Preparation of egg albumin solutions and observations on their optical activity during heat denaturation
by Norman D Cascaden

A THESIS Submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of Master of Science in Chemistry at Montana State College
Montana State University

Abstract:
S.P.L. Sorensen's method for preparing crystalline egg albumin, or solutions thereof, has been modified to permit a faster and more economical production of albumin solutions‘for a study of optical activity changes during heat denaturation

PREPARATION OF EGG ALBUMIN SOLUTIONS AND OBSERVATIONS ON THEIR OPTICAL ACTIVITY DURING HEAT DENATURATION

by

NORMAN D. CASCADEN

A THESIS

Submitted to the Graduate Faculty

in

partial fulfillment of the requirements

for the degree of

Master of Science in Chemistry

at

Montana State College

Approved:

Head, Major Department

Chairman, Examining Committee

Dean, Graduate Division

Bozeman, Montana
May, 1950

TABLE OF CONTENTS

Abstract

S. P. L. Sørensen's method for preparing crystalline egg albumin, or solutions thereof, has been modified to permit a faster and more economical production of albumin solutions for a study of optical activity changes during heat denaturation.

Preparation of Egg Albumin Solutions and Observations on Their Optical Activity During Heat Treatment

Introduction and Historical

When an egg is cooked its internal consistency changes and what once was fluid is after cooking quite solid. The egg has been coagulated. This phenomenon has been known for thousands of years, but it was only more recently that heat coagulation was recognized as being one of many possible ways that a protein could be changed chemically. The term applied now to this change in proteins is denaturation. The phenomenon of heat coagulation of proteins, particularly the albumins of the egg and blood, has been the object of many studies in the last seventy-five years.[17] Denaturation is now being studied intensively in all its aspects and especially so with respect to the effect that denaturation has on the original properties of the protein. However, in spite of all attempts to learn what takes place when the white of an egg, which consists of albumins and globulins, changes from the liquid state to insoluble coagulum, it still can be said that little is definitely known about the actual chemical and physical mechanisms involved.

The few well known facts pertaining to heat coagulation are given as follows:

1 - The liquid part of the egg or the white of the egg on heating turns to a solid state. This is the primal fact of coagulation. 2 - Sulfhydryl (-SH) groups, which are shown with the

sodium nitroprusside test,[24,25,28] appear in the heat denatured egg albumin. These groups are not originally present in the natural white of eggs. 3 - If a solution of egg albumin in a salt-free medium is shifted from its isoelectric point by the addition of acid or base and heated, it remains clear.[22] These are some of the facts that are known about heat denaturation. Many ideas have been offered to explain what occurs during denaturation, but they usually are refuted by someone who could not confirm the results on which they were predicated. An example of this is the case of Wu and Wu[35] who suggested that tyrosine is eliminated when albumin is coagulated by heat, but Mastin and Rees[23] could not confirm the finding of Wu and Wu.

Nevertheless, some constructive work has been done in determining what occurs when albumin is denatured by heat. Though disagreement is to be found in the work of Chick and Martin,[8] Cooper and Neurath,[9] and MacPherson et al.,[22] it does support the idea that two types of linkages are affected by heat during denaturation; first, the pH sensitive, and second, the pH insensitive linkages. They state that the pH sensitive linkages are very possibly salt linkages and result from the carboxyl and amino groupings in the protein molecule. The pH insensitive linkages are declared to be hydrogen bonds between two adjacent peptide chains. The destruction of these bonds by the application of heat causes displacement of the peptide chains in relation to one another. Cooper and Neurath[9] also

suggest the possibility that heat denaturation may cause polymerization of the protein. On the basis of their work, Neurath et al.[27,28] define denaturation as follows: "Denaturation is any non-proteolytic modification of the unique structure of a native protein, giving rise to definite changes in chemical, physical, or biological properties. Denaturation is an intramolecular change of the protein that causes a decrease in solubility, loss of crystallizing ability, increased reactivity of the constituent groups, change in molecular shape, and a change in the rate of enzymatic hydrolysis."

The term "non-proteolytic" as used in the definition by Neurath et al.[27,28] is in the estimation of the writer too limited in meaning with respect to the many changes in composition and structure of proteins that may be the causes of denaturation. It is entirely conceivable that an incipient hydrolysis (a true proteolytic) may be the origin of denaturation. Denaturation in its broadest and most general sense means any change in a protein causing it to have properties unlike those which it had in its natural state. However, since no one knows what properties a protein has in its natural state, the term denaturation can only be applied to proteins once they have been isolated.

The biggest stumbling block to knowing what occurs in the heat denaturation process is our ignorance as to what a protein actually is structurally. The theory of protein structure was

greatly advanced by the work of Emil Fischer and his students,[10] for it was their work with synthesis of polypeptides that gave rise to their idea of peptide linkages as the main linkages in proteins. Fischer's peptide chains are stated to be composed of simple amino acids united through their carboxyl and primary amino groups. Other work seems now to show that in addition to peptide linkages other groups and linkages do possibly exist in the unaltered proteins. Reactive groups now thought to exist in proteins include: aliphatic alcohol group (-OH), phenolic group (OH), imino group (=NH), sulfhydryl (-SH) and disulfide groups (-S-S-) and possibly others.

Following the advocation of polypeptide chains as a major protein linkage, other ideas followed, but as yet none of them has been quite able to displace Fischer's concept. One very serious defect in his theory is that it does not attempt to explain why proteins have a variable solubility, especially when the same amounts of the same amino acids are present in the protein molecule. During the last 30 years many new protein structures have been proposed. Abderhalden et al.[1,2] suggests that proteins are built of cyclic substituted diketopiperazine rings held together by latent valence forces. Miss Wrinch[18,34] has brought out a cyclol pattern that is composed of a series of diazine and triazine hexagons. These cyclol patterns are arranged so that for every triazine there are three diazine groups connected to it. Both theories have been accepted, however, only with reservations.

A resume of the known facts on protein structure and properties follows: (1) Some proteins hydrolyze into only alpha amino acids. About 40-45 such acids have been isolated from either proteins or protoplasmic systems. The general formula of an alpha amino acid is $R-\overset{H}{\underset{NH_2}{C}}-COOH$ and when two amino acids unite a dipeptide is formed viz. $R'-\overset{H}{\underset{NH_2}{C}}-\overset{O}{\overset{\|}{C}}-NH-\overset{H}{\underset{R''}{C}}-COOH$. Each dipeptide contains COOH and NH_2 groups through which the peptide may be lengthened indefinitely. This is the Emil Fischer peptide concept. (2) Other proteins give, on hydrolysis, alpha amino acids and non-protein groups, for example: chromoproteins which possess a chromophore or color group attached on the protein, nucleoproteins which contain nucleic acid attached to a protein, and phosphoproteins which yield o-phosphoric acid as the non protein group. (3) This class of proteins include the artificially synthesized ones as well as those which are decomposition products of the first two kinds mentioned. It is in this third group that meta proteins, mentioned in this paper, are to be found.[12]

Egg albumin, the material used in this study, may be regarded as a peptide chain protein, whose constituent parts may form layers in close physical proximity or, if not, united by secondary valence forces or hydrogen bridges. Therefore, two or more albumin molecules may be held in place by the above forces so that one may consider an albumin system as a giant molecule. This would probably be true of albumin, especially in concentrated solutions.

In the course of this work, it was observed that if an

albumin solution is held at pH 4.5 to 6.5 an insoluble protein precipitates from the solution. This protein goes into solution in a slight excess of either acid or base. Similarly, when a dialyzed albumin solution is neutralized almost to the point where it fails to precipitate when heated, a precipitate separates which also dissolves readily in either acid or base. In this work, this precipitate is referred to as albumin acid meta protein because the properties of this precipitate are like those of the acid meta proteins.[28]

Statement of the Problem Involved

The purpose of this work was to secure a suitable method for the preparation of egg albumin solutions and at the same time to prepare a solution that was comparable in purity, with respect to albumin and water, to ones obtained by the established methods of past investigators. It was hoped that any egg albumin solution so obtained might be suitable for optical activity studies.

Discussion of Methods

The isolation of a specific protein is of interest because of the unusual nature of proteins. A protein is one of the more difficult substances to purify and crystallize. In the first place, a protein may not be heated without some change and thus cannot be crystallized from a hot concentrated solution like some other organic compounds. Secondly, even though crystals of proteins can be obtained, they are invariably hydrated and when absolutely dry pass into the amorphous state.

The first definite references consulted by the author were by Harnack[13] and Osborne[29] who mentioned that Hofmeister prepared egg albumin in crystalline form in the following manner: free egg white was whipped to a froth, let stand for 24 hours with an equal volume of saturated ammonium sulfate and filtered to separate the globulin. The filtrate was allowed to evaporate for several days at room temperature. The substance was recrystallized several times from half-saturated ammonium sulfate solution by evaporation, but not to dryness, as before, until clear crystals unmixed with spheroids were obtained. Harnack[13] however, stated in several of his papers published in Berichte Der Deutschen Chemischen Gesellschaft that Hofmeister never was able to obtain crystalline egg albumin from the dialyzed egg albumin solution. Harnack[14] obtained crystalline albumin by the following method: egg white was treated with a slight excess of dilute solution of acetic acid until all the globulins

precipitated. The suspension of globulin was filtered, and the filtrate was neutralized with an excess of cold saturated sodium carbonate solution. Cold saturated copper sulfate solution was added and, if the copper albuminate did not precipitate out at once in faint green flocs, more sodium carbonate solution was added until it did precipitate. The precipitate was suspended in water and dilute potassium hydroxide was added until all the precipitate dissolved. The albumin was reprecipitated by cautious neutralization with acetic acid. This procedure was repeated until the desired purity was obtained.

Hopkins and Pinkus[15] used acetic acid to neutralize the half-saturated solution of ammonium sulfate solution (using Hofmeister's method) and claimed to have greatly facilitated the formation of albumin crystals. By this means, they stated they were able to secure pure crystals without evaporation and no ammonium sulfate crystals would be present.

Osborne[29] and Osborne and Campbell[30] found, that if they used hydrochloric acid instead of acetic acid, the separation of the crystals occurred more quickly and more completely. Solutions were dialyzed to remove sulfates before the crystals were allowed to form by evaporation.

The following method is that of Sørensen as given by Morrow.[26] It is one of the most common methods used for the preparation of pure egg albumin solutions and was the one used at the beginning of this

work. "Thoroughly stir the whites of 36 fresh eggs with an egual volume of saturated pure ammonium sulfate solution, and filter off the precipitate of ovoglobulin at once. To the clear reddish-yellow filtrate, contained in a precipitating jar, add saturated ammonium sulfate solution until the slight precipitate, which is indicated by a milky turbidity, ceases to dissolve and becomes permanent. Then add 0.2 N sulfuric acid until the mixture has a hydrogen ion concentration of pH 4.58, the optimum for crystallization. During the addition of the sulfuric acid the precipitate is first dissolved; the color of the solution changes from reddish-yellow to yellow, and finally there is formed an amorphous precipitate, which is at first easy, but later more difficult to dissolve by stirring. At about pH 4.6 it should be just possible to dissolve this precipitate. Stir the resulting opalescent solution vigorously; crystallization will usually begin in an hour. Allow the mixture to stand at room temperature from 5 to 6 days, stirring frequently. Then filter over night on three large gravity funnels; should any mother liquor remain above the precipitate next day, remove it with a pipette. Wash each precipitate twice with an ammonium sulfate solution of such concentration that the precipitation of the filtrate is just avoided. Fill each filter completely without stirring up the precipitate, and the washing solution will gradually replace the mother liquor. To determine the concentration of the washing solution, prepare a series of test tubes, each containing 10 cc. of saturated ammonium sulfate

solution and a different volume of distilled water; to each add a few cubic centimeters of the clear filtrate. When, for example, all the tubes up to and including that containing 6.5 cc. of water show turbidity, while all those containing 7 cc. or more of water are clear, it is evident that the concentration of the washing solution should be in the ratio of 10 cc. of ammonium sulfate solution to 7 cc. of distilled water.

"When the second washing solution has filtered over night, remove with a pipette any which has not run through. Scrape the precipitate from the filter papers into a large evaporating dish, using a porcelain spoon. Dissolve the precipitate remaining on the papers with distilled water and add to the evaporating dish; then add with thorough stirring, just enough more distilled water to dissolve the precipitate completely. If necessary, filter the solution and pour it into the precipitating jar; again add saturated ammonium sulfate solution to permanent turbidity, and adjust to a hydrogen-ion concentration of pH 4.58 by the addition of 0.2 N sulfuric acid. Stir the solution vigorously and allow to stand 3 or 4 days. Filter the crystallized egg albumin, wash with an ammonium sulfate solution of the proper concentration, and then dissolve the precipitate in distilled water. Dialyze in a large hardened collodion bag until free from sulfates. Early in the dialysis, observe whether the collodion bag has been sufficiently hardened, by testing the dialysate for the presence of albumin according to the method of

Sørensen.[32] The purified egg albumin solution will preserve its characteristic properties for months if mixed with a large quantity of toluene, shaken frequently, and kept at about 0°C."

Other methods have been suggested since the method of Sørensen was published but the principle has usually been the same. Others have suggested using sodium sulfate in place of ammonium sulfate for the precipitating agent.

La Rosa[19] used the centrifuge merely to shorten the time in separating the crystalline egg albumin. The globulin, likewise, was centrifuged off instead of removing it by filtering. This step alone saved him much time and effort.

The method used in this work, except for slight alterations, is the same as that used by Sørensen and co-workers.

After a review of the methods suggested for making egg albumin solutions, a modification was decided on as being the most convenient for the purposes of this work. Sørensen's method was tried a number of times and suspensions of crystalline albumin were obtained. The crystals were examined under a microscope where long needles were visible, also rosettes made up of needle-like crystals. However, for the purposes of this work only solutions of egg albumin were necessary. By repeated recrystallization and filtrations it was hoped with the modified procedure to obtain solutions of egg albumin, which might be referred to as solutions of pure albumin in water. The modified method gave results comparable to those obtained with Sørensen's

method. The modified method has the advantage of being quicker and more economical.

The modified method is as follows: separate the whites of 60 eggs and dilute with an equal volume of saturated ammonium sulfate solution. The mixture is stirred thoroughly and completely to separate the globulin from the albumin. The mixture is then filtered through large gravity funnels using a coarse filter paper. Filtration takes about 6 to 12 hours and possibly longer depending on the amount of solution and precipitate present. The albumin is a clear yellow filtrate and, unless kept in a cold room, should be protected against bacterial action by the use of toluene. The albumin is precipitated out of solution with an excess solid ammonium sulfate (U.S.P. grade is desirable) which is added to the albumin solution until a heavy white precipitate appears that is permanent on vigorous stirring. Allow to settle out over night in a refrigerator.

Cooling down to 0°C appears to encourage crystallization and a greater percentage of albumin is obtained on the first crystallization under such conditions. The filtrate is treated with more solid ammonium sulfate to saturation and allowed to settle out under the same conditions as before. Almost all the albumin crystallizes out the second time and the filtrate is generally clear when tested for a heat coagulable protein. The albumin is filtered off both times using a large gravity funnel. Filtration takes about 4 - 6 hours depending on the amount of solution present. The albumin