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
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Abstract:
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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. GASCADEN

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Approved:

[Signatures]

Head, Major Department

Chairman, Examining Committee

Dean, Graduate Division

Bozeman, Montana
May, 1950
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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.\(^\text{17}\) 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. Sulphydryl (\(-\text{SH}\)) groups, which are shown with the
sodium nitroprusside test, appear in the heat denatured egg albumin. These groups are not originally present in the natural white of eggs. 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. 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 who suggested that tyrosine is eliminated when albumin is coagulated by heat, but Mastin and Rees 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, Cooper and Neurath, and MacPherson et al., 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.
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-enzymatic 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-enzymatic" 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,\textsuperscript{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 includes aliphatic alcohol group (–OH), phenolic group (OH), imino group (=NH), sulfhydryl (–SH) and disulfide groups (–S–S–) and possibly others.

Following the advocacy 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.\textsuperscript{1,2} suggests that proteins are built of cyclic substituted diketopiperazine rings held together by latent valence forces. Misa Wrinch\textsuperscript{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 50-65 such acids have been isolated from either proteins or protoplastic systems. The general formula of an alpha amino acid is $R-\text{COOH}$ and when two amino acids unite a dipeptide is formed viz $R^1-\text{CH}_2-\text{NH}_2-\text{CH}_2-\text{COOH}$. Each dipeptide contains $\text{COOH}$ and $\text{NH}_2$ 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 $\text{PO}_4$ 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. 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 and Osborne 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 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 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 floc, 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\textsuperscript{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\textsuperscript{29} and Osborne and Campbell\textsuperscript{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.\textsuperscript{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 equal 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 4.56 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
Sörensen. 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.6

Other methods have been suggested since the method of Sörensen 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 Rosa19 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 Sörensen 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. Sörensen'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 Sörensen'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 1 to 6 hours depending on the amount of solution present. The albumin
precipitate is a faint pink color. The precipitate is redissolved in the smallest amount of distilled water, which forms a yellow solution, and then is reprecipitated with solid ammonium sulfate again. The albumin is precipitated three times using the above procedure.

After the third precipitation the albumin is redissolved in the least amount of water — 60 eggs of average size usually makes about two liters of a highly concentrated solution at cold room temperatures. Toluene is used as a preservative and the solution is placed in dialysis tubes for dialyzing. The outside dialyzing water (distilled water) is changed every half-hour for the first five hours, and then every 4 - 6 hours for a total of 24 hours. The solution is filtered, neutralized with dilute sodium hydroxide (about 0.5 N) and again dialyzed.

The water used for dialyzing the second and third time is now changed only as often as indicated by using saturated barium chloride solution as an indicator of the amount of the sulfate going into the water. Usually only four or five changes are needed if a 20 liter container is used for dialyzing purposes. The solution is dialyzed at least three times before it is free enough of sulfate for the purpose of this investigation.
Experimental Procedures and Results

A - Preliminary Studies

After an egg albumin solution had been prepared and dialyzed until apparently free from ammonium sulfate and sulfuric acid, it was heated to determine whether it would be suitable for optical activity measurements. It may be well to mention at this point that MacPherson et al.\textsuperscript{22} had produced such clear salt-free solutions of egg albumin which had pH values of 6.8 and 7.3 and which failed to coagulate when heated.\textsuperscript{*} The dialyzed solution was tested with litmus and nitrazine paper and the pH was noted to be between 4.5 and 5.0. The solution was heated slowly to boiling and kept at that temperature for twenty minutes to ensure complete coagulation, if any should occur. The coagulum which formed was filtered off. The filtrate

\begin{footnotesize}
\begin{itemize}
  \item This thesis evolved out of an observation made by B. L. Johnson, Montana State College, while he was trying to duplicate the heat denaturation properties of egg albumin as described by Dr. R. A. Gortner.\textsuperscript{21} The particular property involved was the reported heat sensitization of an egg albumin solution which had been so thoroughly dialyzed that all electrolytes had been removed. Such a solution as this is reported to exhibit heat sensitization (the first step in heat coagulation) but not the curding or the flocculation phase. However, it is stated in the same reference, that when the heat sensitized egg albumin solution is treated with an electrolyte, for
\end{itemize}
\end{footnotesize}
obtained on treatment with a few drops of sulfuric acid (approximately
\(0.5\ \text{N}\)) and heat gave rise to no further coagulation. This indicated
that the coagulation in the first treatment had been complete.

Additional portions of the above albumin solutions were then
neutralized with sodium hydroxide, the pH being roughly followed with
both litmus and nitrazine papers. In some instances, the Beckman pH
meter was used to determine the final pH at which the coagulation
just failed to appear on heating.

It was suspected that coagulation was dependent on, at least
three factors, namely: the amount of ammonium sulfate, absorbed
sulfuric acid held by the albumin, and the pH. This suspicion came
from the fact that after neutralization with sodium hydroxide and
dialysis, coagulation occurred a lower pH values which tended to

\[\text{example: sodium chloride, the flocculation reaction is initiated.}\]

While Johnson was mainly interested in determining whether heat
sensitization caused a change in optical activity, (the measurement
of this change should be quite possible with a clear sensitized
albumin solution) he made the observation that it was impossible to
dialyze an egg albumin solution to such a point that it would fail
to give the ordinary coagulation reaction. Because these results
seemed to contradict the above mentioned contentions in Gortner's
text, the solution was tested for acid and was found to be definitely
acid. It was then found that repeated dialysis did not lower the
approach neutrality as a possible limiting value. The more times
the solution was neutralized and dialyzed, the lower the pH at
which a coagulum would just fail to appear. This change in pH was
followed by litmus and nitrazine papers.

Alkalinity is known to interfere with heat coagulation.

Exactly what pH values prevent coagulation of egg albumin have not
been definitely established. However, some hydrolysis, as an
inhibiting factor, as well as the formation of alkali metaprotein
may occur. Precipitation is also induced by alkalinity. All these
changes are undesirable as they might be construed as the cause of
any changes in optical activity that might occur during heat sensi-
tization.

It was observed that, when a neutral egg albumin solution

acidity appreciably. The albumin solution was then neutralized to
litmus with sodium hydroxide and dialyzed thoroughly again. The
lack of a positive test for sulfate ions in the dialyzing water was
taken as proof that the dialysis was complete. The solution was
neutralized, dialyzed, and then heated, but the usual coagulation
occurred. From these results it appeared as if no little hope
existed for studying the optical activity of the denatured egg
albumin. Quite by accident, Johnson heated a neutralized solution
of egg albumin and though the solution was heated to boiling no
coagulum appeared. Polarscopic tests were run on this solution and
after repeated dialysis failed to coagulate on heating, a coagulum could always be obtained on adding a few drops of acid (approximately 0.5 N) to the cold solution. This coagulum would dissolve readily in more of the same acid and also in the alkali solution. These results suggest that an alkali metaprotein is produced during the heat sensitization phase, and that this change occurs far below the pH values customarily used in preparing albumin alkali metaproteins.

It was also found that egg albumin retained its characteristic heat coagulating ability in alkaline solution. This finding contradicts the prevalent opinion that alkali prevents heat coagulation. One such solution was found to coagulate at pH 9.5. This may have been due to substances such as ammonium sulfate and sodium sulfate (both suspected to be present in small amounts) which might act as heat coagulation buffering agents. Therefore, it seems likely that alkali metaprotein formation might occur at any pH above the isoelectric point of albumin and that any failure to coagulate could be the numerical values, though small, were measurable and significant. Johnson's findings disagreed with the statements in Cottner's text. Inasmuch as sodium hydroxide was added to neutrality, an electrolyte was present, nevertheless no coagulation took place. He also found that acid conditions favored coagulation on heating. This is in opposition to Neurath's more recent statement that slight departures from the isoelectric point of egg albumin inhibits coagulation.
due to this cause alone.

Since it was thought that the duration of heating might be a factor involved in the failure of egg albumin solutions to coagulate, a properly prepared albumin solution was heated for one and one half hours at the temperature of boiling water (95°C at 1,800 feet elevation) without any perceptible coagulation being noticed. Polarscopic tests made on this solution were no different numerically than those obtained after a heating period of twenty minutes.

It was noted, too, that highly concentrated egg albumin solutions required more time to heat coagulate than was anticipated. Whether this was due to large amounts of albumin being present and therefore to be coagulated, or to the absence of certain coagulating ions, as per Gortner (see footnote), was not investigated.

A final observation was that though albumin solutions were adequately protected from bacterial action by the use of toluene and kept in the cold, a slight precipitate formed gradually which was considered to be probably a metaprotein because of its solubility in acids and alkalis. No work was done to ascertain its exact identity.
B - Determination of weights of albumin in albumin solutions

A means of estimating the concentration of egg albumin present in a specific volume of solution was deemed necessary. To determine the amount of albumin in solution, two approaches were used: first, the weight of denatured albumin was determined by drying, and second, this same weight was estimated by the Kjeldahl method. The first method will be called the dry weight method and the second, the Kjeldahl weight method.

For the dry weight determinations 25 ml. samples of egg albumin solutions were used. Because heat coagulation of these untreated undiluted solutions gave rise to non-filterable flocs, which were equally incapable of being sedimented completely by centrifugation, 25 ml. of physiological saline (9 gr. of NaCl per liter) solution were added as a flocculation aid to each 25 ml. of albumin solution before heating.

Filtration of the flocculum was found impossible but centrifuging at 4000 r.p.m. for 60 minutes yielded almost complete sedimentation and hence this procedure was followed in getting the dry weight of albumin.

To remove the sodium chloride, the sedimented flocs were suspended in distilled water after the removal of the supernaunt solution. They were sedimented a second time under the same conditions. One hundred fifty ml. of distilled water were used in making the above suspension. About 10 ml. of sedimented albumin
and water were left behind each time when removing the supernatant solution. A calculation of the concentration of NaCl revealed that the amount of remaining NaCl was negligible.

The sedimented flocs were transferred quantitatively to weighed evaporating dishes and permitted to dry, first at a low temperature, and then to constant weight at 110°C. The dry weights were then obtained by difference.

For the Kjeldahl method of determining the weight of albumin, 25 ml. of the egg albumin solution were used. To each sample was added 25 ml. of concentrated sulfuric acid, a few boiling stones, 10 grams of Na₂SO₄, 1 gram of copper oxide, and the contents of each sample were digested for ninety minutes or until the digest became a transparent green color. When the clear digest had cooled, 300 ml. of distilled water and 100 ml. of saturated, carbonate-free sodium hydroxide solution were added cautiously to the flask and placed on the Kjeldahl apparatus, which previously had been readied for distillation. The flask was shaken vigorously to start the reaction and then heated until the desired amount had distilled over. The acid in the receiving flask was then back-titrated to determine the ammonia present in the sample. From the ammonia distilled over, the protein was calculated, by multiplying the nitrogen value by 6.38.

The compilation shown in Table 1, Series A and B gives the weights of albumin in 25 ml. of an albumin solution as obtained by both methods.
TABLE 1 - Series A

Albumin by Drying and by Kjeldahl Methods

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Ml. Albumin Solution</th>
<th>Dry Weight Albumin</th>
<th>Ml. Albumin Solution</th>
<th>Ml. Back Titration</th>
<th>Calculated Weight Albumin</th>
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<td>37.52</td>
<td>0.1596</td>
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Normality of acid-0.2995
Normality of base-0.1449
Blank Titrations Ave. Ml.-49.85
Nitrogen-Protein factor-6.38
Indicator: Methyl orange
Table I - Series B

Albumin by Drying and by Kjeldahl Methods

<table>
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<tr>
<th>Sample No.</th>
<th>Ml. Albumin Solution</th>
<th>Dry Weight</th>
<th>Ml. Albumin Solution</th>
<th>Ml. Back Titration</th>
<th>Calculated Weight Albumin</th>
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<td>25</td>
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<td>0.3455</td>
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<tr>
<td>4</td>
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<td>0.3523</td>
<td>25</td>
<td>11.05</td>
<td>0.3424</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
<td>Lost</td>
<td>25</td>
<td>10.8</td>
<td>0.3416</td>
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<tr>
<td>Avg*</td>
<td></td>
<td>0.3367</td>
<td></td>
<td>10.89</td>
<td>0.3438</td>
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Normality of acid-0.0989
Normality of base-0.0997
Blank-19.5 Ml.
Nitrogen-Protein factor-6.38
Indicator- Methyl orange.
Calculations for the Kjeldahl method were made as follows:

\[(V_1 - V_2) \times N \times 0.014 \times 6.28 = \text{grams of protein}\]

\(V_1 = \text{ml. of NaOH in back titrating blank test}\)

\(V_2 = \text{ml. of NaOH in back titrating sample}\)

\(N = \text{normality of NaOH}\)

0.014 = grams of nitrogen per ml. of 1 N solution of \(\text{NH}_3\)

6.28 = nitrogen-protein conversion factor
The difference in the results using both methods for the determination of the weight of albumin in definite volumes of solutions appeared to be due to experimental difficulties. In the dry weight determination, several problems appeared. In centrifuging, no speed was found that sedimented all the denatured albumin. The supernatant liquid, as a result, in spite of all precautions, carried off some of the denatured protein. Because the albumin was denatured in a different container from the one in which it was centrifuged, losses occurred unavoidably in the transfer despite great care. In the case of the Kjeldahl method, the use of methyl orange did not lend itself to high precision in back-titrating. However, the results presented in Table 2, Series A and B were obtained by use of the mixed indicator (brom-cresol green and methyl red) which afforded high precision in obtaining end points. All aspects considered, the Kjeldahl method was probably the more accurate of the two.

C - Polarscopic Determinations

Solutions of egg albumin were dialyzed at least three times for a period of twenty-four hours and also neutralized between each dialysis before they were used for polarscopic determination. These egg albumin solutions were tested with litmus and nitrazine papers to determine their approximate acidity and also samples of them were heated in boiling water to test their ability to coagulate. Twenty-five milliliter portions were transferred to 50 ml, volumetric flasks and filled to the mark with distilled water. Other portions
TABLE 2 - Series A

Optical Activity Change During Heat Denaturation

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Base Used in Back Titration, Ml.</th>
<th>Wt. Acquired in Sample, g</th>
<th>Rotation Observed in Samples of the Same Solution</th>
<th>Rotation Computed in Relation to One Gram Based on Weights Obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>36.41</td>
<td>0.1223</td>
<td>1 -0.5 -0.5 -1.0</td>
<td>1 -4.08 -4.08 -8.17</td>
</tr>
<tr>
<td>2</td>
<td>36.40</td>
<td>0.1224</td>
<td>2 -0.5 -0.5 -1.0</td>
<td>2 -4.08 -4.08 -8.16</td>
</tr>
<tr>
<td>3</td>
<td>36.30</td>
<td>0.1232</td>
<td>3 -0.5 -0.5 -1.0</td>
<td>3 -4.05 -4.05 -8.11</td>
</tr>
<tr>
<td>4</td>
<td>36.10</td>
<td>0.1224</td>
<td>4 -0.5 -0.5 -1.0</td>
<td>4 -4.08 -4.08 -8.16</td>
</tr>
<tr>
<td>5</td>
<td>36.30</td>
<td>0.1232</td>
<td>5 -0.5 -0.5 -1.0</td>
<td>5 -4.05 -4.05 -8.11</td>
</tr>
<tr>
<td>6</td>
<td>36.42</td>
<td>0.1222</td>
<td>6 -0.5 -0.5 -1.0</td>
<td>6 -4.09 -4.09 -8.18</td>
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</table>

Normality of acid = 0.0989
Normality of base = 0.0993
Blank = 50.2
Nitrogen-Protein factor = 6.38
Indicator-Mixed (brom-cresol green and methyl red)
TABLE 2 - Series B
Optical Activity Change During Heat Denaturation

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Ml. Base Used</th>
<th>Calc. Wt., Sample</th>
<th>Rotation-Observed in Samples of the Same Solution</th>
<th>Rotation Computed in Relation to One Gram Based on Weights Obtained</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>39.05</td>
<td>0.0989</td>
<td>1</td>
<td>-0.5</td>
</tr>
<tr>
<td>2</td>
<td>39.10</td>
<td>0.0984</td>
<td>2</td>
<td>-0.5</td>
</tr>
<tr>
<td>3</td>
<td>39.06</td>
<td>0.0983</td>
<td>3</td>
<td>-0.5</td>
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<tr>
<td>4</td>
<td>39.10</td>
<td>0.0984</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>39.08</td>
<td>0.0986</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>39.10</td>
<td>0.0984</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Normality of acid-0.0989
Normality of base-0.0993
Blank-50.2
Nitrogen-Protein factor-6.38
Indicator-Mixed (brom-cresol green and methyl red)21
*Rotation in scale divisions
of the solution were treated with sodium hydroxide (about 0.5 N) until points were reached at which they just failed to coagulate on heating. Samples were then measured out and neutralized in the same manner as above. Half of these samples were placed in water, heated to boiling and kept at this temperature for 20 to 30 minutes. This period was found by tests to be sufficient to coagulate the egg albumin completely. All heated solutions were cooled to room temperature. The various samples, i.e., the unneutralized diluted solutions, the solution which just fail to coagulate on heating, and the heated neutralized diluted solution were placed in a 10 cm. polariscop tube and the readings taken on a Soleil-Ventske saccharimeter. A series of readings was always taken on each sample but little or no variation existed between readings. All readings in this paper are given in Soleil-Ventske 20 scale divisions.
Discussion of the Results

Although the action of heat on proteins has been studied, more work needs to be done before a complete understanding will exist as to what happens chemically and physically when a protein is heat-denatured. Those investigators\(^\text{3,4,5}\) who adhere to the globular hypothesis of protein structure state that the unrolling of the protein into layers of peptides is an integral phase of denaturation. Sulphydryl groups, according to them,\(^\text{24,25,26}\) become free by this unrolling to react with the sodium nitroprusside reagent. Others, like Kuhn\(^\text{16}\) and Yasawa,\(^\text{33}\) state it quite probable that diketopiperazine nuclei are probably formed during this change. Opening of some peptide and other linkages is also possible; conversely the formation of new linkages may lead to denaturation.

It was not the intention in this work to study the chemistry of heat-denaturation, but to establish that optical activity is modified during this phenomenon. However, inasmuch as this investigation demonstrates changes in optical activity, it is rather timely now to speculate as to what such changes might mean. Because all amino acids save glycine are optically active, proteins contain asymmetric carbon atoms. The hydrolysis, therefore, of practically any peptide linkages would, for the same reason, cause an optical change. Because alkali favors racemization, which ends in changes in optical activity, it is incorrect (Barker\(^\text{6}\)) to attribute changes in alkaline solution specifically to the heat-denaturation process.
Such changes could possibly be allocated to alkali metaprotein formation. On the acid side of pH 7 it is impossible to heat-denaturate and obtain a system which is sufficiently clear to study polarisoscopically. But at pH 7, or slightly above, it is possible to find a point where the protein system just barely fails to curd and only at this point is it safe to attempt to demonstrate changes in optical activity caused by or accompanying heat-denaturatation. It is obvious that the optical change noted may be due as readily to the formation of new peptide linkages from existing amine and carboxy groups, as from peptide linkages opened by hydrolysis. The changes in hydration of protein molecules may also be responsible for the variation in optical activity noted, especially would this be true if water were to add by means of primary valence forces. Whether hydrogen bonding would play any significant part in modifying polarisoscopic power is without affirmation at the present time. Though racemization implies an equal dextro and levo change with respect to a particular amino acid, it does not follow that the change is necessarily due to this cause. The final effect, in the-case of racemization, may be more levo or more dextro, but always there will be a change except in one theoretically possible instance. But the chance that new groups of both levo and dextro character of such numerical optical effects being formed to compensate for the racemisation effect is practically nil. This is the exception referred to above, but such a series of changes could, of course,
not be detected by optical methods. Any change actually noted could therefore be due to racemization, one which would be truly of a heat-denaturation kind and not one due to alkali denaturation (i.e., Barker's racemization). The optical change could equally well, of course, be due to hydration or dehydration, or to an actual interchange of groups. Whether it could be due to any unraveling of the globular protein, or to association by virtue of secondary valence forces is highly problematical.

The success of any attempt to isolate and identify any heat racemized amino acids, if racemization is the cause of the result noted, is indeed remote, unless one were to find a protein in which such changes are far more numerous than are now suspected to occur in denaturation (a small effect, it may be noted, is not necessarily a measure of the extent of the reaction). The isolation of the enantiomorph of the opposite sign to the one originally present in the undenatured protein would be more probable if heat-denaturation brought about an interchange of any levo groups about an asymmetric carbon atom.

No conclusion can be drawn at this stage of the study as to what chemical or physical change caused the change in optical activity during heat-denaturation.
Summary

1. Egg albumin solutions were prepared that were similar in purity and properties to those albumin solutions which are prepared by Sorensen method; however, the means of preparing these solutions were modifications of his method.

2. Concentrations of albumin solutions were determined by use of the Kjeldahl method for the determination of total nitrogen, and weighing of dried samples of specific volumes of the albumin solution.

3. By use of a saccharimeter, polariscopic readings were taken in order to show that denaturation by heat is accompanied by a change in optical activity.
Acknowledgements

The author wishes to take this opportunity to express his sincere appreciation and thanks to Dr. Birger L. Johnson for his personal guidance and inspiration during this work, also to the other members of the Chemistry Department of Montana State College for their very helpful suggestions concerning this work. He also wishes to express his appreciation to Miss Doris E. Wilson of the library staff for her valuable assistance.
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<td>Victor E. Kinzler</td>
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Cascaden, N. D.

Preparation of egg albumin solutions.