STRUCTURAL INVESTIGATION OF PEPTIDES AND PROTEINS

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1. Introduction.—Proteins are large molecules of biological origin which on hydrolysis give α-amino- or -imino-acids. This definition and the important Hofmeister–Fischer [1, 2] theory that the amino-acids are present in amide or " peptide " linkage with one another are both of long standing. It is, however, only in recent years that further great advances have been made in the chemistry of these macromolecules. The renewed impetus to the structural investigation of these biologically important substances has come mainly from recent developments and refinements in analytical methods, for the inadequacy of technique has in the past been the chief obstacle in this field.[3] In the past decade or so, interest has, however, also been greatly stimulated by the isolation of many naturally occurring peptides,[4] which, although of varying size and complexity, are in general of much lower molecular weight than proteins. These peptides [4] frequently exhibit some remarkable physiological activity, as hormones, poisons, growth factors, inhibitors, etc. Glutathione,[5] carnosine,[6] and anserine [6] are the oldest and simplest examples, whereas oxytocin [7, 8] and vasopressin [9] (the oxytocic and pressor hormones respectively of the posterior lobe of the pituitary gland), lycomarasmin (the tomato leaf wilting factor),[10] phalloidine,[11] amanitine (the highly toxic substances from the fungus *Aminata Phalloides*),[12] and the several families of antibiotics, gramicidins,[13, 14] tyrocidins,[13, 15] gramicidin S [16] (from *Bacillus brevis*), and polymyxins [17] (from *Bacillus polymyxa*) are recent examples. Not only have

[1] F. Hofmeister, *Ergebn. Physiol.*, 1902, **1,** 159.

[2] E. Fischer, *Chem. Ztg.*, 1902, **26,** 939.

[3] R. L. M. Synge, *Chem. Reviews*, 1943, **32,** 135.

[4] *Idem, Quart. Reviews*, 1949, **3,** 245.

[5] F. G. Hopkins, *J. Biol. Chem.*, 1927, **72,** 185; 1929, **84,** 269; C. R. Harington and T. H. Mead, *Biochem. J.*, 1935, **29,** 1602.

[6] S. W. Fox, *Chem. Reviews*, 1943, **32,** 47.

[7] G. W. Irving, jun., and V. du Vigneaud, *Ann. N.Y. Acad. Sci.*, 1943, **43,** 273.

[8] A. H. Livermore and V. du Vigneaud, *J. Biol. Chem.*, 1949, **180,** 365; V. du Vigneaud and J. G. Pierce, *ibid.*, **186,** 77.

[9] G. W. Irving, H. M. Dyer, and V. du Vigneaud, *J. Amer. Chem. Soc.*, 1941, **63,** 503; A. M. Potts and T. F. Gallacher, *J. Biol. Chem.*, 1944, **154,** 349; V. du Vigneaud, Abstr. Papers 120th Meeting Amer. Chem. Soc., 1951, 13L.

[10] P. A. Plattner and N. Clauson-Kaas, *Experientia*, 1945, **1,** 195.

[11] F. Lynen and H. Wieland, *Annalen*, 1937, **533,** 93; H. Wieland and B. Witkop, *ibid.*, 1940, **543,** 171.

[12] H. Wieland, R. Hallermeyer, and W. Zilg, *ibid.*, 1941, **548,** 1.

[13] R. D. Hotchkiss, *Adv. Enzymology*, 1944, **4,** 153.

[14] J. D. Gregory and L. C. Craig, *J. Biol. Chem.*, 1948, **172,** 839.

[15] R. L. M. Synge and A. Tiselius, *Acta Chem. Scand.*, 1947, **1,** 749; 1949, **3,** 231.

[16] P. G. Sergiev (ed.), " Sovyetskii Gramitsidin i Lecheniye Ran ", Medgiz, Moscow, 1943.

[17] P. H. Long *et al.*, *Ann. N.Y. Acad. Sci.*, 1949, **51,** 853—1000.

they their own intrinsic chemical interest and frequently distinctive structural features, but also they have served as useful models in the study of proteins ; [4] as will be seen later, it has proved possible to extend the methods developed for the elucidation of the structures of the simpler to the much more complex substances.

It is obvious that chemical investigation of a peptide or a protein must include the following stages : (1) isolation in a homogeneous state ; (2) identification and assay of the constituent amino-acids ; and (3) elucidation of the sequence of these building units in the original macromolecule. A few outstanding examples of the separations and isolations achieved recently will illustrate the fine resolving powers of some of the newer analytical tools. L. C. Craig's counter-current distribution technique [18] has been applied to the purification of oxytocin,[8] gramicidins,[14] and bacitracin,[19] etc. The application of starch column chromatography to peptide fractionations has been demonstrated by R. L. M. Synge [20] and by M. Ottesen and C. Villee.[21] The use of ion-exchange columns has been extended to proteins of low molecular weight (lysozyme [22] and ribonuclease [23]). The technique developed for the structural investigations of peptides and proteins is mentioned later.

Precise knowledge of the amino-acid composition of peptides and proteins must form the necessary background to detailed studies of their fine structure. A detailed discussion of this important subject is not within the scope of the present Review and only a brief section on this aspect is included.

It is intended here to discuss the determination of the order of α-amino-acid residues in all substances which are known to contain recurring peptide bonds of the general type —CHR·CO·NH·CHR—. Thus no distinction is made between peptides, which possess comparatively low molecular weight, and proteins. In order to illustrate the scope and limitations of the different methods, recent progress in elucidation of the amino-acid sequences in some peptides and proteins is included. None of the sections which follow is claimed as comprehensive and, in particular, the discussion of enzymic degradation, a large and important field, is very general.

2. Amino-acid Analysis.—It is only ten years since H. B. Vickery [24] stated that satisfactory methods for the determination of only nine amino-acids were known and that many of these depended on quantitative isolation. These classical methods were brought to the maximum degree of refinement by A. C. Chibnall and his co-workers.[25] Since then amino-acid analysis has been completely revolutionised by the development of several

[18] L. C. Craig, *J. Biol. Chem.*, 1944, **155**, 519.
[19] G. G. F. Newton and E. P. Abraham, *Biochem. J.*, 1950, **47**, 257.
[20] *Ibid.*, 1949, **44**, 542.
[21] *Compt. rend. Trav. Lab. Carlsberg*, 1951, **27**, No. 18.
[22] H. H. Tallan and W. H. Stein, *J. Amer. Chem. Soc.*, 1951, **73**, 2976.
[23] C. H. W. Hirs, W. H. Stein, and S. Moore, *ibid.*, p. 1893.
[24] *Ann. N.Y. Acad. Sci.*, 1941, **41**, 87.
[25] A. C. Chibnall, *Proc. Roy. Soc.*, 1942, *B*, **131**, 136 ; A. C. Chibnall, M. W. Rees, and E. F. Williams, *Biochem. J.*, 1943, **37**, 372.

novel micro-methods, which are less tedious and more accurate. As a result, fairly complete analyses of a large number of proteins with almost theoretical nitrogen and weight recoveries are now available [26] and further data are rapidly accumulating. Several comprehensive reviews,[27] some general, some specialised, of the various analytical procedures are now available.

More recently the method of starch chromatography for the quantitative separation of amino-acids has been carefully worked out by S. Moore and W. H. Stein.[28] This method promises to serve as a routine procedure for complete amino-acid analysis. The method has been applied by V. du Vigneaud and his co-workers [29, 30] extensively in studies on oxytocic hormone, by Ottesen and Villee [21] in the study of the peptides released in the enzymic transformation of ovalbumin into plakalbumin and to the amino-acid analysis of proteins.[31] Moore and Stein have further extended their technique to include columns of synthetic ion-exchange resins,[32] which show resolving powers even higher than those of starch columns and are superior to the latter in many other ways.

Mention should also be made of the recent improvement [33] of the isotope-dilution method for amino-acid analysis, which employs *p*-iodobenzenesulphonyl chloride (labelled with ^{131}I). The use of paper-chromatographic and indicator techniques [33] appears to offer advantages over the carrier technique [34] earlier employed.

3. Terminal Amino-acids in Peptides and Proteins.—According to the " polypeptide " chain theory for the structure of peptides and proteins it would be expected that every such chain, unless it is cyclic, would bear at one end a free α-amino-group and at the other a free carboxyl group, in addition to other free amino- and carboxyl groups at the side chains of certain constituent amino-acids. Some of these functional groups might, indeed, be involved in cross linkages and, further, certain of the carboxyl groups might be represented by amide groups (formation of ammonia on hydrolysis).*

[26] G. R. Tristram, *Adv. Protein Chem.*, 1949, **5**, 83.

[27] A. J. P. Martin and R. L. M. Synge, *ibid.*, 1945, **2**, 1 ; E. E. Snell (Microbiological assay of amino-acids), *ibid.*, p. 85 ; *Ann. N.Y. Acad. Sci.*, 1946, **47**, 161 ; M. S. Dunn, *Physiol. Rev.*, 1949, **29**, 219 ; D. Shemin and G. L. Foster (Isotope dilution), *Ann. N.Y. Acad. Sci.*, 1946, **47**, 119 ; R. K. Cannan (Chromatography), *ibid.*, p. 135 ; R. M. Archibald, *ibid.*, p. 181.

[28] *J. Biol. Chem.*, 1948, **176**, 337, 367 ; 1949, **178**, 53.

[29] J. G. Pierce and V. du Vigneaud, *ibid.*, 1950, **182**, 359.

[30] H. Davoll, R. A. Turner, J. G. Pierce, and V. du Vigneaud, *ibid.*, 1951, **193**, 363.

[31] S. Moore and W. H. Stein, *ibid.*, 1949, **178**, 79.

[32] *Ibid.*, 1951, **192**, 663.

[33] A. S. Keston, S. Udenfriend, and M. Levy, *J. Amer. Chem. Soc.*, 1950, **72**, 748.

[34] A. S. Keston, S. Udenfriend, and R. K. Cannan, *ibid.*, 1949, **71**, 249.

* This extremely simplified statement omits the several other chemically reactive functional groups present on the side chains of some amino-acids and other possible cross linkages in peptides and proteins. In proteins, the whole situation is further complicated according to whether the protein is in a " native " or " denatured "

At all events, knowledge of the nature and number of the terminal amino-acids bearing free functional groups affords valuable information for structural investigations. Indeed, several of the naturally occurring peptides [4] and proteins (*e.g.*, insulin [39]) appear to have normal open-chain structures whilst others have cyclic structures (gramicidin S,[16] tyrocidin,[13] phalloidine [11]). The absence of free α-amino- or carboxyl groups can very often be regarded as strong evidence of cyclic structure. Further, the determination of the terminal amino-acids has important implications in the study of the products of partial hydrolysis (section 4) and, when used in combination with the latter technique, it has proved a powerful method for the elucidation of sequences of amino-acid residues in peptide chains of considerable size. (The methods of selective degradation of peptides which rely on the presence of free α-amino- and carboxyl groups on the terminal amino-acids are discussed in section 5.)

Identification of free amino-groups. Determination of the total number of free amino-groups in peptides and proteins by the nitrous acid procedure,[40] by "formol" titration,[41] and by the ninhydrin reaction [42] is well known. Most of the chemical methods which have been suggested for the identification of the amino-acids bearing free amino-groups have aimed at altering by some means the fundamental character of the residues carrying such groups so that, after subsequent hydrolysis of the peptide bonds, they can be distinguished from the others (see, however, section 5). Thus deamination of the terminal amino-acids with nitrosyl chloride [43] has been employed successfully in recent structural investigations of simple di- and tri-peptides released on partial hydrolysis of gramicidin S [44] and insulin [45, 46] (section 4). Most of the chemical approaches, however, involve the reaction of the free amino-groups with some suitable acylating reagent. The terminal amino-acids are then identified as the corresponding acyl derivatives after complete hydrolysis of the molecule. An ideal reagent for this purpose should have the following important attributes. It should condense specifically and quantitatively with amino-groups under conditions which do not

state. Comprehensive reviews are available on denaturation [35] and group reagents [36] for native proteins. The present discussion is confined to the free amino- and carboxyl groups only; in proteins, unless these are denatured, not all of the free amino-groups may be available for the attack of the chemical reagents.[37, 38]

[35] H. Neurath, J. P. Greenstein, F. W. Putnam, and J. O. Erikson, *Chem. Reviews*, 1944, **34**, 157; M. L. Anson, *Adv. Protein Chem.*, 1945, **2**, 361.

[36] R. M. Herriott, *ibid.*, 1947, **3**, 169; H. S. Olcott and H. Fraenkel-Conrat, *Chem. Reviews*, 1947, **41**, 151.

[37] A. M. Pappenheimer, *J. Biol. Chem.*, 1937, **125**, 201; E. Brand, L. J. Saidel, W. H. Goldwater, B. Kassell, and F. J. Ryan, *J. Amer. Chem. Soc.*, 1945, **67**, 1524.

[38] R. R. Porter, *Biochim. Biophys. Acta*, 1948, **2**, 105.

[39] F. Sanger, *Biochem. J.*, 1945, **39**, 507.

[40] D. D. Van Slyke, *J. Biol. Chem.*, 1913, **16**, 121.

[41] J. H. Northrop, *J. Gen. Physiol.*, 1926, **9**, 767.

[42] A. F. Ross and W. M. Stanley, *ibid.*, 1938, **22**, 165.

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

[44] R. Consden, A. H. Gordon, A. J. P. Martin, and R. L. M. Synge, *ibid.*, p. 596.

[45] F. Sanger and H. Tuppy, *ibid.*, 1951, **49**, 463. [46] *Idem*, *ibid.*, p. 481.

cause any degradation or fundamental structural alteration in the peptide or protein under investigation. Secondly, the substituting group should be completely resistant in the conditions employed for the hydrolysis of the peptide bonds. Finally, the acyl derivatives of the amino-acids should lend themselves conveniently to quantitative separation and determination on a small scale. Several acylating reagents [47] have served useful though limited purpose in earlier work. Naphthalene-β-sulphonyl chloride was employed frequently by Fischer, Abderhalden, and their respective co-workers in the structural investigations on silk fibroin and other proteins.[3] The use of chloro-2 : 4-dinitrobenzene was investigated by E. Abderhalden and W. Stix,[48] and the dinitrophenyl (DNP) derivatives of several amino-acids were characterised. Elevated temperatures, however, had to be employed for the preparation of such derivatives.

Recently, F. Sanger [39] has successfully developed the use of fluoro-2 : 4-dinitrobenzene (DNFB) for the identification and estimation of the amino-acids bearing free amino-groups. As this technique has already found wide application in this field, it is discussed here in somewhat greater detail. The reagent enters into reaction with peptides and proteins at room temperature in the presence of sodium hydrogen carbonate, conditions which can be regarded as mild (see, however, below). The reaction apparently goes to completion with free amino-groups. (In DNP-insulin,[39] 93% of the total free amino-groups of the original molecule were estimated to be substituted. Incomplete reaction with ε-amino-groups of lysine in native serum globulins and β-lactoglobulin has been observed by R. R. Porter.[38]) Dinitrophenyl derivatives [39, 49] of most of the amino-acids have been well characterised and, as they are bright yellow, their quantitative determination on a micro-scale is possible colorimetrically. Furthermore, complete separation of their mixtures has been accomplished by partition chromatography. Silica gel columns [49] were originally used for the purpose ; the process was, however, very laborious. Columns of kieselguhr,[50] buffered silica gel,[51] and " Hyflo Supercel " [30, 52] have since been employed. Regeneration of the original amino-acids from the dinitrophenyl derivatives by hydrolysis with barium hydroxide [53] and ammonia [54] has been suggested but an apparent risk here is the partial decomposition of certain amino-acids. However, the reagent is not specific for amino-groups : it reacts also with thiol groups (the nature of the products does not seem to be clear), glyoxaline, and phenolic hydroxyl groups. (The *O*-dinitrophenyl derivatives are, however, colourless and do not interfere with the determination of the coloured compounds.) Its chief drawback in quantitative determinations is partial

[47] See S. W. Fox, *Adv. Protein Chem.*, 1945, **2**, 155.
[48] *Z. physiol. Chem.*, 1923, **129**, 143.
[49] R. R. Porter and F. Sanger, *Biochem. J.*, 1948, **42**, 287.
[50] J. C. Perrone, *Nature*, 1951, **167**, 513.
[51] S. Blackburn, *Biochem. J.*, 1949, **45**, 579.
[52] P. H. Bell, J. F. Bone, J. P. English, C. E. Fellows, K. S. Howard, M. M. Rogers, R. G. Shepherd, and R. Winterbottom, *Ann. N.Y. Acad. Sci.*, 1949, **51**, 897.
[53] G. L. Mills, *Nature*, 1950, **165**, 403.
[54] A. G. Lowther, *ibid.*, 1951, **167**, 767.

destruction of some dinitrophenylamino-acids during hydrolysis of the dinitrophenyl-peptide [39, 44, 49] or -protein. Various conditions of hydrolysis were tried by Sanger [39] and Porter [49] to ascertain recoveries of intact dinitrophenylamino-acids and to apply appropriate corrections. The recoveries were between 70 and 90%. A few dinitrophenylamino-acids were found to be particularly susceptible to acidic conditions. Further, the formation of some coloured artefacts on acid hydrolysis was detected during separations on columns. In spite of these sources of error the technique has added greatly to our knowledge of many peptides and proteins and should be accepted, in the absence of a better method, as a routine procedure in structural investigations.

The first major contribution of the fluorodinitrobenzene technique was to the investigation of the terminal amino-acids of insulin.[39] The results suggested that the protein was built up of four polypeptide chains, two of these having phenylalanine and the others glycine residues at the amino-end. (Further use of this technique in the investigation of the chemistry of insulin is discussed in section 4.) Examples of further applications were soon forthcoming. Gramicidin S [55] was shown to be a cyclic peptide with only the δ-amino-group of ornithine free. Cyclic structures have also been suggested for ovalbumin,[56] tropomyosin,[57] and myosin,[57] because of the failure to isolate α-dinitrophenylamino-acids. End-group assay of a considerable number of proteins [58] including hæmoglobins obtained from different sources has revealed very interesting compositions. The application of this method to the determination of the end group of oxytocin [58] and lysozyme,[59] conalbumin, ovomucoid, and avidin [60] has also been reported.

Although a considerable number of examples have been cited to indicate the value of this technique, mention should be made of the very important observation by F. Weygand and R. Junk [61] during their investigations of the free amino-groups of the " old yellow enzyme " by the use of fluorodinitrobenzene. Whereas the protein itself was found to be stable at the pH of aqueous sodium hydrogen carbonate, the dinitrophenyl derivative, which apparently contains dinitrophenyl-aspartic and -glutamic acid at the end of two chains, was unstable under the standard conditions, fission of these two dinitrophenyl amino-acids occurred, and two more residues were uncovered. However, at a controlled pH of 7 and 5·8, cleavage was avoided. In view of the great lability of certain peptide bonds, caution is essential in interpreting the results of the end-group assay.

The use of [^{131}I]*p*-iodophenylsulphonyl chloride for identification of the terminal amino-acid has been reported by S. F. Velick and S. Udenfriend.[62]

[55] F. Sanger, *Biochem. J.*, 1946, **40**, 261.
[56] R. R. Porter, quoted by Sanger, ref. 58.
[57] K. Bailey, *Biochem. J.*, 1951, **49**, 23.
[58] F. Sanger, *Biochem. Symp.*, 1948, **3**, 21.
[59] F. C. Green and W. A. Schroeder, *J. Amer. Chem. Soc.*, 1951, **73**, 1385.
[60] H. Fraenkel-Conrat and R. R. Porter, *Biochem. J.*, 1951, lxxviii.
[61] *Naturwiss.*, 1951, **38**, 433.
[62] *J. Biol. Chem.*, 1951, **190**, 733; **191**, 233.

It seems to offer the advantages over the dinitrophenyl technique that quantitative isolation of the derivatives is unnecessary and that the acyl derivatives are more resistant to acid hydrolysis. However, the reagent does not appear to react with all the terminal amino-groups and so gives much lower results.

Identification of free carboxyl groups. No direct method is available for the determination of the total number of free carboxyl groups in intact proteins, although titration constants sometimes afford some information.[63] Nor is there a satisfactory method for the identification of the amino-acids bearing free carboxyl groups by attaching a substituent, resistant to subsequent hydrolysis of peptide bonds. Conversion of a free acid group into the benzylamide by reaction, after esterification, with benzylamine has been suggested.[64] The newly formed amide link was shown to have greater resistance to hydrolysis than the peptide bonds but the method does not appear to have found practical application.

Methods have recently become available, worked out independently by A. C. Chibnall and M. W. Rees [65] and C. Fromageot *et al.*,[66] which bring about selective reduction of the free carboxyl groups, after esterification, to the alcohol stage by metal hydrides. Thus, subsequent hydrolysis of the protein yields the amino-acids carrying originally the free carboxyl groups as the corresponding amino-alcohols. The method has been applied to the determination of the free carboxyl groups of insulin.[65, 66]

The use of carboxypeptidase (section 6) for determination of the terminal carboxyl group was first demonstrated in the case of glutathione [67] and has recently been extended to insulin by J. Lens.[68] Although the method is often not completely reliable, it made possible the identification of alanine as the terminal residue in this protein.

4. Partial Hydrolysis.—It has been known for a long time [2] that the different peptide bonds in peptides and proteins are not equally stable to the action of hydrolytic agents such as acids and alkalis and, therefore, it is possible to isolate, by employing comparatively mild conditions, products which are intermediate between the starting materials and amino-acids. Partial hydrolysis thus has far reaching potentialities as a method for the elucidation of detailed structures provided certain conditions can be satisfied. It should, for example, be possible to employ separately a number of selective hydrolytic agents which would preferentially attack different bonds in the original molecule and then to separate and investigate the primary fission products individually. Relatively unselective hydrolysis would obviously produce in one step a very complex mixture of peptides and even more refined techniques would be necessary to effect separations. The first part of this section is devoted to a general discussion

[63] R. M. Herriott, *Adv. Protein Chem.*, 1947, **3**, 169.
[64] E. Abderhalden and H. Brockmann, *Biochem. Z.*, 1930, **225**, 386.
[65] *Biochem. J.*, 1951, **48**, xlvii.
[66] *Biochim. Biophys. Acta*, 1950, **6**, 283.
[67] W. Grassman, H. Dycherhoff, and H. Eibeler, *Z. physikal. Chem.*, 1930, **189**, 112.
[68] *Biochim. Biophys. Acta*, 1949, **3**, 367.

of the conditions of partial hydrolysis, and the second to actual recent progress in elucidation of the arrangement of amino-acids in peptide chains, made possible mainly by improved techniques.

Conditions of hydrolysis. The products of partial hydrolysis of proteins have been reviewed very comprehensively by Synge,[3] up to 1941. It can be seen from this account that our knowledge of the kinetics of the hydrolysis of peptides by chemical means is very inadequate. Extensive investigations by Levene [69, 70] and E. Abderhalden [71] and their respective collaborators added considerably to our knowledge of the relation of the chemical structure to the rate of hydrolysis of peptides, and of the comparative labilities of various bonds to the conditions of acidic and alkaline hydrolysis. However, it seems unlikely even at the present time that any fine control can be exercised over the path of the breakdown of a polypeptide to the final products by the use of these hydrolytic agents.

A very interesting study of the kinetics of the low-temperature acidic hydrolysis of some simple dipeptides was made recently by Synge [72] to compare the results with those obtained earlier by Levene *et al.*[70] on the alkaline hydrolysis of similar peptides. In addition to some important points of general significance in the field, the suspected unfortunate conclusion emerged that " the sequence of the ease of hydrolysis was analogous under both acidic and alkaline conditions ". There appears to be some theoretical justification [3, 73] for the experimental findings [44, 74] that dipeptides are more resistant to hydrolysis than the higher peptides. The hope expressed by Synge,[72] that during hydrolysis of large peptide chains by acid and alkali, although initial attack would probably be at the same bonds, the altered nature of the resulting peptides might then cause differential susceptibilities to the hydrolytic agents, does not seem to have been realised. For example, Sanger and Tuppy [45, 46] found that the alkaline hydrolysis of the B chain (comprising thirty amino-acid residues) of insulin gave only a few new peptides, which had not been previously obtained under acidic conditions. Moreover, the additional disadvantages such as racemisation and partial destruction of the amino-acids by alkali are well known.

Some interesting and encouraging results have been forthcoming from the recent comparative studies of the use of cold concentrated acid and hot dilute acids. (An extensive study of the conditions of acidic hydrolysis was made earlier by K. Felix *et al.*[75] during their investigations of

[69] P. A. Levene and his co-workers, several papers in *J. Biol. Chem.*, *e.g.*, 1924, **61**, 445 ; 1929, **82**, 167.

[70] P. A. Levene, R. E. Steiger, and A. Rothen, *ibid.*, 1932, **97**, 717.

[71] *Z. physiol. Chem.*, 1927, **170**, 134, and subsequent papers, *e.g.*, *ibid.*, pp. 146, 158, 226 ; **176**, 207 ; E. Abderhalden and H. Mahn, *ibid.*, 1927, **169**, 196 *et seq.*

[72] *Biochem. J.*, 1945, **39**, 351.

[73] R. C. G. Moggridge and A. Neuberger, *J.*, 1938, 745 ; A. Neuberger and R. Pitt-Rivers, *J.*, 1939, 122.

[74] H. N. Christensen and D. M. Heysted, *J. Biol. Chem.*, 1945, **158**, 593 ; W. H. Stein, S. Moore, and M. Bergmann, *ibid.*, 1944, **154**, 191.

[75] K. Felix, R. Hirohata, and K. Dirr, *Z. physiol. Chem.*, 1933, **218**, 269.

clupein.) The recent work of Consden *et al.*[44] and Sanger and Tuppy [45, 4] has supported the general conclusion, which emerged from the older work that preference should be given to prolonged partial hydrolysis with cold concentrated acid. No noticeable destruction of amino-acids occurs, and the products of hydrolysis are comparatively authentic because rearrangements are unlikely under these conditions. On the other hand, the hydrolysis at higher temperatures (*e.g.*, 0·1N-hydrochloric acid at 100° for 1 hours) tends to favour the formation of cyclic compounds such as diketopiperazines. These two broad types of conditions of acidic hydrolysis have, however, been shown to possess markedly different specificities.[76, 7] Particularly significant are the observations of S. Blackburn [77] who found that, whereas hydrolysis of wool with cold concentrated hydrochloric acid at 37° released peptides containing glutamic and aspartic acid, boiling dilute acid liberated aspartic acid preferentially. The effect of variations of acid concentration and temperature of hydrolysis was studied. (See also the results of H. van Vunakis and E. A. Kabat [78] on mild acid hydrolysis of hog-blood group A and O substances.) Similarly, very interesting results have been obtained by S. M. Partridge and H. F. Davies [79] in the study of protein hydrolysis with organic acids (acetic, oxalic): aspartic acid is released first. Mention should also be made of the particular lability to acid of the peptide bonds involving the amino-groups of serine and threonine,[80] which presumably occurs with the formation of the oxazoline intermediate.[81]

Important as these newer findings are, their application to specific selective cleavage of large polypeptide chains has still to be demonstrated. Exact knowledge of the kinetics of the hydrolysis of various peptide bonds under different conditions of temperature and hydrogen-ion concentration would prove of great value. A start has been made in this direction by L. Lawrence and W. J. Moore.[82]

Applications to structural investigations. The elucidation of the arrangement of the amino-acid residues in glutathione [5] (γ-L-glutamyl-L-cysteinylglycine) is probably the first, and is now the classical, example of the application of the techniques of the identification of terminal amino-acids (preceding section) and partial hydrolysis to the investigation of the structures of peptides. Fox [47] has already reviewed this work but it is recapitulated here, for it embodies the important principles employed in more recent work discussed below. The terminal glutamic acid with the free α-amino-group was identified [83] by condensation of the glutamic acid-cysteine moiety (a product of partial hydrolysis) with 2 : 3 : 4-trinitrotoluene and by deamination [83] with nitrous acid followed by hydrolysis. Gluta

[76] F. Sanger, *Biochem. J.*, 1949, **45**, 563.
[77] S. Blackburn, *ibid.*, 1950, **47**, xxviii.
[78] *J. Amer. Chem. Soc.*, 1951, **73**, 2977. [79] *Nature*, 1950, **165**, 62.
[80] P. Desnuelle and A. Casal, *Biochem. Biophys. Acta*, 1948, **2**, 64.
[81] M. Bergmann and A. Miekeley, *Z. physiol. Chem.*, 1924, **140**, 128; A. P. Phillip and R. Baltzly, *J. Amer. Chem. Soc.*, 1947, **69**, 200.
[82] *Ibid.*, 1951, **73**, 3973.
[83] J. H. Quastel, C. P. Stewart, and H. E. Tunnicliffe, *Biochem. J.*, 1923, **17**, 586

thione itself was deaminated with nitrous acid by E. C. Kendall *et al.*[84] who were, further, able to isolate the second possible dipeptide fragment (cysteinylglycine) by a modification of the conditions of hydrolysis.[85] Treatment of this dipeptide with trinitrotoluene and subsequent hydrolysis afforded glycine, which established the order of the three amino-acids in the peptide. The Bettzieche–Menger [86, 87] and Schlack–Kumpf [88] techniques (next section) were also employed [89] to designate the terminal glycine with the free carboxyl group.

However, the extensive investigations, during the past several decades, of the complex molecules such as proteins by the method of partial hydrolysis succeeded only in establishing the existence of certain types of linkage in these molecules by the isolation of the relevant dipeptides, their derivatives (naphthalene-β-sulphonyl, etc.), or cyclic anhydrides. Thus the presence of direct linkages of two identical amino-acids in protamines, tussore silk, etc., and the linkage of different amino-acids, *e.g.*, glycine–alanine, glycine–tyrosine, in silk fibroin had been proved. However, no detailed picture of the architecture of any of the proteins studied emerged. As emphasised by Synge,[3] the important obstacles in the development of the knowledge of protein fine structure were those of technique. A full appreciation of this led A. J. P. Martin and R. L. M. Synge [90] to investigate the use of partition chromatography for the separation, detection, and determination of amino-acids and peptides. Various elaborations and extensions not only have resulted in major advances in the chemistry of proteins but have given tremendous stimulus in many other fields of research.[58, 91]

Here it is pertinent to mention only the procedures, based mainly on the principle of partition chromatography, which have been employed recently for the separation and structural analysis of complex mixtures of small peptides invariably encountered in partial hydrolysis. A strikingly successful scheme [43] consists of: (1) ionophoresis to separate the peptides into acid, neutral, and basic groups; (2) fractionation of these individual groups on a number of replicate two-dimensional chromatograms; (3) elution from paper, followed by (*a*) hydrolysis, and (*b*) deamination with nitrosyl chloride and hydrolysis. Further extensions and improvements (particularly of group separations on ion-exchange columns) have been described by Consden *et al.*[92, 93] and Sanger *et al.*[45]

[84] E. C. Kendall, H. L. Mason, and B. F. McKenzie, *J. Biol. Chem.*, 1929, **84**, 657.
[85] *Idem, ibid.*, **88**, 409.
[86] F. Bettzieche and R. Menger, *Z. physiol. Chem.*, 1926, **161**, 37.
[87] F. Bettzieche, *ibid.*, p. 178.
[88] P. Schlack and W. Kumpf, *ibid.*, 1926, **154**, 125.
[89] (*a*) E. C. Kendall, H. L. Mason, and B. F. McKenzie, *J. Biol. Chem.*, 1929, **87**, 55; (*b*) B. H. Nicolet, *ibid.*, 1930, **88**, 389, 395, 403.
[90] *Biochem. J.*, 1941, **35**, 1358.
[91] A. J. P. Martin, *Ann. Reports*, 1948, **45**, 267; *Ann. Rev. Biochem.*, 1950, 517; *Analyt. Chem.*, Annual reviews of analytical techniques, 1949–1951.
[92] R. Consden, A. H. Gordon, and A. J. P. Martin, *Biochem. J.*, 1949, **44**, 548.
[93] R. Consden and A. H. Gordon, *ibid.*, 1950, **46**, 8.

The first application of these novel techniques was to the determination of the sequence of amino-acid residues in the cyclic decapeptide gramicidin S.[16, 44, 94, 95] In addition to two tripeptides, four of the possible five dipeptides were identified in a partial hydrolysate, and the sequence was thus demonstrated without doubt to be valyl-ornithyl-leucyl-phenylalanyl-prolyl. More recent molecular-weight determination [95] and other evidence [4] show this sequence to be repeated twice in the cyclic chain. Investigations of partial hydrolysates of wool have resulted in the identification of nineteen dipeptides [92] containing aspartic or glutamic acid. Further, Consden and Gordon [93] have isolated thirteen peptides of cysteine in wool and have thus obtained additional valuable information on the structure.

The crowning achievement is undoubtedly the assignment of the order to thirty amino-acid residues present in the larger of the two polypeptide chains (B chain [96]) of insulin. Combination of the technique of substituting the free amino-groups in proteins and peptides by the dinitrophenyl group (section 3) and that of "regulated" partial hydrolysis enabled Sanger [76] to obtain information about the sequence of amino-acid residues located near the free amino-groups. The mixtures of dinitrophenylpeptides and their intermediate breakdown products were extracted by organic solvents from the partial hydrolysates and separated on buffered silica gel columns. The structures of the individual dinitrophenylpeptides were then elucidated by partial hydrolysis and estimation of the relative amounts of the liberated amino-acids. In extended investigations, the techniques employed successfully for gramicidin S were applied to the partial hydrolysates of fraction B.[45] Confirmation of the sequences established previously [76] was forthcoming and in addition, a number of new amino-acid sequences emerged. It was not possible, however, to derive the complete sequence of the amino-acid residues by the use of chemical hydrolytic agents alone. Additional data [46] were afforded by the more selective fissions by proteolytic enzymes (section 6).

With further application of these methods our knowledge of the architecture of various physiologically important substances (*e.g.*, gramicidins, tyrocidins) is increasing rapidly. K. Heyns and his co-workers [97] have reported the results of the investigation of several peptides obtained from the partial hydrolysates of gelatin. The structure of clupein is being investigated in greater detail (after the classical work of K. Felix and his co-workers [3, 75]) by F. Sörm *et al.*,[98] who have also suggested [99] the sequence, hydroxytryptophyl-cysteinyl-alanyl-hydroxy*allo*prolyl-alanyl-hydroxy-*allo*prolyl, as the most probable for the cyclic hexapeptide phalloidine.[11]

5. Selective Chemical Degradation.—Lack of control over the course of the breakdown of peptides by hydrolytic methods has prompted many investigators to seek more selective methods of degradation. One main

[94] R. L. M. Synge, *ibid.*, 1945, **39**, 363.
[95] A. R. Battersby and L. C. Craig, *J. Amer. Chem. Soc.*, 1951, **73**, 1887.
[96] F. Sanger, *Biochem. J.*, 1949, **44**, 126.
[97] *Z. physiol. Chem.*, 1951, **287**, 109, 120.
[98] F. Sörm and B. Keil, *Chemická Listy*, 1951, **45**, 279.
[99] Sörm *et al.*, *ibid.*, p. 215.

approach to the problem is the use of certain enzymes and this is discussed in the following section. The chemical approaches * suggested so far have all aimed at the stepwise removal of the terminal amino-acids from a peptide chain. This is readily understandable because the frequent presence of free functional groups at the terminal amino-acids renders these sites particularly accessible to chemical attack. In this respect, the approaches now to be reviewed are similar to those described earlier for the identification of terminal amino-acids ; but their most important distinctive feature is the selective cleavage of the terminal amino-acid residue without alteration of the others.

The older methods have been reviewed fully by Fox [47] and here only a general discussion of these is given in relation to the more recent developments. It is possible to classify into two broad groups all the methods which have been put forward so far. In the first, the terminal peptide bond is rendered labile by a fundamental structural change in the terminal amino-acid. The hypobromite method [100] of degradation from the amino-end, the Bergmann–Zervas [101] and the Bettzieche–Menger [86, 87] methods of degradation from the carboxyl end belong to this group. In the second group, a reagent is attached to the free functional group of the terminal amino-acid and, in a second step, cleavage of the terminal peptide bond is caused by the interaction of the elements of the attached residue with those of the terminal amino-acid : formation of a cyclic intermediate or preferably a stable ring system seems to be the essential feature.

Whatever the principle of the method employed, it should possess the following characteristics. All the reactions employed should proceed quantitatively and specifically. The conditions throughout should be so mild that the main peptide molecule remains unaffected. Further, the more the stages involved, the less is likely to be its value as a stepwise procedure. The majority of the methods devised have not found wide application, perhaps on account of the lack of fulfilment of one or more of these criteria. The methods developed more recently appear largely to satisfy the above conditions and thus promise to be of practical value in structural elucidation of oligopeptides.

Degradation from the amino-end. P. Edman's elegant technique [102] consists of the reaction of phenyl *iso*thiocyanate (phenyl *iso*cyanate was used by earlier workers [103]) with the amino-group of a peptide at a controlled pH (*ca.* 9), to form the phenylthiocarbamyl derivative (I). Cleavage of (I) in

[100] 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.

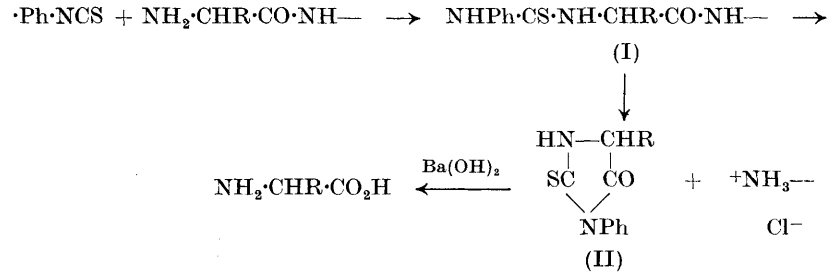
[101] M. Bergmann and L. Zervas, *J. Biol. Chem.*, 1936, **113**, 341.

[102] *Acta Chem. Scand.*, 1950, **4**, 283.

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

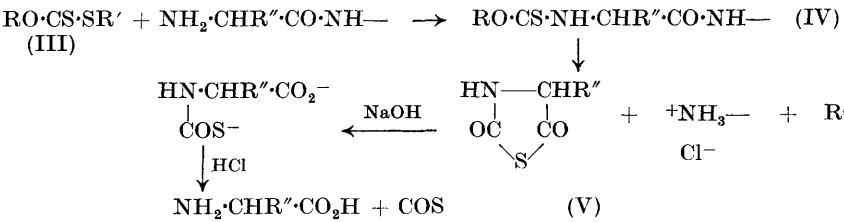
* Although in the above discussion, selective chemical degradation implies the cleavage of peptide bonds, the oxidative fission of —S—S—bridges in insulin employed by Sanger [96] to obtain two polypeptide chains of lower molecular weight is an excellent example of such degradation. The method should prove of general value in structural investigations irrespective of the size of the molecule. Oxidation of cysteine peptides with bromine to facilitate their separation as cysteic acid peptides [93] is also worthy of note.

anhydrous nitromethane saturated with hydrogen chloride affords the phenylthiohydantoin (II) and the hydrochloride of the amino-acid or peptide lacking the terminal amino-acid. The whole process can then be repeated. The method has been adapted to small-scale work and successfully applied to a number of synthetic simple peptides. The main serious limitation in



this method seems to be the very low solubility of the intermediate phenylthiocarbamyl derivatives of peptides and, in consequence, the cleavage of these has to be carried out by employing a suspension in anhydrous acid.

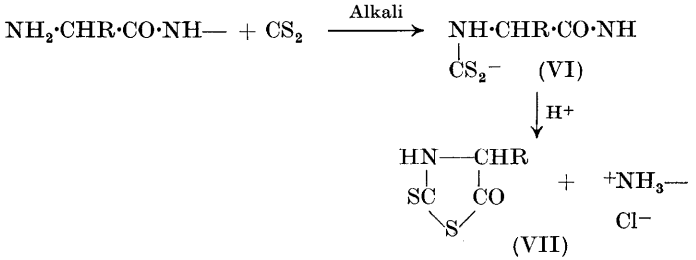
Another method which employs Edman's conditions [102] for the " cleavage step " and appears to offer certain other advantages has been devised in this laboratory.[104] The first stage is the reaction of the sodium salts of peptides at room temperature with alkyl alkoxydithioformates [cf. 105, 106] (dialkyl xanthates) (III). The *N*-thioncarbalkoxy-derivatives (IV) which are thus formed are fairly soluble in organic solvents and can thus be isolated from the reaction mixture. Also, they are more easily cleaved than (I) because they are more soluble in anhydrous inert solvents. Insoluble hydrochlorides of the degraded peptide or amino-acids are thus obtained and the terminal amino-acid is regenerated after short hydrolysis of the soluble cyclic compounds, presumably (V).[105, 107] This method also has been successfully applied to simple synthetic peptides.



A. L. Levy [108] has shown that 4-alkyl-2-thiothiazolid-5-ones (VII) are split off when salts of *N*-dithiocarboxypeptides (VI) are acidified. He has utilised this reaction in a method of degradation in which the initial forma-

[104] H. G. Khorana, *Chem. and Ind.*, 1951, 129; G. W. Kenner and H. G. Khorana, *J.*, 1952, 2076.
[105] P. Aubert and E. B. Knott, *Nature*, 1950, **166**, 1039.
[106] P. Aubert, E. B. Knott, and L. A. Williams, *J.*, 1951, 2185.
[107] P. Aubert, R. A. Jeffreys, and E. B. Knott, *J.*, 1951, 2195.
[108] *J.*, 1950, 404.

tion of the dithiocarboxy-derivatives and their cleavage are carried out consecutively in the same aqueous solution. This involves, therefore, a risk of contamination of the degraded with the original peptide.



The above-mentioned methods have not been applied as yet to relatively complex peptides such as, for example, those in which, in addition to an α-amino-group, a δ-amino group of ornithine or ε-amino-group of lysine is present. Reaction of such groups would probably occur in the normal fashion, to form the corresponding derivatives, but the latter might not interfere in the cleavage step. It is some evidence in favour of this that, unlike the α-isomer, dithiocarboxy-β-alanine amide did not cyclise to give a thiazine derivative.[109] The driving force in the above cleavages seems to reside in the stability of the resulting five-membered rings.

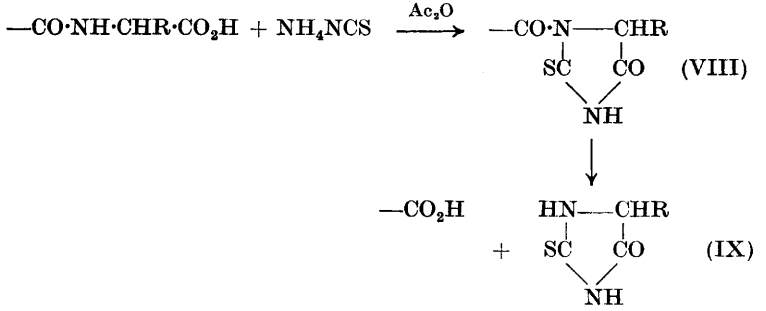
Degradation from the carboxyl end. Selective degradation from the carboxyl end has proved to be relatively difficult and, although several methods have been suggested,[47, 87, 101] none appears to be satisfactory as a stepwise procedure. Most of them suffice probably only for identification of the carboxyl end-group.

Recently P. Schlack and W. Kumpf's method [88] has been re-examined by S. G. Waley and J. Watson [110] and by J. Tibbs,[111] and the limitations of the method in practical application have been indicated. According to this method an *N*-acylpeptide is heated with acetic anhydride and ammonium thiocyanate, to form (VIII) which decomposes readily in alkali to the thiohydantoin (IX) (derived from the terminal amino-acid) and the degraded peptide. The method has been used in the identification of the end-group of glutathione [89b] and of insulin.[110]

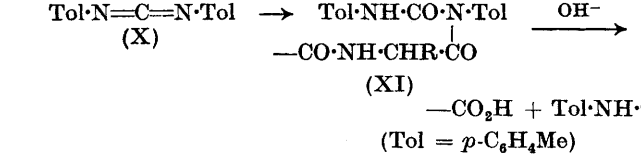
A method which employs mild conditions throughout has been discovered in this laboratory ; [112] often, however, the degraded peptide is contaminated with the original peptide. The first step is the formation of the acylureas (XI) by reaction of *N*-acyl-peptides with di-*p*-tolylcarbodi-imide (X) [113] at room temperature. (The reactions of carbodi-imides with carboxylic acid have been studied extensively by F. Zetzsche and his co-workers.[114]) The

[109] A. C. Davis and A. L. Levy, *J.*, 1951, 2419.
[110] *J.*, 1951, 2394.
[111] *Nature*, 1951, **168**, 911.
[112] H. G. Khorana, *J.*, 1952, 2081.
[113] F. Zetzsche *et al.*, *Ber.*, 1938, **71**, 1512 ; F. Zetzsche and W. Nerger, *Ber.*, 1940, **73**, 467.
[114] F. Zetzsche *et al.*, *Ber.*, 1938, **71**, 1088 *et seq.*

acylureas are immediately split in cold dilute alkali to form (XII) (alkaline hydrolysis regenerates the terminal amino-acid) and the degraded peptide



or amino-acid. The method has been applied to simple synthetic peptides but needs further examination with a wider variety.



Selective chemical methods for degrading peptide chains are needed. One possible approach would be to attach a suitable substituent to the functional groups (*e.g.*, thiol or hydroxyl) to facilitate cleavage of adjacent amide linkages.

6. Enzymic Degradation.—Proteolytic enzymes, which bring about the degradation of peptides and proteins, show high specificity in their attack at different peptide bonds. An exact knowledge of the specificity and the mode of their action is a prerequisite in the application of enzymic degradation as a tool in structural investigations. The " structural environment " necessary for the action of different proteolytic enzymes has been studied during the past several decades. However, the use of impure enzymic preparations and ill-defined substrates has rendered earlier work [115–118] of doubtful value ; the difficulties of obtaining homogeneous macromolecules, such as proteins, and proteolytic enzymes, which are themselves of protein nature, have always been enormous.[119] Comparatively recently, more reliable information has accrued from the pioneer work on the purification and crystallisation of certain proteolytic enzymes [120] and the use of pure synthetic substrates.[121]

[115] E. Fischer and P. Bergell, *Ber.*, 1903, **36**, 2592 ; 1904, **37**, 3103.
[116] E. Fischer and E. Abderhalden, *Z. physiol. Chem.*, 1905, **46**, 52.
[117] E. Waldschmidt-Leitz and A. Harteneck, *ibid.*, 1925, **149**, 203.
[118] W. Grassmann and F. Schneider, *Ergebn. Enzymforsch.*, 1936, **5**, 79.
[119] N. W. Pirie, *Biol. Reviews*, 1940, **15**, 377.
[120] J. H. Northrop, M. Kunitz, and R. M. Herriott, " Crystalline Enzymes ", Columbia Univ. Press, New York, 1948 ; M. L. Anson, *J. Gen. Physiol.*, 1937, **20**, 663.
[121] M. Bergmann, *Naturwiss.*, 1932, **20**, 420 ; *Adv. Enzymology*, 1942, **2**, 49 ; M. Bergmann and J. S. Fruton, *ibid.*, 1941, **1**, 63.

Specificity and classification. The earlier classification [117, 118] (accepted until *ca.* 1935) distinguished between " proteinases " which attacked only proteins of high molecular weight, and " peptidases ", which hydrolysed the peptides of low molecular weight. The experimental findings of Bergmann [121] and his co-workers on synthetic substrates have invalidated this differentiation of proteolytic enzymes. Instead, a broad classification has now become widely accepted which is based on the difference in specificity with regard to the requirement, in the " backbone " of the substrate, of a free α-amino- or carboxyl group adjacent to a sensitive peptide bond. One main group, the " exopeptidases ", acts only on the compounds which have one or both of the above-mentioned groups, whereas " endopeptidases " can hydrolyse internal peptide bonds in proteins and substituted peptides. The exopeptidases have been subdivided into " aminopeptidases " and " carboxypeptidases " depending on the specificity of the requirement of a free α-amino- or carboxyl group respectively. Further, among the aminopeptidases the discovery of the side-chain specificity [122] has led to the recognition of enzymes such as leucine-aminopeptidase. The sub-classification of endopeptidases, however, has proved to be more difficult, as several enzymes from different sources have been found to have similar " backbone " and " side-chain " specificities. This fact has led to the suggestion of the term " homospecific " for these enzymes, as opposed to " heterospecific " for enzymes with different side-chain requirements. Further, members of the homospecific group have also been named after the best known member of that group, *e.g.*, " trypsinases ".

Most proteolytic enzymes hydrolyse peptide bonds involving L-amino-acids but some peptidases have been discovered which split specifically peptides containing D-amino-acids.[123]

In recent years, the activation requirements of certain enzymes of some substances (such as cysteine, cyanide, ascorbic acid, etc.), and metal-ion activation of another group of enzymes, have become generally known. Comprehensive reviews [124] on the mode of action and specificity of the important exopeptidases and endopeptidases have recently become available.

Use in structural investigations. One important danger in the use of the proteolytic enzymes to secure selective fission is the possibility of simultaneous synthesis and thus of amino-acid sequences which are not present in the original molecule. Examples of such rearrangements have become known through the work of Bergmann [125, 126] and his collaborators, who have emphasised the frequency of such reactions. Another example has

[122] K. Linderström-Lang, *Z. physiol. Chem.*, 1929, **182**, 151.

[123] M. Bergmann and J. S. Fruton, *J. Biol. Chem.*, 1937, **117**, 189 ; H. Herken and H. Erksleben, *Z. physiol. Chem.*, 1940, **264**, 251 ; 1941, **269**, 47 ; **270**, 201 ; E. Maschmann, *Naturwiss.*, 1941, **29**, 709 ; *Biochem. Z.*, **308**, 359 ; **309**, 179 ; 1942, **313**, 129 ; E. Waldschmidt-Leitz, *Ergebn. Enzymforsch.*, 1943, **9**, 193.

[124] M. J. Johnson and J. Berger, *Adv. Enzymology*, 1942, **2**, 72 ; E. L. Smith, *ibid.*, 1951, **12**, 191 ; H. Neurath and G. W. Schwert, *Chem. Reviews*, 1950, **46**, 69.

[125] M. Bergmann and H. Fraenkel-Conrat, *J. Biol. Chem.*, 1937, **119**, 707.

[126] O. K. Behrens and M. Bergmann, *ibid.*, 1939, **129**, 587.

been found recently by Watson and Waley [127] during enzymic hydrolysis of tripeptides ; this further points to the generality of such reactions involving different enzymes, for these authors employed trypsin and chymotrypsin whereas in the first well-known example [126] papain was used. In view of the likelihood of such rearrangements, Synge [3] has pointed out the lack of authenticity of the products of enzymic degradation of proteins.

Recently, Sanger [46] used proteolytic enzymes (pepsin, trypsin, and chymotrypsin) in his work on insulin. The possibility of error was minimised by the use of two enzymes and through additional evidence from products of acid hydrolysis.

As one main interest in enzymic degradation is the cleavage of large polypeptide chains to oligopeptides rather than to amino-acids or di- and tri-peptides, a knowledge of the earlier stages of digestion of proteins by proteolytic enzymes would be very desirable. In this connection, the conclusion by A. Tiselius and I. B. Eriksson-Quensel [128] of an " all-or-none " reaction rather than a gradual breakdown during the peptic digestion of egg albumin is disappointing. Further work on different proteins and enzymes has yielded evidence in favour of,[129] as well as against,[130] this type of reaction. It is obvious that the enzymic degradation depends on the nature of the enzyme and the substrate. In fact, there appears to be a rich variety in the mode of degradation brought about by proteolytic enzymes. The formation of hypertensin, the blood-pressure-raising substance from serum protein by the action of " renin " [131] and strepogenin,[132] a growth factor, in tryptic digests of various proteins is an interesting example.

A very significant discovery is the transformation of ovalbumin by a proteinase from *Bacillus subtilis* into a second protein which has been called plakalbumin.[133] This is accompanied by the release of three peptides,[21] containing a total of six amino-acid residues.

Another interesting degradation, which affords valuable information on the structure of silk fibroin, has been achieved by B. Ducker and S. G. Smith : [134] short tryptic hydrolysis yielded a polypeptide of molecular weight approximately 7000, which consisted entirely of glycine, alanine, and serine.

7. Occurrence of D-Amino-acids in Naturally Occurring Peptides.—It has been generally accepted [135, 136] in the past that all the amino-acids occurring in proteins have L-configuration. Although recently some newer methods of analysis have revealed the presence of small amounts of D-isomers in

[127] S. G. Waley and J. Watson, *Nature*, 1951, **167**, 360.

[128] *Biochem. J.*, 1939, **33**, 1752.

[129] D. M. P. Phillips, *ibid.*, 1951, **49**, 506 ; A. Beloff and C. B. Anfinsen, *J. Biol. Chem.*, 1948, **176**, 863.

[130] I. Moring-Claesson, *Biochim. Biophys. Acta*, 1948, **2**, 389.

[131] P. Edman, *Arkiv Kemi, Min., Geol.*, 1945, **22**, *A*, No. 3.

[132] H. Sprince and D. W. Wooley, *J. Amer. Chem. Soc.*, 1945, **67**, 1734.

[133] K. Lindenström-Lang and M. Ottesen, *Comp. rend. Trav. Lab. Carlsberg*, 1949, **26**, 403.

[134] *Nature*, 1950, **165**, 196.

[135] A. Neuberger, *Adv. Protein Chemistry*, 1948, **4**, 297.

[136] G. R. Tristram, *Biochem. Symp.*, 1948, **1**, 33.

protein hydrolysates, these isomers appear to be mere artefacts of hydrolysis and the present evidence is in accord with the earlier view.[136] On the other hand, the occurrence of D-amino-acids in the metabolic products of micro-organisms [137] and several naturally occurring peptides [4] is regarded as established.[138] Thus, ergot alkaloids contain D-proline,[139] gramicidin S [94] contains D-phenylalanine, gramicidins A, B, etc.,[4, 13] and tyrocidins [4, 13] similarly contain D-amino-acid residues.[140, 141] There is also some evidence that both the antipodal forms might be present preformed in the same molecule (*e.g.*, in gramicidins).

G. R. Tristram [136] has discussed the microbiological and enzymic methods for the detection of such isomers in hydrolysates. S. W. Fox [142] also has described a microbiological method for the determination of the sequence of amino-acids. (In this connection, it should be mentioned that the chemical methods of selective degradation discussed in section 5 will probably cause extensive racemisation of the cleaved amino-acids.) In the investigations on peptide antibiotics, the presence of D-isomers has been shown by the isolation of the particular amino-acid or its derivative or the dipeptides containing such isomers from partial hydrolysates (*e.g.*, L-alanyl-D-valine [141] and D-valyl-D-valine from gramicidins [140]). An ultra-micro-method for revealing the presence of D-amino-acids consists of the use of D-amino-acid oxidase of sheep's kidney on a paper chromatogram. The method has been successfully applied by Synge for the identification of D-leucine and D-valine and by T. S. G. Jones for the identification of D-leucine in " aerosporin ", one of the polymyxin antibiotics.[143]

However, it should be pointed out that the presence of D-amino-acid residues in hydrolysates or partial hydrolysates may not always constitute rigorous proof for the presence of such isomers in the original molecule. A. Neuberger has considered at length the possibilities of inversions and racemisations at the asymmetric centres in peptides before the hydrolysis of the peptide bonds ; and Synge has emphasised the need for " extensive model experiments with optically active peptides ".

The Reviewer is indebted to Professor A. R. Todd, F.R.S., for generous encouragement and acknowledges the benefit of discussions with Drs. G. W. Kenner and D. T. Elmore and their valuable help during the preparation of the manuscript.

[137] G. Ivánovics and V. Bruckner, *Z. physiol. Chem.*, 1937, **247**, 281 ; H. N. Rydon, *Biochem. Symp.*, 1948, **1**, 40.
[138] T. S. Work, *ibid.*, p. 61.
[139] W. A. Jacobs and L. Craig, *J. Biol. Chem.*, 1935, **110**, 521.
[140] H. N. Christensen, *ibid.*, 1943, **151**, 319 ; 1944, **154**, 427.
[141] R. L. M. Synge, *Biochem. J.*, 1949, **44**, 542.
[142] S. W. Fox, *J. Amer. Chem. Soc.*, 1951, **73**, 3573.
[143] *Biochem. J.*, 1948, **42**, lix.