RECENT ADVANCES IN THE DETERMINATION OF THE STRUCTURE OF PROTEINS

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INTRODUCTION

In reviewing the development of the methods of research in experimental sciences and their theoretical foundations, we may observe generally that the way in which the contemporary investigator establishes or attempts to establish a scientific truth is often somewhat different from that of the scientist of several generations ago. Physics illustrates in the most distinct manner the conception of the working hypothesis now and some time ago. In considering the idea of a working hypothesis one realizes that every type of research is connected in a more or less fundamental manner with the concept of a working hypothesis. We expect from a working hypothesis that it permits a combination and correlation of facts which have been previously established in a certain realm, but at the same time it must indicate ways how and where to obtain more facts which would make the particular realm most complete and clear.

In trying to reach this goal one may encounter new experimental evidence which strengthens the foundation of the working hypothesis and raises it to the status of a theory. The latter may then serve as a firm basis from which more detailed investigation is undertaken in different directions. On the other hand one possibly meets with experimental evidence which does not fit into the general scheme of the working hypothesis; and when the disagreeing facts are placed in a sufficiently strong position by repeated confirmation, the investigator is bound to abandon the first hypothesis and must endeavor to find a new one which serves his purpose better. As a rule he attempts to

do this as quickly as possible in order to have at his disposal a guiding principle for new research work.

One is used to the idea that in experimental sciences the theory is formed in most cases in a deductive way. A great number of known phenomena is gathered and a mutual relation is discovered. This is as a rule accomplished by one individual of great intuitive ability. In most cases he is in a position to immediately secure experimental corroboration if necessary. Thus a new theory is usually based in most cases on a number of well known facts. Naturally this statement does not hold sway in all cases. It certainly does not apply to philosophy. Likewise it is not to be applied to some findings in natural sciences, particularly physics and chemistry, when these findings have been obtained in a purely speculative way. It suffices to point to the atom theory of Democritus and to the large share of scientific truth it contains, in order to realize that inductive speculation always has its justification and that intuitive investigators foresee things, the experimental corroboration of which may come centuries later. But we wish to exclude these singular instances from our considerations now and return to the scientist who builds his theories on experimental foundations and obtains the confirmation of his assumptions from experimental evidence.

And here in an ever increasing number of cases we see that the scientist proceeds not in a deductive but rather in an axiomatic manner. He does not summarize large scientific evidence. He has only little evidence at his disposal which in itself would not fill the entire volume of a theory. He therefore produces a postulate including in it the little experimental evidence he possesses and proceeding in an inductive manner he develops it into a theory and tries to devise methods and ways for the deciding experimental test. Investigators in other fields may realize that the idea contains something fundamental and they apply it, again possibly in an axiomatic manner, to their particular problems; this procedure may prove the value of the idea as a working hypothesis but it may also contribute to the strengthening of the foundation of the original theory. It is obvious that the postulate plays an extremely important part in a modern

theory. It is sufficient to refer to Planck's quantum theory or to Einstein's theory of relativity in order to illustrate the above contention.

The reference to theories of physics in a paper on a chemical topic is made because the examples cited are particularly striking. Without dwelling upon problems of physical chemistry, we proceed immediately to organic chemistry some phases of which commence to bear resemblance to the axiomatic methods of physics.

The development of organic chemistry of natural products is a classic example of deductive work. The scientist always attempted to obtain an analysis of the compound under investigation. He tried to get complete information on its constituents and then to build up the same original compound with laboratory means and methods. The synthesis was the confirmation of the analysis and the solution of the problem. Many compounds were thus investigated and their constitution determined. But there is still a great number of substances which are being investigated at the present time in a different manner. Among these is the large group of proteins and their derivatives. In order to obtain information on these compounds, scientists do much work on so called models which to our mind represent nothing else but axioms stating that a given structure is postulated for a certain chemical individual or group. In some cases the experimental evidence for an assumed model structure is very small. On the other hand we observe that theories are utilized which were originally devised for different realms of chemistry. All this points to the fact that changed tactics are being adopted in organic chemistry for the elucidation of the structure of compounds occurring in nature. As in physics, nature is called upon to corroborate assumed structures, the most important criterion in the case of proteins being furnished by their biological behavior, e.g., their reaction with enzymes.

One reason may be given for this changed attitude of the natural sciences. The phenomena with which the scientist deals become more and more complicated. In many cases the methods prove to be entirely too crude to aid the investigator in the

elucidation of the unquestionably very fine complexity. It is therefore this lack of adequate methods which prevents him from proceeding in a deductive manner. It is sufficient to mention the entirely different methods used in the experiments of Willstätter and collaborators for the separation of enzymes to make one realize that deductive work alone, based on older methods cannot at present help in solving our problems. This kind of work also calls for a better basic knowledge of the far reaching problem of valence which, as it appears, is extremely difficult to treat from one unitary viewpoint.

This introduction is given in order to show the difficulties which some organic chemists are facing today and to point out that these difficulties seem to be typical of science of our age. It looks as though the broadening of our knowledge of certain important natural phenomena does not proceed very quickly because the number of subordinate phenomena which can be investigated by older methods based on older conceptions is being exhausted. The axiomatic procedure in establishing a working hypothesis is on the other hand not economic since too many probabilities must be taken into consideration before one is established as a certainty by experiment. The necessity arises of inventing new standard methods that would allow the chemist to resume the deductive work. This we believe is the safest and most economic way of producing new working hypotheses. It is needless to emphasize that these remarks do not refer to organic chemistry as a whole but rather to a limited number of investigations. The latter, however, are regarded as symptomatic. It is expected that the presentation of the experimental facts in this paper will make the axiomatic tendency distinctly visible which in organic chemistry pervades also the determination of the structure of the proteins.

ALIPHATIC AND CYCLIC COMPOUNDS AMONG THE PRODUCTS OF PROTEIN DEGRADATION

Research in the composition of proteins has begun rather early. Different proteins were subjected to different treatments and the investigator attempted to obtain information on the original

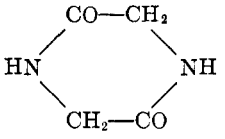
material by studying its cleavage products. These early investigations, some of which possess immediate interest with view to modern protein research, helped to build firm analytical foundations. The real research into the structure of proteins starts with the establishment of the CO·NH– linkage as characteristic for proteins, pronounced nearly simultaneously by F. Hofmeister and E. Fischer. The numerous papers which were published, particularly by E. Fischer and his coworkers, prove that an immense variety of compounds was conceivable and was actually prepared on the basis of this theory. The original assumption was that the amino acids, which were regarded as the components of the proteins, are combined with each other by this CO·NH-bond in a straight chain. Thus the variety of proteins was explained by the great number of possible combinations of the various amino acids in the polypeptides and by the length of the chain of connected amino acids. These conceptions were encouraged by the fact that combinations of high molecular weight were made accessible—a very high molecular weight was generally attributed to the proteins—and that particularly higher polypeptides gave reactions resembling those of the proteins. Some of them show a characteristic behavior on salting out and give the typical color reactions. Moreover a direct experimental corroboration was obtained by the direct isolation of peptides from products of hydrolysis of proteins and by the proof that synthetically prepared peptides were split by proteolytic enzymes. The action of enzymes always was and still is regarded as the most sensitive criterion in the determination of protein structures.

It was, therefore, an interesting task to attempt the preparation of polypeptides with as many amino acids as possible in order to closely approach the tentative structure of the proteins. E. Fischer's (1) peptide with eighteen and E. Abderhalden's (2) peptide with nineteen amino acids are steps undertaken in this direction.

It is not intended to dwell upon this well known peptide theory; it is sufficient to point to its extreme usefulness. In spite of the variety of possible combinations, the idea that ultimately proteins are composed of substances of a comparatively simple

structure was agreeable since it tended to satisfy the scientific desire of simplification.

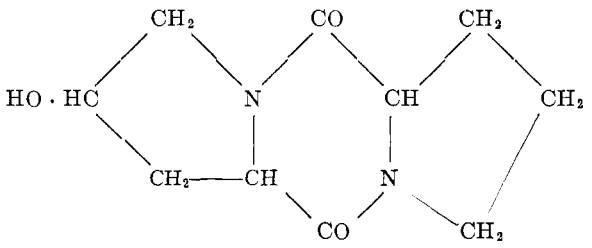
However, it was well known that the cleavage of proteins by chemical means or by peptic and tryptic enzymes has often yielded compounds which were of cyclic structure and not straight polypeptide chains. The pertaining observations were made very early and refer to the occurrence of 2,5-dioxopiperazines among the products of protein degradation. These 2,5-dioxopiperazines are composed of two amino acids; their simplest representative is glycine anhydride of the formula:



The two amino acids may be the same or different. They also may differ in their spatial configuration, thus making a great variety of these cyclic compounds possible. Among the dioxopiperazines the 3,6-diisobutyl-2,5-dioxopiperazine (leucine imide, leucine anhydride) was observed as early as 1849 by Bopp (3). Bopp digested casein with 25 per cent sulfuric acid for a day and allowed a portion of the syrup to stand for 2 months. He knew that the product obtained was a leucine derivative, but the exact formula was determined later by Erlenmeyer (4). Hlasivetz and Habermann (5) found the compound upon treatment under pressure of proteins with bromine in the presence of water, while R. Cohn obtained it on heating casein with concentrated hydrochloric acid (6).

Of course leucine imide is not the only dioxopiperazine found among the products of protein degradation. The mixed anhydrides which were found by E. Fischer and E. Abderhalden (7) were advanced as extremely important evidence in favor of the peptide theory of the proteins. Among these are the methyl dioxopiperazine (alanyl-glycine anhydride) and the glycyl-l-tyrosine anhydride from silk, glycyl-l-leucine anhydride from elastin. d-Isoleucyl-d-valine anhydride was isolated by Dakin (8) from casein. The same author obtained hydroxyprolyl-

proline anhydride from gelatin (9) which possesses an interesting structure since it contains three rings:



In reviewing the work that was done on the isolation of dioxopiperazines, it is always necessary to consider the methods which were used in the individual cases for their isolation in order to be able to judge whether the particular cyclic compound was preformed in the protein or whether a secondary formation of this heterocyclic structure out of an aliphatic one is possible. In pointing to this question we are entering into the last phase of the development of the ideas of the structure of the proteins.

Although dioxopiperazines were obtained from proteins comparatively early, no systematic experiments were carried out aiming at their establishment as elementary nuclei of the protein structure. The polypeptide theory was so well supported that there was no need for the present for considering any such possibilities. It was thought that continued research along the lines of the polypeptide theory would allow to gradually elucidate the protein problem. On the other hand the possibility of the occurrence of preformed dioxopiperazines was not disregarded (10). This is shown best by a statement of E. Fischer, parts of which read in free translation as follows:

I wish to emphasize that the simple amide bond does not represent the only possible linkage within the protein molecule. On the contrary the occurrence of piperazine rings is rather probable. . . . . The numerous hydroxyl groups of hydroxyamino acids are by no means to be regarded as inert. By intramolecular formation of anhydrides they could be transformed into ether and ester groups and the variety would increase still more when one considers the polyamino acids as probable

constituents of proteins. There is no reason for a further extension of these considerations, but I deemed it necessary to point to the various possibilities in order to discourage too onesided ideas which would impede the progress of experimental research.

NEW CONCEPTIONS OF VALENCE AND THEIR APPLICATION TO PROTEIN STRUCTURE

But it was soon realized that the assumption of polypeptide linkages as the basic linkages of protein structure was not sufficient. Still the degradation of proteins did not yet justify the assumption of other formations. An impulse to pursue the problem in a different direction was given by the brilliant researches of Pringsheim, Herzog, Hess, Karrer and others in the domain of carbohydrates. These investigations revealed that the colloid carbohydrates are not necessarily built up of complicated original compounds which are connected by ordinary valencies. Thus the idea made headway that similar conditions might prevail in the proteins. The protein chemists began to regard the proteins and their first degradation products, the peptones and albumoses as mixtures of a number of polypeptides. Similarly Siegfried's kyrines could be identified by Levene and coworkers in some instances as mixtures of polypeptides (11).

This phase of research is extremely important for the chemistry of proteins. The assumption was made that the regular valencies do not suffice, that we have to deal with associations, aggregations and polymerizations (12). Already in 1901 Kossel anticipated that the assumption of ordinary valence linkages would not suffice, in advancing the contention that proteins are particularly inclined to formation of mixed crystals and so called solid solutions. The forces by which the individual elementary complexes are mutually attracted are of an electric nature, but we have at present no means for their characterization. In comparing this valence energy with the classical one we are unable to visualize either its capacity or its intensity factors. Therefore new information in this realm is obtained accidentally when a compound is prepared which shows a tendency of formation of aggregated complexes. But with few very special exceptions we

have no means to predict what simple structure is liable to form protein-like aggregates and just what is the constitution of these associated compounds. These conceptions of aggregation are not limited to the peptides but are applied equally to the cyclic structure of the proteins.

THE THEORIES OF CYCLIC STRUCTURE OF PROTEINS

1. The pyrrole theory

The researches on polysaccharides not only helped in producing new conceptions regarding the forces of attraction between the individual primary complexes but they also fertilized the structural ideas themselves. Several theories were submitted nearly simultaneously, regarding the possible cyclic structure of proteins. N. Troensegaard regards proteins as consisting of pyrrole derivatives. The ideas that the elementary units of proteins are dioxopiperazines in addition to polypeptides is advocated particularly by E. Abderhalden. Other ring systems are also to be taken into consideration. Thus Karrer discusses the possibility of the pyrazine, imidazolone, oxazole and metoxazine oxazoline rings, while in Bergmann's theory oxazoline rings, ester peptides and recently a particular type of polymer dioxopiperazines play an important rôle. An unusual type of anhydride structure is suggested by Ssadikow and Zelinsky (14) but their suggestion is not supported by satisfactory experimental evidence.

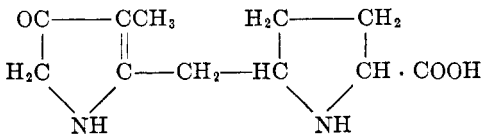
It was known that proteins furnish pyrroles upon dry distillation and pyrrole derivatives are found among the amino acids, the primary components of the proteins (proline, hydroxyproline). Troensegaard (15) assumes, however, that the pyrrole structure is more frequent and occurs to a larger extent. He investigated the gliadin of wheat, casein, gelatin and blood proteins. In order to avoid a hydrolysis of proteins in the presence of water, the proteins were treated in non aqueous solutions. First an acetylation of the protein was carried out, then the acetylated protein was reduced with metallic sodium and amyl alcohol. With special methods a separation into several basic and acid fractions is

effected, most of which possess heterocyclic character and more specifically pyrrole structure. Troensegaard contends that the assumption of the CO·NH– linkage alone is insufficient. Neither the CO nor the NH group reacts with methyl iodide while all fractions of his protein cleavage combine with a large number of methyl groups. Thus the correctness of the polypeptide theory of the proteins must be doubted. Whenever polypeptides are found among the degradation products of proteins, it must be assumed that they are formed in a secondary manner by hydrolysis of pyrrolidone or pyrrolone (keto pyrrole) rings. According to Troensegaard the dioxopiperazine theory can be only partly correct on the following grounds. Dioxopiperazines yield oxygen free piperazines when subjected to reduction with sodium and alcohol. However, only a very small fraction of oxygen free products could be isolated by Troensegaard.

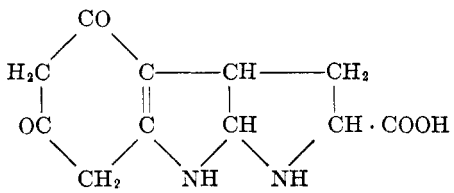
H. D. Dakin (16) also ascribes a more prominent position to the pyrrole derivatives, since comparatively large amounts of proline and hydroxyproline may be obtained from gelatin by extraction. Another interesting corroboration,—but based more on circumstantial evidence—of an extensive occurrence of pyrrole compounds in gelatin is advanced by E. Komm (17). This author finds that the reaction between tryptophane and aldehydes which may be followed up colorimetrically, is promoted by the presence of amino acids which contain pyrrole nuclei. By comparing this with the accelerating action of hydrolyzed gelatin he concludes that approximately 26 per cent of proline + hydroxyproline must be present in it which agrees very satisfactorily with Dakin's figure (14.1 per cent hydroxyproline + 9.5 per cent proline = 23.6 per cent). On the other hand the accelerating action of untreated gelatin is much more pronounced and would correspond to a content of 74 per cent of pyrrole nuclei. It is therefore suggested that labile pyrrole nuclei actually occur in gelatin but are destroyed by acid or alkaline hydrolysis. Only the comparatively resistant proline and hydroxyproline remain intact. It is important that Troensegaard's "proteols" from gelatin show a strongly accelerating influence on the tryptophane aldehyde reaction.

Although the pyrrole ring is assumed as the fundamental element of proteins, yet there is not sufficient evidence as to the composition of the individual fractions. N. Troensegaard and Eug. Fischer (18) isolated an acid from gliadin for which the following two formulas are suggested:

(1)



(2)



In addition biological tests were carried out and it was found that substances resulting from the reduction of acetylated proteins are more or less poisonous, showing an "alkaloid"-like behavior.

It is natural that more individual compounds of defined constitution will have to be isolated and more synthetic and enzymatic work done before Troensegaard's theory is accepted.

2. The dioxopiperazine theory

Considerable work has been done on the dioxopiperazine theory of the proteins. It was mentioned before that homolog dioxopiperazines were repeatedly isolated from degradation products of proteins. They were also the object of thorough synthetic work. It is perhaps desirable to mention some of the more important researches carried out on dioxopiperazines. Their formation from esters of amino acids was first observed by Curtius and Goebel (20). E. Fischer used this method extensively and studied the transformation of dioxopiperazines into dipeptides. The preparation of glycyl-glycine from glycine anhydride by the action of alkali is a classical example of preparation of a simple dipeptide. Numerous papers on the preparation of N, N′-diaryl-

2,5-dioxopiperazines were published by Bischoff, Widmann, Abenius and collaborators (21). With view to recent attempts (22) to isolate higher oxidation stages (tetraoxopiperazine) it may be pointed to related experiments with N,N'-diphenyl 2,5-dioxopiperazine. This compound gives 1,4-diphenyl-2-oxopiperazine when reduced with zinc dust and sulfuric acid in glacial acetic acid. The latter gives on treatment with CrO_3 in glacial acetic acid 1,4-diphenyl-2, 3-dioxopiperazine, and this is oxidizable to N,N'-diphenyltetraoxopiperazine (23). The constitution of 2,5-dioxopiperazines (24) was corroborated both by their oxidation and reduction. Thus F. Mylius (25) obtained dimethyl oxamide on oxidation of sarcosine anhydride (N, N'-dimethyl dioxopiperazine) with potassium permanganate, while E. Hoyer (26) prepared dimethyl piperazine by the reduction of alanine anhydride. Cohn (27) reduced leucine imide and observed the formation of 2,5-dibutyl piperazine. The few references represent only a part of the older literature on the subject of dioxopiperazines.

The question of primary occurrence of dioxopiperazines was raised again by E. Abderhalden and K. Funk (28). The possibility of the occurrence in silk fibroin of polymerization products of a dipeptide from alanine and glycine or the respective anhydride was discussed by R. Brill (29) on the basis of Röntgen spectrograms obtained with silk fibroin and by R. O. Herzog and M. Kobel (30). It was mentioned before that the occurrence of small elementary complexes connected by forces different from the ordinary valency was discussed by several authors. Systematic experiments aiming at the establishment of dioxopiperazine structure in proteins were undertaken by E. Abderhalden and his collaborators. Methods were devised to attack the problem simultaneously from several points. We here wish to give a logical rather than a chronological presentation, of the methods applied and the results obtained. It should be only pointed out that the first report of an attempt at the isolation of dioxopiperazines from proteins by means of additive compounds and the report of a chemical identification of a piperazine derivative obtained from a protein was given by E. Abderhalden in 1923 (31).

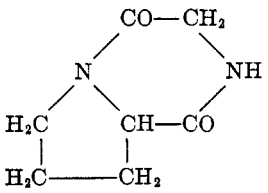
It was very important to obtain more information on the direct isolation of dioxopiperazines from proteins first in order to subject as large a number of proteins as possible to this investigation and secondly to secure the widest variety possible of dioxopiperazines from native proteins. As a rule the general methods used were similar in all cases. The protein was partly hydrolyzed, the product of hydrolysis concentrated in a vacuum and afterwards extracted with various solvents (ethyl acetate, methyl alcohol, acetone, chloroform, ether). The extraction with ethyl acetate gave particularly favorable results. Thus a large number of new and known dioxopiperazines was isolated.

The investigations of E. Abderhalden and coworkers show that extreme care is required in drawing conclusions from the results obtained. The utmost attention must be paid to the method of hydrolysis of the protein and it must be ascertained that the method of treatment does not include a possibility of producing anhydrides out of aliphatic peptides. In the latter case the results could be regarded as indicative of the occurrence of a certain dipeptide combination of two amino acids but would not give any evidence as to the occurrence of preformed dioxopiperazines.

It is not intended to give a list of anhydrides which were isolated by the methods described above. It should be mentioned that while in some cases the anhydrides must have been present in a preformed state, in other cases the possibility of a secondary formation cannot be disregarded. This has been shown clearly first by S. S. Grave, J. T. W. Marshall and H. W. Eckweiler (33), then by P. Brigl (34) and finally by E. Abderhalden and E. Komm (35). Thus leucyl-leucine is transformed into leucine anhydride when heated with water to 200° with a yield of more than 90 per cent of the theory. Glycyl-glycine is transformed into glycine anhydride (2,5-dioxopiperazine) when heated with 0.5 per cent hydrochloric acid under pressure at higher temperatures. Other dipeptides show a similar behavior giving anhydrides on heating with water under pressure. Some dipeptides (e.g., glycyl-tyrosine) are decomposed by this treatment. Tri- and tetrapeptides form anhydrides consisting of two amino acids

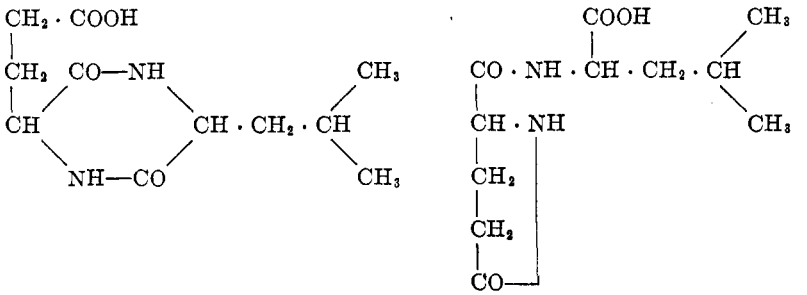
while the amino acids themselves do not give dioxopiperazines under these circumstances or only traces. Thus in judging whether dioxopiperazines are preformed in the proteins studied, we have to exclude all cases where the original method of hydrolysis might have promoted this secondary formation of dioxopiperazines from polypeptides, i.e., all investigations where heating with water or weak acids under pressure or even under reflux was used, or where an opportunity of formation of ester peptides was given, which as it is well known are readily transformed into dioxopiperazines. Treating polypeptides with 70 per cent sulfuric or with concentrated hydrochloric acid does not lead to dioxopiperazines. However, this relation between acid concentration and formation of anhydrides from peptides must be paid further attention, in consideration of the experiments of Kohler (36) who showed that leucine anhydride is obtained from leucine on heating to 220° in the presence of HCl gas and those of E. Abderhalden and K. Funk (28) who observed the formation of a small quantity of leucyl-glycine anhydride on treating leucyl glycine with 25 per cent sulphuric acid for 16 hours.

However, there is evidence that dioxopiperazines exist in a preformed state, having been extracted from proteins which were cleaved by enzymes or by concentrated acids. Salaskin (37) obtained leucine anhydride by the action of gastric juice on oxyhemoglobin and subsequent extraction with ethyl acetate. When edestin was decomposed by pancreatin and the dry residue extracted with ether glycyl proline anhydride was isolated (38):



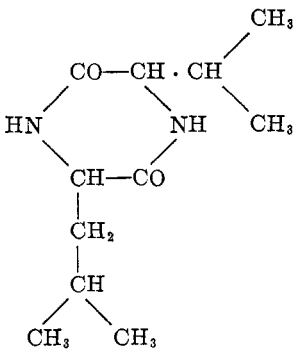
The same anhydride was obtained previously from gelatin by Levene and coworkers (30). But the value of this finding with reference to our consideration is diminished by the fact that gelatin is not a natural protein and that hydrolysis under

pressure and at a high temperature is employed in the manufacture of this product. Leucine imide was also obtained from the product of tryptic cleavage of gliadin. In addition a compound was isolated which seems to be an anhydride of leucine and glutamic acid, for which the following formulas are suggested:



This agrees with a compound obtained synthetically by Abderbalden and Rossner.

A direct hydrolysis of casein with concentrated hydrochloric acid (without esterification) and with subsequent extraction yielded leucine anhydride (28). d-Valyl-l-leucine anhydride was obtained from casein by boiling with 5 and 10 per cent sulfuric acid and subsequent extraction of the dried product (42).



It is improbable that this procedure should have led to a secondary formation of the anhydride.

It is interesting that some dioxopiperazines are rather resistant to acids. (Their behavior toward alkalis will be dealt with later.)

E. Fischer (43) found that leucine anhydride dissolves easily in concentrated acids without decomposition. The ring is split only on prolonged heating. Thus in order to obtain leucylleucine the anhydride is heated in a sealed tube with hydrobromic acid (aqueous solution saturated at 0°) at 100° for ½ hour. Unfortunately the methods used in most of the experiments aiming at the hydrolysis of proteins, do not exclude the possibility of a secondary formation of anhydrides. They are interesting only from the standpoint of possible dipeptide combinations; actually a great variety of such new combinations was observed by E. Abderhalden and E. Komm (44) in experiments which were originally intended to adduce evidence for the occurrence of preformed dioxopiperazines in different proteins.

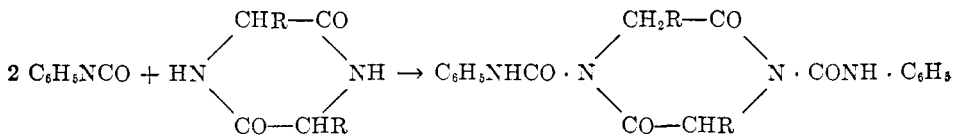
In discussing the isolation of dioxopiperazines from products of enzymatic cleavage, attention should be paid to the work of S. Fränkel (41a) who reports the secondary formation of anhydrides of amino acids which are regarded as true acid anhydrides and not as dioxopiperazines.

Experiments were also carried out aiming at the isolation of dioxopiperazines by combination with certain reagents. Although no definite compounds with dioxopiperazines could be isolated from hydrolyzed silk fibroin either by application of reagents which were to combine with the CO group or by those attacking the NH group (dinitrochlorobenzene) the investigations along these lines may still be successful, when a more specific reagent is found. The action of naphthalene sulfochloride on silk peptone was previously studied by E. Abderhalden and K. Funk (46).

The results obtained by Abderhalden and Stix (45) seemed to indirectly support the dioxopiperazine theory for the following reason. The NH_2 group and the NH group of dioxopiperazines are capable of reacting with dinitrochlorobenzene, while the NH- group of polypeptides is not. The authors found that the amount of dinitrochlorobenzene which entered into reaction with silk peptone was much larger than would be expected from the number of free NH_2 groups.

It is to be pointed in this connection to a paper by M.

Lüdtke (47) in which the reaction between an amino acidanhydride and phenyl isocyanate with formation of a substituted urea is described (48):

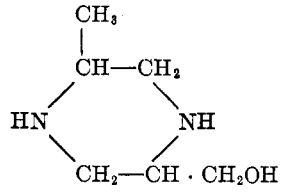


No information is available as yet as to the applicability of this reagent for the isolation of dioxopiperazines from proteins. A paper by Bergmann and Zervas (49) of recent date considers the possibility of application of aldehydes for the isolation of definite higher molecular compounds.

Although the previous experiments encouraged the conception of dioxopiperazine structure, the study of the reduction and oxidation of proteins contributed the more substantial results. Regardless of how dioxopiperazines might be combined with each other or with amino acids and polypeptides respectively, it was possible that a reduction which prevents the splitting of dioxopiperazines might produce volatile piperazines which could be driven out by steam distillation and identified in the distillate. This is actually the case and the formation of homolog piperazines from proteins is a strong point in favor of the dioxopiperazine theory. The first pertaining experiment was carried out by E. Abderhalden and W. Stix (50), who subjected silk peptone to reduction with metallic sodium and amyl alcohol. The distillate obtained gave the typical reactions of piperazines. The yield was small. But this does not indicate that dioxopiperazines are present in a small proportion since the direct treatment of dioxopiperazines in a similar manner leads to very small yields of piperazines. This is evidently due to the low resistance of dioxopiperazines; apparently the alcohol and sodium or sodium alcoholate respectively effect cleavage to a great extent, while only a small portion is reduced.

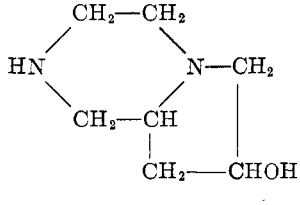
At the same time experiments were carried out on the reduction of a number of synthetic dioxopiperazines. Thus methyl piperazine was prepared from alanyl glycine anhydride; this compound

was of particular interest since the presence of the corresponding anhydride was assumed in silk fibroin. E. Abderhalden, E. Klarmann and E. Schwab subjected glycine anhydride and leucyl glycine anhydride to reduction and obtained the respective piperazines (51). E. Abderhalden and E. Schwab (52) produced from silk peptone in addition to the methyl piperazine previously obtained the 3-methyl-6-hydroxymethyl piperazine corresponding with alanyl-serine anhydride:

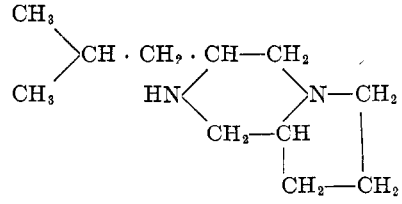


and a piperazine derived from four molecules of amino acids (two molecules of glycine, one of alanine and one of tyrosine). The latter compound was interesting since it did not give Millon's reaction in spite of the presence of tyrosine. However it was shown that the substituted piperazine resulting from the reduction of tyrosine anhydride likewise fails to give Millon's reaction. This points to the fact that the presence of the piperazine nucleus interferes with this test.

The same authors reduced directly untreated gelatin with sodium and alcohol (ethyl or amyl) and by using phenyl isocyanate for the separation of the individual fractions isolated piperazines which are derived from hydroxyproline glycine anhydride (I) and proline leucine anhydride (II) respectively:



I

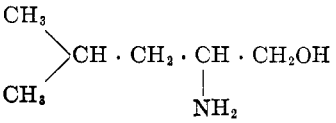


II

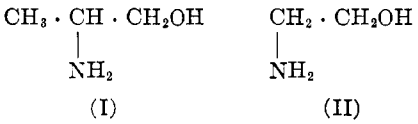
This experiment is however not convincing, since a secondary

formation of dioxopiperazines in gelatin is possible with view to the methods used in its preparation (53).

The behavior of amino acids and polypeptides in the presence of reducing agents of the type described was likewise studied and it was found that in no case the formation of a piperazine takes place. On reduction of dipeptides the formation of amino alcohols with partial deamination is observed. Thus the reduction of leucyl-glycine leads to γ-methyl-α-hydroxymethyl butylamine (leucinol):



At the same time caproic acid forms (54). Upon reduction of d,l-alanyl-glycine α-hydroxymethylethylamine (I) and propionic acid were obtained, while glycyl-glycine yielded hydroxyethylamine (II).



(I) (II)

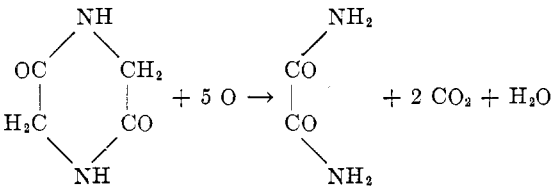
E. Abderhalden and coworkers also took the possibility of primary occurrence of piperazine nuclei into consideration and synthesized a considerable number of piperazine-amino-acid compounds. These experiments will be dealt with later in connection with the other studies on model compounds.

The formation of piperazine from proteins upon reduction certainly points to the probability of the primary occurrence of dioxopiperazines. Abderhalden and coworkers carried out another series of experiments aiming at the establishment of their presence in proteins by oxidation methods. Their results were corroborated by those of S. Goldschmidt and Ch. Steigerwald.

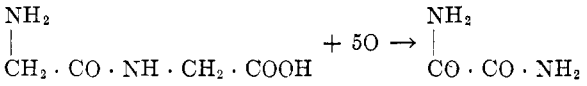
The use of the oxidation method is also based upon a different behavior of peptides, amino acids and dioxopiperazines to oxidizing agents. Oxidations of proteins with various oxidizing

agents were carried out in the past. The results of Loew (35) and Kutscher and Schenk (56) are particularly interesting. These authors found oxamide and oxaminic acid among the products of oxidation of proteins with potassium permanganate. F. Mylius' oxidation of sarcosine anhydride was mentioned before. In order to ascertain whether these oxidation products were indicative of a particular structure E. Abderhalden with E. Klarmann and E. Komm (57) subjected dipeptides, their corresponding anhydrides and silk peptone to oxidation. Zinc permanganate was used which is more convenient than the potassium salt.

All dioxopiperazines yielded oxamide. The dipeptides were decomposed with the exception of glycyl-glycine which also yielded oxamide. In the case of the simplest dioxopiperazine the oxidation takes place according to the equation



Similarly the oxidation of glycyl-glycine is represented by the equation:



It was shown later by E. Abderhalden and E. Komm (58) that oxamide is obtained also on oxidation of polypeptides which contain the glycyl-glycine group. All the other polypeptides did not give oxamide on oxidation, while all amino acid anhydrides (dioxopiperazines) and their 0,0′– or N,N′– substituted derivatives yielded oxamide in a satisfactory quantity.

This method was also used in the oxidation of proteins. Some proteins are very difficultly attacked by permanganate. But oxamide was obtained from gelatin, blood globulin, egg albumen and caseinogen (Hammarsten) in addition to the silk peptone previously mentioned.

It may be added in this connection that oxamide results also on oxidation with hydrogen peroxide.

The experiments of S. Goldschmidt and Ch. Steigerwald (59) belong in this group. The authors realized that only mild methods should be used in the experiments aiming at the elucidation of the structure of the protein molecule. They found that proteins are attacked by alkali hypobromite at 0°; different proteins react with varying amounts of hypobromite during the same length of time. Thus a series of titration curves may be obtained, each being characteristic of a given protein. The age of a protein solution is discernible by means of the titration curve, freshly prepared solutions of some proteins absorbing more hypobromite than older ones. It is assumed that the NH– group is attacked by hypobromite according to the equation:

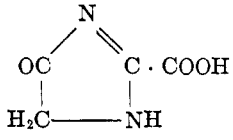
$$—CO \cdot NH \cdot CH\langle \rightarrow CO \cdot NBrCH\langle \xrightarrow{NaOH} CON : C\langle \xrightarrow{H_2O} CONH_2 + OC\langle$$

On the other hand it was found that it is not theNH – group of peptides which reacts in this manner, since glycyl-glycine is completely split by hypobromite giving ammonia and oxalic acid. The probable course of this reaction is the following one:

$$HOOC \cdot CH_2 \cdot NH \cdot CO \cdot CH_2 \cdot NH_2 \rightarrow HOOC \cdot CH_2 \cdot NH \cdot CO \cdot CN \rightarrow$$

$$\rightarrow HOOC \cdot CH_2 \cdot NH_2 + NH_3 + (COOH)_2$$

However, dioxopiperazines react with hypobromite. From glycine anhydride 4-imidazolone-2-carboxylic acid is obtained in a 20 per cent yield; it is extremely sensitive toward alkalis.



Alanine anhydride shows the same behavior. It is concluded from the analogy of behavior of dioxopiperazines and the proteins, that it is very probably the dioxopiperazine ring in the proteins that reacts with hypobromite.

Finally it should be pointed to a paper by Z. Stary (60) who studied the action of bromine in glacial acetic acid and of hydrogen peroxide in a 4N solution of sulfuric acid on human hair and the enzyme action (trypsin) on the resulting degradation products. He mentions that the primary existence of dioxopiperazine nuclei is to be taken into consideration which subsequently under the influence of splitting agents form polypeptides.

Thus the oxidation experiments also suggest the primary occurrence of dioxopiperazines in proteins. It should, however, be kept in mind that oxamide, the formation of which is the criterion of most of the oxidation experiments is also produced on oxidation of some polypeptides, although of a highly specific class. It would be interesting to carry out oxidations of proteins which contain only little glycine in order to exclude the possibility of the interference by the presence of glycyl glycine. The establishing of a quantity of oxamide larger than would correspond to the amount of glycine present in the protein, would show more convincingly that preformed dioxopiperazines are present. However, it must not be disregarded that comparatively few oxidation experiments were carried out on heterocyclic compounds and that oxamide might possibly be formed by oxidation of ring compounds different from dioxopiperazines.

Another series of experiments aiming at the comparative investigation of dioxopiperazines, peptides, amino acids and proteins or their cleavage products respectively, includes the application of color reactions which would be specific for one class but would not be given by other classes. It is perhaps useful to mention in this connection that group distinction in protein chemistry by means of color reactions is well known and extensively applied. Thus ninhydrine (triketohydrindene hydrate) gives color reactions on heating with amino acids, peptones and soluble proteins. On the other hand the biuret reaction is given by proteins, some higher peptides and peptones but not by amino acids. (It is not important that it is given by amino acid amides, since one does not take their occurrence in proteins into consideration.) Both reagents do not give any reaction with

dioxopiperazines. On the other hand precipitated moist copper oxide reacts readily with amino acids, and peptides but not with dioxopiperazines. The combination of the three tests is utilized for a qualitative analysis of a protein compound. But it is not suited for the determination of the occurrence of a dioxopiperazine in a protein.

E. Abderhalden and coworkers started a search for reagents which would be more or less specific for dioxopiperazines. Particularly aromatic nitro compounds (carbonyl reagents) were found to give characteristic reactions with dioxopiperazines. In order to carry out the reaction the reagent and sodium carbonate (in one case sodium alcoholate) is added to the tested solution and boiled for a short time. The following reagents were suggested out of a number of polynitro derivatives: picric acid, m-dinitrobenzene, 1,3,5-dinitrobenzoic acid, m-dinitrostilbene.

Picric acid was used originally by Jaffe as reagent for creatinine (61). T. Sasaki showed that amino acid anhydrides which contain glycine as a constituent also give a positive reaction. However, a number of different compounds, e.g., hydantoins, glucose, malonic ester, acetic ester also give a positive reaction. H. A. Dox (63) showed that the same reaction is given by barbituric acid, but it is negative with mono- or dialkyl barbituric acids. E. Abderhalden realized that the reaction is not absolutely specific, but most of the above named cases could be excluded, since the occurrence of these compounds in proteins is highly improbable. He therefore investigated systematically in collaboration with E. Komm and E. Schwab (64) a considerable number of amino acid anhydrides and found that all with the exception of l-leucyl-d-leucine anhydride gave a positive reaction with picric acid and sodium carbonate. No amino acid, polypeptide or biogenic amine, but all peptones and the majority of proteins gave a positive reaction. It is contended that the negative reaction with some of the proteins is due to their insolubility, although some of them contain reacting groups as shown by their behaviour toward m-dinitrobenzene, the reagent of v. Bittó.

Similar results were obtained with m-dinitrobenzene. All amino acid anhydrides gave a positive reaction, similarly all peptones and proteins, e.g., silk peptone, plant casein, blood globulin, keratin, ricin, legumin, vitellin, gelatin. Neither amino acids nor peptides give the reaction. l-Leucyl-d-leucine anhydride also gives a negative reaction. The authors prefer this reagent to picric acid, since a much more distinct change of color is observed and the reaction takes place more readily.

It is important to note that compounds which are regarded as N,N′– substituted dioxopiperazines give positive color reactions, while the reactions of the O,O′– substituted derivatives are negative.

The results obtained with 1,3,5-dinitrobenzoic acid are largely the same. However, some irregularities were observed. Thus it was found that cystine, cysteine, diglycyl-cystine, diglycyl-piperazine and dialanyl-piperazine gave positive reactions with m-dinitrobenzoic acid although they do not contain the dioxopiperazine ring. m-Dinitrostilbene (2,4) behaves like the other reagents except that no reaction is obtained with cysteine, cystine or its derivatives. On the other hand a few proteins which gave positive reaction with the other reagents give a negative reaction here, e.g., plant casein or egg albumen.

These color reactions represent in spite of their not entirely specific nature another support of the dioxopiperazine theory. Although for themselves they would not be absolutely conclusive yet in conjunction with the direct isolation of anhydrides, and the products isolated from the oxidation and reduction of proteins they contribute to the strengthening of the dioxopiperazine theory.

It must not be disregarded, however, that the color tests when carried out in a modified manner according to Brand and Sandberg (65) fail to give positive results with proteins, while a positive reaction is obtained with ordinary carbonyl compounds and dioxopiperazines using the same method. This means possibly that the short heating with sodium carbonate solution as required in Abderhalden's specifications of the method first

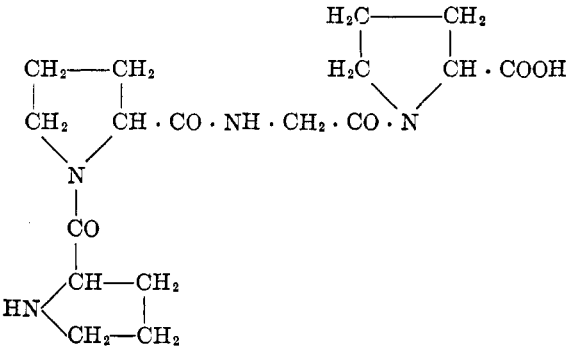
loosens the combination of the dioxopiperazines with some other compounds before the free dioxopiperazine is able to give the positive color reaction.

It is obvious that the investigator attempts to synthesize the compounds which he determines in the natural products. This is particularly important here. It is true that there is considerable evidence for the existence of preformed dioxopiperazines in proteins. But after all they represent only small complexes. Where is the connection with the compounds of high molecular weight?

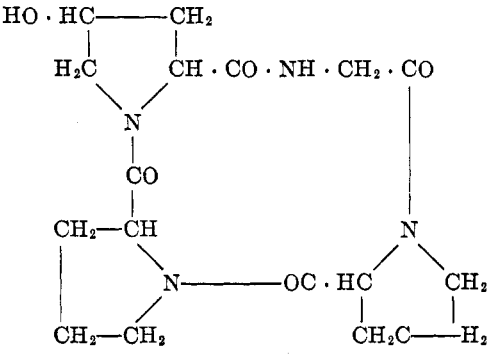
It was mentioned before that, on the basis of the polypeptide theory, attempts were made to arrive at compounds of high molecular weight similar to proteins by connecting individual amino acids to long chains. Similarly, Abderhalden attempted first to prepare compounds consisting of dioxopiperazines and amino acids, in order to realize a possibility of connecting a number of dioxopiperazines with each other. While E. Fischer's method of building long polypeptide chains is comparatively simple, no such method could be devised here. It is necessary that much more information be gathered on the chemical properties of dioxopiperazines, before this problem can be successfully handled. On the other hand it does not further the cause of science when hypotheses are advanced involving forces of attraction which we cannot clearly deal with. Such hypotheses are not justified unless all means of experimental approach have been exhausted and the investigator realizes that he is confronted with entirely unknown conditions. Although it is impossible at the present time to make any statements with reference to the problem of valence within the protein molecule on the basis of analytical research, one may say safely that there is still a considerable amount of experimental work to be done and there is no reason except the still disputable x-ray evidence why a careful study of compounds with true chemical linkages should not be carried out.

Of course such synthetic compounds are tentative models. There is experimental evidence for the existence of amino acid

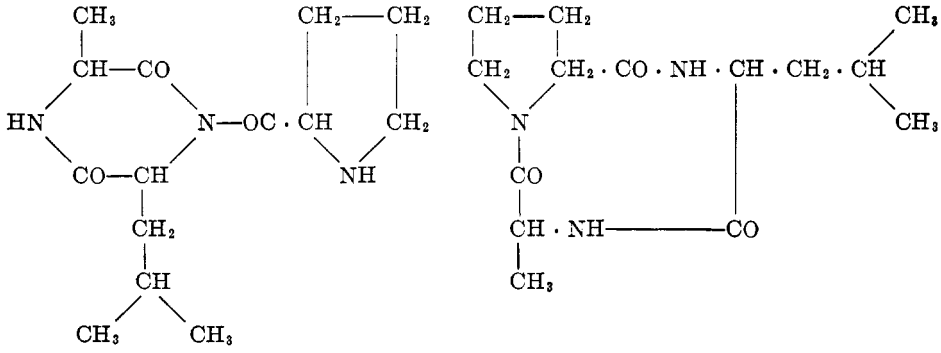
anhydrides containing more than two amino acids, but the structural formula attributed to them is a probable and not an established one. Some of these compounds which were isolated from native proteins and their tentative formulas shall be given as examples: a compound consisting of three molecules of l-proline (66) with the tentative formula:



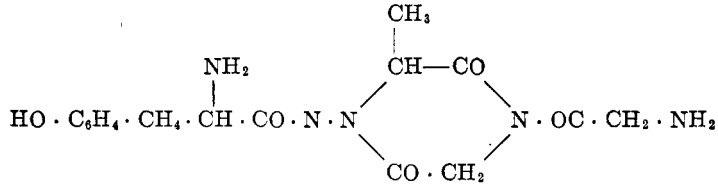
an anhydride consisting of two molecules of l-proline, one molecule of hydroxyproline and one molecule of glycine:



Both were obtained from keratin (goose feathers). From casein an anhydride was produced consisting of one molecule of d-alanine, one molecule of l-leucine and one of l-proline (67) for which the following formulas are suggested:



It was mentioned before that the presence in silk fibroin of an anhydride containing two molecules of glycine, one molecule of d-alanine and one of l-tyrosine is assumed by Abderhalden and Schwab (52).

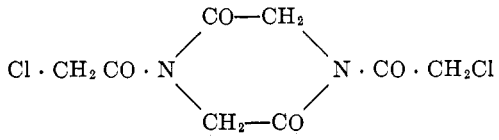


The number of compounds of this type is much larger (68) but the few examples cited, suffice to produce an idea as to the assumed nature of combinations of dioxopiperazines and amino acids.

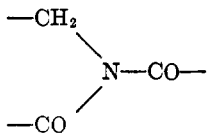
It is noteworthy that upon addition of alkali the amount of NH_2 – nitrogen increases, thus indicating that the ring is opened on one side by this treatment.

All these compounds were found in native proteins. On the other hand the problem was approached also synthetically. The first methods used resembled those of E. Fischer for the preparation of polypeptides. The direct condensation with halogen acyl halides and subsequent amination did not lead to the desired products. E. Abderhalden and E. Klarmann found that when water is excluded a condensation may take place at a higher temperature with formation of the corresponding di-

(halogen acyl)-dioxopiperazines. Thus chloroacetyl chloride reacts at 160° with glycine anhydride in nitrobenzene solution:



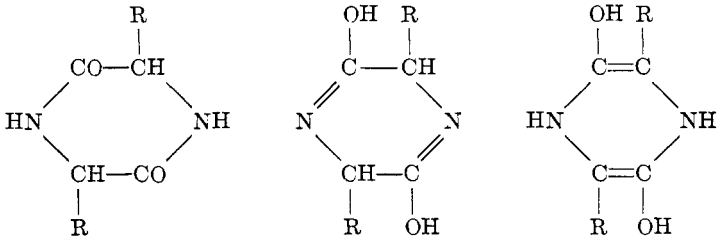
Similarly di-(α-bromoisocaproyl)-dioxopiperazine is obtained (69) on boiling glycine anhydride with the acid chloride. However, it was impossible to replace the halogen by the NH_2 group since in the presence of NH_3 cleavage takes place in all solvents with formation of the acid amide and free dioxopiperazine. It is perhaps interesting to add that according to Karrer (70) these two compounds might be O,O′– substituted derivatives. The easy formation of acid amides from an ester-like compound would then be intelligible since it is known that esters readily produce acid amides in contact with ammonia. On the other hand compounds were prepared synthetically (see page 84) which probably have the structure



and are rather resistant to ammonia.

The synthetic preparation of such compounds is important with view to the reaction toward enzymes. The study of the behavior of dioxopiperazines toward enzymes shows so far, that no cleavage takes place. These investigations will be dealt with later. But it is possible that the nature of the dioxopiperazine nucleus might be so changed by the introduction of an amino acid rest that the resulting compound would be attacked by enzymes. It was therefore attempted to prepare compounds of amino acids and dioxopiperazines using different methods. Before we turn to these investigations we shall first consider the nature of the dioxopiperazine nucleus itself.

The structural formula of the dioxopiperazine nucleus permits the following tautomer structures to be assumed (71):



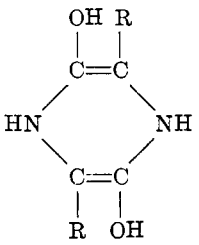
I. II. III.

Actually compounds were obtained under certain conditions which possess an outspoken unsaturated character (72). Thus when glycine anhydride is heated with glycerol in the presence of tyrosine to 190–200°C. for five hours a compound is isolated which has the empirical composition of glycine anhydride. However, in contrast to this, it immediately decolorizes permanganate, gives a positive xanthoprotein reaction and readily allows the introduction of methyl groups by means of diazo methane. The authors assume the formula III as the most probable for this compound. The same treatment in the absence of tyrosine leads to a compound which originally possesses this unsaturated nature but loses it in the course of purification. Similarly heating of d,l-leucyl-glycine anhydride with glycerol in the presence of tyrosine leads to a substance which shows all properties of an unsaturated compound (73). The rôle of tyrosine in this connection is not entirely clear. Rearrangement of the enol form of 2,5-dioxopiperazine into its keto form takes place by heating in aqueous solution to 90–100° (74).

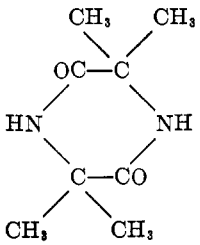
Another method of obtaining dioxopiperazines in the enol form is heating of the respective dipeptides with diphenylamine (75). The enolic anhydrides of d,l-leucyl-glycine, d,l-leucyl-d,l-valine, d,l-alanyl-d,l-alanine, d,l-leucyl-d,l-leucine were prepared in excellent yields. Glycyl-glycine and glycyl-alanine showed a behavior different from that of the other dipeptides. The first gives a difficultly soluble, probably polymer compound with the empirical composition of glycine anhydride. While dioxo-

piperazines in keto form cannot be transformed into the enol form by heating with glycerol or diphenylamine, this transformation is effected by heating with aniline (79).

An interesting proof for the assumed structure (I):



(I)



(II)

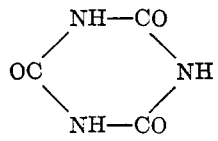
was given by the preparation of the anhydride from α-aminoisobutyryl-α-aminoisobutyric acid (II). This anhydride cannot exist in the previously mentioned enol form on account of its particular structure. Actually the synthetized product gave all anhydride reactions, but unlike the other anhydrides prepared by the same method, it failed to give the xanthoprotein reaction and did not decolorize permanganate. Since it was possible to obtain sarcosine anhydride in the unsaturated form (79), the existence of the $-C=C-$ linkage in enolic dioxopiperazines seems to be established.

Methods were devised which also allow to distinguish between these tautomer compounds in a physical way. E. Abderhalden and R. Haas found that a number of amino acid anhydrides give a characteristic absorption in the ultraviolet, the enol form showing a more pronounced absorption than the keto form. It is important that some proteins also absorb in the ultraviolet. The interesting fact was found that the absorption spectrum of an amino acid depends on the method of its preparation. Thus alanine precipitated from its aqueous solution with alcohol behaves differently from alanine obtained by direct crystallization. Possibly this observation is related to that of E. Fischer (77) that the precipitation with alcohol influences the amino

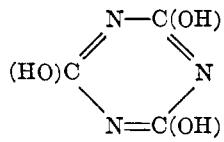
acid so that a preparation of the acid chloride is made possible, while no acid chloride can be made from an acid obtained directly from water. In one case, the enol-keto rearrangement could be observed spectroscopically in d,l-leucyl-glycine anhydride and it was found that the rearrangement is complete after 8 hours (78).

These results are interesting from two points of view. First the ultraviolet absorption of dioxopiperazines may be adduced as evidence for their occurrence in proteins. Secondly it was possible to show that there are labile enol modifications which show a measurable rearrangement.

However, it must be taken into consideration that according to W. Stenström and M. Reinhard (80) the ultraviolet absorption of the proteins is due to the aromatic amino acids. Y. Shibata and T. Asahina (81) also investigated the question of desmotropy of dioxopiperazines. Their investigations are based on those of J. N. Hartley (82). This author studied the possible desmotropy of cyanuric acid for which the following formulas were possible:



I.



II.

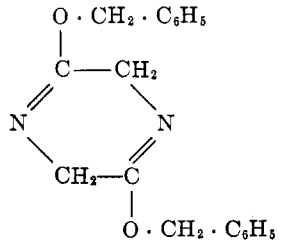
The second formula resembling to a certain degree the benzene nucleus, it was expected that the compound would show absorption in the ultraviolet. Since this was not the case, the conclusion was drawn that the enol tautomer does not exist. Shibata and Asahina prepared dioxopiperazines (glycine anhydride, alanine anhydride, sarcosine anhydride) by heating the corresponding amino acids in glycerol according to Balbiano and Trasciati (83) and Maillard (84) and none of the compounds studied showed an absorption spectrum; the conclusion was therefore drawn that they all exist in the keto form only. There seems to be a discrepancy between these observations and those of Abderhalden and Haas. In any case the continued research

into the desmotropy of dioxopiperazines with application of optical methods seems to promise interesting results.

Some interesting observations have been made on the dioxopiperazines which later will be possibly utilized for structural investigations. Thus the refractive index of the enol form was found to be higher than that of the keto form (85). The optical rotation of solutions of dioxopiperazines decreases under the influence of Röntgen rays and ultraviolet rays, while that of the corresponding peptides remains unchanged. Probably an oxidation takes place due to the formation of ozone under the influence of irradiation.

It is very important that the cleavage of enolic dioxopiperazines leads to unsaturated dipeptides (86). Glycyl-glycine prepared from enolic glycine anhydride behaves differently from that obtained from the keto form. It decolorizes permanganate in the cold, gives a positive xanthoprotein reaction, a positive ninhydrine and negative anhydride reaction and dissolves in aqueous sodium hydroxide giving intensely yellow colored solutions. The yellow color disappears on heating. A similar behavior is shown by the unsaturated form of d,l-leucyl-glycine, which may be prepared by splitting the corresponding anhydride or by heating ordinary leucyl-glycyl-leucine with diphenylamine to 200°. The two forms of leucyl-glycine show a different behavior toward alkali. While the saturated form is entirely unchanged in the presence of 0.2 *N* sodium hydroxide after a lapse of 15 hours, the unsaturated form is split quantitatively after 9 hours.

The observations made on the introduction of the acyl rest into benzylated glycine anhydride would also indicate that the enolic form of dioxopiperazines possesses a higher reactivity. While it was previously found that glycine anhydride must be heated with chloroacetyl chloride in the presence of nitrobenzene to 160° in order to effect a substitution, the dichloroacetyl compound results on heating the dibenzyl dioxopiperazine with chloroacetyl chloride at water bath temperature. According to Karrer (70) the dibenzyl compound is derived from an enolic form for which the following formula is suggested:



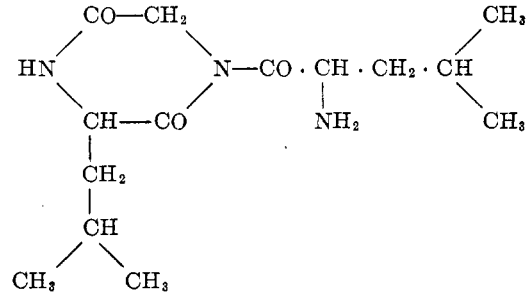
The double bonds are not in the position assumed by Abderhalden and Schwab. With view to these experiments it is not excluded that heating 2,5-dioxopiperazine in nitrobenzene likewise leads to an enol structure, possibly different from that which results when dipeptides are treated in diphenylamine, or dioxopiperazines in aniline.

The existence of labile polypeptides with enolic structure opens a new field of possible relations to amino alcohols, amino aldehydes, etc., and generally to the reactions of proteins in metabolism. It is not excluded that the view of the total degradation of proteins introduced into metabolism will have to be changed. Similarly it might be found that compounds other than amino acids are being absorbed in the gastro-intestinal tract. It is held now with view to the experiments with ferments that the stable modification of dioxopiperazines occurs in proteins which are resistant to the action of enzymes, while the labile enolic modification occurs in the proteins accessible to enzymatic cleavage. It is also possible that the denaturation of proteins by heat depends upon the transition from the enol into the keto modification (32).

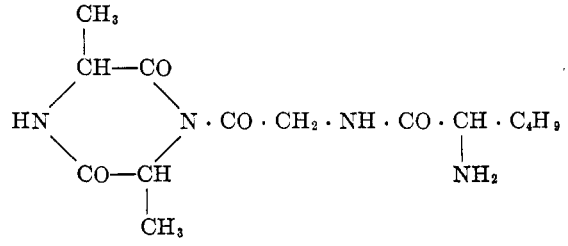
The researches which were carried out by L. Balbiano and later by L. C. Maillard and created new possibilities for the synthetic preparation of dioxopiperazines and their derivatives deserve mention in this place. Every such contribution to the synthesis is highly welcome to the investigator of proteins, since it opens urgently needed new sources of supply, the older methods of preparation of dioxopiperazines being none too convenient. Balbiano heated glycine in a sealed tube to 150–170° and ob-

tained a keratin-like compound which he regards as a polymer glycine anhydride $(C_2H_3ON)_x$. It is interesting that on heating glycyl-glycine with diphenylamine to 185–190° an extremely difficulty soluble compound results (88), which likewise is regarded as a polymer of glycine anhydride. Maillard studied the influence of hot glycerol on amino acids. According to his investigations the composition of the products depends upon the amount of glycerol. Thus either glycine anhydride or the tetrapeptide triglycyl-glycine may form. This formation of a tetrapeptide may have some bearing upon Bergmann's researches which will be dealt with later. In addition a number of different glycine combinations is obtained, e.g., the tripeptide diglycyl-glycine and the hexapeptide pentaglycyl-glycine. Likewise the formation of a polymer compound, possibly cycloheptaglycyl-glycine $(C_2H_3ON)_8$ is observed. This compound seems to possess remarkable properties. It dissolves in concentrated acids and gives upon dilution solutions which first stay clear and give a positive biuret reaction; on standing the solution becomes turbid and the biuret reaction becomes negative. Kaito Shibata (89) also reports the formation of dioxopiperazines on heating proteins with glycerol.

This method was used by Abderhalden and collaborators in the preparation of simple and mixed anhydrides (e.g., d,l-leucyl-glycine anhydride from leucine and glycine) (90). Heating of dipeptides with glycerol likewise leads to anhydrides (91). But it is more important that compounds were obtained synthetically which in all probability consist of combinations of anhydrides and amino acids and resemble those obtained by the degradation of proteins. Thus on heating of d,l-leucine with d,l-leucyl-glycine anhydride the leucyl-(glycyl-leucine) or leucyl-(leucyl-glycine) anhydride respectively is obtained. Similarly heating the tripeptide leucyl-glycyl-leucine leads to a compound to which the following constitution or its tautomer is attributed:



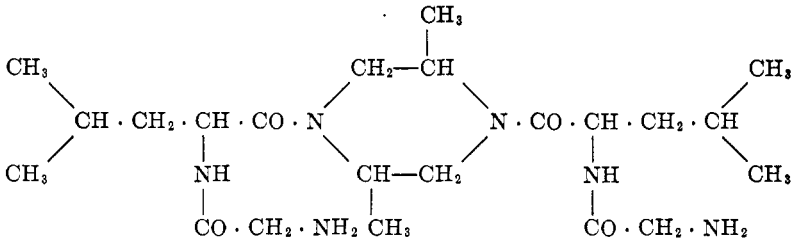
The same compound may be obtained from the methyl ester of leucyl-glycyl-leucine (91). The positive ninhydrine and anhydride reactions confirm its structure. The biuret reaction is negative. Also a dipeptide was brought into reaction with an amino acid anhydride. On heating alanine anhydride directly with leucyl-glycine in aniline to 200°, a compound with the probable structure:



was obtained. This compound also gives positive ninhydrine and anhydride reactions and no biuret reaction. In aqueous solution it behaves like a colloid since it first dissolves clearly and then flocculates on standing. It is intended to study the action of enzymes on compounds of this type.

It was mentioned previously that in addition to the dioxopiperazine ring, the occurrence of the piperazine ring itself was taken into consideration. Numerous experiments were carried out on the introduction of amino acids and polypeptide rests into the piperazine ring and its alkylated derivatives. The compounds thus obtained were exposed to the action of enzymes (92) but in no case a cleavage was observed. The formula of diglycyl-

leucyl-2,5-dimethyl piperazine prepared by Abderhalden and Kohl-Egger (93) is given here in order to convey a general idea of the structure of compounds of this group:



This compound is not attacked by yeast juice.

Abderhalden and coworkers attempted to find more data for the distinction of peptides and anhydrides in proteins in addition to the tests already mentioned (94). The adsorption experiments are very interesting. Solutions (0.05 *N*) of dipeptides and dioxopiperazines were shaken with animal charcoal for 15 minutes; 8.45 per cent of glycyl-glycine or 7.37 per cent of alanyl-glycine are removed by this treatment in contrast to as much as 38.93 per cent glycine anhydride and 37.66 per cent alanyl-glycine anhydride. Further it was found that precipitating agents act differently on amino acids, polypeptides and anhydrides. The comparative cleavage of peptones and proteins by alkali also gives noteworthy results. Experiments on the action of alkali and acid on dioxopiperazines and dipeptides with control of pH were conducted first by M. Lüdtke (95). Abderhalden and Haas showed (96) that the cleavage of dioxopiperazine is an equilibrium reaction:

$$\text{Dioxopiperazine} + H_2O \rightleftarrows \text{dipeptide}$$

Accordingly, it is possible to effect a shift either by addition of freshly precipitated $Cu(OH)_2$ which is bound by the free peptide, or by treatment with yeast juice which produces enzymatic cleavage of the peptide. While glycyl-glycine and leucyl-glycine are perfectly stable at pH 12.4, the corresponding dioxopiperazines are split at this and lower pH. The action of alkali on

silk peptone is accompanied by a slow decrease of pH, but simultaneously the strong picric acid and dinitrobenzoic acid reaction is considerably weakened. The same phenomenon is observed on casein Hammarsten, casein Osborne and beef blood serum. This points to the splitting of preformed dioxopiperazine rings while the alkali is neutralized by the carboxylic group of the peptide formed. There is also a close relation between the picric acid reaction, the formation of free NH_2 groups and the yield of oxamide (97).

It is perhaps desirable to mention in this connection the experiments of Fischer and Schrauth. These authors found that the ease of cleavage of a dioxopiperazine by alkali depends upon the amino acids which constitute the particular dioxopiperazine. While anhydrides with glycine are easily split, neither leucine anhydride nor valine anhydride are attacked by dilute alkali even on standing for 10 days at 37°. In accordance with these findings tyrosine anhydride is split much more difficultly than glycyl tyrosine anhydride. With view to the objections to the dioxopiperazine theory which will be dealt with later it might be interesting to subject one of the proteins from which leucine anhydride was isolated by enzymatic or acid hydrolysis (e.g., hemoglobin) to hydrolysis with weak alkali and establish the presence of this anhydride either by direct isolation or by reduction to diisobutyl piperazine. Such procedure would exclude the possibility of the secondary formation of this anhydride (99). Of course, it would be necessary to establish that enolic leucine anhydride is not attacked by alkali much more easily than the keto form investigated by Fischer and Schrauth. These considerations of the dependence of cleavage upon the constitution of the anhydride should be kept in mind when the problem is discussed, whether the acidity of the stomach or the alkalinity of the intestines are liable to effect the cleavage of dioxopiperazines.

Substitution of dioxopiperazines changes also their resistance to alkali. Thus O,O′-diacetyl-glycine- and -alanine anhydrides are not split by a boiling 5 *N* solution of alkali while the corresponding N,N′- compounds are completely hydrolyzed (100).

In reviewing the dioxopiperazine theory we may say that considerable experimental evidence has been adduced in its favor. On the other hand it was also shown how easily a secondary formation of dioxopiperazines takes place and how careful the investigator must be in the elimination of these possibilities. A modified dioxopiperazine theory based entirely on synthesis is suggested by Bergmann and his collaborators. This will be dealt with later in conjunction with the other theories which are built up on synthetic experiments.

We mentioned before that on the basis of researches into the polysaccharides it was assumed that associations and aggregations of elementary complexes are present in the proteins. However, this is to be regarded as a working hypothesis which cannot be generalized as yet although sporadic experiments are known in which simple compounds polymerize and the polymers show a behavior similar to that of proteins. The conception of proteins as aggregated complexes seemed to be so convincing that the existence of specifically acting disaggregating enzymes was first assumed by Oppenheimer (101). Pepsin was supposed to act in a disaggregating manner only by dissolving the subordinate valences which hold together the elementary complexes. It was regarded as a non-hydrolyzing ferment. This assumption could not be corroborated by experiment. It was also mentioned that a true valence linkage between anhydrides and amino acids was thought of as possible.

We here wish to discuss briefly the experiments which can be adduced in favor of the existence of associated compounds. It was shown first by Pfeiffer (102) that amino acids and polypeptides are capable of forming molecular compounds with neutral salts, e.g., NaCl, $CH_3 \cdot NH \cdot CH_2 \cdot COOH$, H_2O or LiBr, $NH_2 \cdot CH(CH_3)$-$COOH$, H_2O or $ZnCl_2$, $2NH_2 \cdot CH_2COOH$, $2H_2O$, etc. He also showed in collaboration with Angern (103) that not only proteins of high molecular weight but also amino acids are salted out easily, this behavior being independent from the solubility of the amino acid in water. Abderhalden and Sickel (104) observed the formation of mixed crystals of amino acids. Particularly important are the experiments by Pfeiffer and Angern

(105) which show that dioxopiperazines are capable of forming molecular combinations with amino acids, various salts and organic ompounds, e.g., glycine anhydride, 2LiCl, $2.5H_2O$. Sarcosine anhydride gives molecular compounds with tryptophane scatole, anthranilic and p-aminobenzoic acids.

3. The experiments of Waldschmidt-Leitz

An extremely important criterion for the validity of an assumed structural formula is the enzyme test. It is well known that the enzymatic cleavage is very specific and depending upon fine configurational particulars. It indicates that a certain configuration but not that a particular compound occurs in nature (106). The attempts to split dioxopiperazines by enzymes were so far entirely unsuccessful (107). Levene and Meyer found that glycine anhydride is eliminated unchanged in the urine, in contrast to glycyl-glycine, the nitrogen of which is completely eliminated as urea. Abderhalden and Goto observed that a cleavage of 2,5-dioxopiperazines does not take place with pepsin. No enzymatic cleavage of 2,5-dioxopiperazines was observed by Waldschmidt-Leitz and Schäffner (108).

One may expect that the researches of Waldschmidt-Leitz and his collaborators on the separation of enzymes will contribute to the elucidation of the question of the protein structure.

It is well known that the foundation of Fischer's peptide theory, i.e., the conception that the CO·NH linkage is the characteristic linkage of proteins was strengthened particularly by the fact that synthetic peptides were split by proteolytic enzymes. The investigations of Willstätter and his collaborators on the purification and characterization of enzymes made a further progress in this direction possible.

In continuation of Willstätter's researches on the application of fractionated adsorption Waldschmidt-Leitz and Harteneck (109) succeeded in separating trypsin and erepsin the two proteolytic enzymes of the pancreas. Similarly a separation of trypsin and erepsin of the intestines could be effected (110). It could be proved that the two enzymes possess a highly specific action, since all simple dipeptides are split by erepsin of both the pan-

creas and the intestines, while proteins are not attacked by this enzyme. On the other hand trypsin attacks proteins. According to Waldschmidt-Leitz, the following specific enzyme groups must be distinguished (118): 1) erepsin, 2) trypsin not activated, 3) trypsin activated (trypsin + enterokinase), 4) pepsin.

The following table shows the differential behavior of erepsin and trypsin:

SUBSTITUTE	ENZYME			
	Erepsin from		Trypsin	
	Intestines	Pancreas	Activated	Not activated
Alanyl-tyrosine	+	+	−	−
Glycyl-tyrosine	+	+	−	−
Glycyl-glycine	+	+	−	−
Leucyl-glycine	+	+	−	−
Leucyl-alanine	+	+	−	−
Glycyl-alanine	+	+	−	−
Leucyl-glycyl-glycine	+	+	−	−
Peptone (Merck)	−	−	++	+
Clupein (herring)	−	−	++	+
Thymus histone	−	−	+	−
Casein	−	−	+	−
Fibrin	−	−	+	−
Gelatin	−	−	+	−
Gliadin	−	−	+	−
Zein	−	−	+	−
Egg albumen	−	−	+	−
Rhicinus globulin	−	−	+	−

These results are in contrast with those of Fischer and Abderhalden (111) who distinguished between dipeptides which are hydrolyzed by enzymes and those which are not (112). According to Waldschmidt-Leitz, there is no such differentiation among the dipeptides, provided their configuration justifies the assumption that they occur in nature. Similarly all statements on the cleavage by erepsin of peptones, protamines, histones and casein must be revised. The complete hydrolysis of proteins is not feasible with a combination of two enzymes out of the four groups mentioned in contrast to older statements of Fischer and Abderhalden who assume that some enzyme groups may mutually

replace each other (113), or to the researches of Henriques and Gjaldbaek who find that an exhaustive treatment of casein with trypsin may replace the action of pepsin (114). Of course, the contrary results obtained by the older investigators are due to the impurity of enzymes used which represented mixtures with varying amounts of components. The action of enzymes is independent from the order of their application. E. Waldschmidt-Leitz found that the three enzyme groups, trypsin, trypsin + enterokinase, erepsin may be substituted for each other to a certain extent, although the enzymatic action of each is well defined. This was observed by following up the cleavage of casein effected by pepsin and erepsin-free trypsin. The progress of cleavage was estimated by the increase of the number of free carboxyl groups (115). The peptic action could be observed after the tryptic action had taken place. On the other hand when the hydrolysis is carried out with trypsin and erepsin combined, there is no substrate left which would make the peptic action visible. Under the action of pepsin and similarly under the action of non-activated trypsin, the protein molecule is split into large parts. This is indicated by a slight increase of the number of carboxyl groups. The activated trypsin on the other hand produces free amino acids from the protein. The cleavage of proteins by non-activated trypsin shows that trypsin does not occur in an inactive form as a zymogen which is activated by enterokinase. It is rather to be assumed that the activator enterokinase acts as an auxiliary substance for the cleavage of certain compounds (116). It was shown in a recent paper that the composition of the products of cleavage of clupein depends upon the sequence of the enzymes. But whatever enzyme is taken into consideration the cleavage is always one of the CO·NH-linkage. In no case a disaggregation into elementary complexes was observed (117).

These conclusions are in a certain contrast with the results obtained by H. Steudel and collaborators (117a). If the action of a proteolytic enzyme always depends upon the dissolution of the regular CO·NH-linkage, the amount of amino and carboxyl groups should always be the same, regardless of the method of

determination. However, when certain proteins (casein, serum globulin, serum albumin and gluten casein) were subjected to peptic cleavage the number of carboxyl groups formed was several times greater than that of the amino groups. It is impossible as yet to give a definite explanation of this phenomenon It might be caused by the cleavage of linkages different from the CO·NH-linkage, e.g., the ester linkage between a carboxyl group and the hydroxy group of a hydroxy amino acid. However, this would not account for the increase of amino nitrogen. Another explanation would be that groups are freed by peptic cleavage which are less basic than the α-amino group of the simple amino acids, since it is known that only a fraction of the nitrogen of some amino acids (e.g., tryptophane, arginine, lysine) is indicated by the method of Van Slyke.

Judging from the fact that the splitting action of individual enzymes stops at certain intermediate stages of the protein hydrolysis, one is justified in assuming that it will be possible by this method to realize the fractionated hydrolysis of proteins which will permit the isolation of biologically defined elementary complexes. Thus the investigations of Waldschmidt-Leitz corroborate the original idea of true valence linkages without leaving room for the conception of aggregates which are separated into smaller parts by enzymatic action. His objections to the dioxopiperazine theory are based on the same principles. He contends that altogether too much importance is attributed to the possibility of occurrence of dioxopiperazines (118). It is admitted that the anhydride structure may play a rôle in proteinoids like silk or certain skeleton constituents which are resistant to enzymes. But since no cleavage of 2,5-dioxopiperazines is observed under biological conditions (they also are very difficultly split at the pH of the gastro-intestinal tract) it is not to be assumed that they generally occur in proteins. Similar considerations hold for the other suggested cyclic structures, particularly those of Troensegaard and Bergmann.

It seems that this general statement goes too far, since it is based only on the action of enzymes on the simplest dioxopiperazines. As long as we do not have any information on the enzyme

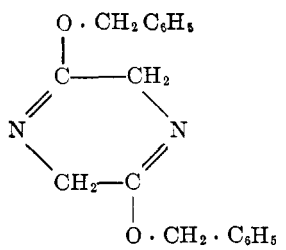
action on the enolic tautomers of both dioxopiperazines and peptides, peptide-anhydride combinations and the other assumed ring structures, it is as yet unjustified to deny their occurrence in proteins. As the interest of the protein chemists is focussed on these researches, it is to be expected that a decision will be reached in the near future.

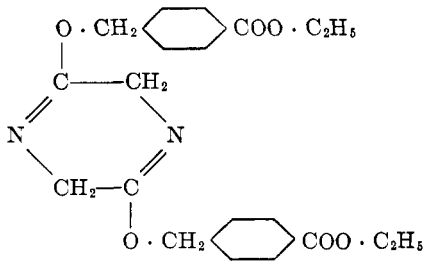
In this connection attention should be paid to the experiments of Levene and coworkers (119) on the action of enzymes on peptides and the circumstances influencing the formation of anhydrides.

4. The synthetic heterocyclic compounds

We still have to deal with the interesting synthetic experiments of Karrer, Bergmann and their collaborators. These investigators attempt to approach the problem in a purely synthetic manner by preparing compounds of given structures which might possibly occur in proteins.

It was previously mentioned that Karrer first pointed to the possibility of existence of enolic forms of dioxopiperazines (70). The pertaining experiments were first carried out with dibenzyl dioxopiperazine derivatives, which were obtained by the action of benzyl chloride and ω-chloro-p-toluic acid ester respectively on the silver compound of glycine anhydride and have the formulas (120):

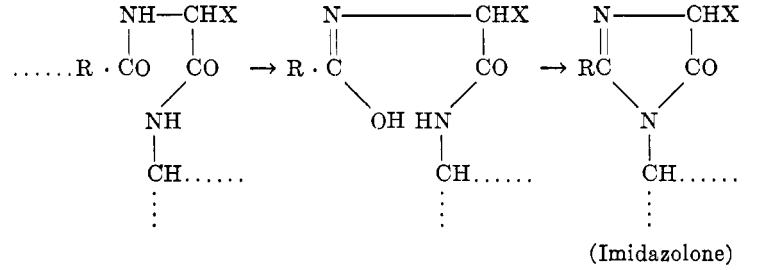




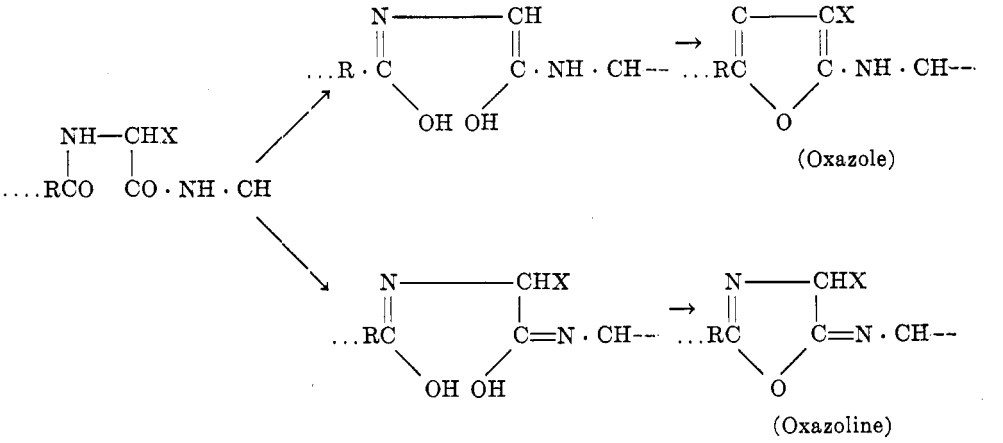
These compounds are particularly interesting with view to their extreme sensitiveness to dilute acids; dilute hydrochloric acid effects a cleavage with formation of glycine and benzyl chloride or the ω-chloro-p-toluic acid respectively. Karrer and coworkers contemplate also other possibilities of formation of anhydrides out of peptides, which are represented by the following equations.

They are characterized by the assumption of enolic rearrangement:

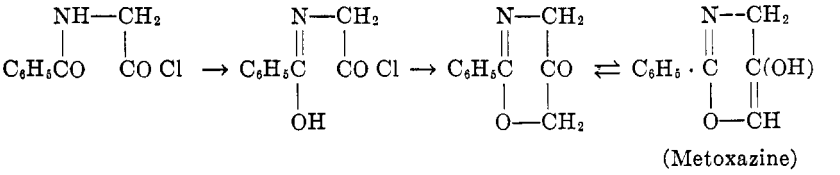
(1)



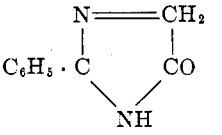
(2)



(3)

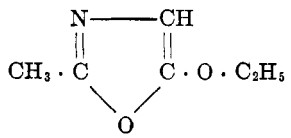


The formation of an imidazolone compound according to 1.) may be seen on the reaction of the hippuric acid amide with PCl_5. Here the 2-phenyl glyoxal-5-one is obtained:



which is decomposed by acids with formation of hippuric acid.

The formation of oxazoles according to 2.) was observed in many cases. The formula of 2-methyl-5-ethoxyoxazole may serve as an example of the compounds of this group:



Phenylhydroxydihydrometoxazine (3) is obtained by the action of diazomethane on hippuryl chloride.

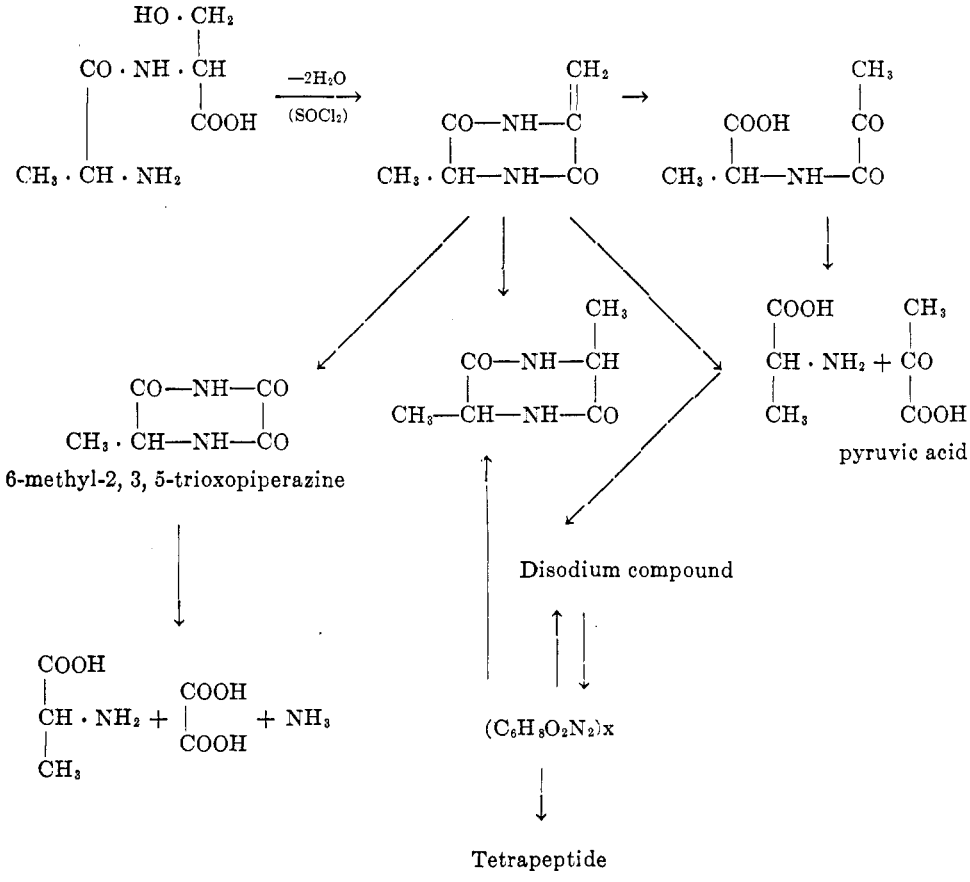
The imidazolones, oxazoles and metoxazines are likewise extremely sensitive to acids. In some cases an acid concentration which is necessary for the activation of pepsin is sufficient to effect the cleavage. If these compounds were built entirely of amino acids, this treatment would lead to peptides. The formation of the ring takes place the more easily the lower the fatty acid, which is combined with the amino acid (121).

The results obtained by Karrer are no doubt extremely interesting, particularly the observation that compounds related to peptides may be obtained by the cleavage of certain ring structures at an extremely slight acid reaction, such as is found in the stomach. Since these researches are not yet directly applicable to the conditions prevailing in natural proteins, it is very desirable that attempts be made to synthetize these ring structures entirely out of amino acids and to study these under conditions which prevail in the gastro-intestinal tract.

5. The iso- and allodioxopiperazines

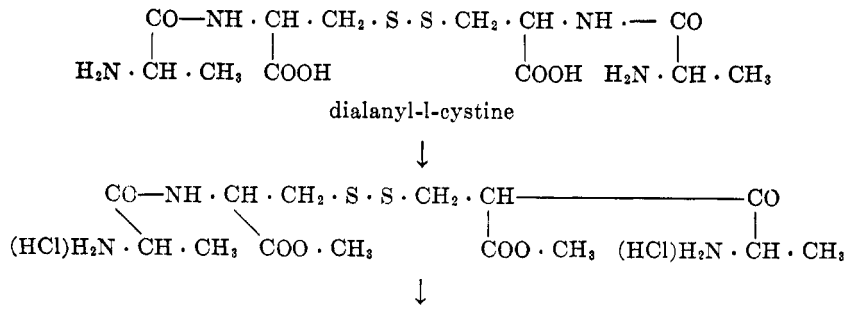
A series of important synthetic investigations was carried out by Bergmann and his collaborators. In continuation of the studies on the rearrangement of compounds which show a behavior similar to peptides they succeeded in isolating compounds which were regarded first as oxazoline derivatives. Later they were recognized as dioxopiperazine derivatives. In contrast to the other dioxopiperazines these compounds show a tendency to polymerization (122). Bergmann showed in collaboration with

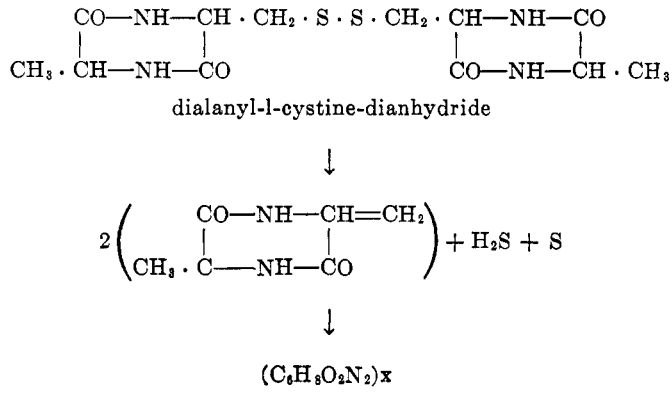
Miekeley and Kann that these compounds are formed from ester chlorides of the dipeptides glycyl-and alanyl-serine on treatment with thionyl chloride. They show a peculiar behavior on treatment with acids and alkalis. With acid one-half of their nitrogen content is split off as ammonia with formation of pyruvic acid. On treatment with alkali and subsequent neutralization polymerization takes place with formation of compounds which are insoluble in water and other solvents. The series of reactions which are involved in these changes may be illustrated by the following equations showing the changes to which alanyl serine may be subjected:



This scheme of reactions shows the following: The catalytic hydrogenation which leads to alanine anhydride proves that the assumed formula of a methylene dioxopiperazine is correct. It also shows that there is a clear relation to pyruvic acid to which an important biological rôle is attributed. Solution in alkali leads to a yellow compound from which a polymer compound is precipitated with acid. This reaction is reversible. The polymer compound yields on acid hydrolysis a tetrapeptide. This would suggest the possibility of four amino acids constituting this compound without any piperazine nuclei. However, this assumption cannot be made since this same polymer gives on reduction alanine anhydride, i.e., a compound with two amino acids only. The determination of molecular weight in phenol corresponds to the formula $(C_6H_8O_2N_2)_2$ (mol. weight 280). However, this is not necessarily the true molecular weight, but possibly that of elementary complexes disrupted by the action of phenol.

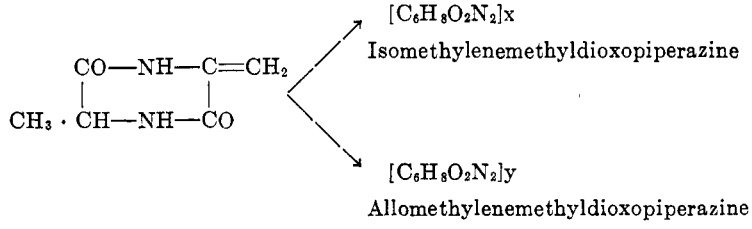
Bergmann and Stather (123) obtained the same compound also in a different way. They started with cystine which according to known methods was transformed into dialanyl-l-cystine and the corresponding diester. From this the new dialanyl-l-cystine dianhydride was obtained. This compound is insoluble in water, but dissolves quickly on addition of alkali. When the solution is neutralized with acid a crystalline product is obtained and simultaneously sulfur and hydrogen sulfide form. The crystalline compound has the composition of a polymer 3-methylene-6-methyl-2,5-dioxopiperazine which is obtained from alanyl serine as described before. The reactions which lead to the formation of this compound are:





It is remarkable that the formation of the two anhydrides in cystine so increases the lability of the sulfur atoms that 71 per cent of the sulfur of that compound is split off, while the $-C-S-$ linkage in dialanyl cystine remains entirely intact under the same conditions. It seems that this reaction is not limited to serine and cystine derivatives but is shown as well by other amino acids containing no hydroxyl or sulfur groups.

In addition to the polymer isodioxopiperazines, another polymer modification has been prepared recently by Bergmann, Miekeley and Kann (123a). They found that it is sufficient to heat methylenemethyldioxopiperazine with a dilute aqueous solution of arginine, to obtain the "allo-form" which is different from the "iso-form" previously obtained. Other weak bases (ammonia, guanidine, etc.) act similarly. Treating with concentrated hydrochloric acid leads to a tetrapeptide which is identical with that obtained from the "iso-form."



Particular importance is attributed to this finding, since it is shown for the first time that the anhydride of two amino acids

which frequently occur in native proteins may be transformed into an isomer form in aqueous solution by an amino acid which likewise occurs in natural proteins. This in turn permits the conclusion that the allodioxopiperazines themselves occur in natural proteins.

The allo-form is chemically very interesting. Upon treatment with formaldehyde a compound results to which the designation monoformal-methylenemethyldioxopiperazine is given. This compound swells in the presence of water, forms a jelly, causes no noticeable depression of the freezing temperature and gives on drying in thin layers films which may be made sensitive to light on treatment with chromate just like gelatin films. It is concluded that compounds which show a colloidal behavior similar to that of gelatin, do not necessarily have to consist of a great number of different amino acids.

In contrast to the ordinary dioxopiperazines (and dibenzalpiperazine) which bind only a small quantity of tannin, the iso- and allodioxopiperazines possess an outspoken capacity to absorb both tannin and dyestuffs (malachite green, acid fuchsin) similar to some natural proteins. The following table illustrates this behavior. The results were obtained by the colorimetric comparison of solutions originally containing a known quantity of malachite green which were shaken with a given quantity of the compound.

Compound	*Adsorption per cent*
Glycine anhydride	0.9
Methylenedioxopiperazine	2.2
Isomethylenedioxopiperazine	35.6
Allomethylenedioxopiperazine	16.7
Methylenemethyldioxopiperazine	6.3
Isomethylenemethyldioxopiperazine	20
Allomethylenemethyldioxopiperazine	60.3
Silk fibroin	100
Zephir wool	97.6
Benzaldehyde compound of methylenedioxopiperazine	98.3

This "super molecular" state of certain dioxopiperazines seems to be related to the state of some natural proteins (gelatin, silk fibroin gliadin). Also the results of the determination of molec-

ular weight of these proteins (200–400) would fit into this conception. Like proteins the iso- and allodioxopiperazines bind dyestuffs and tannins. It is emphasized that the stabiliy of these substances depends upon the presence of the methylene group, while the stability of dioxopiperazines of the proteins in case these are actually present, is probably brought about in a different manner.

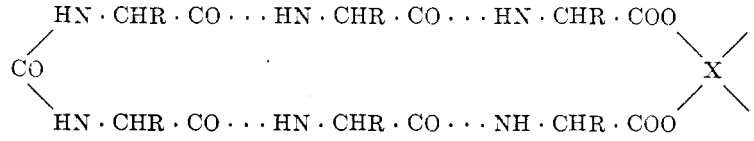
Bergmann's investigations lead to a new conception of the structure of the compounds of high molecular weight (124). The following comparison is used to illustrate these conditions. When a compound enters into the crystallized state, the individual molecules cease to exist since they are now connected with each other by lattice forces. Thus a crystal is to be regarded as a kind of supermolecular structure, it being possible to restore the molecular subdivision through destruction of the crystallized state by vaporization, solution or liquefaction. This supermolecular state is not a structural constant but a form of a state which is determined by the surrounding physical and chemical conditions. Similarly the conclusion that a protein appears to be of a high molecular weight is drawn as a rule from its behavior toward water, although it may be "molecularly disperse" in phenol. Thus the forces which bring about this phenomenon are comparable to the lattice forces, the difference being only a quantitative one. On the other hand, the hydrogenation of the polymer isodioxopiperazines leads to compounds with six carbon atoms while acid hydrolysis and the determinations of molecular weight of the same substance in phenol point to the existence of compounds with twelve carbon atoms. This fact would suggest that there is no sharply defined elementary complex which would first come into appearance in all degradations. This seems to be characteristic of all proteins. There is another resemblance between these polymer isodioxopiperazines and the proteins. The fact that polypeptides result on hydrolysis of proteins makes the assumption possible that the degradation does not lead over the dioxopiperazines first. It is thinkable that the disaggregation by ferments leads directly to polypeptides which are further

split by another enzyme group, just as the acid hydrolysis of the isodioxopiperazines leads to tetrapeptides.

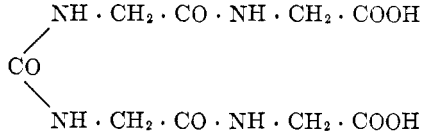
This interesting theory of Bergmann also requires biological corroboration. In its present formulation it would make appear the protein problem clear and reduced to comparatively simple basic ideas.

6. The ureide theory

Finally the ureide linkage is to be mentioned, the occurrence of which is suggested by Brigl and Held (53). These authors base their assumption on the fact that neither the pure peptide nor the dioxopiperazine theories account for the relation of oxygen to nitrogen being in most proteins considerably higher than 1, while according to either theory it should be approximately 1. Therefore, the hypothesis is advanced that proteins contain polypeptide chains of varying lengths which are connected with each other by means of groups containing oxygen. It is possible that they possess ureide structure according to the schematic formula:



The possibility of an ureide structure was discussed repeatedly in the chemical literature (125). Brigl and Held used as a model for their investigation the diureide of the dipeptide glycyl glycine:



The results obtained by fusion with phthalic anhydride, which was suggested by Brigl and Klenk (126) as a possible reagent for the isolation of definite compounds out of proteins and by the application of the previously mentioned hypobromite method of

Goldschmidt and Steigerwald do not disagree with the assumption of the ureide structure of proteins. The experiments with enzymes (pepsin and trypsin), however, gave entirely negative results. It is possible that ureides of other peptides will behave differently.

It is not desired to carry further our presentation of the researches into the structure of proteins. Although it was intended to illustrate the different phases and branches of development as completely as possible this paper does not by any means contain a complete review of all the work that has been done in this realm.

It is obvious that the research into the structure of the proteins is in a constant flux at the present time. There are radical theories which regard even amino acids as secondary products and there are others which view amino acids and polypeptides as elementary compounds related directly to the various cyclic structures. It is impossible at the present time to unconditionally accept any one of the theories described. They all have their weak and strong points. It is possible that the near future will bring fundamental developments in this realm. New facts may become known to which one or another conception may fall prey. Nevertheless a compilation of pertaining facts and ideas at this, as it seems, critical point, may involve psychological and educational interest, since it allows an easier contraposition of the state of scientific progress in given periods.

In considering the possibilities of future development, we believe that the investigator who tries to elucidate the riddles of protein structure is entitled to the same optimism which guided Emil Fischer in his work, as pronounced in a statement, part of which may be given in free translation:

> Nature accomplished her highest achievement in building up proteins and their various derivatives. The belief that she confined herself to only a few types would disagree with all experiences of chemistry and biology. . . . .
>
> If by a lucky accident it should become possible today to prepare a

genuine protein by some brutal reaction, e.g., by fusing amino acids in the presence of a dehydrating agent, and if it were possible to identify this artificial product with a natural one, only little would have been achieved for the chemistry and practically nothing for the biology of proteins. . . . . I should feel inclined to regard it as good luck, that chemistry is forced to create numerous new methods of synthesis, idenfication and isolation and to closely study hundreds of intermediate products, before it solves the protein problem. For ultimately these methods will not only serve in the preparation of all proteins of nature and of more than nature created. They will probably suffice to elucidate the numerous and remarkable products of transformation of proteins which play such an important rôle as enzymes, toxins, and others.

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