCl → amino alcohols</td>
</tr>
<tr>
<td>(24,15)</td>
<td>(2) LiBH$_4$</td>
<td>Same as above. Milder reaction</td>
</tr>
<tr>
<td>Hydrazinolysis</td>
<td>Hydrazine</td>
<td>Protein–COOH + Hydrazine → anhydrous hydrazides of all amino acids but C-terminal + C-terminal amino acid</td>
</tr>
<tr>
<td>(1,52,53,60)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carboxypeptidase</td>
<td>Carboxypeptidase</td>
<td>Enzymatic cleavage of amino acids containing free alpha carboxyl group</td>
</tr>
<tr>
<td>(27,23)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
separation of individual peptide components an extremely difficult one. Further, extensive reconstruction of the parent peptide chain is generally impossible, because of the small size of the fragments, and their great number.

Sanger (76) concluded in his review that, "...probably the chief need in this field is for techniques of specific breakdown of proteins into larger peptides, and for the fractionation of such peptides." The use of proteolytic enzymes furnishes the most obvious approach to the solution of this problem.

The usual approach to the controlled enzymatic degradation of proteins is to submit the protein first to the action of one proteolytic enzyme, separate the peptides obtained, and then submit each peptide separately to further hydrolysis by one or more proteolytic enzymes. This would result in a pyramid of peptides, all obtained by hydrolysis of definite peptide bonds. Characterization of the peptide fragments from each of these enzymatic hydrolysates may then permit the relative alignment of the fragments with respect to one another on the basis of their amino acid compositions. Such alignment is simplified by preliminary determination of the terminal residues of each peptide. In many cases, and particularly with trypsin, the known specificity of the enzyme used, may be used to help to fix the identity of the C-terminal residues.

The proteolytic enzymes most frequently used in the
studies of protein structure, and the type of peptide bond which they usually split, are described in Table III.
TABLE III

PROTEOLYTIC ENZYMES USED IN SEQUENCE DETERMINATIONS

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specificity*</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td>Peptide bonds involving the COOH group of lysine and arginine</td>
<td>ACTH (42)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glucagon (13)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Insulin &quot;B&quot; chain (32)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ribonuclease (3, 34)</td>
</tr>
<tr>
<td>Pepsin</td>
<td>Peptide bonds involving the NH₂ group of tyrosine and phenylalanine</td>
<td>ACTH (42)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Insulin (26)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ribonuclease (3)</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>Peptide bonds involving the COOH groups of tyrosine and phenylalanine</td>
<td>ACTH (42)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Insulin (26)</td>
</tr>
<tr>
<td>Papain</td>
<td>Specificity similar to pepsin</td>
<td>Insulin &quot;A&quot; chain (26)</td>
</tr>
<tr>
<td>Subtilisin</td>
<td>Relatively non-specific</td>
<td>Insulin &quot;B&quot; chain (30)</td>
</tr>
</tbody>
</table>

* The enzymes are arranged in order of decreasing specificity. The peptide bonds listed are those which are most readily hydrolysed by the enzyme. Many cases of "non-specific" hydrolysis have been reported.
CHAPTER II

THE CHEMICAL AND PHYSICAL CHARACTERISTICS OF PEP SIN

Pepsin is the major proteolytic enzyme found in the stomach. It is secreted in an inactive precursor form, pepsinogen, by the glands of the gastric mucosa (31). The activity of this enzyme is characterized by the rapid proteolysis of peptide bonds involving the carboxylic group of tyrosine and phenylalanine (6, 7).

The enzyme was first crystallized from commercial preparations of swine pepsin by Northrup (59). The enzyme is obtained from gastric juice or from pepsinogen, isolated from the gastric mucosa. Purification is accomplished by precipitation with magnesium sulfate at pH 2, dissolution of the precipitate with alkali to a pH less than 4, and reprecipitation by acidification. Crystallization occurs when this precipitate is dissolved at pH 4, 45° C, and the solution allowed to cool. This procedure has been found to lead to considerable decomposition (36) with the formation of free tyrosine and a large number of peptides. Pepsin may also be crystallized from 20% ethanol and is soluble in 65% ethanol (58). Crystalline pepsin has been obtained from the gastric mucosa of a variety of animals (54, 55, 12).
59). Many proteolytic enzymes have recently been isolated from human semen. Among them is an enzyme having an activity resembling pepsin (8, 43).

Pepsin is produced autocatalytically, by acidification of pepsinogen solutions (31, 32). Six peptide bonds are hydrolyzed in the precursor to yield pepsin plus six small peptides. The largest of these peptides has been found to inhibit the activity of pepsin above pH 5.4 by forming a pepsin-inhibitor complex. At a pH less than 3.5, the inhibitor is hydrolysed by pepsin. The activation of pepsinogen to pepsin has been formulated according to the following scheme (31, 32):

Pepsinogen $\rightarrow$ Pepsin-inhibitor compound $\rightarrow$ 5 peptides

\[
\begin{align*}
\text{pH} &> 5.4 \quad \uparrow \quad \text{pH} < 5.4 \\
\text{pepsin} \quad + \quad \text{inhibitor} &\Downarrow \quad \text{pH} < 3.5 \\
\text{pepsin} \quad + \quad \text{hydrolysis products of inhibitor}
\end{align*}
\]

The activation has a maximum rate at pH 2 and is irreversible.

The chemical characteristics of pepsinogen and its derivatives have been determined and are shown in Table IV. From the experimental data outlined in this table, it has been assumed that pepsinogen and pepsin are both single polypeptide chains (2). Since both molecules have the same C-terminal amino acid sequence, and the N-terminal amino acids have been found to be
### TABLE IV

**THE CHEMICAL CHARACTERISTICS OF PEPsinogen AND ITS DERIVATIVES**

<table>
<thead>
<tr>
<th></th>
<th>Pepsinogen</th>
<th>Pepsin</th>
<th>Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E-terminal sequence</strong></td>
<td><em>Leu</em>&lt;sub&gt;33&lt;/sub&gt;<em>,Leu</em>&lt;sub&gt;81&lt;/sub&gt;<em>,Leu</em>&lt;sub&gt;11&lt;/sub&gt;<em>,Leu</em>&lt;sub&gt;61&lt;/sub&gt;*</td>
<td><em>Ileu</em>&lt;sub&gt;11&lt;/sub&gt;<em>&lt;sub&gt;28&lt;/sub&gt;</em>,Gly*&lt;sub&gt;33&lt;/sub&gt;<em>,Asp</em>&lt;sub&gt;62&lt;/sub&gt;<em>,Asp</em>&lt;sub&gt;85&lt;/sub&gt;*</td>
<td><em>Leu</em>&lt;sub&gt;82&lt;/sub&gt;<em>&lt;sub&gt;Clu&lt;/sub&gt;</em></td>
</tr>
<tr>
<td><strong>C-terminal sequence</strong></td>
<td>-Val*&lt;sub&gt;81&lt;/sub&gt;<em>&lt;sub&gt;Leu&lt;/sub&gt;</em>&lt;sub&gt;81&lt;/sub&gt;<em>&lt;sub&gt;Ala&lt;/sub&gt;</em>&lt;sub&gt;81&lt;/sub&gt;</td>
<td>-Val*&lt;sub&gt;33&lt;/sub&gt;<em>&lt;sub&gt;63&lt;/sub&gt;</em>,Leu*&lt;sub&gt;86&lt;/sub&gt;<em>&lt;sub&gt;Ala&lt;/sub&gt;</em>&lt;sub&gt;86&lt;/sub&gt;</td>
<td>unknown</td>
</tr>
<tr>
<td>Phosphorus atoms / mole</td>
<td>1&lt;sup&gt;(21)&lt;/sup&gt;</td>
<td>1&lt;sup&gt;(31)&lt;/sup&gt;</td>
<td>0&lt;sup&gt;(82)&lt;/sup&gt;</td>
</tr>
<tr>
<td>Half-cystines / mole</td>
<td>6&lt;sup&gt;(37)&lt;/sup&gt;</td>
<td>6&lt;sup&gt;(37)&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Lysines / mole</td>
<td>12&lt;sup&gt;(81)&lt;/sup&gt;</td>
<td>1 or 2&lt;sup&gt;(11,12)&lt;/sup&gt;</td>
<td>4&lt;sup&gt;(82)&lt;/sup&gt;</td>
</tr>
<tr>
<td>Molecular weight (a)</td>
<td>42,862&lt;sup&gt;(81)&lt;/sup&gt;</td>
<td>35,298&lt;sup&gt;(11,12)&lt;/sup&gt;</td>
<td>3242&lt;sup&gt;(82)&lt;/sup&gt;</td>
</tr>
<tr>
<td>Molecular weight (b)</td>
<td>42,500&lt;sup&gt;(61)&lt;/sup&gt;</td>
<td>34,500&lt;sup&gt;(18,37,51,57,61,69,78)&lt;/sup&gt;</td>
<td>3100&lt;sup&gt;(32)&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* terminal amino acid
(a) sum of analytical values for amino acid analyses
(b) sum of physical measurement values
different, it appears that the activation of pepsinogen to pepsin is brought about by the proteolysis of several peptides from the N-terminal end of the pepsinogen molecule. The N-terminal amino acid of the pepsin inhibitor is leucine, and the amino acid penultimate to the N-terminal is glutamic acid. This sequence differs from the N-terminal sequence of pepsinogen, indicating that the pepsin inhibitor is not the peptide split from the original N-terminus of the precursor. With this information, the activation may be pictured schematically in the following way:

\[
\text{[Leu,Leu.........]} \quad \text{[Leu,Glu.........]} \quad \text{[Leu,Gly....Leu,Ala]} \\
\text{peptides} \quad \text{inhibitor} \quad \text{pepsin}
\]

\[
\text{pepsin} \quad + \quad \text{inhibitor} \quad + \quad 5 \text{ peptides}
\]

The amino acid compositions of pepsinogen (81), pepsin (11, 12) and inhibitor (82) are shown in Table V.

Isoleucine was found to be the N-terminal amino acid of pepsin by Berriott (33), Blumenfeld and Perlmann (11), and Heirwegh and Edman (28). Williamson and Passmann (85, 62) found the N-terminal amino acid to be leucine. However, the chromatographic systems used by the other investigators to distinguish leucine from isoleucine appears to be more discriminating than the system used by Williamson and Passmann.
<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Residues per mole*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pepainogen (A)</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>46</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>32</td>
</tr>
<tr>
<td>Glycine</td>
<td>36</td>
</tr>
<tr>
<td>Alanine</td>
<td>27</td>
</tr>
<tr>
<td>Valine</td>
<td>27</td>
</tr>
<tr>
<td>Leucine</td>
<td>64</td>
</tr>
<tr>
<td>Isoleucine</td>
<td></td>
</tr>
<tr>
<td>Serine</td>
<td>53</td>
</tr>
<tr>
<td>Threonine</td>
<td>25</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>6</td>
</tr>
<tr>
<td>Methionine</td>
<td>5</td>
</tr>
<tr>
<td>Proline</td>
<td>20</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>0</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>20</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>16</td>
</tr>
<tr>
<td>Tryptophane</td>
<td>4</td>
</tr>
<tr>
<td>Histidine</td>
<td>4</td>
</tr>
<tr>
<td>Lysine</td>
<td>12</td>
</tr>
<tr>
<td>Arginine</td>
<td>3</td>
</tr>
<tr>
<td>Amide NH₃</td>
<td>39</td>
</tr>
<tr>
<td>Total amino acid</td>
<td>400</td>
</tr>
</tbody>
</table>

* rounded off to the nearest integer

(A) & (D) analysis by VanVunakis and Berriott (81) & (82)
(B) analysis by Brand (12)
(C) analysis by Blumenfeld and Perlmann (11)
Williamson and Passmann determined the N-terminal amino acid sequence to be Leu,Gly,Asp,Asp,His,Glu-. The C-terminal amino acid is alanine, and a partial C-terminal amino acid sequence was determined to be -Val,Leu,Ale (33, 63, 86).

Brand (12) first estimated four half-cystines to be present in pepsin. Kern's work indicated the presence of six half-cystines existing as disulfide bridges linking the protein chain at three sites (37). Blumenfeld and Perlmann (11) more recently estimated only four half-cystines.

A unique structural feature of the pepsin molecule, and also of pepsinogen, is the presence of a single phosphate ester of the -O-P-O- diester type (64, 66). This phosphorus can be removed enzymatically by potato phosphatase at pH 5.6. As might be expected, the dephosphorylated pepsin has a slightly higher isoelectric point than pepsin itself (pH 1.7), the mobility differences between the two proteins below pH 4.0 being of the magnitude expected for the removal of one group with one dissociable proton. Since dephosphorylated pepsin is still fully active, it is evident that the O-P-O diester bond is nonessential for the biological activity of these proteins. The structural function of this diester bond must be to link two distant sites of the single peptide chain into a loop, as suggested by Perlmann (66, 67). As yet, only one point of attachment of the diester bond has been determined. Flavin (59, 58) has provided evidence that
the phosphate group in pepsin is esterified to a serine residue in the sequence \(-\text{threonyl-seryl phosphate-glutamyl}\).

Pure pepsin is believed to have an isoelectric point below pH 1, a value which is in accord with the low content of basic amino acids (62, 75, 76). However, considering the pK of the free carboxylic amino acid groups, the isoelectric point can be calculated to be about pH 3.0. McLaren postulated the absorption of chloride ion as the major cause of the low isoelectric point of pepsin.

Studies to determine the portion of the pepsin molecule required for proteolytic activity have been performed by various investigators. These studies have been summarized briefly in Table VI. The data indicates that at least some of the tyrosine residues are involved in the active center of pepsin. The autodigestion studies indicate that the proper spatial configuration of the molecule is also essential, and that the enzymatically active portion of the molecule is probably not located near the N-terminal end.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction of 20 acetyl groups</td>
<td>Inactivation (29)</td>
</tr>
<tr>
<td>Introduction of less than 20 acetyl groups</td>
<td>Slight decrease in activity (29)</td>
</tr>
<tr>
<td>Iodination of all tyrosine residues</td>
<td>Inactivation (30)</td>
</tr>
<tr>
<td>UV oxidation of tyrosine → DOPA</td>
<td>Inactivation (35)</td>
</tr>
<tr>
<td>Reduction of one –S–S– bond</td>
<td>Activity retained (37)</td>
</tr>
<tr>
<td>Reduction of more than one –S–S– bond</td>
<td>Inactivation (37)</td>
</tr>
<tr>
<td>Dephosphorylation</td>
<td>All activity retained (66, 67)</td>
</tr>
<tr>
<td>Autolysis at pH 5.0, 4°C and 37°C, 48 hours</td>
<td>Activity lost. Leu, Phe, and Leu peptides dialysable (87)</td>
</tr>
<tr>
<td>Storage in 1.0 to 6.0 M urea, 1 to 15 days, at 30°C.</td>
<td>No loss in activity (78)</td>
</tr>
<tr>
<td>Storage in 8.0 M urea at 20°C.</td>
<td>Rapid loss in activity (67, 68)</td>
</tr>
<tr>
<td>Autolysis in 8.0 M urea at 37°C, 20 hours</td>
<td>Apparent decrease in activity and recovery of a smaller fragment with 50% higher specific activity (67, 68)</td>
</tr>
</tbody>
</table>
CHAPTER III

THE RATIONALE OF THE PRESENT STUDY

Trypsin has been selected as the tool for the study of the structure of pepsin for several reasons. As previously pointed out, trypsin is, by far, the most specific of the proteolytic enzymes. Trypsin is known to most readily hydrolyze the peptide bonds involving the carboxylic groups of arginine and lysine. According to the amino acid analyses shown in Table V, pepsin contains two arginine residues (11, 12), and either one (1) or two (12) lysine residues. Therefore, the tryptic hydrolyses of pepsin would be expected to result in the formation of either four or five peptides. Since pepsin has a molecular weight of about 35,000, the average peptide weight would be about 7,000, or equivalent to a chain length of 60 to 70 amino acids. Polypeptides of this size are more amenable to amino acid sequence determination than are large proteins.

The original intention of the author was to separate and characterize the peptides obtained from a tryptic hydrolysis of pepsin. In order to determine the number of peptides that would result from this type of enzymatic digestion, a two dimensional chromatogram of the dinitrophenyl derivatives of the E-
terminal amino acids of these peptides was run. Seven dinitrophenyl amino acids were detected on the chromatogram. Two of them, DNP-threonine and DNP-glycine, appeared to be present in smaller amounts than the others (Fig. 1). This number was higher than expected on the basis of trypsin specificity, but many cases of non-specific peptide bond splits, due to the action of trypsin, have been reported.

Fig. 2 illustrates an attempt to separate the peptides of the hydrolysis of pepsin as dinitrophenyl derivatives. A four-hour trypic hydrolysate of pepsin was dinitrophenylated in 50% acetone, and acidified to pH 2 with HCl. The yellow precipitate of DNP-peptides that formed was separated from the supernatant by centrifugation. Two DNP-peptides were found in the supernatant, and their N-terminals were isoleucine and alanine. The dry precipitate was suspended in water and placed on a watercellulose column. A DNP-aspartic acid N-terminal, and the other with a DNP-serine N-terminal, were eluted with 50% ethanol. Then 0.05 M NaHCO\textsubscript{3} in 50% ethanol was used on the eluent and a peptide with an N-terminal isoleucine was eluted. An eluent of 20% pyridine in 50% ethanol contained at least two DNP-peptides, and when 0.05 M sodium hydroxide were used, at least three more DNP-peptides were separated. After eluting with the NaOH, the column still retained some DNP-peptide material.

This preliminary separation indicated that the trypsic
FIGURE 1

A TWO DIMENSIONAL CHROMATOGRAM OF DNP DERIVATIVES
OF THE N-TERMINAL AMINO ACIDS FROM A
TRYPTIC HYDROLYSATE OF PEP3IN

The "toluene" solvent was run first. Both solvents
were run for 16 hours at RT.
Tryptic Hydrolysate Of Pepsin

\[ \text{DNFB in 50% acetone} \]

\[ \text{DNF-Peptides} \]

Evaporate acetone.
Add HCl to pH 2; centrifuge.

Precipitate (Cellulose Column) \[ \rightarrow \text{Supernatant} \]

\[ \text{Extract with Ether} \]

\[ \rightarrow \text{DNF-OH} \]

\[ \text{Extract with Butanol} \]

\[ \rightarrow \text{Aqueous Colorless} \]

\[ \text{DNF-Peptides in Butanol (2)} \]

\[ \text{Extract with 0.1 M NaHCO}_3 \]

\[ \rightarrow \text{DNF-peptide (DNF-Ala)} \]

\[ \text{DNF-Peptide in Butanol (1)} \]

\[ \text{DNF-Peptide in Butanol (1)} \]

\[ \text{(DNF-Ileu)} \]

\[ \text{DNF-Peptides in Butanol (2)} \]

\[ \text{DNP-Peptides retained on column} \]

**FIGURE 2**

**SEPARATION OF DNP DERIVATIVES OF PEPTIDES OBTAINED FROM A FOUR HOUR TRYPIC HYDROLYSATE OF PEPsin**
hydrolysis of pepsin gave rise to at least eleven peptides. This result was puzzling in the light of the known specificity of trypsin. Furthermore, the two peptides with N-terminal alanine, and N-terminal isoleucine, were found to be present in both the supernatant and the precipitate. The possibility then arose that the peptides found in the supernatant were smaller peptides produced from a partial splitting of two larger peptides found in the precipitated fraction. This would indicate an incomplete hydrolysis of pepsin, and a re-evaluation of the idea of enzymatic hydrolysis appeared necessary. The procedures used for obtaining the two dimensional chromatograms, and for the separation of DNP-peptides, will be described later.

A straight chain protein which contains four bonds labile to enzymatic hydrolysis, could theoretically yield fourteen peptides, sometime intermediate to complete hydrolysis. Nine of these peptides would be the products of incomplete enzyme action; i.e. combinations of the five peptides obtained after complete hydrolysis. This can be shown schematically, by considering a protein consisting of five peptides "ABCDE":

\[
\begin{align*}
\text{ABCDE} & \rightarrow A + B + C + D + E \\
\text{ABCDE} & \rightarrow \text{ABCD} + \text{BCDE} + \text{CDE} + \text{DE} + E \\
& \rightarrow \text{ABC} + \text{BCD} + \text{CD} + \text{D} \\
& \rightarrow \text{AB} + \text{BC} + \text{C} \\
& \rightarrow A + B \\
\end{align*}
\]

\text{complete hydrolysis} \quad \text{incomplete hydrolysis}
In the above scheme, the letters, A, B, C, D, E, and the combinations of letters represent peptides obtained by hydrolysis of from one to four peptide bonds in the protein. If the protein ABCDE consists of a single straight chain polypeptide, it will have only one N-terminal amino acid at the end of the "A" peptide. After complete tryptic hydrolysis, there should be four new N-terminal amino acids containing free alpha amino groups in the peptide mixture, one each from B, C, D and E. It then follows that regardless of the extent of hydrolysis, only a total of five N-terminal amino acids should be observed at any one time, even though more than five peptides will be present if the reaction is stopped at any time prior to the time of complete hydrolysis.

With this picture in mind, the results shown in Fig. 3 are not surprising. A four-hour hydrolysate obviously yields some peptides which are products of incomplete hydrolysis. From the two dimensional chromatogram (Fig. 1), it appears that pepsin contains at least six bonds labile to hydrolysis by trypsin, giving rise to seven peptides after complete hydrolysis. In keeping with the above scheme, 27 peptides could theoretically result from a partial tryptic hydrolysate of pepsin. Such a mixture of peptides would be difficult to separate, since the 27 peptides would be the various combinations of seven peptides. A knowledge of the conditions necessary to give optimal hydrolysis of
the protein appears desirable.

Many studies on the structure of proteins with the use of proteolytic enzymes have been reported in the literature. But in only a few of these (13, 34), has the rate of hydrolysis of the protein under study been determined. In neither of these cases have the authors indicated that the conditions used gave optimal hydrolysis of the protein.

In view of the fact that the pH optimum of enzymes in general, and proteolytic enzymes in particular, can vary widely depending on the substrate (16), it seems most important to actually determine the pH that will give the maximum enzymatic digestion of the protein. The most recent studies on the stability of enzymes, with particular reference to trypsin (14, 50), have indicated that the buffer concentration of the medium can have a marked effect on enzymatic stability. These two points, coupled with the fact that both enzyme and substrate concentrations are important factors affecting the rate of hydrolysis of a substrate, justify an exploration into the rate of hydrolysis of pepsin by trypsin.
CHAPTER IV

EXPERIMENTAL PROCEDURE

In the present study, the overall rate of hydrolysis of pepsin by trypsin was measured first by the use of the ninhydrin reagent. This reagent reacts with all free alpha amino groups present in the protein, and gives a reaction with free ammonia as well. All efforts by various investigators to quantitate the color produced with ninhydrin to the actual number of free amino groups freed on hydrolysis of a protein, have failed. Moore and Stein made a reasonable estimation of the number of bonds broken in ribonuclease by trypsin, by comparison of the color produced by ninhydrin with that given by the chymotryptic hydrolysis of the same protein, in which the number of peptide bonds split had already been determined by other means (34).

In an effort to obtain an insight into the relative size of the peptides obtained, a second method for following the rate of hydrolysis was used. This was to measure the amount of trichloroacetic acid soluble peptides, using the Folin-Ciocalteu reagent for tyrosine. Pepsin contains from 16 to 18 tyrosine residues (11, 12), so that the distribution of tyrosine throughout the protein may be expected.
The rate of hydrolysis of each individual peptide bond can be followed if a method is devised for easy separation of the N-terminal amino acids of each of the peptides. Quantitative evaluation of each of these N-terminal amino acids might demonstrate the relative susceptibility of the peptide bonds, in which they are involved, to trypsin hydrolysis. Since the possibility exists that each peptide bond may be hydrolysed optimally at a different pH, this type of study seems more than worthwhile.

Dinitrofluorobenzene (DNFB) is an ideal reagent for labeling of free amino groups present in a protein hydrolysate, and it is the reagent chosen for the present study. After treating the peptides with DNFB, they were completely hydrolysed in constant boiling hydrochloric acid, and the dinitrophenyl (DNP) labeled amino acids were separated by extraction into ether from an acid medium. Paper chromatography was used for separation of the DNP amino acids, in preference to column chromatography, because the number of end groups was not certain, and there were a large number of samples to be dealt with. After paper chromatographic separation, the quantitative estimation was performed by a densitometric procedure.

The rate of hydrolysis of pepsin was determined in 0.2 M phosphate buffers at pH 6.7, 7.5, 8.0 and 8.3. In order to determine the effect of buffer concentration on hydrolysis, 0.4, 0.1, 0.05 and 0.01 M phosphate buffers at pH 7.5 were used.
To see if different buffers exhibited an effect, 0.1 M phosphate was compared with 0.1 M sodium bicarbonate at pH 6.0. Since there was no reason to believe that various buffer concentrations or different buffer solutions would have an effect on the lability of individual peptide bonds to enzymatic hydrolysis, the DNFB method was used only where the pH was varied. The salt concentration of the medium was expected only to affect the stability of the trypsin.

**Pepsin Preparation:** The pepsin used in these experiments was of porcine origin and was recrystallized three times (Armour, Lot No. 108-145). The pepsin was prepared by the method of Northrup (59). Electrophoretic analysis at pH 4.3 in acetate buffer (ionic strength = 0.1) showed the preparation to move essentially as a homogeneous protein. A slower moving fraction represents about 4% of the total protein. The preparation contained 6.5% moisture, 2.7% ash, and 14.4% nitrogen.

The purity of the pepsin preparation was also determined by solubility in 1.5 M MgSO₄, pH 5.0 acetate buffer (Fig. 3). The solution has been used by Northrup and Harriott to separate native and denatured forms of pepsin. Only two major components are detected by the curve. A visual precipitate of the first component is first seen after 12 mg. of the preparation has been added to the solvent. This, in all probability, is the denatured pepsin present in the preparation, since it exhibits
SOLUBILITY OF Pepsin
IN 1.5M MgSO\textsubscript{4}, pH 5.0

14.4\% NITROGEN
2.7\% ASH
6.5\% MOISTURE

FIGURE 3

SOLUBILITY CURVE OF PEPsin PREPARATION

Armour, Lot \# 108-145. The pepsin was shaken with the solvent, centrifuged, and 1.0 ml. of the supernatant was diluted to 25.0 ml. with water. The optical density of this solution was read in a Beckman DU spectrophotometer.
essentially the same solubility characteristics. A slight bending of the curve occurs after 18 mg. of pepsin per ml. of solution has been added, that represents about 5 to 6 per cent of the total absorption. This component corresponds to the 4 per cent of "non-pepsin" protein estimated by electrophoretic analysis. The total pepsin in the sample is calculated to be 87 per cent.

Trypsin Preparation: Lyophilized crystalline trypsin (Lot No. 62330) was obtained from Worthington Biochemical Corporation. The trypsin was prepared by the method of Kunitz and Northrup (39). All salt impurities have been removed by dialysis without loss of enzymatic activity.

Enzymatic Hydrolysis Technique: All glassware used for enzymatic hydrolysis was sterilized in a 250° C. oven for three hours. Solutions were sterilized before being used by heating to 95° C. for 15 to 30 minutes, slowly cooling to room temperature for 30 minutes and then heating again for the same length of time.

The pepsin samples were weighed directly into sterile eight-inch test tubes and dissolved in a volume of sterile buffer solution sufficient to give a concentration of 2.0 mg. pepsin per ml. The samples were incubated in a 37° C. water bath for 30 minutes before trypsin was added. Since pepsin is rapidly denatured above pH 6 (59), the enzyme was permanently inactivated. Trypsin solutions were made up in sterile water in such manner that one ml. of solution would contain 1/50 of the total weight
of pepsin that was to be hydrolysed (Enzyme:substrate ratio of 1:50). One ml. of the trypsin solution was added to the pepsin solution and immediately mixed. An aliquot was taken within one minute after mixing to serve as a blank; other aliquots were taken at various times throughout the digestion period. The aliquots were added directly to a volume of 0.5 M HCl sufficient to give a resultant pH of 1.5 to 2.0, and immediately frozen.

After all the aliquots were collected they were thawed, brought to about pH 5 with bicarbonate, and then buffered to pH 5.5 with 2.4 M, pH 5.5 acetate buffer. The aliquot was then diluted to a final volume that would contain 1 mg. of hydrolysed pepsin per ml.

Preparation of Buffer Solutions: Phosphate buffer solutions were made from 0.20 M solutions of Na₂HPO₄ and NaH₂PO₄. These were mixed to give 0.20 M buffers of pH 6.7, 7.5, 8.0 and 8.3. The pH of all buffers were checked with a Beckman pH meter and were found to be within ± 0.05 pH 0.05 pH units of desired value.

Phosphate buffers of varying concentration were made by diluting 0.40 M phosphate buffer, pH 7.5. In the experiment reported, 0.4 M, 0.1 M, 0.05 M, and 0.01 M buffers were used.

Preparation of Ninhydrin Reagent: Two grams of ninhydrin and 0.2 gm. of hydrindantin were dissolved in 50 ml. of methyl cellulose, and 50 ml. of 2.4 M, pH 5.5 acetate buffer were added (49). The reagent was made fresh, immediately prior to use.

Hydrindantin was prepared by the reduction of ninhydrin
with ascorbic acid (49, 84). To 40 gm. of ninhydrin in one liter 
of water at 90° C, 40 gm. of ascorbic acid in 200 ml. of water at 
40° C, were added. Crystallization of hydrindantin starts im-
mediately and is allowed to proceed for 30 minutes without fur-
ther heating. The solution was allowed to cool under running 
tap water for one hour. The hydrindantin was filtered off, 
washe& well with water and dried over P₂O₅ in vacuum desicator,
protected from light.

**Ninhydrin Reaction:** A 0.5 ml. aliquot of the sample containing 
0.5 mg. of hydrolysed pepsin was added to one milliliter of the 
ninhydrin reagent in a four-inch test tube. The tubes were im-
mersed in boiling water for 15 minutes and then cooled to room 
temperature. They were then diluted to 25.0 ml. with water, and 
the color of each was read with a Klett-Summerson colorimeter, 
using a #56 green filter. The color values were expressed as 
being equivalent to the color produced by a certain concentration 
of leucine.

A 0.10 mg. per ml. solution of leucine in pH 5.5 
acetate buffer was diluted to various concentrations and treated 
in the same manner as the samples for the standard curve. Stand-
ards containing 5 to 50 micrograms of leucine per ml. were used 
to construct the standard curve (Fig. 4).

**Preparation of Folin-Ciocalteu Reagent:** The following reagents 
were used to prepare 2.5 liters of Folin-Ciocalteu reagent:
NINHYDRIN STANDARD CURVE

FIGURE 4

STANDARD CURVE FOR THE NINHYDRIN REACTION

Total dilution is 25.0 ml. A Klett-Summerson colorimeter was used with a #56 filter.
250 gm. sodium tungstate
63 gm. sodium molybdate
1,750 ml. distilled water
105 ml. phosphoric acid (85%)
250 ml. concentrated HCl

The mixture was refluxed for ten hours, cooled, and then 375 gm. of lithium sulfate and 1.3 ml. bromine were added. The mixture was boiled for 20 minutes, or until no more bromine was liberated. This solution was then cooled and diluted to 2.5 liters with water.

**Determination of TCA Soluble Tyrosine:** The amount of trichloroacetic acid soluble tyrosine was determined by use of the Folin-Ciocalteu reagent. A 1.0 ml. aliquot of the digestion sample was added to a 15 ml. centrifuge tube containing 1.0 ml. of 10% trichloroacetic acid. The tube was mixed and then centrifuged for ten minutes. 1.0 ml. of the supernatant was carefully removed from the tube and transferred to a Klett tube containing 2.0 ml. of 1.5 M sodium carbonate. After mixing, 1.0 ml. of Folin-Ciocalteu reagent was added and the tube contents were mixed again. After 20 minutes the color produced was measured in a Klett-Summerson colorimeter, using a 559 filter.

The tyrosine standards were made by dilution of a 0.10 mg. per ml. tyrosine solution in 0.1 M sodium carbonate. Standards ranging from 5 to 45 micrograms tyrosine per ml. were used.
to construct a standard curve (Fig. 5). "Tyrosine Equivalents" were used to plot the standard curve. One "Tyrosine Equivalent" was calculated from the formula:

\[
\text{mg of Tyrosine in sample} = \frac{\text{mg Tyrosine}}{\text{mg pepsin}} \times \text{mg pepsin}
\]

where MW = molecular weight, and mgm. = micrograms.

Treatment of the Hydrolysate with DNFB: An aliquot of the hydrolysis sample containing 8 mg. of hydrolysed pepsin was added to 7 ml. of acetone. The solution was saturated with sodium bicarbonate, and 1.0 ml. of a 5 mg. per ml. solution of dinitrofluorobenzene (DNFB) in acetone was added. The solutions were allowed to react in the dark for three hours and then were made acid to Congo red with HCl. The acetone was evaporated off in a hot water bath and the total volume of the solution reduced to 5 ml. by boiling in a sand bath. Five milliliters of concentrated HCl were added, and the solution was allowed to boil down to a volume of 10 ml. The solutions were then brought to a total volume of 10 ml. with 5.7 N HCl and set up in a sand bath and allowed to reflux for 16 hours. The temperature of the sand bath was kept between 105° and 115° C.

After acid hydrolysis, the samples were cooled, transferred to a separatory funnel, and extracted three times with 10 ml. portions of ether. The ether extracts were dried over
TYROSINE STANDARD CURVE

"Tyrosine Equivalent" = \( \frac{MW(\text{tyr}) \times \text{mg pepsin in sample}}{MW(\text{pepsin})} \)

Solutions were read on a Klett-Summerson colorimeter with a #59 filter. Total dilution = 4.0 ml.
anhydrous sodium sulfate, and after removal of the ether, the residue was subjected to vacuum sublimation to remove the dinitrophenol present (46). The residue was then washed into a six-inch test tube with ether, and the ether evaporated to dryness. The sample was now ready for application to the chromatogram.

The DNP-amino acids present in the hydrolysate were initially identified by a two dimensional chromatographic system (41) described below (Fig. 6). They were further characterized by elution from the two dimensional chromatogram and rechromatographing in other solvent systems.

Paper Chromatography: Whatman #1 paper was used for all systems, and the chromatograms were developed by descending solvent flow in a Chromatocab. A sheet of chromatographic paper 22-1/4" x 18-1/2" was used for the two dimensional chromatograms. Pencil lines were drawn three inches from one side and three inches from the adjacent side of the paper, and the material was applied from a micropipette at the point where the lines intersected. Sheets of paper of various sizes were used for the one dimensional systems, and the substances were applied along a line three inches from the top of the paper.

Authentic DNP-amino acids were placed alongside of all unknown samples, on the one dimensional chromatograms, and on the two dimensional chromatograms, they were placed in the corner of the paper just opposite the sample spot.
2-Dimensional chromatogram of a synthetic mixture of dinitrophenyl amino-acids (approx. 0.02 μM of each)

FIGURE 6

CHROMATOGRAPHIC SYSTEM FOR IDENTIFICATION
OF DNP-AMINO ACIDS (41)
Solvent Systems:

(1) "Toluene" Solvent (9, 41). This was the solvent used to develop the two dimensional chromatograms in the first direction. Toluene-pyridine-ethyleneschlorohydrin were mixed in the volume ratio 5:1.5:3 in a separatory funnel. Three volumes of aqueous 0.8 M ammonia was then poured carefully down the side of the funnel and the two layers allowed to equilibrate for one hour. The aqueous layer was separated and used to saturate the atmosphere of the chromatographic chamber. The chromatogram was developed with the organic layer for a period of 16 hours at room temperature. Total movement of the solvent front was about 18 inches past the point of application of the DNP-amino acids. After development, the chromatograms were allowed to dry three hours before developing in the second direction.

(2) 1.5 M Phosphate Buffer, pH 6.0 (41). This was the solvent used to develop the two dimensional chromatograms in the second direction. It was allowed to develop at right angles to the direction of the "Toluene" solvent. The buffer was prepared by mixing one volume of 1.5 M Na₂HPO₄ with five volumes of 1.5 M NaH₂PO₄. The phosphate buffer moved down the chromatogram about 14 inches in 16 hours.

(3) Amyl Alcohol-Acetic Acid (9). Normal amyl alcohol was saturated with 15% acetic acid in a separatory funnel. The aqueous phase was drawn off and used to equilibrate the chromat-
graphic chamber. The organic layer was used to develop the chromatogram. Chromatograms were allowed to run about 20 hours, during which time the solvent front moved about 10 inches past the point of application of the DNP-amino acids.

(4) Amyl Alcohol-pH 6.0 Phthalate Buffer 0.05 M.
Normal amy! alcohol was saturated with pH 6.0 phthalate buffer. The buffer was prepared by mixing 450 ml. of 0.1 M NaOH with 500 ml. of 0.1 M potassium acid phthalate, and adjusting the pH to 6.0 by the addition of 0.1 M NaOH. The chromatogram paper was washed with the phthalate buffer and air dried prior to use. Chromatograms were developed for 16 hours, in which time the solvent front moved about 14 inches past the point of application of the DNP-amino acids. This was the solvent system that was used to separate the DNP-amino acids for densitometric measurements.

Preparation of Authentic DNP-Amino Acids: Most of 2,4-dinitrophenyl amino acids used in this study were prepared using the method of Sanger (73), by Williamson and Passmann (62). DNP-threonine, DNP-aspartic acid, and DNP-glutamic acid were prepared by the author. About 100 mg. of the amino acid, and 100 mg. of sodium bicarbonate were dissolved in 20 ml. of water, and 300 mg. of 2,4-dinitrofluorobenzene in 20 ml. of acetone was slowly added with constant stirring. The mixture was stirred for three hours at room temperature. The acetone was removed
under reduced pressure, and the residual solution was acidified to pH 2 with 2 M HCl. The precipitate obtained upon acidification was recrystallized from aqueous methanol. Some of the derivatives thus prepared were found to contain large amounts of dinitrophenol. This was removed by low vacuum sublimation, according to Mills (46). The purity of the DNP compounds was established by determination of the Rf in the "Toluene" solvent (9), and by the melting point (70, 73). In both cases, the values that were established agreed with the values found in the literature.

**Densitometry:** There were two major reasons for using densitometry as the method for quantitative evaluation of the DNP-amino acids. First, an automatic scanning recorder for the densitometry of substances on paper strips was available. Secondly, densitometric reading of the chromatograms directly on paper would eliminate the manipulations required if the spots were to be eluted and read spectrophotometrically.

To establish the applicability of densitometry for DNP-amino acids on paper, various quantities of DNP-leucine, DNP-serine, DNP-aspartic acid, and DNP-alanine were chromatographed in 1.5 M phosphate buffer, and the chromatograms were read, with the use of a special adapter for paper strips, in a Beckman DU spectrophotometer at 360 nm. The optical density was read at 6 mm intervals along the paper strip. Fig. 7 is a plot of the optical density readings versus distance from the origin for the
The optical density of the paper chromatograms, developed in pH 6.0 phosphate buffer, was read in a Beckman DU spectrophotometer at 360 μm. The area under the curves was measured by planimetry.
DNP-leucine chromatograms. The area under the curves was obtained with a planimeter. A straight line was obtained when the area under the curve was plotted against the concentration of the spot.

**Apparatus for Densitometry:** The apparatus used has been described by McDonald (45) and consists of six components:

1. A Bausch & Lomb grating monochromator connected to a tungsten filament lamp was used as the light source. The monochromator has entrance and exit slits that can be adjusted from 0 to 1 cm. All lenses in the system are quartz, therefore, they transmit energy throughout the scale range. A wavelength control allowed chopping of the spectrum, but since only a tungsten lamp was available, the wavelengths were restricted to the visible light region. All DNP-amino acids in solution absorb optimally at 360 millimicrons (62, 73), but this wavelength could not be achieved with the setup used. However, a wavelength of 410 millimicrons was found to be satisfactory. Upon leaving the grating system, the light is focused through a quartz lens on a slit 10 mm. long and 1 mm. wide.

2. A Welch motor driven feed system designed to accommodate paper strips, was arranged in front of the light path. The motor was synchronized to run at the same speed as the chart recorder.

3. A Welch blue sensitive photoelectric probe holds
the paper strip against the light slit. The photoelectric tube converts the transmitted light to an electrical current that will vary as an exponential function of the amount of material on the paper.

(4) The electrical current was amplified by a Welch Densichron amplifying unit. This unit has a dial calibrated in optical density units.

(5) After amplification, the current is fed to another electrical circuit, the Welch Logarithmic Converter, which "linearizes" the signal received from the densichron.

(6) The current is then transferred from the converter to a 10 mv. range Minneapolis-Honeywell Brown Electronik strip chart recorder, where a permanent record of the chromatogram is charted on special record paper. A tracing is thus obtained in which the area under the various portions of the curve is directly proportional both to the quantity of the material presented, and to the area over which the material is spread.

Procedure for Densitometry: The mixtures of DNP-amino acids obtained from the hydrolysate aliquots, after sublimation, were dissolved in approximately 0.15 ml. of 95% ethanol, and 50 microliters of this was applied to the paper as a streak, one cm. long. About two microliters were applied at a time, and the spot was dried between applications. The n-amyl alcohol-pH 6.0 phthalate buffer system was used, since this gave the best
separation of all the DNP-amino acids involved. The chromatograms were developed for 16 hours, and air dried for two hours.

The developed patterns were cut into strips two cm. wide, and clamped in place on the paper strip feeder. The light beam was focused on the paper, two cm. behind the origin of application of the DNP-amino acids. The photoelectric probe was put in place over the strip. Using the densichron dial, the paper was set at zero optical density, with monochromator entrance and exit slit widths of 2.5 mm. The log converter was set so that the optical density range from 0 to 2, would be linearized. The recording potentiometer was then balanced on this linear signal. The charts used on the recorder were marked from "0" to "100". At zero optical density the recording pen was set on the "100" line of the chart, and at a density of two, the pen was set on the "0" line.

It was necessary to set the instruments only once in a day's run, with the exception that the papers had to be set at zero optical density each time. The chart and paper strip were started simultaneously, and allowed to run from the point of application of the DNP-amino acids to the solvent front of the chromatogram.

**Evaluation of Densitometer Curves:** Fig. 8 is a pattern obtained from a chromatogram of an equimolar mixture of DNP-amino acids. Three important effects of chromatography can be noted:
FIGURE 8

DENSITOMETRIC TRACING OF A CHROMATOGRAM CONTAINING
EQUIMOLAR AMOUNTS OF DNP AMINO ACIDS AND DINITROPHENOL

The chromatogram was developed with the Amyl alcohol - phthalate buffer solvent system, and read on a recording densitometer.
(a) The maximum optical density of the spot decreases with increasing displacement from the origin. The maximum density of a spot is proportional to the concentration of substance on the paper (10); this correlation, however, can be made only between substances that have moved exactly equal distances.

(b) As can be seen from the base of each spot, the spot area on the paper increases with increasing displacement from the origin.

(c) The tracings obtained from the densitometry of DNP-amino acids on paper are essentially triangular. The area under a tracing, is then approximately equal to the area of a triangle: Area = 1/2h x b, where h is the maximum density or height of the curve, and b is the base of the curve or length of the spot.

In the present method, the area under the curve was evaluated by the method of Moore and Stein (48) where the peak of the curve, and the width of the curve at one-half the peak value, are measured. The product of these two values is taken as the area.

Separation of DNP-Peptides from a Tryptic Hydrolysis of Pepsin:

A separation of the peptides of tryptic hydrolysis was attempted (Fig. 2). The sample was prepared by dissolving 50 mg. of pepsin in 25 ml. of 0.1 M sodium bicarbonate, and adding one mg. of trypsin. The hydrolysis was allowed to proceed for four hours,
then 25 ml. of acetone containing 10 mg. of 2,4-dinitrofluoro- 
benzene was added. The solution was placed in the dark for three 
hours, then made acid to congo red with 2 M HCl. The acetone 
was evaporated off and the precipitate that had formed was sepa-
rated by centrifugation and washed three times with 10 ml. of 
0.05 M HCl, twice with water, and then lyophilized. The washings 
and supernatant were combined and extracted with three 25 ml. 
portions of ether. Some of the yellow color of the aqueous 
portion was transferred to the ether. This was shown by paper 
chromatography to be dinitrophenol, and was discarded. Upon 
shaking the aqueous layer with n-butanol, all remaining yellow 
color was extracted. The butanol was evaporated to dryness under 
reduced pressure, and the residue was taken up in 5.7 M HCl and 
hydrolysed for 16 hours. The DNP-amino acids were then extracted 
and treated as previously described. They were identified by 
paper chromatography using the "Toluene" solvent system.

The precipitated fraction was suspended in water and 
the various fractions were separated on a cellulose column and 
identified. The column procedure used is as follows:

A glass column 25 x 1 cm. was used. Powdered cellulose 
was made into a slurry with water and was packed into the column 
to a height of about 15 cm. The column was washed with 50 ml. 
of water and the sample was added just as the last of the water 
entered the cellulose. The column was washed with 50 ml. more
of water, and then followed by 50 ml. portions of 50% ethanol, and 20% pyridine in 50% ethanol. Then a 100 ml. portion of 0.05 M sodium hydroxide was added.

The DNP-peptide fractions eluted were reduced to a volume of 2 ml. and 2 ml. of concentrated HCl were added. They were allowed to reflux 15 hours, and were treated as other samples, previously described. Identification of the N-terminal amino acids of the peptides was performed only in the "Toluene" solvent.
CHAPTER V

RESULTS AND DISCUSSION

In order to determine the effect of pH on the tryptic hydrolysis of pepsin, at 37° C, the reaction was carried out in 0.2 M phosphate buffers at pH 6.7, 7.5, 8.0 and 8.3. Aliquots of each reaction mixture were removed at various time intervals and the amount of pepsin hydrolysed in each aliquot was determined by two methods.

The results of these determinations are given in Tables IX and X, and shown in Figs. 9, 10, and 11. In Fig. 9, the concentration of leucine, giving a color equivalent to that found in the aliquot treated with ninhydrin, is plotted against hours of hydrolysis. The rate of hydrolysis of the pepsin is seen to proceed most rapidly at pH 8.3. At this pH, five times as much ninhydrin-produced color is obtained at 73 hours as is obtained at zero hours. Digestion in the pH 8.0 buffer produces practically the same effect as the digestion in pH 8.3 buffer. An increase in ninhydrin-produced color of about four times that of the zero hour aliquot is found at pH 6.7 after 73 hours of tryptic digestion. Further digestion was not observed, after 73 hours, at any pH. The addition of more trypsin to the
TRYPTIC HYDROLYSIS OF PEPsin

0.2 M PHOSPHATE BUFFER
- pH 8.3
- pH 8.0
- pH 7.5
- pH 6.7

FIGURE 9
EFFECT OF pH ON THE TRYPTIC HYDROLYSIS OF PEPsin

Hydrolysis measured by the ninhydrin reaction. The enzyme: substrate ratio is 1:50. Temperature = 37°C.
FIGURE 10

EFFECT OF pH ON THE TRYPTIC HYDROLYSIS OF PEPSIN

Hydrolysis was measured by the ninhydrin reaction. The enzyme:substrate ratio is 1:50. Temperature = 37°C.
TRYPTIC HYDROLYSIS OF PEPsin

0.2 M PHOSPHATE BUFFER
- pH 8.3
- pH 8.0
- pH 7.5
- pH 6.7

FIGURE 11
EFFECT OF pH ON THE TRYPTIC HYDROLYSIS OF PEPsin

Hydrolysis was measured by the amount of TCA soluble tyrosine produced. The enzyme:substrate ratio is 1:50. Temperature = 37°C.
hydrolysis mixtures resulted in no further hydrolysis at pH 8.0 and 8.3, while only slight increases in hydrolysis were observed at pH 6.7 and 7.5.

It was expected that the rate of hydrolysis would be different in buffers of different pH. However, an enzymatic reaction occurring at a pH other than optimal, would still be expected to yield the same degree of hydrolysis as observed at the optimal pH, even though the rate of hydrolysis is decreased. This is not found to be true in Fig. 9 and Table IX. The results may possibly be explained on the basis of the known behavior of trypsin. It is known that dissolved trypsin is unstable unless kept in acid solution. As Kunitz and Northrup have shown (38), above pH 6.0 and at elevated temperatures, native trypsin is reversibly changed into an inactive form. The latter is digested by the remaining native trypsin, shifting the equilibrium between active and inactive trypsin. Thus, the activity of the enzyme is decreased. When a protein substrate such as pepsin is present, a reaction competitive to the digestion of the inactive trypsin occurs. The pepsin will also be digested by the active trypsin. If pepsin is present in large concentration, compared to the concentration of inactive trypsin, the rate of destruction of the trypsin activity will be greatly reduced. As the hydrolysis of pepsin continues, the competition of the pepsin and the inactive trypsin, for hydrolysis by active trypsin, is lessened.
the rate of hydrolysis of inactive trypsin will increase, causing a gradual decrease in tryptic activity. The results shown in Fig. 9 may therefore be explained as the result of at least three variables; i.e. (a) increasing activity of the enzyme as the pH is increased to 8.3, (b) decreasing stability of the enzyme as the digestion of the pepsin proceeds, and (c) decreasing stability of the enzyme as the pH is increased above 6.0. From the results obtained, it can be said that the increase in tryptic activity from pH 6.7 to pH 8.3, exceeds the two variables affecting the stability of the trypsin. Factors affecting the ionic state of the substrate may also be involved.

The data used for Fig. 9 was used to construct the graph in Fig. 10. This plot demonstrates the optimal pH of hydrolysis to be pH 3.3.

Fig. 11 shows the effect of pH on the tryptic hydrolysis of pepsin, when the rate was determined by measuring the amount of tyrosine soluble in five per cent trichloroacetic acid. The results shown are similar to the ninhydrin results, in that the total TCA soluble tyrosine released at 73 hours of digestion in pH 6.7 buffer is only about eighty per cent of that released in pH 8.3 buffer. The amount of hydrolysis at pH 8.0 is seen to be identical with that obtained at pH 8.3. The addition of excess enzyme to these digestes, after 73 hours, does not increase the quantity of TCA soluble tyrosine. A total of about 16
"Tyrosine Equivalents" have been released during the hydrolysis at pH 8.0 and 8.3. This indicates that some peptide material, containing tyrosine, is insoluble in trichloroacetic acid.

Since proteolysis of peptide bonds by trypsin involves the liberation of acid groups, the concentration of the buffer used might have an effect on the rate of hydrolysis of pepsin by trypsin. To determine this, the tryptic hydrolysis of pepsin was carried out in phosphate buffers (pH 8.0) of 0.4 M, 0.1 M, 0.05 M and 0.01 M. The results are given in Tables XI and XII, and presented in Figs. 12 and 13. In the 0.01 M buffer, the hydrolysis is almost thirty-five per cent less than the hydrolysis obtained in the 0.4 M buffer. In the 0.1 M and 0.05 M buffers, the hydrolysis observed is within ten per cent of that found when the 0.4 M buffer was used. Phosphate buffer, pH 8.0, is not considered to be an efficient buffer, because the acid to salt ratio is 1 to 20. In this respect then, the production of small amounts of acid during the hydrolysis would be expected to have an effect on the pH of the solution. The pH of the reaction mixtures were measured after 77 hours, and are shown in the following table:

<table>
<thead>
<tr>
<th>Buffer Concentration</th>
<th>pH at 0 hours</th>
<th>pH at 73 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4 M</td>
<td>8.00</td>
<td>7.90</td>
</tr>
<tr>
<td>0.1 M</td>
<td>8.00</td>
<td>7.85</td>
</tr>
<tr>
<td>0.05 M</td>
<td>8.00</td>
<td>7.80</td>
</tr>
<tr>
<td>0.01 M</td>
<td>8.00</td>
<td>7.65</td>
</tr>
</tbody>
</table>
FIGURE 12

EFFECT OF BUFFER CONCENTRATION ON
THE TRYPTIC HYDROLYSIS OF PEP SIN

Hydrolysis was measured by the ninhydrin reaction. The enzyme:substrate ratio is 1:50. Temperature = 37°C.
FIGURE 13

EFFECT OF BUFFER CONCENTRATION ON
THE TRYPsic HYDROLYSIS OF PEPsin

Hydrolysis was measured by the amount of TCA soluble tyrosine produced. The enzyme:substrate ratio is 1:50. Temperature = 37°C.
From the data on the effect of pH on the rate of hydrolysis, the total digestion at pH 7.5 is approximately five per cent lower than the digestion at pH 8.0. If buffer concentration has no real effect on hydrolysis, the total digestion in the 0.01 M phosphate buffer should agree with the total digestion in the 0.4 M buffer within five per cent. The results show the digestion to be much less. Therefore, it seems likely that an effect greater than buffer capacity is operating. This may be the effect of salt concentration on the stability of trypsin.

A result different from that shown in Fig. 12 was noted when the rate of hydrolysis at various buffer concentrations was determined by measuring the amount of trichloroacetic acid soluble tyrosine released (Fig. 13, Table XII). Reactions in the 0.1 M and 0.05 M buffers gave the maximum digestion of pepsin. Hydrolysis in the 0.4 M buffer was about ten per cent less than in the 0.1 M and 0.05 M buffers. Repetition of this experiment gave essentially the same results. A measure of trichloroacetic acid soluble tyrosine is actually a measure of peptide material soluble in trichloroacetic acid. Since hydrolysis in 0.4 M buffer liberates less peptide material from pepsin than does hydrolysis in 0.1 M and 0.05 M buffers, it may be that the increased concentration of salt inhibits proteolysis of certain bonds in pepsin that would ordinarily be hydrolysed in a
less concentrated buffer.

Tryptic hydrolysis of pepsin was carried out in 0.1 M sodium bicarbonate buffer, pH 8.0, for comparison with the phosphate buffer of the same concentration and pH (Table XII). According to the results in Fig. 14, the hydrolysis in bicarbonate was slightly faster and more extensive than in the phosphate buffer. In a corresponding measure of trichloroacetic acid soluble tyrosine, the hydrolysis of pepsin was found to be identical (Fig. 15).

It appears that the rate of hydrolysis of pepsin by trypsin will proceed at an optimal rate in a pH 8.3, 0.1 M bicarbonate buffer. A 0.1 M solution of sodium bicarbonate has a pH of 8.4. This was the solution used for the four-hour tryptic hydrolysate, discussed previously, from which the data shown in Figs. 1 and 2 was obtained. It can now be seen that hydrolysis in this buffer at the end of four hours, is only fifty per cent of the hydrolysis obtained at the end of 73 hours, so that a greater number of peptides should be expected than the number obtained after the complete tryptic hydrolysis of the pepsin.

It should be noted, that regardless of the pH or the concentration of the buffer, the tryptic hydrolysis of pepsin appears to stop at approximately 30 hours, when the reaction is measured by the rate of release of trichloroacetic acid soluble peptides. On the other hand, the tryptic hydrolysis ceases after
FIGURE 14
COMPARISON OF THE RATE OF HYDROLYSIS IN
BICARBONATE AND PHOSPHATE BUFFERS

Hydrolysis was measured by the ninhydrin reaction. The enzyme:substrate ratio is 1:50. Temperature = 37°C.
TRYPTIC HYDROLYSIS OF PEPsin

pH 8.0 BUFFERS

- 0.1 M BICARBONATE

- 0.1 M PHOSPHATE

FIGURE 15

COMPARISON OF THE RATE OF HYDROLYSIS IN
BICARBONATE AND PHOSPHATE BUFFERS

Hydrolysis was measured by the amount of TCA soluble tyrosine produced. The enzyme:substrate ratio is 1:50. Temperature = 37°C.
about 70 hours, when the rate is measured by the ninhydrin re-
action. One possible interpretation of this result may be that
trypsin will hydrolyse the labile bonds in the small peptides,
that are soluble in trichloroacetic acid, faster than it will
hydrolyse the labile bonds in the larger peptides, that may be
insoluble in TCA.

Identification of the N-terminal amino acids of the
peptides from the tryptic hydrolysis of pepsin was performed by
labeling the free amino groups with the DNFB reagent. The hydro-
lysate was treated as previously described to give a final mix-
ture of the DNFB derivatives of the N-terminal amino acids. These
amino acid derivatives were initially identified by two dimen-
sional chromatography (Fig. 1) as being aspartic and glutamic
acids, serine, alanine, threonine, glycine and isoleucine. The
isoleucine is the original N-terminal of pepsin. Two other spots
were found, and were characterized as dinitrophenol and dinitro-
anilines, artifacts arising from the reaction of the amino acids
with DNFB.

All of the spots found on the two dimensional chromato-
gram were eluted with bicarbonate, the eluents acidified and ex-
tracted with ether, and the ether removed under reduced pressure.
The residues were then chromatographed for further identification
in two other solvent systems; n-amyl alcohol saturated with 1%
acetic acid, and n-amy l alcohol saturated with pH 6.0 phthalate buffer. The amyl alcohol-acetic acid system gave tailing of the DNP amino acids aspartic, glutamic and serine, but the amyl alcohol-phthalate buffer system gave more distinct spots, probably because the paper used in this system was prebuffered. All of the DNP-amino acids from the two dimensional chromatogram were identified by chromatographing alongside of an authentic DNP-amino acid sample. All of the spots were found to correspond to the initial identification made in the two dimensional solvent system, except the DNP-glutamic acid spot, which was found only to be a part of the DNP-aspartic acid tailing (Fig. 6). When the DNP-isoleucine spot was run in the amyl alcohol-phthalate buffer system, another DNP-amino acid, DNP-valine, was found on the chromatogram. DNP-valine has an \( R_f \) slightly less than that of DNP-isoleucine, in the "Toluene" solvent, and on the two dimensional chromatogram (Fig. 1), it was thought to be part of the DNP-isoleucine tailing. It was determined qualitatively, that the tryptic hydrolysis of pepsin liberated six new N-terminals. They were the amino acids aspartic, serine, glycine, threonine, alanine, and valine.

A quantitative evaluation of the N-terminal amino acids of the peptides from tryptic hydrolysis of pepsin at pH 6.7, 7.5, 8.0 and 8.3, was performed by densitometry of the DNP-amino acid
derivatives. The amyl alcohol-phthalate buffer solvent system was found to be ideal for separating all the DNP-amino acids involved. All samples and standards were chromatographed in the same period of time. The developed chromatograms were cut into strips and read in the densitometer as previously described. Most of the standards were run in triplicate, and the samples were run in duplicate. Authentic samples of the DNP-derivatives of the amino acids aspartic, serine, glycine, threonine, alanine, valine and isoleucine were used to construct the standard curve. A mixture of the DNP-amino acids, each 0.0025 M, was made in 95% ethanol, and 2, 4, 10, 20, and 40 microliters of this solution were used to establish the standard curve shown in Fig. 16. The data for the standard curve is recorded in Table XIII. A standard deviation of ±16% was found with spots having a concentration greater than 0.01 M.

The standard curve is not linear, because the light path from the monochromator was not wide enough to accommodate the entire width of the DNP-amino acid spot on the paper. The slit was only 1.0 cm. wide. A 0.10 micromolar spot, that had traveled a distance of about 20 cm. on the chromatogram, was about 2.0 cm. wide. If the entire area of the spot can be read on the densitometer, the ratio of concentration to area under the curve of the DNP-amino acid is constant, and a straight line is obtained. But, when the entire spot cannot be read, as is the case at the higher
DENSITOMETRY OF DNP AMINO ACIDS
ON RECORDING DENSITOMETER

STANDARD CURVE

FIGURE 16
STANDARD CURVE FOR DENSITOMETRY
OF DNP AMINO ACIDS

The circles represent the averages of from 14 to 21 values, and the lines across them are the standard deviations.
concentrations shown on the standard curve, this ratio increases, and the slope of the curve increases.

A procedure was devised to eliminate the quantification of the amount of material which must be applied to the paper chromatogram. Isoleucine is the N-terminal amino acid in pepsin. Therefore, one mole of pepsin will contain one mole of N-terminal isoleucine. When one mole of pepsin is hydrolysed by trypsin, new N-terminal amino acids are formed. The concentrations of the new N-terminal amino acids, at any time during hydrolysis, will be less than one mole. If, at the end of hydrolysis, a peptide bond has been completely hydrolysed, the molar concentration of the new N-terminal amino acid formed, will be equal to that of the original isoleucine N-terminal.

Since the present method is a measure of the quantity of the DNP-derivatives, the N-terminal of pepsin can be used as an internal standard. Regardless of the quantity of material applied to the paper chromatogram, the concentration of the DNP-isoleucine spot can be taken as one residue of pepsin. The concentrations of the other DNP-amino acids on the same chromatogram can then be calculated directly as residues of pepsin.

This procedure can be used as long as isoleucine is not one of the new end groups formed by the tryptic hydrolysis of the pepsin. Leucine will also have to be excluded as one of the new N-terminals, since DNP-leucine and DNP-isoleucine have the same
Authentic samples of aspartic acid, serine, glycine, valine, and isoleucine were used to test the recovery of amino acids in the method. A 10.0 ml. solution containing 100 micrograms of each amino acid was treated in the same manner as the hydrolysate samples, with the exception that it was not subjected to acid hydrolysis. The molar ratios of the DNP-amino acids to DNP-leucine were calculated from the concentration of each amino acid derivative found on the chromatogram. The per cent recovery of each amino acid was then calculated from the molar ratio of amino acid to leucine, present in the starting solution. The per cent recovery of each amino acid in two determinations was as follows:

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Recovery</th>
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<tbody>
<tr>
<td></td>
<td>Exp. 1</td>
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<tr>
<td>Aspartic Acid</td>
<td>94%</td>
</tr>
<tr>
<td>Serine</td>
<td>101%</td>
</tr>
<tr>
<td>Glycine</td>
<td>80%</td>
</tr>
<tr>
<td>Alanine</td>
<td>74%</td>
</tr>
<tr>
<td>Valine</td>
<td>98%</td>
</tr>
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</table>

Table VII records the residues of each amino acid N-terminal found at various times throughout the hydrolysis of pepsin by trypsin. The results were found to be much lower than
### Table VII

**The Tryptic Hydrolysis of Pepsin**

Residues of DNP-AmiNo Acid N-Terminal Formed

Per Mole of DNP-Isoleucine

<table>
<thead>
<tr>
<th>pH</th>
<th>Hours of Digestion</th>
<th>Asp</th>
<th>Ser</th>
<th>Gly</th>
<th>Thr</th>
<th>Ala</th>
<th>Val</th>
</tr>
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<tr>
<td>6.7</td>
<td>1</td>
<td>.06</td>
<td>.17</td>
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<td>.22</td>
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<tr>
<td></td>
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<td>.13</td>
<td>.10</td>
<td>.26</td>
<td>.43</td>
</tr>
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<td>.14</td>
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<td>.36</td>
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<td>.44</td>
</tr>
<tr>
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<td>45</td>
<td>.37</td>
<td>.21</td>
<td>.19</td>
<td>.39</td>
<td>.38</td>
<td>.54</td>
</tr>
</tbody>
</table>
anticipated. Several reasons are possible for the low values:

(1) The DNP-amino acids were not corrected for destruction during acid hydrolysis. Various investigators have estimated this destruction to be different for each DNP-amino acid.\(^\text{(70, 73)}\). The actual amount of destruction of each DNP-amino acid varies widely, depending on the conditions used for acid hydrolysis. Since all residue values were calculated on the basis of the amount of DNP-isoleucine present on the chromatograms, correction factors for losses during acid hydrolysis could not be used. All samples were hydrolysed in acid at the same time, so that variation in losses between samples could be avoided.

(2) In the recovery experiment, an average value of 67\% was found for two determinations on each of five amino acids. The method used was not considered to be accurate enough to warrant individual recovery corrections for each amino acid. It was felt that the recovery of all the amino acids involved, should be approximately the same.

(3) The method of using the DNP-isoleucine spot as the internal standard for calculating the residue values of the other amino acids may possibly be at fault. Whether or not another isoleucine or leucine is among the new N-terminals formed is not known, but this would be the best explanation for the low results.

An attempt was made to determine whether or not DNP-
leucine was present in the DNP-isoleucine spot on the chromatograms. The DNP-isoleucine spot was eluted from the paper and hydrolysed for one hour in a sealed tube, with a saturated solution of Ba(OH)$_2$ at 105° C. This procedure converts the DNP-derivatives to free amino acids. The barium was then precipitated with CO$_2$. The supernatant was chromatographed with a tertiary amyl alcohol-water solvent system. This solvent gives excellent separation of the amino acids leucine and isoleucine. A chromatogram of the supernatant appeared to contain two amino acids, corresponding to leucine and isoleucine. However, this result was not conclusive, since the slight traces of salt present in the supernatant retarded the sample spots, making identification uncertain.

From the results in Table VII, it appears that the N-terminal amino acid valine is released to the greatest extent. The aspartic acid, and alanine N-terminals, appear to be present in larger amounts than the threonine, serine, and glycine N-terminals. This order of concentration parallels the order of lability of DNP-amino acids to acid hydrolysis. DNP-valine is one of the most stable DNP-amino acid derivatives, while DNP-glycine is the least stable during acid hydrolysis. This would indicate that all the amino acids would be present to approximately the same extent. If the DNP-isoleucine spot is actually equivalent to two residues of N-terminal amino acids after 45
hours of tryptic digestion, then all the amino acids found should each be approximately equal to one residue of pepsin. This result would indicate that seven bonds have been split in pepsin during tryptic hydrolysis.

It is difficult to point out differences in the amount of each N-terminal amino acid formed. However, if the number of residues of each amino acid found at the end of 45 hours of hydrolysis are totaled, for each pH, the result is seen to parallel the result obtained in Fig. 9. Hydrolysis in the pH 6.7 buffer gives a total of 1.5 residues after 45 hours, while hydrolysis in pH 8.3 buffer gives a total of about 2.1 residues. The pH 7.5 and 8.0 hydrolysates both give a total of 1.9 residues of amino acids.

Pepsin was previously shown to contain only two arginine and either one or two lysine residues (11, 12). Because of the well accepted specificity behavior of trypsin (7, 34, 74, 75) in splitting only those bonds involving the carboxyl groups of these residues, it was expected that three or four bonds in pepsin would be split. The results presented here show clearly that the tryptic hydrolysis of pepsin did not proceed in the expected manner. At least six bonds, and possibly seven, have been split in the pepsin molecule by the action of trypsin.

Thus, a trypsin preparation has split at least two
bonds in pepsin in addition to the number predicted from the
known specificity of trypsin. In order to rule out the possibil-
ity that part of the activity was caused by a proteolytic im-
purity, such as chymotrypsin, the rate of tryptic hydrolysis of
pepsin was determined with the addition of B-phenyl propionate,
an inhibitor of chymotrypsin (13), to the reaction mixture in
phosphate buffer at pH 8.3. This rate of hydrolysis was followed
by the ninhydrin reaction, and was found to be identical to that
observed without the chymotryptic inhibitor.
CHAPTER VI

SUMMARY

The present study has been performed as a necessary prelude to the separation of the peptides obtained from the tryptic hydrolysis of pepsin. Early work, on the separation of DNP-peptides from a dinitrophenylated tryptic hydrolysate, indicated the presence of at least eleven peptides. On the basis of the established behavior of trypsin, only three or four peptides were expected. It was assumed that the action of trypsin was not complete in the tryptic hydrolysate used. This indicated the necessity for an investigation of the rate of the tryptic hydrolysis of pepsin.

The rate of hydrolysis was followed by two methods. The ninhydrin reaction was used to determine the relative number of free amino groups released during the course of the hydrolysis, and the Folin-Ciocalteu reagent was used to measure the release of TCA soluble peptides, containing tyrosine. The relative rates were determined in 0.2 M phosphate buffers at pH 6.7, 7.5, 8.0 and 8.3, and in 0.4 M, 0.1 M, 0.05 M and 0.01 M, pH 8.0 phosphate buffers. In order to determine if the rate would differ in different buffers, hydrolysis in 0.1 M bicarbonate was compared to the hydrolysis in 0.1 M phosphate buffer, at pH 8.0.
The results of this study can be summarized as follows:

(a) Tryptic hydrolysis of pepsin proceeds most rapidly at pH 8.3. Total hydrolysis in pH 6.7 buffer was about twenty per cent less than that found in pH 8.3 buffer.

(b) When the rate of tryptic hydrolysis of pepsin is followed by the ninhydrin reaction, the rate increases with increasing buffer concentration. When the rate is followed by a measure of TCA soluble tyrosine released, optimal hydrolysis occurs in the 0.1 M and 0.05 M buffers.

(c) Bicarbonate buffer, 0.1 M, appears to promote more hydrolysis at pH 8.0, than does 0.1 M phosphate buffer of the same pH, when the hydrolysis is measured by the ninhydrin reaction. When a measure of TCA soluble tyrosine is used to follow the hydrolysis in the two buffers, no difference is noted.

The study indicates that a pH 8.3, 0.1 M bicarbonate buffer will promote optimal tryptic hydrolysis of pepsin.

The N-terminal amino acids of the peptides formed during the tryptic hydrolysis of pepsin were determined by paper chromatography. They were found to be aspartic acid, serine, glycine, threonine, alanine and valine. Isoleucine, the original N-terminal amino acid of pepsin was also found to be present. A quantitative estimation of the DNP-derivatives of the N-terminal amino acids released during the hydrolysis, was made by densitometry of a paper chromatogram of the derivatives. The number of
residues of each DNP-amino acid present in the hydrolysate was determined relative to the quantity of DNP-isoleucine present. This determination was made on the assumption that the DNP-isoleucine spot on the chromatograms had been derived solely from the N-terminal isoleucine of pepsin.

Values of 0.2 to 0.5 residues were found for the N-terminal amino acids, after 45 hours of hydrolysis. The values were not corrected for destruction during acid hydrolysis. The low results could best be explained if another peptide bond, involving the amino group of isoleucine or leucine, had been split in pepsin during hydrolysis by trypsin. This would mean the values calculated for each amino acid after 45 hours of hydrolysis, should be approximately doubled.

The presence of DNP-leucine on the chromatograms, indicates that trypsin has hydrolysed a total of seven peptide bonds in pepsin, each bond being hydrolysed to approximately the same extent.
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### TABLE VIII

**SOLUBILITY OF PEPSEIN IN 1.5 M MgSO₄, pH 5.0**

<table>
<thead>
<tr>
<th>mg. pepsin added per ml. solvent</th>
<th>Optical density at 275 μm of 1/25 dilution of supernatant</th>
</tr>
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<tbody>
<tr>
<td>5.2</td>
<td>0.340</td>
</tr>
<tr>
<td>7.4</td>
<td>0.460</td>
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<tr>
<td>8.9</td>
<td>0.570</td>
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<tr>
<td>10.2</td>
<td>0.655</td>
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<tr>
<td>12.3</td>
<td>0.770</td>
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<td>15.5</td>
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<td>1.095</td>
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<td>1.137</td>
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<tr>
<td>22.5</td>
<td>1.134</td>
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<tr>
<td>24.4</td>
<td>1.098</td>
</tr>
<tr>
<td>30.2</td>
<td>1.052</td>
</tr>
<tr>
<td>36.8</td>
<td>1.034</td>
</tr>
<tr>
<td>pH</td>
<td>6.7</td>
</tr>
<tr>
<td>------</td>
<td>-----</td>
</tr>
<tr>
<td>Hours of digestion</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>13.1</td>
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<tr>
<td>2</td>
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<tr>
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<td>26.5</td>
</tr>
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<td>6</td>
<td>29.3</td>
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<td>52</td>
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<tr>
<td>73</td>
<td>48.5</td>
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</table>

Values expressed in gamma of leucine.
### Table X

**Variation of the Rate of Tryptic Hydrolysis of Pepsin in 0.2 M Phosphate Buffer**

**Measures of TCA Soluble Tyrosine**

<table>
<thead>
<tr>
<th>Hours of digestion</th>
<th>pH = 6.7</th>
<th>7.5</th>
<th>8.0</th>
<th>8.5</th>
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<tbody>
<tr>
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<td>1.0</td>
<td>0.95</td>
<td>0.7</td>
<td>0.95</td>
</tr>
<tr>
<td>2</td>
<td>4.35</td>
<td>4.85</td>
<td>5.2</td>
<td>5.05</td>
</tr>
<tr>
<td>4</td>
<td>5.55</td>
<td>8.0</td>
<td>7.85</td>
<td>9.2</td>
</tr>
<tr>
<td>6</td>
<td>6.55</td>
<td>8.35</td>
<td>9.5</td>
<td>9.3</td>
</tr>
<tr>
<td>12</td>
<td>6.4</td>
<td>13.0</td>
<td>11.9</td>
<td>12.8</td>
</tr>
<tr>
<td>26</td>
<td>10.3</td>
<td>17.2</td>
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<td>52</td>
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<td>16.5</td>
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<tr>
<td>73</td>
<td>12.0</td>
<td>14.3</td>
<td>16.1</td>
<td>15.7</td>
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</tbody>
</table>

Values expressed in "Tyrosine Equivalents".
### TABLE XI

**Variation of the Rate of Tryptic Hydrolysis of Pepsin in pH 8.0 Phosphate Buffers**

**Hemoglobin Reaction**

<table>
<thead>
<tr>
<th>Molarity</th>
<th>Phosphate Buffers</th>
<th>Dicarbonate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.4</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>Hours of digestion</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>14.0</td>
<td>13.0</td>
</tr>
<tr>
<td>2</td>
<td>21.3</td>
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</tr>
<tr>
<td>4</td>
<td>26.4</td>
<td>28.7</td>
</tr>
<tr>
<td>6</td>
<td>31.5</td>
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<td>10</td>
<td>37.7</td>
<td>38.0</td>
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<tr>
<td>24</td>
<td>50.6</td>
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<td>54.0</td>
</tr>
<tr>
<td>77</td>
<td>60.8</td>
<td>59.0</td>
</tr>
<tr>
<td>103</td>
<td>65.0</td>
<td>61.0</td>
</tr>
</tbody>
</table>

Values expressed in gamma leucine.
TABLE XII

VARIATION OF THE RATE OF TRYPIC HYDROLYSIS OF Pepsin
IN pH 8.0 PHOSPHATE BUFFERS
MEASURE OF TCA SOLUBLE TYROSINE

<table>
<thead>
<tr>
<th>Molarity</th>
<th>Phosphate Buffers</th>
<th>Bicarbonate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.4</td>
<td>0.1</td>
</tr>
<tr>
<td>Hours of digestion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.5</td>
<td>2.3</td>
</tr>
<tr>
<td>2</td>
<td>4.3</td>
<td>5.7</td>
</tr>
<tr>
<td>4</td>
<td>6.1</td>
<td>7.6</td>
</tr>
<tr>
<td>6</td>
<td>8.4</td>
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<tr>
<td>103</td>
<td>12.5</td>
<td>13.6</td>
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</table>

Values expressed in "Tyrosine Equivalents".
<table>
<thead>
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<th>um/spt</th>
<th>Asp</th>
<th>Ser</th>
<th>Gly</th>
<th>Thr</th>
<th>Ala</th>
<th>Val</th>
<th>Lleu</th>
<th>Average cm² Area</th>
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<tr>
<td>0.005</td>
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<td>3.6</td>
<td>2.7</td>
<td>2.4</td>
<td>2.1</td>
<td>2.3</td>
<td>2.1</td>
<td>2.3 ± 0.9</td>
</tr>
<tr>
<td>± 30%</td>
<td>3.8</td>
<td>3.2</td>
<td>3.0</td>
<td>2.8</td>
<td>2.3</td>
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<td>0.9</td>
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</tr>
<tr>
<td>(average)</td>
<td>4.2</td>
<td>3.5</td>
<td>3.0</td>
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<td>2.7</td>
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</tr>
<tr>
<td>(average)</td>
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<td>6.1</td>
<td>5.7</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>± 16%</td>
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<td>12.4</td>
<td>10.8</td>
<td>9.9</td>
<td>10.1</td>
<td>8.7</td>
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</tr>
<tr>
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<td>13.7</td>
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<td>17.6</td>
<td></td>
</tr>
<tr>
<td>± 16%</td>
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<td>21.5</td>
<td>21.0</td>
<td>15.9</td>
<td>20.0</td>
<td>17.7</td>
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</tr>
<tr>
<td>(average)</td>
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<td>19.6</td>
<td>18.2</td>
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<td>18.6 ± 1.9</td>
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<td>30.4</td>
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<td>29.1</td>
<td></td>
</tr>
<tr>
<td>± 16%</td>
<td>25.5</td>
<td>24.7</td>
<td>30.4</td>
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<td>29.6</td>
<td>25.6</td>
<td>31.1</td>
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</tr>
<tr>
<td>(average)</td>
<td>26.0</td>
<td>34.6</td>
<td>25.8</td>
<td>29.3</td>
<td>29.4</td>
<td>27.6</td>
<td>30.1</td>
<td>29.0 ± 3.0</td>
</tr>
</tbody>
</table>
The thesis submitted by Frederick W. Parent has been read and approved by three members of the faculty of Loyola University.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form, and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

May 26, 1959

Signature of Advisor