PARTIAL HYDROLYSIS PRODUCTS DERIVED FROM PROTEINS AND THEIR SIGNIFICANCE FOR PROTEIN STRUCTURE

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Received November 19, 1941

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I. Introduction

A change has come about during the past half-century in the chemical significance of the term *protein* (albumin, Eiweisskörper, etc.). While the group of substances referred to, and even their broad classification and nomenclature (*cf.* Kossel (130)), remain unchanged substantially, it is possible to define the class in a more satisfactory way. Schorlemmer (199), in 1879, writing of the "albuminous bodies" said: "All that we know of them is their percentage composition, and that they contain the carbon atoms linked together partly as in the aliphatic compounds and partly as in the aromatic bodies. We do not know their molecular weights, the most simple formula for albumin being, according to Lieberkühn, $C_{72}H_{112}N_{18}SO_{22}$ and, according to Harnack, $C_{204}H_{322}N_{52}S_2O_{66}$."

At the present day a more apt definition would be "large molecules of biological origin yielding on hydrolysis *l*-α-amino- or *l*-α-imino-carboxylic acids." This definition leaves out of account the "peptide" of *d*-glutamic acid obtained from *B. anthracis* etc. by Iványovics and Bruckner (122), as well as the fact that a very large proportion of the proteins yield on more or less violent hydrolytic treatment a wide variety of substances not amino acid in nature. Of recent years the range of these "prosthetic groups" has been extended by the discovery that in many cases "physiologically active" small molecules (aneurin, lactoflavin, various porphyrins, chlorophyll, carotenes, etc.) occur in the living organism bound in a highly specific, if labile, manner to specific proteins. With such discoveries has come an increased appreciation of the significance and versatility of the proteins in biology, and hence an increased interest in their detailed nature.

Such remarks as the following (Pauli (174)) are not often made at the present day: "A comparatively recent epoch ascribed to the proteins the central position in the life process. Today, however, they appear rather as the passive carriers of the life phenomena, determining the important chemical and physico-chemical properties of the medium and the mechanical properties of the tissue or forming the base for certain attached active groups. The true directive participation in the maze of vital chemical reactions on the other hand devolves on scattered specific substances which, in general, belong to entirely different classes of bodies and by which the fields of the chemist and biochemist have been greatly enlarged." Bergmann (39) has dealt with the question in a manner more satisfying to modern biologists: "The major part of the organic substance of the human and animal body consists of proteins. To the proteins belong substances serving rather different biological functions, such as: hair and nails; muscle and skin tissue; tendons; egg white; the casein of milk; the fiber of natural silk; the red substance of the blood; the digestive enzymes, *pepsin* and *trypsin*; the hormone, insulin; and certain viruses. The proteins of one animal or plant species differ from those of all other species. Wherever life phenomena occur, proteins are involved in one way or another. They are, therefore, regarded as being the chemical requisite for life."

The increased precision of our chemical definition of the proteins we owe largely to the detailed evidence that has now accumulated that proteins yield on hydrolysis a limited range of chemically diverse amino acids. The history of the discovery of these in its qualitative aspects has been given by Vickery and Schmidt (230; *cf.* Dunn (60)). The quantitative aspect (amino acid analysis), in regard to both its technique and its results when applied to a number of proteins, has been reviewed by Mitchell and Hamilton (162), although a comprehensive and critical treatment of developments in this field since then has not yet appeared (*cf.* Block (46)). The present time would seem particularly opportune for such a treatment, since the application of "isotope dilution" analysis to amino acids (Rittenberg and Foster (192); *cf.* Schoenheimer *et al.* (198) for the relevant control experiments) would seem to have introduced a technical advance making possible in the future a new level of accuracy in amino acid analysis, which promises to become far more than it has been in the past a routine analytical operation.

Such a review would show that during the past fifty years sufficient proteins (e.g., clupein, wool, zein, gelatin) have had more than 90 per cent of their nitrogen accounted for as known amino acids and would justify the now widely held belief that no other type of primary hydrolysis product need contribute to the structure of a protein.

The Hofmeister–Fischer theory that proteins are built up from amino acid residues in acid–amide or "peptide" linkage with one another has remained since its formulation the basic hypothesis of protein chemistry. As Vickery and Osborne (229) put it: "Beginning with the announcement of the polypeptide hypothesis, the study of the proteins became once more an occupation in which a self-respecting organic chemist might take a part." These authors have re-

viewed the older alternative and supplementary hypotheses. Some more recent (and more speculative) views have been criticized by Pauling and Niemann (173), who conclude: "It is our opinion that the polypeptide chain structure of proteins, with hydrogen bonds and other interatomic forces (weaker than those corresponding to covalent bond formation) acting between peptide chains, parts of chains and side chains, is compatible not only with the chemical and physical properties of proteins, but also with the detailed information about molecular structure in general which has been provided by the experimental and theoretical researches of the last decade."

The main evidence in favor of the peptide hypothesis falls into the following classes: (*1*) Proteins yield on hydrolysis approximately equal numbers of amino and carboxyl groups simultaneously; most of the other chemical and physical properties of "intact" proteins are consistent with what would be expected from peptides of the naturally occurring amino acids. (*2*) Peptide-like simple substances (as well as free amino acids) occur commonly in nature, e.g., glutathione, carnosine, hippuric acid, pantothenic acid, etc. (*3*) Enzymes capable of splitting proteins are also capable of splitting and synthesizing peptide bonds in synthetic compounds. The establishment of the general truth of this we owe largely to Bergmann and his collaborators (for review and references see Bergmann (39)). Especially before synthetic substrates for pepsin had been discovered, this test proved an obstacle to the acceptance of the peptide hypothesis. (*4*) Peptides of known constitution (established by synthesis) have been shown to result from the partial hydrolysis of proteins. It is with evidence of this type and its bearing on the structure of proteins that the present review is concerned. It is hoped to establish that the comparative poverty of the data that have accumulated under this head is due more to the inadequacy of the techniques employed than to any defect in the peptide hypothesis. The newer physical methods for the characterization of proteins, combined with improved amino acid analysis, have made it possible to obtain very precise information as to the number and nature of the amino acid residues in one molecule of a "homogeneous" protein. Inside the framework of this information and the peptide hypothesis, even assuming that the molecule consists of a single *unbranched* peptide chain, the possibilities of isomerism are enormous. Thus, for a molecule containing altogether 288 amino acid residues, equally distributed between twelve different amino acid species, 0.6×10^{300} different isomers are possible. (Fischer (78)) made similar calculations, at a time when less was known of the molecular dimensions of proteins, for a triacontapeptide.) This isomerism is based on the "classical" formula alone; it is obviously extended almost beyond comprehension when the configurational isomerism postulated by Pauling (172), based on secondary valence, is taken into account. Nevertheless, taking into account only this limited isomerism of a schematized albumin molecule, it is clear that the coexistence of a single physical molecule of each isomer is impossible, since the amount of matter involved (10^{280} g.), whatever its relation to the amount of matter in the universe, exceeds the more relevant mass of the earth by a factor of 10^{253}.

Irrespective, however, of the theoretical limits to protein isomerism imposed by an *a priori* argument such as that given above, it is becoming increasingly clear as experimental data accumulate that the proteins exhibit very definite regularities of structure, even permitting in some cases, such as that of insulin (*cf.* Crowfoot and Riley (50)), the use of the concept "molecule" in the same sense as for simple organic compounds.

In the search for such structural regularities, the postulate of regular repetition of amino acid residues along a peptide chain, which appears to have been first explicitly enunciated by Kossel and Pringle (134), has achieved prominence in recent years as a result of the speculation put forward by Bergmann and Niemann (41, 42, 43). They postulate that in proteins any particular amino acid residue recurs in the unbranched peptide chain with a frequency expressible as $2^m \times 3^n$, where m and n are positive integers or zero and, further, that the number of residues of any particular amino acid occurring in one protein molecule is expressible in the same form, as is the total number of all amino acid residues in one protein molecule.

Without attempting to offer a detailed criticism of the theory here, it must be emphasized that the most important experimental evidence that has been produced in its support rests on analyses of the amino acids in complete hydrolysates of proteins. While evidence of this type is important in establishing the totals of the amino acid residues in the molecule, it is clear that the only *direct* evidence in respect of the fundamental postulate of Bergmann and Niemann, the regular recurrence of particular amino acid residues along the peptide chain, can come either from the synthesis of a protein or from the isolation of recognizable protein breakdown products. The second of these approaches seems more profitable at present than the first, and is likely to yield information about the detailed chemical structure of proteins, whatever the fate of the Bergmann–Niemann hypothesis.

Additional importance can be attached to studies of partial breakdown products of proteins, since recent serological studies (*cf.* especially Landsteiner and van der Scheer (141)) suggest that the nature of particular groupings of amino acid residues of greater or less length along a peptide chain may serve as the basis of antigenic specificity. Recently, also, Pauling (172) has formulated a theory of antibody formation and reaction in physicochemical terms. This is based on the configurations that a peptide chain can assume, and it is tentatively suggested that proline and hydroxyproline residues may play an important rôle in determining these configurations. Investigation of partial hydrolysis products of purified antibodies may in the future help to throw light on their mode of action, and on their structural relation to "normal" globulin.

It seems clear that the phenomena of biological specificity which have been studied in such detail by serological techniques are by no means restricted to serology; similar mechanisms may be postulated for enzyme action, specific permeability, reproduction, etc. and in all these fields our accumulating knowledge stresses the rôle of the proteins.

It is the purpose of the present review to enumerate comprehensively and

critically the data that have so far accumulated on the partial hydrolysis products of proteins; to assess as far as possible their significance for our present knowledge of protein structure; and to review, with an eye to future developments, the techniques that have been employed in this field, for it seems probable that in this direction at present lies the way forward towards the goal cited by Bergmann (39) as "to establish the molecular composition and architecture of the various proteins with the same precision as the molecular composition and architecture of simpler molecules have been ascertained," and thereby, one may hope, to establish with greatly increased precision the nature of living matter.

II. Significance and Authenticity of Partial Hydrolysis Products from Proteins

The present review aims at dealing comprehensively with those products that have been found to result from the partial hydrolysis of proteins. The emphasis is laid more on the chemical nature of the products studied than on the kinetics of their formation or the theoretical background that inspired their isolation. Non-isolative investigations are therefore referred to only where they seem to throw light on the phenomena under discussion. In general, compounds fall within the scope of this review when they contain two or more amino acid residues in a molecule. It is possible, especially when dealing with proteins that are not homogeneous and of which the "purity" (*cf.* Pirie (176)) is difficult to assess, that some of the more complex isolation products described, differing in composition from the original material, may arise less from partial hydrolysis than from a fractionation of the different constituent molecular species of the original protein material. This question becomes especially thorny in dealing with the insoluble proteins. Thus, it is generally recognized that wool, as it has usually been subjected to chemical study, consists of at least two different histological components, which have not in the past been readily separable by mechanical means. Since these (the scales and the cortical cells) have definitely distinct physical properties, it seems not unreasonable to expect differences of chemical composition or structure. The question whether the cortical cells of wool differ in composition from the intercellular material has been discussed by Elsworth and Phillips (62), who give references to the earlier literature. This comparatively simple case is cited as an obvious example of a phenomenon that may be expected in all the less readily fractionable protein materials. A more complex example is offered by silk fibroin, and is discussed in this connection below. For the case of casein, reference should be made to Linderstrøm-Lang's thorough investigation of its fractions (152; *cf.* also Holter *et al.* (116) on the course of peptic digestion of these fractions and for clupein the work of Felix *et al.* cited below). Although in most investigations there is good evidence that hydrolysis of the starting material has occurred, it will be appreciated that heterogeneity of the starting material cannot fail to make interpretations of structure more equivocal than they otherwise would be.

Up to this point it has been a tacit assumption that the isolation of compounds

of two or more amino acid residues and more or less understood constitution is to be taken as evidence for the preëxistence of these groupings in the intact protein. The *a priori* nature of this assumption should be noted. The recent discovery of Behrens and Bergmann (38) that papain is capable of carrying out synthesis and hydrolysis of peptide bonds simultaneously in the same reaction mixture casts doubt at least on the significance of products obtained from proteins through the action of papain. Other enzymes also must be under suspicion, and the whole question of the significance of products of enzyme action must be treated with reserve until the specificity of enzymes in proteolysis has been more adequately established. The researches of Bergmann and his collaborators (*cf.* Bergmann (39)), using synthetic substrates, have taken us a long step in this direction.

With non-enzymic hydrolytic agents, the only established synthetic action seems to be the formation of diketopiperazines from dipeptides etc. on heating with acids or water. High temperatures seem to favor ring formation, which does not appear to take place in concentrated acids at low temperatures (*cf.* Abderhalden and Funk (17) and Abderhalden and Komm (22, 23)). Hopkins (117) noted hydrolysis and diketopiperazine formation on boiling glutathione with water. Synthesis from free amino acids treated with acid or alkali has never been found in numerous control experiments. It seems therefore legitimate to regard products isolated from partial acid or alkali protein hydrolysates as of significance in establishing protein structure until contrary evidence is produced. It is satisfactory that in the past protein chemists, in studies of this sort, have more often employed partial acid hydrolysis than enzymic hydrolysis.

Attention must also be paid to artifacts arising in the course of isolation of the hydrolysis products. Although the balance of evidence suggests that diketopiperazines found in protein hydrolysates are not preformed in proteins (this whole question and Abderhalden's diketopiperazine theory of protein structure are dealt with in detail by Vickery and Osborne (229)), it seems likely that these compounds arise by secondary condensation of amino acid residues already linked to each other by one bond. This is, in fact, the basis of Fischer and Abderhalden's (80, 81, 82) esterification method of separating dipeptides from higher peptides and free amino acids. Nevertheless, free amino acids under certain conditions can and do condense to form diketopiperazines,—e.g., upon distillation of their esters (Fischer) or when simply heated, especially with alcohols, or under dehydrating conditions. Thus Dakin's isoleucine valine anhydride from casein (52) and hydroxyproline proline anhydride from gelatin (53) were obtained only after prolonged refluxing of free amino acid mixtures in butyl alcohol, and must be regarded as artifacts (*cf.* Osborne, Leavenworth and Nolan (171)).

It is obvious that the *yield* of any particular partial hydrolysis product is of fundamental importance in any interpretation of protein structure. Workers in this field have in many cases entirely omitted reference to yields, thus robbing their work of most of any value that it otherwise might have. In the present review special emphasis is placed on yield, which is noted wherever possible.

These yields should be studied in connection with tables of the amino acid composition of proteins, to be found in reference works such as those of Mitchell and Hamilton (162), Jordan Lloyd and Shore (126), etc. It should be noted that in nearly every case the isolation of any particular partial hydrolysis product must have been accompanied by considerable losses. Rittenberg and Foster's isotope dilution method (192) seems likely to prove itself of incalculable value in this field.

With regard to the authenticity of partial hydrolysis products of proteins, it would clearly be desirable to apply the same criteria as those applied to the naturally occurring amino acids by Vickery and Schmidt (230). These are: (*1*) In order that an amino acid shall be accepted as a definite product of the hydrolysis of proteins, it must also have been isolated by some worker other than its discoverer. (*2*) Its constitution must have been established by synthesis and by demonstration of identity between the synthetic product and the racemized natural product, or by actual resolution of the synthetic product and preparation of the optically active natural isomer. If these criteria are adopted, remarkably few of the partial hydrolysis products of proteins described below could be called authentic, since of the minority the constitution of which has been established by synthesis, few would conform to criterion 1.

It should be noted that synthesis in this field is very laborious, although the carbobenzoxy technique of Bergmann and collaborators marks a big step forward towards its standardization. In general, it is necessary that the synthetic product should be made directly from optically active amino acids, since any simple peptide containing n amino acid residues has 2^n optical stereoisomers, a mixture of which in unknown proportions arises when synthesis is carried through using racemic amino acids as starting material. The whole problem is complicated by the fact that the methods employed in isolating the natural product often lead to its partial racemization.

It seems true that polypeptides as a class do not crystallize at all well. Organic chemists in the past have attached an almost fetish-like significance to crystallinity, and it seems opportune to point out that, in comparing synthetic with natural products, crystal form and melting point are not the only criteria that can be used.

In characterizing a partial hydrolysis product of a protein, an essential criterion is a quantitative analysis of the amino acids resulting on total hydrolysis. This information is of value even where (as in the phosphopeptone of Damodaran and Ramachandran (57)) too many amino acid residues are present for an immediate synthetic approach. A complete amino acid analysis of any partial hydrolysis product isolated should in general be technically easier than the analysis of the original protein. Far too many workers in this field have been content with the inconclusive data of elementary analysis.

In these more complicated cases, various methods of analytic elucidation of structure are available. These are discussed in a subsequent section of this review. Some of them have been employed in attempts to elucidate the structure of intact proteins directly; in this field, beyond the general conclusion that

amino acid side chains in most cases react as though they were free, not much of significance has been established; Gurin and Clarke (109), however, have shown by benzenesulfonylation and subsequent acid hydrolysis that a large proportion of the ϵ-amino groups of the lysine residues of gelatin are free, thus confirming the negative evidence obtained by previous workers who had failed to isolate lysine from hydrolysates of proteins that had been deaminated with nitrous acid. Hess and Sullivan (114) have obtained evidence of this sort for free cystine amino groups in a number of proteins, and Jensen and Evans (123) have obtained positive evidence for free phenylalanine amino groups in insulin. Dirr and Felix's (58) demonstration of a free arginine carboxyl group in clupein should also be noted.

Finally, mention must be made of the interesting, if not directly interpretable in terms of structure, studies of Dakin and collaborators (51, 54, 55) on the racemization of amino acid residues in proteins by the action of dilute alkali. In the cases of duck and hen egg albumins, which, though serologically distinguishable, do not show significant differences in amino acid composition, Dakin and Dale observed significant differences in the degree of racemization of histidine, leucine, and aspartic acid. The model experiments on peptides of the simpler monoamino acids carried out subsequently by Levene and his collaborators have tended to confirm Dakin's view that amino acid residues having a free $-NH_2$ or $-COOH$ group escape racemization, but it is probable that in the case of the proteins other more complicated factors are involved.

III. Details of Investigations of Particular Proteins

A. Silk Fibroin

In addition to being the first protein material from which peptides of known constitution were isolated, this group of arthropod proteins, for which the amino acid analysis suggests a relatively simple structure, has been more closely investigated by methods of partial hydrolysis than any other group of proteins.[1]

Fischer (76), in 1902, delivered a lecture at Karlsbad in which he described how he and Bergell had isolated from a hydrolysate of silk fibroin, prepared by the successive action of hydrochloric acid, trypsin, and baryta, a dipeptide as its naphthalenesulfonyl derivative, which was thought to be glycylalanine: "Diese Beobachtung ist, wie leicht ersichtlich, von allgemeiner Bedeutung, weil sie die Möglichkeit beweist, kristallisierbare Produkte zu gewinnen, die zwischen den Peptonen und Aminosäuren stehen."

Fischer and Bergell subsequently (84) announced that they had prepared synthetically the naphthalenesulfonyl derivative of *l*-alanylglycine and of glycyl-*l*-alanine, and found neither to correspond exactly in properties with the material of natural origin, which they suggested might be a mixture of the two compounds in question. Further, there were difficulties in repeating exactly the original preparation. However, Fischer and Abderhalden (81) made use of their method

[1] In the following, "silk fibroin" or "fibroin" refers to material from *Bombyx mori*, unless otherwise specified.

(80) of converting dipeptides to diketopiperazines through their esters. In this way they succeeded in isolating from digests of silk fibroin (prepared with sulfuric acid followed by trypsin, or by hydrochloric acid alone) crystalline glycine *l*-alanine anhydride, which they identified completely with the synthetic product. In the case of the hydrochloric acid hydrolysate, the weight of diketopiperazine isolated amounted to 12 per cent of the weight of fibroin used. Glycyl-*l*-alanine or *l*-alanylglycine could both act as precursors of the anhydride; its formation from free amino acids in the course of esterification was excluded by control experiments. The authors inclined to the view that the anhydride was formed from glycyl-*l*-alanine since, unlike the material in the digest, *l*-alanylglycine was readily split by the tryptic preparation used by them. They noted further the isolation of a small amount of glycine tyrosine anhydride. In a subsequent paper by Fischer and Abderhalden (82) this product, obtained from hydrochloric acid digest of silk fibroin (in the mother liquors from the glycine-*l*-alanine anhydride), was formally identified with glycine *l*-tyrosine anhydride obtained synthetically. The yield on one occasion was 4.2 per cent of the fibroin used, but on a second only 0.18 per cent.

In a further paper, Fischer and Abderhalden (83) returned to the original approach of Fischer and Bergell; they treated a partial hydrochloric acid hydrolysate of fibroin with phosphotungstic acid. From the syrupy material not precipitated by this reagent they isolated, by naphthalenesulfonylation, an amount of naphthalenesulfonylglycyl-*l*-alanine (formally identified with synthetic material) equivalent, as glycylalanine, to 6 per cent of the fibroin used in the preparation. As a further proof of structure it was shown that acid hydrolysis of this compound yielded naphthalenesulfonylglycine. By using esterification instead of naphthalenesulfonylation, large quantities of glycine *l*-alanine anhydride were obtained, together with smaller amounts of glycine *l*-tyrosine anhydride. In the mother liquors was also found a small amount of material having the elementary composition of alanine serine anhydride, which was not further characterized.

From the phosphotungstic acid precipitate they isolated, by fractional precipitation with alcohol, a non-crystalline material which appeared to be a tetrapeptide containing 2 molecules of glycine, 1 molecule of alanine, and 1 molecule of tyrosine. On partial hydrolysis, followed by esterification, some glycine *l*-alanine anhydride and glycine *l*-tyrosine anhydride were obtained. Subsequently, Fischer (77) prepared by synthesis one of the possible isomers of this tetrapeptide —glycyl-*l*-alanylglycyl-*l*-tyrosine—which proved to be non-crystalline and in other ways very similar to the material from silk, although it was less readily precipitated by ammonium sulfate.

Abderhalden (2) obtained from a digest of silk fibroin (70 per cent sulfuric acid for 4 days at 18°C.) 0.4 per cent of glycyl-*l*-tyrosine by direct crystallization with alcohol after removal of the sulfuric acid. Later (3) better yields (4 to 5 per cent) were obtained, together with 3.8 per cent of *l*-alanylglycine, which was adequately characterized and compared with synthetic material. Still later (4) 8 per cent of *l*-alanylglycine was found to result from a partial hydrolysis of

fibroin, carried out in Hofmann-La Roche's Basel factory, and detailed directions for the preparation were given.

Abderhalden and Suwa (35) applied the diketopiperazine procedure to partial acid hydrolysates of various silks. From Canton silk they obtained 2 to 5 per cent of glycine *l*-alanine anhydride and 1 to 3 per cent of glycine *l*-tyrosine anhydride, whereas New-Chwang silk gave 6 per cent of *l*-alanine anhydride, together with small amounts of glycine *l*-alanine anhydride. Indian tussore silk gave 2 to 5 per cent of *l*-alanine anhydride.

Abderhalden (5, 6) obtained various yields of *l*-alanylglycine from partial acid hydrolysates of Canton and Bengal silks. He consistently obtained in the mother liquors an amorphous material, which was purified by precipitation with phosphotungstic acid, followed by fractionation with alcohol. The final product was microcrystalline; on one occasion 5.6 g. was obtained from 3 kg. of fibroin. It yielded on complete hydrolysis equimolecular amounts of glycine, *l*-alanine, and *l*-tyrosine, and had the molecular weight of the corresponding tripeptide. It agreed in properties with synthetic *l*-alanylglycyl-*l*-tyrosine, although the synthetic material had a rather lower rotation and was completely amorphous. This may have been due to partial racemization in the course of synthesis. Naphthalenesulfonylation followed by acid hydrolysis resulted in glycine, naphthalenesulfonylalanine, and *o*-naphthalenesulfonyltyrosine, although the yields were very poor. A similar investigation of "silk peptone" by Abderhalden and Funk (18) had led to poor yields of acylated products.

After the war, Abderhalden (7; *cf.* 8) returned to the study of the hydrolysis products of silk fibroin and reported, in addition to a new amino acid analysis, the isolation of 5 per cent of glycine *l*-alanine anhydride from a partial acid hydrolysate, together with glycine *l*-tyrosine anhydride and a product giving on complete hydrolysis serine, glycine, and alanine. Later (10) he obtained 8.5 per cent of glycine *l*-alanine anhydride from a partial hydrolysate with sulfuric acid ("silk peptone").

Abderhalden and Stix (33) attempted, without reaching any definite conclusion, to allocate the free amino groups of "silk peptone" by coupling with dinitrophenyl chloride, followed by acid hydrolysis. The same authors (34) studied the results of reducing "silk peptone" with sodium in amyl alcohol, and obtained piperazines (unidentified). Abderhalden and Schwab (28) claimed to have identified in this material, using ethyl alcohol for the reduction, piperazines in unspecified yield corresponding to glycine alanine anhydride, alanine serine anhydride, and an "anhydride of glycylalanylglycyltyrosine." Only incomplete elementary analyses of the hydrochlorides of the bases were given, and no comparisons were made with synthetic material. The further claim was made, based on equally inadequate evidence, to have isolated the precursors of the two last-mentioned piperazines directly from a preparation of "silk peptone." The importance of these results for Abderhalden's diketopiperazine theory of protein structure has been stressed by Vickery and Osborne (229).

Abderhalden and Heyns (19) subjected tussore silk to partial acid hydrolysis, and obtained 0.28 per cent of *l*-alanyl-*l*-tyrosine, from which a dibenzoyl deriva-

tive was obtained which yielded on acid hydrolysis tyrosine, benzoic acid, and benzoylalanine. The material was satisfactorily identified with the synthetic peptide. A portion of the silk which had not readily dissolved in the 70 per cent sulfuric acid used for the hydrolysis yielded a product which was thought to be alanylalanylalanylglycine. It had approximately 25 per cent of its nitrogen as amino nitrogen, and on complete hydrolysis followed by benzoylation it yielded 3 molecules of benzoylalanine and 1 molecule of hippuric acid; on benzoylation followed by acid hydrolysis, benzoylalanine was obtained. Successive condensation with benzylamine, acid hydrolysis, and condensation with phenyl isocyanate (*cf.* Abderhalden and Brockman (16)) yielded the phenylureido compound of glycine benzylamide; this was regarded as demonstrating that the free carboxyl group of the peptide belonged to its glycine residue.

These experiments, together with Abderhalden and Suwa's isolation of alanine anhydride from tussore silk, suggest that this material has a very different structure from that of *Bombyx mori* fibroin, from which no product suggesting the direct linkage of alanine with alanine or tyrosine has yet been isolated.

Goldschmidt, Martin, and Heidinger (98; *cf.* Goldschmidt and Strauss (99)) reported the isolation from fibroin after treatment with alkaline hypobromite of an insoluble product giving on hydrolysis only glycine and alanine in the molecular ratio 1:3. This product, representing a considerable proportion of the weight of fibroin used, was reported to give the same x-ray diffraction spacings as the original fibroin.

In a further paper (Goldschmidt, Freyss, and Strauss (97)), the degradation of fibroin by 5 *N* hydrochloric acid at 25°C. was studied. After 80 to 160 hr. about half the nitrogen in solution was amino nitrogen, but insoluble material equivalent to about 30 per cent of the original fibroin remained. This gave on complete hydrolysis 20–32 per cent of glycine, 57–68 per cent of alanine, and 6–11 per cent of tyrosine. It was claimed that no other amino acids were present, and it was suggested that the material consisted of alanine, glycine, and tyrosine in the molecular ratio 13:8:1. X-ray powder photographs of the material revealed the main spacings present in untreated silk fibroin. In none of these studies by Goldschmidt and collaborators can the chemical identification of alanine be regarded as satisfactory.

Uchino (225) has made a comparative study of the partial degradation of silk fibroin by various methods. Upon heating glycerol at 180–190°C. partial solution resulted, giving a 2.5 per cent yield of glycine alanine anhydride. Sulfuric acid (100 per cent) in the cold effected very little degradation to products of low molecular weight. Hydrolysis with dilute acid in the autoclave led to liberation of 35–48 per cent of the nitrogen of the fibroin as amino nitrogen. No further increase in this value occurred on prolonging the treatment, but the biuret reaction became progressively weaker and disappeared. This was attributed to simultaneous anhydrization of dipeptides and hydrolysis of higher peptides, giving no net change of amino nitrogen. Dilute alkali in the autoclave gave no such limiting value to the ratio of amino nitrogen to total nitrogen. Among the better characterized products obtained from the dilute acid hy-

drolysates by direct crystallization and by extraction with organic solvents were glycine alanine anhydride and glycine tyrosine anhydride. A nitrogen balance was constructed for the various fractions obtained, and the distribution of glycine, alanine, and tyrosine in the fractions was studied. It was noted that partial hydrolysis products of silk fibroin lost amino nitrogen on heating with glycerol at 170°C.

Grant and Lewis (104) repeated and confirmed Abderhalden and Steinbeck's (32) preparation of "silk peptone". This material had 17–64 per cent of its nitrogen in the form of amino nitrogen, and its tyrosine content was not significantly different from that of the original silk fibroin. Much shorter treatment of the fibroin (1 hr.) with 70 per cent sulfuric acid at 30°C. resulted, however, in a water-insoluble fraction (22–40 per cent of the weight of the fibroin used), having 1.7–3.0 per cent of its nitrogen in the form of amino nitrogen, and 6.4–8.0 per cent as tyrosine nitrogen; from the mother liquors of this a fraction was obtained by alcohol precipitation (10–27 per cent of the weight of fibroin used), having 10–17 per cent of its nitrogen as amino nitrogen and only 1.0–2.3 per cent as tyrosine nitrogen. Similar divergences in the tyrosine content of fractions from silk fibroin are indicated by Kaneko and Komatsu (127).

Although they do not constitute a rigid refutation, it is difficult to see how Grant and Lewis' results can be reconciled with Bergmann and Niemann's (43) theory of the structure of silk fibroin, which demands that every second, fourth, and sixteenth consecutive residue of the peptide chain shall be respectively glycine, alanine, and tyrosine. Similarly, the materials isolated by Abderhalden and Bahn, though obtained in small yield and not finally identified, were definitely more deficient in glycine and alanine, and in one case richer in tyrosine, than any product that could be expected to result from hydrolytic breakdown of the structure postulated by Bergmann and Niemann. The results of Goldschmidt and collaborators raise further difficulties. Moreover, difficulty has been experienced by those who have studied fibroin by the methods of x-ray diffraction in correlating the unit cell weight with the chemical data, and workers in this field (*cf.* Bergmann and Niemann's paper for references) have been inclined to regard silk fibroin as composed of "crystallites" embedded in an "encrusting material" of different composition.

It appears, moreover, that there is a mistake in Bergmann and Niemann's calculations. In the paper in question, they state: "The mean molecular weight of the amino acids formed by the complete hydrolysis of fibroin was estimated to be 102, which in turn leads to a value of 84 for the average residue weight." The accompanying calculation (table 1) is based on the values for glycine, alanine, tyrosine, arginine, lysine, and histidine employed by Bergmann and Niemann, supplemented by the figures of Abderhalden (7) for leucine, phenylalanine, proline, and serine. The figures in brackets incorporate estimates of the serine and threonine content of silk fibroin obtained by the use of periodic acid (Martin and Synge (158, 159)). Assuming that silk fibroin contains 18.5 per cent of nitrogen, it is seen that the amino acid values employed in the calculation, including the newer values for serine and threonine, account for 90 per cent of

the nitrogen of fibroin. By dividing the total of column 1 by the total of column 3 the average molecular weight of the determined amino acids of fibroin is found to be 90.0 (92.2), leading to an average residue weight of 72 (74.2). This is significantly different from the value 84 used by Bergmann and Niemann, and implies that the glycine residues have a frequency not of 2 but of 2.38 (2.31); the frequency of the other residues must be altered in the same proportion.

Recently, Meyer *et al.* (160) have criticized Bergmann's figure for the tyrosine of silk fibroin, and have proposed a lower figure. They found no alteration in the tyrosine content of the residue from partial dispersion of fibroin in lithium bromide solution. They regard the fact that there is no change in the x-ray photograph given by fibroin when it is coupled with diazobenzenesulfonic acid

TABLE 1

Amino acid composition of silk fibroin

AMINO ACID	(1) WEIGHT	(2) MOLECULAR WEIGHT	(3) GRAM-MOLECULES PER 100 G. OF PROTEIN	(4) GRAMS OF NITROGEN PER 100 G. OF PROTEIN
	per cent			
Glycine	43.8	75	0.5840	8.18
Alanine	26.4	89	0.2966	4.15
Tyrosine	13.2	181	0.0729	1.02
Arginine	0.95	174	0.0055	0.31
Lysine	0.25	146	0.0017	0.05
Histidine	0.07	155	0.0005	0.02
Leucine	2.5	131	0.0191	0.27
Phenylalanine	1.5	165	0.0091	0.13
Proline	1.0	115	0.0087	0.12
Serine	1.8 (17.4)	105	0.0171 (0.1655)	(2.32)
Threonine	(1.0)	119	(0.0084)	(0.12)
Total	91.47 (108.07)		1.0152 (1.1720)	(16.69)

as evidence for the absence of tyrosine from the "crystallites" responsible for the photograph.

In spite, therefore, of the fact that the best-characterized partial hydrolysis products of fibroin, often isolated in quite significant yields, are entirely consistent with Bergmann and Niemann's theory of its structure, it seems that the theory will have to be substantially modified, very probably in the direction of recognizing microheterogeneity in silk fibroin.

B. PROTAMINES AND HISTONES

After silk fibroin, the group of basic proteins (protamines) derived from the ripe sperm of certain fish has probably received the closest investigation by the methods of partial hydrolysis. The complete literature on these substances and the related "histones" up to 1927 was surveyed by Albrecht Kossel (131),

who had himself inspired most of the experimental work. Of the protamines, clupein and salmine have received most attention. Their amino acid composition is very simple, there being present in a complete hydrolysate 2 molecules of arginine to 1 molecule of monoamino acids. No basic amino acids other than arginine are present.

Kossel (133), in 1898, wrote: "Erhitzt man die Protamine mit verdünnter Schwefelsäure zum Sieden, so entstehen zuerst Produkte welche ihnen hinsichtlich ihrer Eigenschaften noch nahestehen und welche man als Peptone der Protamine—'Protone'—zu betrachten hat; aus diesen gehen dann durch weitere Zersetzung die Basen hervor. . . ."

These "protones" were recognized as having sulfates more soluble in water than those of the original protamines, and to be in general less readily precipitable, e.g., by proteins.

About the same time, Kossel and Mathews (133) studied the action of proteolytic enzymes on the protamines, and obtained from sturine, by the action of a tryptic preparation, a basic material giving a crystalline double salt with silver nitrate. Its elementary composition suggested that it might be formed from 1 molecule of histidine and 2 molecules of lysine minus 1 molecule of water, but the amount of material available was insufficient for further investigation.

Goto (101) extended the work on "protone". He prepared a copper salt from the material from clupein, studied the optical rotation, and measured the molecular weight cryoscopically and ebullioscopically. This was found to be 384–443. As pointed out by Dakin and West (56), these results are certainly low, as such results always are for chain molecules except in the most dilute solution. An experimental study of the deviations in molecular-weight determinations on chain molecules of known molecular weight and constitution has been made by Meyer and Lühdemann (161). Goto also prepared double salts of "clupeone" with platinum chloride. Arginine determinations showed no significant differences from those carried out on the original clupein.

Kossel and Dakin (132) obtained a "protone" by the action of intestinal mucosa extract for 18 months on clupein sulfate. This was separated from the ornithine, urea, monoamino acids etc. simultaneously formed by silver–baryta precipitation. The material proved different from Goto's product, having only 69.7 per cent of its nitrogen as arginine nitrogen. Complete acid hydrolysis yielded ornithine, which was identified as its phenylhydantoin. Similar observations on material prepared from clupein by partial alkaline hydrolysis were made by Kossel and Weiss (137) and extended to gelatin (138).

Kossel and Pringle (134) described further work with "clupeone". The material was fractionated by precipitation with excess picric acid in neutral and acid solution, by precipitation with silver–baryta, and as its copper salt. None of the various fractions had an arginine content significantly different from that of the original clupein. The monoamino acid fraction from a complete hydrolysate of one of these preparations of "clupeone" was found to consist of a mixture of proline with other amino acids.

Thus so far, no partial hydrolysis product of clupein had yet been demon-

strated to be significantly different in amino acid composition from the original substance, and Kossel and Pringle inclined to the view that clupein consists of a regular arrangement of arginine (a) and monoamino acid residues (m) along a peptide chain, thus:

—aamaamaamaamaamaamaamaamaam—

Kossel and Weiss (136) obtained a crystalline picrolonate from a partial acid hydrolysate of clupein. This material was converted to the corresponding hydrochloride, and fractionated by precipitation with alcohol, and with phosphotungstic acid. The arginine content of some of the resulting fractions was a little lower than that of the original clupein. The same material (Hirayama (115)) yielded a crystalline naphthalenesulfonyl derivative, containing 3.9 naphthalenesulfonyl residues per nine nitrogen atoms. Clupein, naphthalenesulfonylated under the same conditions, took up only 1.3 residues per nine nitrogen atoms, although benzenesulfonylation introduced 2.4 residues per nine nitrogen atoms.

Nelson-Gerhardt (166) obtained evidence (from molecular-weight determinations, and from increase of formol titration on further hydrolysis with acid) for the occurrence of peptides consisting entirely of monoamino acids in a partial acid hydrolysate of salmine. The protamine was hydrolyzed with 6 per cent sulfuric acid at 140°C. for 2 hr., and the resulting solution was freed from arginine and arginine peptides by the silver–baryta procedure before investigation.

Such peptides could not be expected on the basis of Kossel and Pringle's view of the structure of clupein and salmine.

Gross (107), working with clupein, confirmed Nelson-Gerhardt's results, and also obtained evidence for the occurrence of arginine peptides under the same conditions of hydrolysis. The material obtained from the hydrolysate by silver–baryta precipitation was fractionated by precipitation with phosphotungstic acid in 33 per cent alcohol. In this way amorphous material was obtained containing only about 3 per cent of its nitrogen in the form of amino nitrogen (Van Slyke). Twenty grams of clupein gave 125 mg. of nitrogen in this form (after analytical samples had been removed). In a molecular-weight determination, the material did not raise the boiling point of water. On total hydrolysis, 23.8 per cent of the nitrogen was found to be amino nitrogen and 96 per cent of the nitrogen was precipitable by silver–baryta, and was identified as arginine. Gross suggested that the material was either a peptide consisting entirely of arginine residues (eight or more) or else arginine anhydride. He established that free arginine did not polymerize on autoclaving with dilute sulfuric acid.

All the partial acid hydrolyses of protamines described above were carried out with dilute acid at high temperatures: Kossel and Staudt (135) hydrolyzed clupein with 70 per cent sulfuric acid at 37°C. for several days, and obtained a basic material (precipitated as an oil with flavianic acid in unspecified yield) which gave only arginine on complete hydrolysis, and which, on the basis of amino nitrogen and molecular-weight determinations, they concluded was

arginylarginine contaminated with a little free arginine. It could not be identified with the (unhydrolyzable) "arginylarginine" of Fischer and Suzuki (89), the non-peptide structure of which was later established by Bergmann and Zervas (44) (*cf.* Edlbacher and Bonem (61)). Kossel and Staudt's product was amorphous, and they could prepare no crystalline derivatives from it.

All the work on protamines described above was carried out by Kossel and his school. Since Kossel's death, the most important work of this character on protamines has been that of Felix and collaborators.

Felix and Dirr (67) fractionated clupein methyl ester hydrochloride (prepared without apparent degradation by using methyl alcoholic hydrogen chloride) and obtained preparations of widely differing methoxyl content (0.76–3.20 per cent), which suggested that clupein is a heterogeneous material. Dirr and Felix (59) repeated the work of Kossel and Staudt on the isolation of arginylarginine from clupein. Starting with 50 g. of the (unfractionated) methyl ester hydrochloride, they obtained 5 g. of crystalline arginylarginine dipicrate. The actual quantity of arginylarginine in the hydrolysate was estimated (by the amount of oily flavianate precipitated) as equivalent to 45 per cent of the arginine in the clupein used. The arginylarginine was further converted to its amorphous methyl and ethyl ester hydrochlorides, which appeared to retain excess of alcohol. Unlike Kossel and Staudt's preparation, that of Dirr and Felix gave no biuret reaction.

Felix, Inouye and Dirr (72) investigated the products of the action of trypsin-kinase on clupein B (*cf.* Felix and Dirr (67)). By fractionation with alcohol, methyl alcoholic hydrogen chloride, picric acid, and flavianic acid the following products were obtained: (*1*) A dipeptide of arginine and serine in the form of a crystalline picrate (1 g. of picrate from 30 g. of clupein methyl ester hydrochloride). This had the correct elementary composition and amino nitrogen content, and on hydrolysis yielded equimolecular quantities of arginine and serine. (*2*) A dipeptide of arginine and alanine as its crystalline picrate (1.5 g. of picrate from 30 g. of ester hydrochloride), which was characterized in the same way as the previous compound. (*3*) Three and a half grams of arginylarginine dipicrate (*cf.* Dirr and Felix (59)) from 64 g. of ester hydrochloride. (*4*) A dipeptide of arginine and hydroxyproline as a crystalline flavianate (1 g. of flavianate from 64 g. of ester hydrochloride). This gave the expected quantity of arginine on acid hydrolysis, and hydroxyproline was also isolated. Since the dipeptide had a free amino group, it was assigned the structure arginylhydroxyproline. (*5*) Two grams (from 64 g. of ester hydrochloride) of the crystalline flavianate of a dipeptide of arginine and valine. Valine was formally identified in an acid hydrolysate.

Although the constitution of none of the above products was established by synthesis, the evidence that they are dipeptides of the stated amino acids can be regarded as satisfactory. Considerable losses were obviously inherent in the methods used for their isolation.

Felix, Hirohata, and Dirr (71) studied the hydrolysis of clupein by boiling 2 *N* hydrochloric acid. Study of the liberation of urea by arginase at pH 8

and pH 9.3 after different times of hydrolysis led to no very definite results. This was an attempt (*cf.* Felix, Dirr, and Hoff (68)) to discriminate between free arginine and arginine in peptide linkage having its carboxyl free. From a 16-hr. hydrolysate, arginylarginine was obtained as its dipicrate, and it was found also to form a crystalline dipicrolonate. From an 8-hr. hydrolysate of 50 g. of clupein ester hydrochloride were obtained: (*1*) Two and six-tenths grams of the crystalline picrolonate of what appeared to be a tripeptide containing 2 molecules of arginine and 1 molecule of a monoamino acid having perhaps a five-carbon-atom skeleton. This picrolonate was possibly the same as Kossel and Weiss' (136) product, as it had the same melting point (*cf.* Hirayama (115)). (*2*) Five and eight-tenths grams of the crystalline picrolonate of a substance having 6.7 per cent of its nitrogen in the form of amino nitrogen, and giving a positive biuret reaction. Acid hydrolysis resulted only in arginine. The substance gave a crystalline picrate. It appeared to be arginylarginylarginylarginine. Attempts to prepare its hydrochloride led to rupture of the molecule.

Felix and Mager (74) slightly modified the methyl ester fractionation of clupein, and conducted an amino acid analysis on the clupein C fraction obtained in this way. A small amount of hydroxyproline was found in the monoamino acid fraction, and it was concluded that the molecule contained 2 molecules of serine, 2 molecules of alanine, 4 molecules of (proline + hydroxyproline), 3 molecules of valine, and 22 molecules of arginine. Since no free amino group exists in clupein, although formol-titratable nitrogen was found (*cf.* Sørensen (216) for the formol-titration behavior of proline), it was concluded that the terminal residue of the peptide chain was prolyl or hydroxyprolyl, and the formula

$$\begin{array}{r} \text{m'aaaammaaaammaaaammaaaammaaaammaa} \\ \text{NH}\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\text{COOH} \end{array}$$

where m' = proline or hydroxyproline residue,

m = monoamino acid residue (including proline and hydroxyproline), and

a = arginine residue,

was given as being consistent with all the partial hydrolysis products isolated from clupein as well as with Gross' and Nelson-Gerhardt's demonstrations of the presence of peptides of monoamino acids.

Studies of the enzymic hydrolysis of protamines have also been made by Takemura (223), Rogozinski (193), Felix and Lang (73), and Waldschmidt-Leitz *et al.* (231, 232, 233, 234). None of the partial hydrolysis products obtained in these studies was subjected to complete amino acid analysis. Waldschmidt-Leitz (231) gives a review of the work of his school on the protamines.

The partial hydrolysis of calf thymus histone has also been investigated. On digestion of this material with pepsin hydrochloride Kossel and Pringle (134) obtained a product which they named "histopeptone". This was isolated from the neutralized digest by precipitation with sodium picrate, and could further

be precipitated with silver–baryta. Sixteen and five-tenths grams of histopeptone sulfate were obtained from 80 g. of the air-dry histone. The histopeptone had a higher nitrogen content than the original histone, and gave no ammonia on acid hydrolysis. On total acid hydrolysis, the histidine and lysine contents of the histopeptone were found to be about twice those of the original histone. Only 27 per cent of the nitrogen of the hydrolysate was not precipitable by phosphotungstic acid. The mother liquors from the preparation of the histopeptone yielded a fraction richer in monoamino acids than the original histone.

Felix (64) confirmed Kossel's work on the histopeptone from thymus. Of the nitrogen of the histone 30.1 per cent was obtained as histopeptone, which gave on hydrolysis the same base and monoamino acid figures as those found by Kossel. Attempts at fractionation by silver–baryta precipitation at different pH's suggested that the material was homogeneous.

C. PARTIAL HYDROLYSIS PRODUCTS CONTAINING BASIC AMINO ACIDS DERIVED FROM OTHER PROTEINS: THE "KYRINE" QUESTION

Siegfried (200) reported investigations of the hydrolysis of gelatin (and "gelatin peptone") by 12.5 per cent hydrochloric acid at 38°C. The optical rotation of the hydrolysate was found to be constant after 120 hr. Precipitation with phosphotungstic acid gave 12 g. of basic material from 400 g. of gelatin. In working up the material, considerable losses were encouraged, in order to achieve fractionation. The platinum salt was employed for this purpose. A number of preparations of the sulfate of the base were analyzed, and were in agreement with the formula $(C_{21}H_{39}N_9O_8)_2(H_2SO_4)_5$. The base formed a crystalline phosphotungstate. "Für die das Sulfat bildende Base schlage ich den Namen *Kyrin* vor (*τὸ κῦρος*, der Kern einer Sache im bildlichen Sinne) und zwar für die aus Glutin entstehende Base den Namen *Glutokyrin*." Naphthalenesulfonylation gave a crystalline product, incorporating five naphthalenesulfonyl groups per "molecule" (21 carbon atoms) of base. Acid hydrolysis with hydrochloric acid gave a little ammonia, with sulfuric acid none; two-thirds of the nitrogen was precipitated by phosphotungstic acid after acid hydrolysis. This appeared to consist of equimolecular amounts of arginine and lysine. Histidine was absent. From the phosphotungstic acid filtrate, glutamic acid was isolated, and was thought to account for about one-third of the nitrogen present in this. The rest was thought to be glycine. It was shown that a complex of 1 molecule of arginine + 1 molecule of lysine + 1 molecule of glutamic acid + 2 molecules of glycine − 4 molecules of water agreed with the elementary composition of the material. Siegfried summed up as follows: "Wenn sich entsprechende nicht basische Komplexe auffinden lassen, die ähnliche Widerstandskraft gegen Hydrolyse besitzen, wird man diese neben dem Kyrin zu ordnen haben. Zerfällt aber das Proteinmolekül bei der Reaktion, bei welcher das Kyrin erhalten bleibt, in Kyrin und die einfachen, letzten Spaltungsprodukte, die Amidosäure etc., so wird dem basischen Kern, dem Kyrin, eine Sonderstellung zuzuweisen sein, in diesem Falle wird es *der* Kern sein."

Later Siegfried (201) reported a more detailed investigation of the mono-

amino acid fraction of "glutokyrin". Much less glutamic acid than the "theoretical" was obtained. After removal of this, glycine in unspecified yield was formally identified. At the same time (202) he described the preparation of a similar "kyrine" from casein, and noted that fibrin also yielded a "kyrine". In the case of casein, a rather more complex series of precipitations was found necessary. Free proline was isolated in the course of the preparation. Eventually the "kyrine" was obtained as its sulfate, to which the formula $C_{23}H_{47}N_9O_8 \cdot 3H_2SO_4$ was assigned on the basis of elementary composition. The yield was not specified. The material gave no ammonia on acid hydrolysis, and was free from histidine. From 84 to 85 per cent of the nitrogen of its acid hydrolysate was precipitable by phosphotungstic acid; this was made up of approximately 2 molecules of lysine to 1 molecule of arginine. In the monoamino acid fraction arginine was isolated, though in low yield. The composition 2 molecules of lysine + 1 molecule of arginine + 1 molecule of glutamic acid was tentatively assigned to the "kyrine". The material was optically inactive. Both the material from casein and that from gelatin gave a biuret reaction.

Skraup and Zwerger (214) criticized Siegfried's work, asserting that free lysine, arginine, and histidine were present in the product. They characterized the lysine as its picrate and as its platinum double salt. Since, however, they had used for the hydrolysis concentrated hydrochloric acid for 42 hr. at 100°C., conditions usually reckoned to give complete hydrolysis, little weight can be attached to this criticism. Later Skraup and Witt (212) repeated Siegfried's work on casein, using the same conditions of hydrolysis as Siegfried. It was found that little fractionation resulted from the precipitation of "kyrine sulfate" with alcohol; fractionation with phosphotungstic acid, potassium mercuric iodide, and picric acid led, however, to fractions of differing elementary composition, and evidence was obtained that these fractions had different contents of basic amino acids.

Siegfried (203) replied to the criticisms of Skraup and Zwerger, and gave further details of the properties of the phosphotungstates and picrates of "caseinokyrine". He gave details of the elementary composition of "fibrinokyrine", which became constant after nine reprecipitations of the sulfate. No yields were stated. In a further paper (204) he described the application of the phosphotungstic acid fractionation of Skraup and Witt to "caseinokyrine"; he was unable in this way to alter its elementary composition. By applying his carbamino reaction to "caseinokyrine" and "fibrinokyrine", he found that the intact "kyrines" reacted with smaller proportions of carbon dioxide than did their hydrolysates. This was taken as further evidence of the complex character of the "kyrines".

Levene and Birchard (148) repeated the work of Siegfried with gelatin employing the amino nitrogen determination of Van Slyke. A qualitative examination of the hydrolysis products of the "kyrine" showed that arginine, lysine, and glutamic acid were definitely present, probably also proline and perhaps glycine and alanine. The amino nitrogen content of the "kyrine" before hydrolysis was 32 per cent of the total nitrogen, rising to 65 per cent

afterwards. It was thus impossible that the "kyrine" was a homogeneous pentapeptide. On "purification" with silver–baryta, there was found in the precipitable fraction what appeared on similar evidence to be a dipeptide of arginine and glutamic acid. However, the ratio of amino nitrogen to total nitrogen in the filtrate from this rose from 23 per cent to 36 per cent on further treatment (five times) with silver–baryta, suggesting that the product was not homogeneous.

Levene and van der Scheer (150) also investigated the "kyrine" from casein. After fractionation with silver–baryta, the fractions were further fractionated with phosphotungstic acid in the hot and in the cold. In one of the four resulting fractions there was obtained an unspecified yield of a substance showing a tendency to crystallize. Its amino nitrogen before hydrolysis was 50 per cent of the total nitrogen, rising to 75 per cent after hydrolysis. Lysine was the only basic hydrolysis product; in addition to this there appeared to be an amino acid and an imino acid present. The elementary composition agreed with that of a tripeptide of lysine, valine, and hydroxyproline. A little valine, but no hydroxyproline, was obtained from the hydrolysate. The possibility of other lysine peptides of similar make-up being present was not excluded.

Siegfried (205) applied the silver–baryta precipitation to "glutokyrine" and investigated the fractions by carbamino and formol titrations. Results similar to those of Levene were obtained, and no satisfactory evidence was obtained that the silver–baryta precipitation induces breakage of peptide bonds (*cf.* Todorowic (224)). Siegfried and Schunke (207) investigated the question further, and obtained evidence from the Van Slyke and carbamino figures, though not from the formol titration, suggesting that hydrolysis might be effected by the silver–baryta treatment. Baryta alone did not appear to effect this. The naphthalenesulfonylation of "kyrine" was also studied.

Luck (156), in an investigation of the origin of the ammonia liberated by casein on acid hydrolysis, found that about one-third of this was not liberated by tryptic digestion. Isolative investigations suggested that this trypsin-resistant ammonia precursor in the digest yielded largely glutamic acid, lysine, and ammonia on acid hydrolysis. Naphthalenesulfonylation before hydrolysis led to the isolation of what was probably dinaphthalenesulfonyllysine, and the product (40 g. from 1200 g. of casein) may have been very impure lysylglutamine. Such a compound might clearly, in its deamidated condition, be a constituent of Siegfried's "caseinokyrine".

Haurowitz (110) studied the partial hydrolysis of globin by acid. A preparation was made in the same way as Kirbach's "kyrine" (128), and was shown by silver–baryta precipitation not to be homogeneous. The course of hydrolysis of globin by 70 per cent sulfuric acid at 40°C. was studied by formol titration. By precipitation with mercuric sulfate from a 3-day hydrolysate, followed by reprecipitation with silver–baryta, followed by alcohol precipitation, 6.76 g. of material was obtained from 165 g. of horse hemoglobin. Free amino acids and diketopiperazines were shown to be absent from this preparation, and it was further tested for homogeneity by electrodialysis and by fractional precipitation

with silver–baryta. Hydrolysis gave 69 per cent of the total nitrogen as histidine, and 31 per cent as monoamino acid nitrogen (of which proline was 17.8 per cent and tyrosine 0.3 per cent). Amino nitrogen (Van Slyke) was 18 per cent of total nitrogen before hydrolysis, and 51 per cent after hydrolysis. Proline was demonstrated in the hydrolysate through its hydantoin. An ester distillation of the monoamino acid fraction suggested that mainly leucine was also present. The material was not split by the enzymes with which it was tested.

Haurowitz's work confirms an earlier report by Abderhalden (1) of the isolation of material rich in histidine and leucine from a partial acid hydrolysate of hemoglobin by precipitation with mercury. Abderhalden gave no experimental details.

Grassmann and Lang (105) reinvestigated "glutokyrine". They confirmed most of the earlier work, although they could not detect glutamic acid among its hydrolysis products. A little glycine was isolated as its ester hydrochloride. Determinations of amino nitrogen and esterification experiments suggested that the material is a mixture of di- and tri-peptides of basic amino acids. Hydrolysis after deamination with nitrous acid gave no lysine, but the yield of arginine was not seriously diminished, suggesting that at least one amino group of the lysine was free, and that most of the arginine amino groups were involved in peptide linkage. Enzyme experiments were also carried out. It was concluded ". . . dass im Glutokyrin ein Gemisch niedriger basischer Peptide vorliegt, die in bezug auf das gegenseitige Verhältnis der Basen, der Monoaminosäuren und der Iminosäuren und auch hinsichtlich ihres allgemeinen Aufbauplanes weitgehend untereinander übereinstimmen, während vermutlich die einzelnen Monoaminosäuren und Iminosäuren sich scheinbar unregelmässig innerhalb der einzelnen Peptide vertreten können."

Grassmann and Riederle (106) emphasized the importance of *yield* in peptide isolations. By applying the silver–baryta precipitation to 30 g. of "glutokyrine sulfate" they obtained: (*1*) in the precipitate, 4.2 g. of material giving on acid hydrolysis arginine as the only base; (*2*) in the filtrate, 6 g. of material giving only lysine.

Fraction 1 appeared to be a dipeptide of arginine with a monoamino acid (not glycine or glutamic acid). Determinations of amino nitrogen showed little proline and hydroxyproline could be present.

From base content, and from determinations of amino nitrogen on the original material and its basic and monoamino fractions after hydrolysis, it was concluded that fraction 2 was a tripeptide of lysine, glycine, and proline. Since it had two free amino groups, a terminal prolyl residue was excluded. A glycerol extract of kidney split one peptide bond, liberating a carboxyl but no amino group; therefore this bond involved the proline imino group. Free lysine was simultaneously produced, and the resulting dipeptide, prolylglycine, yielded one amino group on acid hydrolysis. From 125 g. of gelatin there was obtained 6 g. of this tripeptide, which thus represents a very appreciable fraction of the total

lysine of gelatin and, as well as being entirely consistent with the isolation of glycine proline anhydride from partial hydrolysates of gelatin referred to in the next section, justifies the drawing of conclusions as to the structure of gelatin.

D. NON-BASIC PARTIAL HYDROLYSIS PRODUCTS OF OTHER PROTEINS

1. Keratins

Abderhalden and Suzuki (36) investigated the products of treating goose feathers with 70 per cent sulfuric acid for 5 days at room temperature. After exact removal of the sulfuric acid with baryta, the aqueous solution was evaporated to dryness and exhaustively extracted with boiling methyl alcohol. This extract gave products in unspecified yield which were insoluble in water and in other organic solvents. Apart from the biuret reaction they did not give the usual protein color reactions, and gave on complete hydrolysis with acid only glycine and *l*-proline. Two grams of one fraction of this material on hydrolysis gave 0.39 g. of glycine and 1.75 g. of proline. The whole preparation was repeated twice with similar results. An attempt to allocate a terminal amino or imino group by naphthalenesulfonylation was unsuccessful.

Abderhalden (9) later isolated a crystalline product from the same fraction. In addition to glycine and proline it gave hydroxyproline and was considered to be an anhydride formed from 2 molecules of proline, 1 molecule of hydroxyproline, and 1 molecule of glycine by elimination of 4 molecules of water.

Abderhalden and Komm (20) investigated the products obtained by the partial acid hydrolysis of pig bristles. No yields of the products isolated were stated, and, although the stated amino acids were identified after complete hydrolysis in each case, no experimental details of the identifications were recorded. Dilute acid hydrolysis in the autoclave yielded: (*1*) glycine *l*-alanine anhydride; (*2*) a diketopiperazine consisting of leucine and proline, which had a considerably lower rotation than the synthetic product of Fischer and Reif (86); (*3*) *l*-proline *l*-valine anhydride; (*4*) a complex product containing leucine, glutamic acid, glycine, and proline; (*5*) a product consisting of proline, leucine, and alanine, which had no free amino nitrogen, but which gave one-third of its nitrogen as amino nitrogen after standing for a short while in *N* sodium hydroxide. The authors speculate at length as to the possible structure of this compound.

An idea of the yields of the above substances is given by the remark: "Immerhin hatten wir in jedem Falle Mühe für die einzelnen Bestimmungen und die totale Hydrolyse genügend Material zusammenzubekommen." The starting material was 400 g. of bristles. Hydrolysis of the bristles with 70 per cent sulfuric acid at room temperature and at 37°C. led to different products: (*1*) a crystalline optically active compound having one-half of its nitrogen as amino nitrogen and yielding leucine and serine on hydrolysis; (*2*) an amorphous fraction having one-third of its nitrogen as amino nitrogen and yielding cystine, leucine, valine, glycine, and proline on hydrolysis; (*3*) a crystalline substance giving only valine on acid hydrolysis; (*4*) further amorphous material containing cystine, leucine, valine, and glycine.

Abderhalden and Komm (21) by dilute acid hydrolysis of pig bristles obtained in unstated yield equally inadequately characterized products as follows: (*1*) a crystalline compound yielding on hydrolysis 2 molecules of hydroxyproline and 1 molecule of glycine; (*2*) a crystalline compound thought to be leucine isoleucine anhydride; (*3*) a compound having the elementary composition of glycine alanine anhydride; its melting point was lower than that of authentic glycine *l*-alanine anhydride. "Es war nicht genügend Substanz zur weiteren Untersuchung vorhanden."

The same authors (26) obtained: (*1*) from dog hair subjected to dilute acid hydrolysis in the autoclave an unspecified yield of a crystalline product corresponding in elementary composition to alanine glycine anhydride: (*2*) from pig bristles treated in the same way: (*a*) alanine leucine anhydride (0.1 per cent yield of crude product). This had a much lower rotation than that of the synthetic material (*cf.* Fischer and Abderhalden (83)); (*b*) an unspecified yield of a further product giving alanine and leucine on acid hydrolysis, and having a lower melting point; (*c*) an unspecified yield of a crystalline product giving alanine and phenylalanine on hydrolysis. The oxidation of "keratinpepton" from sheep's wool by zinc permanganate was also studied (*cf.* the section on silk fibroin above).

2. Gelatin

The only adequately characterized non-basic partial hydrolysis product of gelatin that has been described is the anhydride of glycine and proline. This was obtained by Levene and coworkers from tryptic digests. Its subsequent isolation in good yield from acid hydrolysates (Gawrilow and Lawrowsky (96); Konuma (129)) suggests that direct linkage of glycine to proline already exists in the gelatin, and is not an artifact due to enzyme action. Grassmann and Riederle's (106) isolation of lysylprolylglycine from gelatin serves as a confirmation of this.

Levene (142) described the preparation of a crystalline product from a tryptic digest of gelatin, and gave the melting point and elementary composition of the compound and its picrate. He concluded: "Hence it is probable that the composition of the substance is $C_7H_{10}N_2O_2$, and that it is in some way related to proline." Levene and Wallace (151) gave experimental details of the preparation (for which precipitation with phosphotungstic acid was used) but reached no further conclusion as to the nature of the compound. Levene and Beatty (145, 146) investigated a preparation which had a melting point of 182–183°C., and recognized glycine and proline as its hydrolysis products. One gram gave 0.370 g. of glycine and 0.400 g. of proline. They therefore suggested that the product was glycine proline anhydride.

Fischer and Reif (86) synthesized glycine *l*-proline anhydride. Its specific rotation, $[\alpha]_D$ (water), was 216–217°, and its melting point was 203–213°C. "Im übrigen ist die Aehnlichkeit, soweit sich nach den kurzen Angaben von Levene und Beatty beurtheilen lässt, recht gross."

Levene (143) returned to the question and gave more adequate experimental

details. On tryptic digestion of gelatin for 8 months, he obtained 5–6 g. of the product from 1 kg. of gelatin. The new preparation had a melting point of 178–180°C. and a specific rotation, $[\alpha]_D$ (water), of $-55°$. Extraction of this product with alcohol–ether yielded material of lower melting point (168–170°C.) and rotation ($-5.7°$). By using a shorter period of digestion (24 days) a product with higher melting point (212°C.) and rotation ($-169°$) was obtained. Levene concluded that the prolonged action of the alkaline digestion medium induced racemization.

Gawrilow and Lawrowsky (96) subjected gelatin to hydrolysis by dilute sulfuric acid at 180°C. The neutralized hydrolysate was extracted with ethyl acetate. In the extract (90 g. from 500 g. of gelatin) was obtained 50 g. of crystalline material, and recrystallization of this from isoamyl alcohol–ether gave a product having a melting point of 188°C. and $[\alpha]_D$ (water) of $-9.2°$. Acid hydrolysis of this yielded proline and glycine, and the material was considered to be racemized glycine proline anhydride, referred to at length above. A small quantity of crystalline material having a melting point of 195–199°C. was obtained from the mother liquors. This was thought to be glycine leucine anhydride, though its melting point was lower than that of the authentic material (*cf.* Fischer and Abderhalden (82)) and the products of its hydrolysis were not satisfactorily identified (*cf.* also Ssadikow (219)).

Konuma (129) made a study of the kinetics of partial hydrolysis of gelatin, obtaining results similar to those of Uchino (225) with silk fibroin (*vide supra*). By heating gelatin with 0.15 *N* hydrochloric acid at 170°C., Konuma isolated a 1.43 per cent yield of glycine proline anhydride having a melting point of 207°C. and a specific rotation, $[\alpha]_D$ (water), of $-185.4°$. A similar yield of the same compound (racemized) was obtained from a partial hydrolysate of gelatin by alkali.

Fodor *et al.* (90, 91, 92, 94) have subjected gelatin and its enzymic hydrolysis products to the action of boiling acetic anhydride and of glycerol at high temperatures. They claim to have isolated compounds of proline and hydroxyproline with glycine and alanine, and have studied the enzymic digestion of these products. Their views as to the structure of the products in question are supported mainly by elementary analyses and by molecular-weight determinations, to which latter, as pointed out above, little significance can be attached. No adequate characterization of the amino acids resulting from hydrolysis of these products was carried out. More interest would attach to the assertion that they contain only the monoamino acids stated if it had been demonstrated that basic amino acids etc. were absent from them.

3. Elastin

Fischer and Abderhalden (82) hydrolyzed elastin with 70 per cent sulfuric acid at room temperature and at 37°C., and obtained by the esterification procedure a yield of more than 5 per cent of crude glycine *l*-leucine anhydride. This was purified by recrystallization and formally identified with the synthetic product.

The same authors (83) reported further work on a partial hydrolysate of elastin prepared in the same way. After a precipitation with phosphotungstic acid, there was found in the filtrate from this a 0.34 per cent yield of *l*-alanyl-*l*-leucine, from which alanine and leucine were identified after hydrolysis. The material was identified with synthetic *l*-alanyl-*l*-leucine.

A hydrolysate of elastin made with concentrated hydrochloric acid at 36°C. was also investigated by the esterification method, and in addition to the previously isolated glycine *l*-leucine anhydride, a small yield of partly racemized *l*-alanine *l*-leucine anhydride was obtained. This was formally identified with synthetic material, and was shown to yield alanine and leucine on hydrolysis. From the amorphous anhydride material in the mother liquors there was obtained: (*1*) An amorphous preparation giving alanine and proline on acid hydrolysis, which was thought to be alanine proline anhydride. *dl*-Alanine *dl*-proline anhydride had already been synthesized (Fischer and Suzuki (88)), and had proved to be crystalline. (*2*) An amorphous preparation which could be crystallized only by sublimation. This appeared to be glycine valine anhydride, glycine being definitely, and valine probably, identified as its hydrolysis products. Synthetic glycine *l*-valine anhydride was shown to exhibit the same peculiar crystallization phenomena (*cf.* Fischer and Scheibler (87)).

Abderhalden (1) reported the isolation of *l*-leucyl-*l*-alanine in unspecified yield from the mother liquors of the *l*-alanyl-*l*-leucine obtained above. It was formally identified with the synthetic product, and alanine and leucine were recognized as its hydrolysis products.

Abderhalden (2) hydrolyzed elastin with saturated baryta solution at 90°C. for 10 hr. After removal of baryta, the solution was treated with phosphotungstic acid, and the filtrate from this yielded optically inactive crystalline material (2.2 per cent yield). This gave glycine and leucine on acid hydrolysis, and a study of its asymmetric hydrolysis by yeast press-juice suggested that *dl*-leucylglycine rather than glycyl-*dl*-leucine was present. The possible occurrence of glycylleucine in the mother liquors was suggested.

4. Plant proteins

Fischer and Abderhalden (83) hydrolyzed gliadin with 70 per cent sulfuric acid at room temperature and at 37°C. The hydrolysate, after precipitation with phosphotungstic acid, yielded from the filtrate 0.9 per cent of *l*-leucyl-*l*-glutamic acid (through its silver salt). The product was identified with synthetic material, and gave leucine and glutamic acid on hydrolysis.

Osborne and Clapp (169) refluxed gliadin with 20 per cent sulfuric acid for 13 hr. After exact removal of the sulfuric acid with baryta, the hydrolysate was concentrated and allowed to crystallize. The first crystals (0.4 per cent yield) agreed in elementary composition with the monohydrate of a dipeptide of proline and phenylalanine, formed a copper salt (which was characterized crystallographically), and on hydrolysis yielded proline and phenylalanine. Fischer and Luniak (85) synthesized *l*-prolyl-*l*-phenylalanine, and showed that it was identical with the product of Osborne and Clapp.

Abderhalden (8) digested 500 g. of gliadin with pancreatin for 14 days. Extraction of the neutralized and dried residue with ethyl acetate gave crystals. "Ihre Menge reichte zu einer genauen Identifizierung nicht aus." The material, on the basis of melting point, specific rotation, and nitrogen content, was considered to be *l*-leucine *l*-proline anhydride (*cf.* Fischer and Reif (86)). In the mother liquors from this he found glycine *l*-proline anhydride contaminated with a compound of proline with an aromatic amino acid, perhaps phenylalanine. It was finally purified by crystallization from acetone, and had a melting point of 209°C. and specific rotation, $[\alpha]_D$ (water), of $-206.5°$ (*cf.* section on gelatin above), as well as the correct elementary composition. The yield was 0.6 per cent.

Abderhalden (1) hydrolyzed cottonseed globulin with 70 per cent sulfuric acid for 5 days at 20°C. By precipitation with phosphotungstic acid, followed by precipitation with mercuric sulfate, followed by fractional reprecipitation with phosphotungstic acid, he isolated from 1 kg. of protein 16.5 g. of a preparation giving tryptophan and glutamic acid on hydrolysis and 22.5 g. of a product giving tryptophan, leucine, and glutamic acid. Both preparations were amorphous. Molecular-weight determinations suggested that they were di- and tri-peptides, respectively. In the filtrate from the mercury precipitation, there was obtained by fractionation first with phosphotungstic acid and then with alcohol, an unspecified yield of an amorphous product which gave on hydrolysis roughly equimolecular amounts of glycine, leucine, and tyrosine. Molecular-weight determinations suggested that it was a tripeptide.

5. Casein and the phosphoproteins

Fischer and Abderhalden (79), in an attempt to isolate proline from enzymic protein digests, noted that in a tryptic digest of casein no free proline was demonstrable, but that the whole of the proline and phenylalanine were present in a combination of polypeptide type precipitable by phosphotungstic acid, and could be liberated from this material by acid hydrolysis together with alanine, leucine, glutamic acid, and aspartic acid. The same phenomenon was noted with edestin, hemoglobin, serum globulin, egg albumin, and fibrin. In edestin and serum globulin the glycine behaved in the same way. No attempt was made to fractionate or characterize the polypeptide material.

Hunter (119) extracted a tryptic digest of casein with butyl alcohol after various digestion periods, and obtained a fraction soluble in butyl alcohol which appeared to consist largely of proline peptides and diketopiperazines. Definite evidence was obtained of condensation of amino acids (loss of amino nitrogen) during the heating with butyl alcohol, but this peptide fraction could not all have had its origin in this secondary manner.

Muldrew (mentioned by Hunter (120)) fractionated the peptic digestion products of casein and showed that the "primary proteose" fraction was lacking in tyrosine and tryptophan. Hunter commented: "It is an obvious advantage in dealing with a highly complex combination of amino acids to be able to localise even two of them in one particular fragment of the whole."

Jones and Gersdorff (125) fractionated casein by digestion for 1 hr. with pepsin hydrochloride. There were obtained the following fractions:

Fraction A (precipitated during digestion)	21.9 per cent of casein
Fraction B (precipitated on bringing digest to pH 6)	12.7 per cent of casein
Fraction C (not precipitated)	65.4 per cent of casein

Fractions A and B contained nearly all the phosphorus of the casein, while all the cystine that could be demonstrated was in fraction C. Significant differences in the amino acid contents of the three fractions were established, especially for tryptophan and lysine.

In an earlier paper the same authors (124) showed by the Sullivan method that free cystine is completely liberated at an early stage in the hydrolysis of casein by boiling acid, whereas no free cystine is formed from casein by the action of pepsin hydrochloride.

Rimington (187) analyzed the product obtained from casein by precipitation with acetic acid after dephosphorylation of the protein with dilute sodium hydroxide. He found little difference in its amino acid content from that of the original casein, if the loss of amide nitrogen was allowed for. Plimmer and Lawton (177) compared the amino acid content of this "depocasein" (representing 55 per cent of the original casein) with that of the original casein and, like Rimington, found little difference. Later, Macara and Plimmer (157) compared the "depocasein" with the "primary proteose" ("depocaseose") obtained from its mother liquor, and found no very considerable differences in the distribution of a large number of the constituent amino acids except in the case of tyrosine.

Abderhalden (8) obtained 0.6 per cent of leucine valine anhydride from a digest of casein prepared by the action of 70 per cent sulfuric acid at 18°C. This was accompanied by a trace of alanine phenylalanine anhydride. Later Abderhalden (10) obtained 0.25 per cent of the same leucine valine anhydride from a hydrolysate of casein prepared with hot dilute sulfuric acid. By extraction with methyl alcohol there was also obtained from the same hydrolysate an unspecified yield of a dipeptide which was identified as partly racemized *l*-alanyl-*l*-leucine. In the mother liquors from this was obtained 0.35 per cent of a crystalline material which gave *dl*-proline, *l*-leucine, and *l*-alanine on acid hydrolysis; this was thought to be some sort of tripeptide anhydride.

Abderhalden and Sickel (30) reported the isolation from a tryptic digest of casein[2] of 0.28 per cent of a crystalline material which gave tyrosine and proline on acid hydrolysis but which showed deviations from the expected behavior of a dipeptide. The authors recorded extensive observations on the chemical behavior of this compound, and in a subsequent paper (31) described the syn-

[2] It is of interest for the history of the discovery of the amino acids to note that Abderhalden (6) obtained from a tryptic digest of casein, by precipitation with mercuric sulfate, a sulfur-rich crystalline material which looked like leucine but which was more soluble in water. On the basis of present-day knowledge it can be deduced with certainty that this was an impure specimen of methionine.

thesis of a racemic tyrosylproline that showed many properties in common with the natural product, although certain differences were noted. ". . . kann man doch nicht mit Sicherheit annehmen, dass einzig und allein optische Isomerien die oben genannten Verschiedenheiten im Verhalten bedingen."

Abderhalden and Kröner (27) attempted the separation of peptides by distillation of the esters of their benzoyl derivatives. Although benzoyl-*dl*-leucylglycine ethyl ester distilled unchanged, derivatives of other peptides decomposed on distillation, as did the benzoylated and esterified product of a digest of casein (prepared with sodium hydroxide solution acting at 37°C. until 40 per cent of the nitrogen present was amino nitrogen). However, extraction of the same benzoylated and esterified material with butyl alcohol, followed by a series of fractional precipitations with organic solvents, led to a 1.1 per cent yield of an apparently homogeneous product which on acid hydrolysis gave benzoylleucine and glutamic acid and was thought to be benzoylleucylglutamic acid dimethyl ester.

Dakin (52) described the use of his butyl alcohol extraction method for the isolation of amino acids, and applied it to an acid hydrolysate of casein. In the extract were found 2 to 6 per cent of amino acid anhydrides, of which a considerable proportion (1 per cent on the weight of casein used) was identified as *l*-isoleucine *l*-valine anhydride. The probable secondary origin of these compounds from free amino acids has been referred to above.

The earlier literature on the phosphorus-containing group of casein has been reviewed by Rimington and Kay (191) with special reference to the action of enzymes and to the discovery that a phosphorus-rich material—"paranuclein"—is precipitated during the early stages of the peptic digestion of casein. Of these earlier authors Salkowski (194) noticed that his "paranucleinsäure", obtained by iron precipitation, did not give the Adamkiewicz reaction. Reh (186) isolated a "polypeptidphosphorsäure" by precipitation with uranyl acetate from a peptic digest of casein. After acid hydrolysis it was subjected to amino acid analysis. In addition to large quantities of ammonia (in part no doubt originating from decomposition of serine; *cf.* Damodaran and Ramachandran (57)) it gave large quantities of glutamic acid and lysine, and then, in order of diminishing magnitude, smaller quantities of isoleucine, valine, leucine, aspartic acid, proline, histidine, arginine, phenylalanine, alanine, and traces of tyrosine. A product differing markedly in composition from the original casein had thus been isolated. It is of special interest as forming a possible link in the relation of Luck's lysine-glutamine compound mentioned in section C (above) and the phosphoserine-glutamic acid-isoleucine compound of Damodaran and Ramachandran referred to below.

Rimington and Kay also mention the isolation of a phosphorus-rich diffusible material from a tryptic digest of caseinogen. Later Rimington (188) described the preparation of this substance by successive precipitations with lead, uranium, and copper. The final product had constant composition, and contained about 50 per cent of the organic phosphorus of the tryptic digest. One-ninth

of the total nitrogen was amino nitrogen. The free "phosphopeptone" and its barium and brucine salts were prepared. Rimington (189) next investigated its amino acid composition. Calcium salt precipitation from the hydrolysate with alcohol did not lead to the isolation of glutamic acid, although it was claimed that hydroxyglutamic acid was then isolated through its silver salt. From the material not precipitated by alcohol, it was claimed that hydroxyaminobutyric acid and serine had been obtained. A constitution of the "phosphopeptone" as an enneapeptide triphosphoric acid was suggested.

Meanwhile the Posternaks were pursuing parallel researches. S. Posternak (178; *cf.* 179 and 215) mentioned the isolation of phosphorus-rich "lactotyrines" from tryptic digests of casein, and claimed that "lactotyrine α" had the composition 3 molecules of glutamic acid, 1 molecule of aspartic acid, 4 molecules of isoleucine, 7 molecules of serine, and 4 molecules of phosphoric acid.

S. Posternak and T. Posternak (181) described the isolation of "ovotyrines" from egg-yolk by peptic followed by tryptic digestion (*cf.* Levene and Alsberg (144)). They noted (182) in a hydrolysate of "ovotyrine" the presence of pyruvic acid, phosphoric acid, ammonia, arginine, histidine, and lysine. The serine isolated was optically active. They concluded that the phosphorus of ovovitellin existed as serine phosphoric ester.

S. Posternak (180) stated that it was impossible by tryptic digestion to get the nitrogen/organic phosphorus atomic ratio below 3.5 in the product derived from casein; his new preparation gave the same amino acids as he had obtained previously, and he suggested that Rimington's silver salt of hydroxyglutamic acid might be the silver compound of basic amino acids, and that his hydroxyaminobutyric acid was a mixture of serine with isoleucine.

S. Posternak and T. Posternak (183) noted that the "tyrines" on boiling with acid yield far more ammonia than does free serine under the same conditions. They noted also that ammonia evolution occurred on treatment with alkali, and suggested that peptides of phosphoserine were dephosphorylated to some extent by dehydration, giving peptides of α-aminoacrylic acid as intermediates.

Lipmann and Levene (154) studied the hydrolysis of vitellinic acid. They found that the same proportion of ammonia was formed on acid hydrolysis, whether or not the material had first been dephosphorylated by treatment with alkali. By partial hydrolysis with 2 *N* hydrochloric acid for 10 hr., optically active serine phosphoric acid was isolated as its barium salt. Thirty-eight per cent of the organic phosphorus of the vitellinic acid separated as inorganic phosphate during the hydrolysis; of the rest, 60 per cent was isolated in the form of serine phosphoric ester.

Lipmann (153) isolated serine phosphoric ester directly from an acid hydrolysate of casein, as well as from casein "phosphopeptone." Thirty per cent of the phosphorus of the casein was obtained in this form.

Levene and Hill (149) described the isolation from a tryptic digest of casein of an unspecified yield of a dipeptide phosphoric acid yielding serine and glutamic acid on acid hydrolysis. The order of the amino acids was not determined.

Schmidt (197) supplemented his earlier communication (196) with an account of the isolation of an unspecified yield of phosphorus-rich material from a tryptic casein digest by lead acetate precipitation followed by precipitation of the barium salt by alcohol. The material had the atomic ratio nitrogen/phosphorus = 2, and in its hydrolysate only glutamic acid and serine could be demonstrated in addition to phosphoric acid. Barium salts with a higher nitrogen/phosphorus ratio gave in addition "ein Leucin" on hydrolysis.

Sorimati (217) studied the liberation of amino nitrogen and inorganic phosphorus from casein and its phosphopeptone under various conditions of hydrolysis, and described the preparation of 7 g. of the barium salt of the glutamic acid serine phosphoric ester dipeptide by direct acid hydrolysis of 200 g. of casein. This compound had the correct elementary composition, and one-half of its nitrogen was amino nitrogen. On acid hydrolysis it yielded serine phosphoric ester; glutamic acid was not isolated. The action of phosphatases on these various products was studied.

Herd (111) made kinetic studies of the enzymic and alkaline hydrolysis of "paranuclein" from casein; she also (112) studied similar digestion products of other phosphoproteins (as well as proteins that had been artificially phosphorylated). (*Cf.* Herd (113)).

Rapoport (185) claimed that the phosphorus/serine molecular ratio in casein was 4:3, and that other residues than serine must be involved in the linkage of organic phosphorus. He determined serine colorimetrically in the hydrolysate after deamination to glyceric acid. A 1:1 ratio of phosphorus to serine was claimed for vitellinic acid.

Similar studies of the tryptic digestion of casein were made by Grabar (102), who subsequently (103) studied a phosphorus-rich fraction obtained in the early stages of tryptic digestion. He noted that it gave a negative reaction for tryptophan and a very faint Millon reaction, and prepared its insoluble copper derivative.

Damodaran and Ramachandran (57) digested the "paranuclein" of casein with trypsin. The digest was precipitated with lead, and the precipitated material was redigested with trypsin and precipitated as its barium salt with alcohol. This led to a "phosphopeptone" of constant composition, having one-tenth of its nitrogen in the form of amino nitrogen. Amino acid analysis of this decapeptide showed that it was composed of 4 molecules of serine, 3 molecules of glutamic acid, and 3 molecules of isoleucine, and contained three phosphoric acid residues, presumably bound to the serine residues in ester linkage. Considerable decomposition of the serine during acid hydrolysis was noted, the main products being glyceric acid and ammonia. No other amino acid could be isolated from the material. After dephosphorylation by alkali, the material could be hydrolyzed by trypsin, so it is presumably to the presence of the phosphoric acid residues that it owes its trypsin-resistance.

Products of similar qualitative amino acid composition have been isolated from casein by Lowndes *et al.* (155) and by Rimington (190), who subjected his original material to a reinvestigation.

IV. Conclusions

No general picture of protein structure can be said to emerge from the studies reviewed above. The most complete structural investigation is undoubtedly that of clupein, and even for this substance Felix's proposed formula, although entirely consistent with the manifold experimental data that have accumulated, is by no means rigidly established. Any attempt to construct a formula for silk fibroin is made difficult by the questionable homogeneity of this substance. Except in the protamines, tussore silk, and vitellinic acid, few cases of direct linkage of two amino acid residues of the same species have been established; direct association of different amino acids has been proved in a number of cases, of which the following seem to be the outstanding examples: (*1*) glycine–alanine and glycine–tyrosine in silk fibroin; (*2*) glycine–leucine and alanine–leucine in elastin; (*3*) proline–phenylalanine and leucine–glutamic acid in gliadin; (*4*) lysine–proline–glycine in gelatin; and (*5*) valine–leucine, isoleucine–glutamic acid–serine (phosphoric ester) in casein.

The isolation of such groupings in significant yield does suggest some regularity of structure such as that put forward by Bergmann and Niemann, rather than statistically random occurrence of amino acid residues along a peptide chain. At the present stage there is little justification for further speculation.

It is clear that future progress in this field is bound up with the improvement of technique. The techniques employed for the separations described in this review have proved themselves over and over again to be inadequate. It is time that this was generally recognized, and in concluding the present review I shall deal briefly with the technical aspects of peptide separation and point out some of the probable lines of advance.

For hydrolysis, concentrated acids acting at low temperatures seem to be at present the most desirable reagents. The disadvantages of the use of alkali are well known. Hydrolysis at high temperatures favors diketopiperazine formation and probably other changes. The isolation of a diketopiperazine yields no information, such as is given by a dipeptide, as to the order of the component residues in the peptide chain.

The work of Levene and Abderhalden and their respective collaborators has made it clear that different peptide bonds show very different stabilities towards acid and alkaline hydrolysis. One may tentatively picture that the course of hydrolysis of a protein involves first the preferential splitting of certain bonds; immediately this has occurred the effect of the liberated $-NH_3^+$ and $-COO^-$ will come into action: the bond adjacent to a free α-amino group is likely to acquire increased resistance to hydrolysis by acids (*cf.* Moggridge and Neuberger (163) and Neuberger and Pitt Rivers (167) on the acid hydrolysis of the *N*-acetylmethylglucosaminides). Converse effects may be expected with alkali, and it is possible that charged groups in the side chains of amino acid residues may play a similar rôle to those of the main chain in determining hydrolysis. On this view it would be expected that dipeptides would have a special resistance to hydrolysis, and there appears to be some experimental evidence in favor of

this. It is clear that the passage from the intact protein to its ultimate hydrolysis products may take a number of different paths. Very little is known as to the extent to which the path taken may be altered by varying the conditions of hydrolysis. Nevertheless, hydrolysis by acid or alkali, however carefully controlled, does not seem likely to show the same selectivity in the splitting of peptide bonds as enzymes are known to possess, and it is to be hoped that the more accurate determination of the specificity of proteolytic enzymes in hydrolysis and synthesis will soon permit their unequivocal use in the interpretation of protein structure.

Non-isolative studies of partial hydrolysates and partial hydrolysis products have proved of value. The Van Slyke determination of amino nitrogen (in spite of its anomalous results with such compounds as glycylglycine (Van Slyke (226)) and glutathione (*cf.* Hopkins (117)) has certainly not been used to full effect in the studies reviewed above. The ninhydrin–carbon dioxide reaction of Van Slyke and Dillon (227) (*cf.* Christensen, West, and Dimick (49)) promises to prove of value in detecting free amino acids; there is also available the Sullivan reaction in the special case of cystine. It seems probable also that hydroxyamino acids such as serine and threonine may be estimated by their reaction with periodic acid (*cf.* Nicolet and Shinn (168) and Martin and Synge (159)) when both the OH and NH_2 groups of the residue are free.

Where the methods employed in the past for the separation of recognizable partial hydrolysis products of proteins have not been purely empirical (e.g., direct crystallization, precipitation with organic solvents), they have usually depended on a crude analogy between the behavior of the free amino acids and of peptides containing these residues (e.g., precipitation with phosphotungstic, picric, picrolonic, or flavianic acid or with the salts of metals such as mercury, copper, iron, uranium, barium, silver, lead, etc.). In this connection it is odd that cuprous oxide, which is a selective precipitant for compounds containing a potential thiol group (Hopkins (117); *cf.* Pirie (175)), has not so far been employed in this field.

It is likely that in the future solid-phase separations, which are by their nature slow and unreliable (especially when the compounds concerned do not readily crystallize), will give way to methods based on diffusion, adsorption (*cf.* Felix and Lang (73), who tested the use of permutit in the separation of the peptic hydrolysis products of gelatin), electrophoresis (*cf.* Albanese (37)), liquid extraction, distillation, etc. To such methods, whether based on kinetic or equilibrium properties, counter-current technique can be applied, and the most delicate separations effected. Liquid extraction has been used (e.g., by Ssadikow) for isolating diketopiperazines from partial protein hydrolysates, and Hunter (119) employed Dakin's butyl extraction method on a partial hydrolysate of casein. Chemical substitution of the partial hydrolysis products, and the application of counter-current methods (*cf.* Martin and Synge (158)) may extend the applicability of liquid–liquid extraction. Abderhalden and Kröner (27) were unsuccessful in distilling the esters of benzoylpeptides, but Gurin (108) had more success with the ethyl and butyl esters of *N*-benzenesulfonyl peptides,

and he gives references to the previous literature on volatile derivatives of amino acids. Molecular distillation will prove a more satisfactory purification technique when counter-current principles have been applied to it. Methods depending on specific chemical reactions, such as Fischer and Abderhalden's condensation of dipeptide ethyl esters to diketopiperazines, are likely also to find employment.

When isolation, structural characterization, and synthesis of a partial hydrolysis product have been carried through, the 'isotope dilution' method of Rittenberg and Foster (192) is likely, as already pointed out, to prove of great value in its quantitative determination in the partial hydrolysate.

A number of methods have been used for the elucidation of the order of the amino acid residues in simple peptides. Fischer employed naphthalenesulfonylation followed by acid hydrolysis, which yields the amino acid having a free NH_2 group in the form of its acid-resistant naphthalenesulfonyl derivative. Poor yields are often encountered in the naphthalenesulfonylation. Benzoylation has been employed in the same way, although Abderhalden and Bahn have shown that some benzamino acids, e.g., benzoylserine, are fairly readily hydrolyzed by acid. Methylation and condensation with dinitrophenyl chloride have also been employed for the allocation of free amino groups.

Schlack and Kumpf (195) worked out a stepwise degradation procedure in which the amino acid residue bearing the carboxyl group of the peptide is removed as its thiohydantoin by successive treatment with ammonium isothiocyanate in acetic anhydride and with alkali.

Bergmann, Kann, and Miekeley (40) allocated the free amino group by condensation with phenyl isocyanate. Subsequent acid hydrolysis gives this terminal amino acid residue as its phenylhydantoin. Abderhalden and Brockman (16) elaborated this method to deal also with the free carboxyl group by condensation with benzylamine. Goldschmidt and Wiberg (100) have employed hypobromite to obtain the amino acid with free amino group as the next lower nitrile of the homologous series.

Bergmann and Zervas (45) have worked out a method of stepwise degradation of benzoyl peptides through the carbobenzoxy derivative of the azide, which removes the residue carrying the free carboxyl group as the next lower aldehyde in the homologous series, and leaves the rest of the peptide in the form of its amide.

Nitrous acid may be employed for selective deamination of the residue carrying the free amino group. Racemization with alkali or, as suggested by Cahill and Burton (48), with ketene may also prove useful for structural elucidation, and serological methods are likely in the future to be a valuable aid.

To conclude, it seems that the main obstacle to progress in the study of protein structure by the methods of organic chemistry is inadequacy of technique rather than any theoretical difficulty. It is likely that the development of new methods of work in this field will lead us to a very much clearer understanding of the proteins.

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