The Influence of Varying Hydrogen Ion Activity and Protein Concentration upon the Stability of the Molecules in Certain Protein Solutions.

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Introduction

Proteins, as definite chemical individuals, exhibit determinable molecular weights in aqueous solution. This thesis embodies the measurements of such molecular weights at varying degrees of acidity and alkalinity and at varying levels of protein concentration. These measurements were made to determine the effects of certain variables which might conceivably affect the stability of proteins. The proteins studied were the hemocyanins or respiratory proteins of the blood of the snail, Helix pomatia, and the king crab, Limulus polyphemus. An understanding of the purpose of such measurements requires a brief statement of certain difficulties encountered by those who regard the proteins as chemical individuals. A brief orienting statement of the points of view which obtain today in the physical chemistry of the proteins precedes the presentation of some of the experimental evidence which has led to the work described.

Points of View in the Physical Chemistry of the Proteins.

The principles underlying the constitution of the proteins are comparatively simple and the view that they

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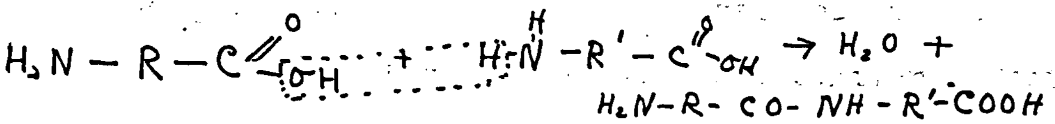
behave as would the condensation products of a number of molecules of various amphoteric amino-acids is generally accepted. (1). Two amino-acids may, under certain, conditions, condense to form a polypeptide by a reaction of the type



$$H_2N-R-C\overset{O}{\leqslant}OH + H-\overset{H}{N}-R'-C\overset{O}{\leqslant}OH \rightarrow H_2O + H_2N-R-CO-NH-R'-COOH$$

in which R and R' are characteristic of the particular amino-acids involved. By a series of similar condensations very large molecules may theoretically be produced. The specific characteristics of the naturally occurring proteins are attributed to the proportions and arrangement in their molecules of the twenty-odd stepping stones which result from their decomposition and from which it is assumed they are built. The processes of digestion involve the splitting of the proteins by repeated hydrolyses through a series of poorly characterized substances known as proteoses, peptones, and polypeptides to, in some instances, the amino-acids.

It has long been recognized that the behavior of the proteins indicates that in aqueous solution they must be in the colloidal state. Many of them form opalescent solutions which exhibit the Tyndall cone. They diffuse but slowly and only rarely pass through collodion or parchment paper membranes. The knowledge that the particles present in the colloidal sols of inorganic materials as, e.g. gold, are aggregates of a very great number of atoms or molecules has led several investigators to suggest the possibility that the solute particles encountered in the protein solutions are also aggregates of much smaller units, the true molecules, held together by secondary valences. Further, as many of the properties of the inorganic colloids are dependent upon the extent of an interface between the colloid particles and the dispersion medium, it was for a time felt that similar effects were conditioning the properties of the protein solutions. A restricted group of workers still maintains that the binding of acids and alkalies by the proteins may be ascribed to adsorption at the interface.(2).

The pioneer work of Bugarzsky and Lieberman (3) on the electrochemical behavior fp protein solutions, however, indicated that the proteins, large though their molecules be, are nevertheless capable of functioning as amphoteric electrolytes by virtue of the NH_2 and COOH groups which they contain. Summaries of the more recent work have been published by T. B. Robertson (4), Wolfgang Pauli (5), Jacques Loeb (6), E. J. Cohn (7), and others. It is now quite generally accepted that the binding of acids and of bases which occurs in protein solutions is due to direct combination rather than to processes fp adsorption at more or less hypothetical interfaces. Thus the proteins are both weak acids and weak bases and exhibit, each at a definite hydrogen ion activity called the isoelectric point, a condition in which their solutions show minimal ionization, stability, viscosity, osmotic pressure, and a minimal ability to bind acid or alkali. Gels of proteins also show minimal swelling at their isoelectric points. Critical experiments have in general failed to demonstrate any ability of the proteins to adsorb both ions of salts from their aqueous solutions. Certain properties of protein solutions are, however, to be ascribed to the large magnitude and resultant low rate of diffusion of the molecules which must exist therein. As has been pointed out by Jacques Loeb (6), these properties are a large part of the viscosities of their solutions, and the osmotic pressures and membrane potentials which arise when their solutions are separated from solutions of electrolytes by collodion membranes. The swelling of gels is probably another such property. These properties may be ascribed to the colloidal behavior of the protein molecules, while many of the other properties of the solutions of the proteins are similar in nature to the properties of crystalloidal substances in aqueous solution. <u>Use of the Term "Solution."</u> In what has just been written the term "protein solution" has been used frequently. It is believed that this is in accordance with the practice of those chemists whose investigations in this field have been most significant. Definite proof that at least certain proteins may enter into true solution and exhibit many of the properties of crystalloids therein has been offered by J. Loeb (6). It seems likely that the most fruitful method will be to regard this as true until all experimental work suggested by this point of view shall have exhausted the possibilities and left a residue of unexplained facts.

Two criteria are generally held to betray the fact that the stability of certain sols, for example, colloidal gold, is due to the presence of an electrical double layer about the particles, the removal of which destroys the electrostatic barrier to the coalescence of the particles due to cohesive forces. (A). Small quantities of salts suffice to remove the charge adjacent to the colloid and lead to precipitation. (B). The active ion of the salt has always the opposite sign of charge to that of the particles. (8). Neither of these criteria hold for most solutions of proteins. Neither gelatin chloride, isoelectric gelatin, nor sodium gelatinate can be precipitated by solutions of magnesium chloride as concentrated as three molar, while sulphates always prove to be much better precipitants than the chlorides, regardless of the sign of the protein ion. These facts have led the organizers of the field of colloid chemistry to set many of the proteins apart in a class known as the hydrophilic colloids. These are defined as colloids which have a certain affinity for or relation to water so that they are soluble in water. (9). Boiled egg white has, however, undergone a chemical alteration of unknown nature called denaturation. By virtue of this change it loses its solubility in water and its sols become unstable so that they are readily coagulated by salts added in small quantities. ~~The sign of~~

The sign of the particles of the egg white determines which of the ions of the precipitating salt shall be effective in the precipitation. The denaturation has thus changed the egg albumin from a hydrophilic or water-soluble colloid to one which lacks solubility in water and which is accordingly called hydrophobic. In what follows, only solutions of undenatured hydrophilic proteins will be considered. Further evidence that the problem of the solubility of proteins belongs to the general theory of solution rather than to a colloid chemistry which ascribes it to the accumulation of stabilizing charges of preferentially adsorbed ions at the interfaces of dispersed groups of protein molecules and water, has been afforded by the work of E. J. Cohn (10) on the solubility of casein. He has been able to show that the solubility of casein in alkali is completely determined by (a) the solubility of the casein ~~compound~~ molecule and (b) the concentration of the disodium casein compound. More recently, Cohn (11) has shown that the solvent action of a neutral salt upon oxyhemoglobin is identical with the solvent action of a neutral salt upon any sparingly soluble bi-bivalent or uni-quadrivalent inorganic compound, and that "the solubility of oxyhemoglobin in phosphate solutions of varying ionic strength has been defined by the equation: $\log S/S_0 = \frac{2\sqrt{\mu}}{1+1.5\sqrt{\mu}}$ in which μ is the ionic strength and S_0 is the solubility in the absence of salt."

The Possibility of Aggregate Formation: Uniformity or Non-Uniformity. If, then, proteins be studied from the point of view that they form true solutions in water or salt solutions, efforts to determine their molecular weights would be expected. It would, however, be necessary to inquire whether all of the molecules of the protein solute are in the simplest chemical form or whether there are present in varying proportions under varying conditions aggregates of these molecules. By this is meant not aggregates of a very large number of small units to attain colloidal dimensions, but rather aggregates of a small (1-2-3-4) number of very large molecules. A similar problem is frequently encountered in inorganic chemistry. Water is regarded as a mixture in varying proportions of monohydrol (H_2O), dihydrol, $(H_2O)_2$ and trihydrol $(H_2O)_3$. Aqueous solutions of inorganic chemicals are frequently known to contain the solute as aggregates of the simplest possible molecules, and association occurs even more frequently when organic liquids are employed as solvents. This fact complicates greatly the determinations of the molecular weights of solutes by measurements of the osmotic pressures, vapor pressures, freezing-point depressions, or boiling-point elevations of their solutions. Benzoic acid is present as C_6H_5COOH in aqueous solution and as $(C_6H_5COOH)_2$ in benzene solution. Thus from the chemical standpoint one would say that benzoic acid has the molecular weight of 114, and that it dissolves without association in water, but that association into double molecules occurs when it is dissolved in benzene. But for the physicist, who is concerned with the relative numbers of the molecules and their relative masses in the systems investigated, the molecular weight of benzoic acid in benzene would be 228. The properties of the two varieties of molecules might conceivably be quite different. If methods were available for determining the densities, shapes, and dimensions of the molecules in a solution, the physical molecular weight of the solute could be readily determined, and this weight in grams per liter of solution would be the quantity required to make a solution of such concentration that the number of dispersed particles of solute per 22.4 liters of solution would equal the Avogadro number; the molecular weight so determined would be that which the solute exhibits <u>under the conditions studied</u>. This value might be a multiple of the value exhibited by the same substance under other conditions. From the standpoint of the physicist the molecular weight of a substance might then be a variable quantity, changing with the environment. As aggregate formation involves the formation of new substances, recognized by the appearance of new sets of properties, aggregates should be distinguished from each other by suitable names, as for example acetaldehyde, metaldehyde, and paraldehyde.

The absence of aggregate formation can be determined with certainty when the molecular weight as obtained for a material agrees exactly with the minimal molecular weight calculated from the percentage composition determined from the direct analysis of the dried substance. When, however, the molecular weight determined for a material in solution is a multiple of the minimal molecular weight calculated from the composition, there is always the possibility that, had another solvent been chosen in which to carry out the molecular weight determination, the value obtained might have agreed with the minimal molecular weight. Dextrose, $C_6H_{12}O_6$, has in water a molecular weight which is six times the minimal value calculated from the percentage of carbon or hydrogen. Corroborative evidence from the chemical behavior of the compound leads to the development of a structural formula for dextrose which indicates that its molecular weight is six times that of formaldehyde CH_2O which has the same composition. When the corroborative evidence of structure is difficult to secure, it is scarcely possible to be certain that physical measurements give the true chemical molecular weights of the solute in a solution when the value obtained is a multiple of the minimal molecular weight obtained by chemical analysis. The best that can be done is to accompany the physically determined molecular weights by a statement of the exact conditions under which they were obtained.

This somewhat prolonged discussion of an elementary matter has been included because the difficulties indicated appear in the determinations of the molecular weights of the proteins. The large size of their molecules greatly lessens the accuracy with which minimal molecular weights can be calculated. The hydrolysis of such large molecules as must exist in protein solutions would be capable of producing only a slight alteration in the resulting analyses for carbon, hydrogen, or nitrogen. Minimal molecular weights can, however, be calculated from the percentage of such constituents as the iron in hemoglobin, the copper in hemocyanin, sulphur and phosphorus in other proteins, and from the amounts of certain amino-acids in the hydrolyzates of various proteins. Even here the chances of error are great. The minimal molecular weights obtained in this way, as well as by a consideration of the combining weights with acid, alkali, carbon monoxide or carbon dioxide, were calculated by E. J. Cohn, J. L. Hendry and A. M. Prentiss (12) in 1925 for all proteins for which sufficient data existed. Their results are given in Table I which also gives the molecular weights obtained by osmotic pressure measurements.

Table I

Probable Molecular Weights of Proteins (1925).

Protein	Minimal Molecular Weight	Osmotic Pressure Molecular Weight	Probable Molecular Weight
Egg Albumin	33800 Tryptophane 2 Sulphide S 5 Tyrosine 8 Sulphur 17 Acid-binding 27	34000	33800
Serum albumin	5000	45000	45000
Hemoglobin	16666	48000	50000
Edestin	29000		58000
Hemocyanin, Limulus	22700		68100
Pseudoglobulin	27000	80000	81000
Gelatin	10300	96000	
Casein	12800		192000

The results of the ultracentrifugal method of Svedberg(13) have, as will be shown later, necessitated the revision of some of these values.

The colligative property methods for the determination of molecular weights are, in this field, practically limited to but one, measurement~~s~~ of the osmotic pressure. These methods are rendered difficult of interpretation because of the reactivity and multivalent character of the proteins. As these compounds react with acids or with bases at reactions on the acid and alkaline sides of their isoelectric points, the osmotic pressures rise on either side of these characterizing hydrogen ion activities. A further error is introduced by the membrane equilibria set up because of the inability of the protein ions to diffuse through the membranes. Finally, the presence of traces of impurities produces very large errors. Granting the possibility of correctly extimating the minimal molecular weight and the osmotic pressure molecular weight of a protein, there is no available corroboratory evidence to be derived from a structural formula based upon the chemical behavior of the particular protein to enable a decision to be made between the two values in the event that a discrepancy exists.

Should aggregate formation be known to occur in a solution of a protein, it is conceivable that there may be present a mixture of non-aggregated and aggregated molecules. The proportion in which the constituents of such a mixture are present might readily vary with the concentration or activity of the protein molecules, with the reaction of the solution, and with the presence of such other substances as salts. Protein solutions may therefore have one or more different solutes, the molecular weights of which are multiples one of another. Besides the simple aggregates which must be regarded as the result of the coalescence of two or more protein molecules, there is another possibility for the formation of protein molecules the weights of which are nearly multiples one of the other. If a protein should be symmetrically split by hydrolysis, two new proteins might be produced, the weights of each of which would be practically one half that of the original protein. The weights of the H and OH ions introduced are negligible in comparison with the weights of the proteins. If multiple linkages are split or if the hydrolysis occurs unsymmetrically, the ratio of the weights of the various proteins involved would still be expressed by small whole numbers. The recent work of Waldschmidt-Leitz (14) indicates enzymic hydrolysis to occur in ways specific for each enzyme, and to be partly dependent upon the size of the molecules and upon the nature of the groups adjacent to the linkage at which the splitting occurs. He also finds the weight of his experimental evidence to be against the belief that proteins consist largely of aggregates by secondary valences of some simple enzyme-resistant groups, such as diketopiperazines.

If the aqueous solution of a protein could be shown to contain solutes of molecular weights which are practically multiples one of another, a decision as to the type of relation existing between them (simple aggregation or hydrolysis) would be made only with difficulty if at all.

In Part I of this thesis is presented some of the evidence which has led to the belief that the solutions of many proteins contain more than one kind of solute. It is not intended to represent a complete summary of what is known in this field, but is rather to be considered only as indicating certain of the difficulties which have led to the suggestion that the proteins may participitate in either or both types of reaction - - simple polymerization and hydrolysis.

Although the evidence points toward the possibility that the hypothesis of a polydisperse structure may surmount the difficulties encountered in the study of certain protein solutions, it does not in itself prove that such a condition actually obtains. A direct experimental test is offered by the ultracentrifugal methods. (13). As the first studies by the aid of these methods tended to indicate the protein solutions examined to contain but one solute of definite molecular weight, it was thought advisable to extend the measurements on such uniform protein solutions to varied environments. The results of these measurements are described in Part II.

Part I

Experimental Indications of the Non-Uniformity of Protein Solutions.

A protein solution may contain more than one kind of protein molecule either because the original source contained a mixture of proteins and the methods employed for their separation were imperfect, or it may have originally contained but one protein unstable under the conditions employed. The following examples of difficulties encountered in several phases of protein chemistry are presented merely because they inclined the author to the belief that instability (even though there be no "denaturation") is a marked feature of the behavior of protein solutions, and thus led to the investigations reported in Part II. They represent merely a small portion of the accumulating evidence that the belief that protein solutions are in all cases monodisperse is naive and unnecessary. At the same time, the investigation of the conditions affecting the stability of the protein molecules as attempted for specific proteins in Part II may indicate that the fact that certain protein solutions contain more than one solute need not necessarily lead to the conclusion that they are beyond the scope of treatment by the usual physical-chemical methods.

A. Gelatin. As the method of preparing gelatin involves the hydrolysis of collagen it is not surprizing to find that the product is usually a mixture of at least two products of hydrolysis. That this is true has been indicated by several workers. S. B. Schryver (15), for example, has attempted the fractionation of the material, and J. Northrop (16) has succeeded in effecting a partial separation of its constituents. At least two values for the isoelectric point of gelatin have been reported by reliable workers, the variations being ascribed to variations in the source of the material. (E. O. Kraemer) (17). That the constituents of the mixtures known as gelatin are capable of entering into reactions one with another with the production of new compounds, or with the interconversion of one into the other has been rendered likely by the experiments of D. C. Carpenter (18) on the effects of salts upon the optical rotation of gelatin solutions. The influence of temperature upon a possible equilibrium has been pointed out by C. R. Smith (18a) and others. Thus there is abundant evidence that the study of gelatin is complicated by certain variables which affect the stability of the compounds which make up the mixture known by that name, That gelatin is scarcely " a suitable material for researches which are intended to be of theoretical significance because it is doubtful whether it is a chemical individual " has been noted by Bayliss (8). Nevertheless, the greater part of Loeb's important book (6) on " Proteins and the Theory of Colloidal Behavior " was devoted to experiments with this material. It was possible to successfully develop a theory for the swelling, osmotic pressure and viscosity of gelatin on each side of the assumed isoelectric point (4.7). That Loeb was not unmindful of the possibility of the existence of varied molecular species in gelatin solutions under certain conditions is indicated by the following quotations:

> " It is undoubtedly true that salts precipitate proteins and that precipitation is due to an increase in aggregation, but the salting out of gelatin from its aqueous solution is not determined by the ion with the opposite sign of charge to that of the protein ion, while we have seen

that the depressing effect of a salt on the osmotic pressure of gelatin solutions is determined by the ion with the opposite sign of charge to that of the protein ion. In other words, the salting out of gelatin from its aqueous solution is a process of an entirely different character from the lowering of the osmotic pressure of a protein solution by a neutral salt. It is, therefore, impossible to explain the latter process by the former." (page 164).

"Since jelly formation of gelatin is a reversible process, we should expect that two opposite processes always take place simultaneously in a gelatin solution on standing, namely, first, the formation of solid particles of jelly through the aggregation of previously isolated gelatin molecules and ions as soon as their oily groups come into close contact, and second, the dissolution of such aggregates (micellae) back into isolated molecules and ions, due to heat agitation." (page 283). " The temperature at which the two opposite processes - the formation and the melting of micelles - occur equally rapidly in a 2% solution of gelatin chloride at pH 2.7 lies near 35 degrees C., according to Davis and Oakes (Journal of the American Chemical Society, <u>44</u>, 464 <u>1922</u>) near 38 degrees C. " (page 283).

" This probably explains a phenomenon which has puzzled the writer for a long time, namely, that the osmotic pressure of gelatin solutions of the same pH and concentration of originally isoelectric gelatin occasionally showed variations for which he could not account. It now becomes probable that this was due to a factor which was not taken into consideration, namely, that on standing at room temperatures a gradual transformation of isolated molecules or ions into larger aggregates takes place which must diminish the osmotic pressure but increase the viscosity." (page 302).

" the fact that very often solid submicroscopic particles are formed in protein solutions does not contradict the crystalloidal nature of their solubility, since aggregate formation may occur in the aqueous solutions of true crystalloids, e.g. cane sugar, if the proper concentration is reached." (page 367).

The author felt that it would be more profitable to study equilibria of the type postulated upon proteins obtained by methods which involved less alteration that the drastic ones employed for the production of gelatin. Results obtained would thus have more relation to the proteins as they occur in living matter. Further, any investigation of the variables affecting the stability of what are usually regarded as pure proteins would have greater significance for the general theory of the physical chemistry of protein solutions. The proper choice of proteins for this study might also eliminate the phenomenon of gel formation which complicates somewhat the study of gelatin.

B. Edestin. As a representative of the crystalline plant globulins, edestin appeared a more suitable material, as it is quite generally regarded as a definite chemical individual. It exhibits, however, certain behavior which necessitates an explanation which it has not yet received. According to Hitchcock (19), the isoelectric point of edestin appears to vary with the nature of the buffer used in the determination. Its solubility in salt solutions increases with the amount of the protein in the solution studied. This phenomenon - strange for a definite compound - was described as early as 1905 by Sir W. B. Hardy (20). It has been verified by the writer and occurs even when buffer solutions are employed as solvents. Thus it cannot be said that differing quantities of the protein give to a constant volume of solvent different reactions, and so account for the alteration in solubility of the protein. Edestin crystals were stirred until equilibrium was attained in each of two flasks containing the same volume of the same phosphate buffer solution. One flask contained five times as much edestin as the other.

After filtering the solutions from the undissolved saturating body, the nitrogen was determined in each solution by the method of Kjeldahl. The solubility of the edestin in the case in which the larger amount was present was 1.75 mg. of nitrogen per 100 cc., while in the other case it was only 0.35 mg. of nitrogen per 100 cc. This dependence of the solubility of the edestin upon the amount of the saturating body present in the system was noted repeatedly in the course of certain work upon the solubility of this protein in buffer solvents of independently varied hydrogen ion activity and ionic strength. It was also readily seen qualitatively in the gross diminution of the volumes of the insoluble material on repeated washings with fresh solvent in two flasks containing initially widely different amounts of edestin for a given volume of solvent.

C. Serum Globulin. The same phenomenon - dependence of the solubility upon the amount of the saturating body in the system - was also observed by Sørensen (21) in the study of fractions prepared during attempts to prepare the serum globulins. It has been designated as the Hardy - Mellanby phenomenon, since it had been noted by both Hardy (20) and by Mellanby (22) in 1905. Sørensen ascribed it to the presence of two or more distinct proteins of different solubility in the preparation studied. Thus if a preparation contained a a large proportion of a protein of low solubility and a small proportion of one of greater solubility, the total apparent solubility measured in terms of the miligrams of nitrogen per 100 cc. of solvent might readily be expected to vary with the amount of the mixture taken. This would be the case so, long as insufficient of the more soluble material were taken to saturate the solvent. Sørensen (23) attempted unsuccessfully by repeated fractionations with ammonium sulphate to separate pseudo - and euglobulins in the effort to obtain proteins of a definite solubility independent of the amount of protein present in the system. He has suggested that the saturating body in his experiments was a compound of pseudo- and euglobulin.

The Hardy- Mellanby phenomenon which has been encountered in the case of edestin, serum globulin, and also muscle globulin preparations has recently been studied in great detail by Wolfgang Ostwald (24), A. V. Buzagh (25) and others. These authors attempt to make it a criterion of the colloidal state of matter, and attach great importance to adsorption in explaining its mechanism in some cases. Ostwald propounds a general "Bodenkörper Regel" which states that the solubility or peptizability of a colloidal substance increases with the amount of the solid - substance left in the solid phase in the system. Previous to the recent work of Ostwald, the phenomenon had been noted by Eric Müller (26) in studies on the solubility of chromium hydroxide. He suggested that the formation of polymers of different solubilities might furnish an explanation of this behavior.

D. Hemoglobin. One other instance may be given of a difficulty encountered in physiology which points to the possibility of protein aggregate formation. This is met in studying the process by which hemoglobin combines with oxygen. The dissociation curves of oxyhemoglobin as obtained by Hüfner (27) and by Barcroft (28) by using dialyzed hemoglobin are rectangular hyperbolas, while Bohr's (29) curves obtained in the presence of salts are of a different shape. The effect of acid upon the shape of the curves is the same as is that of salts, but more marked. An equation

$$y = 100 \; Kx^n/(1+Kx^n) \qquad (1)$$

derived by A. V. Hill (30) on the hypothesis that the electrolytes cause an aggregation of molecules of hemoglobin, causing the reaction to become of a higher order than unimolecular, applies well to the experimental data. y is the percentage saturation of hemoglobin with oxygen, x the oxygen pressure, while K and n are constants. Hill attached no physical meaning to these constants, but Barcroft regards K as the equilibrium constant and n as the average number of molecules of hemoglobin in each aggregate. That aggregate formation may lead to a change in the order of the reaction implies, as pointed out by Bayliss (8) the fact that the hemoglobin molecules and aggregates must behave as different chemical individuals. Most of the values of n used by Hill are fractional, and the explanation is given that there are present a number of aggregates of hemoglobin containing different numbers of molecules, so that the net result is a combination of different orders of reaction above the unimolecular one. The significance of the constants in this equation is by no means clear as yet. Direct measurements of the particle size in oxyhemoglobin solutions, dialyzed and in the presence of salts and of acids, are necessary to confirm the possibility of aggregate formation. Some of these measurements have already been made as noted in Part II.

In view of the evidence here presented that protein solutions frequently contain more than one molecular species, a series of direct measurements of the molecular weight (in the physical sense) and the particle size would be desirable in order to indicate (1) the uniformity or non-uniformity of the molecules in protein solutions and (2) the influence of certain variables upon the stability of certain molecular species found therein. In Part II a method for making such measurements is described, and the results thus far obtained in the study of the variables affecting the stability of the proteins are reviewed.

Part II

Determinations of the Stability of Certain Proteins under the Influence of Varying Protein Concentration and Hydrogen Ion Activity.

1. The Method. The ultracentrifugal method for the determination of the size of particles or molecules in colloidal sus-

pensions or solutions was developed by The Svedberg (31) for the purpose of obtaining curves of the distribution of particle sizes in such colloidal sols as those of gold. The theory of the method has been fully elucidated by Herman Rinde (32) in a dissertation, "The Distribution of the Sizes of Particles in Gold Sols Prepared According to the Nuclear Method." It was based upon an attempt ot utilize and extend the principles of Perrin's work on the settling of coarsely dispersed suspensions of gamboge under the influence of gravity. This work dimonstrated the applicability of Stokes' law to the determination of particle sizes.

Upon every particle settling in a gravity field, two opposing forces are acting (13). One is that due to gravity and is expressed by $V(\rho_p - \rho)g$, in which V is the volume of the particle, g the force due to gravity, ρ_p the density of the particle, and ρ the density of the medium in which the settling occurs. The other is the frictional resistance, which may be expressed as kdx/dt, if k represent a frictional coefficient, and dx/dt the velocity of sedimentation.

Experience demonstrated, however, that this reasoning applies only to relatively large particles. Smaller ones do not settle down, but come rather to an equilibrium because of their "molecular" diffusion which tends to maintain a state of uniform distribution. Studies of the concentrations at various heights in the resulting sedimentation equilibrium showed that an application of equations derived by Einstein (33) and by Perrin (34) permitted a calculation of the size of the particles. Even when the particles were not uniform, some knowledge of the distribution of sizes could be obtained in this way.

The gravity method was only applicable to very coarse suspensions, and for lyophilic colloids it was necessary to increase the directive force by replacing the gravity field by a powerful centrifugal field. The ratio of settling must be measured by optical means during the centrifugation. This is possible if the material studied has a strong light absorption in the visible or ultraviolet range. If light is sent through windows in the centrifuge case across a cell bored in the rotor and containing the solution investigated, the light absorption varies in the different parts of the cell in accordance with the distribution of concentration of the absorbing material. This is readily recorded photographically and by comparing the densities on the photographic plate with a concentration - density scale made upon an adjacent portion of the same plate by photographing dilutions of the same solution, the concentrations may be determined at any time at any position in the cell. The Svedberg constructed a series of ultracentrifuges capable of developing centrifugal fields of various magnitudes. The first produced only a field 150 times that due to gravity. A second developed one about 7000 g, and a more recent one, operated at 42000 r.p.m. by an oilturbine, (35) produces approximately 100,000 times the gravity field.

The use of centrifugal forces involves the replacement of g in the equations by an expression for the centrifugal field, $\omega^2 x$ in which ω is the angular acceleration and x is the distance from the axis of rotation at which the force is acting. ~~As~~ In dealing with the molecules of proteins, the assumption that the molecules are spherical would be unwarranted. The attempt to estimate the radius of the particle must be replaced by an attempt to determine the "physical molecular weight" which may or may not be identical with the "chemical ultimate molecular weight" which characterizes the protein. The force m.a tending to move the molecules (micelle or aggregate) to the periphery will then be $m.\omega^2 x$ in which m the apparent mass of the particle is either Vol. $(\rho_p - \rho)$ or $M(1-V\rho)$, M being the molecular weight, V the partial specific volume, and ρ the density of the solvent. Thus the volume of solvent displaced multiplied by the density and subtracted from the mass of the particle must give its apparent mass. In most of the experiments ρ is taken as the density of water at the temperature at which the experiments are made. V was determined pycnometrically by the aid of the equation

$$V = \frac{w - (l - h)}{\rho h} \qquad (2)$$

where w is the weight of solvent in the pycnometer, l the weight of solution, h the weight of the protein, and ρ the density of the solvent.

The frictional resistance is expressed as f dx/dt, and the fundamental equation is, therefore,

$$M(1-V\rho)\omega^2 x = f\, dx/dt \qquad (3)$$

The frictional force of a colloid particle of any shape affects the rate at which it diffuses. From measured diffusion constants D the frictional force may be calculated by the equation

$$f = RT/D \qquad (4)$$

Therefore the final equation becomes

$$M(1-V\rho)\omega^2 x = RT/D \cdot dx/dt \qquad (5)$$

and the molecular weight becomes

$$M = \frac{RT\,\frac{dx}{dt}}{D(1-V\rho)\omega^2 x} \qquad (6)$$

This equation may be used when the boundary is sharp and moves at a measurable rate under the influence of the centrufugal field. D must be known and may be determined by independent methods of measurement, or, by a method to be explained later, from the blurring of the otherwise sharp boundary which it occasions.

When the centrifugal force was only about 12000 times the gravity field most protein solutions failed to exhibit a sharp boundary moving outward. Instead, an equilibrium between centrifugation peripherally and diffusion centrally was obtained after two to three days, resulting in a definite distribution of protein concentrations from the central to the peripheral part of the cell. By photographic methods, the equilibrium concentration was determined and recorded graphically at each part of the cell. The calculation of the molecular weight was then made according to an equation which has been developed independently by both kinetic and thermodynamic

Considerations,

$$M = \frac{2RT \ln \frac{c_2}{c_1}}{(1 - V\rho)\,\omega^2 (x_2 - x_1)(x_2 + x_1)} \qquad (7)$$

in which c_1 and c_2 are the protein concentrations at the distances x_1 and x_2 respectively from the axis of rotation.

By applying these two methods - sedimentation velocity and sedimentation equilibrium - to the study of protein solutions both electrolyte free and in the presence of dilute buffer solutions, Svedberg (13) and his collaborators have arrived at the following values:

Table VI

Protein	Molecular Weight
Egg albumin	34,500
Hemoglobin	68,100
Serum albumin	67,500
Serum globulin	103,600
Phycocyan	105,500
Phycoerythrin	208,000
Limulus hemocyanin	2,040,000
Helix hemocyanin	5,005,000

Results obtained by the two methods usually check very well for a given protein. Thus,

Table VII

Protein	Molecular Weight by	
	Velocity	Equilibrium
Carbon monoxide hemoglobin	68,000	67,700
Phycocyan	105,000	105,900
Helix hemocyanin	4,900,000	5,000,000