FRACTIONATION OF COMPONENT 1 (PREALBUMIN) FROM DIETHYLSTILBESTROL-INJECTED COCKEREL SERUM

by

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INTRODUCTION

The increase in calcium, phosphorus, and total protein of laying hen and diethylstilbestrol-injected cockerel serum associated with the appearance of electrophoretic components 1 and 6 has been noted by several workers. A relationship between egg formation and these electrophoretic components has been proposed.

Since a considerable amount of these components may be removed by means of ether extraction, these substances should provide suitable material for the investigation of the chemical structure of phospholipoprotein. Attempts have been made to purify these fractions but these attempts have not been altogether successful.

This investigation was initiated to design methods for fractionating component 1 from the "Mixed Fraction" and "Bottom Fraction" of diethylstilbestrol-injected cockerel and laying hen serum using a combination of preparatory ultracentrifugation, salt fractionation, preparatory column electrophoresis, and moving boundary electrophoresis.

LITERATURE REVIEW

The increase in the calcium, phosphorus, and total protein of chicken sera during egg production has been reported by several workers (1,14,17,18,33). Hughes, Titus, and Smits (18) and others showed that the calcium content of the blood serum of hens during time of egg production was two to three times as

high as the serum calcium values in non-laying hens.

Calcium exists in blood serum in two forms: filtrable and non-filtrable calcium. By the method of Nicholas (32), Correl and Hughes (9) were able to show that in the laying hen, the filtrable calcium remains constant regardless of the great increase in total calcium content. This was confirmed later by McDonald and Riddle (25).

In the serum of cockerels and non-laying hens, the total phosphorus was essentially equal to that of the lipid and acid soluble fractions. On the other hand, Roepke and Hughes (33) found that the total phosphorus in the sera of laying hens was much greater than the sum of the lipoid and acid soluble fraction, and they concluded that this extra phosphoprotein possessed properties similar to those of vitellin. This was demonstrated simultaneously and independently by Laskowski (20).

In 1938, Laskowski (21) observed the appearance of a serum-vitellin in the blood of non-laying hens injected with estrogen, and noted that this serum-vitellin contained much of the extra phosphorus. Studying the changes in calcium, phosphorus, and nitrogen content of pigeon plasma during the reproductive cycle or after the injection of estrogens, McDonald and Riddle (25) showed that there were no significant differences in either filtrable calcium, filtrable inorganic phosphorus, or non-protein nitrogen. However, non-filtrable calcium, non-filtrable inorganic phosphorus, lipid phosphorus, and protein phosphorus increased markedly during the period of egg production. Similar

and even greater increases resulted from the administration of estrogen in normal pigeons. A small increase in the plasma protein nitrogen was also noted under these conditions.

Differences in the electrophoretic patterns of hen, cockerel, laying hen, and estrogen-injected cockerel sera have been demonstrated by Deutsch and Goodloe (10) and Moore (31). A fast-moving fraction, component 1, moving ahead of the albumin, in the pattern of the laying hens, was noted. Further study by Brandt, Clegg, and Andrews (2) on the comparison of the electrophoretic pattern of the non-laying adult hen and adult rooster with that of the laying hen demonstrated the appearance of this component at the start of egg production and indicated a possible relationship to egg formation. A remarkable similarity between the electrophoretic pattern of the serum proteins of the diethylstilbestrol-injected cockerel and that of the laying hen was shown by Clegg et al. (8). The appearance of fast-moving component 1 and the increase in size of the slower-moving component 6 indicated that the injection of diethylstilbestrol will cause changes associated with egg production in the serum proteins of male birds. The appearance of these electrophoretic components in the serum of the laying hen and the diethylstilbestrol-treated cockerel was paralleled by variations in other serum components.

By using a method for measuring the P^{32} distribution in the various components of protein mixture, Clegg and Hein (7) found that electrophoretic components 1 and 6 of the laying hen and

diethylstilbestrol-injected rooster blood serum exhibited a very high phosphorus activity. McDonald and Riddle (25) showed that the calcium-combining capacity of the phosphoprotein, serum vitellin, was eight to nine times greater than that of other plasma proteins. By combination of ultrafiltrate and radiochemical techniques (32,15), Hein and Clegg (16) demonstrated a parallel between the rise of both total and non-filtrable calcium and components 1 and 6 in the sera of both laying and diethylstilbestrol-treated cockerels. Later, they (6) further indicated that the non-filtrable calcium was present in combination with the fast-moving high phosphorus-containing component 1 by showing that the presence of calcium ion in the buffer reduced drastically the mobility of the prealbumin component in the laying hen and diethylstilbestrol-injected cockerel sera so that it migrated in the area considered as β-globulin instead of the area faster than albumin.

Moore (31) found that the ether extraction method of McFarlane (26) removed the extra or enlarged components found in the sera of laying and hormone-treated birds. Ericson (11) also showed that the electrophoretic pattern of cockerel blood sera was not affected by extraction with cold ether, while the phosphorus content and the amount of prealbumin and globulin in the serum from diethylstilbestrol-injected roosters were greatly reduced by ether extraction. He also evaluated the lipid and phosphorus contents of serum samples of laying hens, diethylstilbestrol-injected roosters, and cockerels before and after

ether extraction and indicated that those components that increase in amount or appear in the diethylstilbestrol-injected chicken serum contained a high amount of total phosphorus and lipid and may be considered lipoproteins which bind the increased calcium so that the total diffusible calcium remained constant. According to Chargoff (5) phosphorus plays an important role in the binding of lipids to protein.

Isolation of the high lipid and phosphorus containing protein fractions has been performed. Laskowski (19) obtained a phosphoprotein fraction as a precipitate by dilution of laying hen plasma. McKinley et al. (28,29) also presented evidence for the occurrence of phosphoprotein in the material precipitated on dilution from the serum of estrogenized pullets. More recently, McIndoe (27) prepared the precipitable lipoprotein by dilution of laying hen plasma. This lipoprotein was constant in composition but examination in the analytical ultracentrifuge showed that there are two components, one a light lipoprotein and the other a dense lipoprotein. Using salt-fractionation, ultracentrifuge and preparatory electrophoresis, Misra (30) has purified component 6 to the extent that it is homogeneous to electrophoretic and ultracentrifuge analysis, although gradient ion exchange chromatography indicated a slight heterogeneity.

MATERIALS AND METHODS

Birds and Blood Samples

The serum used in this work was obtained from the blood of Leghorn hens or White Rock cockerels. They were from one to two years old and were fed a commercial chick starter or grower at the Kansas State University Poultry Farm.

In the case of the laying hens, non-laying hens and cockerels, approximately 10 ml of blood were taken from the wing-vein of each bird. The cockerels, treated with intramuscular injection of 3 mg of diethylstilbestrol in 0.2 ml propylene glycol daily for seven days, were sacrificed and the blood collected in 50 ml centrifuge tubes.

The blood was allowed to clot at 38° C for two to three hours and then centrifuged at approximately 3,000 r.p.m. for 20 minutes at 0° C. The serum was decanted and stored at 5 to 7° C.

Preparatory Ultracentrifuge

In this investigation ultracentrifugation techniques were employed in the initial isolation step to remove component 6. A Spinco Preparative Ultracentrifuge Model L, manufactured by Beckman Instrument, Inc., Spinco Division, Belmont, California was used.

The chicken blood serum, which had been stored overnight at 5 to 7° C before centrifugation, was placed into plastic tubes of 13 ml capacity. The tubes were filled completely and

capped tightly with aluminum caps. Three fractions were formed by centrifugation which was carried out for 16 hours under refrigeration, at 40,000 r.p.m. (rotor number 40).

These three fractions were "Top Fraction," "Middle Fraction," and "Bottom Fraction" as shown in Plate I in the Experimental and Results section (p.12). The "Top Fraction," as found by Misra (30), was essentially component 6. The "Middle Fraction" and "Bottom Fraction" were mixed and designated as "Mixed Fraction." When the "Bottom Fraction" only was desirable, the "Middle Fraction" was decanted and the red, semi-solid "Bottom Fraction" remained in the tubes.

Buffers

Borate chloride buffer at pH 8.6 and at pH 4.5, and borate chloride buffer containing calcium ions at pH 8.6 were those most extensively used in this investigation. The composition of the borate chloride buffer was: 0.05 M potassium chloride, 0.05 M boric acid, and 0.012 M sodium hydroxide. The pH was adjusted with hydrochloric acid and/or sodium hydroxide to values of 8.6 and 4.5, respectively. The buffer containing calcium was made as follows: 340 mg of calcium carbonate was dissolved in the borate chloride buffer by bringing the buffer to pH 3 with concentrated hydrochloric acid, and then the pH was adjusted to 8.6 with 30 per cent sodium hydroxide. For the purpose of separating component 1 from the diethylstilbestrol-injected cockerel serum components, the borate chloride buffer

at pH 8.6 was generally superior to the other buffer solutions commonly used in the electrophoretic investigation of serum protein (2).

Before electrophoresis, the serum sample was dissolved, diluted, and dialyzed against the suitable buffer. The borate chloride buffer containing calcium ion was used to reduce the mobility of the prealbumin component, component 1, as described by Clegg et al. (6), while the borate chloride buffer at pH 4.5 was employed to carry out salt fractionation.

Moving Boundary Electrophoresis

In moving boundary electrophoresis, the components are only partially separated, and therefore in this investigation moving boundary electrophoresis was used as an important criterium of purity for substances as well as a guide for separation by other procedures.

In this investigation the electrophoresis was conducted in an Aminco Portable Electrophoresis Apparatus, manufactured by the American Instrument Company, Inc., Silver Spring, Maryland, U.S.A. This technique has been described by McColloch (24), Stanley (37), Brandt (3), and Ericson (12). The highly sensitive optical method using refractive index observation was introduced to observe the migration of the boundaries and their resolution into several components. A cell capacity of 11 ml was employed for electrophoretic analysis of large samples of protein solution (5-10 ml).

Prior to electrophoresis, the sample was diluted with and dialyzed against the suitable buffer over night at 5 to 7° C to give the final concentration of approximately 1 per cent protein. After running the electrophoresis at 20 ma. for a desired period at 0° C, the ascending and descending boundaries were photographed with the Schlieren Lens System on Kodak Contrast Panchromatic Sheet Film.

The Aminco negatives were then developed in D-11 developer for two minutes, rinsed in Kodak Indicator Stop Bath about 30 seconds, then fixed 5 to 10 minutes with Kodak Acid Fixer and finally washed 20 to 30 minutes in running tap water. All steps were carried out at 20° C.

LKB Column Electrophoresis

Moving boundary electrophoresis is a suitable method for evaluating separation by other procedures, but for separation purposes, it cannot compete with zone electrophoresis, in which each component is allowed to form a zone separated from the others by empty regions. In order to stabilize those zones formed, the tube is packed with a stabilizing medium such as starch, glass wool, asbestos fibre, or glass powder.

A good supporting medium for zone electrophoresis should stabilize as large a volume of the liquid medium as possible, should not adsorb the sample substances, and should not give off any material to the solution. In addition, such a column must not contain foreign materials, must be strongly hydrophilic,

must not contain ionized groups, and be in a form such that uniform packing is possible.

Irreversible adsorption, especially of the basic proteins, has always been a problem in zone electrophoresis. Flodin et al. (13) has found that electrophoresis with vertical columns of cellulose powder not only did not have this trouble but also fulfilled those requirements mentioned above.

The column used here for the separation of the serum sample was a LKB column, electrophoresis type 3340, manufactured by LKB Produkter, Stockholm, Sweden. The source of cellulose powder was surgical cotton. Preparation of the cellulose powder, packing and operation of the column has been described by Misra (30).

After electrophoresis had been carried out for the desired period of time, the column was eluted with the same buffer as that used in packing the column. The eluted fractions were collected in an automatic fraction collector. The protein concentration of each fraction was determined in a Beckman spectrophotometer, model DU, at a wavelength of 280 millimicron.

EXPERIMENTAL AND RESULTS

The relationship between lipoprotein components 1 and 6, egg production by the laying hen, and diethylstilbestrol-treatment of the cockerel, has been demonstrated extensively (2,8,11,15,7,16,6,30,12).

By using ultracentrifugation techniques, Misra (30) purified component 6 from laying hen serum to the extent that it was

homogeneous to electrophoretic and ultracentrifuge analysis. He also found that the "Mixed Fraction" obtained after removing the ultracentrifuge "Top Fraction" indicated the presence of electrophoretic component 1 and a greatly reduced amount of component 6 ("Top Fraction").

Due to the great similarity between the electrophoretic pattern of the serum proteins of the diethylstilbestrol-injected cockerel and that of the laying hen, it was decided to use diethylstilbestrol-injected cockerel serum as the starting material for the preparation of component 1.

Preliminary Fractionation Using Ultracentrifuge

For the purpose of preliminary fractionation of serum obtained from the diethylstilbestrol-treated cockerel, ultracentrifuge technique was employed.

For each experiment six diethylstilbestrol-injected cockerels were sacrificed, the blood allowed to clot at 38° C for two to three hours, and about 200 ml of sera were prepared by mild centrifugation (3,000 r.p.m.) of the blood clots under refrigeration for 20 minutes. The serum was decanted and stored at 5 to 7° C overnight.

For centrifugation, the blood serum was put into plastic tubes of 13 ml capacity, completely filled and capped tightly with aluminum caps. The tubes were then placed in a number 40 rotor and the serum samples were centrifuged for 16 hours at 40,000 r.p.m. at 0° Centigrade.

After ultracentrifugation, these serum samples were observed to be separated into three main fractions as shown in the diagram (Plate I).

The "Top Fraction" was a creamy, pale yellow, semi-solid substance. The "Middle Fraction" was a very pale yellow solution. The "Bottom Fraction" was a sticky, semi-solid substance, red in color. The cockerel serum fractions obtained in this manner were very similar to those of the laying hen serum observed by Misra (30).

The "Top Fraction" was removed very carefully with a spatula while that remaining on the surface was removed by means of a syringe. The other fractions were mixed and designated as the "Mixed Fraction." When the "Bottom Fraction" only was desired, the "Middle Fraction" was decanted thoroughly and the "Bottom Fraction" remained in the tube.

"Mixed Fraction" of Diethylstilbestrol-injected Cockerel or Laying Hen Serum

Salt Fractionation and Moving Boundary Electrophoretic Analysis. It has been shown by Misra (30) that the mixed fraction of laying hen serum contained component 1 and a greatly reduced amount of component 6. An attempt was made to fractionate component 1 from the "Mixed Fraction" of serum obtained from diethylstilbestrol-treated cockerel by means of the combined techniques of salt fractionation and moving boundary electrophoresis. The procedures used are outlined in Table 1.

EXPLANATION OF PLATE I

A schematic diagram of diethylstilbestrol-injected cockerel serum fractions obtained upon centrifugation at 40,000 r.p.m. at 0° C for 16 hours in a Spinco Ultracentrifuge, Model L.

PLATE I

DIETHYLSTILBESTROL-INJECTED COCKEREL SERUM FRACTIONS

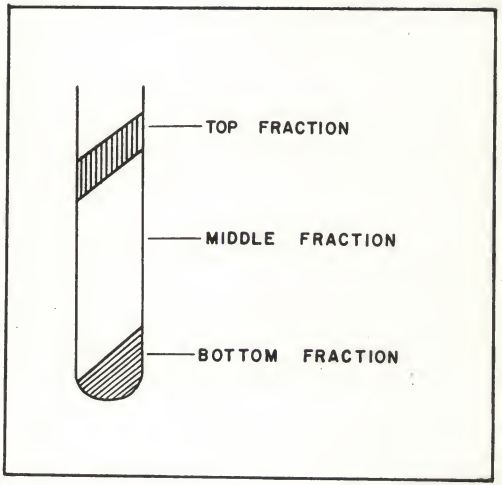
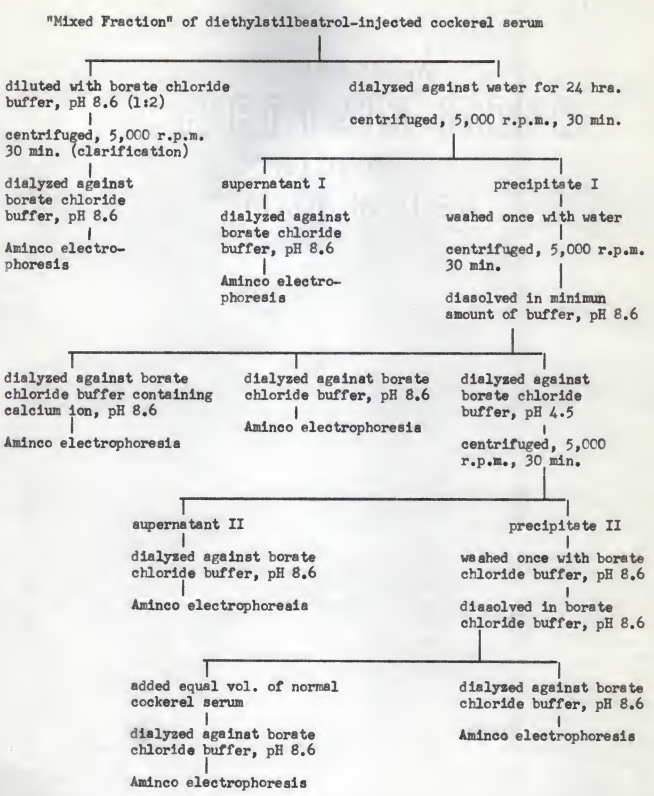


Table 1


"Mixed Fraction" of diethylstilbestrol-injected cockerel serum
diluted with borate chloride buffer, pH 8.6 (1:2)
centrifuged, 5,000 r.p.m. 30 min. (clarification)
dialyzed against borate chloride buffer, pH 8.6
Aminco electrophoresis
dialyzed against water for 24 hrs.
centrifuged, 5,000 r.p.m., 30 min.
supernatant I
dialyzed against borate chloride buffer, pH 8.6
Aminco electrophoresis
precipitate I
washed once with water
centrifuged, 5,000 r.p.m. 30 min.
diasolved in minimun amount of buffer, pH 8.6
dialyzed against borate chloride buffer containing calcium ion, pH 8.6
Aminco electrophoresis
dialyzed against borate chloride buffer, pH 8.6
Aminco electrophoresis
dialyzed against borate chloride buffer, pH 4.5
centrifuged, 5,000 r.p.m., 30 min.
supernatant II
dialyzed against borate chloride buffer, pH 8.6
Aminco electrophoresis
precipitate II
washed once with borate chloride buffer, pH 8.6
diasolved in borate chloride buffer, pH 8.6
added equal vol. of normal cockerel serum
dialyzed against borate chloride buffer, pH 8.6
Aminco electrophoresis
dialyzed against borate chloride buffer, pH 8.6
Aminco electrophoresis

About 180 ml of the "Mixed Fraction" obtained from the serum of six diethylstilbestrol-treated cockerels were stored at 5 to 7° C before use. Five ml of this "Mixed Fraction" was diluted with 10 ml of borate chloride buffer at pH 8.6 and dialyzed against the same buffer overnight. This dilution resulted in a final concentration of approximately 1 per cent protein. The dialysis was carried out in order to bring the ionic composition of both the serum and the buffer solution to nearly identical values.

The moving boundary electrophoresis of this diluted "Mixed Fraction" was conducted for 81 minutes, employing a current of 20 ma. After the analysis, the electrophoretic pattern was photographed on Kodak Contrast Process Panchromatic Sheet Film. The result was given in Plate II (A).

For the purpose of comparison, the whole serum from the non-laying hen and the normal cockerel, and the "Mixed Fraction" obtained from serum of the laying hen were subjected to dilution and dialysis against borate chloride buffer at pH 8.6 and then to electrophoretic analysis conducted in the same manner as described previously for the "Mixed Fraction" of diethylstilbestrol-injected serum.

The results were shown in Plate II (B, C, and D, respectively).

It is frequently difficult to see the visible light of the optical system when diethylstilbestrol-injected cockerel serum is employed. In order to eliminate this difficulty, clarification

EXPLANATION OF PLATE II

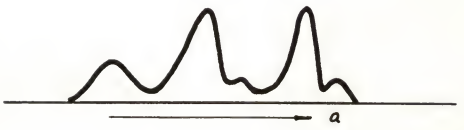
A comparison of moving boundary electrophoresis patterns of: (A) "Mixed Fraction" obtained from the diethylstilbestrol-injected cockerel serum, (B) "Mixed Fraction" obtained from the laying hen serum, (C) Whole serum obtained from the non-laying hen, and (D) Whole serum obtained from the normal cockerel.

The "Mixed Fraction" was obtained from the whole sera by means of centrifugation at 40,000 r.p.m. for 16 hours, removing the "Top Fraction," and mixing the "Bottom Fraction" and "Middle Fraction."

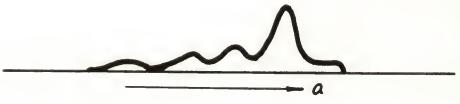
The electrophoretic analyses were conducted under the condition of:

Buffer	Borate chloride buffer, 0.05M, pH 8.6.
Time	81 min.
Temperature	0° C.
Current	20 ma.

PLATE II

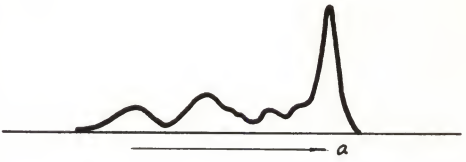


A



B

C



D

was carried out by means of high speed centrifugation, either before or after dialysis. In the case of the "Mixed Fraction" of diethylstilbestrol-injected cockerel serum, centrifugation at 5,000 r.p.m. for 30 minutes gave satisfactory clarification.

The electrophoretic pattern of the "Mixed Fraction" of laying hen serum (Plate II, B) was very close to that of the "Mixed Fraction" of diethylstilbestrol-injected cockerel serum (Plate II, A) in that they both possessed five protein components with component 1 moving ahead of the albumin. A higher concentration of component 6 in the "Mixed Fraction" of diethylstilbestrol-injected cockerel serum was the only difference noticed. As shown in Plate II (C and D), the electrophoretic pattern of normal cockerel serum was almost identical to that of the non-laying hen serum. None of them contained the prealbumin component. Since the protein component content of normal cockerel serum was not as variable as that of the hen serum, and was free from prealbumin component, it was used in this investigation as a guide to detect the presence of component 1 by combining the normal cockerel serum with the sample to be tested. The appearance of the prealbumin component in the electrophoretic pattern of the combined serum would show the presence of component 1 in the sample.

In this part of the isolation work, the "Mixed Fraction" of the diethylstilbestrol-injected cockerel serum was used as the starting material, and the electrophoretic pattern of this fraction was used as a standard.

The remainder of the "Mixed Fraction" was dialyzed against water for 24 hours. The precipitate formed was centrifuged at 5,000 r.p.m. for 30 minutes at 0° C and designated as precipitate I, while the supernatant was designated as supernatant I. Precipitate I had a yellow, translucent, waxy appearance and was a well-packed precipitate. Supernatant I was decanted and precipitate I was washed once with five ml of water, and the precipitate was then dissolved in a minimum amount of borate chloride buffer at pH 8.6.

The buffer solution of precipitate I was divided into three portions, and the first 6 ml portion and supernatant I were dialyzed against borate chloride buffer at pH 8.6. The second 6 ml portion was dialyzed against the borate chloride buffer containing calcium ion at pH 8.6. After 24 hours of dialysis, with one change of buffer, moving boundary electrophoreses of these three samples were performed. The results obtained are shown in Plate III (A, B, and C, respectively).

The presence of component 1 and a reduced amount of component 6 in precipitate I was indicated from the electrophoretic pattern of precipitate I (Plate III, A) and also by the other two patterns as follows: (1) The resemblance of the electrophoretic pattern of supernatant I to that of the normal cockerel serum (Plate II, D), (2) The absence of the prealbumin component in the pattern of supernatant I (Plate III, C), and (3) The reduction in mobility of the fast-moving component of precipitate I on addition of calcium ion (Plate III, B).

EXPLANATION OF PLATE III

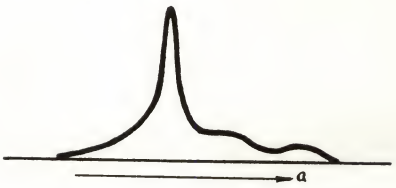
Moving boundary electrophoresis patterns of: (A) Precipitate I on dialysis against borate chloride buffer at pH 8.6, (B) Precipitate I on dialysis against borate chloride buffer containing calcium ion at pH 8.6, and (C) Supernatant I on dialysis against borate chloride buffer at pH 8.6.

Precipitate I and supernatant I were obtained by dialysis of the "Mixed Fraction" from diethylstilbestrol-injected cockerel serum against water.

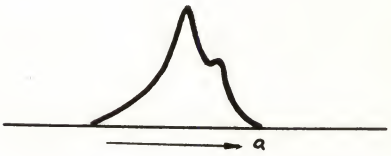
The electrophoretic analyses were conducted under the condition of:

Buffer	Borate chloride buffer at pH 8.6 with or without calcium ion.
Time	81 min.
Temperature	0° C.
Current	20 ma.

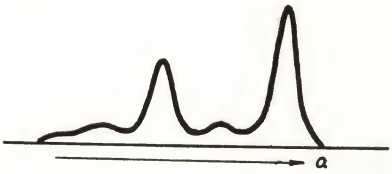
PLATE III



A



B



C

The rest of the precipitate I solution was subjected to dialysis at pH 4.5 in 0.05 molar borate chloride buffer to carry out further salt fractionation. The yellow waxy precipitate formed after dialysis for two days, was separated from the supernatant by centrifugation at 5,000 r.p.m. for 30 minutes under refrigeration. This precipitate was designated as precipitate II and the supernatant as supernatant II. Precipitate II was washed once with the buffer and then dissolved in a minimum amount of borate chloride buffer at pH 8.6.

Six ml of precipitate II solution and supernatant II were dialyzed against borate chloride buffer at pH 8.6 for 24 hours. An equal amount of normal cockerel serum was added to the remainder of the precipitate II solution, and this mixture was dialyzed against borate chloride buffer at pH 8.6 in the same manner as the other two samples. After dialysis, moving boundary electrophoreses were conducted for these three samples for 81 minutes, at a current of 20 ma., and the electrophoretic pattern photographed on Kodak Contrast Process Panchromatic Sheet Film. The results were given in Plate IV (A, B, and C, respectively).

The electrophoretic pattern of supernatant II showed four components (Plate IV, B), that of precipitate II showed two components (Plate IV, A) while that of the combined mixture of precipitate II and normal cockerel whole serum showed a great similarity to that of the "Mixed Fraction" of diethylstilbestrol-injected cockerel serum (Plate IV, C), especially with respect to the presence of the prealbumin fraction and the greatly increased amount of component 6 to the electrophoretic pattern of normal cockerel serum.

EXPLANATION OF PLATE IV

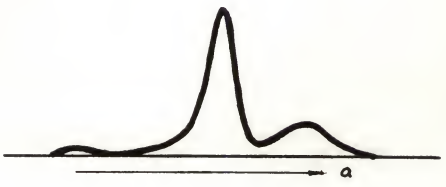
Moving boundary electrophoresis patterns of: (A) Precipitate II on dialysis against borate chloride buffer at pH 8.6, (B) Supernatant II on dialysis against borate chloride buffer at pH 8.6, and (C) Combined mixture of precipitate II and normal cockerel whole serum on dialysis against borate chloride buffer at pH 8.6.

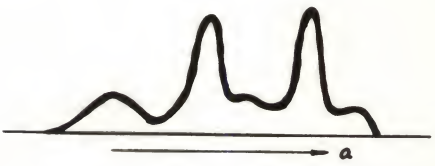
Precipitate II and supernatant II were obtained by dialysis of the precipitate I obtained from diethylstilbestrol-injected cockerel serum against borate chloride buffer at pH 4.5.

The electrophoretic analyses were conducted under the conditions of:

Buffer	Borate chloride buffer at pH 8.6.
Time	81 min.
Temperature	0° C.
Current	20 ma.

PLATE IV



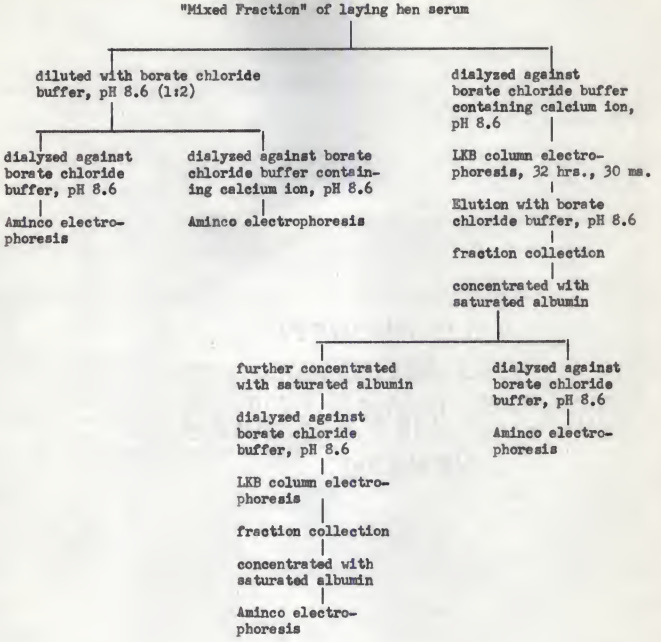


From these results it was noted that component 1 or prealbumin component of laying hen and diethylstilbestrol-injected cockerel serum had been separated, but that a considerable amount of another component remained as a contaminant. From the results as illustrated in Plate IV (C), it may be assumed that this contaminant was component 6.

LKB Column Electrophoresis and Moving Boundary Electrophoretic Analysis. Clegg et al. (6) have shown that the presence of calcium ion in the buffer drastically reduced the mobility of component 1 in that it migrated in the area considered as β-globulin instead of the prealbumin area. Based on this investigation, the following isolation of component 1 was carried out on the LKB electrophoretic column, and moving boundary electrophoretic analysis was used as a means of evaluating this preparative electrophoresis. The preparation of the supporting medium (cellulose powder), packing and operation of the column has been described by Misra (30). Here the "Mixed Fraction" of laying hen serum was used as the starting material. The preparation procedure is shown in Table 2.

Ten ml of the "Mixed Fraction" of laying hen serum was prepared as described previously and divided into two equal portions. The first 5 ml was diluted with 10 ml of borate chloride buffer at pH 8.6 and then further divided into two equal portions. These two portions were dialyzed against borate chloride buffer at pH 8.6 and borate chloride buffer containing calcium ion at pH 8.6, respectively, and were analyzed electrophoretically in an

Table 2


"Mixed Fraction" of laying hen serum
diluted with borate chloride buffer, pH 8.6 (1:2)
dialyzed against borate chloride buffer, pH 8.6
Aminco electrophoresis
dialyzed against borate chloride buffer containing calcium ion, pH 8.6
Aminco electrophoresis
dialyzed against borate chloride buffer containing calcium ion, pH 8.6
LKB column electrophoresis, 32 hrs., 30 ma.
Elution with borate chloride buffer, pH 8.6
fraction collection
concentrated with saturated albumin
further concentrated with saturated albumin
dialyzed against borate chloride buffer, pH 8.6
LKB column electrophoresis
fraction collection
concentrated with saturated albumin
Aminco electrophoresis
dialyzed against borate chloride buffer, pH 8.6
Aminco electrophoresis

Aminco Portable Electrophoresis Apparatus. The electrophoretic patterns shown in Plate V (A and B) were used as controls for later experiments in column electrophoresis.

The second 5 ml portion of the original "Mixed Fraction" was dialyzed against borate chloride buffer containing calcium ion at pH 8.6. After 24 hours of dialysis the preparatory electrophoresis of 4 ml of this dialyzed sample was carried out for 32 hours at a current of 30 ma. at 15° C. The borate chloride buffer, 0.05 M, pH 8.6, was used and renewed once after 16 hours of dialysis.

The column was eluted with the same buffer at a flow rate of 10 ml per hour as soon as the separation electrophoresis was completed. A RodiRic automatic fraction collector was used and about 80 fractions (5 ml per fraction) of the eluent were collected. The operation of this collector is described in the instruction manual (38).

Using borate chloride buffer, pH 8.6, 0.05 M as a blank, protein concentration of the eluted fractions was determined with a Beckman Spectrophotometer model DU at a wavelength of 280 millicrons. Absorbance of the fractions was plotted against the number of fractions as shown in Plate VI.

The electrophoretic separation pattern of laying hen serum dialyzed against borate chloride buffer containing calcium ion at pH 8.6 obtained on column electrophoresis showed two peaks with component 1 moving in the area behind the albumin, designated as "Q." This was in agreement with that of the moving

EXPLANATION OF PLATE V

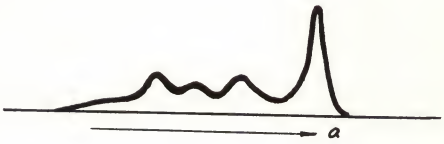
Moving boundary electrophoresis patterns of (A) "Mixed Fraction" of laying hen serum on dialysis against borate chloride buffer at pH 8.6, and (B) "Mixed Fraction" of laying hen serum on dialysis against borate chloride buffer containing calcium ion at pH 8.6.

The "Mixed Fraction" was obtained from the whole sera by means of centrifugation at 40,000 r.p.m. for 16 hours, removing the "Top Fraction," and mixing the "Bottom Fraction" and "Middle Fraction."

The electrophoretic analyses were conducted under the conditions of:

Buffer	Borate chloride buffer at pH 8.6 with or without calcium ion.
Time	81 min.
Temperature	0° C.
Current	20 ma.

PLATE V



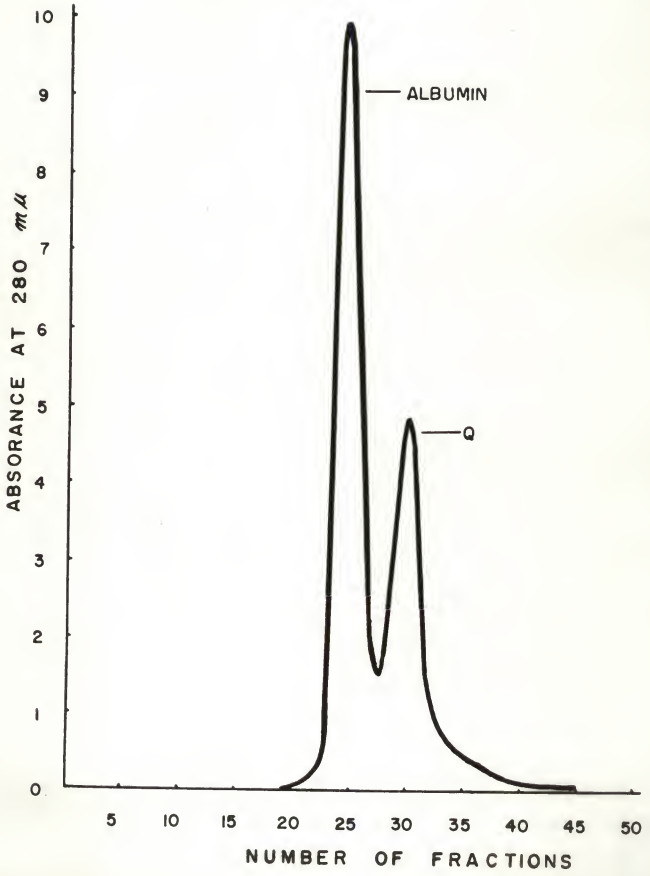
EXPLANATION OF PLATE VI

Column electrophoresis pattern of "Mixed Fraction" obtained from laying hen serum on dialysis against borate chloride buffer containing calcium ion at pH 8.6.

The separation was carried out for 32 hours at a current of 30 ma. at 5 to 7° C, using borate chloride buffer containing calcium ion at pH 8.6. Five ml fractions per 30 min. were eluted with the same buffer, and their protein concentration determined at a wavelength of 280 millimicrons with the Beckman Spectrophotometer Model DU.

The diagram was prepared from the data in Table 1 in the Appendix.

PLATE VI


ALBUMIN
Q
ABSORANCE AT 280 mμ
0
1
2
3
4
5
6
7
8
9
10
5
10
15
20
25
30
35
40
45
50
NUMBER OF FRACTIONS

boundary electrophoresis. As "Q" component included fractions 28, 29, 30, 31, and 32, these five fractions were combined and dialyzed against saturated albumin to raise the concentration.

Six ml of this concentrated component "Q" was then subjected to dialysis against borate chloride buffer, pH 8.6 and analyzed by moving boundary electrophoresis. The result, as shown in Plate VII, indicated two peaks. These two peaks, however, were too short to give a clear picture because the concentration procedure was not satisfactory. Although this procedure appeared satisfactory as a means of separation, equipment was not available to process large amounts of serum by this method. Therefore this procedure was discontinued.

"Bottom Fraction" of Diethylstilbestrol-injected Cockerel Serum

Salt Fractionation and Moving Boundary Electrophoretic Analysis. It has been shown earlier in this section, that the "Mixed Fraction" of diethylstilbestrol-injected cockerel serum was not a good source of component 1, as the contamination in the final product was rather high. Therefore, the "Bottom Fraction" was compared with the "Middle Fraction" in an attempt to find a better source of component 1. The moving boundary electrophoresis was conducted on the "Middle Fraction" and "Bottom Fraction" of the diethylstilbestrol-injected cockerel serum, respectively, in the following manner. The whole serum obtained from the normal cockerel was diluted with two times the volume of borate chloride buffer at pH 8.6. Eleven ml of this diluted

EXPLANATION OF PLATE VII

Moving boundary electrophoresis pattern of component Q (as described previously in Plate VI) on dialysis against borate chloride buffer at pH 8.6.

The electrophoretic analyses were conducted under the condition of:

Buffer	Borate chloride buffer at pH 8.6.
Time	81 min.
Temperature	0° C.
Current	20 ma.

PLATE VII

normal cockerel serum was added to the "Bottom Fraction" prepared from 13 ml of the diethylstilbestrol-injected cockerel whole serum by means of ultracentrifuge procedure, and mixed thoroughly so that the semi-solid "Bottom Fraction" went into solution completely. This combined serum was then dialyzed against the borate chloride buffer at pH 8.6.

Ten ml of the "Middle Fraction" was diluted with borate chloride buffer at pH 8.6 to give a total volume of 25 ml. The diluted fraction was subjected to dialysis against the same buffer. After dialysis for 24 hours, electrophoretic analysis of this diluted "Middle Fraction" and the combined serum mentioned above was conducted. The electrophoretic patterns are illustrated in Plate VIII (A and B).

The large area of prealbumin fraction present in the electrophoretic pattern of the combined serum (Plate VIII, A) was noted. Other than this, the electrophoretic pattern of the combined serum was very close to that of the normal cockerel serum. In the case of the "Middle Fraction," the electrophoretic pattern showed two distinctive components — a decreased amount of albumin and a large component in the patterns where component 6 was usually found. These results indicated the presence of a large amount of component 1 and a greatly reduced amount of component 6 in the "Bottom Fraction" of diethylstilbestrol-injected cockerel serum.

The second part of the isolation work was based on this finding and the "Bottom Fraction" of the diethylstilbestrol-

EXPLANATION OF PLATE VIII

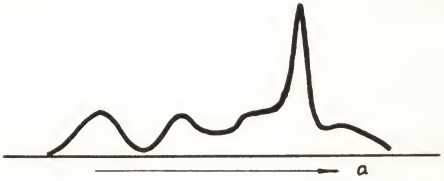
Moving boundary electrophoresis patterns of: (A) Combined mixture of "Bottom Fraction" from diethylstilbestrol-injected cockerel serum and normal cockerel whole serum on dialysis against borate chloride buffer at pH 8.6, and (B) "Middle Fraction" from diethylstilbestrol-injected cockerel serum on dialysis against borate chloride buffer at pH 8.6.

"Middle Fraction" and "Bottom Fraction" were obtained from the whole sera by means of centrifugation at 40,000 r.p.m for 16 hours, removing the "Top Fraction" and separating the "Middle Fraction" from the "Bottom Fraction" by decantating the liquid "Middle Fraction" and leaving the semi-solid "Bottom Fraction" in the tube.

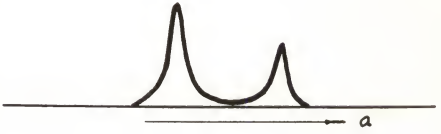
The electrophoretic analyses were conducted under the condition of:

Buffer	Borate chloride buffer at pH 8.6.
Time	81 min.
Temperature	0° C.
Current	20 ma.

PLATE VIII



A



B

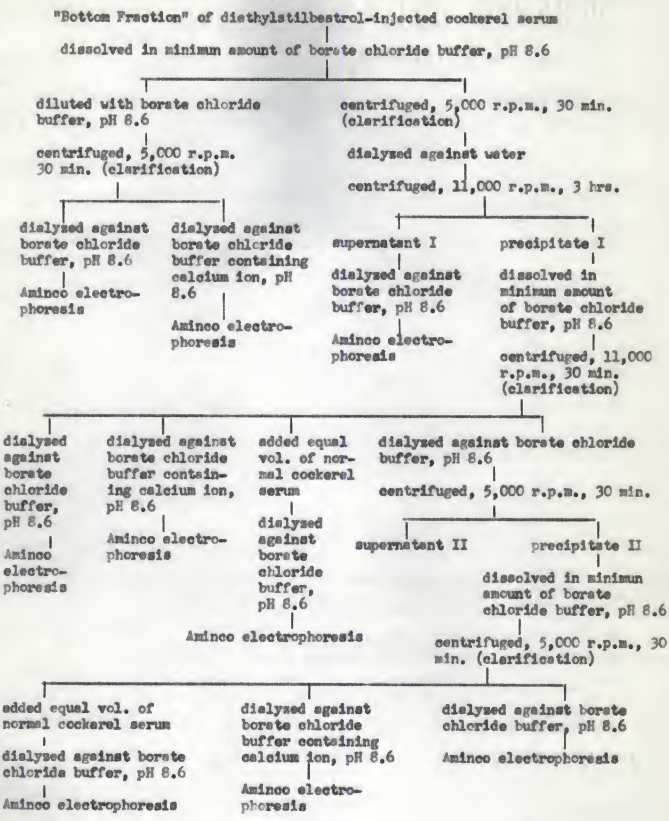
injected cockerel serum was used as a starting material. The procedure used is outlined in Table 3.

For each experiment, six diethylstilbestrol-injected cockerels were sacrificed and approximately 200-230 ml of the serum was obtained from the blood. The blood serum was then put into 20 plastic tubes of 13 ml capacity and centrifuged at 40,000 r.p.m. for 16 hours. "Mixed Fraction" was prepared from one of the tubes and "Bottom Fraction" from the remainder of the tubes as described previously.

The "Mixed Fraction" was diluted with two times the volume of borate chloride buffer at pH 8.6 and divided into two equal portions. The first portion was dialyzed against borate chloride buffer at pH 8.6 while the other portion was dialyzed against borate chloride buffer containing calcium ion at pH 8.6.

The "Bottom Fraction" in the other 19 tubes was dissolved in a minimum amount of borate chloride buffer at pH 8.6 to give a total volume of approximately 95 ml. Five ml of this diluted "Bottom Fraction" was then further diluted with twice the volume of the same buffer and clarified by centrifugation at 5,000 r.p.m. for 30 minutes. This centrifuged "Bottom Fraction" was divided into two portions. The first portion was dialyzed against borate chloride buffer at pH 8.6 while the second portion was dialyzed against borate buffer containing calcium ion at pH 8.6. After 24 hours of dialysis, electrophoretic analyses of all four samples were conducted. The results are illustrated in Plate IX (A, B, C, and D).

Table 3


"Bottom Fraction" of diethylstilbestrol-injected cockerel serum
dissolved in minimum amount of borate chloride buffer, pH 8.6
diluted with borate chloride buffer, pH 8.6
centrifuged, 5,000 r.p.m. 30 min. (clarification)
dialysed against borate chloride buffer, pH 8.6
Aminco electrophoresis
dialysed against borate chloride buffer containing calcium ion, pH 8.6
Aminco electrophoresis
centrifuged, 5,000 r.p.m., 30 min. (clarification)
dialysed against water
centrifuged, 11,000 r.p.m., 3 hrs.
supernatant I
dialysed against borate chloride buffer, pH 8.6
Aminco electrophoresis
precipitate I
dissolved in minimum amount of borate chloride buffer, pH 8.6
centrifuged, 11,000 r.p.m., 30 min. (clarification)
dialysed against borate chloride buffer, pH 8.6
Aminco electrophoresis
dialysed against borate chloride buffer containing calcium ion, pH 8.6
Aminco electrophoresis
added equal vol. of normal cockerel serum
dialysed against borate chloride buffer, pH 8.6
Aminco electrophoresis
dialysed against borate chloride buffer, pH 8.6
centrifuged, 5,000 r.p.m., 30 min.
supernatant II
precipitate II
dissolved in minimum amount of borate chloride buffer, pH 8.6
centrifuged, 5,000 r.p.m., 30 min. (clarification)
added equal vol. of normal cockerel serum
dialysed against borate chloride buffer, pH 8.6
Aminco electrophoresis
dialysed against borate chloride buffer containing calcium ion, pH 8.6
Aminco electrophoresis
dialysed against borate chloride buffer, pH 8.6
Aminco electrophoresis

EXPLANATION OF PLATE IX

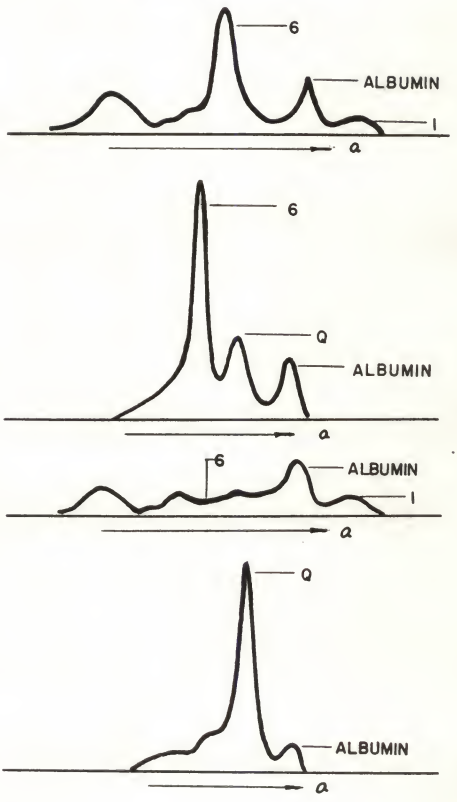
Moving boundary electrophoresis patterns of: (A) "Mixed Fraction" on dialysis against borate chloride buffer at pH 8.6, (B) "Mixed Fraction" on dialysis against calcium containing borate chloride buffer at pH 8.6, (C) "Bottom Fraction" on dialysis against borate chloride buffer at pH 8.6, and (D) "Bottom Fraction" on dialysis against calcium containing borate chloride buffer at pH 8.6.

The "Middle Fraction" and "Bottom Fraction" were obtained from the whole sera of diethylstilbestrol-injected cockerel by means of preparatory ultracentrifugation as described previously.

The electrophoretic analyses were conducted under the condition of:

Buffer	Borate chloride buffer at pH 8.6 with or without calcium ion.
Time	81 min.
Temperature	0° C.
Current	20 ma.

PLATE IX



The large area of prealbumin component 1, in the "Bottom Fraction" of diethylstilbestrol-injected cockerel serum (Plate IX, C) was comparable to that of the "Mixed Fraction" (Plate IX, A). The removal of component 6 and a considerable amount of albumin was observed. The electrophoretic pattern of those samples which were dialyzed against the calcium-containing buffer (Plate IX, B and D) emphasized the finding that the first component was the prealbumin of the original serum. The letter "Q" designated the area where component 1 was present after being dialyzed against borate chloride buffer containing calcium ion. This is in agreement with the observation that when sufficient calcium ion is present, component 1 will appear in the β-globulin area of the electrophoretic pattern.

The remainder of the diluted "Bottom Fraction" was clarified by centrifugation at 5,000 r.p.m. for 30 minutes and then dialyzed against water. In order to separate the precipitate formed after 24 hours of dialysis, centrifugation at 11,000 r.p.m. for three hours was applied. The precipitate was designated as precipitate I and the supernatant was designated as supernatant I. Precipitate I was dissolved in a minimum amount of borate chloride buffer at pH 8.6, clarified by ultracentrifugation at 11,000 r.p.m. for 30 minutes, and the clear solution divided into four portions. The first 6 ml portion and supernatant I were dialyzed against borate chloride buffer containing calcium ion at pH 8.6 while the third 3 ml portion of the solution was added to an equal amount of normal cockerel serum and then dialyzed

against borate chloride buffer at pH 8.6. After 24 hours of dialysis, moving boundary electrophoresis of all four samples was conducted, with results as illustrated in Plate X (A, B, C, and D, respectively).

The complete disappearance of the prealbumin component in the electrophoretic pattern of supernatant I (Plate X, B) was noted, while Plate X (A) showed a great increase in amount of component 1 in precipitate I owing to its being highly concentrated during the fractionation steps. The large area of component 1 shown in Plate X (C) also gave a very strong evidence of this fact. As has been shown in Plate X (D), the fractionation step also reduced the albumin concentration to a considerable extent.

The remainder of the clarified precipitate I solution was dialyzed against borate chloride buffer, 0.05 M, pH 4.5. After two to three days of dialysis, a pale yellow, waxy precipitate was formed and designated as precipitate II. Precipitate II was separated from supernatant II by means of centrifugation at 5,000 r.p.m. for 30 minutes. Precipitate II was then dissolved in a minimum amount of borate chloride buffer at pH 8.6 and clarified by centrifugation at 5,000 r.p.m. for 30 minutes.

Six ml of precipitate II solution was dialyzed against borate chloride buffer at pH 8.6 and the other six ml was dialyzed against borate chloride buffer containing calcium ion at pH 8.6. An equal amount of normal cockerel serum was added to the rest of the precipitate II and this mixture was then

EXPLANATION OF PLATE X

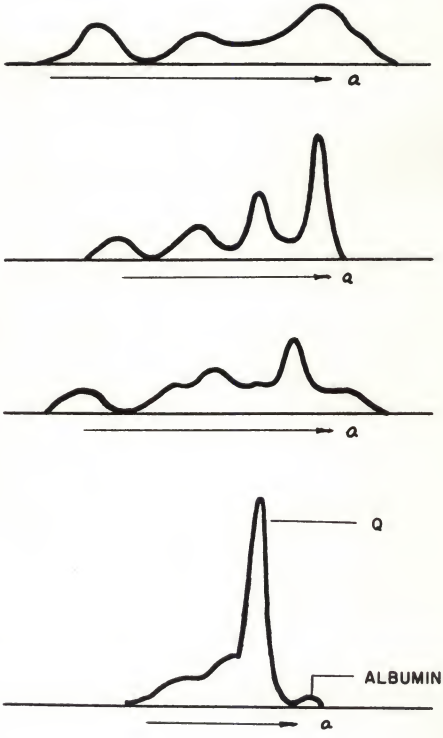
Moving boundary electrophoresis patterns of: (A) Precipitate I on dialysis against borate chloride buffer at pH 8.6, (B) Supernatant I on dialysis against borate chloride buffer at pH 8.6, (C) Combined mixture of precipitate I and normal cockerel whole serum on dialysis against borate chloride buffer at pH 8.6, and (D) Precipitate I on dialysis against calcium containing borate chloride buffer at pH 8.6.

Precipitate I and supernatant I were obtained by dialysis of the "Mixed Fraction" from diethylstilbestrol-injected cockerel serum against water.

The electrophoretic analyses were conducted under the condition of:

Buffer	Borate chloride buffer at pH 8.6 with or without calcium ion.
Time	81 min.
Temperature	0° C.
Current	20 ma.

PLATE X



EXPLANATION OF PLATE XI

Moving boundary electrophoresis patterns of: (A) Precipitate II on dialysis against borate chloride buffer at pH 8.6, (B) Precipitate II on dialysis against calcium containing borate chloride buffer at pH 8.6, and (C) Combined mixture of precipitate II and normal cockerel serum on dialysis against borate chloride buffer at pH 8.6.

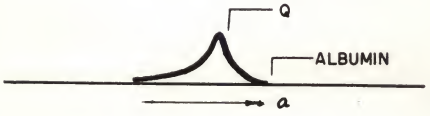
Precipitate II and supernatant II were obtained by dialysis of the precipitate I obtained from diethylstilbestrol-injected cockerel serum against borate chloride buffer at pH 4.5.

The electrophoretic analyses were conducted under the condition of:

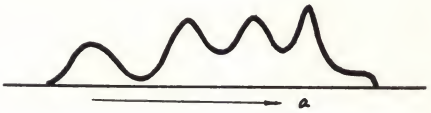
Buffer	Borate chloride buffer at pH 8.6 with or without calcium ion.
Time	81 min.
Temperature	0° C.
Current	20 ma.

PLATE XI

A



B



C

dialyzed against borate chloride buffer at pH 8.6. After dialysis for 24 hours, electrophoretic analysis of these three samples was conducted and patterns as shown in Plate XI (A, B, and C, respectively), were obtained.

As is shown in Plate XI (A), precipitate II contained two peaks. It was apparent that the fast-moving component was component 1 according to the following criteria: (1) The appearance of the component in prealbumin position (comparable to that of the normal cockerel serum), (2) The absence of an albumin component in the electrophoretic pattern of precipitate II which had been dialyzed against the borate chloride buffer containing calcium ion at pH 8.6.

From these results it was concluded that the "Bottom Fraction" of the diethylstilbestrol-injected cockerel serum was a better source for the separation of component 1 than the "Mixed Fraction" as the contamination by the other component was rather low for component 1 separated in this manner.

DISCUSSION

The appearance of two new major phospholipoprotein components in laying hen serum and the great similarity of the electrophoretic pattern of the sera of the laying hen to the pattern obtained with the sera of diethylstilbestrol-injected cockerels are well known. These two components have been designated as components 1 and 6, respectively, with the former migrating at a rate faster than that of the albumin, while the

latter migrated in the area where β-globulin is normally observed in the electrophoretic pattern.

The isolation of components 1 and 6 is important since this will provide suitable material for the investigation of the chemical structure of phospholipoproteins. Such investigations may help in understanding the problem of yolk formation and in determining the function of these substances in the laying hen and the diethylstilbestrol-injected cockerel. Numerous investigations have dealt with the problem of separation of phosphoproteins and lipoproteins, but none have been entirely successful.

Recently, Misra (30) purified component 6 from laying hen serum to the extent that it was homogenous to electrophoresis and ultracentrifuge analysis, although gradient ion exchange chromatography indicated a slight heterogeneity. In the present investigation, the combined technique of preparatory ultracentrifugation, salt fractionation, and moving boundary electrophoresis, component 1 has been separated from diethylstilbestrol-injected cockerel serum with only very slight contamination.

The "Mixed Fraction" was first used as the starting material and subjected to isoelectric precipitation with water (precipitate I). Moving boundary electrophoresis of the precipitate showed the presence of component 1 in a less complicated system. Precipitate II was obtained by further salt fractionation of precipitate I with borate chloride buffer at pH 4.5. The

electrophoretic analysis of precipitate II revealed the separation of component 1 with a considerable amount of component 6 as a contaminant.

As the "Bottom Fraction" of diethylstilbestrol-injected cockerel serum contained a large amount of component 1 and a greatly reduced amount of component 6, it was employed as the source for the separation of component 1 in later work. The fractionation procedures were carried out in the same manner as mentioned above and the electrophoretic analysis of the precipitate II obtained from the "Bottom Fraction" indicated a better separation.

The terms "light" and "dense" phospholipoproteins have been used by McIndole (29) to describe two components in precipitable lipophosphoprotein (which was obtained by isoelectric precipitation of the laying hen plasma) observed by means of the analytical ultracentrifuge. Using the ultracentrifuge, Schjeide (35) has also obtained a large amount of lipoprotein from laying hen serum corresponding to a lipoprotein and a lighter component. It is believed that component 6 is the light phospholipoprotein and component 1 the dense phospholipoprotein.

There are no general methods which permit the isolation of lipoproteins from tissue. Each lipoprotein must be treated as a special case and studied individually. Salt fractionation has been extensively employed for the separation of lipovitellin from egg yolk, but this method is not very satisfactory for the separation of component 1 from either the "Mixed Fraction" or

the "Bottom Fraction" of diethylstilbestrol-injected cockerel serum. Preparatory electrophoretic separation using an LKB column could be an excellent method for this purpose if a better method of concentrating the combined fractions than dialysis against saturated albumin could be employed.

Based on the present investigation, the isolation of somponent 1 from the "Bottom Fraction" of diethylstilbestrol-injected cockerel serum in a relatively pure form can be accomplished by employing column electrophoresis and a suitable concentrating apparatus.

Buffer solution of the "Bottom Fraction" may be subjected first to dialysis against borate chloride buffer containing calcium ion to reduce the mobility of component 1 and then to column electrophoresis. The component migrating behind albumin would be eluted and concentrated with a suitable concentrating apparatus. Further column electrophoresis would be conducted for this concentrated sample after being dialyzed against borate chloride buffer, the first zone eluted being the relatively pure component 1. By repeating this process, a large amount of component 1 may be obtained.

SUMMARY

(1) The "Bottom Fraction," obtained by means of preparatory ultracentrifugation, contained a large amount of component 1 and a small amount of component 6, in addition to other components.

(2) Using a combined technique of salt fractionation and moving boundary electrophoresis, component 1 has been separated in the form of precipitate II from both the "Mixed Fraction" and the "Bottom Fraction" of diethylstilbestrol-injected cockerel serum. The "Bottom Fraction" was a better source for the isolation, as the contamination by other components was rather low.

(3) LKB column electrophoresis of the "Mixed Fraction" of laying hen serum which had been dialyzed against borate chloride buffer containing calcium ion was performed. Hydrolyzed cotton cellulose was used as the supporting medium. Preparatory electrophoresis could be a better separation method for component 1 if a suitable concentrating apparatus was available.

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APPENDIX

Table 4

Protein concentration of five ml fractions in terms of optical density at 280 mu. as determined with a Beckman spectrophotometer, model DU. (These fractions were obtained from the "Mixed Fraction" of laying hen serum after being dialyzed against calcium containing borate chloride buffer, pH 8.6, carried out in LKB column electrophoresis apparatus for 32 hours at a current of 30 ma, and eluted with the same buffer at a flow rate of five ml per 30 min.)

No. of fractions	Optical density at 280 mu.	No. of fractions	Optical density at 280 mu.	No. of fractions	Optical density at 280 mu.
1	0.065	16	0.049	31	4.400
2	0.060	17	0.061	32	1.190
3	0.060	18	0.067	33	0.799
4	0.048	19	0.059	34	0.555
5	0.070	20	0.072	35	0.399
6	0.078	21	0.079	36	0.317
7	0.061	22	0.100	37	0.244
8	0.062	23	0.424	38	0.196
9	0.071	24	6.500	39	0.167
10	0.056	25	10.000	40	0.137
11	0.061	26	7.320	41	0.112
12	0.063	27	3.660	42	0.101
13	0.052	28	1.770	43	0.074
14	0.060	29	3.310	44	0.065
15	0.062	30	4.950	45	0.075

FRACTIONATION OF COMPONENT 1 (PREALBUMIN) FROM DIETHYLSTILBESTROL-INJECTED COCKEREL SERUM

by

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An increase in calcium, phosphorus, and total protein in chicken serum during the reproductive cycle or after the injection of diethylstilbestrol is well known. It has been reported that these higher values for calcium, phosphorus, and protein were directly associated with the appearance of component 1 (prealbumin) and the increase in size of component 6.

The isolation of components 1 and 6 is important in the sense that this will provide material suitable for the investigation of the chemical structure of phospholipoprotein, as well as the elucidation of the problem of yolk formation.

Due to the great similarity of the electrophoretic pattern of diethylstilbestrol-injected cockerel serum to that of laying hen serum, the serum of diethylstilbestrol-injected cockerel serum was used in this investigation.

By applying a combination of preparatory untracentrifugation, salt fractionation and moving boundary electrophoresis, component 1 was separated from both the "Mixed Fraction" and the "Bottom Fraction" of diethylstilbestrol-injected cockerel serum in the form of precipitate II. The electrophoretic analysis of the precipitate II obtained from the "Bottom Fraction" indicated that the "Bottom Fraction" was a better source of material for the isolation of component 1.

Based on the present investigation, the isolation of component 1 from the "Bottom Fraction" of diethylstilbestrol-injected cockerel serum in a relatively pure form can be accomplished by employing column electrophoresis and a suitable concentrating apparatus.