Biochemistry

AND

Human Metabolism

BY

BURNHAM S. WALKER, M.D., Ph.D.

Professor of Biochemistry
Boston University School of Medicine

WILLIAM C. BOYD, Ph.D.

Professor of Immunochemistry
Boston University School of Medicine

ISAAC ASIMOV, Ph.D.

Assistant Professor of Biochemistry
Boston University School of Medicine

WITH A FOREWORD BY

JOHN T. EDSALL, M.D.

Professor of Biological Chemistry
Harvard University

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Foreword

This book will certainly win adequate recognition without the need of any foreword from a colleague. However, I am glad that Drs. Walker, Boyd and Asimov have given me the opportunity to read it in manuscript, and to record the belief that it has a freshness and vitality, in its general outlook and in the pattern of the presentation, which give it a distinctive place among all the texts of biochemistry for medical students of which I am aware. Naturally, as a protein chemist, I must express my enthusiasm for the decision of the authors to put proteins first, in the prime position, where they ought to be. Moreover, they have succeeded in saying a good many true and important things about proteins and amino acids which have not yet found their way into most of the elementary books.

It is unnecessary here to comment in detail on the rest of the book, the general organization of which seems to me admirable. One may call particular attention to the discussion of genetics, for the interpenetration of biochemistry and genetics has been one of the great scientific developments of the last fifteen years. Moreover, medical students generally know far too little about genetics; it is a vital subject for the practicing physician to know today, and it will become even more vital in the generation to come.

An important feature of the book to me is its combined treatment of fundamental biochemistry and many of the clinical applications. This should help to awaken more medical students to the potential clinical significance of what they are studying, even in the more abstract portions of biochemistry, and to remind the student in the clinic later on of the foundations of the methods which he is applying. The theory and practical applications are interwoven so that each reinforces the other. For this, and for many other contributions, one may confidently wish and expect that this book will serve its purpose well.

JOHN T. EDSALL

Preface

The events and motivations that led to the construction of this book were neither few nor simple. Of them, however, one and only one need be of any concern to the reader. That refers to our intention to prepare a book on biochemistry intended primarily for the medical student. This intention has constantly been in our minds and has dictated our every decision in the matter of what to include in the book and how to include it.

A biochemistry text for medical students must of necessity differ from a biochemistry text for biochemists in certain important particulars. Much organic chemistry may be sacrificed with profit, considering that the space thus saved may be utilized for greater detail in clinical applications of biochemistry. The classical order of topics can be, and has been, altered in order that proteins may be taken up first, as that group of chemical substances is of prime importance to medical biochemistry. The chemistry of carbohydrates and lipids can be, and has been, taken up as an introduction to tissue chemistry. Human biochemistry has been emphasized almost to the exclusion of other branches of biochemistry.

We have not hesitated to stress the current uncertainties in biochemical theory. In fact, we have gone out of our way to do so, and where one or more of the authors have felt sufficiently venturesome have even taken sides. This is a deliberate attempt to impress upon the student that biochemistry is not a closed science that can make statements with finality which need only be memorized to remain in good repute for a lifetime. Rather, we show biochemistry to be a growing, expanding science which, particularly today, sometimes changes with bewildering rapidity. The physician, therefore, must understand that the biochemistry he is taught is merely the basis for the biochemistry he must continually teach himself as the years pass.

No attention has been given to historical priority in choosing articles for citation. The choice has been, in most instances, in favor of reviews or of relatively recent papers which themselves cite the original work.

That the book may contain errors we are painfully aware. To those who point such out to us we will be grateful.

We wish to express our thanks to the numerous friends and colleagues, both at Boston University and elsewhere, who have read and criticized portions of this manuscript.

One of us (W. C. B.) wishes to express his gratitude for permission from Little, Brown and Company to make use of certain material which appeared in his book, *Genetics and the Races of Man.*

Another (I. A.) wishes to thank the National Cancer Institute, National Institutes of Health, U. S. Public Health Service for its support from teaching and research grants over the period during which this book was written.

The preparation of the typescript was done by Miss Georgiana M. Curtin.

B. S. W.
W. C. B.
I. A.

Contents

PART VI. APPENDICES

PART I

Structure

CHAPTER 1

Proteins and Amino Acids

Just as it is necessary when beginning the study of medicine to learn first of all the gross anatomy of the human body, so in beginning the study of the chemistry of life processes it is necessary to study first of all the structural chemistry of living matter. The characteristic material of living matter is called *protein*. The term was first applied to complex nitrogenous substances found in plant and animal tissues by Mulder in 1838. The word comes from Greek roots meaning "preeminence". If we compare a living cell with a machine, then we can compare the protein with the steel and brass out of which the engine is constructed. Other substances, to be discussed later, are to be found in living tissue, and many of them have indispensable functions there; but the material which is most essential to life itself—indeed, which is life itself—is the tissue protein.

All living cells contain protein, carbohydrate, lipids, water, and inorganic ions, linked together in various ways to form compounds and complexes. Most of our chemical study of these materials comes only after the cells have been taken apart and the individual constituents isolated. The method of taking the cell apart often influences the result. For instance, if we represent the various constituents of a cell by the letters, A, B, C, D . . . , and assume that by rough treatment of some sort we split the cell up into fragments which can be dealt with chemically, it is easy to see that we might come out with complexes such as AB, BC, ABC, CD, CDE . . . , depending on the methods used and the severity of the treatment. This does in fact happen and it is always difficult to make sure that what has been isolated is a reproducible material which actually represents a constituent of the cell, for if at one time we obtain AB from the cell and another time BC or ABC, our chemical analyses in the two instances will be somewhat contradictory, or at least inconsistent. Indeed, since there is every reason to suspect that the protein in the cell is rarely if ever free, the idea of pure protein is in itself an idealization and represents something which, probably, is not to be found in the average tissue. But, like many other abstract ideas, it is a very useful one; and we shall

find it very valuable to examine the properties of purified proteins and to try to apply this knowledge to the behavior of the living cell.

That the concept of protein is not entirely an imaginary one has been demonstrated by the isolation of proteins with reproducible properties from various types of tissue. It is true that the situation is not as simple as it was thought to be by Mulder, who believed that there was only one protein which combined with sulfur or phosphorus or both to give various compounds, and that it was these which were present in the tissues of plants and animals. Indeed, we know that there are a very large number of proteins although we have not obtained very many of them in a pure state. It is not usually to be expected that even a single organ will contain but one sort of protein, for diversity of chemical structure is required by the manifold functions performed by most of the organs of the human body. We can not state, for instance, that there is such a thing as a "kidney protein" because the kidney contains proteins of various types. So do most of the tissues. However, there are proteins more or less characteristic of certain types of tissues and our ability to isolate them in more or less pure form justifies our naming them for the tissue from which they come or for some special property or activity which they exhibit.

DEFINITION OF PROTEIN

It is at once difficult and easy accurately to define a protein. It is easy in the sense that it is not difficult to give the reader a fairly clear idea of what we mean by the word. It is difficult in the sense that we find difficulty in being absolutely precise about our definition. Suppose we simply say for the time being that proteins are large molecules, of molecular weight of the order of several thousand to several million, occurring in the tissues of plants and animals and containing carbon, hydrogen, oxygen, nitrogen, and sometimes other elements, and constructed largely from *amino acids*, which will be discussed later in this chapter. This definition serves to differentiate proteins from all the other compounds we shall study in this book.

PROPERTIES OF PROTEINS

In discussing the properties of the proteins we shall want to say something about their behavior *in vivo*—that is, in the intact cell—and *in vitro*—that is, as they behave in the laboratory after we have isolated them.

Lability

One of the most striking characteristics of the proteins is that they are extremely sensitive to change and it is difficult to keep them from change during chemical manipulation. Probably one of the first and most serious

changes which takes place in a cell when the organism of which it forms a part has died is alteration in the constituent proteins. These changes soon reach the stage where they are irreversible and the cell is then itself dead. As an example of this lability we may consider the properties of the egg albumin, which forms the principal protein of the white of the hen's egg. This protein can be isolated from the egg and obtained in crystalline form, but as a rule only if the egg is less than 24 hours old. If the egg is older than this, great difficulty is experienced in trying to crystallize the protein. The egg is still viable; it will still hatch, but nevertheless something has happened to the albumin during the first twenty-four hours the egg has been outside the body of the hen. And keeping the egg at body temperature, instead of arresting this process, merely accelerates it. After the albumin has been crystallized, it will keep in the form of crystals covered with mother liquor for a considerable time, but nevertheless at room temperature or even at icebox temperatures slow changes take place, so that the solubility of the crystals in water gradually becomes less, and after a few years they may become completely insoluble, showing that even when the protein is in the form of crystals and relatively pure, something has happened to it. Crystals of the blood protein hemoglobin, even when kept in the icebox, lose their characteristic properties even more rapidly than does egg albumin.

In order to isolate proteins with as little damage as possible the temperature must be kept low—lower than body temperature whenever conditions permit; otherwise random thermal agitation of the atoms will cause some denaturation. Also, enzymes which are originally present in the impure preparation may begin to act on the protein before they are separated from it. The pH[1] should be maintained as near that of the environment of the native protein as possible. The dielectric constant of the medium should be kept as high as possible, which means that other things being equal, an aqueous medium is best. Organic solvents such as alcohol should be used only with great caution and preferably at low temperatures. They appear to damage certain proteins only slightly but may rapidly damage others. The use of high salt concentrations to precipitate proteins, though traditional, should be avoided when possible. In some cases this seems to result in very slight damage but ultimately damage always does result. Some proteins are damaged immediately beyond repair by this procedure.

Solubility

Some proteins are insoluble in all ordinary solvents; others are more or less soluble in various mixtures of water and other compounds. Pure water will dissolve some proteins; in other cases it acts as a better solvent if

[1] The symbol pH is defined on page 19.

salts are present. Some proteins will dissolve best in mixtures of water and less polar solvents such as alcohol. In general, the more polar the solvent the greater its power of dissolving proteins, which suggests that the protein is itself polar in nature. If sufficient salt, especially a salt such as ammonium sulfate, is dissolved in a protein solution, the protein becomes less soluble and most proteins are completely precipitated. There are certain general rules about the solubility of proteins which may now be stated.

1. A protein is least soluble in the neighborhood of its isoelectric point. The term "isoelectric point" will be defined on page 17. The pH of minimum solubility varies with the nature and concentration of the salt which is used. Unless the salt concentration is very dilute, the pH of minimum solubility is generally found to be somewhat different from the true isoelectric point of the protein.

2. Solubility of proteins in water without salt varies a great deal. Serum albumin dissolves in water readily and it seems to be miscible with water in all proportions. Other proteins are soluble only if salt is present; some, like edestin from hempseed, require concentrations of neutral salt of the order of 5 per cent to get them into solution.

3. The solubility of a protein in water or other solvents depends upon the nature of the amino acids of which it is composed. Thus proteins rich in non-polar groups such as paraffin side chains, benzene rings, or pyrrolidine rings tend to dissolve better in alcohol-water mixtures than in water; whereas those poor in non-polar groups but rich in polar (electrically charged) groups tend to be precipitated even by small amounts of alcohol or acetone.

4. Proteins which are insoluble in water but have large numbers of charged groups become more soluble in the presence of neutral salts or other dipolar ions.

5. Proteins are usually more soluble when combined with acids or bases than in the neutral state. This will be discussed immediately below.

6. Formation of salts between proteins and another protein or between a protein and ion may result in compounds which are more or less soluble. Thus, protamines form a compound with insulin which is less soluble than either protamine or insulin. Protamines also form insoluble salts with casein.

Amphoteric Behavior of Proteins

Most proteins can behave either as acids or bases and are thus called amphoteric. Consequently it is possible to dissolve these proteins either in dilute acid or dilute alkali, forming a salt in either case. This amphoteric behavior of proteins is of great importance to understanding other proper-

ties in general. For instance, whether a protein combines with an anion or a cation depends upon the pH of a solution or in other words, on which side of the isoelectric point of the protein we find ourselves. This was well shown in the classical experiment of Jacques Loeb, described on page 18.

The amphoteric behavior of proteins is due to the presence of acidic and basic groups in their molecules (p. 28). Some of these groups are ordinarily charged, positively or negatively as the case may be, and thus account for the presence on the surface of the molecule of fixed charges. Except at the isoelectric points, the positive and negative charges do not usually balance exactly, so the molecule has an over-all net charge which is positive or negative, depending on pH. The distributions of the two sorts of charges are seldom the same, so negative charges will predominate in one part of the molecule and positive charges in another. The effect of this is the same as the localization of a positive charge of varying magnitude on one part of the molecule and a negative charge on another part. This causes the protein molecule, even at the isoelectric point, to behave like an electric dipole. Therefore, protein molecules in solution will orient themselves in an electric field.

A dipolar ion will tend to orient itself in an electric field, with the end which is predominantly negative pointing towards the positive pole and the positive end towards the negative pole.

The dielectric constant of a solution can be interpreted as being almost entirely a measure of the number of molecules oriented by the electric field. This orientation is hindered by frictional forces which vary with the size and shape of the molecule. Hence, a study of the dielectric constant of a protein solution subjected to alternating electric fields of various frequencies gives information about the physical characteristics of the protein molecules (21).

Ability to Form Complexes

Proteins, having a fair number of charged groups which may be of either sign, can and do form complexes of various sorts. Some of them form insoluble salts with anions or with cations. For instance, the vegetable protein, edestin, is not only relatively insoluble itself but forms a relatively insoluble hydrochloride. Some proteins which form soluble sodium or potassium salts form insoluble calcium salts. Others form insoluble zinc salts. A large number of substances form insoluble compounds with nearly all proteins and are thus used as tests for proteins. Among these we may list tannic acid, picric acid, phosphotungstic acid, and trichloracetic acid. These will be mentioned under tests for proteins. The process of tanning leather seems to be a process of formation of insoluble complexes with various agents such as tannic acid, chromic acid, and so forth. Proteins may also form

insoluble complexes with other proteins. A very well known example is the complex of insulin and the simple protein, protamine. The combination of insulin and protamine is used clinically because the insolubility of the complex results in its liberating insulin into the circulation more slowly than if the hormone were injected by itself.

EXTRACTION AND PURIFICATION OF PROTEINS

In order to make chemical studies of proteins, it is necessary to extract them from the tissues in which they occur and follow this by purification procedures. In the case of blood, the proteins of the plasma are already in solution and it is only necessary to add something to prevent the clotting of the blood, if we wish to study also the fibrinogen, or to take the serum which separates from the clot in case we are not interested in the fibrinogen. But blood is a rather special tissue, although one of great importance, and it is somewhat easier to study than most of the others. If we have tissue consisting of cells, connective tissue, fat, and so forth, and wish to study one of the proteins contained therein, one of our first problems is how to break up the cells so as to get out the proteins. To break up the cells of the tissue, several methods are available. Alternate freezing and thawing will usually accomplish it; or grinding with sand in some sort of mill, or the action of intense sound waves of high frequency ("ultrasonic vibration"). In a given case one of these may have some advantage over the other. It is probable that none is quite ideal. In some cases simple mincing of the tissue, followed by extraction with dilute salt solution will extract a large amount of protein present. This is perhaps the mildest of the methods of extraction.

The purification procedure: concept of purification. It is somewhat more difficult to establish the purity of a protein than in the case of simpler chemical compounds, although even in these cases it is not always as easy as might be supposed. In a case of simple organic compounds which can be crystallized readily, it is usual to repeat crystallization until one or several of the properties of the substance remain unchanged after further crystallization. For instance, it is customary to follow the melting point and crystallize until the product no longer changes its melting point after further crystallizations. Elementary analyses (C, H, N, and so forth) also help one to follow the degree of purity attained. But some compounds such as cholesterol, for instance, even after repeated crystallization, still contain impurities which are very difficult to remove. To understand how this may occur consider the distillation of ethyl alcohol from beer, wine, and other products of alcoholic fermentation. Distillation to constant boiling point is usually a good method of fractionating liquids and obtaining pure components. But the reader will recall that if this is attempted in the case of

mixtures of ethyl alcohol and water, the constant boiling mixture as finally obtained contains between 95 and 96 per cent alcohol and no further distillation will improve the purity because a *mixture* with a constant boiling point has been obtained. Other methods must be relied upon to remove the rest of the water. A similar situation can arise in crystallization if compounds are formed which separate out in the form of crystals and the solubility of the compound is less than that of either component. There is good reason to think that this does often happen with protein materials. The usual definition of "pure" for an organic substance is a substance which consists of a single molecular species—that is, all of its molecules are exactly alike. This concept, though satisfactory enough for most organic compounds, especially those dissolved in non-polar solvents, is not at all satisfactory when applied to proteins—even ideally. In fact there is very good evidence in many cases that it is not so. Repeatedly crystallized material can be shown to be non-homogeneous. In the case of proteins we are inclined to rely upon physical and relatively crude criteria such as solubility, the sedimentation constant as determined in the ultracentrifuge (see below), and the electrophoretic mobility as determined in the Tiselius electrophoresis apparatus. It is desirable that supposedly pure protein should conform to the phase rule of solubility, that is, if the amount which goes into solution is plotted against the amount added, a straight line of definite slope should be obtained up to a certain point after which this line abruptly becomes horizontal and does not change its slope thereafter. The point at which the line changes its slope, of course, represents the point at which saturation is obtained. (See figure 1.)

It is natural to suppose that all the molecules of a protein which fulfills a single function in a single tissue are exactly alike. But this is not necessarily so. Human serum albumin, after careful separation and repeated crystallization, is homogeneous electrophoretically, ultracentrifugally, and immunologically. But amino acid analysis suggests that there is only half as much tryptophane present as would be needed for one tryptophane residue to be present in each molecule.

Even when there is no difference in structure between the different molecules of a purified protein, it is unlikely that all of them are at any one time in the same state. Thus Cohn and Edsall (3) calculate that in the case of the hemoglobin studied by them at pH 6.4, the isoelectric point, only 22.4 per cent of the molecules possessed zero net charge. They estimated that 21.2 per cent possessed a net charge of -1. and 0.03 per cent, a net charge of -7.

Salting out. After the protein has been obtained in the form of a solution from the cells, the next procedure is to purify it. A number of different possibilities present themselves, some better than others. One of the older

methods consists in what is called "salting out"—that is, the addition of a sufficient concentration of a neutral salt such as sodium chloride, magnesium chloride, or ammonium sulfate, the latter being the one most commonly used, to precipitate the protein and throw it out of solution. We may consider that salting out depends on the monopolization of so much of the water by the more polar salt that not enough is free to keep the protein in solution. If the precipitate is then filtered off, it may be redissolved and the process repeated. This will often result in considerable purification because small amounts of other proteins, particularly if they are relatively soluble, will be left behind in the filtrate so that eventually a relatively pure protein

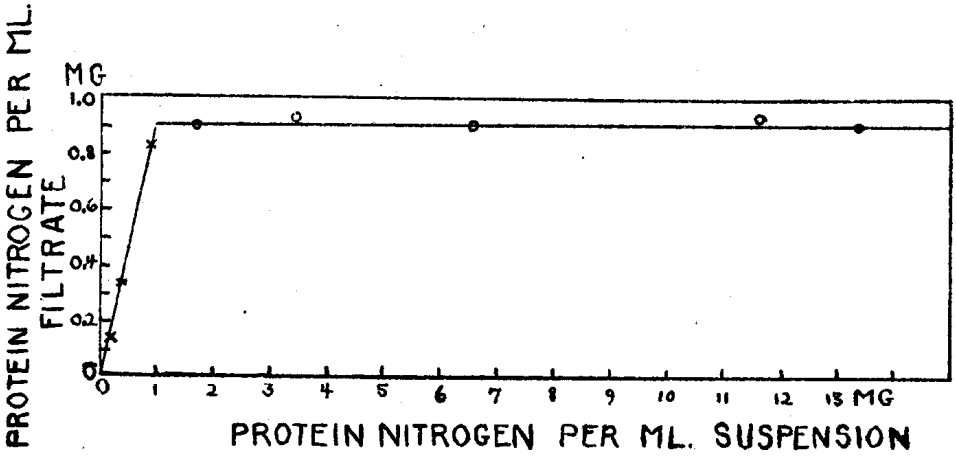

FIG. 1. Solubility of crystalline chymotrypsinogen in one-quarter saturated ammonium sulfate at 10 C in the presence of increasing quantities of chymotrypsinogen in the solid phase (Northrop).

preparation will result. Crystallization can be achieved in many cases by bringing salt concentrations to the right concentration and adjusting the pH to a value in the vicinity of the isoelectric point. Such methods have allowed the crystallization of egg albumin, serum albumin, and various other proteins. However, the addition of high concentrations of salts to proteins is not without danger and in many cases, without doubt, results in denaturation, which may be mild or in some cases pronounced.

It has been shown that the salting out of a protein depends upon the character of the protein and also upon the particular neutral salt which is used. The characteristics of salting out with ammonium sulfate for a number of proteins are shown in figure 2 taken from Cohn (3) and for a number of salts in figure 2a.

Crystallization. It has been possible to purify many proteins by crystallization. The most successful of the early procedures were based on the prin-