



# ADVANCES IN PROTEIN CHEMISTRY

Volume V

M. L. Anson &  
John T. Edsall

**ADVANCES IN PROTEIN CHEMISTRY**

**VOLUME V**

This Page Intentionally Left Blank

# ADVANCES IN PROTEIN CHEMISTRY

EDITED BY

M. L. ANSON

*Continental Foods, Inc.,  
Hoboken, New Jersey*

JOHN T. EDSALL

*Harvard Medical School,  
Boston, Massachusetts*

*Associate Editor for the British Isles*

KENNETH BAILEY

*Trinity College,  
Cambridge, England*

VOLUME V



1949

ACADEMIC PRESS INC., PUBLISHERS  
NEW YORK, N. Y.

COPYRIGHT © 1949 BY ACADEMIC PRESS INC.

ALL RIGHTS RESERVED

NO PART OF THIS BOOK MAY BE REPRODUCED IN ANY FORM  
BY PHOTOSTAT, MICROFILM, OR ANY OTHER MEANS,  
WITHOUT WRITTEN PERMISSION FROM THE PUBLISHERS.

ACADEMIC PRESS INC.

111 FIFTH AVENUE

NEW YORK 3, N. Y.

*United Kingdom Edition*

Published by

ACADEMIC PRESS INC. (LONDON) LTD.

BERKELEY SQUARE HOUSE, LONDON W. 1

First Printing, 1949

Second Printing, 1963

PRINTED IN THE UNITED STATES OF AMERICA

CONTRIBUTORS TO VOLUME V

- JAMES B. ALLISON, *Rutgers University, New Brunswick, New Jersey*
- ALBERT CLAUDE, *The Rockefeller Institute for Medical Research, New York, New York\**
- JOSEPH S. FRUTON, *Department of Physiological Chemistry, Yale University, New Haven, Connecticut*
- K. H. GUSTAVSON, *Swedish Tanning Research Institute, Stockholm, Sweden*
- J. W. H. LUGG, *Department of Biochemistry, University of Melbourne, Melbourne, Victoria, Australia*
- HAROLD P. LUNDGREN, *Western Regional Research Laboratory, U. S. Department of Agriculture, Albany, California*
- THOMAS L. McMEEKIN, *Eastern Regional Research Laboratory, U. S. Department of Agriculture, Philadelphia, Pennsylvania*
- B. DAVID POLIS, *Eastern Regional Research Laboratory, U. S. Department of Agriculture, Philadelphia, Pennsylvania*
- G. R. TRISTRAM, *Sir William Dunn Institute of Biochemistry, University of Cambridge, Cambridge, England*

\* Present Address: *Institute Jules Bordet, Bruxelles, Belgium.*

This Page Intentionally Left Blank

## CONTENTS

CONTRIBUTORS TO VOLUME V. . . . . v

### The Synthesis of Peptides

BY JOSEPH S. FRUTON, *Department of Physiological Chemistry, Yale University, New Haven, Connecticut*

I. Introduction and Nomenclature. . . . .	1
II. General Methods of Peptide Synthesis. . . . .	5
III. Special Aspects of Peptide Synthesis. . . . .	34
References . . . . .	75

### Amino Acid Composition of Purified Proteins

BY G. R. TRISTRAM, *Sir William Dunn Institute of Biochemistry, University of Cambridge, Cambridge, England*

I. Introduction . . . . .	84
II. Purposes of Amino Acid Analysis . . . . .	85
III. Methods of Analysis. . . . .	86
IV. Establishment of Accuracy and Specificity of Methods of Analysis. . . . .	99
V. Comparison of Analytical Methods . . . . .	106
VI. Structure of Proteins as Revealed by Amino Acid Analysis . . . . .	125
VII. Amino Acid Composition of Certain Proteins . . . . .	129
VIII. General Discussion . . . . .	141
IX. Conclusion and Summary. . . . .	148
References . . . . .	148

### Biological Evaluation of Proteins

BY JAMES B. ALLISON, *Rutgers University, New Brunswick, New Jersey*

I. Introduction . . . . .	155
II. Evaluation through Nitrogen Balance . . . . .	157
III. Evaluation through Growth. . . . .	173
IV. Evaluation through Tissue Regeneration. . . . .	180
V. Evaluation through Amino Acid Analysis . . . . .	192
VI. Summary. . . . .	195
References . . . . .	196

### Milk Proteins

BY THOMAS L. McMEEKIN AND B. DAVID POLIS, *Eastern Regional Research Laboratory, Philadelphia, Pennsylvania*

I. Introduction . . . . .	202
II. Protein Distribution in Milk . . . . .	202
III. Separation and Properties of Milk Proteins. . . . .	203
IV. Proteins of Whey . . . . .	210

V. Amino Acid Composition of Milk Proteins . . . . .	219
VI. Enzymes in Milk . . . . .	219
VII. Relationship of Milk Proteins to Serum Proteins . . . . .	223
References . . . . .	225

#### Plant Proteins

By J. W. H. LUGG, *Department of Biochemistry, University of Melbourne, Melbourne, Victoria, Australia*

I. Introduction . . . . .	230
II. Bulk Proteins of Plants, Plant Organs, etc. . . . .	232
III. "Individual" Proteins of Plants. . . . .	259
IV. Modes of Occurrence of Protein in Plants. . . . .	265
V. Protein Metabolism in Plants. . . . .	269
VI. Conclusion . . . . .	294
References . . . . .	295

#### Synthetic Fibers Made from Proteins

By HAROLD P. LUNDGREN, *Western Regional Research Laboratory, U. S. Department of Agriculture, Albany, California*

I. Introduction . . . . .	305
II. General Considerations of Proteins Chain Behavior . . . . .	307
III. Preparation of Fibers from Proteins . . . . .	311
IV. Molecular Basis for Mechanical Properties of Fibers Made from Proteins . . . . .	327
V. Summary and Conclusion. . . . .	345
References . . . . .	346

#### Some Protein-Chemical Aspects of Tanning Processes

By K. H. GUSTAVSON, *Swedish Tanning Research Institute, Stockholm, Sweden*

I. Introduction . . . . .	354
II. Chemistry of Collagen. . . . .	356
III. Keratolysis and Action of Alkali on Hide. . . . .	375
IV. General Aspects of Tanning. . . . .	378
V. Reaction of Chromium Compounds with Collagen (Chrome Tanning) . . . . .	379
VI. Vegetable-Tanning Process. . . . .	394
VII. Reaction of Condensed Sulfo Acids (Syntans) with Collagen . . . . .	402
VIII. Tanning Power of Aldehydes . . . . .	405
IX. Quinone Tannage . . . . .	411
X. General Comments . . . . .	413
References . . . . .	415

#### Proteins, Lipids, and Nucleic Acids in Cell Structures and Functions

By ALBERT CLAUDE, *The Rockefeller Institute for Medical Research, New York, New York*

I. Introduction . . . . .	423
II. The Cell . . . . .	425
III. The Nucleus . . . . .	426
IV. The Cytoplasm . . . . .	428
V. Constitution and Duplication of Living Matter . . . . .	433

**CONTENTS**

**ix**

VI. Phospholipids in Cell Structures and Functions . . . . .	434
VII. Proteins . . . . .	436
VIII. Nucleic Acids. . . . .	437
References . . . . .	439
<b>AUTHOR INDEX . . . . .</b>	<b>441</b>
<b>SUBJECT INDEX. . . . .</b>	<b>466</b>

This Page Intentionally Left Blank

# The Synthesis of Peptides

By JOSEPH S. FRUTON

*Department of Physiological Chemistry, Yale University, New Haven, Connecticut*

## CONTENTS

	<i>Page</i>
I. Introduction and Nomenclature.....	1
II. General Methods of Peptide Synthesis.....	5
1. Synthesis of Dipeptides by Partial Hydrolysis of Diketopiperazines.....	5
2. Condensation of Peptide Esters.....	6
3. Synthesis of Peptide Derivatives by Means of Acylamino Acid Chlorides and Azides.....	9
4. Use of Amino Acid Chlorides in Peptide Synthesis.....	11
5. Use of $\alpha$ -Halogen Acyl Halides in Peptide Synthesis.....	11
6. "Azlactone" Method for Synthesis of Peptides.....	15
7. Condensation of Keto Acids and Amides.....	19
8. Use of <i>N</i> -Carbonic Acid Anhydrides of Amino Acids in Peptide Synthesis.....	21
9. Use of Toluenesulfonylamino Acids in Peptide Synthesis.....	23
10. Use of Azidoacyl Chlorides in Peptide Synthesis.....	24
11. "Carbobenzyo" Method of Peptide Synthesis.....	25
12. Use of Phthalylamino Acids in Peptide Synthesis.....	32
13. Enzymatic Synthesis of Peptide Derivatives.....	33
III. Special Aspects of Peptide Synthesis.....	34
1. Peptides of Glycine, Alanine, Valine, Leucine, and Isoleucine.....	35
2. Peptides of Aspartic and Glutamic Acids.....	41
3. Peptides of Phenylalanine and Tyrosine.....	46
4. Peptides of Cystine and Cysteine.....	49
5. Peptides of Serine.....	55
6. Peptides of Lysine and Other Diamino Acids.....	58
7. Peptides of Arginine.....	62
8. Peptides of Histidine.....	65
9. Peptides of Proline and Hydroxyproline.....	67
10. Peptides of Tryptophan.....	72
11. Peptides of Methionine.....	73
12. Peptides of Other Amino Acids.....	73
References.....	75

## I. INTRODUCTION AND NOMENCLATURE

The objectives of the search for satisfactory methods of peptide synthesis were clearly stated by Fischer and Fournau (149) in the

memorable paper which initiated the systematic exploration of this field of study:

"Der Gedanke, die aus den Proteinstoffen durch Hydrolyse entstehenden Aminosäuren durch Anhydridbildung wieder zu grösseren Komplexen zu vereinigen, ist schon seit längerer Zeit von verschiedenen Forschern experimentell behandelt worden. Wir erinnern nur an die Anhydride von Schaal (262), ihre Verwandlung einerseits in den kolloidalen Polyasparaginharnstoff von Grimaux (199), anderseits in die Polyasparatsäuren von H. Schiff (263), ferner an die Versuche von Schützenberger (270) über die Vereinigung verschiedener Aminosäuren (Leucine und Leuceine) mit Harnstoff durch Erhitzen mit Phosphorsäureanhydrid, an die ähnlichen Beobachtungen Lillienfelds (238) über die Wirkung von Kaliumbisulfat, Formaldehyd und anderen Kondensationsmitteln auf ein Gemisch von Aminosäureestern und endlich an die Angaben von Balbiano und Trasciatti (39) über die Verwandlung des Glycocolls in ein hornartiges Anhydrid durch Erhitzen mit Glycerin. Aber alle von ihnen beschriebenen Produkte sind amorphe, schwer characterisierbare Substanzen, über deren Struktur man ebensowenig wie über den Grad ihrer Verwandtschaft mit den natürlichen Proteinstoffen etwas sagen kann.

"Will man auf diesem schwierigen Gebiete zu sicheren Resultaten kommen, so wird man zuerst eine Methode finden müssen, welche es gestattet, successive und mit definierbaren Zwischenstufen die Moleküle verschiedener Aminosäuren anhydridartig aneinander zu reihen."

Since the enunciation of this view, it has become abundantly clear that one of the principal contributions of the organic chemist to the study of the structure and reactions of proteins has been indeed the development of several techniques for the synthesis of compounds in which amino acids are joined to one another "anhydridartig" by means of acid amide linkages. The services of peptide synthesis to protein chemistry have been many and various. The finding of synthetic peptides and peptide derivatives which are hydrolyzed by crystalline enzymes specifically adapted to the hydrolysis of proteins has buttressed the theory, first expressed by Hofmeister (217) and Fischer (126), that in proteins the peptide bond represents the most general type of linkage between the individual amino acid residues.\* In addition, modern methods of peptide synthesis have made possible the preparation of special peptides of low molecular weight and known chemical structure

\* In stressing the importance of the peptide bond in the structure of proteins, it is not intended to exclude the possibility that other types of covalent linkage may also play a significant role in the architecture of the protein molecule (99,135). Of particular interest in connection with the search for labile bonds which may be involved in the phenomena associated with protein denaturation is the recent suggestion of Linderstrøm-Lang and Jacobsen (239) that cysteine residues of proteins might participate in the formation of thiazoline rings. Similar consideration might be given to the participation of serine (or threonine) residues in oxazoline groupings (46). The recent synthesis, by Ehrensward and Davidsson (119), of labile peptides with thioiminoether linkages is a further experimental approach in this direction.

for use as models in the examination of several physical and chemical properties of proteins. Thus, the use of synthetic peptides has notably facilitated the interpretation of data on the acid-base relationships of proteins (101). Furthermore, in the study of the reactions of proteins with various chemical reagents, parallel experiments with peptides or peptide derivatives have frequently clarified the results observed with proteins. Of the numerous examples in the recent literature one may cite the study of the iodination of tyrosine-containing proteins (212), or the investigation of the action of mustard gas (250) and the nitrogen mustard gases (191) on proteins.

Apart from their importance in providing simple models for study of the enzymatic degradation, physical properties, or chemical reactions of proteins, the newer techniques of peptide synthesis have been invaluable in the final proof of the chemical structure of several physiologically important substances, such as glutathione (202) and carnosine (272). The discovery that the antibiotics gramicidin, tyrocidine, and gramicidin S are peptides (219,282) and the recent reports that there is, in pancreatic hydrolyzates of several proteins, a factor (or factors) of peptide nature which promotes the growth of certain microorganisms and the rat (299,302) offer further opportunity for the fruitful use of the methods of peptide synthesis to establish the chemical structure and to study the physiological action of peptides of biological interest.

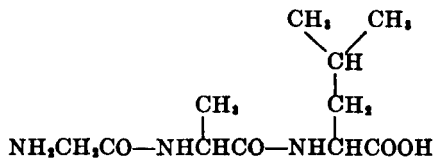
The development, in recent years, of new methods for the separation of amino acids and peptides (103,242) has led to a renewed interest in the products of the partial hydrolysis of proteins (281). It is clear, however, that, whatever methods are used to separate and identify peptides obtained from proteins, the conclusive evidence for the identity of such peptides must come from the comparison of the isolated material with synthetic peptides of known structure. This is the procedure that allowed Fischer and Luniak (159) to establish definitely that the peptide isolated by Osborne and Clapp (253) from a gliadin hydrolyzate was indeed L-prolyl-L-phenylalanine. In a similar manner, Stein, Moore, and Bergmann (278) demonstrated the presence of glycyl-L-alanine and L-alanylglycine in partial hydrolyzates of silk fibroin.

Enough has been said in the foregoing to justify the importance attached to peptide synthesis as a tool in the study of proteins. The purpose of this review is to survey the available methods for the synthesis of peptides. These methods will be evaluated, whenever possible, with regard to their relative difficulty, their adaptability to meet the many problems encountered in amino acid chemistry, and the yield and purity of the products of synthesis. Although the preparation of peptides containing nonprotein amino acids will occasionally be men-

tioned, primary emphasis will be placed on the synthesis of peptides of amino acids definitely known to be formed upon protein hydrolysis.

In what follows, the configuration of the amino acids will be given in accordance with the report of the Editorial Board of the *Journal of Biological Chemistry* (284). The amino acid residues will, in general, be designated by adding the suffix "yl" to the roots of the names of the free amino acids. Thus, for glycine, the term will be glycylyl; for proline, prolyl; for tyrosine, tyrosyl; and so forth. Several departures from this rule will be noted, however. The peptides of cysteine will not be designated "cysteyl" but "cysteinyl" peptides, and for the cystine peptides, the designation will be "cystinyl" rather than "cystyl." In the case of peptides of glutamine, the amino acid residue will be termed "glutaminylyl" to differentiate it from "glutamyl," which refers to glutamic acid. Similarly, for asparagine peptides, the term will be "asparaginylyl" and, for aspartic acid peptides, it will be "aspartyl." In addition, the term "tryptophanyl" will be used to designate the amino acid residue of tryptophan.

Since this nomenclature implies that an amino acid has been converted to an acyl group, it follows that the amino acid residues in a polypeptide should be listed in the sequence of substitution at the amino group of the adjacent amino acid. The tripeptide glycylyl-L-alanyl-L-leucine has, therefore, the following formula:



A brief discussion of the configurational relationships of peptides appears necessary at this point since frequent mention will be found in the literature of racemic peptides which contain more than one optically active amino acid. It is clear that, in the case of a racemic dipeptide containing two optically active amino acids, *i.e.*, DL-leucyl-DL-alanine, four isomers are possible: (a) D-leucyl-D-alanine, (b) L-leucyl-L-alanine, (c) L-leucyl-D-alanine, and (d) D-leucyl-L-alanine. Two racemates may be expected; one composed of forms *a* and *b*, and another composed of forms *c* and *d*. Separation of the two racemates may sometimes be achieved by taking advantage of their differences in solubility. It is then customary to designate the less soluble form by the letter "A" and the more soluble racemate by the letter "B."

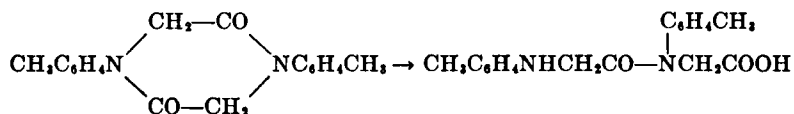
Reports will be encountered in the literature of the synthesis of peptides containing more than one optically active amino acid and in which one amino acid residue is present in a single configuration, while another is given as the DL form, *e.g.*, DL-alanylglycyl-L-glutamic acid. Such preparations are clearly mixtures of diastereoisomers. When attempts to separate the two forms are not successful, three possibilities may be envisaged: (a) the two isomers have very similar solubilities, thus making their separation by fractional crystallization difficult; (b)

they are isomorphic and form mixed crystals; or (c) they form addition compounds in stoichiometric proportions. Since it cannot be predicted beforehand whether, after the synthesis of a mixture of such diastereoisomers, a pure peptide can be isolated, it appears preferable, whenever possible, to perform peptide syntheses with optically active amino acids under conditions where racemization is avoided. Furthermore, in the study of the specificity of proteolytic enzymes or of the properties of model compounds related to proteins, it is usually desirable to have peptides containing optically active, rather than the racemic, forms of the amino acids. Greater interest attaches itself, therefore, to methods which permit the synthesis of such optically active peptides.

## II. GENERAL METHODS OF PEPTIDE SYNTHESIS

### 1. *Synthesis of Dipeptides by Partial Hydrolysis of Diketopiperazines*

In 1888 Abenius and Widmark (38) found that ditolyldiketopiperazine could be partially cleaved by acid hydrolysis:



An analogous reaction of aliphatic compounds was not observed until 1901, when Fischer and Fourneau (149) hydrolyzed glycine anhydride by heating it briefly with concentrated hydrochloric acid and thus obtained glycyglycine, the simplest representative of the group of substances under discussion in this review. The method used by Fischer and Fourneau still is a convenient method for making this dipeptide, especially in view of the recent development of an excellent procedure for the synthesis of glycine anhydride from glycine (261). Fischer noted, however, that brief acid hydrolysis of DL-alanine anhydride and of DL-leucine anhydride did not yield the expected dipeptides (125). Fischer later found that brief hydrolysis with dilute sodium hydroxide at room temperature would also yield dipeptides from diketopiperazines (132). In addition to glycyglycine, one of the two possible racemic forms of DL-alanyl-DL-alanine was prepared in this way. It soon became clear, however, that the method was not generally applicable to the synthesis of dipeptides containing optically active amino acids, for, when Fischer attempted to prepare L-alanyl-L-alanine from the corresponding anhydride, the product which resulted was partially racemic (153). Similarly, treatment of L-tyrosine anhydride with alkali caused appreciable racemization (166). As was shown by the later systematic studies of Levene (237) and Bergmann (82), among others, the racemization of optically

active diketopiperazines is favored by alkali. It is obvious, therefore, that this procedure is essentially limited to work with glycine anhydride. In fact, it is frequently convenient to treat glycine anhydride with sodium hydroxide and to use the resulting solution of the sodium salt of glycylglycine for condensation reactions involving the amino group of the dipeptide.

The racemization of diketopiperazines, so pronounced in alkaline media, is notably less in acid. In some cases, it is possible, therefore, to achieve a satisfactory synthesis of optically active dipeptides by cautious treatment with hydrochloric acid. Thus, Greenstein (195) was able to make L-cysteinyl-L-cysteine from L-cysteine anhydride by hydrolysis of the latter with concentrated hydrochloric acid at room temperature. It must be added, however, that some diketopiperazines, such as histidine anhydride and tyrosine anhydride, are fairly stable when they are heated with strong acid, while alanine anhydride and leucine anhydride require prolonged treatment with hot concentrated acid to effect cleavage of the ring.

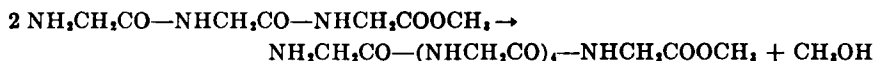
To the difficulties encountered in the partial cleavage of diketopiperazines containing like amino acid residues, must be added those met in the splitting of diketopiperazines derived from two different amino acids. In the latter case, two different dipeptides may result, as in the hydrolysis of glycyl-DL-leucine diketopiperazine, which yielded a mixture of glycyl-DL-leucine and DL-leucylglycine (166). The separation of such mixtures is usually difficult. If, however, the formation of only one of the two possible dipeptides is favored, this difficulty may be eluded. One of several instances in which a mixed dipeptide could be prepared in pure form by hydrolysis of a diketopiperazine was described by Bergmann and Tietzman (68), who obtained L-prolyl-L-phenylalanine from L-prolyl-L-phenylalanine diketopiperazine. The dipeptide had the same rotation as that of the product obtained by Fischer and Luniak (159), who coupled L-prolyl chloride with L-phenylalanine ethyl ester, and then saponified the dipeptide ester.

As the first available method for the synthesis of free peptides, the procedure discussed in this section has considerable historical interest. It is clear from the foregoing, however, that it has many limitations. Despite the occasional successes of this method, and its value as an adjunct to other synthetic procedures, it may be expected that, even for the synthesis of dipeptides, preference will be given to other methods.

## 2. *Condensation of Peptide Esters*

Curtius noted in 1883 (105) that glycine ethyl ester undergoes spontaneous transformation to yield glycine anhydride and a substance

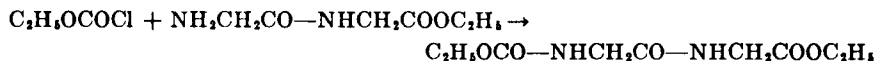
which gives a positive biuret test. For this reason, the latter product was termed "biuret base." Later studies by Curtius (107) showed that, if moisture is excluded, the formation of the anhydride is suppressed and the "biuret base," which he formulated as (triglycyl)glycine ethyl ester, is the chief product. In a similar manner, Fischer (134) converted (diglycyl)glycine methyl ester to (pentaglycyl)glycine methyl ester by heating the former at 100°.



More recently Pacsu and Wilson (254) and Frankel and Katchalski (176) have shown that, under suitable conditions, long-chain polycondensation products may be obtained from amino acid and peptide esters. The materials obtained represented mixtures of homologous peptide esters containing 20 to 100 amino acid units.

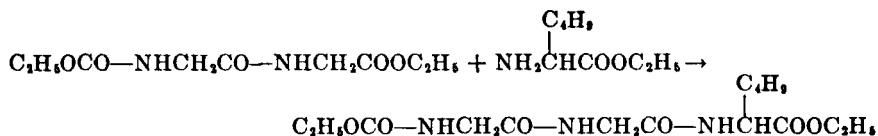
It may be noted at this point that, in general, amino acid esters and dipeptide esters readily yield diketopiperazines rather than polycondensation products. In fact, when a diketopiperazine composed of like amino acid residues is desired, it is most convenient to prepare it from the corresponding amino acid ester with ammonia in alcohol. In this manner, there have been synthesized a variety of diketopiperazines, such as histidine anhydride, lysine anhydride, and serine anhydride (172). Proline methyl ester, in particular, cyclizes with great ease (223). For the preparation of diketopiperazines derived from two different amino acids, treatment of the dipeptide ester with ammonia in alcohol usually leads to the desired product. Examples of this procedure are, among others, the synthesis of L-leucyl-L-alanine diketopiperazine from L-leucyl-L-alanine methyl ester (136) and glycyl-L-valine diketopiperazine from glycyl-L-valine methyl ester (164). A modification of this method, employing a lithium hydroxide solution saturated with carbon dioxide, was useful for the preparation of L-glutamylglycine diketopiperazine from  $\alpha$ -L-glutamylglycine ethyl ester (78).

Fischer's first attempts to develop a general method of peptide synthesis led him to prepare the carbethoxy derivative of glycyglycine ethyl ester by treatment of the dipeptide ester with ethyl chlorocarbonate (149).



It was his hope, in this way, to introduce a substituent which would protect the reactive amino group from further attack in the course of condensation reactions and which could also be removed without hydrolysis

of the peptide bonds of the synthetic product. When the carbethoxy peptide noted above was heated for 36 hours with DL-leucine ethyl ester, ethyl alcohol was eliminated, and the resulting product was carbethoxyglycylglycylleucine ethyl ester (124).

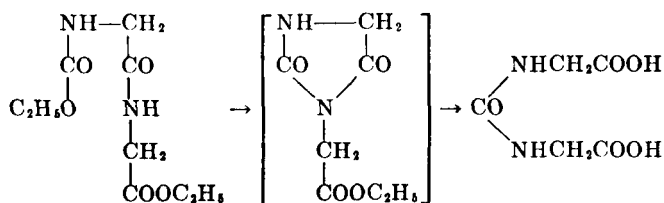


Fischer soon realized, however, that such condensation reactions are increasingly difficult as the peptide chain is lengthened and that even in the preparation of smaller peptides the yields are low. For this reason he turned his efforts to the development of other methods of peptide synthesis.

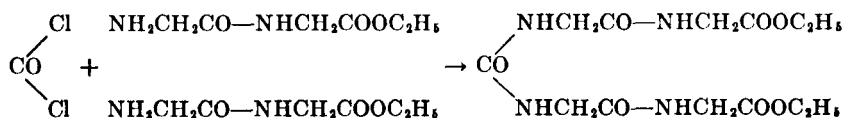
Of some interest in this connection is the behavior of carbethoxyglycylglycine ethyl ester on prolonged hydrolysis with alkali. Fischer (124) obtained a product which he formulated as glycylglycine carbamino acid:



It appeared, therefore, that the carbethoxy group could be removed from the acylated peptide ester without cleavage of the peptide bond. However, the complex nature of the reaction led Fischer to abandon further study of its mechanism. It remained for Wessely (296) to show that the product obtained by Fischer was actually carbonyl-bis-glycine, and the following sequence of reactions was suggested to explain its formation:



It may be added that, in the same paper in which he described the hydrolysis of the carbethoxyglycylglycine ethyl ester, Fischer also reported the synthesis of carbonyl-bis-glycylglycine by the treatment of the dipeptide ester with phosgene (in toluene), followed by saponification:

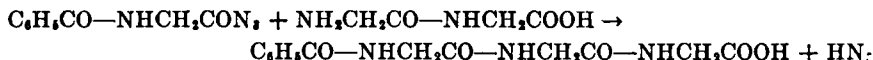


Such compounds may also be prepared by the reaction of the sodium salts of amino acids or peptides with phosgene.

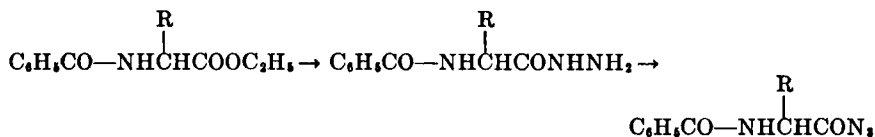
### 3. *Synthesis of Peptide Derivatives by Means of Acylamino Acid Chlorides and Azides*

While working on the synthesis of hippuric acid, Curtius found, in 1881 (104), that one of the products of the interaction of benzoyl chloride and glycine silver was the substance benzoylglycylglycine. As pointed out by Fischer (129) in the course of a polemic with Curtius, this reaction is rather complex in nature, and, although it may be considered to represent the first recorded synthesis of a well-defined peptide derivative, the method is not suitable for general application.

Curtius' studies, during the period 1890-1900, on the reactions of hydrazides and azides, led him to use these in the synthesis of peptide derivatives. After the report by Fischer and Fournau (149) that dipeptides could be made by the partial hydrolysis of diketopiperazines, Curtius (106) described the use of azides of benzoylamino acids or peptides according to the following reaction, illustrated for the case of the synthesis of benzoylglycylglycylglycine:



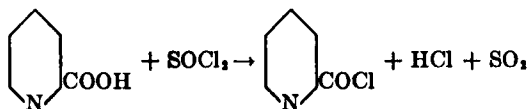
In order to obtain the azide, the corresponding ester was treated with hydrazine hydrate, thus yielding a hydrazide, which was in turn converted to the azide by means of nitrous acid:



These reactions proceeded smoothly and, frequently, with excellent yield. Curtius and Levy (111) were able to make benzoyl(tetraglycyl)glycine ethyl ester by the condensation of benzoyl(diglycyl)glycine azide with glycylglycine ethyl ester, and with his collaborators, Curtius extended this method to the synthesis of benzoylated peptides containing alanine (110), aspartic acid (108), and aminobutyric acid (109).

As was noted in the previous section of this review, Fischer's first attempts to develop a general method of peptide synthesis led him to prepare the carbethoxy derivative of glycylglycine ethyl ester (144). When he concluded that condensation of such esters with esters of amino acids was not feasible as a general procedure, he decided to convert the carbethoxyamino acids to the corresponding acid chlorides by means of

thionyl chloride (127), a reaction which had been found by Meyer (246) to be suitable for the preparation of the acid chloride of pyridine carboxylic acid:



By warming carbethoxyglycine or carbethoxyglycylglycine with thionyl chloride at 35–40°, noncrystalline products were obtained which were used directly for coupling in ethereal or in chloroform solution with amino acid or peptide esters. From the resulting carbethoxy peptide esters, the corresponding acids could be prepared by saponification. In several cases, these acids could be converted to acid chlorides with thionyl chloride, and the peptide chain lengthened by coupling with amino acid or peptide esters.

In principle, the above methods of Curtius and Fischer provide the basis for the further development of the techniques of peptide synthesis. All the subsequent procedures for lengthening the peptide chain have involved the conversion of the carboxyl group of an amino acid into forms which permit reaction with the amino group of another amino acid. Of the various derivatives of carboxylic acids which have proved useful for this purpose, the azides and chlorides have been of the most general value. Indeed, in many cases, the older azide method of Curtius is preferable to the chloride method, particularly in coupling reactions involving acyl peptides (*cf.* page 26).

Although Fischer showed in 1905 (132) that it was possible to convert free amino acids to acid chlorides, it was realized that, in order to permit smooth coupling reactions, the amino group had to be blocked by acylation, or otherwise modified to avoid complicated side reactions in the course of the conversion of the carboxyl group to an acid chloride. In addition to the carbethoxy and benzoyl groups mentioned above, a variety of acyl substituents were introduced into peptide chemistry. In addition to others to be discussed later may be mentioned the naphthalenesulfonyl (144), phenylureido (170), benzenesulfonyl (145), and methanesulfonyl (207) groups. However, as long as it was necessary to remove an acyl substituent by hydrolysis, it could not be used in the synthesis of free peptides, since attempts to eliminate the acyl group in this manner invariably led to either partial or complete cleavage of the linkages between the amino acids. The essential problem of peptide synthesis thus became the development of methods which would obviate the necessity for the hydrolytic removal of an acyl substituent at the end of a series of coupling reactions.

It should be added, however, that the methods discussed in this section have been of considerable value in the preparation of benzoyl peptides, as well as amides of benzoylamino acids and benzoyl peptides, which were required for studies of the specificity of proteolytic enzymes (50,244).

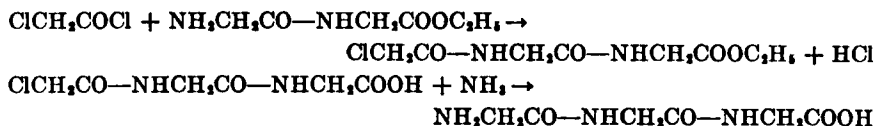
#### 4. *Use of Amino Acid Chlorides in Peptide Synthesis*

As noted earlier, Fischer, in 1905 (132), described a method of peptide synthesis which obviated the necessity for blocking the amino group of an amino acid prior to the conversion of the carboxyl group to an acid chloride. This method takes advantage of the fact that several amino acids, when shaken with phosphorus pentachloride and acetyl chloride, are readily converted into crystalline hydrochlorides of the amino acid chlorides. In this manner, Fischer prepared the acid chlorides of DL-leucine, L-alanine, DL-phenylalanine, and L-proline. Peptide synthesis was effected, in the case of L-alanyl-glycine (133), by the addition of L-alanyl chloride hydrochloride to a solution of glycine ethyl ester in dry chloroform, neutralization of the hydrochloric acid by the addition of sodium methylate, and the saponification of the dipeptide ester with alkali. The yields obtained by this procedure are not too satisfactory, since, as can readily be seen, one of the possible side reactions is the formation of a diketopiperazine from the dipeptide ester. Only isolated instances of its application can therefore be cited. Fischer and Luniak (159) used L-prolyl chloride to make L-prolyl-L-phenylalanine and Abderhalden and Kempe (19) made L-tryptophyl-glycine by means of L-tryptophyl chloride. Havestadt and Fricke (206) have reported the synthesis of DL-alanyl-L-histidine through the coupling of DL-alanyl chloride with L-histidine methyl ester but, as pointed out by Hunt and du Vigneaud (220), the identity of their product is open to some doubt. It is clear, therefore, that, except for a few cases, the amino acid chlorides are not suitable reagents in peptide synthesis.

#### 5. *Use of $\alpha$ -Halogen Acyl Halides in Peptide Synthesis*

The greatest of Fischer's many important contributions to protein chemistry may be said to have been the invention of the first of several methods now available to circumvent the difficulty encountered in the removal, by hydrolysis, of an acyl substituent of a peptide without cleavage of the peptide itself. The first report of this method came in 1903, when Fischer and Otto (160) described the synthesis of glycyl-glycyl-glycine. Chloroacetyl chloride and glycyl-glycine ethyl ester were coupled, and the product was saponified to yield chloroacetylglycyl-glycine. The next step was the introduction of the free amino group

of the peptide by treatment of the chloroacetyl dipeptide with 25% ammonia at 100°:



It was later noted that the amination could be carried out more effectively by allowing the reaction mixture to stand at room temperature for two to three days.

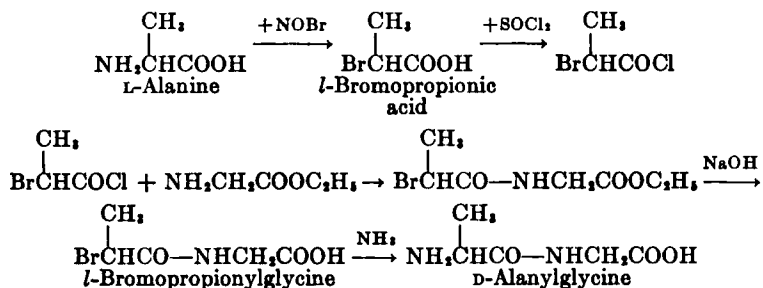
This ingenious method was applied in a similar manner to the condensation of  $\alpha$ -bromopropionyl bromide with glycylglycine ethyl ester, thus leading to the synthesis of DL-alanylglycylglycine (128). In rapid succession, there followed a memorable series of papers which described the extension of this method to the synthesis of peptides of DL-leucine (132), DL-proline (169), DL-phenylalanine (130), and other amino acids. In Table I are listed the various halogen acyl halides used in connection with these syntheses.

TABLE I  
*Halogen Acyl Halides Used in Peptide Synthesis*

Amino acid residue	Halogen acyl halide
Glycyl.....	Chloroacetyl chloride Bromoacetyl bromide
Alanyl.....	$\alpha$ -Bromopropionyl bromide (or chloride)
Valyl.....	$\alpha$ -Bromoisovaleryl chloride
Leucyl.....	$\alpha$ -Bromoisocaproyl chloride
Isoleucyl.....	$\alpha$ -Bromo- $\beta$ -methyl- $\beta$ -ethylpropionyl chloride
Phenylalanyl.....	$\alpha$ -Bromo- $\beta$ -phenylpropionyl chloride
Prolyl.....	$\alpha,\delta$ -Dibromovaleryl chloride

One of the principal difficulties of this method arose from the fact that those members of the group of  $\alpha$ -halogen acyl halides in which the acyl group was propionyl or larger could occur in at least two stereoisomeric forms (135). Thus, if the racemate were to be used in a reaction with an optically active amino acid or peptide, there would be produced a mixture of diastereoisomers. Except in rare instances, where the differences in the rates of reaction for the two isomeric acid halides are considerable, approximately equal amounts of the diastereoisomers would result, thus making the separation by fractional crystallization a laborious operation of dubious outcome. Accordingly, Fischer set about to prepare halogen acyl halides from optically active  $\alpha$ -halogen acids. With Warburg (131) he prepared the optically active *l*-bromopropionyl

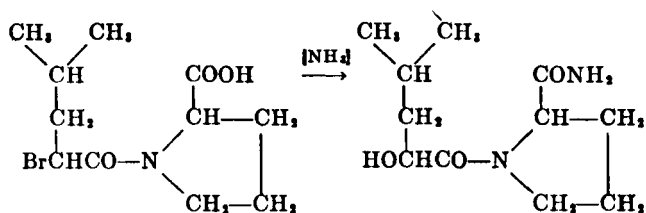
chloride by treatment of levorotatory  $\alpha$ -bromopropionic acid with thionyl chloride. This acid was obtained in two ways: either by resolution of the synthetic *dl* acid, or by treatment of L-alanine with nitrosyl bromide according to the method of Walden (293). Fischer assumed that a change of configuration ("Walden inversion") had taken place in the course of the latter reaction. Subsequent work (180) showed, however, that the *l*-bromopropionic acid obtained from L-alanine retains the configuration of the amino acid. The coupling of the *l*-bromopropionyl chloride with glycine ethyl ester, followed by saponification of the coupling product, gave a substance which was then subjected to amination to yield the peptide now recognized to be D-alanylglycine. Actually Walden inversion had occurred during the amination of the bromopropionylglycine rather than during the synthesis of the bromo acid, as Fischer had assumed (136, 252a). Despite this error, Fischer was, in general, correct in the conclusion that, in order to synthesize peptides containing amino acids of the L series ("natural" amino acids) it was necessary, in the halogen acyl halide method, to prepare halogen acids from amino acids of the D-series ("unnatural" amino acids). These D-amino acids could be obtained only by the resolution of synthetic racemic products, and Fischer and his collaborators devised a number of excellent, albeit time-consuming, procedures for achieving this end. The synthesis of D-alanylglycine from L-alanine and glycine ethyl ester may be summarized in the following scheme:



With the aid of several optically active  $\alpha$ -halogen acyl halides, Fischer, and later Abderhalden, succeeded in synthesizing an impressive series of peptides, in which all the components were either glycine or higher amino acids of the L or D series. The most notable achievements in this regard were the synthesis of the octadecapeptide L-leucyl-(triglycyl)-L-leucyl-(triglycyl)-L-leucyl(octaglycyl)glycine which Fischer made in 1907 (138) and of a nonadecapeptide prepared by Abderhalden and Fodor in 1916 (10). In the preparation of long peptides of this type, special care had to be taken in the conversion of halogen acyl peptides to

the corresponding chlorides. Fischer effected this reaction by means of phosphorus pentachloride and acetyl chloride, as in the case of  $\alpha$ -*D*-bromoisocaprylglycylglycylglycine. The resulting chloride could then be coupled with (pentaglycyl)glycine in alkaline solution. The bromoisocaprylpeptide was then aminated with liquid ammonia, since aqueous ammonia was not effective, and the decapeptide was coupled with  $\alpha$ -*D*-bromoisocaprylglycylglycylglycyl chloride. Needless to say, as the chain length increased, the experimental difficulties became more serious. Furthermore, by coupling optically active halogen acyl halides with amino acids such as *L*-phenylalanine, *L*-tyrosine, *L*-histidine, *L*-cystine, etc., or peptides of the *L* series such as glycyl-*L*-tyrosine, a large variety of interesting products were obtained by Fischer and Abderhalden, among others, and used for the study of the specificity of proteolytic enzymes.

Despite the ingenuity and wide applicability of this method, its many difficulties greatly limit its general application. In addition to the labor involved in the synthesis of optically active peptides, the method has disadvantages in several other respects. First, the reaction of *N*-(halogen acyl) hydroxyamino acids with phosphorus pentachloride is complicated and, even if the hydroxyl group is blocked, as in the case of *O*-carbomethoxy-*N*-chloroacetyl-*L*-tyrosine, chlorination of the carboxyl group may result in complete racemization (141). Surprising deviations from the expected result were also observed in the course of the amination of several halogen acylamino acids. Thus, when Fischer attempted to prepare *L*-leucyl-*L*-proline he found that, on treatment of  $\alpha$ -*D*-bromoisocapryl-*L*-proline with ammonia, there resulted, not the expected dipeptide, but rather the substance  $\alpha$ -hydroxyisocapryl-*L*-prolinamide (162):



Later, it was found that bromoisocapryl-*N*-phenylglycine undergoes a similar reaction on treatment with ammonia (151):

