corrections in this study were less significant than the errors introduced by using percentages of water determined by the oven-dry method as criteria of accuracy. In any event, the final practical answer to this problem is the incorporation of a temperature-compensation feature in the Wheatstone bridge.

The intimate mixing and temperature rise resulting from agitation with an Oster mixer are both beneficial, in that both help toward transferring water from the cottonseed meal sample to the mixed solvent and subsequently saturating the alcohol-acetone-water system with salt. As shown by the results, very nearly reproducible temperatures can be obtained by cooling the warm mixture for 5 minutes in a running tap water bath.

Close inspection of Figure 1 indicates the possibility of some curvature in the relationship at lower percentages of water. This was disregarded in the statistical treatment of the data, the analysis being made by linear regression (*3*). This appears justifiable in view of the degree of accuracy and precision found. If the indicated curvature at lower percentages of water is real, the curved portion could be eliminated by adding an appropriate amount of water to the stock supply of alcohol-acetone mixed solvent.

Further research on this method is in progress. The bridge of a Model RB-26 Solu-Bridge (Drawing A230E, Industrial Instruments, Inc.) has been modified (by changing four of the bridge resistances to the following values in ohms: *R*1, 1230; *R*2, 1000; *R*3, 874; *R*4, 560), so that the full scale represents 0.2 to 1.0 millimho. The Model RB-26 is a small, portable, battery-operated, 1000-cycle instrument with an adjustable temperature compensator. By suitable calibration, the scale has been modified to read percentage of water directly and the temperature compensator modified to apply to alcohol-acetone-water–sodium chloride systems. A pipet-type conductivity cell is being used with this modified bridge.

Acknowledgment

The authors are grateful for the cooperation and assistance of the following personnel of the Cottonseed Products Research Laboratory, Texas Engineering Experiment Station: A. C. Wamble, W. B. Harris, J. C. Creagor, and Beverly Osborne. The technical assistance of J. W. Jernigan is acknowledged. Except as noted, the experimental work was performed in the laboratories of the Department of Chemistry, A&M College of Texas.

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Received for review December 27, 1955. Accepted March 30, 1956. Analytical Chemistry Section, 11th Southwest Regional Meeting, ACS, Houston, Tex., December 1, 1955. Data obtained in research conducted cooperatively by the Cotton Research Committee of Texas and Texas Engineering Experiment Station.

EGG PROTEINS

Separation of Egg White Proteins by Paper Electrophoresis

ROBERT JOHN EVANS and SELMA L. BANDEMER

Department of Agricultural Chemistry, Michigan State University of Agriculture and Applied Science, East Lansing, Mich.

The need for a rapid method for the quantitative determination of the individual egg white proteins led to a study of their separation by paper electrophoresis. A procedure was developed whereby the proteins in whole egg white were separated by the ridgepole technique of Durrum, using a pH 8.6 diethylbarbiturate buffer of 0.05 ionic strength. The separated proteins were dyed with bromophenol blue, the color was eluted with dilute sodium hydroxide solution, and absorbance was determined at 590 mμ in the spectrophotometer. Fresh egg white protein contained 65.2% ovalbumin (48.4% A_1, 12.6% A_2, and 4.2% A_3), 11.2% ovomucoid plus ovoglobulin, 17.0% conalbumin, 2.1% nonmobile protein, and 4.5% lysozyme. The method is ideal for studying possible changes in egg white proteins during storage of shell eggs, because of its speed and the reproducibility of results.

PAPER ELECTROPHORESIS offers a new technique for the study of proteins. It is less expensive and more rapid than the conventional moving-boundary electrophoresis method and has been successfully used, especially for the separation and study of blood proteins. Tiselius and Flodin (*22*), Block, Durrum, and Zweig (*3*), and McDonald (*15*) have reviewed the literature on the general subject of paper electrophoresis.

Longsworth, Cannan, and MacInnes (*14*) were the first to make an extensive study of the protein of egg white by means of moving-boundary electrophoresis. Bain and Deutsch (*2*), Forsythe and Foster (*10*), and Csonka and Jones (*6*) have also used the Tiselius moving-boundary electrophoresis method for the study of egg white proteins.

Nothing has been published on the use of paper electrophoresis for the separation of egg white proteins. This method, however, should be useful in the quantitative study of the proteins of egg white, if these proteins can be satisfactorily separated by this means. The purposes of the experiments reported herein were to study the behavior of egg white proteins when subjected to

electrophoresis on paper under different conditions and to work out a satisfactory method for separating the different egg white proteins by means of paper electrophoresis.

Preparation of Egg White Proteins

Ovalbumin. Ovalbumin was prepared essentially by the procedure of Sörensen and Höyrup (*21*). To the whites of 24 eggs, which had been broken up with the Waring Blendor, was added an equal volume of saturated ammonium sulfate solution, and the insoluble material (ovomucin, ovoglobulins, and some denatured proteins) was filtered off. Ovalbumin 1 was obtained from the filtrate by crystallizing three times with ammonium sulfate. Ovalbumin 2 was crystallized twice and the ammonium sulfate removed by dialysis. Ovalbumin 3 was prepared from the filtrate remaining after the second precipitation of the ovoglobulins by crystallizing three times with ammonium sulfate. Ovalbumin 4 was a sample of commercial ovalbumin (Nutritional Biochemicals Corp.) obtained for comparative purposes. It was labeled as two times crystallized and salt-free.

Conalbumin. Two methods were used to prepare conalbumin. The first was essentially the method of Longsworth, Cannan, and MacInnes (*14*). The filtrate from the first ovalbumin precipitate was adjusted to pH 3.0 with a dilute sulfuric acid solution. Precipitation was allowed to take place overnight in the refrigerator. The precipitate was filtered off and suspended in 200 ml. of water. Solid ammonium sulfate was added to give a 1.5*M* solution, the pH adjusted to 3.0, and precipitation allowed to take place overnight. Conalbumin 1 was precipitated three times in all. Conalbumins 2 and 3 were prepared from different ovalbumin filtrates as described in Table I.

The second method of preparation was that of Warner and Weber (*23*) up to a point. To the filtrate from the ovalbumin crystallization were added 8 grams of ammonium sulfate for every 100 ml. of filtrate. The precipitated conalbumin 2a was filtered off and dissolved in water, and the solution was dialyzed against distilled water in the cold. The solution was filtered, the pH adjusted to 6.0, and sodium chloride added to 0.02*M*. An 0.02*M* solution of sodium chloride in 50% ethyl alcohol was added with mechanical stirring at 2° C. until a concentration of 20% by volume of ethyl alcohol was reached. The precipitate was filtered off and dried under vacuum.

Ovoglobulin. The material that precipitated in half-saturated ammonium sulfate solution was suspended in an 0.25% sodium chloride solution to dissolve the globulins, and insoluble material was removed by filtration. The ovoglobulins were twice precipitated with half-saturated ammonium sulfate, and dissolved in 0.25% sodium chloride solution, and the salts were removed by dialysis in the cold. Ovoglobulin 1 stayed in solution during dialysis and ovoglobulin 2 precipitated.

Ovomucoid. Ammonium sulfate was added to the filtrate from the conalbumin precipitation until the solution was saturated. The precipitated ovomucoid was dissolved in distilled water, and the solutions were dialyzed in the cold until all ammonium sulfate was removed. Ovomucoid 1 was prepared from the filtrate from the first conalbumin 1 precipitation, and ovomucoid 2 from the second. The filtrates from the other conalbumin precipitations were combined and dialyzed. Ovomucoid 3 was obtained by evaporating part of the dialyzate to dryness under vacuum. The vacuum broke, and the ovomucoid partially spoiled before it dried. Ovomucoids 4 and 5 were prepared by precipitation from other parts of the dialyzate by the addition of absolute ethyl alcohol.

Lysozyme. The lysozyme was a commercial preparation obtained from Nutritional Biochemicals Corp.

All preparations were dried in vacuo in the cold unless otherwise indicated. Samples were studied electrophoretically before drying as well as after, and no changes in mobility took place during drying. No attempt was made to obtain uncontaminated preparations of the proteins, because their principal purpose was to identify the different spots obtained by paper electrophoresis of whole egg white.

Methods of Paper Electrophoresis

Two types of filter paper electrophoresis cells were used in the studies reported herein. One utilized the closed strip technique with the paper sandwiched between two glass plates. The apparatus was obtained from Microchemical Specialties Co. (Misco). The unit consists of two plastic end cells joined by a 16 × 8 × 2 inch tray. Each end cell has a carbon electrode separated from the paper by a partition which does not quite reach the bottom of the cell. Whatman filter paper No. 3MM, cut into 6 × 22½ inch pieces, was used.

The filter paper, wetted with the buffer to be used, was placed on a plate-glass plate cut to fit in the tray rather than on the ordinary window-glass plate furnished with the apparatus. Six spots of protein solution were placed at 1-inch intervals along a line drawn across the middle of the filter paper sheet. The plate was put in the tray with equal lengths of filter paper extending into each cell and was covered with two ordinary plates of window glass, and the entire apparatus was covered with a third, which rested on the uppermost plate but did not quite touch the plastic cells. Six 580-gram bottles were used to weight the top plate down and to put a constant weight on the sheet of filter paper. Buffer was added to each cell to a level just above the electrodes, and the levels in the two cells were made equal by means of a siphon tube. A 150-volt direct current (2.8 volts per cm.) was applied for 24 hours at a temperature of approximately 25° C. The paper was then removed and dried at 100° C. for 10 minutes in a special rack which held the paper nearly horizontal while drying. The paper was placed for 10 minutes in a saturated solution of mercuric chloride in ethyl alcohol containing 0.1% of bromophenol blue and then washed for 10 minutes each with four changes of 0.5% acetic acid solution (*12*). The papers were finally dried for 10 minutes at 100° C. and cut lengthwise into six 1-inch strips. The paper strips were "scanned" in a Welch Densichron densitometer using the white light source. Absorbance was then plotted against distance traveled.

The other apparatus used was of the ridgepole type and was the Spinco Model R paper electrophoresis cell manufactured by the Specialized Instruments Corp. The apparatus was described by Williams, Pickels, and Durrum (*26*), and directions for its use were given by Block, Durrum, and Zweig (*3*). Ten-microliter samples were used. A 5-ma. constant direct current (2.2 to 2.7 volts per cm.) was applied over the eight strips for 16 hours at room temperature (25° C.). Whatman No. 3MM paper strips were used. At the end of the electrophoresis run the paper strips were dried at 125° C. for 30 minutes and stained by placing for 16 hours in a solution containing 0.1 gram of bromophenol blue and 50 grams of zinc sulfate heptahydrate made to 1 liter with 5% acetic acid. The strips were rinsed twice with 5% acetic acid for 6 minutes each and once for 6 minutes with a solution of 3.0 grams of sodium acetate trihydrate diluted to 1 liter with 5% acetic acid. They were then blotted between two sheets of filter paper and dried for 15 minutes at 125° C.

Buffers

Phosphate buffers with a molarity of 0.05 were used for most of the studies in which the horizontal closed strip (Misco) apparatus was used. Five hundredths molar solutions of dibasic sodium phosphate and monobasic potassium phosphate were prepared, and mixed together in different proportions to give buffers with pH values of 4.5, 5.0, 5.6, 6.0, 6.8, 7.0, 7.8, 8.6, and 9.1. The desired pH was obtained by adding one solution to the other until it was in-

dicated on the Beckman Model G pH meter.

A diethylbarbiturate (barbital) buffer of pH 8.6 was obtained by diluting 10.309 grams of sodium diethylbarbiturate and 1.8419 grams of diethylbarbituric acid to 1000 ml. with distilled water. The buffer was 0.05*M* in sodium diethylbarbiturate and 0.01*M* in diethylbarbituric acid.

Acetate buffer was prepared by mixing 1000 ml. of an acetic acid solution, containing 18 ml. of glacial acetic acid, with 600 ml. of a solution containing 24.606 grams of anhydrous sodium acetate per liter. This buffer had a molarity of 0.30 and a pH of 4.3. The acetate buffer of pH 5.0 was prepared by using more of the sodium acetate solution and less of the acetic acid and adjusting with the pH meter.

The pH 8.6 borate buffer was prepared by adding 0.05*M* sodium hydroxide solution to a solution 0.05*M* in both boric acid and potassium chloride until the desired pH was reached, as indicated by the Beckman pH meter.

Effect of pH of Buffer on Separation of Egg White Proteins

Most of the paper electrophoresis separations of blood proteins have been done with a diethylbarbiturate buffer of pH 8.6 and 0.05 ionic strength. Several types of buffers have been used for the separation of egg white proteins by the conventional moving - boundary electrophoresis method, but Forsythe and Foster (*10*) obtained best resolution at pH 7.8 using a phosphate buffer of 0.05 ionic strength.

The first problem was to determine some of the conditions necessary in order to obtain the best separations of egg white proteins by filter paper electrophoresis. All of the early work was done with the Misco sandwich-type horizontal electrophoresis cell. Whatman No. 1 filter paper was too thin and did not give as satisfactory separations as did Whatman No. 3MM paper. The thicker paper was used for all the work reported herein. Because a wide range of pH values can be obtained with phosphate buffers, they were used for the most part in the study to determine the pH at which best separation of egg white proteins was obtained. In order to avoid heating the paper and possible denaturation of the proteins, the relatively low voltage of 150 volts was applied for 24 hours. The proteins moved more slowly and spread less with 0.05*M* phosphate buffers than with those of lower strength.

The data were evaluated by plotting the distance traveled by the protein against the absorbance of the blue protein–bromophenol blue complex as measured on the Densichron. The distances from the point of application to the peak of the curve for each protein fraction were then measured, and the mobilities were calculated by the equation of Tiselius and Flodin (*22*). The equation is

$$\mu(\text{obsd.}) = \frac{d}{tV/l}$$

where d is the observed distance in centimeters that the protein spots migrated, t is the time in seconds that electrophoresis progressed, V is the voltage across the paper, and l is the length of the paper. The significance of mobility as determined in this way has been questioned (*15*), but it is used here only for comparative purposes. Because the time, the voltage, and the length of the paper were the same for all the determinations, the equation as it was used was reduced to

$$\mu(\text{obsd.}) = \frac{d}{2.44 \times 10^5}$$

and the mobility is simply a function of the distance traveled by the protein.

Studies were conducted with the egg white protein preparations, described earlier, using the 0.3*M* acetate buffer of pH 4.3 and 0.05*M* phosphate buffers of pH 5.6 and 7.8. The egg white proteins were dissolved in distilled water to give a concentration of 5%, and 30 µl. of each one were used. The results are given in Table I.

Single well-defined spots were obtained in all three buffers with ovalbumins 1 and 3 which had been crystallized three times and not dialyzed and with the commercial ovalbumin which was twice crystallized and salt-free. Ovalbumin 2 gave, in addition, another weak spot that corresponded with one of the conalbumin spots at pH 4.3 and 5.6 and was evidently still contaminated with some conalbumin.

The three conalbumin preparations described in Table I were prepared by acid precipitation. They were not completely soluble at the 5% level in distilled water, and sodium hydroxide solution had to be added to put them in solution. Whether the two peaks obtained at pH 4.3 and pH 5.6 are natural for conalbumin or are caused by a change in the conalbumin is not known. That these preparations of conalbumin were changed during isolation is shown by the decreased solubility in water and by the difference in mobility at pH 7.8 between the three preparations and the conalbumin impurity in ovalbumin 2.

Longsworth, Cannan, and MacInnes (*14*) obtained three ovoglobulin peaks in their moving-boundary electrophoresis study of egg white proteins. These were designated as G_1, G_2, and G_3. Ovoglobulin G_1 was found by Alderton, Ward, and Fevold (*1*) to be identical with lysozyme. Commercial lysozyme had a greater mobility with each of the three buffers than did the lysozyme present in ovoglobulin 1 or 2 (Table I). In fact, one might question the presence of lysozyme in ovoglobulin 2 from the paper electrophoresis peaks at pH 4.3 and 5.6. Two components were separated in ovoglobulin 1 at pH 4.3, three at pH 5.6, and four at pH 7.8. Just where the fourth component fits into the

Table I. Mobilities of Egg White Protein Preparations

(Mobility × 10^5)

Protein	How Prepared	Acetate Buffer, pH 4.3	Phosphate Buffer, pH 5.6	Phosphate Buffer, pH 7.8
Ovalbumin 1	3 × crystallized, not dialyzed	1.2[a]	−4.1	−7.0
Ovalbumin 2	2 × crystallized, dialyzed	1.3, 3.4	−3.6, 1.2	−7.1, −1.2
Ovalbumin 3	3 × crystallized, second batch	1.3	−4.0	−7.0
Ovalbumin 4	Commercial	1.7	−4.0	−7.1
Conalbumin 1	3 × precipitated, first batch	1.8, 3.2	0.3, 1.2	−2.2
Conalbumin 2	3 × precipitated, second batch	1.8, 3.2	0.5, 1.3	−2.2
Conalbumin 3	Recoveries	1.8, 3.3	0, 1.2	−1.8
Ovoglobulin 1	Sol. after dialysis	3.3, 6.1	0.1, 1.7, 4.1	−3.6, −2.6, −1.6, 3.6
Ovoglobulin 2	Pptd. by dialysis	1.1, 3.6	0.4, 0.8, 2.6	−3.4, −2.4, −1.2, 2.6
Lysozyme	Commercial	7.8	5.9	4.4
Ovomucoid 1	Dried in vacuo	0, 3.2	0	−2.8
Ovomucoid 2	Dried in vacuo, second batch	0.5, 3.3	−3.6, −2.2, −0.7	−3.4, −2.4
Ovomucoid 3	Partially spoiled	2.0, 3.4, 5.4	−4.4, −4.0, −2.2, 0.3, 3.7, 4.1	−7.1, −6.7, −5.9, −3.3, −2.5, 3.2
Ovomucoid 4	Ethyl alcohol precipitated	0, 0.9	−2.5, −1.3, 0, 0.5	−4.1, −3.2, 0
Ovomucoid 5	Ethyl alcohol precipitated	0, 0.8, 1.7, 3.8	−1.6, −1.1, 0, 3.0	−3.8, −2.4, 0, 0.4, 2.5

[a] Each value is mobility of one protein in preparation, no matter how small the amount, if it gave a distinct peak when data were plotted.

scheme of Longsworth, Cannan, and MacInnes (*14*) is not known, nor is it certain which peak, if any, is the extra one, but the peak with a mobility of −1.2 or −1.6 may be conalbumin present as an impurity in the preparations.

Five preparations of ovomucoid were used. Ovomucoid 1 was obtained in largest quantity and appears to be a more homogeneous product, although it gave two electrophoretic peaks at pH 4.3. Ovomucoids 3, 4, and 5, which were prepared from the last conalbumin filtrates, contained a number of contaminants, as indicated by the extra peaks for ovomucoids 4 and 5. During the drying of ovomucoid 3 the vacuum desiccator leaked, the vacuum was released, the preparation warmed up, and some decomposition took place as evidenced by the odor. Compounds were formed in the process with mobilities similar to those of ovalbumin and lysozyme.

In another experiment the Misco sandwich-type cell was used with 0.05*M* phosphate buffers of pH 4.5, 5.6, 6.8, 7.8, and 9.1, a 0.30*M* acetate buffer of pH 4.3, and a 0.06*M* barbital buffer of pH 8.6. Ovalbumin 1, conalbumin 1, ovoglobulin 1, and ovomucoid 1 were the proteins studied.

An additional experiment was conducted using the Misco paper electrophoresis cell. Four egg white protein preparations were used: ovalbumin 2, conalbumin 2a, ovoglobulin 1, and ovomucoid 1. Eight different buffers were used: 0.05*M* phosphate buffers of pH 5.0, 6.0, 7.0, 7.8, and 8.6, 0.30*M* acetate buffer of pH 5.0, and 0.05*M* borate and 0.06*M* barbital buffers of pH 8.6.

The combined data from all three experiments using the 0.05*M* phosphate and 0.30*M* acetate buffers are presented in Table II. The data for conalbumin 2a, which was used only in the last experiment and those from the impurity of conalbumin in ovalbumin 2, are the only conalbumin data included. Conalbumin 1 needed considerable alkali to put it in solution and so gave erroneous values. The only pH which was the same in all three studies was 7.8. The mobilities obtained in the three studies agree reasonably well. Two studies made with the pH 5.6 buffer are in good agreement except for lysozyme.

Ovalbumin 2 was contaminated with some conalbumin, and the peak for the conalbumin contaminant came at the same point as that for conalbumin 2a. Ovoglobulin 1 contained two major peaks—ovoglobulin and lysozyme. With increasing pH of the buffers there was a decrease in positive mobilities and an increase in the negative ones, except for lysozyme. For some reason the mobility of lysozyme was very erratic. About the only thing consistent about it was that it was always positive.

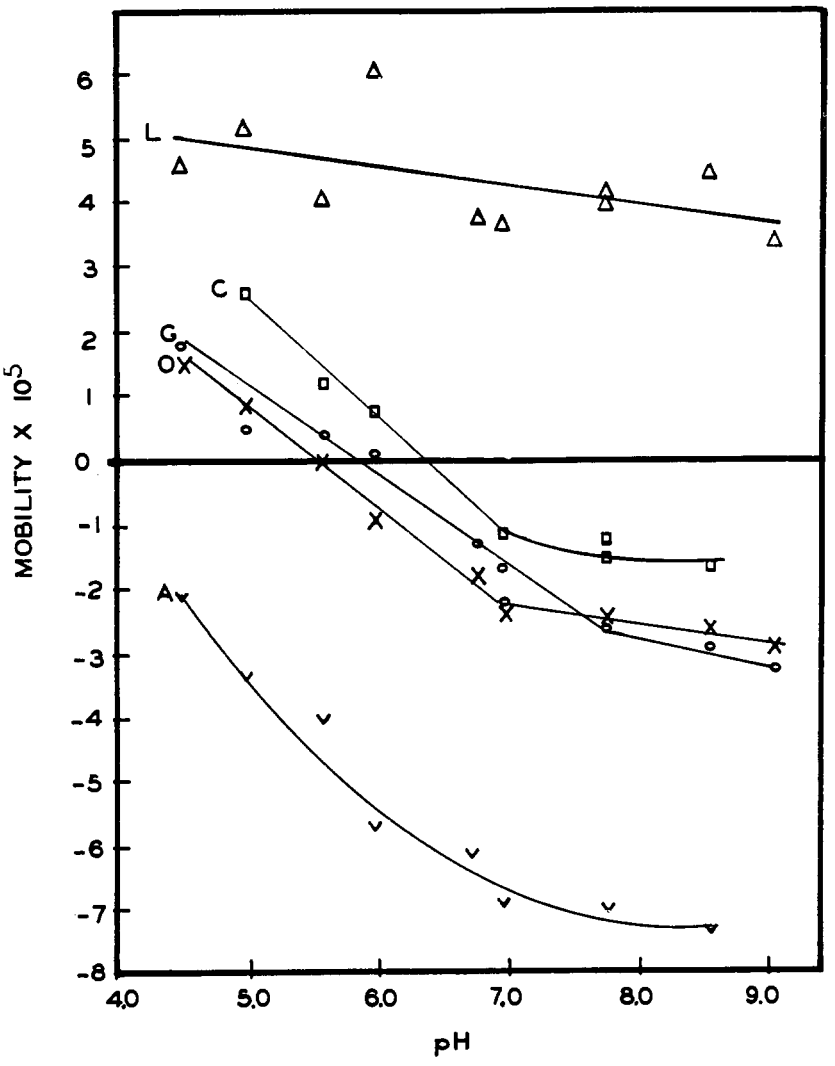

Figure 1. Paper electrophoretic mobilities of egg white proteins as functions of pH

0.05M phosphate buffers used. A. Ovalbumin C. Conalbumin G. Ovoglobulin O. Ovomucoid L. Lysozyme

The relationship of pH to mobility for the isolated egg white proteins is shown graphically in Figure 1. Phosphate buffers were used. The curves are not perfect by any means, but, except for lysozyme, most of the points are not far off the curves. The curves are nearly straight lines in the region between pH 4.5 and 7.0 with approximately a 45 degree slope (except for lysozyme), but above this pH region they rapidly level off.

Conventional moving-boundary electrophoresis is used to determine the

Table II. Change in Mobility of Egg White Proteins with pH Using 0.05*M* Phosphate and 0.3*M* Acetate Buffers

(Major peaks only given)

	Mobility × 10⁵				
pH	Ovalbumin	Ovomucoid	Ovoglobulin	Conalbumin	Lysozyme
4.5	−2.1	1.5	1.8	...	4.6
5.0	−3.4	0.9	0.5	2.6	5.2
5.6	−4.0	0	0.4	...	5.6
	−4.0	0	0.4	1.2	4.1
6.0	−5.7	−0.9	0.1	0.8	6.1
6.8	−6.1	−1.8	−1.3	...	3.8
7.0	−6.9	−2.4	−1.7, −2.2	−1.1	3.7
7.8	−6.9	−2.3	−2.4	...	4.0
	−7.0	−2.4	−2.6	−1.2	4.0
	−7.1	−2.5	−2.6	−1.5	4.2
8.6	−7.3	−2.6	−2.9	−1.6	4.5
9.1	−7.9	−2.9	−3.2	...	3.4
4.3[a]	1.3	3.2	3.3	3.4	7.8
5.0[a]	−1.6	0.9	2.4	2.6	6.3

[a] Acetate buffers.

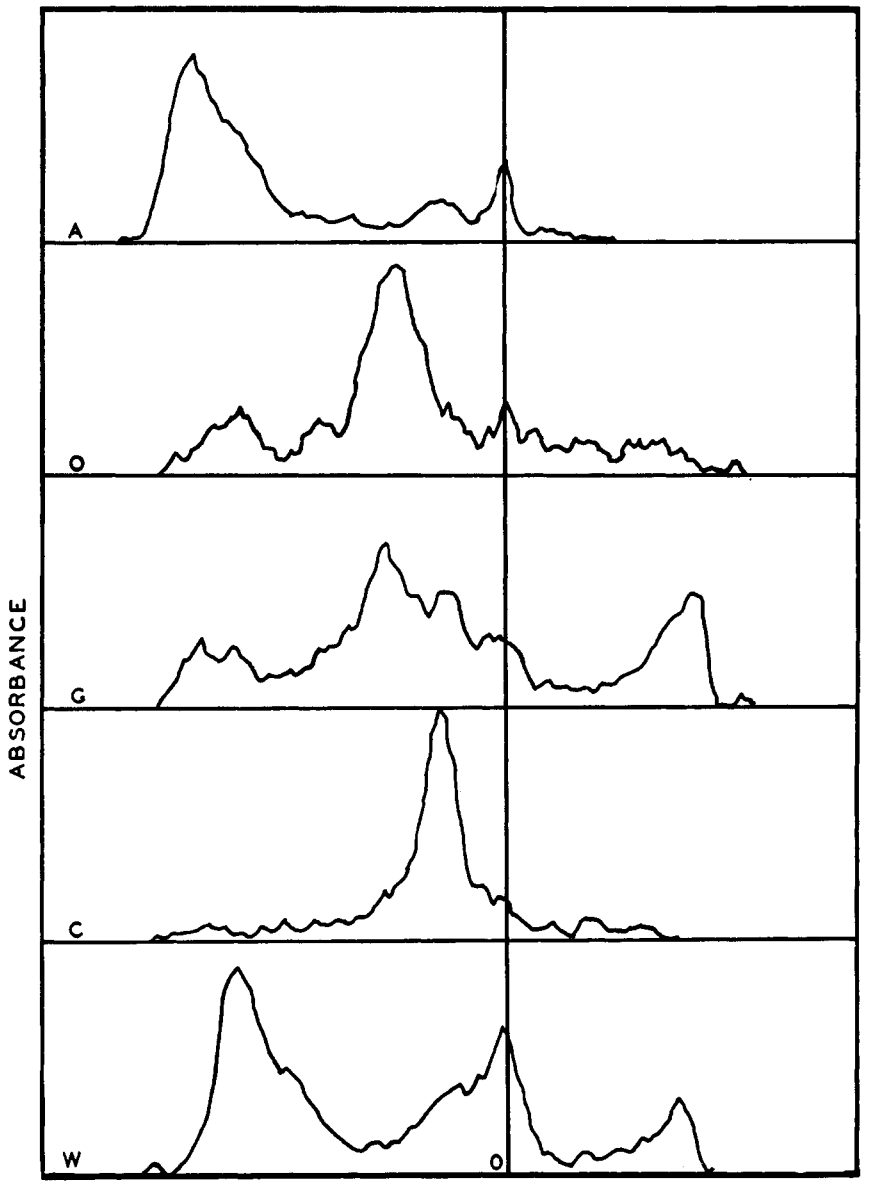

Figure 2. Paper electrophoretic patterns of egg white and isolated egg white proteins

Determined by plotting distance traveled against absorbance of blue compound formed by reaction of protein with bromophenol blue. Misco sandwich-type horizontal paper electrophoresis cell filled with 0.05M phosphate buffer of pH 7.8 used

A. Ovalbumin
O. Ovomucoid
G. Ovoglobulin and lysozyme
C. Conalbumin
W. Whole egg white

isoelectric points of proteins. The isoelectric points of the egg white proteins were determined with paper electrophoresis by estimating the pH where the curve for each protein crossed the line of zero mobility in Figure 1. The isoelectric points obtained are presented in Table III along with isoelectric points read from the curve given by Longsworth, Cannan, and MacInnes (*14*) in their Figure 8. Some interesting observations were made. The isoelectric point of 5.9 for ovoglobulin is just between their values of 5.7 and 6.1 for ovoglobulins G_3 and G_2. The isoelectric point of 6.4 for conalbumin 2a was considerably higher than those of 5.8 and 6.0 of Longsworth and coworkers (*14*). Conalbumin 2a behaved differently from conalbumin 1, which was prepared in the same way that Longsworth and coworkers (*14*) prepared their sample. Longsworth and coworkers (*14*) used phosphate buffers above pH 6.12 and acetate buffers below pH 5.33. Different isolectric points were obtained for ovalbumin using phosphate and acetate buffers (Table III). An isoelectric point considerably less than 4.5 (the lowest pH studied with phosphate) was indicated with phosphate buffers. A straight line connecting the mobilities of ovalbumin at pH 4.3 and 5.0 in 0.3*M* acetate buffers crossed the zero line to give an isoelectric point of 4.6, which agrees well with that calculated from Longsworth's data. The significance of the different isoelectric points in phosphate and in acetate buffers is not known, but a higher isoelectric point was obtained for ovomucoid with phosphate buffers than Longsworth and coworkers (*14*) obtained with acetate buffers.

What influence does the nature of the buffer have on the mobilities of egg white proteins when the pH is the same? Much preliminary work was done to find the best concentrations of the different buffers for these experiments. The molarities were chosen for the buffers after much trial and error. A comparison of mobilities of the different egg white proteins at pH 5.0 using phosphate and acetate buffers and at pH 8.6 using barbital, phosphate, and borate buffers is given in Table IV. Ovalbumin moved twice as fast in the phosphate as in the acetate at pH 5.0. Ovomucoid and conalbumin moved at the same rate in either buffer. Lysozyme moved a little faster in the acetate buffer. Ovoglobulin moved five times as fast in the acetate as in the phosphate buffer. The reasons for the differences are not known, but the differences probably account for some of the discrepancies between the isoelectric points of different egg white proteins determined by Longsworth and coworkers (*14*) and those determined in the present study. Of course, the nature of the buffer may affect protein mobility very differently in paper electrophoresis than in free moving-boundary electrophoresis.

Phosphate, borate, and barbital buffers of 0.05 molarity were compared at pH 8.6. Mobilities of ovalbumin, ovomucoid, and ovoglobulin were similar in the phosphate and in the barbital buffers but were greater in the borate buffer. The mobility of conalbumin was twice as great in the phosphate buffer as in barbital, with the value in borate lying between these two. Lysozyme had the same mobility in borate

Table III. Isoelectric Points of Egg White Proteins

	Calcd. from Figure 1	*Calcd. from Longsworth (14)*
Ovalbumin (phosphate buffer)	<4.5 (3.4 extrapolated)	...
Ovalbumin (acetate buffer)	4.6	4.6
Ovomucoid	5.6	4.3 (acetate buffer)
Ovoglobulin	5.9	5.7, 6.1
Conalbumin	6.4	5.8, 6.0
Lysozyme	>9.1	>7.8

and barbital buffers, but moved slightly faster in the phosphate.

Reference to Tables II and IV shows that none of the phosphate, borate, or barbital buffers used gave satisfactory separation of ovomucoid and ovoglobulin. Ovomucoid and ovoglobulin were separated in acetate buffer of pH 5.0, but ovoglobulin was not separated from conalbumin (Table IV).

Typical filter paper electrophoresis curves are presented in Figures 2 and 3 for whole egg white and for the different isolated egg white proteins with pH 7.8 phosphate buffer and pH 8.6 barbital buffer. The absorbance as determined with the Densichron densitometer is plotted against distance from the point of application. Separation of the different proteins of egg white was not especially good with the pH 7.8 phosphate buffer (Figure 2). Ovalbumin and lysozyme were well separated from the other proteins, but ovomucoid and ovoglobulin gave peaks at the same point, and conalbumin was not completely separated from them. Ovalbumin 2 was used in the study presented in Figure 2, and the peak of the conalbumin contained therein was plainly evident. The ovoglobulin preparation seemed to contain some conalbumin and some ovalbumin. The high peak at the origin is really a distortion, because a drop of egg white was added at the origin, and the very small portion which did not move was dyed dark blue. The Densichron had an orifice 3 mm. in diameter and measured almost the entire spot at the origin, but with the proteins that migrated and spread across the paper, each reading was only a very small portion of the spot.

Separation of the egg white proteins appeared to be much better with the pH 8.6 barbital buffer (Figure 3) than with the pH 7.8 phosphate one. The ovomucoid and ovoglobulin peaks still overlapped, but conalbumin was better separated from them.

All of the foregoing studies were made using the Misco sandwich-type horizontal paper electrophoresis apparatus and a constant voltage to measure mobility. Separations were not clear-cut enough for quantitative use, and some protein from the paper stayed on the glass plates. Accordingly, the Spinco paper electrophoresis cell which uses the ridgepole principle of Durrum (7) was tried. The apparatus has been standardized for blood protein analyses using a constant current and a barbital buffer of pH 8.6 and 0.05 ionic strength. The first studies with the Spinco apparatus compared pH 7.8 phosphate buffer with pH 8.6 barbital buffer, using a constant current of 5 ma. over 8 strips for 16 hours. Migration and separation of the egg white proteins were good with the barbital buffer. Separation was distinct with the 0.05*M* phosphate buffer, but migration was not far enough to give a clear-cut separation. Increasing the time of resolution to 24 hours increased migration only slightly and resulted in less clear-cut separation because of wider bands. Increasing the current to 10 or 15 ma. increased the migration of the proteins but not to the extent obtained with barbital buffer, and separation was not as good. Decreasing the molarity of the phosphate buffer to 0.025 increased the move-

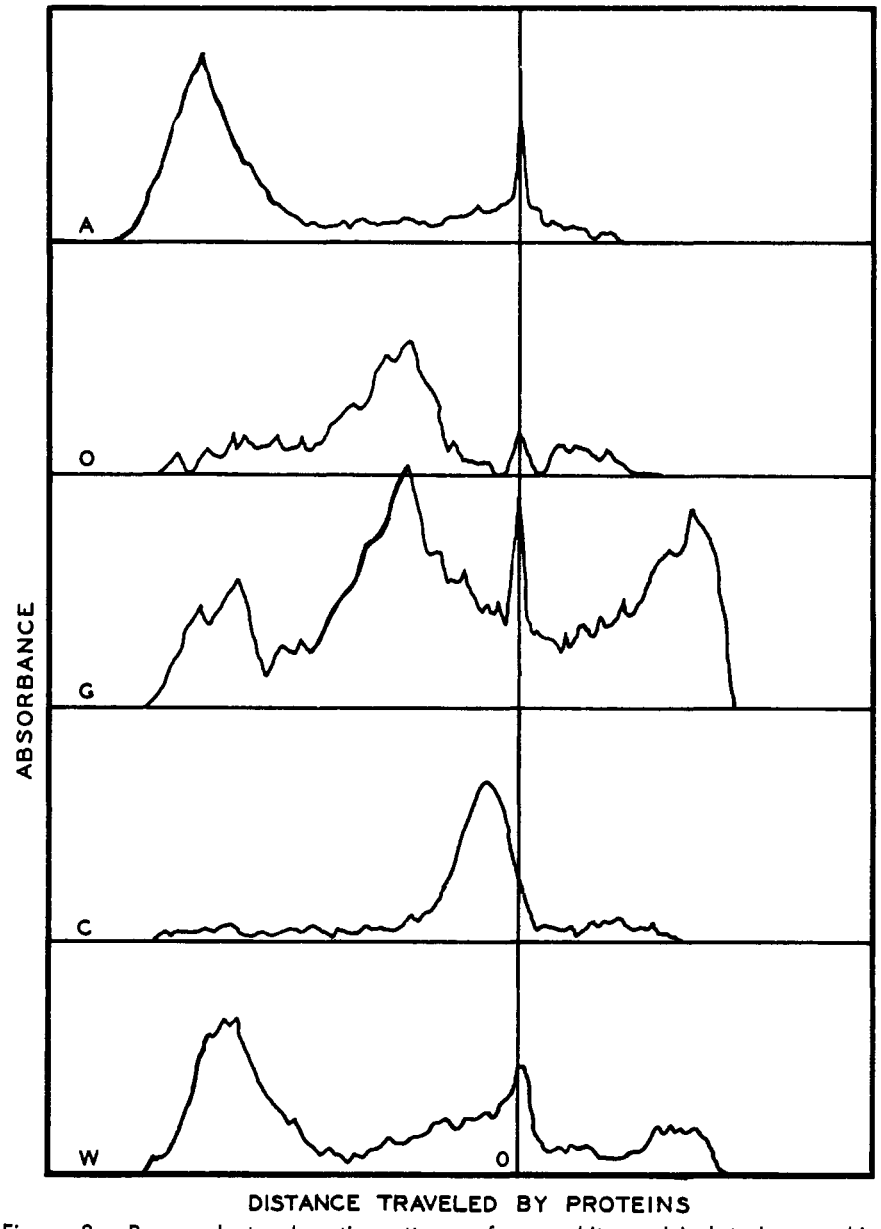

Figure 3. Paper electrophoretic patterns of egg white and isolated egg white proteins

Determined by plotting distance traveled against absorbance of blue compound formed by reaction of proteins with bromophenol blue. Misco sandwich-type horizontal paper electrophoresis cell filled with 0.05*M* barbital buffer of pH 8.6 used

A. Ovalbumin
O. Ovomucoid
G. Ovoglobulin and lysozyme
C. Conalbumin
W. Whole egg white

Table IV. Comparison of Mobilities Using Different Buffers at Same pH

pH	Buffer	Molarity	Mobility × 10^5 Ovalbumin	Ovomucoid	Ovoglobulin	Conalbumin	Lysozyme
5.0	Phosphate	0.05	−3.4	0.9	0.5	2.6	5.2
	Acetate	0.30	−1.6	0.9	2.4	2.6	6.3
8.6	Phosphate	0.05	−7.3	−2.6	−2.9	−1.6	4.5
	Borate	0.05	−8.8	−3.7	−3.0	−1.1	4.0
	Barbital 1	0.05	−7.3	−2.5	−2.2	...	3.3
	Barbital 2	0.05	−7.1	−2.4	−2.5	−0.8	4.0

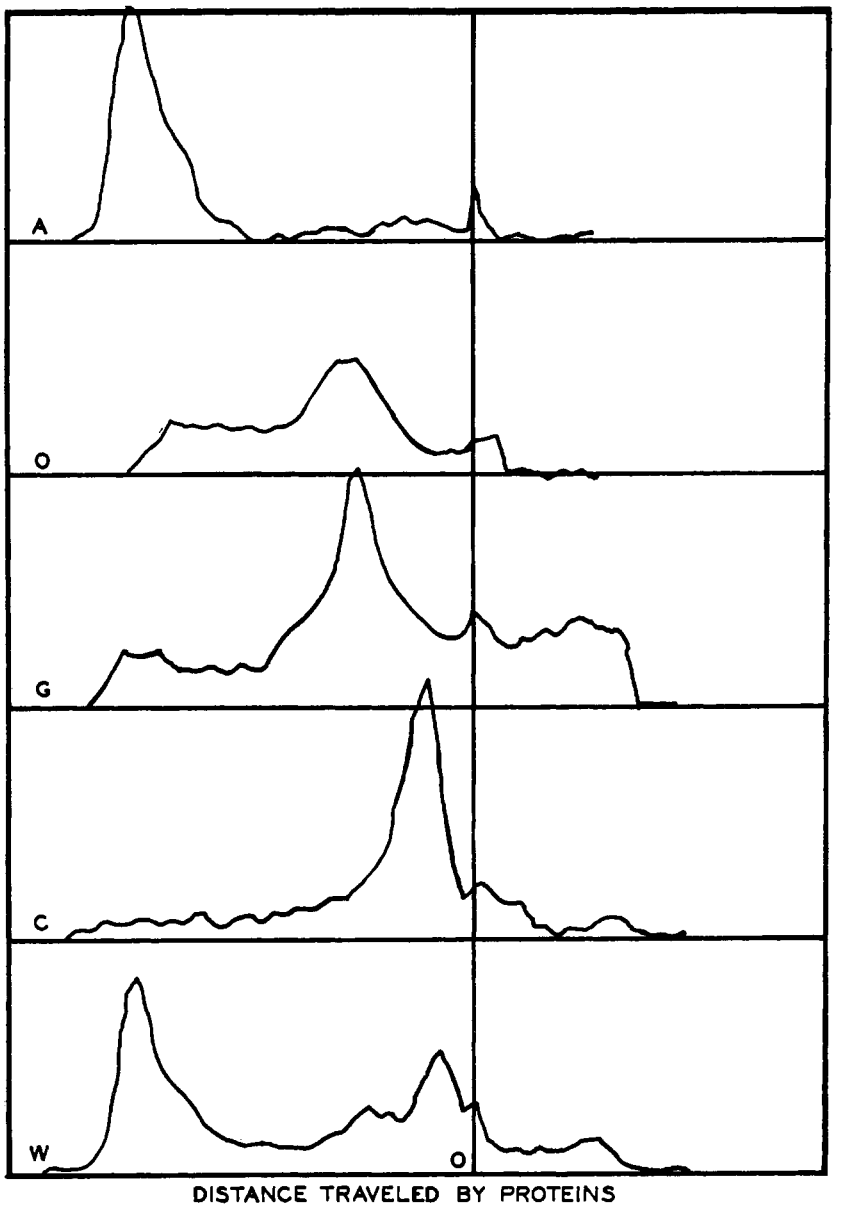

Figure 4. Paper electrophoretic patterns of egg white and isolated egg white proteins

Determined by plotting distance traveled against absorbance of blue compound formed by reaction of protein with bromophenol blue. Spinco ridgepole paper electrophoresis cell filled with 0.05M barbital buffer of pH 8.6 used A. Ovalbumin O. Ovomucoid G. Ovoglobulin and lysozyme C. Conalbumin W. Whole egg white

ment of the proteins but decreased the resolution, because the strips were more diffuse and wider.

Therefore pH 8.6 barbital buffer was used in all the rest of the studies with the Durrum ridgepole-type paper electrophoresis cell, because it gave better separations of the proteins than did the pH 7.8 phosphate buffer, even though ovomucoid and ovoglobulin were still not separated. Distinct, well separated peaks for ovalbumin, ovomucoid plus ovoglobulin, conalbumin, lysozyme, and a nonmobile protein are present in the egg white curve obtained by plotting Densichron readings against distance traveled and given in Figure 4. Separations are not shown as well on the graphs as on the papers themselves (Figure 5). Bands for ovalbumins A_1, A_2, and A_3, ovomucoid plus ovoglobulin, conalbumin, the nonmobile protein, and lysozyme are present on the paper strip and are easily separated from each other by cutting the strip into sections.

Quantitative Determination of Egg White Proteins

Method. Fresh eggs were broken out and the yolks and whites separated. The whites of the eggs to be studied were put together and broken up in the Waring Blendor until the whites were well mixed, by turning the blender on and off rapidly. Ten-microliter portions of the mixed egg white were added to the strips by means of a special stripper (furnished with the Spinco electrophoresis cell). Electrophoresis was carried out as described under methods of paper electrophoresis.

The electrophoresis papers were stained with bromophenol blue, the excess dye was washed out with dilute acetic acid, and the papers were fixed by washing with a sodium acetate solution in acetic acid and dried. Each strip was cut into seven parts to separate ovalbumin A_1, ovalbumin A_2, ovalbumin A_3, ovomucoid plus ovoglobulin, conalbumin, nonmobile protein, and lysozyme. Each portion of the strip was

Figure 5. Separation of egg white proteins by filter paper electrophoresis

Spinco ridgepole paper electrophoresis cell filled with 0.05M barbital buffer of pH 8.6 and current of 5 ma. for 16 hours. Filter paper strips stained with bromophenol blue
A. Ovalbumin O. Ovomucoid G. Ovoglobulin and lysozyme C. Conalbumin W. Whole egg white

placed in a 100-ml. beaker, and 25 ml. of 0.01*N* sodium hydroxide solution were added. The color was extracted for 1 hour with occasional swirling to get the paper completely in the solvent and to mix the extracted color. After the color was all extracted and well mixed, the absorbance was measured in the Beckman Model B spectrophotometer at a wave length of 590 mμ. A standard curve was obtained by spotting egg white on filter paper strips, developing, and extracting the color in the same way that was done for the electrophoresis papers. A straight-line curve of absorbance plotted against egg white concentration was obtained, showing that the color formed by the reaction of egg white proteins with bromophenol blue follows Beer's law and absorbance is directly proportional to the amount of protein present. Although a standard curve was prepared, it was found unnecessary to use it, because absorbance can be used directly in the calculations, thus omitting one extra step. A typical calculation follows:

Protein	Absorbance	% of Total Protein Absorbance × 100/1.114
A_1	0.541	48.6
A_2	0.132	11.8
A_3	0.050	4.5
O + G	0.128	11.5
C	0.190	17.1
N	0.023	2.1
L	0.050	4.5
Total	1.114	100.1

Results and Discussion. Data obtained from the extraction of 46 separate strips are presented in Table V. Each sample was prepared by compositing the whites of two to four eggs. The eggs were obtained from the Michigan State University poultry farm and were produced by the laying flock, which is kept on a standard laying ration.

The separation of the three ovalbumin fractions was not as clear-cut as the other separations, but the sum of the three, or the total ovalbumin, was separated from the other egg white proteins very well. The separation of ovalbumin into three fractions was less satisfactory in the whites of the older eggs than in the fresh ones. The average composition of ovalbumin from fresh eggs was 74.2% A_1, 19.3% A_2, and 6.4% A_3, which compares very favorably with the 76% A_1, 18% A_2, and 6% A_3 reported by Cann (*4*), but it is slightly different from the 85% A_1, 14% A_2, and trace of A_3 obtained by Perlmann (*17*). Ovalbumin in whites of fresh eggs obtained at a different time was composed of 71.4% A_1, 21.2% A_2, and 7.3% A_3, but in eggs from the same dozen that had been stored for 3 months in the refrigerator the distribution was 69.1% A_1, 23.4% A_2, and 7.5% A_3. Whites from eggs over a year old contained 60.6% A_1, 24.3% A_2, and 15.2% A_3 in the ovalbumin. There appeared to be a difference in the composition of ovalbumin from eggs of different hens or of eggs laid at different times of the year and also a change during storage of shell eggs, but conditions were not well enough controlled to draw any definite conclusions. Experiments are now under way to study individual hen variation and the changes in protein composition during storage of shell eggs.

There appeared to be slightly less ovalbumin in the white proteins of stored eggs than of fresh ones and a higher concentration of ovomucoid plus ovoglobulin and a lower concentration of conalbumin in the white protein of old eggs than of fresh ones.

The protein distribution in fresh eggs as determined by paper electrophoresis and presented in Table V agrees in most respects with that determined by other workers in other ways. Other things besides method of determination may account for some of the differences. Composition may be affected by the age of the egg. Csonka and Jones (*6*) showed that eggs from birds of different breeds had different egg white protein distributions, which could be changed by changes in the ration. They obtained values as follows: ovalbumin 58.0 to 62.8%, ovomucoid 12.6 to 17.5%, total globulins (including lysozyme) 9.5 to 14.8%, conalbumin 12.3 to 15.2%, and globulin G_1 (lysozyme) 2.4 to 6.5%.

Ovalbumin percentages presented in Table V compare favorably with those of other investigators (Table VI).

The ovomucoid plus ovoglobulin percentages are low for the most part when compared with literature values (Table VI). Ovomucoid and ovoglobulin have been separated by moving-boundary electrophoresis in some cases, and the percentages of each are given in Table

Table V. Protein Composition of Egg White Determined by Paper Electrophoresis

Protein	% of Total Egg White Protein: Fresh[a]	From 3-mo.-old eggs[a]	From same batch as 3-mo.-old eggs[b]	From eggs over year old[c]
Ovalbumin A_1	48.4	42.5	44.8	35.8
Ovalbumin A_2	12.6	14.4	13.3	14.4
Ovalbumin A_3	4.2	4.6	4.6	8.9
Ovalbumin (total)	65.2	61.5	62.7	59.1
Ovomucoid + ovoglobulin	11.2	12.7	10.8	16.8
Conalbumin	17.0	19.2	20.9	18.0
Nonmobile protein	2.1	2.4	1.8	1.8
Lysozyme	4.5	4.2	3.8	4.4

[a] Av. of 20 separate runs. [b] Av. of 4 separate runs. [c] Av. of 2 separate runs.

Table VI. Protein Composition of Egg White

Method	Reference	% of Total Egg White Protein: Ovalbumin	Ovomucoid + ovoglobulin	Ovomucoid	Ovoglobulin	Conalbumin	Lysozyme
Paper electrophoresis	Table V	59.1–65.2	10.8–16.8	...	...	17.0–20.9	3.8–4.5
Chemical separation	Sörensen (*20*)	69.5	19.6[a]	12.9	...	9.0	...
Moving-boundary electrophoresis	Bain (*2*)	60.5	15.0	...	...	22.0	...
	Csonka (*6*)	58.0–62.8	...	12.6–17.5	9.5–14.8	12.3–15.2	2.4–6.5
	Forsythe (*9*)	67.3	16.3	...	...	16.2	...
	Forsythe (*10*)	64.9	17.9	9.2	8.7	13.8	3.4
	Longsworth (*14*)	60.0	22.9	14.0	8.9	13.8	2.8
	Sober (*19*)	56.2	...	...	...	22.8	...
Chromatographic separation	Sober (*19*)	68.5	...	...	...	18.3	...
Immunological	Cohn (*5*)	67	...	...	...	16.8	...
	Wetter (*24*)	...	...	11.3	...	...	...
	Wetter (*25*)	...	...	...	...	...	3.7
Trypsin inhibitor	MacDonnell (*8, 16*)	...	...	11	...	...	...
Bacteriological	Feeney (*8, 16*)	...	...	...	...	...	2.8–3.5
Iron-binding	Feeney (*8*)	...	...	...	...	14	...
	MacDonnell (*16*)	...	...	...	...	12	...

[a] Includes lysozyme.

VI. From the published curves it is doubtful if the separation of ovomucoid from ovoglobulins G_2 and G_3 is very good. More specific methods, such as by inhibition of trypsin activity and immunological methods, appear to be more satisfactory.

Compared with the usually accepted values for the conalbumin content of egg white proteins, the values given in Table V are high. There appears to be considerable variation among the four groups of eggs studied. Several of the reported values for conalbumin in egg white protein fall within the range given in Table V or are very near to those values (Table VI).

Sörensen (*20*) isolated 1.9% and Forsythe and Foster (*11*) 1.1% of ovomucin from egg white protein. Lanni and others (*13*) have identified their egg white inhibitor of influenza virus hemagglutination as a fraction of ovomucin and found 1.6% of it to be present in egg white protein. Values of 1.8 to 2.4% were found for the nonmobile protein as given in Table V. The nonmobile protein might be assumed to be ovomucin, because it was present in amounts similar to those reported for ovomucin in egg white proteins, and because of the insolubility of ovomucin in egg white. However, this fraction cannot be ovomucin unless ovomucin behaves differently with paper electrophoresis than with moving-boundary electrophoresis, because according to Sharp and others (*18*) the egg white inhibitor contains three fractions with mobilities of -10, -6.7, and -3.5×10^{-5} sq. cm. sec.$^{-1}$ volt^{-1} in phosphate buffer of pH 7.2 and 0.1 ionic strength. The nonmobile protein may thus be some proteins that were denatured by the brief action of the Waring Blendor on the egg white proteins. On the other hand, it could be that ovomucin reacts with the filter paper, so that it is not moved by the electrophoretic force. Whether or not this was the case could not be determined because of the lack of a pure sample of ovomucin.

Lysozyme values obtained by filter paper electrophoresis (Table V) are in reasonably good agreement with literature values (Table VI).

The method described in this paper for the separation of egg white proteins by the Durrum ridgepole method of filter paper electrophoresis appears to give reasonable and reproducible results. A comparison of the quantitative data obtained indicates that they are within limits of values obtained by other methods. Until absolute methods are worked out for the separation and quantitative determination of the egg white proteins, this method appears to give at least as satisfactory results as any other one in use. It is relatively rapid. The speed and reproducibility of the method made it ideal for studying changes that may take place in egg whites during the storage of shell eggs. Ovoglobulins and ovomucoid are not separated by this procedure and some other technique must be used if separate determination is desired.

Summary

Ovalbumin, ovoglobulins, conalbumin, and ovomucoid were separated from egg white by fractional precipitation with ammonium sulfate solution. The crude proteins were purified by recrystallization or other means. The ovoglobulin fraction contained both ovoglobulins and lysozyme.

Mobilities of ovalbumin, ovoglobulin, ovomucoid, conalbumin, and lysozyme were determined between pH 4.3 and 9.1 using a horizontal sandwich-type paper electrophoresis cell with a constant low voltage of 150. Most measurements were made using 0.05*M* phosphate buffers. Diffuse spots were obtained when phosphate buffers of lower strength were used, and resolution was poor. As the pH increased the positive mobilities of the proteins decreased and changed to negative, and the negative ones increased. Mobilities of egg white proteins were compared at pH 5.0 in phosphate and acetate buffers and at pH 8.0 in phosphate, borate, and barbital buffers. The mobility pattern was different in phosphate than in acetate at pH 5.0. The isoelectric point of ovalbumin in acetate buffer was 4.6, but in phosphate buffer it was less than 4.5. The patterns of mobility at pH 8.0 were nearly the same in phosphate, borate, and barbital buffers, except for conalbumin, which moved twice as fast in phosphate as in barbital and at a rate in between the two in borate.

The best separation of the egg white proteins was obtained with pH 8.6 barbital buffer of 0.05 ionic strength. This buffer was used with the ridgepole paper electrophoresis cell, and good separations of all of the proteins occurred, except that ovomucoid and ovoglobulin could not be separated from each other.

Quantitative separations of the egg white proteins were made by cutting out the bands of each protein after the paper strip had been dyed with bromophenol blue and the excess dye washed out with dilute acetic acid. The color was eluted from the sections of each strip with dilute sodium hydroxide solution and the eluted color read in the Beckman Model B spectrophotometer at a wave length of 590 mμ. Percentages of the different proteins in egg white were then calculated. Fresh egg white protein contained 65.2% ovalbumin (48.4% A_1, 12.6% A_2, and 4.2% A_3), 11.2% ovomucoid plus ovoglobulin, 17.0% conalbumin, 2.1% nonmobile protein, and 4.5% lysozyme.

Acknowledgment

Eggs used in this work were kindly furnished by J. A. Davidson of the Poultry Department.

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Received for review January 3, 1956. Accepted April 6, 1956. Journal article 1865, Michigan Agricultural Experiment Station.