THE SEPARATION OF THE HIGH-PHOSPHORUS, FAST-MOVING COMPONENT OF EGG YOLK

by

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TABLE OF CONTENTS

INTRODUCTION

The yolk of hens egg has been the subject of much interest, primarily because it contains proteins, lipids, and vitamins that are essential to good nutrition. In addition, the protein and lipid materials are associated rather loosely and to date very little is known concerning these protein-lipid combinations.

The chief problem has been to separate and purify the yolk constituents without altering these substances. The type of protein found in egg yolk is classified with a group of compounds that are extremely difficult to study because they are unstable to heat and high concentrations of immiscible solvents and are difficult to keep in solution unless the buffers used contain a relatively high salt concentration. This picture is further complicated by the fact that until recently few investigators have realized the importance of maintaining low temperatures when studying these substances.

Four components have been reported in the electrophoretic patterns of the yolk of fresh hens eggs. Two high phosphorus-containing fractions were apparent; a small leading peak which contained 40 percent of the total phosphorus and only ten to eleven percent of the total components, and an extremely slow moving peak that contained 50 percent of the phosphorus and represented 70 percent of total components. The present investigation was undertaken to explore means of separating the high phosphorus-containing leading fraction from the yolk. In these exploratory studies salt fractionation, alcohol fractionation

by electrophoretic methods were attempted. Salt fractionation proved to be more satisfactory compared to the very poor results obtained using ethanol as the fractionating agent. Since yolk proteins precipitate in low ionic strength buffer solutions, almost all the buffers employed in the electrophoreses were of a relatively high salt concentration and this made the use of electrophoretic methods unsatisfactory, because the salt rather than the protein carried the current.

REVIEW OF LITERATURE

As early as the middle 1800's Lehman and Messerchmidt (9) reported a protein-like material that precipitated when egg yolk was diluted with water. This precipitate dissolved in sodium chloride and reprecipitated upon dilution. It was not until 1842 that Dumas and Calhours named this precipitate Vitellin and later in 1865 Hoppe and Seyler showed that the protein, Vitellin, was a conjugate of protein and lipid.

The presence of another protein, in addition to Vitellin, was reported by Valenciennes and Fremy (9) (1854). They termed this protein an "albumin". Osborne and Campbell (17) isolated vitellin from egg yolk in 1900 and concluded that phosphorus was not present in this substance which they called a "para nucleoproteid". They also showed that solutions of sodium chloride dissolved large amounts of protein from egg yolk and that the dissolved substance exhibited the properties of a globulin. They found sulfur but no phosphorus in their preparations.

Shortly after the reports of Osborne and Campbell, livetin was isolated from egg yolk by Plimmer (18) in 1908. Plimmer found that livetin contained 0.1 percent phosphorus and vitellin contained 1 percent phosphorus. He suggested that livetin may be vitellin without the phosphorus-containing portion, but finally concluded that egg yolk contains two very closely related proteins. Plimmer observed livetin when he studied the aqueus solution discarded in the procedure as outlined by Osborne and Campbell for the preparation of vitellin. He found that the solution gave a marked biuret reaction, and that a voluminous coagulated precipitate was formed when the solution was boiled and then acidified with acetic acid. This substance was first regarded as unprecipitated vitellin, but it contained only 0.1 percent phosphorus as compared to the 1.0 percent for vitellin; consequently it was regarded as a second-and at that time undescribed-protein in the yolk of hens egg. He proposed the name livetin. In 1928 Kay and Marshall (14), in attempts to confirm Plimmer's work, thought that the complete separation of the white from the yolk was necessary in order to be sure that the proteins of the white did not add to this coagulum reported by Plimmer. To accomplish this, the yolks were allowed to roll down a strip of clean cloth to remove adhering white and then were washed several times with distilled water. They made several qualitative and quantitative tests on this material and concluded that the non-vitellin protein of egg yolk was approximately 90 percent homogeneous by the then-known criteria for protein characterization.

Vitellin of hens egg was prepared in 1931 by Calvery and White (2) using essentially the same method adopted earlier by Osborne and Campbell for the separation of the chief protein of egg yolk. The method consists of precipitating the protein with water and removing the supernatant by decantation. The precipitate was redissolved in 10 percent sodium chloride and reprecipitated by dilution with water. The precipitate was then suspended in 80 percent alcohol, dried, and assayed for nitrogen, phosphorus, and amino acids; although they used more modern methods, the nitrogen, phosphorus and sulfur results agreed well with those of Plimmer. In 1941 Chargoff (3) obtained a white fluffy powder which he called lipovitellin. In this procedure the yolk was extracted with ether and precipitated by diluting the extracted yolk with tap water. Chargoff also showed that the phosphatides occurring in these yolks in the free state were similar to the lipids accompanying the vitellin fraction reported by earlier workers in this field.

The preparation of the yolk lipoprotein, lipovitellin, was undertaken in 1945 by Alderton and Fevold (1). They passed diluted egg yolk through a Sharples centrifuge and extracted the precipitate with cold ether. Upon analysis the precipitate was found to contain lipovitellin and other materials removable by 10 percent sodium chloride solutions. The analytical data agreed well with that already published; approximately 18 percent of the egg yolk solids appeared to be lipovitellin whereas 17 percent was reported by earlier workers.

Work more directly related to the present investigation was published in 1948 by Mecham and Olcott (16). These investigators prepared a phosphoprotein containing at least 40 percent of the total protein phosphorus of egg yolk. The name phosvitin was proposed for this new phosphoprotein. One year later these same investigators, preparing this phosphoprotein by salt fractionation found that upon analysis this substance contained 57.5 percent phosphorus. Essentially, these investigators precipitated the yolk solution with 0.4 M $MgSO_4$ and found that this precipitate contained approximately 80 percent of the total phosphorus of egg yolk and only 10 to 12 percent of the total yolk protein. Phosvitin was extracted from this precipitate employing 0.4 M $(NH_4)_2 SO_4$ at pH 4. Ethyl ether at room temperature was added to coagulate the non-phosvitin material. Electrophoretic analyses of this new protein in sodium citrate buffer and sodium acetate buffers at pH 4.6 produced two peaks with mobilities in the citrate buffer of -12×10^{-5} units for the larger peak and -9×10^{-5} for the smaller.

Shepherd and Hottle (19) studied the composition of the livetin fraction by the use of electrophoretic analysis. After several ether extractions clear preparations of the livetin of hen's egg was obtained and upon analysis three peaks were observed.

In 1949 Young and Phinney (23) tried several methods to fractionate the yolk proteins of the salmon's egg as well as the hen's egg. By precipitating three to four times by means of dialysis against water at low temperatures, lipovitellin could be obtained which upon electrophoretic analysis produced three

components in buffers of sodium chloride and sodium citrate at pH 8.5. Other physical characteristics of the proteins of the salmon's egg were reported which do not concern us at present. Young and Phinney reported that lipovitellin was readily denatured in contact with water at 20° C.

Radioactive isotopes were first used in connection with yolk proteins by Hahn and Hevesy (10) in 1938. They use it purely as an analytical tool. Labeled sodium phosphate was injected into a hen and after 28 to 34 hours yolks were removed from the ovary. Eggs from the oviduct were of very low activity. They reported that labeled phosphorus did not exchange with the lecithin phosphate but does exchange readily with bone phosphate.

This same year Entenman et al (8) studied the phospholipid metabolism using radioactive phosphorus as an investigating tool. The amounts of labeled phospholipid found in egg yolk from birds fed radioactive phosporus were examined at six and twelve hours after administration of the phosphorus. The amount P^{32} deposited increased with time elasped from three to twelve hours, which was the time intervals that were examined in this experiment. More P^{32} was found in the larger yolks than in the smaller ones; however no relation between activity and size of yolks was observed.

In 1941 Chargoff (4), in attempts to confirm the results of one of his previous papers examined the origin of the phosphatides deposited by the laying hen in egg yolk following the injection of radioactive phosphorus. The phosphorus compounds (free and combined phosphatides, vitellin, and inorganic phosphate) were isolated from the yolks of eggs laid in the course of eight days

following the injection of radioactive sodium phosphate. The rates of formation of "free" lecithin and cephelin and of the combined phosphatides accompanying the vitellin fraction were the same.

In 1952 Hein and Clegg (12) reported a modified electrophoresis cell for determining labeled phosphoproteins. They modified the ascending arm of the center cell of a Tiseleus Electrophoresis apparatus to measure the activity of the peaks as they passed a window made 0.9 mm thick so that beta particles greater than 0.7 M.E.V. from radioactive phosphorus could be counted. This same cell was used to determine the radioactive phosphorus distribution in the serum proteins of the chicken, Clegg and Hein (5). Satisfactory results were observed.

The distribution of radioactive phosphorus in the electrophoretic components of egg yolk was studied in 1955 by Clegg et al. (6). It was observed that four components were present in both the glycine phosphate buffer pH 9.4 and the borate citrate buffer of pH 7.5. A fast-moving, high-phosphorus component contained approximately 39 percent of the total phosphorus and only nine to eleven percent of the total amount of material measured, and a fourth slow-moving peak that contained 40 percent of the total phosphorus and comprised 70 percent of the total components were observed.

In more recent work the changes in embryo yolk phosphoproteins have been investigated (Johnston, 13). Fertile eggs from hens fed radioactive phosphorus were incubated and observed on the seventh, fourteenth, and nineteenth day of incubation.

Electrophoretic patterns of the seven day embryo yolk proteins were essentially the same as those of fresh yolk. Four peaks were still evident, the amount of phosphorus in each peak, and the component percent of various fractions were similar to that of fresh yolks. In the fourteenth day embryo yolk preparations, only three components were present when analyzed electrophoretically. Two of these peaks still contained relatively large amounts of phosphorus. The 19 day embryo yolk preparation produced only two peaks and no major phosphorus-containing protein was evident as compared to earlier stages. In fact, very little of the original phosphorus was left in the yolk preparations at the end of 19 days of incubation.

As is evident from the above discussion, several investigators have prepared vitellin from egg yolk. Essentially, each of those investigators used the same general method, which consisted of dissolving the yolk in 10 percent sodium chloride, extracting the lipid with ether, and then pouring the solution into a large volume of water, whereby vitellin was precipitated. Plimmer prepared a second protein from the aqueus supernatant discarded in earlier procedures.

More recently Chargoff (3) in 1942 and Alderton and Fevold (1) in 1945 prepared lipovitellin. These workers separated the solid materials from the rest of the yolk, extracted the solids with ether, and upon analysis of this solid found that it contained lipovitellin. In 1948 Olcott and Mecham isolated the principal phosphoprotein from egg yolk and they called this phosphoprotein phosvitin. Yolk was precipitated and the precipitate

was extracted with $(NH_4)_2SO_4$ to obtain the phosvitin.

Electrophoresis was used as a means of studying the yolk of hen's egg by Young and Phinney (23) in 1951. These investigators reported only three components. These components were distributed in the proportions of 4.6, 10.2, 85.1 percent for hen's egg yolk.

The distribution of radioactive phosphorus in the electrophoretic patterns of egg yolk was studied in 1955 by Clegg and Hein. These investigators reported four components as compared to three reported earlier by Young and Phinney. In the work of Clegg and Hein, a fast moving component containing 39 percent of the total phosphorus and only 11 percent of the component percent of total was reported. There were two minor peaks and a fourth peak comprising 70 percent of the total and containing 40 percent of the phosphorus.

MATERIALS AND METHODS

In the present work, egg yolks were processed to obtain clear solutions and then attempts were made to separate the fast-moving, high-phosphorus component by salt fractionation, alcohol fractionation and by electrophoresis.

In both the alcohol and the salt precipitation methods, after the yolk solutions were precipitated and the two phases separated, the supernatant was dialyzed directly against the buffer solutions. However, it was necessary to dialyze the precipitate against a solution of high salt concentration before dialyzing against the buffer solutions in order to obtain a

relatively clear solution.

The supernatants and precipitates were analyzed by the Aminco electrophoresis apparatus and by starch gel electrophoresis. The standard methods for determining nitrogen and phosphorus were employed. Since eggs obtained from hens fed radioactive phosphorus were used in some experiments, radio-assay was also employed.

Production of Radioactive Eggs

Approximately 0.5 m.c. of P^{32} was pipetted into mineral oil-coated gelatin capsules and administered by mouth to laying hens confined in special cages. Since the eggs laid during the first nine days did not contain enough activity to produce any measurable count in the special equipment designed to measure activity, these eggs were discarded.

The P^{32} employed in this investigation was contained in a weak solution of H_3PO_4 obtained from the Oak Ridge National Laboratories. All necessary precautions were taken for safety measures. No cages, feeders, or hens were handled without rubber gloves and protective clothing.

Preparation of Yolk Solutions

Fresh yolks were broken and carried through the preparations as described by Clegg et al. (6). The yolks were rolled on cheesecloth to remove all adhering white and then transferred to 100 ml graduated cylinders and diluted six times with a 15 percent glycine solution (pH 7.5). The yolks and the glycine solution were mixed

gently until a homogeneous solution resulted, and then 50 ml aliquots of the yolk solution were shaken with two volumes of cold ether which had been saturated with water. The resulting mixture was allowed to stand for one hour. The ether was decanted and this extraction procedure was repeated six times over a period of 18 to 24 hours. After the extraction, the clear water layer and the white fluffy precipitate contained in the water layer were transferred to a dialysis sack and allowed to dialyze against a solution of 15 percent glycine-10 percent sodium chloride. When the yolk mixture was dialyzed against the solution of 15 percent glycine-10 percent sodium chloride, ether was expelled and at hourly intervals the ether was decanted by pinching the dialysis tubing between the fingers at the ether-yolk layer and draining the ether into a container. When all of the ether had been removed, the yolk solution was faintly cloudy. This solution was left an additional six to eight hours to dialyze against the sodium chloride-glycine solution and the resulting yolk solution was then dialyzed against one of the following buffers:

(1) Glycine-phosphate buffer: 0.053M disodium phosphate and 0.05 M glycine adjusted to pH 9.4 with NaOH.

(2) Borate citrate buffer: 0.05 M sodium tetraborate and 0.10 M sodium citrate adjusted to pH 7.5 with citric acid.

(3) Bicarbonate buffer: 0.1 N sodium hydrogen carbonate and 0.1 N sodium hydroxide adjusted to pH 9.8.

Yolk solutions dialyzed against any of the three buffers usually became very clear. In a few cases these yolk solutions which were dialyzed against the glycine-phosphate buffer were too turbid for

use in electrophoresis work. The borate citrate and the bicarbonate buffers produced solutions which were clear enough for electrophoretic analysis.

Precipitation Procedures

Alcohol Fractionation. The clear yolk solutions, prepared as described in the previous section, were dialyzed against solutions containing various concentrations of ethanol by volume. In each case the desired alcohol concentration was prepared and the yolk solutions dialyzed against the alcohol-buffer mixture which had been cooled to 7° C. The concentrations of ethanol employed were one percent, five percent, ten percent and fifteen percent.

Approximately 40 ml of egg yolk solution which had been previously dialyzed against either the borate citrate (pH 7.5) or the glycine-phosphate buffer (pH 9.4), was left in contact with this alcohol-buffer solution for a period of 24 hours. In several cases the pH of the solution appeared to be an important factor in causing precipitation and this observation will be discussed subsequently. At the end of the 24 hour period of dialysis the sacks that contained precipitates were removed and the total contents drained into centrifuge tubes. These tubes were placed in a centrifuge which had been placed in a cold room and centrifuged at high speed for 15 minutes. At the conclusion of centrifugation, the supernatant was decanted into cellophane sacks and allowed to dialyze directly against the prescribed buffers. It was shown in preliminary work that the precipitate from this procedure would

not redissolve if allowed to dialyze against the buffer; therefore, the precipitate was dialyzed for three to four days against a solution of 15 percent glycine-10 percent sodium chloride. At the end of this time the precipitated mixture had become relatively clear and was then dialyzed against one of the buffers as a preparation for further analysis.

Salt Fractionation. Clear yolk solutions obtained by the method previously described were used in this fractionating procedure. In preparing the 0.6 M $MgSO_4$ solution that was used in this work, the volume of the yolk solutions was included in the calculated volume of water. Fresh yolk solutions were poured into cellophane sacks and placed in this cold solution of 0.6 M $MgSO_4$ for 24 hours. At the end of this time, a white, fluffy precipitate was apparent. The entire contents of the cellophane sacks were transferred to centrifuge tubes and centrifuged for 15 minutes at high speed. At the end of the centrifugation the white precipitate had settled to the bottom of the tube and the clear supernatant liquid was decanted, measured, and transferred to another cellophane sack. The supernatant was dialyzed directly against glycine-phosphate buffer. The precipitate was mixed with 15 ml of 15 percent glycine solution, in order to make the mixture easier to transfer and also to facilitate redissolving. Three to four days of dialysis against the solution of 10 percent sodium chloride-15 percent glycine were required to redissolve the precipitate and obtain a solution clear enough for electrophoretic analysis. When the solutions had become clear, they were transferred to the appropriate buffer solutions and allowed to dialyze

for one to two days.

Electrophoretic Techniques

In this investigation an attempt was made to use electrophoresis to separate the components of egg yolk. Starch granules, starch gel, and filter paper were used as supporting media for these electrophoretic separations.

The starch granules were from potato starch, obtained from E. H. Sargent Company. The container was a plastic tray, as shown in Plate I, made with troughs approximately 20 mm in width, 5 mm in depth, and 10 inches in length. These trays were graduated in centimeters in order to mark the point of application and also to note the distance the components had moved. Plastic strips were used to cover the trough.

Starch granules were mixed with one of the buffers described in section on preparation of yolk solutions, until a very thick paste was obtained. This paste was introduced into the trays and smoothed with a spatula, so that no air pockets were left along the base of the tray. If, by pressing this starch paste, liquid could be observed, more dry potato starch was added to take up this moisture. This process was continued until the paste was of such a consistency that it could be sliced with a sharp razor blade, without becoming soft upon the application of pressure. For the electrophoresis run the yolk solutions were mixed with dry potato starch to make a paste of the same consistency as the starch in the tray. In some runs bromphenol blue was added at this point in order to visually observe the movement of the bands.

A very thin slice of the starch in the tray was cut out with two razor blades which had been especially prepared for this procedure, and the yolk-starch mixture was added to the slit with a spatula. A plastic cover was put in place to prevent evaporation. Special care was taken to fit the cover firmly over the starch tray so that the cover was in contact with the starch. This prevented moisture from condensing on the cover and falling back onto the column. Strips of filter paper were cut and fitted into the tray at both ends as a wick for the connection to the buffer reservoirs. The starch granules were in direct contact with these filter paper leads. Platinum electrodes were placed in the buffer vessels and connected to a power supply of 50 volts and 20 m.A. for varying lengths of time.

Preparation of Starch Gels

Potato starch was treated as described by Smithies (20), for the starch gel electrophoresis, 300 grams of potato starch were suspended in a solution containing 600 ml of acetone and 6 ml of concentrated HCl for 45 minutes at 38.5° C. This starch mixture was stirred at regular intervals during this time. At the end of the 45 minutes, 150 ml of 1M sodium acetate was added to stop the reaction. With the use of suction and a Buchner funnel the starch granules were washed several times with distilled water and allowed to stand over night in fresh distilled water at a temperature of approximately 7° C. The granules were then washed several times with distilled water, mixed with 500 ml of acetone and filtered through a Buchner funnel. The starch was then placed in a tray

EXPLANATION OF PLATE I

Apparatus for starch gel electrophoresis

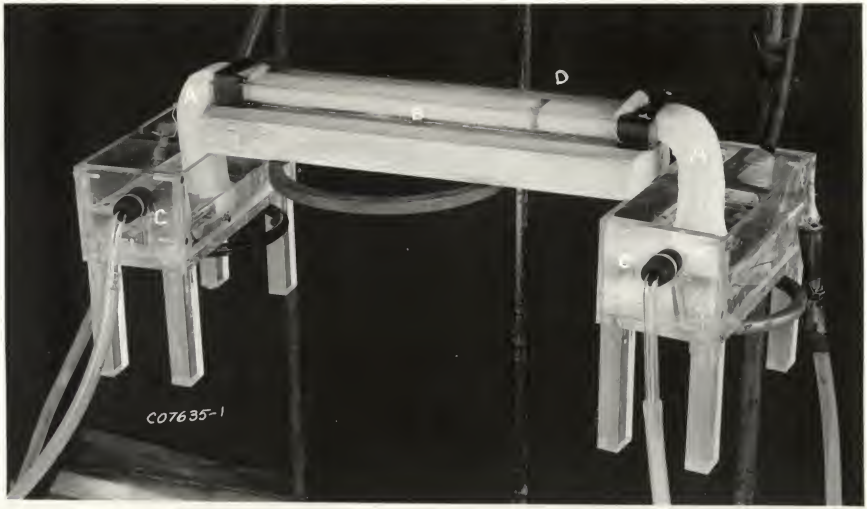
A. Filter paper wicks

B. Starch gel in tray

C. Buffer troughs

D. Slit cut in starch gel

PLATE I



and dried in an oven for one day at 45° C. This starch was used to prepare the starch gel in the following section.

Since this treated starch raised the pH of the buffer solutions by ½ pH unit, each of the buffers employed were previously adjusted to ½ pH unit higher than was normally employed. One hundred ml of the buffer was mixed with 15 grams of the treated starch in an Erlenmeyer flask and heated with constant stirring in a boiling water bath. When this starch solution became thick, it was swirled vigorously to prevent lumps from forming. The solution was then further heated and stirred with a thermometer in order that the temperature could be noted readily. At about 70° C. the thick white paste changed to a transparent fluid and was further heated to 75° C. At this point the starch was removed from the bath and mild suction was applied to remove air bubbles from the starch. When all the air bubbles were gone the solution became very clear. This starch solution was quickly poured into plastic trays, shown in Plate I, which were exactly as described in the previous section and without delay were covered with a thin film of polystyrene which had been oiled with mineral oil. The plastic top was used to press out excess starch, making the top level with the sides of the tray. When this starch was allowed to cool, it became a rigid, white, opaque gel that could be handled, sliced and stained.

Yolk solutions were mixed with starch granules as stated before or mixed directly with one of the several stains and poured into a slit (D of Plate I) which had been cut into the gel with a sharp razor blade. The polystyrene covered the tray during the

entire electrophoresis to prevent drying and shrinkage. At the completion of the run, the gel was sliced and dyed with bromphenol blue for five minutes, and then washed with 0.5 percent acetic acid solution. This staining procedure made the protein bands visible. In this starch gel electrophoresis, the length of time and the voltage were varied depending on the results desired.

Staining Techniques

Yolk proteins which were separated on starch gel were stained with bromphenol blue because better results were obtained with this dye. However, attempts were made to use Sudan Black B, Nile Blue A, and Amido Black 10B.

When yolk solutions were analyzed electrophoretically on starch gel and the resulting gel stained with Amido Black 10B, relatively good results were obtained; however, this dye did not give bands as sharp as were visible when bromphenol blue was employed. Amido Black 10B was obtained from the Hartman-Leddon Company in Philadelphia, Pennsylvania. The dye solution was prepared as follows: a solution was prepared consisting of 45 percent by weight of ethanol, 45 percent by weight of acetic acid, and ten percent by weight of water. To this solution was added Amido Black 10B in excess so that the solution became saturated. A wash solution was prepared in the same manner as the solution containing the dye. Paper strips or starch gels were placed in this solution for five to ten minutes and washed several times with the wash solution.

The bromphenol blue dye was obtained from the Fisher Scientific Company. To prepare the dye solution, ethanol was saturated with solid mercuric chloride and the clear saturated solution was decanted. One gram of the bromphenol blue dye was added to each liter of this solution. In the staining procedure, paper strips or starch gels were placed in this solution and allowed to stain with frequent shaking for a period of five minutes. At the end of the staining period the dye solution was decanted into a container and the starch gels or paper strips were washed several times with a solution of 0.5 percent by volume of acetic acid. Of all the stains used, this stain gave the best results, for starch gel.

The Nile Blue A was obtained from the National Aniline Division of E. H. Sargent and Company. This dye is certified for use in fat staining. To stain the section of the starch gel which was assumed to be of lipid nature, a 0.1 percent alcoholic solution by weight of this dye was mixed with nine parts of distilled water and filtered before use. This solution was made dilute enough so the lipid would adsorb the dye from solution. If a more concentrated solution was used, the gel or paper adsorbed so much that several days of washing were necessary to remove excess dye. When the dilute solution was used, no wash solution was necessary.

Sudan Black B was used in attempts to stain starch gels after electrophoresis runs. This staining solution was prepared by making a saturated solution of this dye in 50 percent by volume of ethanol and 50 percent by volume of water. The saturated

solution was decanted and the filter paper or gels allowed to remain in this solution for three to four hours. A wash solution consisted of a ten percent solution of acetic acid. This stain did not prove very successful in dyeing yolk proteins on starch gel.

Apparatus for Filter Paper Electrophoresis

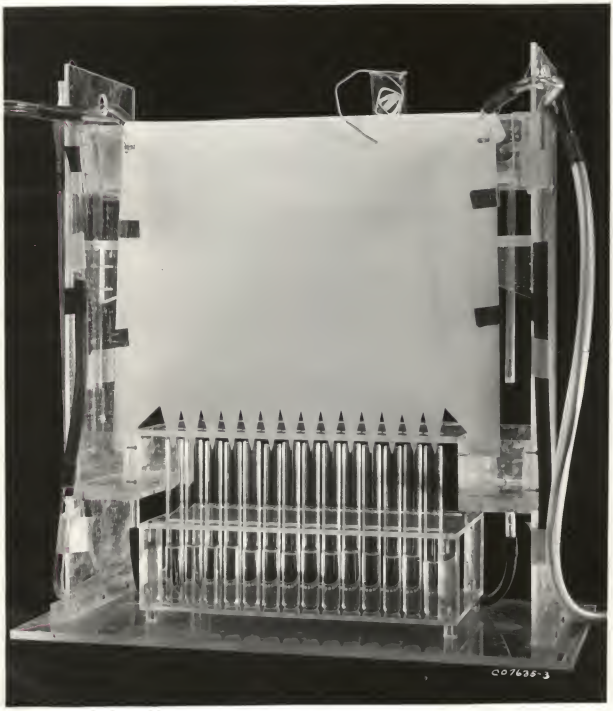
Plate II is the apparatus used for the filter paper type electrophoresis. This is essentially the apparatus used by Durrum, et al. (7). A sheet of paper was folded and small drip points were cut as shown in Plate II. The filter paper curtain was supported by the frame and the edges of the paper were rolled, slits were cut, and thin platinum electrodes were introduced into these slits. Buffer solution was allowed to flow continuously into a trough, which is shown at the top of the diagram. Buffer also flowed continuously from glass tips near the top on to the platinum electrodes. The excess buffer drained into the buffer trays at the base of the apparatus and was constantly being replaced to prevent a pH gradient from developing across the curtain. In all runs the buffer was allowed to flow for approximately six hours before the current was applied. The current was then allowed to run for 24 hours before applying the sample, in order that the whole system would come to equilibrium.

The sample was introduced by placing egg yolk solutions in the small beaker shown on top of the apparatus, and allowed to run down a very thin strip of filter paper, which was used as a wick, onto the curtain where the applied voltage caused the components

EXPLANATION OF PLATE II

Hanging curtain type electrophoresis apparatus for attempt separation of egg yolk components.

PLATE II



of varied charge to separate across the hanging curtain. In all runs 20 m.a. and 100 v. were applied for 72 hours and the buffer flowed continuously. As the fractions separated and moved down the curtain, they were collected in the test tubes. Samples in each tube could then be analyzed for nitrogen and phosphorus or total protein.

Analytical Procedures

The nitrogen was determined by the micro Kjeldahl method described by Hawk et al. (11). The yolk samples were diluted one to ten in a volumetric flask with borate-citrate buffer. One ml aliquots of the sample and one ml of concentrated H_2SO_4 was added to micro Kjeldahl flasks together with a small amount of powdered $CuSO_4$-K_2SO_4 catalyst. The solutions were digested to blackness, cooled and decolorized with 30 percent H_2O_2. These solutions were heated again until white fumes developed. The solutions were then cooled and five ml of distilled water were added. The Kjeldahl apparatus was washed with five ml of water; the samples were introduced, the flasks were rinsed with ten ml of water and this rinse solution was also added to the apparatus. Seven ml of 30 percent NaOH were added, and the ammonia was distilled from the sample into five ml of four percent boric acid. The indicator was prepared as described by Sobel et al. (21). Ten ml of .03 percent methyl red were mixed with 1.0 ml of .08 percent methyl blue. The solutions were titrated against standard H_2SO_4 and in all cases blanks and standards were run simultaneously with the samples. The standard contained 0.6082 mg

of nitrogen per ml and it, too, was diluted one to ten and run through the entire procedure. The amount of nitrogen was calculated as follows:

$$\frac{\text{-ml of acid for sample}}{\text{ml of acid for standard}} \times 0.6082 \text{ mg } N_2/\text{ml} = \text{mg } N_2/\text{ml}$$

The phosphorus content of the samples was determined colorimetrically as described by the method of Fiske and Subbarow as described in Hawk et al. (11), p. 583. One ml of the sample was diluted to ten ml with borate-citrate buffer and two ml aliquots were transferred to Kjeldahl flasks. Two and one-half ml of 5N H_2SO_4 were added to each flask and the solutions were then heated to blackness, cooled and decolorized with 30 percent H_2O_2. The solutions were heated again to expell excess H_2O_2. Five ml of water were added and the solutions boiled for approximately five minutes. These solutions were then cooled, transferred to 25 ml volumetric flasks together with 2.5 ml of ammonium molybdate and one ml of one, two, four amino napthol sulfonic acid. The resulting solution was diluted to volume with water, allowed to stand for five minutes. The amount of phosphorus was determined in the Klett-Sommerson Colorimeter using filter No. 640. The phosphorus standard contained 0.08 mg phosphorus per ml of solution. In each case the standard was treated in the same manner as the samples and the calculations made as follows:

$$\frac{0.08}{\text{reading for standard}} = \frac{X}{\text{reading for sample}} \quad X = \text{mg P/ml sample}$$

The total radioactive phosphorus was determined by pipetting 0.1 ml of the yolk solution on to metal discs and drying them in a hood under the heat lamp. The supernatant and the original

fresh yolk were determined separately. The precipitate was counted in like manner after it was redissolved. All samples were assayed in the end window Berkeley Scaler Model 2001. For the radioactive phosphorus content in the starch gels approximately 0.1 ml of the radioactive yolk was introduced in the gel slit at the beginning of the electrophoresis analysis and the electrophoresis allowed to proceed for 40 hours. At the completion of the run one half of the gel was stained with bromphenol blue to mark the bands and the other half was sliced to coincide with these bands. The unstained slices of the gel were to decrease the thickness of the slice, measured with a millimeter rule and counted for radioactive phosphorus content in a Berkeley Scaler.

RESULTS AND DISCUSSION

In the present work attempts were made to separate the fast moving, high phosphorus-containing fraction of egg yolk from the egg solution by means of ethanol fractionation and a salt precipitation procedure, essentially that used by Mecham and Olcott, (16). In addition electrophoresis was used in attempts to separate the yolk components and was also used as an analytical tool for the fractions obtained from the salt precipitation procedure.

Ethanol Fractionation

Preliminary experiments were carried out to observe the effect of ethanol on the total yolk components. For example,

the yolk solutions prepared as described in the section on Preparation of Yolk Solutions and dialyzed against borate citrate buffer containing ten percent ethanol by volume for 24 hours. At the end of this 24 hour period a precipitate was visible. This precipitate was not separated from the supernatant, but the cellophane sack and contents were dialyzed against a solution of ten percent sodium chloride-1.5 percent glycine for two to three days in order to redissolve the precipitate. The resulting mixture was transferred to a fresh solution of the original buffer for approximately one day, after which the solution was suitable for electrophoretic analysis. Figure 1. of Plate III is a representative pattern of the ascending boundary of whole fresh yolk in borate-citrate buffer and Figure 2. of Plate III is the pattern of the whole yolk which was precipitated with ten percent ethanol and redissolved in sodium chloride-glycine. Note that although both Figure 1. and Figure 2. of Plate III have a fast moving peak, the distribution of the remainder of the pattern has been changed. For example, the separation of the second peak (B of Figure 2) and the third peak (C of Figure 2) was more clearly defined than the same peak in Figure 1. In addition, the complextion of the pattern between peaks C and the salt boundary has changed considerably. In general the components in the ethanol treated system moved farther than did the components of the untreated system during the same period of time. In spite of these differences both patterns exhibit fast moving peaks and the ethanol experiments were carried out in attempts to separate this peak from the remainder of the pattern.

EXPLANATION OF PLATE III

Fig. 1. Ascending pattern of whole yolk in borate-citrate buffer run for period of 300 minutes.

Fig. 2. Ascending pattern of yolk precipitated with ten percent ethanol redissolved in sodium chloride glycine solution and analyzed electrophoretically for 300 minutes employing borate-citrate buffer.

PLATE III

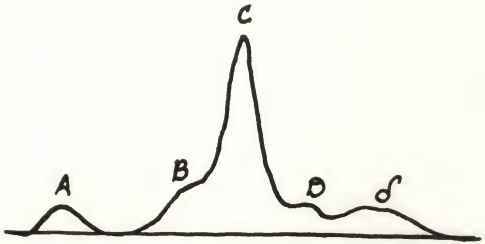


Fig. 1.

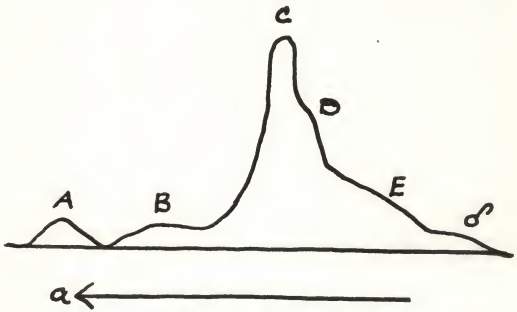


Fig. 2.

Redissolving the precipitate formed during the ethanol treatment was a major problem and deserves special mention. Attempts to redissolve the precipitate in the various buffers employed were unsuccessful. After many combinations were tried, it was discovered that, in some cases, it was possible to redissolve the precipitate by dialysis against a solution of 10 percent sodium chloride-15 percent glycine before dialysis against the buffer employed in the electrophoresis. This method of dissolving the precipitate was employed in all subsequent separations involving ethanol.

After the yolks were processed to obtain clear solutions as described in section on preparation of egg yolk solutions, these solutions were introduced into cellophane sacks and allowed to dialyze against various concentrations of ethanol in borate-citrate buffer for 24 hours at a temperature of 5-7° C. No attempts were made to adjust the ethanol concentration. Approximately 40 ml of the yolk solution were dialyzed against 1000 ml of the buffer-ethanol mixture. This means that the resulting ethanol concentration was slightly less than that recorded below, but for convenience the ethanol concentration was recorded as the concentration of the original ethanol-buffer mixture. At the completion of the dialysis the mixture in the cellophane sacks was transferred to centrifuge tubes and centrifuged for 15 minutes at a temperature of 5-7° C. The supernatant was decanted and dialyzed against one of the buffer solutions. The precipitate was transferred to a dialysis sack and in each case was dialyzed for three to four days against a solution of 10 percent sodium chloride-15

percent glycine so that this precipitate would redissolve.

It was not practical to explore all concentrations of ethanol and in this work one percent, 5 percent, 10 percent, 15 percent ethanol solutions were employed. When yolk solutions contained in glycine-phosphate buffer (pH 9.4) were dialyzed against a glycine-phosphate buffer containing one percent ethanol (by volume) for a period of two to three days, no precipitate occurred; therefore, fresh yolk solutions were prepared and dialyzed against a glycine-phosphate buffer adjusted to pH's of 4, 5, 6, 7, 7.5 and 8.5. These solutions were then transferred to the buffer solution containing one percent ethanol. A precipitate appeared at pH seven or below, but the yolk remained in solution at pH 7.5 and above. The small amount of precipitate which resulted in these runs never redissolved when dialyzed for long periods of time against a 10 percent sodium chloride-15 percent glycine solution and the supernatant remained so cloudy that it could not be analyzed electrophoretically.

Experience has shown that yolk mixtures which have been dialyzed against borate-citrate buffer produce clearer solutions for electrophoretic work, and they did not present a problem in determining phosphorus and nitrogen; therefore, this buffer was employed in the following work, instead of the glycine-phosphate buffer.

Yolk solutions were dialyzed against five percent ethanol solution in 95 percent borate-citrate buffer at pH 7.5 for a period of two or three days, and no precipitate occurred. Yolk solutions were allowed to dialyze against borate-citrate buffers varying from pH four to nine for a period of 24 hours and then

transferred to the 5 percent ethanol solution and 95 percent borate-citrate buffer. The yolk solutions which had previously dialyzed against borate-citrate of pH four precipitated. The yolk solutions which had dialyzed against borate-citrate buffer of pH six became cloudy and the others showed no change. The supernatant from this 5 percent ethanol precipitation procedure was dialyzed against borate-citrate for 24 hours and then analyzed electrophoretically using the Aminco electrophoresis apparatus. The ascending pattern is shown in Figure 2. of Plate IV, for comparison Figure 1. of Plate IV is the pattern of the 10 percent ethanol treated sample, that was redissolved and analyzed employing the borate-citrate buffer. The precipitate from this procedure did not redissolve in 10 percent sodium chloride-15 percent glycine solution; therefore, no electrophoretic analysis was possible.

Fresh yolk solutions which had been dialyzed against a borate-citrate buffer pH 7.5 were transferred to a borate-citrate buffer of the same pH containing 10 percent ethanol by volume, and allowed to dialyze against this solution for 24 hours. At the end of this time a considerable amount of precipitate was apparent. The yolks were centrifuged in the cold and the supernatant was decanted and dialyzed against a fresh solution of the borate-citrate buffer pH 7.5. The precipitate was allowed to redissolve in a solution of 10 percent sodium chloride-15 percent glycine. When the supernatant from the 10 percent ethanol precipitation had dialyzed for 24 to 36 hours, it was analyzed electrophoretically and the ascending pattern is shown in Figure 3. of Plate IV. The precipitate from this procedure was suitable for electrophoretic analysis

EXPLANATION OF PLATE IV

Fig. 1. Ascending pattern of whole yolk after ethanol treatment.

Fig. 2. Ascending pattern of supernatant from five percent ethanol treatment.

Fig. 3. Ascending pattern of supernatant from ten percent ethanol precipitated sample.

Fig. 4. Ascending pattern of precipitate (redissolved) from ten percent ethanol precipitate sample.

Fig. 5. Ascending pattern of supernatant from 15 percent ethanol precipitated sample.

Fig. 6. Ascending pattern of precipitate (redissolved) from 15 percent ethanol precipitated sample.

All of these patterns were analyzed in borate-citrate buffer for a period of 300 minutes.

PLATE IV

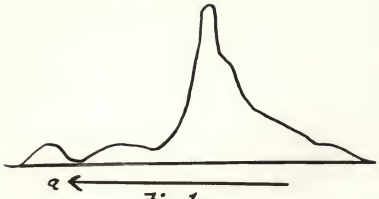


Fig. 1.

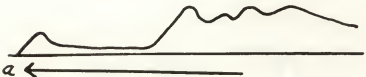


Fig. 2

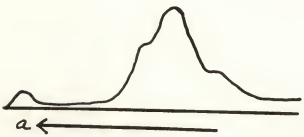


Fig. 3

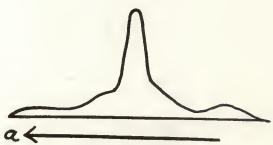


Fig. 4.

Fig. 5.

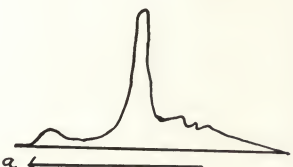


Fig. 6.

after three to four days dialysis against the solution of 10 percent sodium chloride-15 percent glycine. This ascending pattern is shown in Figure 4. of Plate IV. In the 10 percent ethanol precipitation procedure which was employed, a precipitate resulted when dialyzed against a solution of any pH from four to nine; however, for this series only the supernatant and precipitate from the pH 7.5 area were employed. Previous experiments did not appear to warrant further investigation at this point. Three concentrations of ethanol (1 percent, 5 percent, 10 percent) were employed and in every experiment the 10 percent concentration produced considerably more precipitate.

In another series of experiments a buffer solution of pH 7.5 containing 15 percent ethanol by volume was prepared and yolk solutions which had been dialyzed against borate-citrate buffer were placed in this ethanol buffer solution for 24 hours. The mixture was then centrifuged and the supernatant was allowed to dialyze directly against a fresh borate-citrate buffer. The precipitate was allowed to redissolve in the solution of 10 percent sodium chloride-15 percent glycine and then dialyzed against the same buffer. The electrophoretic ascending pattern of the supernatant is shown in Figure 5. of Plate IV. It required about three to four days of dialysis to redissolve the precipitate. After it was redissolved it was also analyzed electrophoretically. The ascending pattern Figure 6. of Plate IV shows more of the protein material appears in the precipitate than in the supernatant.

These attempts at ethanol fractionation did not produce components which were essentially homogeneous, nor was the fast moving

fraction normally present in fresh yolk preparations, successfully separated. In most cases, it was extremely difficult to redissolve the precipitated protein, which was necessary in order to obtain a solution clear enough for electrophoretic analysis. It also appeared that ethanol treatment of the yolk proteins affects the movement of the components and the electrophoretic distribution of the resulting material. The fractions were observed to move faster after treatment with ethanol and in all cases there was a large percentage loss in total nitrogen and phosphorus (see appendix). The increase in solubility of certain proteins upon the addition of salt has been known for some time. A better ethanol precipitation procedure may have resulted if the buffer employed was of a lower salt concentration. However, it has been shown that a decrease in the concentration of these buffers presents the problem of the egg yolk proteins precipitating even if immiscible solvents are excluded. All these factors contributed to the decision to discontinue further attempts at ethanol fractionation and to explore means of fractionating yolk solutions employing salt precipitation and electrophoretic separation.

Salt Fractionation

The phosphorus distribution in the electrophoretic components of fresh egg yolk has been reported by Clegg et al. (6). In the present work efforts were directed toward separating the fast-moving, high phosphorus-containing fraction from these yolk preparations. Mecham and Olcott (16) reported in 1948 that $MgSO_4$ precipitated materials from egg yolk which contained a large percentage

of phosphorus. These investigators extracted a phosphoprotein from this precipitated material which they called "Phosvitin". Since by their conclusion "phosvitin" contained almost 60 percent of the phosphorus of egg yolk, $MgSO_4$ as a precipitating agent was suggested for use in the present work for separating the fast-moving, high phosphorus-containing fraction which Clegg et al (6) had reported.

In this series of experiments, yolk solutions were prepared as described in the section of the preparation of yolk solutions, and were then dialyzed against glycine-phosphate buffer for two to three days. These solutions were then dialyzed against a solution of 0.6 M $MgSO_4$ for a period of 24 hours. The resulting mixture was carried through the same procedure as outlined on page 13 in Precipitation Procedures. The supernatant and the precipitate were then prepared for electrophoretic analysis.

Previous experiments had shown that yolks which had been dialyzed against borate-citrate buffer gave a clearer solution for electrophoretic work than those which had been dialyzed against the glycine-phosphate buffer. However, in this salt precipitation procedure, two problems had to be solved before the solutions were ready for electrophoresis.

When the precipitate from this procedure was redissolved in 10 percent sodium chloride-15 percent glycine and allowed to dialyze directly against the borate-citrate buffer the solutions always became very turbid and in some cases reprecipitated. This difficulty was not encountered if the redissolved precipitate was allowed to dialyze against the glycine-phosphate buffer and later

transferred to the borate-citrate buffer for dialysis prior to electrophoresis.

Attempts to precipitate yolk solutions which had previously been dialyzing against borate-citrate presented another problem. For example, if yolk solutions were transferred from the borate-citrate buffer (pH 7.5) directly to the 0.6 M $MgSO_4$ and allowed to dialyze for 24 hours, no precipitate appeared. Since the pH of the buffer was thought to be a factor, buffers were adjusted to different pH values from 6.0 to 8.5 and yolk solutions were allowed to dialyze against these buffers for a period of two to three days and then transferred to the 0.6 M $MgSO_4$ solutions for 24 hours. At the end of this time none of these solutions precipitated.

Since the yolk solutions which had previously dialyzed against the borate-citrate buffer would not precipitate in $MgSO_4$, the glycine-phosphate buffer was employed for dialysis prior to precipitation with the $MgSO_4$.

In brief, the borate-citrate buffer gave solutions which were better for electrophoretic analysis, but no precipitation occurred when these solutions were dialyzed against $MgSO_4$. The glycine-phosphate buffer solutions were not well suited to electrophoretic analysis, but did give a precipitate when dialyzed against $MgSO_4$ for a period of 24 hours. Therefore, in all experiments, the yolk solutions were dialyzed against glycine-phosphate (pH 9.4) for two to three days prior to dialysis against the 0.6 M $MgSO_4$. These solutions were then transferred to the $MgSO_4$ solution, precipitated in a 24 hour period, and then centrifuged.

The resulting supernatant was dialyzed directly against borate-citrate for two to three days and then analyzed electrophoretically on the Aminco type apparatus. The ascending pattern is shown in Figure 2. of Plate V. The precipitate from this procedure was redissolved in a solution of 10 percent sodium chloride-15 percent glycine and then transferred to the glycine-phosphate buffer for 24 hours. The precipitate was then allowed to dialyze against borate-citrate in preparation of the electrophoretic analysis. The ascending pattern is shown in Figure 3. of Plate V.

Olcott and Mecham in their work precipitated the yolk prior to ether extraction. Therefore, in order to present a good comparison, the yolks were mixed with a solution of the glycine-phosphate buffer (pH 9.4) instead of mixing with 15 percent glycine as described by Clegg et al. (6). This resulting mixture was then treated with 0.6 M $MgSO_4$ to precipitate the solution and the mixture was then centrifuged. Both the precipitate and the supernatant were extracted with ether. The precipitate resulting from this procedure required three to four days of dialysis against a solution of 10 percent sodium chloride-15 percent glycine before it dissolved. Upon redissolving it was dialyzed for 36 hours against the glycine-phosphate buffer and then transferred to the borate-citrate buffer for 24 hours to electrophoresis. The ascending pattern of the electrophoretic pattern is shown in Figure 2. of Plate VI. After the supernatant was ether extracted it was dialyzed against the borate-citrate buffer for 24 hours and analyzed electrophoretically. The ascending pattern is shown in Figure 1. of Plate VI.

EXPLANATION OF PLATE V

Fig. 1. Ascending pattern of whole yolk.

Fig. 2. Ascending pattern of supernatant from $MgSO_4$ precipitated sample.

Fig. 3. Ascending pattern of precipitate resulting from $MgSO_4$ precipitated sample.

All three patterns were run for 300 minutes employing borate-citrate buffer.

PLATE V

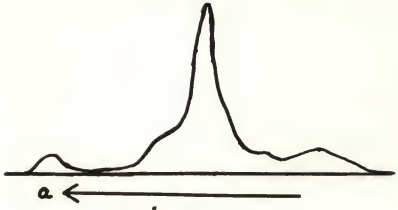


Fig. 1.

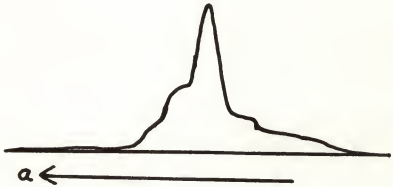


Fig. 2.

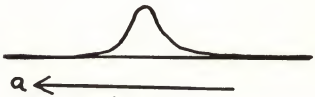


Fig. 3.

EXPLANATION OF PLATE VI

Fig. 1. Ascending electrophoretic pattern of supernatant from sample precipitated with $MgSO_4$ prior to ether extraction.

Fig. 2. Ascending pattern of precipitate (redissolved) from sample precipitate with $MgSO_4$ prior to ether extraction.

Each run for 300 minutes employing the borate-citrate buffer.

PLATE VI

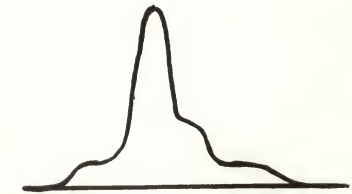


Fig. 1.

Fig. 2.

Note that the patterns are essentially the same whether the yolks were ether extracted before precipitation or precipitated and later extracted with ether. The procedure in which the yolks were mixed with glycine-phosphate buffer and then ether extracted was so time consuming that the remaining work employed yolks that were extracted by the usual method prior to precipitation.

The analytical results of the phosphorus content of the supernatant and precipitate are shown in Table 1. Radioassay was employed to determine the amount of phosphorus in these fractions. Radioactive eggs were obtained as described in the section on Production of Radioactive Eggs, and were processed by the same procedure as normal eggs. In employing the P^{32} as an analytical tool, 0.1 ml of solution of each fraction was pipetted on to metal discs, dried under a heat lamp, and counted in the end window Berkeley Scaler Model 2001. The results of the radioassay shows that a very high percentage of the phosphorus was found in the precipitate. According to these results the amount of P^{32} in the precipitate ranged from 70 to 83.2 percent.

Table 1. Radioassay of yolk fractions.

Fraction	Counts/Min.	% Total
	(A)	
Whole Yolk	1707	100
Sup.	450	26.3
Ppt.	1405	83.2
	(B)	
Whole Yolk	1440	100
Sup.	367	25
Ppt.	978	70

Table 1. Radioassay of yolk fractions. (concl.)

Fraction	Counts/Min.	% Total
	(C)	
Whole Yolk	1133	100
Sup.	100	11
Ppt.	872	77
	(D)	
Whole Yolk	1300	100
Sup.	----	---
Ppt.	998	77.3

Starch Gel Electrophoresis

The apparatus and trays that were employed in starch gel electrophoresis were illustrated in a previous section and the method of preparing starch gel has been described. Approximately 0.5 ml of yolk solution was introduced into the slits cut into the gel and the electrophoretic analysis was allowed to proceed for 40 hours at 240 volts and 20 m.a. In all starch gel electrophoreses the glycine-phosphate buffer was employed because yolk solutions in borate-citrate buffer moved too slowly for satisfactory work in these experiments. At the completion of the run the gel was removed from the trays and sliced lengthwise as shown in Plate VII. Each slice of the gel was placed in a solution of bromphenol blue and allowed to stain for five minutes. The excess stain was removed by washing with 0.5 percent solution of acetic acid, and the gels were placed in a fresh solution of the 0.5 percent acetic acid for two to six hours. At the end of the washing period protein bands were visible. A representative stained gel

is shown in Plate X, in the appendix. In each run there were three bromphenol blue-stained components and an additional white section which preceded these three components. There was a residual pale blue color in front of the white section which covered the entire starch gel. This residual blue was of a lesser shade than the bromphenol blue-stained bands. Electrophoresis runs were made of varying lengths of time to attempt to eliminate the blue color preceding the white section, but the blue always remained. Upon staining freshly prepared starch gel with bromphenol blue and washing with 0.5 percent acetic acid for 24 to 36 hours a residual blue remained on the starch gel although it had not been exposed to protein; therefore, the residual blue in actual runs could not be avoided. In attempts to stain the white section several dyes were employed: Sudan III, Amido Black and Nile Blue. When a starch gel which had been exposed to the egg yolk solution was left in a 0.1 percent Nile blue for 16 to 20 hours, a distinct purple color above the very dark blue background was evident. In confirming the fact that the white section was stained, a starch electrophoresis was run and the gel sliced into two sections; one was stained with bromphenol blue and the other with the 0.1 percent Nile blue. Upon comparing the position of the white section from the bromphenol blue slice to that stained with 0.1 percent Nile blue it was concluded that the white section was stained a darker color than the remainder of the starch gel.

When egg yolk solutions were precipitated with 0.6 M $MgSO_4$ and the supernatant and precipitate separated by centrifugation, the supernatant was dialyzed against glycine-phosphate for 24 to

EXPLANATION OF PLATE VII

Starch gel showing slicing procedure for purpose of staining in two sections.

PLATE VII

36 hours and then analyzed electrophoretically on starch gel. The white section was not present on the gel that was stained with bromphenol blue. This gel with the white section absent is shown as Figure 2. of Plate VIII. The precipitate from this same procedure was mixed with a 15 percent glycine solution and dialyzed for three to four days against a solution of 15 percent glycine-10 percent sodium chloride to redissolve it. Upon redissolving, the precipitate was dialyzed against the glycine phosphate buffer for one day and then run on the starch gel for 40 hours. At the end of the run no blue protein bands were visible when the gel was stained with bromphenol blue, but the white was present and had moved the same distance as it had previously when whole yolk was analyzed on the starch gel. This picture of the white section only is Figure 3. of Plate VIII. Figure 1. of Plate VIII is whole yolk in starch gel which is shown for comparison.

In determining the amount of phosphorus associated with each band, starch gels were set up as before and approximately 0.5 ml of radioactive yolk solution was introduced into the gel slit and allowed to proceed for 40 hours. At the completion of the run, one-half of the gel was stained with bromphenol blue and the other half was placed beside this stained gel and the unstained gel was sliced (crosswise) to coincide with the bromphenol blue-stained bands. Figure 4. of Plate VIII, shows how the gels were sliced for this radio-assay. These starch slices were dried at room temperature and measured with a millimeter rule as accurately as possible to determine the area. These starch gels were then placed in metal discs and assayed in a Berkeley Scaler Model No.

EXPLANATION OF PLATE VIII

Fig. 1. Whole yolk in starch gel stained with bromphenol blue.

Fig. 2. Supernatant only from $MgSO_4$ precipitated sample.

Fig. 3. Precipitate only from $MgSO_4$ precipitated sample.

Fig. 4. A normal run showing divisions for cutting for radioassay each run for 40 hours at 240 volts, 20 m.a. employing glycine phosphate buffer.

PLATE VIII

Fig. 1.

Fig. 2.

Fig. 3.

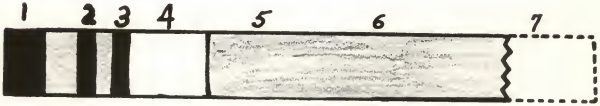


Fig. 4.

2001. The table of results is shown in Table 2. In proportion to the amount of area the leading component (white section) contained considerably more phosphorus than any other section that was assayed. This is additional evidence that a large percentage of phosphorus is present in a band which does not stain normally with bromphenol blue. The fact that this portion takes the Nile blue stain is evidence that it may contain some fatty material.

Table 2. Distribution of radioactivity in starch gel slices.

Section	Area MM^3	Counts/min.
	(A)	
1	144	85
2	144	94
3	144	77
4	144	214
5	144	15
	(B)	
1	156	110
2	156	67
3	156	68
4	156	160
5	156	19
	(C)	
1	180	102
2	180	83
3	180	78
4	180	186
5	180	23

Preliminary Experiments with Modification of Techniques

As further preliminary experiments in different phases of electrophoresis, filter paper, starch granules in trays and starch granule columns were employed as supporting media. Although none

of these gave satisfactory results, they are reported to point out to future investigators the difficulties which were encountered.

Filter paper electrophoresis has been employed by several investigators for the separation of protein material. Durrum et al (7) in 1951, used the hanging curtain continuous flow type electrophoresis for the separation of components of human blood serum. In moving boundary electrophoresis a satisfactory separation of chicken serum is effected in about one hour. Since yolk components separate in about one hour employing the bicarbonate buffer on Schliern type electrophoresis, this buffer was employed in the exploratory filter paper electrophoresis.

In attempts to separate egg yolk components, the apparatus as illustrated in Plate III was used. In this procedure, as has been noted in explanation, the bicarbonate buffer flows continuously. In these experiments, several runs were made for 72 hours at 20 m.a. In order to be able to observe the separation visually, bromphenol blue dye was mixed with the sample 24 hours prior to the run and the excess dye was removed by dialysis against fresh buffer. A picture of a typical run is shown in Plate IX. Note that in this and all other runs the yolk components moved down the curtain in a narrow, slightly curved path, rather than a full separation. Since the pattern is shown to contain very concentrated protein components, it is believed that when the components move down the curtain, they come in contact with the filter paper and that these components are adsorbed thus preventing a satisfactory separation. This may be confirmed by running a straight filter paper electrophoresis. In this case most of the substance remained at the point of application.

EXPLANATION OF PLATE IX

Electrophoresis run on yolk on Filter Paper. For period of 72 hours, 20 m.a. employing the Bicarbonate Buffer.

PLATE IX

In another phase of preliminary work, starch granules were tried as a supporting medium for electrophoresis work. The starch was introduced into trays, which have been illustrated in Plate I, and egg yolk samples added to a very narrow slit. In all these runs a glycine-phosphate buffer was employed primarily because the components moved extremely slowly in the borate-citrate buffer. In this type electrophoresis it was observed that the components did migrate; however, no satisfactory method of staining was found and the bands could not be observed. When thin strips of filter paper were pressed on top of the starch column in order to get an imprint of the protein bands, dried in an oven and stained with bromphenol blue, it was observed that most of the components had diffused. No satisfactory results were obtained with this procedure.

Starch granules in a vertical column were also used for electrophoresis. The column consisted of a cellophane sack with firmly packed starch granules connected to the buffer reservoir by means of a polyethylene tube. The major problem in this procedure was diffusion of the components and the drying of the cellophane tubing. This drying was overcome by greasing the outside of the cellophane sack. In all runs, the proteins migrated faster on one side of the column than on the other; therefore, no complete bands were obtained.

In conclusion, it may be stated that of the procedures investigated, salt precipitation gave the best results. The $MgSO_4$ did precipitate a fraction that could be redissolved and was essentially electrophoretically homogeneous at the pH employed. The

resulting supernatant showed the absence of the normal fast moving peak of the whole yolk preparations. The precipitated fraction contained approximately 80 percent of the phosphorus of the yolk as measured by means of radionietric methods.

Although the salt precipitation procedure gave a separation, an attempt was made to employ ethanol as a fractionating agent. These experiments with ethanol were discontinued because the fractions which resulted were never electrophoretically homogeneous and the ethanol treatment appeared to change the electrophoretic distribution of the yolk components.

Redissolving the precipitate was one of the major problems in this investigation. The precipitates from the ethanol procedure and the salt fractionation method were very difficult to dissolve. In the case of precipitates from the 10 percent ethanol and the 15 percent ethanol procedure, dialysis for two to three days against a solution of 10 percent sodium chloride-15 percent glycine did redissolve the precipitate. The precipitate resulting from the salt fractionation procedure required a longer period of dialysis for complete solution. In every case the precipitate was mixed with a solution of the 15 percent glycine to facilitate the dissolving. Even after the precipitate was redissolved, it would reprecipitate if it was allowed to dialyze directly against the borate-citrate buffer; however, if it was dialyzed against the glycine-phosphate buffer for 24 to 36 hours and then transferred to the borate citrate buffer it gave a clear solution. A period of approximately ten days elasped from the time the yolk was obtained until the precipitate from this

procedure was clear enough for the last stage of investigation, which was the electrophoretic analysis. The above sequence was the result of trial and error and accounted for much of the time spent on this investigation.

For additional electrophoretic analysis of the supernatant and the precipitate from the salt precipitation procedure, starch gel was employed as the supporting medium. In analyzing whole yolk on starch gel, it was evident that four fractions were present, a leading fraction that washed out white when stained with bromphenol blue and three additional bands that stained blue with this dye. When the supernatant was analyzed, the white band was not present, but when the precipitate was run in the gel the white band was the only band visible. In later experiments, this band was successfully stained with Nile blue.

The use of filter paper as a supporting medium for electrophoretic analysis was unsatisfactory because of the extensive adsorption of the yolk components on the filter paper, starch granules in cellophane sacks as columns for electrophoresis always produced very diffused bands, that moved faster on one side of the column than on the other. An earlier problem of the sacks drying out was solved by applying a thin film of grease over the outside of the cellophane sack. Starch granules in trays were used, but as in the case of the starch columns the bands were quite diffused.

SUMMARY

Several methods for the separation of the fast moving high phosphorus containing fraction of egg yolk were investigated.

Ethanol fractionation proved unsatisfactory because the fractions which resulted were not electrophoretically homogeneous, and the ethanol treatment appeared to change the electrophoretic distribution of the yolk components.

Salt precipitation employing $MgSO_4$ did eliminate the fast-moving fraction on the normal yolk pattern. A method of redissolving the precipitate as developed and satisfactory electrophoretic patterns of both the precipitate and supernatant were obtained employing the moving boundary type electrophoresis. Analytical results using radioactive eggs showed that between 77 percent and 83 percent of the phosphorus was associated with the precipitate.

Starch gel was employed as a supporting medium for the electrophoretic analysis as the precipitate and the supernatant from the salt fractionation procedure. The supernatant gave three bands that stained with bromphenol blue dye. The precipitate produced a band that washed out white when bromphenol blue was employed as the stain. This white section had moved the same relative distance for the same period of time of electrophoresis as the leading white band in the electrophoretic analysis of whole yolk on starch gel. After several attempts with different stains, the white band was successfully stained with Nile blue. Analytical results employing radioactive yolk on the starch gels showed that a relatively large amount of phosphorus was associated with this leading white band.

Although preliminary electrophoretic experiments were carried out employing starch granules in trays, filter paper, and starch

granules in cellophane columns, none of these produced very satisfactory results.

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APPENDIX

Table 3. Nitrogen and phosphorus analysis of fractions from 10 percent and 15 percent alcohol solutions.

Composition	Nitrogen	Phosphorus	Phosphorus-Nitrogen Ratio
	I		
Whole Yolk	3.666	.3540	.0965
10% Precipitate	.8872	.0883	.0995
10% Supernatant	1.1814	.1314	.1112
15% Precipitate	1.3832	.1390	.1005
15% Supernatant	.5472	.0780	.1425
	II		
Whole Yolk	1.6907	.3051	.1823
10% Precipitate	.6603	.0824	.1247
10% Supernatant	.7078	.1062	.1500
Whole Yolk	2.2250	.3239	.1439
15% Precipitate	1.3371	.1386	.1036
15% Supernatant	.4712	.0758	.1608
	III		
Whole Yolk	2.2508	.2703	.1200
10% Precipitate	1.0959	.1565	.1425
10% Supernatant	1.0855	.1607	.1481
Whole Yolk	2.6260	.2787	.1610
15% Precipitate	1.3996	.2331	.1667
15% Supernatant	.8253	.1369	.1659
	IV		
Whole Yolk	2.3644	.2865	.1211
10% Precipitate	.9266	.1489	.1609
10% Supernatant	1.2183	.1931	.1586
Whole Yolk	2.4374	.3127	.1285
15% Precipitate	1.9904	.2312	.1172
15% Supernatant	.7421	.1290	.1740

EXPLANATION OF PLATE X

Actual photograph of yolk run on starch gel as contrasted to previous diagrams. Forty hour run employing glycine-phosphate buffer.

PLATE X

THE SEPARATION OF THE HIGH-PHOSPHORUS, FAST-MOVING COMPONENT OF EGG YOLK

BY

HERMAN THOMAS MILLER

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Two methods were employed to separate the high-phosphorus, fast-moving component of egg yolk. Salt fractionation and ethanol precipitation were attempted and starch gel electrophoresis was used to analyze the resulting fractions.

Ethanol solutions of 1, 5, 10, and 15 percent were used. The 1 percent gave only a cloudy solution of the yolk components. Employing 5 percent ethanol, a precipitate resulted, but no means of redissolving this precipitate were found; therefore, only the supernatant was analyzed electrophoretically. The 10 percent and 15 percent ethanol solutions gave precipitates which were redissolved with a solution of 10 percent sodium chloride-15 percent glycine. The precipitates and the supernatants from both concentrations were analyzed electrophoretically.

The experiments with ethanol were discontinued because no fraction was obtained which was electrophoretically homogeneous and the ethanol seemed to change the distribution of the electrophoretic components.

Salt fractionation employing $MgSO_4$ did precipitate the high-phosphorus component of egg yolk, and the precipitate contained between 77 and 83 percent of the phosphorus of the yolk. Starch gels were employed to further analyze the precipitate and supernatant. The precipitate contained a fraction that could not be stained with bromphenol blue dye. However, a solution of Nile Blue stain dyed this band a very dark blue, which indicates that this fraction may contain some fatty material. The supernatant contained three fractions which stained with the bromphenol blue stain.

Radioactive yolk was run in the starch gels after which the gels were sliced and the individual bands were assayed for P^{32}. The leading band contained considerably more phosphorus than any other band. Since this leading band on the gel contained most of the phosphorus, the leading peak was absent from the electrophoretic pattern of the supernatant and the precipitate contained 77-83 percent of the yolk phosphorus, it was concluded that the fast-moving, high-phosphorus component of egg yolk was separated.