PEPTIDES: METHODS OF SYNTHESIS AND TERMINAL-RESIDUE STUDIES

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Introduction

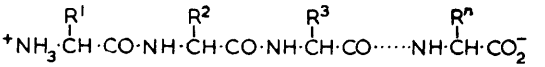
The very great biological importance of the macromolecular proteins ($M > \sim 10{,}000$) and the closely related natural peptides ($M < \sim 10{,}000$) has made the study of their structure of outstanding interest. This study is of extreme technical difficulty because of the intractable nature of the substances: they are, for example, non-volatile, insoluble in the normal organic solvents of low dielectric constant, and are often of a very fragile nature.

Purified proteins and peptides can be hydrolysed by acids, alkalis, and enzymes to mixtures of α-amino-acids, $^{+}NH_3 \cdot CHR \cdot CO_2^{-}$, in yields accounting for virtually the whole of the parent compound. There are nineteen different natural α-amino-acids of frequent occurrence, and a number of rarer examples, their R-side chains varying widely in complexity and reactivity. The nineteen common examples are listed in Table 1.

With all these amino-acids, other than glycine, the α-carbon atom is asymmetric and the compounds occur, in almost all cases, in the L-configuration.*

The amino-acids are less fragile than the proteins and natural peptides but are otherwise very intractable, being crystalline solids melting with decomposition at indefinite very high temperatures, insoluble in organic solvents. These awkward properties, which result from the ion-dipole structure of these compounds, have impeded investigation. Nevertheless, specialised techniques have been devised permitting the synthesis of all the common and most of the uncommon natural α-amino-acids.

The fundamental problem of the manner of the linkage between the α-amino-acid residues in proteins has been intensively studied and work in many fields, biological, physical, and chemical, during the last half-century has established the correctness of the hypothesis, due to Fischer [1] and to Hofmeister,[2] that the linkage is of the amide type and that the fundamental structural feature of the molecules of the natural substances is the polypeptide chain:



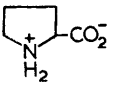
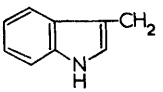
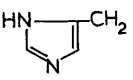
[1] E. Fischer and E. Fourneau, *Ber.*, 1901, **34**, 2868. See also E. Fischer, *Ber.*, (*a*) 1902, **35**, 1095; (*b*) 1903, **36**, 2094.

[2] F. Hofmeister, *Ergebn. Physiol.*, 1902, **1**, 759.

* For the international rules on designation of the stereochemistry of amino-acids see *J.*, 1952, 3522.

These structures are always written conventionally as above : the $^{+}NH_3 \cdot CHR' \cdot CO \cdot$ and the $\cdot NH \cdot CHR^n \cdot CO_2^-$ residue are called respectively *N*-terminal and *C*-terminal. Peptides are named as amino-acyl substitution products of the *C*-terminal residue.

TABLE 1

R	Name of amino-acid $^{+}NH_3 \cdot CHR \cdot CO_2^-$	Reactivity	
H	Glycine	Neutral,	non-polar
Me	Alanine	,,	,,
Pr^i	Valine	,,	,,
Bu^i	Leucine	,,	,,
Bu^s	Isoleucine	,,	,,
	Proline	,,	,,
$Ph \cdot CH_2$	Phenylalanine	,,	,,
$HS \cdot CH_2$	Cysteine	,,	S-containing
$^{-}O_2C \cdot CH(^{+}NH_3) \cdot CH_2 \cdot S \cdot S \cdot CH_2$	Cystine	,,	,,
$MeS \cdot CH_2 \cdot CH_2$	Methionine	,,	,,
	Tryptophan	,,	
$H_2N \cdot C(=NH) \cdot NH \cdot CH_2 \cdot CH_2 \cdot CH_2$	Arginine	Basic	
	Histidine	,,	
$NH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2$	Lysine	,,	
$HO_2C \cdot CH_2$	Aspartic acid *	Acidic	
$HO_2C \cdot CH_2 \cdot CH_2$	Glutamic acid *	,,	
$HO \cdot CH_2$	Serine	Neutral,	polar
$Me \cdot CH(OH) \cdot$	Threonine	,,	,,
$p\text{-}HO \cdot C_6H_4 \cdot CH_2$	Tyrosine	,,	,,

* The carboxyl groups in the side chains of aspartic and glutamic acid frequently occur in Nature as the corresponding amides (asparagine and glutamine).

The classical pattern of the elucidation of the structure of a naturally occurring organic compound is the following sequence of operations : isolation, proof of purity, molecular-weight determination, analysis, degradation, synthesis, comparison of synthetic and natural specimens. Satisfactory techniques for the study of polypeptide chains by stepwise degradation to, and synthesis from, α-amino-acid units are essential requisites for the organic chemical investigation of protein and peptide structure.

Such is the complexity of the substances and such the technical difficulty imposed by their peculiar properties that it is only comparatively recently

that any individual protein or larger natural peptide has been available in sufficient purity to permit the reasonable interpretation of the results of α-amino-acid analysis and polypeptide degradation, and to give a clearly defined objective for synthetic work.

The most complex natural product of this class for which all the stages of the classical pattern of investigation have yet been successfully carried out is the peptide pituitary hormone, oxytocin, a nonapeptide amide. Of the substances of high molecular weight, only for insulin monomer (*M* 6000) and α-corticotropin (*M* 4500) has the investigation been carried as far as the successful completion of degradative studies, resulting in the determination of the complete structural formulæ and opening the way for synthetic attacks.

Despite the unavailability, until recently, of suitable purified proteins and natural peptides, their synthesis and stepwise degradation have been actively studied for many years, by using model substances. The synthetic studies were initiated first.

Synthesis of peptides

The aim of peptide synthesis is to permit the building, normally from the natural, optically active, α-amino-acids, of polypeptide chains of any desired length, containing any desired range of component residues in any desired order: racemisation of the components must be avoided. These are exacting requirements, especially in view of the ion-dipolar properties of the amino-acids and peptides, and completely satisfactory procedures have yet to be devised. (For reviews see Fruton [3] and Shapiro.[4])

Methods based on $Y{\cdot}NH{\cdot}CHR^1{\cdot}CO{\cdot}X + NH_2{\cdot}CHR^2{\cdot}CO_2Z$: "XYZ" **Methods.**—Fischer,[1] having achieved the first peptide synthesis in 1901 by preparing glycylglycine by partial hydrolysis of diketopiperazine, in 1902—03 devised a scheme which, generalised, gives a plan of attack on the problem of stepwise synthesis of peptides which has formed the basis of most subsequent work. The stages in the general synthesis of a dipeptide from the component amino-acids are:

(1) Synthesis of the "carboxyl component", in which the amino-group is protected and the carboxyl group modified for greater reactivity:

$$^+NH_3{\cdot}CHR^1{\cdot}CO_2^- \xrightarrow{NaOH} NH_2CHR^1{\cdot}CO_2Na \xrightarrow{YCl} Y{\cdot}NH{\cdot}CHR^1{\cdot}CO_2H$$
$$\xrightarrow[-OH]{+X} Y{\cdot}NH{\cdot}CHR^1{\cdot}COX$$

(2) Synthesis of the "amino-component", in which the amino-group is free and the carboxyl group masked:

$$^+NH_3{\cdot}CHR^2{\cdot}CO_2^- \xrightarrow[-H_2O]{ZOH} NH_2{\cdot}CHR^2{\cdot}CO_2Z$$

(3) Condensation, followed by removal of Y and Z:

$$Y{\cdot}NH{\cdot}CHR^1{\cdot}COX + NH_2{\cdot}CHR^2{\cdot}CO_2Z \xrightarrow{+H_2O} Y{\cdot}NH{\cdot}CHR^1{\cdot}CO{\cdot}NH{\cdot}CHR^2{\cdot}CO_2Z$$
$$\longrightarrow {}^+NH_3{\cdot}CHR^1{\cdot}CO{\cdot}NH{\cdot}CHR^2{\cdot}CO_2^-$$

[3] J. S. Fruton, *Adv. Protein Chem.*, 1949, **5**, 1.
[4] B. L. Shapiro, *Chem. and Ind.*, 1952, 1119.

Provided that selective methods are available for the removal of the protecting groups Y and Z, the process can be extended so that, in principle, the protected polypeptide

$$Y\cdot NH\cdot CHR^1\cdot CO\cdot NH\cdot CHR^2\cdot CO\cdots\cdots NH\cdot CHR^n\cdot CO_2Z$$

can be prepared and the last protecting groups Y and Z are then removed in a final stage.

Reasonably suitable groups Z and X were already known and were early applied. Fischer used (i) Z = Na, condensing the amino-component with the carboxyl component under Schotten–Baumann conditions and removing Z by acidification, and (ii) Z = Me or Et, condensing the amino-component as ester, in an organic solvent, with the carboxyl component in the presence of an organic base, the Z group then being removed by careful hydrolysis, *e.g.*, by alkali in aqueous dioxan.[5]

For the group X, Fischer used ethoxyl initially. These esters can be prepared under mild conditions but are not very reactive in the subsequent condensation, and Fischer [1b] turned to the use of acid chlorides (X = Cl). These compounds, though very reactive in the condensation, have the disadvantage of requiring strenuous conditions [6] for their preparation. In 1902, Curtius [7] introduced his azide method ($X = N_3$), the most attractive of the early methods. The group is readily introduced in aqueous media by the successive action of hydrazine and nitrous acid on the ester and is sufficiently reactive in the condensation, which is usually performed on the amino-component ester in an organic solvent.

Suitable groups Y susceptible to specific removal in the later stages were much more difficult to find: this proved the main initial problem, so much so that thirty years elapsed before any large measure of success was achieved. (The emphasis of research has changed somewhat, in recent years, towards the study of new X and, to a smaller extent, Z groups.) The Y groups originally tried were carbethoxyl (Fischer) and benzoyl (Curtius), but all attempts to remove these groups, by hydrolysis, from the protected peptide led to the simultaneous hydrolysis of the peptide bond.

At this stage (1903), Fischer and Otto [8] devised an alternative to the XYZ approach, the α-chloro-acyl chloride method of peptide synthesis. The method is only applicable to peptides with unreactive side chains, R, and has other drawbacks, and though Fischer in 1907 used it to prepare an eighteen-residue polypeptide,[9] it is of historical interest only.

Fischer then turned again to the study of the potentially much more versatile XYZ method, seeking Y groups which could be removed by other than hydrolytic means. In 1915 he [10] found that a *N*-toluenesulphonyl group could be removed by reductive fission with hot hydriodic acid. The

[5] Cf. (Sir) C. R. Harington and T. H. Mead, *Biochem. J.*, 1935, **29**, 1602.
[6] E. Fischer, *Ber.*, 1905, **38**, 605.
[7] T. Curtius, *Ber.*, 1902, **35**, 3226.
[8] E. Fischer and K. Otto, *Ber.*, 1903, **36**, 2106.
[9] E. Fischer, *Ber.*, 1907, **40**, 1754.
[10] *Idem. Ber.*, 1915, **48**, 93.

successful application of this discovery to peptide synthesis, delayed by war and Fischer's death in 1919, was made in 1926 by Schoenheimer.[11] The drastic conditions were a handicap to the reduction method in its original form.*

The reductive removal of Y groups was studied by Bergmann and Zervas [13] who in 1932 achieved an advance of the highest importance by introducing the benzyloxycarbonyl group $CH_2Ph{\cdot}O{\cdot}CO{\cdot}$ (often written CBz, derived from the older terminology, carbobenzyloxy) : the group is removed by catalytic hydrogenation. This discovery opened a new phase in peptide synthesis. In its original form the process was not available for the preparation of peptides containing cystine (or cysteine) because of the poisoning of hydrogenation catalysts by sulphur. This difficulty has been overcome in three ways, two of which were quickly announced : (i) Harington and Mead [5] in 1935 used phosphonium iodide ; (ii) Sifferd and du Vigneaud [14] in the same year showed that the benzyloxycarbonyl group could be removed, gently and without side reactions, by sodium in liquid ammonia ; (iii) much more recently (1952) Ben-Ishai and Berger [15], and independently Boissonnas and Preitner,[16] have found that hydrogen bromide in cold acetic acid is a very good reagent for this purpose. The last method is very valuable when it is desired to remove the benzyloxycarbonyl group specifically from a compound

$$Ph{\cdot}CH_2{\cdot}O{\cdot}CO{\cdot}NH{\cdot}CHR^1{\cdot}CO{\cdot}NH{\cdot}CHR^2{\cdot}CO_2Z$$

to prepare the free amino-compound for use in further synthesis, and when the group Z (*e.g.*, benzyl, see below) is liable to hydrogenolysis.

Several modifications to the benzyloxycarbonyl group have been studied recently, usually with a view to improving the solubility relations, melting points, and yields, *e.g.*, the 4-bromo-[17] and 4-nitro-benzyloxycarbonyl group,[18, 19] and the allyloxycarbonyl system.[20] No extensive use has yet been made of the first and the last of these modifications, but the nitro-derivative has been used [19] in devising an important technique for the preparation of the hitherto inaccessible arginyl peptides (see below).

Ehrensvärd [21] described a more distant variant of the benzyloxycarbonyl group, the phenylthiocarbonyl group, PhS·CO·, removable by hydrolysis and

[11] R. Schoenheimer, *Z. physiol. Chem.*, 1926, **154**, 203.
[12] V. du Vigneaud and O. K. Behrens, *J. Biol. Chem.*, 1937, **117**, 27.
[13] M. Bergmann and L. Zervas, *Ber.*, 1932, **65**, 1192.
[14] R. H. Sifferd and V. du Vigneaud, *J. Biol. Chem.*, 1935, **108**, 753.
[15] D. Ben-Ishai and A. Berger, *J. Org. Chem.*, 1952, **17**, 1564.
[16] R. A. Boissonnas and G. Preitner, *Helv. Chim. Acta*, 1952, **35**, 2240 (see also ref. 39).
[17] D. M. Channing, P. B. Turner, and G. T. Young, *Nature*, 1951, **167**, 487.
[18] F. H. Carpenter and D. T. Gish, *J. Amer. Chem. Soc.*, 1952, **74**, 3818 ; 1953, **75**, 950.
[19] D. T. Gish and F. H. Carpenter, *ibid.*, p. 5872.
[20] C. M. Stevens and R. Watanabe, *ibid.*, 1950, **72**, 725.
[21] G. C. H. Ehrensvärd, *Nature*, 1947, **159**, 500.

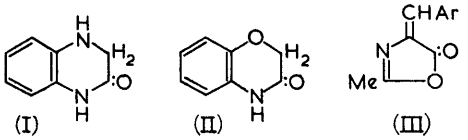
* In 1937 du Vigneaud and Behrens[12] found that the *N*-toluenesulphonyl group could be smoothly removed from nitrogen by reduction with sodium in liquid ammonia.

precipitation with cold aqueous-alcoholic lead acetate. The method has been severely criticised,[22] however, on the grounds that the removal procedure leads to extensive formation of hydantoins.

Though, since 1932, studies of the Y group have largely centred on the benzyloxycarbonyl group, several quite different systems have also been investigated.

King *et al.*[23] and Sheehan *et al.*[24] independently introduced, and have since developed, the use of the phthaloyl protecting group, which is gently removable by hydrazine, as phthalazide. (Schumann and Boissonas [25] suggested the use of phenylhydrazine.)

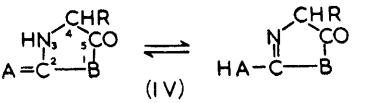
Holley and Holley,[26] advocate the use of (i) the chloroacetyl group, removable by treatment with *o*-phenylenediamine and neutral hydrolysis



[to give (I)], and (ii) the *o*-nitrophenoxyacetyl group removable, after reduction, by neutral hydrolysis [to give (II)]. These are processes of considerable promise. In 1953 Waley [27a] and in 1954 King *et al.*[27b] utilised in peptide synthesis the fact that the formyl group, readily introduced by the direct action of formic acid on an amino-acid, can be removed by treatment with hydrogen chloride in cold alcohol. (For a review of Y groups see ref. 28.)

Y–X **Groups joined in a Cyclic System.** The first (1926) of these was the azlactone (oxazolone) method due to Bergmann *et al.*,[28a] who used compounds of type (III). Though important in its day, this method has not been much used since the discovery of the benzyloxycarbonyl method.

More likely to be of importance in the future is a series of newer methods based on the general tautomeric heterocyclic system (IV) where A and B may be oxygen or sulphur. These 4-substituted 2-hydroxy(or mercapto)-



oxazol-5-ones (or -thiazolones) have been much studied during the past 12 years, mainly as a result of research on penicillin. They react with

[22] A. Lindenmann, N. H. Khan, and K. Hofmann, *J. Amer. Chem. Soc.*, 1952, **74**, 476.

[23] F. E. King and D. A. A. Kidd. *Nature*, 1948, **162**, 776; *J.*, 1949, 3315.

[24] J. C. Sheehan and V. S. Frank, *J. Amer. Chem. Soc.*, 1949, **71**, 1856.

[25] I. Schumann and R. A. Boissonnas, *Helv. Chim. Acta*, 1952, **35**, 2235.

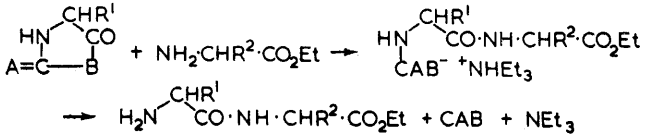
[26] R. W. Holley and A. D. Holley, *J. Amer. Chem. Soc.*, 1952, **74**, 3069.

[27] (*a*) S. G. Waley, *Chem. and Ind.*, 1953, 107; (*b*) F. E. King, J. W. Clark-Lewis, D. A. A. Kidd, and G. R. Smith, *J.*, 1954, 1039.

[28] R. A. Boissonnas and G. Preitner, *Helv. Chim. Acta*, 1953, **36**, 875.

[28a] M. Bergmann, F. Stern, and C. Witte, *Annalen*, 1926, **449**, 227.

amino-esters in the presence of triethylamine, to yield open-chain compounds which can be decomposed to give peptide esters:



The first work of this kind was that of Heilbron, and Cook, and Levy,[29] in 1949–50, working with A = B = S and achieving the decomposition with dry methanolic hydrogen chloride. With R^1 other than H there is a danger of racemisation at position 4 and a tendency for the decomposition to regenerate the original mercaptothiazolone and amino-acid ester (see, however, Davis and Levy [30]).

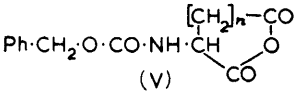
Leggett Bailey [31] has obtained encouraging results with the series A = B = O and A = S, B = O, while Khorana [32] has studied the series A = O, B = S.

These methods, though promising (especially that with A = B = S and A = B = O), have not yet been extensively used.

Modern Work on X Groups. In nearly all work up to 1950 on stepwise peptide synthesis, either acid chlorides (X = Cl) or azides ($X = N_3$) were used. Recently several important new X groups of a rather different type have been introduced. The system R·CO·X may be regarded as the mixed anhydride of the acids $R{\cdot}CO_2H$ and HX, and one of the most promising new lines consists in the replacement of the "classical" HX acids, hydrochloric and hydrazoic, by oxy-acids HO·X′, *i.e.*, using the mixed anhydride Y·NH·CHR¹·CO·O·X′ (for a review see ref. 33).

Such a compound can react with the amino-component $NH_2{\cdot}CHR^2{\cdot}CO_2Z$ in two ways, to form (1) the desired dipeptide protected by both Y and Z, together with HOX′, or (2) the "monomers" $Y{\cdot}NH{\cdot}CHR^1{\cdot}CO_2H$ and $X'{\cdot}NH{\cdot}CHR^2{\cdot}CO_2Z$. This makes critical the choice of suitable (*a*) X′ groups and (*b*) physical conditions.

The first account (1934) of such X groups was by Bergmann *et al.*, [34] who used compounds of type (V), specifically anhydrides of benzylocarbonyl-



(V)

aspartic ($n = 1$) and -glutamic acid ($n = 2$). These react with amino-esters, in organic solvents, to give either or both of the possible products, α- and β-aspartyl or α- and γ-glutamyl peptides, depending on the conditions,

[29] (Sir) I. M. Heilbron, *J.*, 1949, 2099; A. H. Cook and A. L. Levy, *J.*, 1950, 646, 651.

[30] A. C. Davis and A. L. Levy, *J.*, 1951, 2419.

[31] J. Leggett Bailey, *J.*, 1950, 3461.

[32] H. G. Khorana, *Chem. and Ind.*, 1951, 129.

[33] G. W. Kenner, *Chem. Soc. Special Publ.*, No. 2, 1955, p. 103.

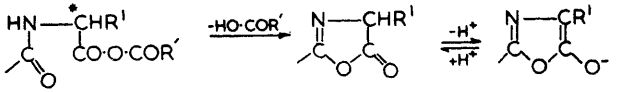
[34] M. Bergmann and L. Zervas, *Ber.*, 1932, **65**, 1192.

and on the nature of R^2. Because of the ambiguity of the ring opening in the peptide coupling reaction, these particular mixed anhydrides are not now used directly in peptide synthesis (though they have very important indirect uses—see below).

Wieland and Sehring,[35] in 1950, devised the first reasonably successful synthesis based on intermolecular mixed anhydrides, using X = OBz (*i.e.*, mixed anhydrides with benzoic acid). They found that this system gave, predominantly, the desired protected dipeptide.

Vaughan and his collaborators [36] have studied the variation of yields of the alternative products in the reaction between Y·NH·CHR^1·CO·O·CO·R′ and amines as a function of R′ and recommend for peptide synthesis R′ = Bu^i or OEt, *i.e.*, mixed anhydrides with *iso*valeric acid or ethyl hydrogen carbonate. (The latter grouping was studied and advocated independently by Boissonnas [37] in 1951.) These two anhydride systems have been much used. They usually give high yields.

With mixed anhydrides involving carboxylic components and incorporating the system ·CO·NH·*CHR^1·CO·O·CO· there is grave danger of racemisation at the α-position (marked with an asterisk) (see Vaughan [38]), especially if the component is a peptide derivative Y·NH·CHR·CO·NH·*CHR^1·CO·OX′ instead of a simple benzyloxycarbonylamino-acid compound. This is probably due [33] to the intervention of the reactions :



Mixed anhydrides with esters of inorganic polybasic acids, capable of reaction, in non-aqueous solvents, with amino-acid esters, have been recently discovered. The most important class have X = O·P(OEt)$_2$. This X group was introduced by Anderson, Welcher, and Young [39] in 1951 in the form of diethyl chlorophosphite and was improved in the next year [40] by the use of tetraethyl pyrophosphite, (EtO)$_2$P·O·P(OEt)$_2$, as a source of the ·O·P(OEt)$_2$ fragment and of diethyl phosphite as a solvent for the reaction ; this constituted a very powerful method, though one somewhat liable to racemise the carboxyl component. [Vaughan [41] suggested the corresponding group X = O·As(OEt)$_2$, and Sheehan and Frank [42] have used a quinquevalent phosphorus derivative where X = ·O·PO(O·CH$_2$Ph)$_2$.]

Some mixed anhydrides with polybasic inorganic acids having ionisable

[35] T. Wieland and R. Sehring, *Annalen*, 1950, **569**, 122.

[36] J. R. Vaughan, *J. Amer. Chem. Soc.*, 1951, **73**, 3547 ; J. R. Vaughan and R. L. Osato, *ibid.*, p. 5553 ; 1952, **74**, 676.

[37] R. A. Boissonnas, *Helv. Chim. Acta*, 1951, **34**, 874.

[38] J. R. Vaughan, *J. Amer. Chem. Soc.*, 1952, **74**, 6137 ; J. R. Vaughan and J. A. Eichler, *ibid.*, 1953, **75**, 5556.

[39] G. W. Anderson, A. D. Welcher, and R. W. Young, *ibid.*, 1951, **73**, 501 ; cf. G. W. Anderson, J. Blodinger, R. W. Young, and A. D. Welcher, *ibid.*, 1952, **74**, 5304 ; G. W. Anderson and R. W. Young, *ibid.*, p. 5307.

[40] G. W. Anderson, J. Blodinger, and A. D. Welcher, *ibid.*, p. 5309.

[41] J. R. Vaughan, *ibid.*, 1951, **73**, 1389.

[42] J. C. Sheehan and V. S. Frank, *ibid.*, 1950, **72**, 1312.

hydrogen atoms after anhydride formation have been examined recently, since such compounds, Y·NH·CHR^1·CO·O·X″·OH, are soluble in water and permit the peptide coupling reaction to proceed directly with an amino-acid (or peptide) in aqueous alkali (*i.e.*, with Z = Na or another metal). This avoids the use of esterified amino-components and the necessity of subsequent hydrolysis during which losses may occur.[43, 44]

The first method (1949) of this kind was that of Chantrenne,[45] who used X = ·O·P·O(OPh)OH ; though his procedure for the introduction of the grouping was rather cumbersome and yields were not very high, the method is of interest in that, employing phosphoric esters and operating in water, it may well be related to peptide biosynthesis.

The second and more practicable synthesis of this type is due to Kenner and his collaborators,[46] who employed X = ·O·SO_3^-. This group is introduced by treating the lithium salt of the potential carboxyl component with the complex of sulphur trioxide and *NN*-dimethylformamide. The coupling

$$NH_2{\cdot}CHR^1{\cdot}CO_2^-\ Li^+ + {}^-O_3S{\cdot}O{\cdot}CH{=}NMe_2^+ \longrightarrow NH_2{\cdot}CHR^1{\cdot}CO{\cdot}O{\cdot}SO_3^-\ Li^+ + OHC{\cdot}NMe_2$$

reaction can be carried out with the free amino-component in aqueous dimethylformamide but there is danger of (*a*) loss of yield by hydrolysis of the mixed anhydride and (*b*) racemisation. Both these difficulties can be avoided by working in anhydrous dimethylformamide and coupling to an amino-acid (or peptide) ester.

Kenner and his collaborators,[47] seeking X-groups capable of reacting with free amino-acids and peptides in aqueous solution but resistant to hydrolysis and to racemisation, have developed an observation by Wieland *et al.*,[48] that aryl thiolester groups will act as X groups, X = ·SPh (the use of cyanomethyl esters, X = ·O·CH_2·CN, has also been studied [49]). Wieland used phenyl thiolesters prepared by the action of thiophenol on compounds Y·NH·CHR^1·CO·O·CO_2Et. Kenner uses *p*-nitrophenyl thiolesters which, because the *p*-nitro-group increases the acidity of the parent thiol, react much faster with amino-compounds than do unsubstituted thiols and permit reaction in cold neutral aqueous dioxan. Preparation of the *p*-nitrophenyl thiolesters by the Wieland procedure gave a completely racemised product, so an ingenious new method was devised, employing tri-*p*-nitrophenyl phosphotrithioite in dimethylformamide, which gives the thiolesters of benzyloxycarbonylamino-acids (and -peptides) quantitatively and unracemised—nor is there racemisation in the subsequent coupling reaction. This is a most promising line, especially as the aryl thiolester group is

[43] J. I. Harris and J. S. Fruton, *J. Biol. Chem.*, 1951, **191**, 143.
[44] C. W. Roberts, *J. Amer. Chem. Soc.*, 1954, **76**, 6203.
[45] H. Chantrenne, *Nature*, 1949, **164**, 576 ; *Biochim. Biophys. Acta*, 1950, **4**, 484.
[46] G. W. Kenner and R. J. Stedman, *J.*, 1952, 2069.
[47] J. A. Farrington, G. W. Kenner, and J. M. Turner, *Chem. and Ind.*, 1955, 601.
[48] T. Wieland, W. Schafer, and E. Bokelmann, *Annalen*, 1951, **573**, 99.
[49] R. Schwyzer, B. Iselin, and M. Feurer, *Angew. Chem.*, 1954, **66**, 747 ; *Helv. Chim. Acta*, 1955, **38**, 83, 1508.

stable to hydrogen bromide in acetic acid which removes benzyloxycarbonyl groups. This is of importance in the preparation of cyclic peptides (see below).

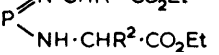
Sheehan and Hess [50] have condensed free carboxyl components with free amino-components in aqueous tetrahydrofuran, in the presence of di*cyclo*hexylcarbodi-imide, which is converted into di*cyclo*hexylurea in the process. Khorana, who had previously [51a] noted the reaction of carbodi-imides with the carboxyl group of amino-acids and peptides in degradation experiments, reports [51b] that in independent studies of the synthetic procedure he finds that the products are contaminated by acylureas. The process has the advantage of aqueous conditions and freedom from racemisation.

Methods based on X′·NH·CHR²·CO_2Z.—A variation on the general XYZ method has been developed recently. In this an X′ group which, in the mixed oxyacid anhydride of the carboxyl component, Y·NH·CHR′·CO·OX′, might promote peptide formation by the methods indicated above, is instead linked, amide-wise, to the amino-group of the second component and allowed to react with a free carboxyl group in the first component :

$$\text{Y·NH·CHR}^1\text{·CO}_2\text{H} + \text{X′·NH·CHR}^2\text{·CO}_2\text{Z} \xrightarrow{-\text{HOX′}} \text{Y·NH·CHR}^1\text{·CO·NH·CHR}^2\text{·CO}_2\text{Z}$$

The first, and still the most important, example of this procedure was due to Anderson, Welcher, and Young,[39] who in 1951 showed that their diethyl phosphite group could be used either as an orthodox mixed anhydride (see above) or as the amide $(EtO)_2P$·NH·CHR²·CO_2Z (Vaughan used his diethyl arsenite grouping [41] similarly). By this modified procedure,[40] with tetraethyl pyrophosphite, the course of the condensation can be changed from an orthodox XYZ method [X = OX′ = ·$OP(OEt)_2$; " anhydride procedure "] to an X′·NH·CHR²·CO_2Z method [X′ = ·$P(OEt)_2$; " amide procedure "] by varying the order in which the reagents are added to the diethyl phosphite medium. The great advantage of the " amide procedure," in this very important case, is that, in it, the danger of racemisation at the *C*-terminal α-position of a peptide carboxyl component is reduced : it is present if the corresponding " anhydride procedure " is used. (Because of this, du Vigneaud *et al.*[52] used the " amide procedure " at two critical stages of the oxytocin synthesis ; see below.)

Goldschmidt and his school, since 1952, have introduced two new groups related to the X′–N type ; these involve the use, as the second component of compounds of the types, (*a*) [53] α-cyanato-esters, OCN·CHR²·CO_2Et, and (*b*),[54] following Süss,[55] phosphorazo-compounds of type (VI). These are



(VI)

[50] J. C. Sheehan and G. P. Hess, *J. Amer. Chem. Soc.*, 1955, **77**, 1067.
[51] H. G. Khorana, (*a*) *J.*, 1952, 2081 ; (*b*) *Chem. and Ind.*, 1955, 1087.
[52] V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, and P. G. Katsoyannis, *J. Amer. Chem. Soc.*, 1954, **76**, 3115.
[53] S. Goldschmidt and M. Wick, *Annalen*, 1952, **575**, 217.
[54] S. Goldschmidt and H. Lautenschlager, *ibid.*, 1953, **580**, 68.
[55] O. Süss, *ibid.*, 1951, **572**, 96.

interesting new departures, but the methods are not yet reliable in the matter of racemisation.

North and Young [56] have studied racemisation of the carboxyl component in the synthesis of acetyl-L-leucylglycine ester in relation to the group X. The use of the small acetyl Y group makes the test very severe. They found extensive racemisation with X = Cl, O·COBu^i, and O·P(OEt)$_2$, and in the "amide-procedure" with X′ = (EtO)$_2$P or phosphorazo. Configuration was retained with the Curtius grouping (X = N_3).

Modern Work on Z-Groups.—Bergmann,[57] in 1935, wishing to utilise the favourable solubility relations of the amino-esters and to avoid the hydrolysis (prone to side reactions [43, 44]) needed with methyl and ethyl esters, considered the possibility of using benzyl esters and found that this group could be removed by catalytic hydrogenation. du Vigneaud and Behrens [12] showed in 1937 that such benzyl esters were also liable to reductive fission by sodium in liquid ammonia. Early developments were held up by poor yields in the preparation of the benzyl esters. Preparative methods have, however, been greatly improved recently,[58] and the use of benzyl esters in this way is now extremely promising; for example, it permits a very clean final stage removal of Y and Z [44] with *N*-benzyloxycarbonyl-peptide benzyl esters.

Use of Amino-Acids with Reactive Side Chains in Peptide Synthesis.—These amino-acids give rise to special problems in peptide synthesis.

(i) *Acidic side chains.* (*a*) Glutamic acid. The difficulties arise when this substance is to be used as a carboxyl component: it is important to be able to use either the α- or the γ-carboxyl group, unequivocally, since both α- and γ-glutamyl peptides are known in Nature.

Peptide formation through the γ-carboxyl group has been achieved by using benzyloxycarbonylglutamic anhydride which, as Bergmann *et al.*[59] showed, when treated with benzyl alcohol, gives largely the α-monobenzyl ester, leaving the γ-carboxyl group free for reaction. This ester is obtained purer (Sachs and Brand [59a]) from dibenzyl glutamate by partial reduction with hydrogen iodide in acetic acid to the α-monoester followed by *N*-benzyloxycarbonylation.

Probably the best route to peptides using the α-carboxyl group of glutamic acid comes from an observation by King and his co-workers [60] several years ago that phthaloylglutamic anhydride on treatment with benzyl (or methyl) alcohol gives the γ-ester, leaving the α-carboxyl group free for reaction.

[56] M. B. North and G. T. Young, *Chem. and Ind.*, 1955, 1597.

[57] See M. Bergmann, L. Zervas, and W. F. Ross, *J. Biol. Chem.*, 1935, **111**, 245.

[58] H. K. Miller and H. Waelsch, *J. Amer. Chem. Soc.*, 1952, **74**, 1092; D. Ben-Ishai and A. Berger, *J. Org. Chem.*, 1952, **17**, 1564; B. F. Erlanger and R. M. Hall, *J. Amer. Chem. Soc.*, 1954, **76**, 5781; J. D. Cipera and R. V. V. Nicholls, *Chem. and Ind.*, 1955, 16.

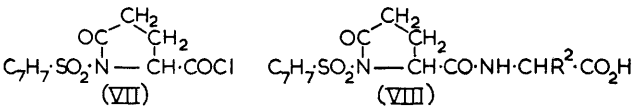
[59] M. Bergmann, L. Zervas, and L. Salzmann, *Ber.*, 1933, **66**, 1288.

[59a] H. Sachs and E. Brand, *J. Amer. Chem. Soc.*, 1953, **75**, 4610.

[60] F. E. King and D. A. A. Kidd, *J.*, 1949, 3315; F. E. King, B. S. Jackson, and D. A. A. Kidd, *J.*, 1951, 243.

Le Quesne and Young [61] explored other routes leading to α- and γ-glutamyl peptides. They treated benzyloxycarbonyl glutamic anhydride with ethyl alcohol and separated the resulting mixture of α- and γ-monoethyl esters into its components by fractional extraction with aqueous alkali. They also prepared the γ-monoethyl ester (by cold alcoholic hydrogen chloride) and converted this into the γ-azide for use in the synthesis of model peptides (Hegedüs [62] had independently prepared the γ-azide). Sachs and Brand [63] found, however, that the γ-azide gave mixtures of α- and γ-peptides.

The γ-amide of glutamic acid, glutamine, is of frequent occurrence in proteins and natural peptides and its incorporation in peptide synthesis is most important. Difficulties arise especially when it is necessary to use a glutamine residue as a carboxyl component. These have recently been overcome by Swan and du Vigneaud,[64] and independently by Rudinger,[65] using the following processes which involve several stages previously studied by Harington and Moggridge.[66] *N*-Toluene-*p*-sulphonylglutamic acid, on treatment with phosphorus pentachloride, yields 5-oxo-1-toluene-*p*-sulphonylpyrrolidine-2-carbonyl chloride (VII). This is used as the carboxyl component in a peptide synthesis, giving the acylamino-acid (VIII) which on



(VII) (VIII)

ring-opening with aqueous ammonia yields the toluenesulphonylglutaminyl peptide. The protecting group is then removed by sodium in liquid ammonia.

Another route to glutamine peptides has been recently announced.[67] This follows from the observation that by using controlled low temperatures it is possible to act upon glutamine hydrazide (available from the methyl ester) with nitrous acid without affecting the γ-amide grouping. This makes it possible to use the benzyloxycarbonyl-azide method for glutamine peptides.

(*b*) Aspartic acid. The problems here are similar to those encountered with glutamic acid.

Unequivocal reaction through the β-carboxyl group can be achieved from the α-benzyl ester, prepared [59] by the action of the alcohol on benzyloxycarbonylaspartic anhydride.

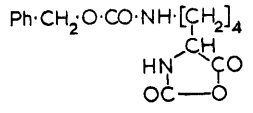
An equally clear-cut general route to reaction through the α-carboxyl group does not appear to have been described.[68]

[61] W. J. le Quesne and G. T. Young, *J.*, 1950, 1954, 1959.
[62] B. Hegedüs, *Helv. Chim. Acta*, 1948, **31**, 737.
[63] H. Sachs and E. Brand, *J. Amer. Chem. Soc.*, 1954, **76**, 1815.
[64] J. M. Swan and V. du Vigneaud, *ibid.*, p. 3110.
[65] J. Rudinger, *Coll. Czech. Chem. Comm.*, 1954, **19**, 235, 244; J. Rudinger and H. Czurbova, *ibid.*, p. 286.
[66] (Sir) C. R. Harington and R. C. G. Moggridge, *J.*, 1940, 706.
[67] E. Sondheimer and R. W. Holley, *J. Amer. Chem. Soc.*, 1954, **76**, 2816.
[68] W. D. John and G. T. Young, *J.*, 1954, 2870.

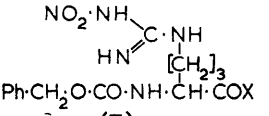
The β-amide, asparagine, can be brought into normal peptide synthesis (du Vigneaud), either as an amino-component,[64] or as a carboxyl component in the presence of tetraethyl pyrophosphite, without complicating reactions at the β-position.[69]

The conditions for the isomerisation of esters of α- and β-aspartyl (and α- and γ-glutamyl) peptides under alkaline hydrolysis have been studied.[70]

(ii) *Basic side chains.* (*a*) Lysine. Complications arise if lysine is to be used as the α-amino-component. It is then necessary to block the ε-amino-group specifically. This was achieved by Bergmann, Zervas, and Ross,[57] as early as 1935, using $\alpha\varepsilon$-dibenzenzyloxycarbonyl-lysyl chloride ; they took advantage of the tendency of α-benzyloxycarbonylamino-acid chlorides to lose benzyl chloride with the formation of hydroxyoxazolones, yielding (IX)



(IX)



(X)

which can be readily hydrolysed to N^{ε}-benzyloxycarbonyl-lysine. (This process has been adapted by Synge [71] for the $\alpha\delta$-diamino-acid, ornithine : this substance, though not a protein constituent, is found in some natural peptides.)

(*b*) Arginine. " Of the protein amino-acids, arginine presents perhaps the greatest difficulties in peptide synthesis " (Fruton [3]). The main reason for this is the strongly basic δ-guanidino-group. This can be sufficiently masked by nitration to permit peptide coupling to the α-amino-group, and the substituent nitro-group can be later removed, with some difficulty, by hydrogenation (Bergmann *et al.*[72]).

Satisfactory means for the introduction of arginine into peptide synthesis as the carboxyl component have been much more difficult to find. The logical development in this connection, from the discovery of the usefulness of nitro-arginine, is the preparation of the substances of type (X). This route was impracticable for years because of the impossibility of converting N^{α}-benzyloxycarbonyl-N^{ε}-nitroarginine into the corresponding acyl chloride or azide, owing to complicating side reactions involving the guanidino-group. In 1953, however, Hofmann, Rheiner, and Peckham [73] found that the mixed anhydride with ethyl carbonate could be formed and condensed with amino-acid esters.

A different approach was realised by Gish and Carpenter [19] in the same year. They utilised the fact that the guanidino-group, because of its strong basicity, retains the positively charged, unreactive guanidinium form under

[69] V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, and P. G. Katsoyannis, *J. Amer. Chem. Soc.*, 1954, **76,** 3115.

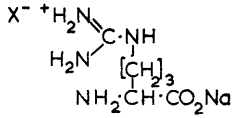
[70] A. R. Battersby and J. C. Robinson, *J.*, 1955, 259.

[71] R. L. M. Synge, *Biochem. J.*, 1948, **42,** 99.

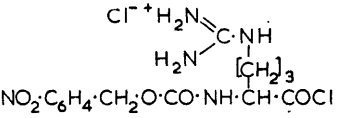
[72] M. Bergmann, L. Zervas, and H. Rinke, *Z. physiol. Chem.*, 1934, **224,** 40.

[73] K. Hofmann, A. Rheiner, and W. D. Peckham, *J. Amer. Chem. Soc.*, 1953, **75,** 6083 ; 1956, **78,** 238.

alkaline conditions strong enough to break up the α-amino-carboxyl ion-dipole. The synthesis is carried out under carefully controlled alkaline



(XI)



(XII)

conditions, *i.e.*, with the salt (XI). This is converted into the N^{α}-4-nitrobenzyloxycarbonyl derivative (yields in this case are much better than in the normal unsubstituted benzyloxycarbonyl method) with which thionyl chloride gives the chloride (XII). This reacts as a normal carboxyl component with amino-esters in dimethylformamide containing triethylamine.

This process was later used by du Vigneaud, Gish, and Katsoyannis,[74] in the probable synthesis of the peptide hormone arginine-vasopressin (see below).

(*c*) Histidine. The basic glyoxaline ring system does not interfere with the use of histidine methyl ester as an amino-component. Until 1954, however, no satisfactory process had been found for using histidine as a carboxyl component, owing to substitution and ring-opening side reactions, Holley and Sondheimer,[75] by a simple modification of standard procedure have succeeded in isolating N^{α}-benzyloxycarbonyl-L-histidine azide in good yield and in condensing it with amino-esters in ether; careful hydrolysis and catalytic hydrogenation gave fully optically active L-histidyl peptides.

Thiol and Disulphide Side Chains.—Preparative chemistry involving cystine (Cy·S·S·Cy) and cysteine (Cy·SH) has been clarified largely by the work of du Vigneaud and his collaborators during the past 25 years, mainly by the use of the sodium–liquid ammonia reagent, in which the following reactions have been achieved :

(1) [76] $Cy{\cdot}S{\cdot}S{\cdot}Cy \longrightarrow 2Cy{\cdot}SH$ (as $Cy{\cdot}SNa$)

(1*a*) [77] Reaction (1) can be reversed by aeration in aqueous solution.

(2) [76] $Cy{\cdot}SH + CH_2PhCl \longrightarrow Cy{\cdot}S{\cdot}CH_2Ph$

(3) [78] $Cy{\cdot}S{\cdot}CH_2Ph \longrightarrow Cy{\cdot}SH + PhMe$

(4) [12] $p\text{-}C_6H_4Me{\cdot}SO_2{\cdot}NHR \longrightarrow NH_2R + p\text{-}C_6H_4Me{\cdot}SH$

(5) [14] $CH_2Ph{\cdot}O{\cdot}CO{\cdot}NHR \longrightarrow NH_2R + CO_2 + PhMe$

(6) [12] $R{\cdot}CO_2{\cdot}CH_2Ph \longrightarrow R{\cdot}CO_2H + PhMe$

Reactions 1, 1*a*, 2, and 3 make it possible to start from cysteine or cystine, prepare the stable *S*-benzylcysteine, carry out synthetic work with this, and finally regenerate the cysteine or cystine residue at will.

Cyclic Peptides.—Several natural peptides are cyclic, without free terminal

[74] V. du Vigneaud, D. T. Gish, and P. G. Katsoyannis, *J. Amer. Chem. Soc.*, 1954, **76**, 4751.

[75] R. W. Holley and E. Sondheimer, *ibid.*, p. 1326.

[76] V. du Vigneaud, L. F. Audrieth, and H. S. Loring, *ibid.*, 1930, **52**, 4500.

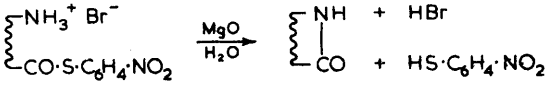
[77] V. du Vigneaud and R. R. Sealock, *J. Pharmacol. Exp. Therap.*, 1934, **54**, 433; S. Gordon and V. du Vigneaud, *Proc. Soc. Exp. Biol. Med.*, 1953, **84**, 723.

[78] H. S. Loring and V. du Vigneaud, *J. Biol. Chem.*, 1935, **111**, 385.

α-amino- or α-carboxy-groups, *e.g.*, gramicidin-S, a cyclic decapeptide containing a single, thirty-atom ring system.

Attempts to synthesise such cyclic peptides have been made, and several cyclic tripeptides obtained,[79] by methods depending on the idea that a peptide derivative of the type, $NH_2{\cdot}CHR^1{\cdot}CO{\cdots}NH{\cdot}CHR^n{\cdot}CO{\cdot}X$, at very high dilution, is more likely to lose HX intramolecularly than intermolecularly.

Such *cyclo*tripeptides must have their amide groups in the unnatural *cis*-conformation. It is not until five residues are present that the natural *trans*-amide conformation can be retained on cyclisation. Kenner and his collaborators have therefore applied their methods to the synthesis of cyclic pentapeptides and recently announced[80] the successful preparation of *cyclo*glycyl-L-leucylglycyl-L-leucylglycyl. The open-chain benzyloxycarbonyl-pentapeptide was prepared by the sulphuric anhydride method and was converted into the *p*-nitrophenyl thiolester. The benzyloxycarbonyl group was removed, without affecting the aryl thiolester group, by hydrogen bromide in acetic acid and the resulting thiolester hydrobromide kept at room temperature for 18 hours in an aqueous suspension of magnesium



oxide. The cyclised pentapeptide was obtained, after purification by counter-current distribution, in 44% yield—a very remarkable achievement.

*cyclo*Hexaglycyl has been prepared during polymerisation studies by Bamford and his colleagues[81] (see below).

Examples of Syntheses of Natural Peptides.—The first important natural peptide to be synthesised was glutathione. This substance was isolated by Hopkins[82] in 1921 and its structure was determined by degradative studies, notably by Quastel, Stewart, and Tunnicliffe,[83] and Kendall, Mason, and McKenzie,[84] as γ-L-glutamyl-L-cysteylglycine.

The first synthesis was due to Harington and Mead,[5] who used benzyloxycarbonylamino-acyl chloride–ethylamino-ester condensations, working with cystyl and unprotected cysteyl residues and removing the benzyloxycarbonyl groups by phosphonium iodide.

In 1936, du Vigneaud and Miller[85] carried out a second synthesis, based on the same type of condensation but using cysteyl residues protected by *S*-benzylation which was effected in the sodium–liquid ammonia reagent.

[79] R. A. Boissonnas and I. Schumann, *Helv. Chim. Acta*, 1952, **35**, 2229; M. Winitz and J. S. Fruton, *J. Amer. Chem. Soc.*, 1953, **75**, 3041; H. Brockman, H. Tummes, and F. A. von Metzsch, *Naturwiss.*, 1954, **41**, 37; J. C. Sheehan and W. L. Richardson, *J. Amer. Chem. Soc.*, 1954, **76**, 6329.

[80] J. A. Farrington, G. W. Kenner, and J. M. Turner, *Chem. and Ind.*, 1955, 602.

[81] D. G. H. Ballard, C. H. Bamford, and F. J. Weymouth, *Proc. Roy. Soc.*, 1954, *A*, **227**, 155; D. G. H. Ballard and C. H. Bamford, *Chem. Soc. Special Publ.*, No. 2, 1955, p. 25.

[82] (Sir) F. G. Hopkins, *Biochem. J.*, 1921, **15**, 286.

[83] J. H. Quastel, C. P. Stewart, and H. E. Tunnicliffe, *ibid.*, 1923, **17**, 580.

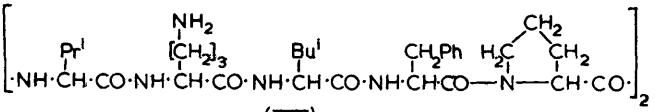
[84] E. C. Kendall, H. L. Mason, and B. F. McKenzie, *J. Biol. Chem.*, 1929, **84**, 657; 1930, **88**, 409.

[85] V. du Vigneaud and G. L. Miller, *ibid.*, 1936, **116**, 469.

This reagent was also used for the reductive removal of both the benzyloxycarbonyl and the *S*-benzyl group.

Hegedüs [62] has described another synthesis, using a benzyloxycarbonylaminoacylazide procedure, and recently Rudinger and Šorm [86] have used anhydro-*S*-benzyl-*N*-carboxycysteine, and Goldschmidt and Jutz [87] the cyanato- and the phosphorazo-method in further glutathione syntheses.

A more complex natural peptide to which a synthetic approach has been made is the antibacterial substance gramidicin-S. This was isolated by Gause and Brazhnikova [88] in 1943. Degradative structural studies (Consden, Gordon, Martin, and Synge [89]) and molecular-weight measurements indicate that the substance is the *cyclo*decapeptide (XIII).



(XIII)

Harris and Work [90] succeeded in preparing the corresponding open-chain pentapeptide by using the orthodox benzyloxycarbonylamino-azide route. Schumann and Boissonnas [91] also report the preparation of this pentapeptide, using the mixed anhydride with ethyl hydrogen carbonate throughout; it appears very probable that considerable racemisation occurred in this synthesis.

Erlanger, Sachs, and Brand [92] have succeeded in the remarkable task of preparing the corresponding open-chain decapeptide L-valyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-prolyl-L-valyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline. They used the carbonate mixed anhydride method for dipeptide intermediates only, reserving the azide route for higher peptides to avoid racemisation. It would be very interesting to see if this decapeptide could be cyclised by the new Kenner procedure.

The most remarkable achievement so far attained in this field is the synthesis of the peptide pituitary hormone, oxytocin, by du Vigneaud and his collaborators,[93, 94] announced in 1953 and fully reported in 1954 (for a review see ref. 95).

The isolation of the pure natural product and the degradative studies,

[86] J. Rudinger and F. Šorm, *Coll. Czech. Chem. Comm.*, 1951, **16**, 214.

[87] S. Goldschmidt and C. Jutz, *Chem. Ber.*, 1953, **86**, 1116.

[88] G. F. Grause and M. G. Brazhnikova, "Sovyetskii Gramitsidin i Lecheniye Ran", Ed. P. G. Sergier, Moscow, 1943.

[89] R. Consden, A. H. Gordon, A. J. P. Martin, and R. L. M. Synge, *Biochem. J.*, 1946, **40**, xliii; 1947, **41**, 596.

[90] J. I. Harris and T. S. Work, *ibid.*, 1950, **46**, 196, 582.

[91] I. Schumann and R. A. Boissonnas, *Helv. Chim. Acta*, 1952, **35**, 2237.

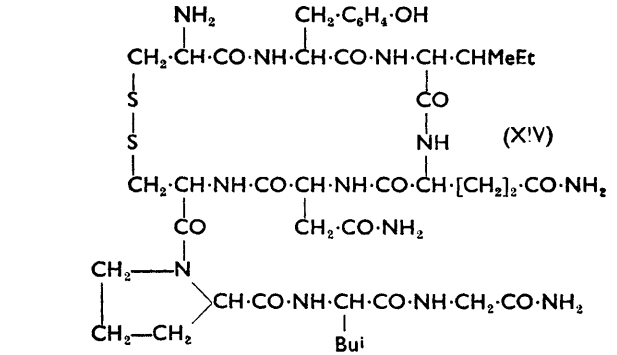
[92] B. F. Erlanger, H. Sachs, and E. Brand, *J. Amer. Chem. Soc.*, 1954, **76**, 1806.

[93] V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, and P. G. Katsoyannis, *ibid.*, 1953, **75**, 4879.

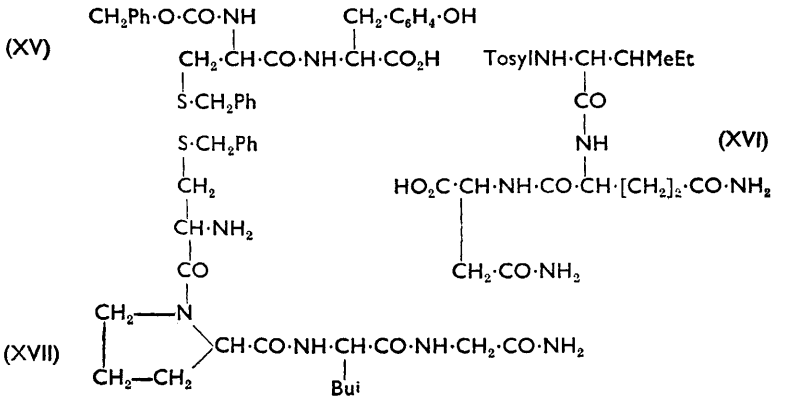
[94] C. Ressler and V. du Vigneaud, *ibid.*, 1954, **76**, 3107; J. M. Swan and V. du Vigneaud, *ibid.*, p. 3110; P. G. Katsoyannis and V. du Vigneaud, *ibid.*, p. 3113; V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, and P. G. Katsoyannis, *ibid.*, p. 3115.

[95] V. du Vigneaud, *Chem. Soc. Special Publ.*, No. 2, 1955, p. 49.

carried out in du Vigneaud's laboratory, indicated that the substance is a nonapeptide amide containing a 20-atom " loop " closed by a cystine disulphide bridge (XIV). The three peptide derivatives (all in the L-configuration) *S*-benzyl-*N*-carbobenzyloxycysteyltyrosine (XV), *N*-toluene-*p*-sulphonyl*iso*leucylglutaminylasparagine (XVI), and *S*-benzylcysteylprolylleucylglycine amide (XVII) were prepared, a wide range of techniques being



(XIV)



(XV) (XVI)

(XVII)

used. Peptide linkage between the last two was effected by the tetraethyl pyrophosphite " amide procedure ". The *N*-toluene-*p*-sulphonyl and the *S*-benzyl group of the resulting protected heptapeptide were removed, and the *S*-benzyl group replaced, all in the sodium–liquid ammonia reagent, and the product was condensed (again by the tetraethyl pyrophosphite " amide procedure ") with the dipeptide (XV). Protecting groups were removed as before and the disulphide bridge was formed by aeration. The identity of the synthetic material with the natural hormone was rigorously proved by physical, chemical, and biological tests.*

[95a] R. A. Boissonnas, St. Guttmann, P.-A. Jaquenoud, and J.-P. Waller, *Helv Chim. Acta*, 1955, **38**, 1491.

[95b] J. Rudinger, J. Honzl, and M. Zaorat, *Coll. Czech. Chem. Comm.*, 1956, **21**, 202.

* A second synthesis has just been announced [95a] which makes extensive use of N_3 and $\cdot O \cdot CO_2Et$ groups and of removal of benzyloxycarbonyl groups by hydrobromic acid in acetic acid. A third is due to Rudinger and his collaborators.[95b]

The probable synthesis of two other peptide pituitary hormones, closely related to oxytocin, has been announced from du Vigneaud's laboratory, namely, lysine-vasopressin [96] (differing from oxytocin in having phenylalanine for *iso*leucine, and lysine for leucine) and arginine-vasopressin [74] (differing from lysine-vasopressin in having arginine for lysine). Full details are not yet available (cf. du Vigneaud [97]).

The 20-atom "loop" of α-amino-acid residues, closed by the cystine disulphide bridge, is also an important feature of the insulin monomer (*M* 6000) elucidated by degradative studies by Sanger *et al.*[98] It is possible, now, to think of planning a synthetical attack upon this important protein molecule (see Harington and du Vigneaud [99]).

Polymerisation Techniques in Polypeptide Synthesis.—Stepwise synthesis of peptides becomes increasingly difficult as the number of amino-acid residues in the polypeptide increases. Polypeptides of high molecular weight (~ 10,000) have, however, been synthesised by polymerisation techniques, starting with monomers based on either single amino-acids or di- or tri-peptides.

Such processes are not likely to lead to proteins or natural higher peptides because, in these substances, the peptide chains comprise wide ranges of kinds of α-amino-acids and the various residues are arranged along the chains in highly specific and complicated orders. Polymerisation syntheses have most usually been applied to single kinds of amino-acid residues, giving polypeptide chains comprising residues of only one kind (*e.g.*, polyglycylglycine). Even when co-polymerisation techniques are invoked, permitting the incorporation of several kinds of residues in a polymer, there is no control of the order in which they are assembled along the resulting chain. Polymerisation based on di- or tri-peptide monomers only permits the assembly of two or three kinds of residues in the simplest possible order. Despite this limitation, polymerisation techniques have been of great importance in the preparation of polypeptide model substances of high molecular weight, which closely resemble fibrous proteins. The study of the physical properties of these polymers, especially their *X*-ray diffraction and infrared absorption, has greatly helped the elucidation of the structure of the native proteins.

The following polymerisation processes have been used (for a review up to 1951, see Katchalski [100]).

(a) *Methods based on amino-acid monomers.* Leuchs [101] (1906—08)

[96] V. du Vigneaud, H. C. Lawler, and E. A. Popenoe, *J. Amer. Chem. Soc.*, 1953, **75**, 4880.

[97] V. du Vigneaud, *Chem. Soc. Special Publ.*, No. 2, 1955, p. 66.

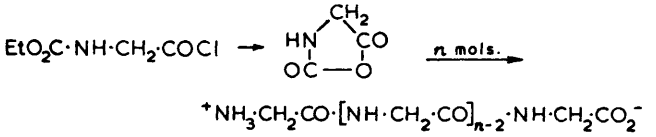
[98] F. Sanger and H. Tuppy, *Biochem. J.*, 1951, **49**, 481; F. Sanger and E. O. P. Thompson, *ibid.*, 1953, **53**, 353, 366; A. P. Ryle, F. Sanger, L. F. Smith, and R. Kitai, *ibid.*, 1955, **60**, 541; H. Brown, F. Sanger, and R. Kitai, *ibid.*, p. 556.

[99] (Sir) Charles Harington, *Chem. Soc. Special Publ.*, No. 2, 1955, p. 64; V. du Vigneaud, *ibid.*, p. 65.

[100] E. Katchalski, *Adv. Protein Chem.*, 1951, **6**, 123.

[101] H. Leuchs, *Ber.*, 1906, **39**, 857; H. Leuchs and W. Manasse, *Ber.*, 1907, **40**, 3235; H. Leuchs and W. Geiger, *Ber.*, 1908, **41**, 1721.

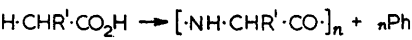
obtained a polyglycine by the annexed route, *via* anhydro-*N*-carboxyglycine; the first step, giving "anhydro-*N*-carboxyglycine" (2-hydroxyoxazol-5-one), is effected by heat and the second in the presence of an amine and



a trace of water. The process was further studied by Curtius [102] and by Wessely [103] and their collaborators, and was being developed by Robinson, Goldsworthy, and Springall in 1938—39. Woodward and Schramm [104] later used the method to produce co-polymers of leucine and phenylalanine. Since 1948 this method has been developed, largely by wor kers of Courtauld's research group, into the most important of the polymerisation techniques. A standard procedure for working in nitrobenzene solution has been devised,[105] and the kinetics of the reaction have been studied.[106] Initiation of polymerisation of anhydro-*N*-carboxyamino-acids by alkali halides (instead of organic bases) leads to polymerisation by a different mechanism and yields cyclic peptides (see Bamford and his collaborators [81]).

Bresler and Selezneva [107] have reported a novel preparation of a polyalanine by treating lactamide with sodium:

and Noguchi [108] has found that amino-acids, *N*-substituted by the Ehrensvärd phenylthiocarbonyl group,[21] can be made to undergo polycondensation with the elimination of thiophenol and carbon dioxide:



(b) *Methods based on di- and tri-peptide monomers.* In 1906, Fischer [109] noted that when glycylglycylglycine methyl ester is heated it yields, by elimination of methyl alcohol, the hexapeptide pentaglycylglycine methyl ester. Similar results were obtained on using alanylglycylglycine ester. Pacsu and Wilson [110] showed that on prolonged heating the reaction goes further, to give polypeptides by repetition of the tripeptide sequence. The kinetics of the process have been studied by Rees, Tong, and Young.[111]

[102] T. Curtius and W. Sieber, *Ber.*, 1922, **55**, 1543.

[103] F. Wessely, *Z. physiol. Chem.*, 1926, **157**, 91; F. Wessely and F. Sigmund, *ibid.*, 1927, **159**, 102; **170**, 167.

[104] R. B. Woodward and C. H. Schramm, *J. Amer. Chem. Soc.*, 1947, **69**, 1551.

[105] W. E. Hanby, S. G. Waley, and J. Watson, *J.*, 1950, 3009.

[106] S. G. Waley and J. Watson, *Proc. Roy. Soc.*, 1949, *A*, **199**, 499; D. G. H. Ballard and C. H. Bamford, *ibid.*, 1954, *A*, **223**, 495.

[107] S. E. Bresler and N. A. Selezneva, *Zhur. obshchei Khim.*, 1950, **20**, 356.

[108] J. Noguchi and T. Hayakawa, *J. Amer. Chem. Soc.*, 1954, **76**, 2846.

[109] E. Fischer, *Ber.*, 1906, **39**, 471.

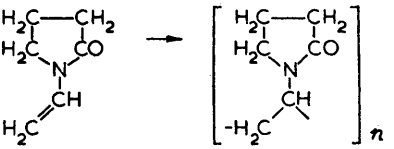
[110] E. Pacsu and E. Wilson, jun., *J. Org. Chem.*, 1942, **7**, 117, 126.

[111] P. S. Rees, D. F Tong, and G. T. Young, *J.*, 1954, 662.

Magee and Hofmann [112] found that when the hydrazide of glycylglycylglycine is treated with nitrous acid the corresponding azide is produced in good yield without complicating side reaction at the free *N*-terminal α-amino-group. Treatment of the azide with alkali leads, by the elimination of hydrazoic acid, to polycondensation.

Polycondensations corresponding to those with the above tripeptide derivatives have recently been observed with dipeptide chlorides [113] and, under the influence of the enzyme cathepsin-C, with dipeptide amides,[114] while Noguchi [108] and Hayakawa successfully applied their method to dipeptide, as well as amino-acid, monomers.

A synthetic polymer material which, though not containing the polypeptide system, nevertheless has remarkable resemblances to some proteins, is the poly-*N*-vinylpyrrolidone (XVIII), synthesised during the war by



(XVIII)

Reppe.[115] This substance has been successfully used as a blood-plasma protein substitute in blood transfusion and in prolonging the effect of, *e.g.*, insulin and penicillin.

Degradation of peptides: terminal residue studies

The sequence of the amino-acid residues in polypeptide chains has a profound effect on physical and physiological properties. The sequences in natural substances are highly specific, follow no known laws, and must be determined experimentally. There are indications that the *N*-terminal are more important than the *C*-terminal sequences for biological activity.

The residue sequence in a single polypeptide chain of any length could, in principle, be determined by the repeated application of a single efficient process for the removal and identification of either the *N*- or the *C*-terminal residue. In practice, it is not feasible to carry out the complete stepwise degradation of a long polypeptide chain in this way, even employing both *N*- and *C*-residue studies, because of the cumulative effect of incomplete reaction. Such a polypeptide must first be partially hydrolysed to oligopeptides, these separated and individually subjected to sequence determination, and the results pieced together to give the structure of the original long chain. A further difficulty, often arising, is that the molecules of many proteins are composed of several long polypeptide chains, cross-linked, *e.g.*, by -S–S-bridges. In such cases the cross linkages must be broken and the individual long polypeptide chains separated as a first step to degradative

[112] M. Z. Magee and K. Hofmann, *J. Amer. Chem. Soc.*, 1949, **71**, 1515.
[113] M. Frankel, Y. Liwschitz, and A. Zilka, *ibid.*, 1954, **76**, 2814.
[114] J. S. Fruton, W. R. Hearn, V. M. Inpam, D. S. Wiggans, and M. Winitz, *J. Biol. Chem.*, 1953, **264**, 891.
[115] See B.I.O.S. Final Report, No. 354, 1945, item 22.

studies. These necessary and very difficult preliminary tasks are outside the scope of the present discussion which is confined to terminal residue studies.[116] These are of two types, chemical and enzymic.

The *chemical methods* involve: (i) modification of the terminal residue to differentiate it and, if possible, to render labile the amide bond joining it to the adjacent residue; (ii) breaking of this amide bond; and (iii) isolation and identification of the modified terminal residue. Only if the conditions in stage (ii) can be such that the other peptide bonds are unaffected can the process be repeated on the same specimen. The conditions in stage (i) must not cause any peptide bond fission, or promote rearrangement of residues in the chain. Yields should be quantitative. These requirements are difficult to meet. The development of methods has, so far, been more successful for *N*- than for *C*-terminal residues.

The *enzymic methods* depend on the existence of exopeptidases,[117] enzymes catalysing specifically the hydrolysis from peptides of terminal residues. These are classified, according to their *N*- or *C* specificity, as amino- or carboxy-peptidases. Their use gives, in principle, the most attractive method of terminal-residue identification. No preliminary chemical modification of the peptide is needed, and the cleavage of the terminal residue is done under the mildest conditions and is progressive, as the enzyme operates on the shortened residual peptide. The difficulties are practical: for such work the enzyme should be quite homogeneous, free from any other enzymes or even inactive proteins. This is, in general, extremely difficult to achieve. Moreover, the enzyme should be rigidly specific to *N*- or to *C*-terminal residues and should show (i) no endopeptidase[117] activity (hydrolysis of peptide bonds remote from the chain ends), (ii) no tendency to promote transpeptidation (synthesis of new peptide bonds), (iii) no R-side-chain specificity (the rate of hydrolysis of the terminal residue should be independent of its nature). No actual amino- or carboxy-peptidase preparations are completely satisfactory in all these respects, least so with regard to side-chain specificity, though modern techniques have permitted the production of carboxy- and, more recently, amino-peptidase in a highly homogeneous, pure form.

***N*-Terminal Residue Studies.**—The two most important current methods are chemical: Sanger's *N*-2 : 4-dinitrophenyl (DNP) method, and Edman's phenylthiohydantoin (phenyl *iso*thiocyanate) method. The recent isolation of amino-peptidase in a state of high purity (Smith and his co-workers[118]) makes its use in *N*-terminal residue studies likely to become important.

(a) *Chemical methods involving total hydrolysis in stage* (ii). (α) DNP method. When an amino-group is exposed to the action of 1-fluoro-2 : 4-

[116] Reviews: S. W. Fox, *Adv. Protein Chem.*, 1945, **2**, 155; F. Sanger, *ibid.*, 1952, **7**, 2; P. Desnuelle, *Adv. Enzymology*, 1953, **14**, 278; H. Fraenkel-Conrat, J. I. Harris, and A. L. Levy in "Methods of Biochemical Analysis", ed. D. Glick, Interscience Publ., New York, 1954, Vol. II, p. 359; M. Rovery and P. Desnuelle, *Bull. Soc. Chim. biol.*, 1954, **36**, No. 1, p. 95; M. Jutisz, *ibid.*, p. 109.

[117] See H. Neurath and G. W. Schwert, *Chem. Rev.*, 1950, **46**, 69.

[118] P. H. Spackman, E. L. Smith, and D. M. Brown, *J. Biol. Chem.*, 1955, **212**, 255; E. L. Smith and D. H. Spackman, *ibid.*, p. 271.

dinitrobenzene (FDNB) under mild, alkaline conditions, it reacts almost quantitatively to form the bright yellow 2 : 4-dinitrophenyl (DNP) derivative. The resulting Ar–N bond is very stable under acid hydrolytic conditions.[119] (Probable forerunners of the DNP method were the use in peptide acylation of 1-chloro-2 : 4-dinitrobenzene [120] and 2 : 3 : 4-trinitrotoluene,[121] which require strenuous conditions and do not give quantitative results.)

When a peptide is treated with 1-fluoro-2 : 4-dinitrobenzene and later hydrolysed, then, any side-chain reactions being neglected, the resulting mixture consists of the dinitrophenyl derivative of the *N*-terminal amino-acid together with the other component amino-acids. The dinitrophenyl-amino-acid is usually separated by extraction with an organic solvent, identified (by control hydrolysis and partition chromatography if necessary), and estimated by means of its strong ultraviolet absorption at ~ 350 mμ.

Reactive R-side-chains may cause complications. In these chains, amino-groups give dinitrophenyl derivatives, thiol groups react in a complex manner (in sequence work involving cystine–cysteine residues these must be kept in the disulphide cystine state or further oxidised to cysteic acid, Cy·SO_3H), phenolic hydroxy-groups give dinitrophenoxy-derivatives which are fortunately colourless, and the cyclised side-chain and amino-system in dinitrophenylproline shift the strong absorption region to ~ 390 mμ.

Porter and Sanger,[122] and Levy and Chung,[123] have studied the conditions and corrections needed for the preparation and hydrolysis of dinitrophenyl-peptides. Sanger and Tuppy [124] have described the separation and estimation procedure (two-dimensional paper chromatography) developed for the successful insulin sequence investigation; and Levy [125] has given an account of a somewhat different technique, employing the same principle, later used on α-corticotropin.

The outstanding successes of the dinitrophenyl method are in the sequence determinations in insulin, B-chain [126] (30 residues), A-chain [127] (21 residues), oxytocin [128] (9 residues), and α-corticotropin [129] (39 residues).

(β) *p*-Iodophenylsulphonyl method.[130] This is closely related to the dinitrophenyl method. The peptide is acylated by the radioactive *p*-iodophenylsulphonyl group, *p*-^{131}I·C_6H_4·SO_2·, introduced as the chloride. The

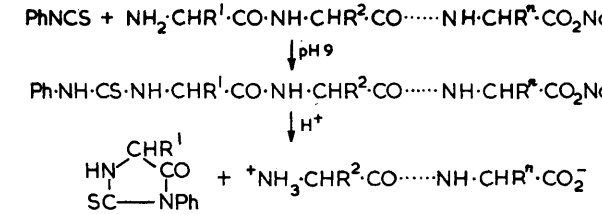
[119] F. Sanger, *Biochem. J.*, 1945, **39**, 507.
[120] E. Abderhalden and W. Stix, *Z. physiol. Chem.*, 1923, **129**, 143.
[121] G. Barger and F. Tutin, *Biochem. J.*, 1918, **12**, 402.
[122] R. R. Porter and F. Sanger, *ibid.*, 1948, **42**, 287; R. R. Porter in "Methods of Medical Research", ed. R. W. Gerard, Year Book Publ., Chicago, 1950, Vol. III, p. 256.
[123] A. L. Levy and D. Chung, *J. Amer. Chem. Soc.*, 1955, **77**, 2899.
[124] F. Sanger and H. Tuppy, *Biochem. J.*, 1951, **49**, 463.
[125] A. L. Levy, *Nature*, 1954, **174**, 126.
[126] F. Sanger and H. Tuppy, *Biochem. J.*, 1951, **49**, 481.
[127] F. Sanger and E. O. P. Thompson, *ibid.*, 1953, **53**, 353, 366.
[128] H. Tuppy, *Biochim. Biophys. Acta*, 1953, **11**, 449.
[129] C. H. Li, I. I. Geschwind, R. D. Cole, I. D. Raacke, J. I. Harris, and J. S. Dixon, *Nature*, 1955, **176**, 687.
[130] S. Udenfriend and S. F. Velick, *J. Biol. Chem.*, 1951, **190**, 733; **191**, 233.
[131] R. E. Bowman, *J.*, 1950, 1349.

resulting sulphonamide is stable to acid hydrolysis and the acyl derivative of the *N*-terminal amino-acid can be detected by its radioactivity and estimated by the isotope-dilution procedure. However, the method seems to give rather low results.

(γ) Methylation method. *N*-Terminal amino-groups can be dimethylated by treatment with aqueous formaldehyde and simultaneous catalytic hydrogenation.[131] After hydrolysis the *NN*-dimethylamino-acid can be separated by partition chromatography, the lack of reaction with ninhydrin being used for detection.[132]

(δ) Deamination methods. Several techniques involving the drastic step of cleaving the N–C bond in the *N*-terminal residue have been described. The following reagents have been used: hypobromous acid;[133] nitrous acid;[83] nitrosyl chloride;[134] ninhydrin, then hydrogen peroxide–vanadium pentoxide.[135] Side reactions usually occur. Extensive use of these methods seems unlikely.

(b) *Stepwise chemical methods.* (α) Phenylthiohydantoin (PTH) method. Edman [136] showed that when a peptide is treated with phenyl *iso*thiocyanate at pH 9 the phenylthiocarbamoyl (PTC) derivative of the *N*-terminal amino-group is formed. On treatment with hydrogen chloride in nitromethane or, better, acetic acid, or with 0·1N-hydrochloric acid at 75°,[137] the *N*-terminal residue is cleaved specifically from the peptide as the 5-R^1-3-phenyl-2-thiohydantoin. The reactions are virtually quantitative. The cyclisation may be followed by the shift of the ultraviolet absorption maximum from 240 (phenylthiocarbamoyl-peptide) to 270 mμ (phenylthiohydantoin).[138]



The thiohydantoin and the shortened peptide can be separated by partition between organic and aqueous solvents. The thiohydantoin may be identified by hydrolysis by baryta [136] or acid [139] to the amino-acid, or by direct paper chromatography, spots being located by suppression of the

[132] V. M. Ingram, *J. Biol. Chem.*, 1953, **202**, 193.

[133] S. Goldschmidt, E. Wiberg, F. Nagel, and K. Martin, *Annalen*, 1927, **456**, 1; S. Goldschmidt and K. Strauss, *ibid.*, 1929, **471**, 1; *Ber.*, 1930, **63**, 1218.

[134] R. Consden, A. H. Gordon, and A. J. P. Martin, *Biochem. J.*, 1947, **41**, 590.

[135] F. Turba in "The Chemical Structure of Proteins", ed. G. E. W. Wolstenholme and M. P. Cameron, Churchill, London, 1953, p. 142.

[136] P. Edman, *Acta Chem. Scand.*, 1950, **4**, 283; 1953, **7**, 700.

[137] M. Ottesen and A. Wollenberger, *Nature*, 1952, **170**, 801; *Compt. rend. Trav. Lab. Carlsberg*, 1953, **28**, 463.

[138] B. Dahlerup-Peterson, K. Linderstrøm-Lang, and M. Ottesen, *Acta Chem. Scand.*, 1952, **6**, 1135.

[139] A. L. Levy, *Biochim. Biophys. Acta*, 1954, **15**, 589.

starch–iodine colour,[140] or by the colour reaction with Grote's solution.[141] The residual shortened peptide may be subjected to a second degradation.

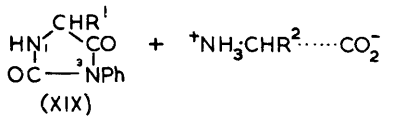
A general Edman procedure applicable to all common amino-acids, save perhaps cystine, on a microscale has been worked out by Fraenkel-Conrat and Harris.[142]

Notable achievements of the method are in the sequence determinations on oxytocin,[143] arginine- and lysine-vasopressin [144] (each of 9 residues), α-[129] and β-corticotropin,[145] and corticotropin-A,[146] (each of 39 residues).

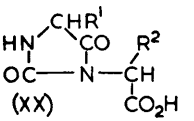
Fox and his collaborators [147] have used the phenyl *iso*thiocyanate (and *iso*cyanate) reaction followed by complete hydrolysis of the phenylthiocarbamoyl-peptide to the thiohydantoin and residual amino-acids. The latter are subjected to bioassay, in which the thiohydantoin is inert. Comparison with a bioassay on the hydrolysate of the unsubstituted peptide identifies the *N*-terminal residue. The process seems rather laborious but has been used in studies on various corticotropin fractions [147] and on lysozyme.[148]

(β) Other methods based on thiohydantoins and hydantoins. Replacement of Edman's phenyl *iso*thiocyanate by the coloured 4-dimethylamino-3 : 5-dinitrophenyl *iso*thiocyanate has been investigated ; [149] so has the use of 3-unsubstituted derivatives.[150]

The forerunner of the Edman procedure was the early use of phenyl *iso*cyanate which reacts with a peptide to form the phenylcarbamoyl derivative from which the *N*-terminal residue is removed as the 3-phenylhydantoin (XIX).[151]



(XIX)



(XX)

[140] J. Sjoquist, *Acta Chem. Scand.*, 1953, **7**, 447.

[141] W. A. Landmann, M. P. Drake, and W. F. White, *J. Amer. Chem. Soc.*, 1953, **75**, 3638.

[142] H. Fraenkel-Conrat and J. I. Harris, *ibid.*, 1954, **76**, 6058.

[143] V. du Vigneaud, C. Ressler, and S. Trippett, *J. Biol. Chem.*, 1953, **205**, 949.

[144] E. A. Popenoe and V. du Vigneaud, *ibid.*, p. 133.

[145] P. H. Bell, *J. Amer. Chem. Soc.*, 1954, **76**, 5565.

[146] W. F. White and W. A. Landmann, *ibid.*, 1955, **77**, 1711.

[147] S. W. Fox, T. L. Hurst, and K. F. Itscher, *ibid.*, 1951, **73**, 3573 ; S. W. Fox, T. L. Hurst, and C. Warner, *ibid.*, 1954, **76**, 1154.

[148] D. de Fontaine and S. W. Fox, *ibid.*, p. 3701.

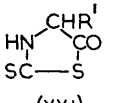
[149] W. S. Reith and N. M. Waldron, *Biochem. J.*, 1954, **56**, 116.

[150] D. T. Elmore and P. A. Toseland, *J.*, 1954, 4533.

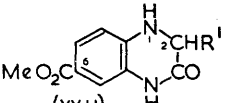
[151] M. Bergmann, A. Miekeley, and E. Kann, *Annalen*, 1927, **458**, 56 ; E. Abderhalden and H. Brockmann, *Biochem. Z.*, 1930, **225**, 386.

The generation, from peptides, of hydantoins 3-substituted by other groups (*e.g.*, the coloured *p*-phenylazophenyl [152] and 4-dimethylamino-3 : 5-dinitrophenyl group [153]) has also been studied. The *N*-terminal and the adjacent residue can be split from an ethoxycarbonyl peptide as the 3-hydantoinylacetic acid derivative (XX).[154] The final identification of the *N*-terminal residue seems ambiguous, however.

(γ) Methods based on mercapto- and hydroxy-thiazolones. Levy [155] noted that when salts of *N*-dithiocarboxy-peptides were acidified the R^1-residue tends to be split off as the 4-R^1-2-mercaptothiazol-5-one (XXI), which could be extracted by organic solvents for regeneration and identification of the amino-acid.



(XXI)



(XXII)

Kenner and Khorana [156] carried out a similar process *via* the 4-R^1-2-hydroxythiazol-5-one.

(δ) Methods based on tetrahydro-oxoquinoxalines. Holley and Holley [157] treated peptides with methyl 4-fluoro-3-nitrobenzoate, reduced the product catalytically, and cleaved the *N*-terminal residue by cold hydrochloric acid as the quinoxaline derivative (XXII). A similar process has been carried out on dinitrophenyl-peptides.[158]

None of these chemical methods, other than the Edman method, has been widely used.

(c) *Enzymic method.* The recent isolation of highly purified amino-peptidase from pig kidney [118] may well open a new phase in the study of *N*-terminal residues. The enzyme does show marked side-chain specificity, *e.g.*, glycine, serine, and glutamic acid are split off very slowly, but has already been used on corticotropin-A.[159] This work has shown the critical importance of the *N*-terminal sequence for the biological activity of this hormone.

***C*-Terminal Residue Studies.**—The most important method is that based on the use of carboxypeptidase. Many chemical methods have been described : none of them is yet really satisfactory ; probably the most promising is the modern version of the Schlack–Kumpf thiohydantoin process.

[152] F. Turba, ref. 135, p. 143.

[153] G. G. Evans and W. S. Reith, *Biochem. J.*, 1954, **56,** 111.

[154] F. Wesseley, K. Schlögl, and G. Korger, *Nature*, 1952, **169,** 708 ; *Monatsh.*, 1952, **83,** 1157.

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[156] H. G. Khorana and G. W. Kenner, *J.*, 1952, 2076.

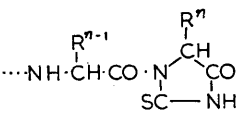
[157] R. W. Holley and A. D. Holley, *J. Amer. Chem. Soc.*, 1952, **74,** 1110.

[158] M. Jutisz, *Bull. Soc. Chim. biol.*, 1954, **36,** p. 108.

[159] W. F. White and W. A. Landmann, *J. Amer. Chem. Soc.*, 1955, **77,** 1711 ; K. Hofmann and A. Jöhl, *ibid.*, p. 2914 ; W. F. White, *ibid.*, p. 4691.

Enzymic method. Highly purified crystalline carboxypeptidase, from ox pancreas, has been available for some years.[160] Dangers of contamination by endopeptidases have been overcome by the observation [161] that carboxypeptidase activity is not affected by di*iso*propyl phosphorofluoridate which inhibits the activity of pancreatic endopeptidases. This enzyme gives the best current method for specific cleavage of a *C*-terminal residue (for reviews see refs. 117, 162); subsequent identification of the residue is made either on the free amino-acid or on its dinitrophenyl derivative. It has the drawback of marked side-chain specificity. Fission is fastest with aromatic R^n-side-chain residues, is very slow with basic or acidic residues, and does not occur with proline; moreover, the enzyme is without action on *C*-terminal residues in the amide form, as in oxytocin and the vasopressins. The enzyme has been used in studies of residue sequence on, *e.g.*, insulin,[163] α-[129] and β-corticotropin,[145] corticotropin-A,[146] and tobacco mosaic virus.[164] In all cases studied so far, the loss of the *C*-terminal residue does not impair biological activity,[162]

Chemical methods. (α) Thiohydantoin method. Schlack and Kumpf [165] showed that when an acylpeptide is treated with hot acetic anhydride and ammonium thiocyanate, it is converted into the corresponding 1-acylpeptidyl-5-R^n-2-thiohydantoin (XXIII) in ~80% yield; the R^n residue can be split by brief alkaline hydrolysis, without significant fission of peptide bonds, as the 5-R^n-2-thiohydantoin, which is extracted by an organic solvent and identified. Waley and Watson, and Tibbs,[166] improved the later stages of the process and reduced the scale, hydrolysing the extracted R^n-thiohydantoin to the free amino-acid and identifying it by paper chromatography.



(XXIII)

Kenner, Khorana, and Stedman [167] improved the first step, avoiding the very vigorous acetic anhydride treatment by the use of diphenyl phosphor-*iso*thiocyanatidate, $(PhO)_2PO{\cdot}NCS$, in dimethylformamide. They followed the hydrolysis of the thiohydantoin spectroscopically [max. at 260 (1-acyl system) and 278 mμ (1-unsubstituted system)]. *C*-Terminal serine and proline seem resistant to this treatment.[168] A somewhat analogous process involving mild treatment with diaryl carbodi-imides, R·N:C:N·R, and the separation of the R^n residue as the open chain $R{\cdot}NH{\cdot}CO{\cdot}NH{\cdot}CHR^n{\cdot}CO{\cdot}NH{\cdot}R$·, has been studied.[51a] Here, however, poor yields cause contamination of products.

(β) Methods depending on the separation of the R^n-residue as $R^n{\cdot}CO{\cdot}R'$.

[160] M. L. Anson, *J. Gen. Physiol.*, 1937, **20**, 663.
[161] H. Neurath and J. A. Gladner, *Biochim. Biophys. Acta*, 1952, **9**, 335.
[162] J. I. Harris, *Chem. Soc. Special Publ.*, No. 2, 1955, p. 71.
[163] J. Lens, *Biochim. Biophys. Acta*. 1949, **3**, 367; J. I. Harris, *J. Amer. Chem. Soc.*, 1952, **74**, 2944.
[164] J. I. Harris and C. A. Knight, *Nature*, 1952, **170**, 613.
[165] P. Schlack and W. Kumpf, *Z. physiol. Chem.*, 1926, **154**, 125.
[166] S. G. Waley and J. Watson, *J.*, 1951, 2394; J. Tibbs, *Nature*, 1951, **168**, 911.
[167] G. W. Kenner, H. G. Khorana, and R. J. Stedman, *J.*, 1953, 673.
[168] R. A. Turner and G. Schmerzler, *Biochim. Biophys. Acta*, 1954, **13**, 553.

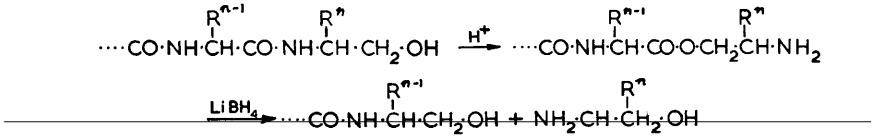
The earliest stepwise methods were of this class ($R^1 = CHPh_2$, *via* ···CO·NH·CHR^n·CPh$_2$·OH from PhMgBr and the peptide ester,[169] $R^1 = H$, *via* ···CO·NH·CHR^n·NH$_2$, (*a*) from Curtius rearrangement [170] of the azide ···CO·NH·CHR^n·CO·N$_3$ and (*b*), recently, from a Lossen reaction [171] with ···CO·NH·CHR^n·CO·NH·OH) but yields are very poor.

Boissonnas has described [172] an elegant microscale technique for $R^1 = H$, *via* ···CO·NH·CHR^n·OMe which is obtained on anodic decarboxylation and methoxylation of the acyl peptide.

(*γ*) Reductive (α-amino-alcohol) method. Chibnall and Fromageot and their co-workers showed that treatment of peptide esters [173] in tetrahydrofuran with lithium borohydride, or free peptides [174] in 4-ethylmorpholine with lithium aluminium hydride converted the terminal carboxyl group into an alcohol group, apparently without affecting the peptide bonds. Subsequent hydrolysis yields the R^n-residue as the amino-alcohol, NH$_2$·CHR^n·CH$_2$·OH, and all other residues as the free amino-acids. The amino-alcohols do not give the ninhydrin test and can be identified by partition chromatography of the dinitrophenyl derivatives.[175]

Lithium aluminium hydride seems liable to cause some cleavage of peptide bonds, and the borohydride appears preferable.[176]

An interesting route for liberating the amino-alcohol stepwise has been investigated recently,[177, 178] This depends on the acid-catalysed N⟶O migration of acyl groups, as illustrated. Complications arise through N⟶O migration at serine residues and through reductive chain fission with higher peptides, the low solubility of which necessitates high reaction temperatures.[177]



[169] F. Bettzieche and R. Menger, *Z. physiol. Chem.*, 1926, **161**, 37; F. Bettzieche, *ibid.*, p. 178.

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[171] T. Wieland in "The Chemical Structure of Proteins", ed. G. E. W. Wolstenholme and M. P. Cameron, Churchill, London, 1953, p. 146.

[172] R. A. Boissonnas, *Nature*, 1953, **171**, 304; *Helv. Chim. Acta*, 1952, **35**, 2226; see, also, R. A. Turner and G. Schmerzler, *J. Amer. Chem. Soc.*, 1954, **76**, 949.

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[177] J. C. Crawhall and D. F. Elliott, *Biochem. J.*, 1955, **61**, 264; D. F. Elliott, *ibid.*, 1952, **50**, 542.

[178] J. Leggett Bailey, *ibid.*, 1955, **60**, 173.

(δ) Hydrazinolysis and ammonolysis. Akabori *et al.*,[179] noting that hydrazine cleaves amide bonds, $R{\cdot}CO{\cdot}NHR' \longrightarrow R{\cdot}CO{\cdot}NH{\cdot}NH_2 + NH_2R'$, but does not attack free carboxyl groups, studied the hydrazinolysis of peptides. This should yield the *C*-terminal residue as the free amino-acid and all the other residues as the amino-acid hydrazides, thus permitting the identification of the *C*-terminal residue (though at the expense of the rest of the specimen). In practice, the drastic attack of hot hydrazine is not always so clear-cut : it appears [180] that several amino-acids are completely destroyed.

The corresponding reaction with ammonia has been investigated [180] and found liable to similar limitations due to destructive degradation.

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