CHEMISTRY AND BIOLOGY OF Proteins

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FOREWORD

When the author was invited to write a book on Proteins, he was hesitant for a long time to embark upon such a difficult project. What finally induced him to write this book was a request by Indiana University to give a one-semester course on proteins for graduate students in the College of Arts and Sciences. No suitable modern textbook on proteins was available. The few good textbooks on proteins written before 1940 were out of print. It was the author's first task, therefore, to write a textbook which would be understandable to graduate students of chemistry and of the biological sciences.

It has been attempted, moreover, to give a *uniform outline of the present state of the protein problem.* Although the chemistry and the biology of proteins is the subject of many thousands of investigations, the results of experimental work have rarely been correlated systematically with each other. True, each experiment in the protein domain opens new questions and we are still far from a definite solution of the protein problem. Nevertheless, it was considered possible and useful to incorporate the results of both old and recent experiments into a unified concept of the nature of the protein molecule and of its reactions and functions.

Completeness cannot be attained in a book of this kind. In writing the text the needs of those interested in the *fundamental aspects* were kept in mind. Therefore the main topics discussed are (1) protein structure, (2) biological activity of proteins, and (3) biosynthesis of proteins in the living cell. The fundamental importance of the structural problem need not be stressed. Likewise all will agree that protein synthesis is one of the main problems of the biological sciences, inasmuch as growth and reproduction consist essentially of the formation of new protein. Many readers, however, may wonder at the large space devoted in this book to enzymes, hormones and immunologically active proteins. To them it must be recalled, therefore, that the most stable proteins are found among the enzyme proteins, that these enzymes crystallize with particular ease, and that they are more uniform than many of the proteins which lack biological activity. The same is true for many of the hormone proteins. Antigens and antibodies, on the other hand, have been treated in some detail because antibody synthesis is, essentially, a modified protein synthesis; antigens, consequently, are valuable tools

which can be used to modify the normal course of protein synthesis. During the last few years a large number of biologically active proteins has been discovered in protein fractions which previously had been regarded as inactive; obviously biological activity cannot be detected unless suitable methods of assay are available. There is no doubt, therefore, that many more biologically active proteins will be discovered in the future.

In a monograph on proteins it is difficult to draw a definite border line between the fundamental and the less important aspects of the problem. It was felt, however, that the technology of proteins could not be treated in this book. Likewise, an account of protein metabolism in different species of the animal and vegetable kingdoms would surpass the limits of this treatise. Readers desirous of orienting themselves in special problems of protein metabolism are referred to numerous review articles in *Advances in Protein Chemistry* and *Annual Reviews of Biochemistry*. Advanced students interested in details of the physical chemistry of proteins will find a wealth of information in the classical book of Edwin J. Cohn and John T. Edsall on "Proteins, Amino Acids and Peptides" (Reinhold Publishing Corporation, New York, 1943).

While the customary textbooks do not endeavor to give an extensive bibliography, it has been attempted to substantiate each statement by quoting the original sources. The numerous references given were not collected *ad hoc*, but were selected from data assembled by the author through many years. By the citation of approximately 1500 original papers the book goes well beyond the usual limits of a textbook. It is hoped that the numerous references will render the book valuable to research workers in biochemistry and in related fields.

When the manuscript of this book was completed, the author felt the necessity of submitting some of the chapters to experts in the topics treated, and to ask them for their criticism. An appeal to several colleagues in protein research resulted in a response which surpassed by far the author's expectations. Dr. Henry B. Bull, Professor of Physiological Chemistry, Northwestern University, Dr. John T. Edsall, Professor of Biochemistry, Harvard Medical School, and Dr. Hans Neurath, Professor of Physical Biochemistry, Duke University, kindly offered to read the whole manuscript, and to send to the author their written comments. Because of this generous help, use could be made of a series of most valuable critical remarks and suggestions, and many pitfalls could be avoided. The author is deeply indebted to Dr. Bull, Dr. Edsall and Dr. Neurath for their invaluable assistance and for the many hours spent on the improvement of his book. The author also wishes to express his best thanks to Dr. Choh H. Li, Professor of Experi-

mental Biology, University of California, for comments on the chapter on hormones.

The author's particular thanks, however, are due to Mr. Charles F. Crampton, Department of Chemistry, Indiana University, who had the difficult task of reading the first draft of the manuscript and of testing it for style and for clarity of presentation. Many sentences and paragraphs were remodelled with Mr. Crampton's help, and several gaps in the deductions were discovered and closed.

In spite of the ample and generous aid of so many colleagues it is unavoidable that the book should contain errors. It is hoped that these are neither too numerous nor too serious, and that the readers will call them to the attention of the author. Obviously none of the above-named helpers is to be blamed for these errors. The only person responsible for them and for the mode of presentation is the author.

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CHAPTER I

Role of Proteins in Biology

Proteins have a unique significance in biology in that they compose the *indispensable substrate of living matter*. To be sure, living organisms also contain carbohydrates and lipids, frequently in even greater abundance than proteins. Thus, green plants are rather poor in proteins, but rich in cellulose, a carbohydrate. There are, however, essential differences between proteins and the other cellular constituents. Wherever the phenomena of *growth* and *reproduction* are seen, proteins are primarily involved. In nucleated cells multiplication is initiated by the nucleus, which contains proteins closely associated with nucleic acids. In bacteria, where there is no visible nucleus, the proteins and nucleoproteins form the bulk of the living substance. Still further down in the order of organisms, we find that viruses consist mainly of proteins and nucleoproteins and that the simplest of them are free of lipids and carbohydrates.

In addition to their role in the functions of growth and multiplication, another important feature of the proteins is their *specificity*, which distinguishes them from other cellular constituents. While we do not know of species-specific lipids, we do know that the proteins of each species of the animal or plant kingdom, and even the proteins of bacteria and of viruses, are typical for the particular species. In some cases we are even able to find variations among the proteins of individuals of the same species. Evidently, proteins must be considered the essential agents in the transmittal of hereditary traits.

A third characteristic property of proteins, in which they differ from other compounds present in the cell, is the phenomenon of *denaturation*. Native proteins are denatured by the same physical or chemical agents which kill or injure living cells. However, denaturation is caused not only by heat, acids or alkalis, and other drastic treatments, but also by the action at room temperature of aqueous solutions of urea and of similar "innocuous" substances. The obvious conclusion to be drawn from such behavior is that the structure of the native protein molecules is so unstable that they are even susceptible to alteration by the impacts of dissolved urea molecules. None of the other cellular constituents exhibits a similar lability.

Proteins have various *functions* in the cell and in organisms. Some of them compose such inert materials as hair, horn, or bones. Also the *contractile substance* of the muscle fiber is a protein. It is somewhat

remarkable in that it possesses the capacity to transform chemical energy into mechanical energy. It is thus responsible for the motility of the higher organism. Similarly, in the lower organisms, the movement of flagellae, the coordinated stroking of cilia, and ameboid movement are all ascribed to contractile protein particles. Serving other functions, the *enzymes*, the highly important catalysts of living organisms, are proteins, as are also the respiratory pigments, the *hormones* of the pancreas, the thyroid, and the pituitary gland, the *antibodies*, and the *toxins* of certain bacteria. It is evident that the importance of proteins for the living organism can scarcely be overemphasized.

It is clear, moreover, from the multiple functions of proteins and from their lability that their molecular structure is, in all probability, extremely complicated. We are far from being able to describe the details of this structure. Only in a few instances has it been possible to gain an insight into the arrangement of portions of the protein molecule. The greatest deficiency in our knowledge of proteins lies in our inability to correlate the enzymatic or the hormonal functions of certain of them with a definite chemical arrangement. We do not yet know what molecular groups are responsible for the proteolytic power of crystalline trypsin or for the hormonal activity of crystalline insulin.

These shortcomings in our understanding of proteins are closely connected with the fact that the properties of a protein depend not only on its chemical composition, on the kind and order of the amino acids forming the peptide chains, but also on the *spatial arrangement* of these chains. The properties of a molecule consisting of a closely folded, long peptide chain are quite different from those of the same molecule containing the same peptide chain in an expanded state. In other words, the properties of a protein molecule depend to a large extent on the three-dimensional structure assumed by its peptide chains. In contrast to most other threadlike macromolecules the peptide chains of the proteins are able to maintain their specific constellation over long periods of time.

The specific arrangement of the peptide chains is of the greatest importance for the properties of the protein macromolecule. The solubility of proteins, their serological behavior, their enzymatic and hormonal activities, and many other biological properties depend on the molecular groups present in the surface of the protein molecule, and hence on the spatial arrangement and the mode of folding of the peptide chains. It is, therefore, one of the principal endeavors of protein chemistry to elucidate the *internal structure* of the protein macromolecule and the distribution of functional groups within the large protein molecule. Only in this way will we be able to correlate the biological functions of the proteins with definite molecular structures.

CHAPTER II

Isolation, Purification, and Determination of Proteins

A. Methods of Isolation. Since most proteins are extremely sensitive to heat, acids, bases, organic solvents, and, in some instances, even to distilled water, the methods generally employed for the isolation of other types of organic compounds can hardly be applied in protein chemistry. The *insoluble cellular proteins* can easily be prepared by extracting the cell with water and with organic solvents to remove fats, carbohydrates, and the soluble proteins. Since some of these soluble proteins are dissolved only in the presence of neutral salts, an extraction by dilute solutions of NaCl or sodium bicarbonate is frequently necessary. If the insoluble protein is resistant to proteolytic enzymes a further purification is achieved by treatment with pepsin at pH 1–2 or with trypsin at pH 8–9. In this way keratin, the insoluble protein of cornified tissues such as horn and hair, can be prepared.

Owing to their insolubility, keratin, collagen, fibroin, and other similar proteins cannot be purified by crystallization. It is impossible to ascertain, therefore, whether the preparations obtained by the usual methods are of a uniform nature or are mixtures of several different proteins.

The isolation and purification of *soluble proteins* involves their extraction from the cells by suitable solvents and their precipitation by altering the concentration of salts and/or of hydrogen ions, or by adding organic solvents. In many instances it has been possible to obtain *crystalline* precipitates by these procedures.

In order *to avoid denaturation* of the protein to be isolated one must work at low temperatures, since solutions of many proteins are subject to denaturation even at room temperature. This is especially true for solutions of proteins in salt-free water. The rate of denaturation is reduced by the addition of neutral salts. For example, crystalline trypsin can be stored in a saturated solution of magnesium sulfate (1). The rate of denaturation is also reduced by storing the protein solution at *low temperatures*. Refrigerators, refrigerated centrifuges, and cold rooms, therefore, form an important part of the equipment required for the preparation of proteins. At low temperatures a second important danger is also reduced, that of bacterial decomposition. Protein solutions form an excellent nutritional medium for bacteria and invariably become infected and are destroyed if kept at room temperature. Owing

to the thermolability of protein solutions, they may not be sterilized by heat. Bacterial infection and growth can be inhibited by the addition of disinfectants, but these disinfectants form compounds with the proteins and alter their physicochemical properties. Most of the common disinfectants are denaturing agents and, therefore, cannot be used in preparing native proteins. Bacteria can be removed from protein solutions by centrifugation at high speed or by filtration through Seitz or Berkefeld filters or through filter pads.* The disadvantage of the filtration is that small portions of the protein are adsorbed to the porous filter mass and are lost by denaturation (3). Thus, the adsorption of gliadin to fritted-glass diaphragms was proved by measuring the electrokinetic potential of the glass diaphragms (3).

The best method for storing protein solutions over long periods of time is to keep them in a deep-freeze unit at approximately $-10°C$. At these temperatures bacteria cannot multiply, making it unnecessary to sterilize the solutions. Antibody solutions and enzyme solutions have been stored in the author's laboratory in the frozen state for many months, some of them for many years without any appreciable loss of their biological activity. One must avoid generalizations, however, since some of the solutions examined lost their antibody activity upon repeated thawing and freezing, although the temperature never was higher than 0°C. (4). Likewise lipoproteins are denatured by freezing and thawing (2). An increase in the activity of zymase is observed when the enzyme solution is frozen and subsequently thawed, the higher activity probably being due to a disaggregation of the enzyme particles in the solution (5). On the other hand it has been found that ovalbumin solutions, on ageing, showed higher turbidity, owing to the aggregation of the ovalbumin molecules, while the crystallizability of the protein was not affected by ageing (6).

The extraction of soluble proteins from tissue cells can only follow the *destruction of the cellular membranes*, since these are impermeable to the massive protein molecules. The destruction of cells can be accomplished mechanically by grinding them with sand or with kieselguhr. In these methods a part of the protein may be adsorbed to the silicate particles, and some denaturation may occur. It is preferable, therefore, to destroy the cells by suitable mills (Latapie mill, Potter-Elvehjem homogenizer). The cellular structure is also destroyed by the action of organic solvents such as alcohol, acetone, or *glycerol*. If the concentration of glycerol does not exceed 85% a large portion of the soluble protein passes into the glycerol extract. In this way hydrolytic enzymes can be

* In the Harvard Department of Physical Chemistry, Hormann or Republic filter pads are used (2).

extracted from the pancreas and other organs. Glycerol extracts are rather stable at room temperature. It would appear that the rate of denaturation is reduced by the loose association of glycerol molecules with the protein, due to the polar hydroxyl groups of glycerol. The same polar groups are responsible for the mutual attraction of glycerol molecules and for the high viscosity of this solvent. The preparation of pure proteins from their solutions in glycerol is very difficult and at present finds infrequent application. The Willstätter school used acetone for the destruction of cells. Use was made of the fact that denaturation of many proteins occurs very slowly in a medium containing more than 80–90% acetone. It is necessary, therefore, to mince the organ to be extracted or to grind it in a meat chopper and then to place the pulp into a large excess of acetone. The advantage of this procedure is that not only the cellular membranes are destroyed, but also that most of the lipids are extracted by the acetone. They can be removed quantitatively by a subsequent treatment with ether. The extracted residue is dried by spreading it over filter paper and is then extracted with water or with dilute solutions of salts or buffers. While many proteins and important enzymes withstand the action of acetone, other less stable proteins are denatured by the action of this solvent.

The simplest and best method for the destruction of the cellular membranes is disintegration by repeated *freezing and thawing*. Since pure ice particles are formed, the concentration of the salts in the cellular liquid increases during freezing, and the cellular membranes burst owing to the heightened osmotic pressure. Mechanical lesions of the cellular membranes by the ice particles may also be involved in the destruction of the cells. Similar osmotic effects are achieved by grinding cells or tissues with dry neutral salts.

The extracts prepared by any of the methods described above are centrifuged to remove insoluble particles. Salts, glycerol, and other substances can be removed by *dialysis* against distilled water. Some proteins are insoluble in salt-free water and are obtained directly as precipitates by dialysis. Thus crystals of horse oxyhemoglobin are formed when oxygen is passed through a solution of reduced horse hemoglobin (7,8). The tendency of rat oxyhemoglobin to crystallize is so great that crystals are formed when rat erythrocytes are mixed with five to ten volumes of water. Many of the vegetable proteins and the euglobulins of blood serum are insoluble in salt-free water and are obtained as precipitates upon prolonged dialysis.

In the past dialysis was performed in *membranes* of animal origin (gut), in parchment paper, or in collodion membranes. The disadvantage of animal membranes is their inhomogeneity, while that of parch-

ment paper and of collodion is their high content of sulfuric and nitric acid ester groups. Many proteins are denatured by adsorption to these groups. The best material at present for dialyzers is cellulose, which is obtainable commercially as *Cellophane*. The only unfortunate feature of Cellophane is the small size of its pores, which renders the rate of diffusion very low, increasing the time required for dialysis and hence the danger of bacterial infection. It is advisable, therefore, to perform the dialysis in the refrigerator. The pore diameter of Cellophane can be increased by treating the membrane with aqueous solutions of zinc chloride. It would considerably facilitate laboratory work involving dialysis if Cellophane membranes of higher permeability were commercially obtainable. The most convenient material for small-scale laboratory experiments is Cellophane tubing; dialyzers of different sizes can easily be prepared by tieing off the tubing at one or at both ends to form sacs of the desired size. Dialysis time can be reduced by a factor of approximately one fourth by stirring the outer liquid. A further increase in the diffusion rate of electrolytes can be achieved by *electrodialysis;* however, one has to avoid acid and alkaline reactions near the surface of the membranes which separate the protein solution from the anodic and the cathodic liquids, respectively. A considerable portion of the protein is denatured when the reaction becomes strongly acid or alkaline.

While some proteins precipitate from their solutions upon dialysis, others are precipitated by the addition of neutral salts to their solutions (*"salting-out" method*). The usual procedure consists first of adjusting the pH of the solution to the isoelectric reaction of the particular protein, for the solubility of proteins has its minimum value here. A saturated solution of the salt or solid salt is next added to the stirred protein solution until a slight opalescence is observed. The solution is then kept at room temperature or in the refrigerator. If the concentration of salts is very high, bacterial multiplication is sharply reduced, so that one can frequently work at room temperature. The salts most frequently used for the precipitation of proteins are sodium sulfate or ammonium sulfate. Thus, if isoelectric solutions of serum albumin or ovalbumin are treated with ammonium sulfate (9) or with sodium sulfate (10) crystals of the albumins are slowly formed and settle at the bottom of the beaker containing the solution. The actual formation of the crystals is due to the slow evaporation of the solution. If an excess of the salt is added amorphous precipitates of the proteins are produced. Crystals can be obtained in such instances by dialysis of the protein solution against a saturated solution of ammonium sulfate.

Crystallization can also be achieved by *increasing the concentration of protein solutions*. While solutions of more stable substances are concen-

trated by evaporation on the steam bath or under reduced pressure, none of these methods can be applied in dealing with proteins. Heating on the steam bath would obviously denature the protein. It is surprising, however, that some proteins such as trypsin (1) or ribonuclease (11) are resistant to short heating at definite pH and ionic strength. Distillation at reduced pressure cannot be used for concentrating protein solutions, since foaming occurs under these conditions. Small volumes of protein solutions can easily be concentrated in desiccators under slightly reduced pressure over large amounts of water-binding substances. If it is intended to reduce the volume of larger amounts of protein solution, the solution can be placed in a Cellophane bag which is suspended in air in front of an electric fan (pervaporation). As the water evaporates from the surface of the bag, the protein solution becomes more concentrated (12). An increase in protein concentration is also achieved by applying positive or negative pressure during the dialysis (13).

Another method which can be applied to large volumes of protein solutions consists of freezing the solution solid and then thawing it very slowly, without any stirring or shaking. Crystals of ice nearly devoid of protein rise to the surface of the solution, while the bulk of the protein is concentrated in the solution in the lower part of the vessel. The concentrated protein solution is siphoned off and can be submitted repeatedly to the same procedure until high concentrations of protein are obtained. Human hemoglobin was obtained in this way in crystalline form by the author (14). A very efficient method for concentrating proteins is *freeze-drying* (15). A block of the frozen protein solution is exposed to a high vacuum in the presence of a substance which binds water. Sublimation of the ice from the surface of the block takes place. The protein is obtained as an air-dry powder (lyophile method) (16). While most proteins are thus obtained in the native state, some proteins are irreversibly inactivated or denatured by freezing, as was mentioned above.

One of the oldest methods for the preparation of crystalline proteins is the cautious addition of *ethanol* or *acetone* to the cold protein solution. Crystalline oxyhemoglobin was obtained from the blood of many animals in this way by Hoppe-Seyler, and also by Hüfner, toward the end of the last century. While these older methods were purely empirical and their results more or less fortuitous, the systematic purification of proteins by means of organic solvents was recently introduced and successfully elaborated by Cohn, Edsall, Oncley, and co-workers (17). The methodical application of ethanol and other organic solvents is based on the fact that these solvents reduce the dielectric constant of the aqueous protein solutions. The opposite effect, an increase in the dielectric constant, is achieved by the addition of glycine. Since the solubility of proteins