THE ability of egg white to heat-coagulate is the basis for its use in many food products. This study is an attempt to determine some of the various factors which affect the heat coagulation of egg white and how and why these factors affect this coagulation. In this study the criteria of optical density and gelation are used as the measures of coagulation.

Slosberg et al. (1948) observed that the pH of the egg white at the time of heat treatment was important and that egg white was more stable to heat at pH 6.5 than at pH 8.5. These observations appear contrary to those of Barmore (1936), who reported that lowering the pH reduced the coagulation temperature. On the other hand, Cotterill and Winter (1934a) reported a greater optical density in egg white from thermostabilized seven-day old eggs than from thermostabilized fresh eggs. They attributed this increase in optical density to the higher pH of the egg white of the older eggs. Payawal et al. (1946) reported that by using an instantaneous method of heat treatment, egg white was denatured in the temperature range of 58 to 62°C (136.4 to 143.6°F.).

Slosberg et al. (1948) also reported that angel cakes baked from egg white which was heated to 138°F (59°C.) momentarily and then cooled, had reduced volumes and longer whip times than did cakes baked from unheated egg white. They also reported that temperatures below 136°F (57.9°C.) were detrimental if the time of heating was long enough. Clinger et al. (1951) also reported that lower cake volumes and increased whip times were the result of pasteurizing commercial frozen egg white at 57°C (134.6°F.) for four minutes. They also reported that angel cakes baked with pasteurized egg white from grade A eggs had volumes only slightly lower than those baked with unpasteurized controls and greater volumes than the cakes baked with the pasteurized commercial egg white.

Funk (1950), Goeresline et al. (1950), and Carlín and Foth (1952) reported that the thermostabilization of commercial eggs resulted in an egg white which had an increased whip time and a decreased cake volume.

Sucrose has long been known to retard the heat coagulation of egg white (Barcroft and Rutzler, 1931; Barmore, 1936; and Lowe, 1955). Further information was added to this subject by Slosberg et al. (1948), who reported that the addition of sucrose to egg white increased its time and temperature of coagulation rather than preventing it altogether.

MATERIALS AND METHODS

Liquid "yolk-free" egg white obtained from eggs held at 13 ± 2°C. (53 ± 5°F.) for 24 to 48 hours was used in these experiments. This egg white was blended to a uniform viscosity and frozen until needed.

Optical Density and Gelation Score Measurements. To obtain the optical density and gelation score measurements, the egg white was adjusted to the desired pH with HCl or NaOH and centrifuged for 15 minutes at about 1,000 times the force of gravity.

1 Contributions from the Missouri Agricultural Experiment Station. Journal Series Number 2469. Approved by Director.
Heat Coagulation of Egg White

A five ml aliquot of the supernatant of each sample was pipetted into a 12X100 mm test tube positioned in a test tube rack. This rack with the filled test tubes was then placed in a constant temperature water bath equipped with a device to constantly agitate the water. At the specified time, the tubes were removed from the water bath, cooled and the optical density determined with a Bausch and Lomb Spectronic 20 spectrophotometer at a wave length of 550 mµ using water as the standard.

After the optical density was recorded, the gelation score was determined by inverting the tube and arbitrarily judging the amount of gelation by the rate of flow, or lack of flow, of the egg white from the tube. The arbitrary score of 0 indicates no gelling and a score of 4 indicates complete gelling, with scores of 1, 2, and 3 being intermediate.

Heat Treatment. The heat treatment of the egg white was carried out in a laboratory pasteurizer similar to the one used by Winter et al. (1946) in their pasteurization studies. Two water-jacketed condensers served as the preheater. The water circulating through these condensers was heated in a constant temperature water bath to 40°C (120°F). The pasteurizer proper consisted of a stirrer-equipped constant temperature water bath, into which five coiled four-foot lengths of thin walled six mm. glass tubing were immersed. These coils were connected by rubber tubing and served as the heat exchanger. Two similar coils of glass tubing were immersed in an ice bath and served as the cooler. The samples were collected in Erlenmeyer flasks connected to a vacuum pump which drew the egg white through the system. The amount of vacuum was adjusted so that the egg white passed through the pasteurizer in three minutes. Because partial restrictions were built up in the system during the pasteurization, the vacuum was continuously adjusted to a constant flow of egg white into the flask.

The samples were drawn through the pasteurizer in their pH sequence and were separated by an air space between each sample. To reduce the error due to mixing of the samples, the first 50 ml of each sample to pass through the pasteurizer was discarded.

Surface Tension and Viscosity Measurements. The surface tension was measured with a direct reading Du Nouy Tensiometer. A constant temperature of 21°C (70°F) was maintained for all readings and distilled water served as a standard for determining the correction factor. Each surface tension value recorded is an average of at least three readings. A new surface was poured for each reading and the ring was blotted and flamed between readings.

The viscosity of the egg white was measured using a number 200 Oswald-Fenske type viscosimeter having a bore of approximately one mm. diameter. The viscosity measurements were carried out at a constant temperature using water as the standard for calibrating the viscosimeter.

Paper Electrophoresis Studies. A Spinco model R paper electrophoresis apparatus was used to qualitatively determine the ability of the individual proteins of the egg white to migrate electrophoretically after the various treatments. The method used was essentially that of Evans and Bandemer (1956). A 0.006 ml sample of egg white was placed on the filter paper strips saturated with a sodium diethylbarbiturate-diethylbarbituric acid buffer of pH 8.6 and ionic strength of 0.075. A two and one-half ma. current was applied across the strips for 16 hours. The strips were dyed with a bromphenol blue dye.
(Spinco B-1) and observed.

**Angel Cake Preparation.** Angel cakes were prepared and baked according to the procedure described by Gardner (1960). After cooling, the cake volume was measured by the seed displacement method. The whip time, the time required for the egg white to be whipped to a medium peak, was determined using a Kitchen Aid mixer at speed 10 (high speed).

**EXPERIMENTS AND RESULTS**

**Exp. 1 Optical Density and Gelation Score Study.** One of the variables in the coagulation of egg white is the pH of heating. The measurements of optical density and gelation score as described above were used to investigate this variable. The pH range of 5.0 to 10.5 was studied at temperatures of 58.0°C and 56.0°C (136.4°F and 132.8°F) for various time intervals. The egg white was centrifuged after pH adjustment and before heating to prevent erratic optical density measurements due to the curding which occurred in some of the samples when heated. This curding occurred in the samples heated at below pH 6.0. The centrifugation had little effect on the resultant optical density or gelation score.

The effect of the pH of heating at 58°C (136.4°F) for 5, 15, 30, and 60 minutes on the optical density and gelation score of the egg white, along with the effect of the pH (without heating) on the optical density of the egg white is shown in Figure 1. This graph shows that the optical density of the unheated control varies with the pH, having a maximum at about pH 6.5. However, this variation in the unheated controls is not as great as it is in the heat treated samples. The curves for the various times of heating (Figure 1) have a common minimum at about pH 8.75 and a common maximum at about 9.5. The major difference between these time of heating curves is their degree of opacity; the samples heated for the longer period of time being more opaque than those heated for the shorter period of time.

The five minute heating curve shows a rapid increase in optical density between pH 6.0 and 7.0. This rapid increase is found in all the heating time curves, but moves to higher pH values as the time of heating is lengthened.

The gelation score of egg white heated for 30 minutes at 58.0°C (136.4°F), when plotted against pH, follows the same pattern as the optical density curves. That is, it has a maximum at pH 9.5 and a minimum at pH 8.75. However, this curve differs from the optical density curve at the lower pH values by having another maximum at about pH 7.75 and another minimum at about pH 6.0. Heating the egg white for 30 minutes at 58.0°C (136.4°F) in the region of pH 6.0, as shown in Figure 1, caused no gelation, even though the egg white was completely opaque.

When this experiment was repeated at 56.0°C (132.8°F), the same types of curves were obtained as at the higher temperature. The main difference between the two temperatures was the degree of opacity and gelation.

**Exp. 2 Physical and Functional Property Study.** In order to study the effect of the pH of heating on some of the other physical properties as well as some of the functional properties of the egg white, larger samples were needed. These samples were prepared with the laboratory pasteurizer described above.

The physical properties studied in this experiment were: the optical density of the egg white after heat treatment, before and after readjustment back to pH 9.0; the surface tension; the viscosity; and the electrophoretic mobility. The functional properties studied were the angel cake volume.
The degree of openness for the longer period of time is more noticeable than those heated for a shorter period of time.

The heating curve shows a change in the optical density below pH 9.5. This rapid increase in the gelation score is due to changes in the f values as the pH is reduced.

The effect of egg white heating at 58°C (136.4°F) on the optical density curves shows the same trend as the pH values at pH 9.5 and above.

However, this increase in the optical density curves is caused by having an increase in the pH from 7.75 to 8.0. Heating for 60 minutes at 58°C caused a region of pH 6.0 to 6.5 to cause no gelation of the sample, which was completed.

The samples were repeated at the same temperature as at the highest difference between the two samples was the degree of openness.

For the study of the effect of the other physical properties such as the volume ratio and the whip time, the heat treatments were carried out in the pH range of 7.0 to 9.5 for three minutes at three temperatures; 58.0, 60.0, and 62.0°C (136.4, 140.0, and 143.6°F.).

The data for the effect of the pH of heating on the cake volume ratio, whip time, surface tension, and viscosity for these three temperatures are presented graphically in Figures 2, 3, and 4. Each point on these graphs represents the average of three trials.

Heat treating egg white in the laboratory pasteurizer at these three temperatures yielded optical density vs. pH curves which were in agreement with those of the optical density and gelation score study. Adapting the pH of the heat treated samples to 9.0 did not alter their optical density. However, all the unheated con-
control samples, when readjusted to pH 9.0, had the same optical density as the pH 9.0 sample.

The angel cake volume ratio and the whip time were affected by the pH at which the egg white was heated. Heating the egg white for three minutes at 58.0°C (136.4°F) at the low pH values (pH 7.0-8.0) and at the higher pH levels (above pH 9), as shown in Figure 2, reduced the cake volume ratio and increased the whip time. However, in the region of pH 8.75 the cake volume ratio of the heat-treated egg white was significantly increased over that of the unheated controls while the whip time was decreased.

Heating the egg white at 58.0°C (136.4°F) for three minutes at these pH values had no effect on the surface tension and lowered the viscosity only slightly. Likewise, the electrophoretic studies of these samples revealed little change in the electrophoretic mobility of the individual egg white proteins with the exception of lysozyme which showed a decreased mobility in the sample heated at pH 9.5.

The cake volume ratios were depressed and the whip times were greatly increased by heating the egg white for three minutes at 60.0°C (140.0°F). It can be observed in Figure 3 that these adverse effects were greatest at the higher pH values.

In contrast to the lower temperatures, heating the egg white at 66.0°C.
minutes at these pH
values, however, the surface tension and viscosity only slightly
affected the surface tension and viscosity. The surface tensions of the heated egg white were greater than that of the un-
heated controls. As can be seen in Figure 3, this higher surface tension increases slightly with increasing pH. At this
temperature the viscosity was also increased by the heat treatment.

The electrophoretic studies of the egg white heated at the various pH values for three minutes at 60.0°C (140.0°F) revealed a decrease in lysozyme mobility at pH 9.0 and above. The sample heated at pH 9.5 contained no detectable mobile lysozyme. Heating the egg white at the lower pH values resulted in a decrease in

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Fig. 3

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the conalbumin mobility. The samples heated at pH 7.0 and 7.5 showed little mobility of this fraction. However, the conalbumin mobility increased with increasing pH, with essentially all of this fraction being mobile at pH 8.5.

Heating the egg white at the various pH values for three minutes at 62.0°C (143.6°F) resulted in cake volume ratios and whip times similar to those obtained by heating at 60.0°C (140.0°F). At this higher temperature these adverse effects were enhanced, especially at the higher pH values (Figure 4). The cakes baked

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from the egg white heated at these higher pH values not only had a reduced volume but also tended to pull away from the
sides of the pan during the baking process.

The surface tension of the egg white heated at 62.0°C (143.6°F) also increased at the higher pH values. Likewise, the viscosity of the egg white heated at this temperature, as can be seen in Figure 4, was greatly influenced by the pH of heating. Heating the egg white at pH 7.0 through 8.0 had little effect on the viscosity. However, at the pH values above 8.0, the viscosity of the heated egg white was greatly increased.

The electrophoretic behavior of the egg white heated at 62.0°C (143.6°F) can be seen in Figure 5. These patterns are similar to those of the egg white heated at 60.0°C (140.0°F). However, heating at this higher temperature caused further reductions in the mobility of the conalbumin and lysozyme.

**Exp. 3 Sucrose Study.** The effect of sucrose on the heat coagulation of egg white as a function of the pH of heating was studied using optical density and gelation score as the criteria of coagulation. Two concentrations of sucrose were used in this study. The first concentration, 10% (10 grams sucrose per 90 ml of egg white), was studied at two temperatures, 57.5 and 59.5°C (135.5 and 139.1°F). The second concentration, 50% (100 grams sucrose per 100 ml of egg white) was studied at 72.5°C (162.5°F).

The results of these experiments indi-
HEAT TREATED (62° C. - 3 MIN.)

Fig. 5. Paper electrophoresis strips of heat treated egg white (62°C. — 3 min.) at various pH values.

Experiments indicated that heating egg white containing 10% sucrose at 57.5°C. (135.5°F.) was comparable to heating egg white at 56.0°C. (132.8°F.) when optical density was used as the criterion. However the egg white plus 10% sucrose was more resistant to gelation at 57.5°C. (135.5°F.) than pure egg white at 56.0°C. (132.8°F.). Contrarily, heating egg white containing 10% sucrose at 59.5°C. (139.1°F.) gave approximately the same results as heating pure egg white at 58.0°C. (136.4°F.) when measured by either optical density or gelation score.

In order to obtain the same amplitude of coagulation with egg white containing 50% sucrose, a temperature of 72.5°C. (162.5°F.) was used. At this temperature the concentration of sucrose the pH vs. optical density and the pH vs. gelation core curves deviated from those of the unheated egg white. As shown in Figure 4, this egg white-sucrose mixture had little increase in optical density or gelation score when heated at above pH 8.5 for times as long as one hour. However, heating at this temperature for an hour caused some caramelization in the higher pH egg white. It can also be seen in Figure 6 that at the pH values between 5.0 and 8.5, this egg white-sucrose mixture was more sensitive to the heat than at the higher pH values when measured by either optical density or gelation score. This change from no apparent effect on the egg white-sucrose mixture to a high optical density and gelation score effect was abrupt at about pH 8.5.

DISCUSSION

The time and the temperature of heating are of the utmost importance in the study of the heat coagulation of proteins. To study other variables, these must be held constant and a time and temperature should be chosen which will affect the system in a measurable way.

The control curve in Figure 1 represents the unheated egg white and the optical density change is due to the pH alone. These findings are in agreement with Cotterill et al. (1959).
The variation in the coagulation of egg white due to the pH of heating is in agreement with the findings reported by Osborne and Campbell (1900), Chick and Martin (1910), and Lepeshkin (1922) for ovalbumin. Preliminary experiments have shown that the differences obtained in this experiment were due to hydrogen ion concentration rather than ionic strength or dilution.

The results of these effects of the pH of heating experiments clarify some of the seeming contradictions in the literature. Slosberg et al. (1948) reported that egg white was more stable to heat at pH 5.3 than at pH 8.5. This is in good agreement with the results obtained in this study, when the gelation score is used as the criterion for stability. On the other hand, Barmore (1936) reported that lowering the pH reduced time. This is a result obtained by optical density of the gelatin in samples when gelatin for coagulation in 15 to 8.75. It is reported an increase in the pH at which the gelatin precipitated at a higher time when considered.

The irregular maxima and minima of pH and curves is what mixture of solubility represents the components of least stable and values at which stable. Since p. denatured: point, it could be curves representer some of the maxima and gelation probably due to involving the zone is increment major egg white which is isoelectric range and become theoretically in.

However, this is reported isoelectric point of 10.5 (Fevold, 1965). The other maxima curve, the optical density was explained by the theory. This ra
Heat Coagulation of Egg White

The pH reduces the coagulation temperature. This is also in agreement with the results obtained in this study when the optical density is used as a criterion for coagulation in the pH range of 5.0 to 8.75, or when gelation score is used as the criterion for coagulation in the pH range of 4.75 to 8.75. Cotterill and Winter (1950a) reported an increase in optical density as the pH at which the egg white was heated was increased. Again, the results of the experiments reported here confirm these findings when the pH range of 8.5 to 9.5 is considered.

The irregular shape, the presence of maxima and minima, of the optical density vs. pH and gelation score vs. pH curves is what could be expected for a mixture of soluble proteins. The maxima represent the pH values at which the components of the protein system are least stable and the minima represent pH values at which they are the most heat stable. Since proteins are generally most easily denatured at or near their isoelectric point, it could be that the maxima of these curves represent isoelectric denaturation of some of the individual proteins.

The maximum in the optical density and gelation score curves at pH 9.5 is probably due to a change in the egg white involving the lysozyme fraction. Lysozyme is incriminated because it is the only major egg white protein (quantitatively) which is isoelectric in the alkaline pH range and because it was easily electrophoretically immobilized at this pH. However, this cannot be exactly considered isoelectric denaturation since the reported isoelectric point of lysozyme is 10.5 (Fevold, 1951).

The other maximum in the optical density curve, the area of rapid increase in optical density with decreasing pH, can be explained by the isoelectric denaturation theory. This rapid increase in the curve for the five minute heating at 58°C (136.4°F.), occurred at about pH 5.9, the reported isoelectric point of conalbumin (Longworth et al., 1940). This indicates that the conalbumin fraction is probably responsible for this rapid increase in optical density. The optical density probably does not decrease on the acid side of the isoelectric point of conalbumin because of the overshadowing by the denaturation of the ovalbumin which is isoelectric at pH 4.6.

The minimum in both the optical density and the gelation score curves at pH 8.75 indicates that in this pH area the proteins of egg white are in a relatively heat stable state. This again, is in agreement with the isoelectric denaturation theory in that this pH is almost midway between the isoelectric points of the proteins of egg white. The major egg white proteins with isoelectric points nearest this pH are conalbumin (pH 5.9) and lysozyme (pH 10.5). The resistance of the egg white to gelation at pH 6.0 and above pH 10.0 is not apparent.

The heat treatment temperatures used in these studies caused some coagulation, in the form of a coagulated foam, to occur in the first two pasteurizer coils necessitating changing the coils after every third sample. The remaining coils contained little coagulated protein. This foam probably was caused by the loss of the dissolved carbon dioxide at the higher temperatures.

The effect of heating the egg white at 58°C (136.4°F.) on the angel cake volume ratio was somewhat surprising, especially at pH 8.75, where the cake volume ratio was, in all three trials, significantly greater than the control cake volume ratios. It is significant to note that this is the same pH at which the egg white was resistant to heat when measured by optical density and gelation score and also the pH region
which other investigators have found to be important in the egg white system. Cotterill and Winter (1954b) postulated that in this pH region the lysozyme-ovomucin complex dissociation was enhanced and Meeham et al. (1961) reported that hard cooked eggs peel easily when the egg white has reached a pH of about 8.9. These reports both imply protein complex dissociation in this pH region. Thus, the heat catalyzed dissociation of a protein complex at this pH could be the mechanism for the improved performance of the egg white. This improvement is realizable because the proteins are most resistant to heat at this pH.

At the pH values removed from 8.75 and at the higher temperatures the cake volume was decreased by the heat treatment because of the destruction of the proteins. At the lower pH values the destruction of conalbumin by the heat was probably responsible for the lower cake volumes while at the higher pH values the destruction of lysozyme can be implicated.

The effect of the heat treatment on the whip time was essentially the same as on cake volume ratio and can be explained by the same mechanisms.

The decrease in cake volume ratio and increase in whip time of the egg white reported by Slosberg et al. (1948), Funk (1950), Goresline et al. (1950), Carlin and Foth (1952), and by Clinger et al. (1951), for their respective heat treatments was probably due to the same factors as were the detrimental effects obtained in this experiment when the egg white was heated above pH 9.0. The finding of Clinger et al. (1951), that the egg white of grade A eggs was not as adversely affected by heat treatment as commercial egg white is probably because the egg white of the grade A eggs was closer to the 8.75 pH region.

The surface tension and viscosity data show that the proteins are affected by the higher temperatures; however, neither of these parameters can be correlated with any of the other properties of the heat treated egg white.

The electrophoretic patterns indicate that heating at the lower pH values immobilizes the conalbumin, while heating at the higher pH values immobilizes the lysozyme. While the immobilization of lysozyme can be, at least in part, correlated with the decrease in cake volume ratio and increase in whip time, a preliminary experiment in which purified lysozyme was added to the heat treated egg white in concentration as high as normally found in egg white failed to show that this protein was responsible for these adverse affects, even though the added lysozyme was shown to be electrophoretically mobile. However, further experimentation may show that the lysozyme must be added under certain conditions to improve the function of the egg white.

The increase in temperature necessary to coagulate the egg white-sucrose mixture is in agreement with the work of Bancroft and Rutzler (1931), Barmore (1936), and Slosberg et al. (1948). However, this study also shows that the degree of this protection against heat is pH dependent.

The abrupt change in the optical density and gelation score of egg white containing equal its weight in sucrose demonstrates the importance of a lower pH in foods in which egg white is used as a coagulation agent.

**SUMMARY**

A study of some factors which affect the coagulation of egg white has been conducted. Heating egg white in test tubes for various times and temperatures resulted in a gelation score curve with minima at pH 8.75 and 6.0, and maxima at pH 9.5 and 7.75 and optical density curves with
Heat Coagulation of Egg White

minimum at pH 8.75 and maxima pH 9.5
and at lower pH values.

Heat-treating the egg white at pH 8.75
for three minutes at 58°C. (136.4°F.) im-
proved its performance in cake volume
ratio and whipping time. However, at pH
values removed from 8.75 and at higher
temperatures these functional properties
were adversely affected. This decrease in
function is correlated with the heat de-
struction of conalbumin at the lower pH
values and lysozyme at the higher pH
values.

The addition of sucrose to egg white
increased its time and/or temperature of
couagulation. This increase is pH depend-
ent and is greater at above pH 8.5.

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NEWS AND NOTES

(Continued from page 405)

Purdue Notes

Dr. Frederick N. Andrews has been named
Vice-President of Research and Dean of the
Graduate School at Purdue University. He will

(Continued on page 428)
STUDIES ON OVALBUMIN

I. DENATURATION BY HEAT, AND THE HETEROGENEITY OF OVALBUMIN

By M. B. SMITH*

[Manuscript received June 21, 1963]

Summary

The denaturation of ovalbumin by heat at pH 2-3 has been studied by following the changes in optical rotation, viscosity, solubility at the isoelectric point, and amount of aggregated protein observed in the ultracentrifuge. The laevorotation and reduced viscosity of heated solutions of ovalbumin increase with ionic strength and protein concentration. This effect is related to the state of aggregation of the denatured protein and not to the extent of denaturation as measured by changes in solubility. The initial denaturation step is irreversible but does not involve the extensive unfolding observed in urea solutions.

The presence is demonstrated, in a number of ovalbumin preparations, of a fraction which is more resistant to heat denaturation. This is shown to be a more stable form of ovalbumin ("8-ovalbumin") and its occurrence is shown to be connected with the storage history of the eggs used for the ovalbumin preparations.

I. INTRODUCTION

Crystalline ovalbumin has served as a standard protein for many investigations since its isolation from egg white by F. Hofmeister in 1890. It is easily prepared, readily purified by repeated crystallizations, and is stable on storage. Although electrophoretically heterogeneous (Longsworth 1939; Cann 1949) the three components have been shown by Perlman (1952) to differ only in the number of phosphate groups but are otherwise identical in composition. No other evidence of heterogeneity has been reported.

The coagulation of ovalbumin solutions by heat was one of the earliest observed examples of protein denaturation, and subsequently loss of solubility in salt solutions at or near the isoelectric point has been used frequently as an index of denaturation (e.g. Gibba, Bier, and Nord 1952). Other workers have demonstrated the irreversibility of heat denaturation and have shown that even in the absence of coagulation there is pronounced aggregation, indicated by changes in such hydrodynamic properties as viscosity (Loughlin and Lewis 1932; Bull 1940), sedimentation-diffusion (MacPherson, Heidelberger, and Moore 1945), light scattering (Bier and Nord 1940), and flow birefringence (Fredericq 1947). The influence of pH and ionic strength on the aggregation has been studied by Foster and Samsa (1951) and by Foster and Rhee (1952).

In studies on the denaturation of proteins by urea carried out in this Laboratory by McKenzie, Smith, and Wake (1963), the usefulness of following a particular denaturation process by means of several different techniques was demonstrated.

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In the work reported in this paper a similar approach has been made to the heat denaturation of ovalbumin in the acid pH region, in order to assess the relative importance of the aggregation reaction during denaturation and to enable the effects of heat to be compared with those previously reported for urea.

During the course of this work it became evident that the ovalbumin contained a minor component which had a much slower rate of denaturation. On isolating this fraction by selective heat denaturation, it was found to be distinguishable from “whole” ovalbumin only by its relative resistance to denaturation. The experiments which indicated the nature and occurrence of this form of ovalbumin are reported here.

II. Methods

(a) Preparation of Ovalbumin

Ovalbumin was prepared from commercially “fresh” hen eggs by the method of Kekwick and Cannan (1936) and recrystallized twice from a sodium sulphate solution at pH 4.7. Stock solutions containing 10–15% ovalbumin were prepared by exhaustive dialysis against distilled water, followed by centrifugation to remove traces of surface-denatured protein. The concentration was determined from semi-micro Kjeldahl nitrogen estimations (McKenzie and Wallace 1954) assuming 15.7% nitrogen in ovalbumin (Steven and Tristram 1958).

(b) Optical Rotation

A Schmidt and Haensch visual polarimeter reading directly to 0.01° was used to measure rotations. Unless otherwise stated, readings were made at 50°C, at the wavelength of the sodium D line. Water from a constant-temperature bath was pumped through the jackets of all-glass, 2-ml cells, controlling the temperature of the sample to 0.1 degC. A blank reading was taken for each cell filled with water at 50°C. The results are expressed as specific rotation.

(c) Viscosity

U-tube viscometers having a flow time of approximately 45 sec for water at 50°C and a working volume of 12 ml were used in a water-bath controlled to 0.1 degC. Flow times were measured to 0.1 sec with a stop-watch. The results are expressed as reduced viscosity, \((\eta - 1)/c\), where \(\eta\) = relative viscosity, and \(c\) is protein concentration in g/dl. The relative viscosity was taken as the ratio of the flow times of solution and solvent, no correction being made for differences in density.

(d) Solubility

A method similar to that of Gibbs (1954) was used, but with some modifications. Heated ovalbumin solution (1 ml) was run into 10 ml of cold “stopping solution” (0.5M sodium chloride, 0.1M sodium acetate-acetic acid buffer, pH 4.7). After mixing and standing for 15 min, the insoluble protein was centrifuged off and the relative concentration of soluble protein determined by ultraviolet absorption at 278 m\(\mu\) in a 10-mm cell, correcting for scattering by subtracting the absorption at
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320 mp. The corrected absorption was expressed as a percentage of the absorption of an unheated portion of the solution treated in the same way, giving “percentage soluble protein”; and, by difference, “percentage insoluble protein”.

(c) Sedimentation Measurements

Measurements of sedimentation coefficients and amounts of slow and fast components were made with a Spinco model E ultracentrifuge fitted with a phaseplate and automatic temperature control. Runs were made near 20°C in 12-mm cells at a speed of 59,780 r.p.m. Sedimentation coefficients were determined as described by Schachman (1957) and corrected to the value in water at 20°C. The area of the slow peak was measured with a planimeter on a tracing of the enlarged pattern and expressed as a percentage of the area of the peak given by an unheated portion of the solution. No correction was made for the relative enhancement of the area of the slow component (Johnston and Ogston 1946) or for change in concentration due to sectorial dilution. The first correction was found to be small in this system and the second was avoided by measuring the area of the peak at the same relative position in the cell for each run. The “percentage fast component” was obtained by difference.

III. Results

(a) Effects of Ionic Strength on Optical Rotation and Viscosity

Figures 1(a) and 1(b) show the changes in viscosity and optical rotation of 1 and 2% ovalbumin solutions at pH 3.0 while heating at 50°C in the presence of different concentrations of sodium chloride. There is a marked increase in both reduced viscosity and specific laevorotation as the ionic strength is increased. At concentrations over 0.1M sodium chloride the viscosity rises very steeply on heating, the solutions become opalescent and, at 2% protein concentration, finally gel.
Increasing the ionic strength apparently caused an increase in the degree of aggregation of the heated protein. Thus after heating a 1% solution of ovalbumin in 0.02M NaCl for 360 min at 50°C, cooling, and then adjusting to 0.1M NaCl, the reduced viscosity of the heated solution rapidly rose from 0.04 to 0.28. The sedimentation patterns [Figs. 2(a) and 2(b)] show a change in the sedimentation rate and heterogeneity of the fast (aggregated) component which also indicates an increase in the degree of aggregation. In a similar experiment, a 1% ovalbumin solution was heated in the absence of salt for 600 min at 50°C and after cooling was adjusted to 0.1M salt. The specific rotation, measured at 20°C, then changed rapidly from −40° to −52° and continued to decrease slowly. The sedimentation patterns are shown in Figures 2(c) and 2(d). [Due to the charge effects caused by the absence of salt there was incomplete resolution in Fig. 2(c).] In both experiments the slow component had a sedimentation rate near that of the native protein.

The changes in reduced viscosity and specific rotation which occur on heating at pH 2 and 45°C [Figs. 3(a) and 3(b)], and the effects of increasing salt concentration, are similar to those at pH 3 and 50°C. That these effects are due to differences in a
the degree of ovalbumin denaturation and heterogeneity in 0.5 M NaCl, the sedimentation rate and an increase in secondary aggregation process and not to differences in the amount of denaturation is shown in Figure 4, A, where the percentage insoluble protein at pH 4.7, ionic strength 0.5, is plotted against the time of heating at 45°C, pH 2.0. Here there is no significant difference between a solution containing no salt and one with 0.05M salt. The effect of protein concentration is also small when solubility change is measured, as shown in Figure 4, B, for 1 and 2% ovalbumin solutions in 0.02M NaCl at pH 3.0, heated at 50°C.

Fig. 4.—(a) Reduced viscosity and (b) specific rotation of 1% ovalbumin solutions heated at 45°C, pH 2.0: A, no salt; B, 0.02M NaCl; C, 0.05M NaCl; D, 0.1M NaCl.

(b) Primary Denaturation Process at pH 3.0 and 50°C

Since neither reduced viscosity nor specific rotation showed any indication of reaching a final steady value after heating for 600 min (and even after 1300 min in two cases), neither of these techniques could be used for measuring reaction rates.
In any case, being dependent on the state of aggregation of the denatured protein, as shown above, they could not validly be used to follow the primary denaturation process.

Solubility change at the isoelectric point has been used by Gibbs, Bier, and Nord (1952) to measure the kinetics of the heat denaturation of ovalbumin, and this measurement, as shown in Figure 4, is independent of ionic strength and protein concentration, and therefore probably does indicate the true extent of denaturation.

It was found that the amount of insoluble protein was the same whether the sample was removed and immediately added to the stopping solution or whether it was kept in the cold for 24 hr before the solubility was determined, i.e. the denaturation is not reversible. This meant that it was reasonable also to measure the extent of denaturation by the change in the relative amounts of slow- and fast-moving components on ultracentrifugation. The effect of heating 1 and 2% solutions of ovalbumin in 0.02M NaCl at pH 3 and 50°C for 10 hr on the amount of fast component present is shown in Figure 5. There is no significant difference between the results obtained for the two solutions.

The sedimentation coefficients of the fast component were in the range 6.8–8.0 at this salt concentration, and increased to 18–21 as the concentration was raised to 0.1M. The rates for the slow component were generally lower than the corresponding values for native ovalbumin (in 0.02M NaCl a time-extrapolated value of S_{20,w} = 2.75 at 1% concentration was obtained) but showed some variation in different experiments.

(c) Stable Fraction of Ovalbumin

It is apparent from Figures 4 and 5 that the changes in solubility and amount of fast component do not approach completion but that the curves tend to level off at a value less than 80%. This effect appeared to have two possible explanations: either the products of the reaction were inhibitory or there was a second component present which either did not denature or denatured more slowly.
OVALBUMIN DENATURATION AND HETEROGENEITY

A 2% ovalbumin solution in 0.02M NaCl at pH 3 was heated at 50°C for 7 hr and the denatured protein removed by precipitation at pH 4.7. The soluble protein (about 25% of the original) was salted-out with sodium sulphate, dissolved, and dialysed until free from salts. This fraction, adjusted to 1% protein concentration in 0.02M NaCl at pH 3, showed a decrease in specific rotation of only 9° on heating for 180 min at 50°C compared with a decrease of 26° for the original ovalbumin, and the sedimentation pattern of the heated solution showed only a trace of fast component. The rise in viscosity on heating this fraction was also small compared with the viscosity change of a similar solution of unfractionated ovalbumin. This showed that in a heated solution at pH 3 there was a fraction of the ovalbumin which was relatively resistant to denaturation; but there was no evidence to show whether this material was originally present in the native ovalbumin or whether it was formed by a side reaction on heating.

<table>
<thead>
<tr>
<th>Preparation No.</th>
<th>Specific Rotation (deg)</th>
<th>Reduced Viscosity (dL/g)</th>
<th>Insoluble Protein (%)</th>
<th>Fast Component (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-53*</td>
<td>0.065*</td>
<td>75*</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>-51*</td>
<td>0.17</td>
<td>64*</td>
<td>57*</td>
</tr>
<tr>
<td>3</td>
<td>-52</td>
<td>0.44</td>
<td>69</td>
<td>73</td>
</tr>
<tr>
<td>5</td>
<td>-74</td>
<td>1.20</td>
<td>79</td>
<td>72</td>
</tr>
<tr>
<td>8</td>
<td>-51</td>
<td>0.21</td>
<td>45</td>
<td>37</td>
</tr>
</tbody>
</table>

* In 0.02M NaCl.

Several large preparations of the stable fraction were made, the conditions of denaturation being heating for 16 hr at 50°C, pH 3. The protein soluble at pH 4.7 was completely recovered by lyophilization of the dialysed solution. Preliminary characterization by sedimentation, optical rotation, paper electrophoresis, and ultraviolet absorption showed only slight differences from native ovalbumin. This fraction is therefore regarded as a distinct type of ovalbumin and called S-ovalbumin (stable ovalbumin).*

(d) Occurrence of S-Ovalbumin

When the measurements of the extent of denaturation by heat at pH 3 were repeated with different preparations of ovalbumin, an unexpected variability was disclosed. Table 1 summarizes the results obtained with six different preparations, giving the values for specific rotation, reduced viscosity, solubility, and amount of fast component after heating for 300 min at 50°C.

* In an earlier communication (Smith and Back 1962) the stable fraction was referred to as "ovalbumin-X".
Since repeated measurements on the same preparation were consistent and showed only small variations, the assumption was made that different preparations contained different amounts of either S-ovalbumin or its precursor (if S-ovalbumin is formed by heating). These differences could, in turn, be due either to variations in the eggs or to some unknown factor affecting the preparation. To examine these possibilities, four lots of eggs were obtained from different sources, ovalbumin was prepared from each lot by a strictly uniform procedure, and the reactivity of each lot of ovalbumin determined by measurements of viscosity, optical rotation, and solubility on heating at 50°C, pH 3. The results are shown in Table 2. The percentage of S-ovalbumin was calculated on the assumption that the amount of insoluble protein produced after 300 min was 97% and 17% for ovalbumin and S-ovalbumin respectively.

<table>
<thead>
<tr>
<th>Source of Eggs</th>
<th>Specific Rotation (deg)</th>
<th>Reduced Viscosity (dl/g)</th>
<th>Insoluble Protein (%)</th>
<th>Estimated S-Ovalbumin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laying boxes</td>
<td>73</td>
<td>1.75</td>
<td>93</td>
<td>5</td>
</tr>
<tr>
<td>Poultry farm, after packing</td>
<td>70</td>
<td>0.56</td>
<td>79</td>
<td>23</td>
</tr>
<tr>
<td>Retail store</td>
<td>80</td>
<td>0.41</td>
<td>65</td>
<td>40</td>
</tr>
<tr>
<td>After 6 months, cold storage</td>
<td>44</td>
<td>0.15</td>
<td>32</td>
<td>81</td>
</tr>
</tbody>
</table>

### IV. Discussion

The ovalbumin preparations which were used for this study of heat denaturation have been shown to be mixtures of two components which differ in their rates of denaturation. Because of this a quantitative evaluation of the results is not justified, but it is possible to make some general observations on the process of heat denaturation.

As other workers have shown, aggregation plays an important part in the overall reaction. Even at pH 3, where aggregation is minimal and no visible precipitation occurs, most of the denatured protein (as measured by the change in solubility) appears as a fast-moving component on ultracentrifugation.

It has generally been considered that aggregation occurs as a secondary process during denaturation. In the denaturation of ovalbumin by urea the two stages of unfolding and aggregation may be distinguished relatively easily in the changes in optical rotation and viscosity. Thus the rapid change in these properties which occurs in 7M urea at pH 3 has been shown by sedimentation and diffusion measurements to be due to a rapid unfolding, and there is no subsequent aggregation (McKenzie, Smith, and Wake 1955, 1963). At pH 7 in urea the initial unfolding is followed by a slow increase in viscosity and the appearance of a fast component in
the ultracentrifuge pattern, indicating aggregation. In all cases the sedimentation rate of the slowest component indicated that a considerable increase in asymmetry had occurred.

In the present measurements of heat denaturation there is little evidence for the presence of an unfolded monomer. The sedimentation rates of the slow component were generally less than that of the native protein at pH 3 but the results were erratic. The cause of this variation is unknown.

Charlwood and Ens (1957) found that there was a slight decrease in sedimentation rate for ovalbumin when the pH was brought from 6 to below 3.5. This has been confirmed, but since the change is reversible and there is no tendency for aggregation at pH 3 at temperatures below 30°C it does not represent the initial denaturation step. The primary reaction may be assisted by this slight expansion occurring at low pH, but it does not involve the extensive disorganization of the molecule which occurs in urea solutions. The effect of ionic strength on the specific rotation suggests that a secondary unfolding occurs during or after aggregation and that the extent of this unfolding depends on the size of the aggregate. This could be regarded as a type of micelle formation, with a reduction in the stabilizing energy of the hydrophobic bonds due to the changed environment of part of the protein molecule (Kauzmann 1959).

Although in this work heat denaturation has been studied for only one protein under a limited range of conditions, it is reasonable to point out that there are obvious differences from denaturation by urea.

The discovery of a less reactive form of ovalbumin has been an unexpected outcome of this work, and appears to be an unusual, if not unique, example of the detection of heterogeneity through measurement of denaturation rates.

It was considered important to find the reason for the variability of different ovalbumin preparations, before examining the properties of the two ovalbumins in detail. The measurements given in Table 2 suggest a relation between the age of the egg and the amount of S-ovalbumin in the preparation.

V. Acknowledgments

The advice and encouragement given by Dr. H. A. McKenzie during the early stages of this work is gratefully acknowledged. Miss Joan Back and Mr. A. G. Mackinlay assisted with some of the measurements and made many valuable suggestions.

VI. References