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SYNTHESIS AND DEGRADATION OF PROTEINS IN THE LABORATORY AND IN METABOLISM[1]

By MAX BERGMANN

KAISER WILHELM-INSTITUT FÜR LEDERFORSCHUNG IN DRESDEN

EVER since men have interested themselves in the study of the chemistry of vital processes the proteins have exercised a peculiar fascination. Many of our most distinguished investigators have been engaged in problems associated with their chemistry and metabolism. I need mention only such names as Dakin, Levene, Osborne and van Slyke among others in order to recall to your minds the achievements of modern protein chemistry in this country. Nevertheless, we are still far from an exact knowledge of the structure of a single protein molecule.

The foundations of our modern knowledge of proteins were laid for the most part by Emil Fischer and by Albrecht Kossel. These investigators provided the methods by which we separate and recognize the individual products resulting from the hydrolysis of proteins. To Fischer we owe the method of welding together the constituents of proteins by laboratory methods to form peptide structures resembling in character the proteins themselves. Fischer succeeded in synthesizing an octadecapeptide containing glycine and leucine. The latter striking synthetic achievement itself indicates, however, the unfortunate limitations of Fischer's methods, the application of which is confined almost exclusively to peptides containing the simplest amino-acids and the monoamino-monocarboxylic acids. Such peptides do not contain the free amino, carboxyl and guanidino groups, which are present in the natural proteins; they do not in fact contain the more complicated and therefore more interesting amino-acids.

If it is desired, therefore, to imitate the synthesis

[1] Lecture given November 17, 1933, in the Rockefeller Institute for Medical Research, New York.

of the natural proteins and thoroughly to explain the mode of action of the proteolytic enzymes it is evidently necessary to devise a method of synthesis which is more generally applicable than the classical method of Fischer. The search for such a synthesis has occupied chemists for a long time. My colleague, Dr. L. Zervas, and myself have recently been able to solve the problem in the following way.

The essential feature of this method is the nature of the residue which we use to block the amino group. This group must be of an acid nature, as its function consists in abolishing the basic properties of the amino group; moreover, it must be of such character as to be readily and completely removable from the amino group in the course of the synthesis, under conditions in which the rest of the molecule remains intact. Of the many substituents tried, the carbobenzoxy group proved the most suitable.

The free benzyl-carbonic acid (I) itself is unknown. But its acid chloride (II), readily obtainable by the interaction of benzyl-alcohol and phosgene, can be condensed with any amino-acid to yield the corresponding carbobenzoxy derivative (III). The carbobenzoxy amino-acids are beautifully crystalline substances which can be easily transformed into their acid chlorides (IV) or azides, and these in turn can be condensed with other amino-acids to yield carbobenzoxy peptides (V). Most important, however, is the fact that the carbobenzoxy residue can be split off without the employment of a hydrolytic agent, so that it can be eliminated from a carbobenzoxy peptide without danger of splitting the peptide bond. The removal of the carbobenzoxy residue is accomplished by catalytic hydrogenation in presence of palladium black, under which conditions the benzyl residue is eliminated as toluene and the resulting carboxy-amino derivative spontaneously loses carbon dioxide to give the free peptide (VI). The last step of the process proceeds with ease and in most cases almost quantitatively.

$$C_7H_7 \cdot O \cdot CO \cdot OH \quad \text{(I)}$$
$$C_7H_7 \cdot O \cdot CO \cdot Cl \quad \text{(II)}$$
$$C_7H_7 \cdot O \cdot CO \cdot NH \cdot CH(R) \cdot COOH \quad \text{(III)}$$
$$C_7H_7 \cdot O \cdot CO \cdot NH \cdot CH(R) \cdot CO \cdot Cl \quad \text{(IV)}$$
$$C_7H_7 \cdot O \cdot CO \cdot NH \cdot CH(R) \cdot CO \cdot NH \cdot CH(R') \cdot COOH \quad \text{(V)}$$
$$C_7H_8 + CO_2 + NH_2 \cdot CH(R) \cdot CO \cdot NH \cdot CH(R') \cdot COOH \quad \text{(VI)}$$

A further and very important advantage of the carbobenzoxy method is its applicability to optically active amino-acids. The carbobenzoxy-amino acids, in contrast to other acyl derivatives of amino-acids, are surprisingly stable towards racemizing influences. With the aid of the carbobenzoxy method, in fact, the preparation of many optically active peptides, which have hitherto been practically inaccessible, becomes a simple matter.

In presence of sulfur compounds like cystin the catalytic hydrogenation unfortunately does not work. For the synthesis of cystin-peptides another method of elimination of the carbobenzoxy residue is available.

There is only one amino-acid with which the benzylcarbonato method fails, and that is arginine; for the latter we have to devise a modified process. The difficulty in the case of arginine consists not only in the presence of more than one basic group, but particularly in the very strongly basic nature of the guanidino group, which masks the acid character of the carboxyl. In some instances it is impossible to alter the carboxyl group without simultaneously affecting the guanidino group. In order to render arginine suitable for the synthesis of peptides it was therefore necessary to neutralize its basic properties by a readily removable acid group. To this end we have modified the application of the carbobenzoxy method to arginine by first converting the latter into nitroarginine; the latter compound behaves like a simple monoamino-acid. The process may be illustrated by an example. For the synthesis of glycyl-l-arginine, carbobenzoxy glycine chloride is coupled with nitroarginine (VII) and the product (VIII) is subjected to catalytic hydrogenation; by this means the carbobenzoxy residue and the nitro group are removed in one operation and glycyl-l-arginine (IX) results.

$$\underset{\displaystyle NO_2}{NH} \cdot C(=NH) \cdot NH \cdot (CH_2)_3 \cdot \underset{\displaystyle NH_2}{CH} \cdot COOH \quad \text{(VII)}$$

$$\underset{\displaystyle NO_2}{NH} \cdot C(=NH) \cdot NH \cdot (CH_2)_3 \cdot \underset{\displaystyle NH \cdot CO \cdot CH_2 \cdot NH \cdot CO \cdot O \cdot C_7H_7}{CH} \cdot COOH \quad \text{(VIII)}$$

$$NH_2 \cdot C(=NH) \cdot NH \cdot (CH_2)_3 \cdot \underset{\displaystyle NH \cdot CO \cdot CH_2 \cdot NH_2}{CH} \cdot COOH \quad \text{(IX)}$$

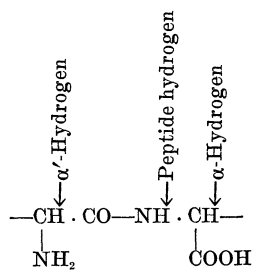
With the help of the carbobenzoxy method, it is now possible to bring into peptide combination aminodicarboxylic acids, diamino-acids, proline, cystine and serine, so that for the first time the different possibilities of combination of the amino-acids within the proteins and the behavior of the latter towards proteolytic enzymes can be accurately studied.

The following list contains a selection of dipeptides which we have been able to prepare during the past year by means of the carbobenzoxy method:

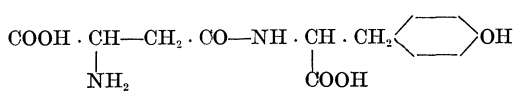
l-Glutamyl-l-glutamic acid
l-Lysyl-l-glutamic acid
l-Lysyl-l-aspartic acid
l-Lysyl-glycine
l-Lysyl-l-histidine
l-Glutamyl-l-tyrosine
l-Tyrosyl-l-tyrosine
l-Tyrosyl-l-aspartic acid
Glycyl-l-arginine

We have subjected all these dipeptides to the action of dipeptidase, in order to ascertain whether the effi-

cacy of the enzyme is influenced by the presence of free amino, guanidino, carboxyl or phenolic hydroxyl groups. This is not the case. All these dipeptides containing complex amino-acids are split by dipeptidase in precisely the same manner as the dipeptides of the simple amino-acids. We can therefore say with complete certainty *that every dipeptide containing natural α-amino-acids and normal peptide linkages will be hydrolyzed by dipeptidase.* In other words, dipeptidase is capable of hydrolyzing the peptide linkage so long as the latter is immediately adjacent to a free amino group on the one side and a free carboxyl on the other, according to the following scheme:

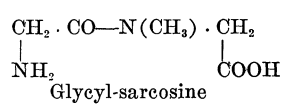


If the amino group or the carboxyl are further removed from the peptide linkage, as for instance in the case of β-l-asparagyl-l-tyrosine, the action of dipeptidase fails.

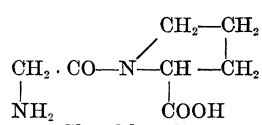


In order to obtain a deeper insight into the mechanism of the action of dipeptidase we have undertaken a series of further problems.

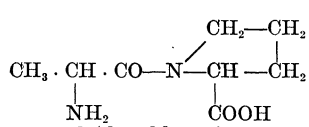
The first of these is the question whether the peptide hydrogen is of importance for the action of the enzyme. We have been able to obtain an answer to this question by investigating the action of dipeptidase on the following dipeptides:



Glycyl-sarcosine



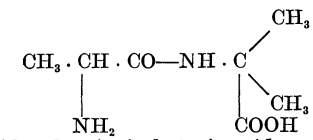
Glycyl-l-proline



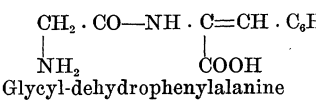
l-Alanyl-l-proline

These three dipeptides are all resistant to the action of dipeptidase. This has already been shown for glycyl-sarcosine by the earlier work of Levene and Simms[2] with erepsin. It appears, therefore, that *the presence of the peptide hydrogen atom is indispensable for the action of this enzyme.*

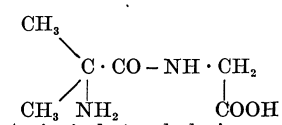
We next studied the question of the influence of the hydrogen atoms in the α- and α'-positions on the action of dipeptidase on dipeptides, such as:



l-Alanyl-aminoisobutyric acid



Glycyl-dehydrophenylalanine



Aminoisobutyryl-glycine

None of these peptides was attacked by dipeptidase, so that we can assume that *the hydrogen atoms in the α- and α'-position are important to the action of dipeptidase.*[3]

In order to restrict the field of our discussion still further we may recall the fact that dipeptidase only attacks those dipeptides which are built up from the natural optically active amino-acids, which, as is well known, all possess the same spatial configuration. The presence in a dipeptide of merely one amino-acid of the opposite configuration is sufficient to inhibit the action of dipeptidase. We can therefore add the statement that it is not sufficient that hydrogen atoms should be present in the α- and α'-positions; *these hydrogen atoms must occupy a definite position in the spatial configuration of the molecule.*

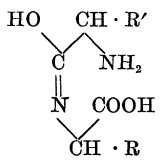
We have now been able to collect so many data that we are able to present an effective picture of the mechanism of the action of dipeptidase. When the enzyme combines with a dipeptide, the peptide linkage is rearranged into its imide form—

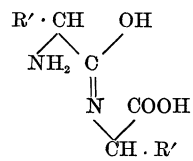
$$-C(OH)=N-$$

The imide form of the dipeptides can occur in two stereomeric arrangements X and XI.

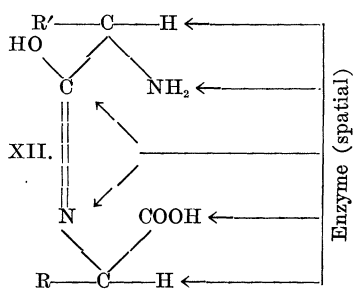
[2] *Jour. Biol. Chem.*, lxii: 711, 1925.

[3] P. A. Levene, R. E. Steiger and L. W. Bass (*Jour. Biol. Chem.*, lxxxii: 155, 1929) have shown that Glycyl-α-aminoisobutyric acid and Glycyl-d-l-phenylmethyl-aminoacetic acid are not split by erepsin. The same applies to Glycyl-d-isovaline and Glycyl-d-l-isovaline as has been shown by Levene and by myself.

X. 

XI. 

Of these, only X is attacked by dipeptidase, because the enzyme requires the simultaneous presence of a free carboxyl and a free amino group, which must exist (as in X but not in XI) in close spatial proximity. The combination of the enzyme with these groups forces the atoms of the dipeptide into a relatively rigid arrangement in space; the enzyme brings about a kind of "ring"-formation. In consequence of the spatial structure of the amino-acids the α- and α′-hydrogen atoms of the dipeptide lie on the same side of the plane of the peptide "ring." The enzyme can thus approach both of these hydrogen atoms simultaneously. The enzyme may therefore be regarded as combining chemically with at least three different atomic groups of the dipeptide, that is to say with the carboxyl, the amino group and the peptide linkage. The presence of hydrogen atoms in the α- and α′-positions, and in the correct spatial configuration, is also essential.

XII. 

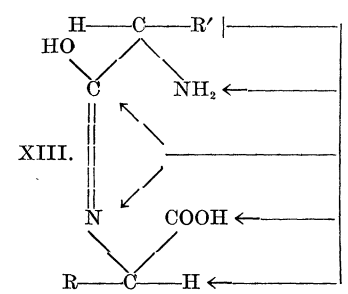
We can now understand why it is that glycine dipeptides are split by dipeptidase, although glycine contains no asymmetric carbon atoms; the reason is that the α-carbon atom of glycine carries two hydrogen atoms, one of which must always occupy the position required by the enzyme in accordance with the scheme which has been outlined.

Since the time of Emil Fischer the view has been generally accepted that the enzymes have specifically different spatial structures. It was in order to express this generalization that Fischer proposed his classical analogy of the lock and key. Now it becomes possible for us, as the result of our more recent investigations, to form a clearer idea of the reasons for the structural specificity of the enzyme dipeptidase. The enzyme itself must contain a whole series of chemically different atomic groups in a definite spatial arrangement; these groups must be so situated that they are capable of entering into combination with the carboxyl, the amino group and the peptide bond of the dipeptides. If the enzyme is brought into contact with a stereoisomeride of the natural peptide it finds the significant atomic groups of the dipeptide in an unsuitable arrangement; the active groups of the enzyme are unable to combine with all those of the dipeptide, and the latter therefore can not be split (XIII).

Hitherto I have spoken of the way in which, with the help of the carbobenzoxy method it is possible to penetrate more deeply into the chemistry of the dipeptides and of their appropriate enzymes, the dipeptidases; this same method can however be used for the investigation of polypeptides and of the enzymes which hydrolyze them.

It is well known that polypeptides containing three or more amino-acids are not attacked by dipeptidase. The polypeptides have indeed their own appropriate enzymes, the polypeptidases; these are usually separated into two main groups: the carboxypolypeptidases, which attack the peptide from the carboxyl end by splitting off the amino-acid whose carboxyl group is free, and the amino-polypeptidases, which attack the polypeptide chain from the amino end.

First of all I should like to speak of the carboxypolypeptidase of the pancreas, and from the outset I

XIII. 

must point out that this enzyme is not strictly entitled to the name of polypeptidase; its field of action is not limited to true polypeptides. It has been known, for instance, for some time past that this enzyme hydrolyzes chloracetyl-l-tyrosine (XIV) with especial ease; besides this we have found that it is also able to hydrolyze pyruvoyl-phenylalanine (XV) and that even genuine dipeptides, such as d-tyrosyl-l-arginine (XVI) and l-tyrosyl-l-tyrosine (XVII) are susceptible to its attack. It is thus possible to state that the substrate for the carboxy-polypeptidase must contain a free carboxyl group adjacent to the peptide linkage, but that the second peptide component of the substrate need possess not a hydrogen atom in α′-position. In this respect the polypeptidases differ fundamentally from dipeptidase. The presence of an amino group adjacent to the peptide linkage does not inhibit the action of polypeptidase in all cases, as is shown by the examples of tyrosyl-arginine and tyrosyl-tyrosine mentioned above. There are, however, only

a few dipeptides which are split by polypeptidase; the majority remain unattacked.

A simple experiment shows that carboxy-polypeptidase is unable to act in the absence of peptide hydrogen; whereas, as already mentioned, chloracetyl-l-tyrosine (XIV) is readily split by the enzyme, chloracetyl-l-methyltyrosine (XVIII) is quite resistant to its action.

XIV. $Cl \cdot CH_2 \cdot CO{-}NH \cdot CH(CH_2{-}C_6H_4{-}OH) \cdot COOH$

XV. $CH_3 \cdot CO \cdot CO{-}NH \cdot CH(CH_2{-}C_6H_5) \cdot COOH$

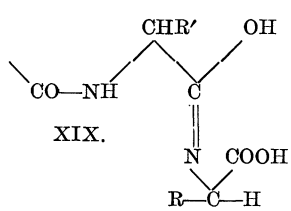
XVI. $HO{-}C_6H_4{-}CH_2 \cdot CH(NH_2) \cdot CO{-}NH \cdot CH((CH_2)_3 \cdot N_3CH_4) \cdot COOH$

XVII. $HO{-}C_6H_4{-}CH_2 \cdot CH(NH_2) \cdot CO{-}NH \cdot CH(CH_2{-}C_6H_4{-}OH) \cdot COOH$

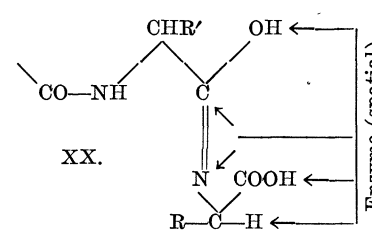
XVIII. $Cl \cdot CH_2 \cdot CO{-}N(CH_3) \cdot CH(CH_2{-}C_6H_4{-}OH) \cdot COOH$

The indispensability of the peptide hydrogen atom and of the free carboxyl group, as well as the inhibitive influence of a free amino group on the action of carboxy-polypeptidase lead to the following interpretation of its mode of action: The enzyme first combines with the peptide in its imide form (shown, for the case of polypeptides, by XIX); an intermediate molecular compound XX being produced.

XIX.



XX.



Only those dipeptides which, like tyrosylamino acids, can exist in the form represented by XI, are split by carboxypolypeptidase. On the other hand, dipeptides which assume the form X are not attacked by carboxypolypeptidase.

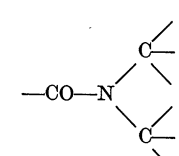
The same considerations hold good for the amino-polypeptidases. These are by no means limited in their action to true polypeptides; they split the amides of simple amino-acids and we have also found that they are able to hydrolyze certain dipeptides. Of the latter I may mention glycyl-l-proline and l-alanyl-l-proline.

This indicates that amino-polypeptidase does not require the presence of peptide hydrogen; its mode of action must be quite different from that of dipeptidase and carboxy-polypeptidase.

You will see that the possibility of the synthesis of selected peptides seems to open the way to an understanding of the action of these enzymes as complete as that which we already possess in the case of dipeptidase.

The higher we go, however, in the series of proteolytic enzymes the more restricted becomes our knowledge. I am thinking now of the so-called proteinases, that is to say, the enzymes which attack genuine proteins. To this class belong pepsin, trypsin, papain and kathepsin. We are almost entirely ignorant of the mode of action of these enzymes; it is the exploration of this very group, however, which is of the most fundamental significance for the elucidation of the structure of the genuine proteins. Are the proteins merely polypeptides with chains of enormous length, which are broken by the proteinases into smaller fragments, or may the protein molecule perhaps contain unrecognized linkages on which the proteinases exercise a specific action? We can not answer these questions because it has not yet been possible to obtain a single substrate of known structure on which to study the mode of action of proteinases.

The problem of proteinases and of the structure of the genuine proteins is of such outstanding importance that we have explored many different methods of attack. In the first place, we have attempted to obtain more precise information as to whether the proteinases are able to attack specific linkages in the protein molecule. One species of amino-acid is known to occur in protein which is not capable of entering into a normal peptide linkage, namely l-proline and l-hydroxy-proline, which in contrast with all other natural amino-acids contain no amino group but have their nitrogen in the imino condition. When proline is converted into a peptide through its nitrogen atom, therefore, no ordinary peptide linkage is formed but a linkage in which the nitrogen is in the tertiary condition.



proline peptide linkage

It seems to us of interest to find out whether such proline-peptide linkages occur in proteins and if so whether they are hydrolyzed by proteinases.

The ordinary method of following analytically the hydrolysis of the usual peptide linkage is accompanied by the liberation of one amino group and one carboxyl. The amino group can be estimated by the method of van Slyke, the carboxyl by titration in alcoholic solution according to Willstätter and Waldschmidt-Leitz. The analytical study of the digestion of proteins has as yet given no grounds for suspicion that amino and carboxyl groups can be liberated in any other proportion; this ratio has indeed been regarded as a criterion of true proteolysis.

Our synthetic proline peptides were the first examples of peptide compounds in which this law of equivalence was not fulfilled. In the process of hydrolysis of glycyl-prolines the carboxyl group of the glycine is liberated and can be titrated in alcoholic solution; simultaneously, the imino group of the proline is set free, but this group can not be determined by the method of van Slyke. In this case, therefore, the law of equivalence breaks down. We have been able to show that such proline-peptide linkages occur in gelatine and that they are not attacked by the pancreatic proteinase.

Gelatine contains about 25 per cent. of proline and hydroxy-proline. The process of digestion of gelatine, first by pancreatic proteinase and then by the enzymes of the intestinal mucosa has been investigated in detail. The digestion by the proteinase results in a rapid increase in the free amino and carboxyl groups, these free groups increase in equivalent proportions. During the second stage of the digestion with the intestinal enzymes the increase in free carboxyl groups predominates greatly over the increase in amino groups; this latter observation affords the first example of digestion of a protein in which the law of equivalence does not hold good.

Our experiments have provided us with the proof that large amounts of proline and hydroxyproline occur in the gelatine molecule linked through their nitrogen atoms, that is to say that the proline in gelatine is present for the most part in a linkage analogous to that of our synthetic proline peptides. On the other hand, our results also show without doubt that these proline peptide linkages are not hydrolyzed by pancreatic proteinase. It is therefore evident that the explanation of the mode of action of proteinase must be sought in another direction.

It has indeed been quite impossible up to the present to find any amino-acids, even of exceptional structure, simple derivatives of which are capable of acting as substrates for proteinases. We are, therefore, driven to the conclusion that it may perhaps only be the length of the peptide chain which decides its susceptibility to attack by these enzymes. It thus becomes one of the most important tasks of contemporary protein chemistry to synthesize polypeptides of large molecular weight and to investigate their behavior towards proteinases. Emil Fischer's octadecapeptide contains fifteen glycine residues and three leucine residues and is thus composed only of two different simple amino-acids; the peptides of glycine, which occurs fifteen times in the molecule, are well known to be particularly resistant to enzymic hydrolysis. It is, therefore, not surprising that polypeptides of the above type, in so far as their costliness has permitted them to be used for experiment, have shown themselves to be resistant to hydrolysis by proteinase.

In this matter the direction to be followed by further work is clearly indicated. The carbobenzoxy method provides the possibility of building up long polypeptide chains containing all the naturally occurring amino-acids. We are thus able now to offer the proteinases a substrate which resembles their natural substrate much more closely than did the simple polypeptides prepared by Fischer. We believe indeed that we may be able in the near future to express a definite opinion regarding the behavior of the proteinases towards peptide chains of high molecular weight and that we shall even be able definitely to sort out the modes of action of the different proteinases.

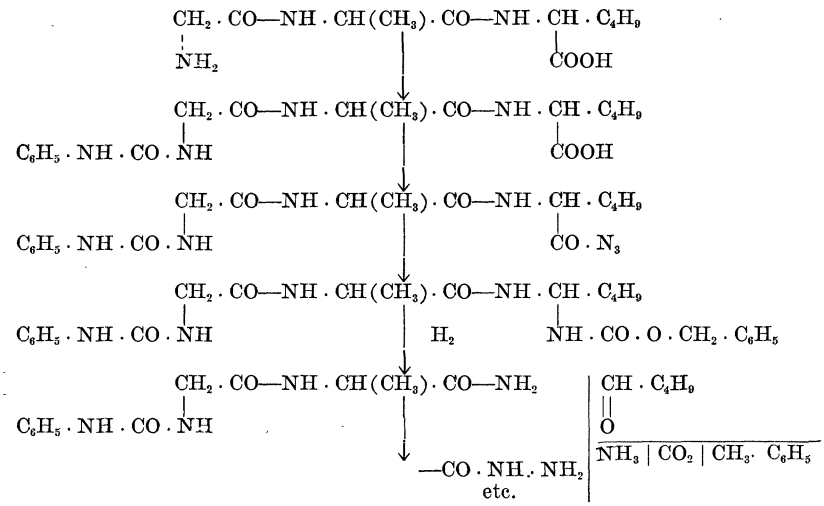
Even after this task has been performed there will remain another fundamental problem, the solution of which is essential to the progress of modern protein chemistry. In order completely to elucidate the structure of the genuine proteins and that of the polypeptides which result from their digestion by proteinases, it is necessary to possess a method for the analytical determination of the mode of arrangement of the constituent amino-acids in the molecules of the proteins and polypeptides, respectively. The importance of this is evident when we consider that the properties of the complete molecule must depend upon the way in which its component parts are arranged. If a protein is subjected to step-wise enzymic degradation the specific mode of action and place of attack of the enzyme can only be explained if the precise arrangement of the component amino-acids in the protein or in its degradation products is known.

The available methods for the analysis of polypeptides and proteins, involving hydrolysis with acids or alkalies, suffer from the disadvantage that all the peptide linkages are simultaneously ruptured, with the resulting formation of difficultly separable mixtures of amino-acids. For our purpose it was necessary to find means of progressively degrading a polypeptide chain such that one amino-acid after an-

other could be detached by procedures which leave the normal peptide linkage intact.

L. Zervas and I have worked out such a method, which I should like to illustrate by the simplest example which we have studied, namely, the tripeptide, glycyl-l-alanyl-l-leucine. The first step is to block the free amino group by substitution. As a substituent we chose in the first place phenyl-isocyanate and prepared a phenylureide of the tripeptide. By thus neutralizing its basic function the tripeptide is converted into a true acid; from this the methyl ester can be prepared under very mild conditions with the aid of diazomethane. The ester is treated with the equivalent amount of hydrazine to yield the hydrazide which in turn is converted according to the method of Curtius into the azide. Such azides on treatment with alcohols readily yield urethanes. The essential point of our method consists in the fact that we choose benzyl alcohol and thus obtain a benzyl-urethane. Now we hydrogenate catalytically this urethane and are able to obtain on the one hand toluene and isovaleric aldehyde and on the other the phenylureide of glycylalaninamide. The original leucine has thus been split off in the form of the easily identifiable aldehyde containing one less carbon atom.

The remaining substituted peptide-amide can now be further treated with an equivalent amount of hydrazine, again converted into a peptide-hydrazide and through the azide with benzyl alcohol into the benzyl-urethane, etc.; the alanine is finally split off as acetaldehyde and can thus be easily identified. In this manner we are able to break down a peptide chain step by step from the carboxyl end obtaining the successive amino-acids in the form of easily recognizable derivatives.[4]



The above work was undertaken in view of the urgent need of improved analytic and synthetic methods for the study of proteins and proteolytic enzymes. Now that some progress has been made in this direction a general plan of attack on the problems of protein constitution lies at hand.

OBITUARY

WILLIAM MORRIS DAVIS

ON the fifth of February last there closed a life which profoundly affected the development of two earth sciences, geology and geography. For more than half a century William Morris Davis was a distinguished leader in these related fields. At the end of this period he possessed, in his eighty-fourth year, the same enthusiasm of purpose, the same flexibility of mind, the same penetrating powers of reasoning which made his career remarkable in the annals of American science. Only a few weeks before his death he crossed the continent during an exceptionally severe winter to deliver the Maiben lecture before the Boston meeting of the American Association for the Advancement of Science. When he lay down to rest his writing table was filled with work actively in progress.

Professor Davis enriched the science of geology by a long succession of able papers based on original studies covering a remarkable range of subjects. In 1882 there began a series of fifteen articles and monographs on the Triassic formations of the Connecticut Valley, out of which came our first full knowledge

[4] This process can no doubt be successfully applied to other classes of compound. Its application to sugars and saccharides is contemplated.